THE DEVELOPMENT OF NOVEL BACTERIAL CELL DIVISION PROTEIN FTSZ INHIBITORS TO BROADEN THEIR ANTIBACTERIAL SPECTRUM

GAO WEI

MPhil

The Hong Kong Polytechnic University

2019

The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

The Development of Novel Bacterial Cell Division Protein FtsZ Inhibitors to Broaden Their Antibacterial Spectrum

GAO Wei

A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of Master of Philosophy

March 2019

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

Signature:_____

Name: GAO Wei

Abstract

Antibacterial resistance has become a new global threat for public health, due to the lack of effective strategies to solve it. For example, methicillin-resistant *S. aureus* (MRSA) which is the most common resistant bacteria, can cause infections with a high rate of mortality. Therefore, there is an urgent need to develop some new types of antibiotics with new drug target and new mechanism of action.

FtsZ protein is a bacterial cell division protein, which plays a very important role in the process of cell division. It has been approved to be an attractive drug target for antibiotic drug discovery. There have been many different types of FtsZ inhibitors from natural products to small molecules. However, most of these FtsZ inhibitors only have antibacterial activity against *S. aureus*, or they have little effect on resistant bacteria and Gram-negative bacteria. In this study, we investigated the synergistic effect of FtsZ inhibitor and beta-lactam antibiotics, and we also developed a series of compounds with broad antibacterial spectrum through structure modification and double warhead strategy.

Firstly, we confirmed that the target of compound **F332** was FtsZ protein by genetic study and docking study, although the compound **F332** was considered as a potential FtsZ inhibitor in our previous study. Then we investigated the synergistic effect of **F332** together with beta-lactam antibiotics. The MIC result showed that combination of drugs had synergistic effect and **F332** could restore the efficacy of beta-lactam antibiotics *in vitro*. The highest synergy percentage was the combination of the compound **F332** used together with methicillin, which was as high as more than 80% *in vitro*. These results indicated that the FtsZ inhibitor **F332** could restore the efficacy of beta-lactam antibiotics to treat MRSA *in vitro*.

In the next stage, we tried to develop broad spectrum FtsZ inhibitors through the structure modification of **F332**. We designed and synthesized a series of compounds, and the antibacterial activities of all compounds were evaluated by the MIC test. Some compounds had the antibacterial activity against both *S. aureus* and *E. coli*, and the antibacterial spectrum was broadened by adding a amidine group. Furthermore, when one fluorine was replaced by different phenol, the antibacterial activity would increase. The compound **32** and **38** exhibited stronger antibacterial activity against both of *S. aureus* and *E. coli*, and the MIC of their antibacterial activity against *S. aureus* was 4 μg/mL, which increased 8-fold comparing with compound **6**, while the antibacterial activity against *E. coli* was 16 μg/mL and it also increased by 2-fold. These results indicated that the amidine group could be a start point for structure modification to develop more broad-spectrum FtsZ inhibitors with better antibacterial activity.

In order to develop more antibiotics for Gram-negative bacterial strains, we tried to use the double warhead strategy to connect two scaffolds of **F332** and triclosan by different linkers. The advantage of this method was that it could broaden the antibacterial spectrum of **F332** and it could also reduce the toxicity of triclosan. We designed and synthesized a series of new compounds and the antibacterial activities of these compounds were evaluated by the MIC test. The MIC result of the compound **60** exhibited improved antibacterial activity against both *S. aureus* and *E. coli* with the MIC of less than 0.25 μg/mL. Furthermore, it also demonstrated excellent antibacterial activity against other Gram-negative bacterial strains with the MIC of less than 0.5 μg/mL, including *K. pneumoniae*, *K. oxytoca* and *E. cloacae*. These results indicated that the double warhead strategy of using the scaffold of FtsZ inhibitors provides a possible method for developing new antibiotics to treat Gram-negative bacterial strains.

Publications

1. H. K. Lui,* **W. Gao**,* K. C. Cheung,* W. Jin, N. Sun, Jason W. Y. Kan, Iris L. K. Wong, J. Chiou, D. Lin, Edward W. C. Chan, Y.-C. Leung, T. H. Chan, S. Chen, K.-F. Chan, K.-Y. Wong. "Boosting the efficacy of anti-MRSA β-lactam antibiotics via an easily accessible, non-cytotoxic and orally bioavailable FtsZ inhibitor", ***Eur. J. Med. Chem.***, 2019, *163*, 95-115.
2. W. Jin, C. Xu, Q. Cheng, X. L. Qi, **W. Gao**, Z. Zheng, E. W. C. Chan, Y. C. Leung, T. H. Chan, K. Y. Wong, S. Chen, and K. F. Chan. "Investigation of Synergistic Antimicrobial Effects of the Drug Combinations of Meropenem and 1,2-Benzisoselenazol-3(2h)-One Derivatives on Carbapenem-Resistant Enterobacteriaceae Producing Ndm-1", ***Eur. J. Med. Chem.,*** 2018, *155*, 285-302.

Acknowledgements

From the bottom of my heart, I hereby express my deep gratitude to my chief supervisor Dr. Kin-fai Chan for his enthusiastic supervision in all aspects during my whole MPhil study period. I am greatly inspired by his valuable suggestions and comments regarding my research project. It is really grateful that he provides me with a meaningful project and he teaches me to think critically about problems.

I truly express my gratitude to my co-supervisor Professor Kwok-yin Wong for his invaluable support, gracious encouragement and guidance. I also would like to express my appreciation to all colleagues in Professor Kwok-yin Wong's research group, who provide technical support for biological studies, which are indispensable in this project.

Moreover, I would like to extend my sincere appreciation to Professor Sheng Chen for his valuable comments and suggestions. I am also deeply grateful to Dr. Jiachi Chiou for her assistance in my bacterial cell experiment. Furthermore, I would express my thanks to Prof. Yun-chung Leung and Prof. Tak Hang Chan for their support and guidance in this project.

Also, I need to thank Dr. Siu-Cheong Yan for his assistance in NMR spectroscopy, and Dr. Pui-Kin So for his efforts in high-resolution mass spectrometry. I also thank Dr. Lai-king Wong for her assistance and expertise with the cytotoxicity experiment.

In addition, I would like to thank Mr. Qipeng Cheng for his assistance in MIC test, and I also thank Miss Xuemei Yang and Mr. Lianwei Ye for their gracious assistance in gene sequence analysis. I would also like to express my gratitude to other colleagues, Dr. Wenbin Jin, Mr. Ping Zeng, Mr. Jun Liu, Mr. K. H. Lai, Mr. Xuezhen

Zhu, Mr. Zhen Liu, Dr. Jian Yuan, Dr. Ning Sun, Dr. Ruichao Li, Dr. Dachuan Lin, Ms. Ning Dong, Ms. Miaomiao Wang, Ms. Chen Xu, Ms. Miaomiao Xie, Mr. Kaichao Chen, Ms. Kathy Po and Mr. Bill Chan for their encouragement and assistance throughout my study period.

Furthermore, I would give my utmost thanks to my family and best friends for their persistent love, unceasing support and endless encouragement.

Last, but not the least, I would like to thank the support from the Hong Kong Research Grants Council, the Hong Kong Polytechnic University and the State Key Laboratory of Chemical Biology and Drug Discovery for offering the stipend on this project.

Table of contents

List of Abbreviations

ACN	Acetonitrile
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
CDC	Center for Disease Control and Prevention
CRE	Carbapenem-Resistant *Enterobacteriaceae*
CLSI	Clinical and Laboratory Standards Institute
DCM	Dichloromethane
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium*
E. faecalis	*Enterococcus faecalis*
ESI-MS	Electrospray ionization mass spectrometry
FICI	Fractional inhibitory concentration index
FtsZ	Filamenting temperature sensitive strain Z
HAI	Health care-associated infections
IC_{50}	Half maximal inhibitory concentration
IDSA	The Infectious Diseases Society of America

K_2CO_3	Potassium carbonate
LB	Luria-Bertani medium
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MRM	Meropenem
MRSA	Methicillin-resistant *Staphylococcus Aureus*
MDR	Multidrug resistant
MW	Molecular weight
S. aureus	*Staphylococcus Aureus*
SAR	Structure activity relationship
TLC	Thin layer chromatography
VRE	Vancomycin resistant *enterococci*
WHO	World Health Organization

Chapter 1. Introduction

1.1. Antibiotics and antibiotic resistance

There is a long history for the human to control and cure bacterial infections by various well-documented traditional methods, such as by using herbs, honey and plants.(Sengupta et al., 2013) There is also evidence that some modern antibiotics can be detected in ancient human, for example, tetracyclines can be found in the human skeleton in Nubia. (Bassett et al., 1980) Pyocyanase was the first antibiotic drug used in hospitals, but it is no longer used today. It was discovered in *Pseudomonas aeruginosa* in the 1890s, and it can inhibit the growth of other bacteria. (Gould, 2016)

The modern age of using antimicrobial agent as a chemotherapy started with the development of antibacterial dyes. Paul Ehrlich was interested in the stain research and he found some stains were highly toxic to bacteria. (Schwartz, 2004) In 1909, he discovered Salvarsan, a chemical containing arsenic element. This agent performed well on the treatment of infections caused by syphilis. From then on, the door for human using modern antimicrobial agents to treat infections was opened. Ehrlich was also interested in immunology, and he worked together with other scientists to develop diphtheria antitoxin, which was very important in the history of antibacterial therapy. There was a method to treat endocarditis called anti-streptococcal serum, which was reported by William Osler. (Pruitt, 1982) The bacteria should be isolated first from blood cultures, and then they were injected into the horse, and finally, the horse serum was used on patients.

But the first time for the human to use real antibiotic is penicillin, which was

discovered by Alexander Fleming in the 1920s. (Piddock, 2012) This antibiotic saved so many soldiers' lives during the Second World War. From then on, humans started to use modern antibiotics to control and treat bacterial infections. Nearly everyone knows this story about the discovery of penicillin by Alexander Fleming, but there may be someone earlier than him. The growth of bacteria can be inhibited in the culture fluid covered in mould, which was reported by Sir John Scott Burdon-Sanderson in 1870. Then, *Penicillium glaucium* was found to have antimicrobial activity on human tissues, as reported by Joseph Lister. It was found that saddle sores were treated by using mould propagated on the saddles in Arab. Ernest Duchesne took this mould and identified it as Penicillium notatum in 1897, and then he successfully used it to treat pigs with typhoid.(Gould, 2016) According to the above results, Fleming knew that there was a potential benefit in penicillin, but he also knew that there was a big challenge to translate a drug substance from the laboratory to the market. In 1940, he published a paper on the penicillin purification. This useful technology facilitated the use of penicillin in humans for treating infections since 1945, although it had many problems, such as very poor bioavailability and short half-life.(Aminov, 2010) During the period of the development of penicillin, sulfanilamide was also produced in 1908, and it was used together with the antimicrobial dyes developed by Ehrlich. This drug was generated in 1931 and it was used to treat bacterial infections. It was popular because of its successful treatment of a boy with staphylococcal septicaemia in 1933. Two years later, it was found that this drug could be metabolized to sulphanilamide in the body, so the other component from the dye was not necessary for the effect of this drug. This is a milestone for coming into the sulphonamide age.(SHAMBAUGH, 1966)

From then on, the golden age of antibiotic discovery started, and many new classes of antibiotics were discovered in the following decades. In the early stage, the source of antibiotic agents came from natural microorganisms, such as the

discovery of streptomycin from *Streptomyces griseus* in 1944. Eli Lilly was very smart to obtain help from Christian missionaries, who brought back soil samples from exotic temples. In 1952, vancomycin was extracted from a soil sample of *Streptomyces orientalis* and it became in clinical use in 1958.(Levine, 2006)

In the beginning, these antibiotics were effective enough to help people reduce pain and even rescue their lives, but with the wide use of them, the resistance problem became a concern for people.(Spellberg and Gilbert, 2014) Although new classes of antibiotics were continuously approved by FDA, bacteria were evolving all the time to develop resistant ability. With the time flying, resistance problem became more and more apparent, and researchers engaged in new strategy to protect existing drugs to fight against this problem. Methicillin is a narrow-spectrum β-lactam antibiotic of the penicillin class, which was developed by Beecham in 1959. It was first used as penicillinase-resistant β-lactam antibiotics in 1961, and the drug-like properties were better than penicillin, such as broad antimicrobial spectrum and pharmacokinetics.

In 1962, the most well-known resistant bacterial strain was emerged, methicillin-resistant *Staphylococcus aureus* (MRSA), which was first found in the UK and then identified in the USA.(Sengupta et al., 2013) However, the antibiotic resistance not only occurred in methicillin, but also in nearly all antibiotics. Another penicillin-type of beta-lactam antibiotics is cephalosporins, which was developed in 1960s.(Russell, 1975)

Nalidixic acid, licensed in 1967, was used to treat urinary tract infections (Emmerson and Jones, 2003) and Vancomycin is used to treat a number of bacterial infections since the 1970s, such as skin infections, bloodstream infections and bone and joint infections, and especially the infections caused by MRSA.(Liu et al., 2011)

Vancomycin is still being used today as the first-line agent to treat serious infections. At first, it was thought that it is hard to generate antibiotic resistance. However, the vancomycin resistant strains were found in coagulase-negative *staphylococci* in less than 10 years.(Sengupta et al., 2013)

With more and more study on beta-lactamase, people started to develop beta-lactamase inhibitors(Drawz and Bonomo, 2010) to improve the efficacy of beta-lactam antibiotics. For example, clavulanic acid, which was used together with amoxicillin to form a combination drug. It was first discovered as a side-product in 1976 from *Streptomyces clavuligerus* cultures. Ciprofloxacin is a representative of fluoroquinolones, and it was discovered in the 1980s. But there were still many new quinolones failed to go into the market, because of the antibacterial activity in clinical trials or serious adverse effects.

Thienamycin, a precursor of the carbapenem antibiotic, was transferred to imipenem in order to obtain very good antibacterial activity *in vitro* and *in vivo*. However, it failed in human trials because of its short half-life. Further study showed that it could be rapidly metabolized by dehydropepidase, a newly identified enzyme in the kidney. In order to solve this problem, it was used together with cilastatin and its half-life increased obviously. This combination of drugs was approved in the United Kingdom by the end of 1980. Furthermore, another carbapenems named meropenem with a similar antimicrobial spectrum was approved in 1995, but it has fewer adverse effects on human.(Papp-Wallace et al., 2011)

Due to the good effect of the combination with beta-lactamase inhibitors, more and more beta-lactamase inhibitors, such as tazobactam and sulbactam, are used together with other agents to improve their activity against bacterial strains.(Drawz

and Bonomo, 2010) Tazobactam was used together with piperacillin to form a combination drug, which was approved by FDA in 1993. This combination of drugs has been used widely in the United Kingdom as an alternative strategy to replace the use of cephalosporins.

Teicoplanin, a glycopeptide antibiotics, was discovered from Actinoplanes teichomyceticus. It was licensed in Europe in the 1990s,(Greenwood, 1988) but it just had low activity against *staphylococci* and was just used to treat glycopeptide-resistant strains. However, due to its easier administration, it is still used today. Dalbavancin was used to do clinical trials in 2007, but failure was announced in 2014. Another glycopeptide was approved for treating skin and soft tissue infections by FDA as a single use at the same time.

Cycloserine was the first antibiotics of oxazolidinones, for treating plant diseases. Cycloserine was first used to treat *Tuberculosis* in 1956. Linezolid, another oxazolidinone antibiotics that was licensed in 2000, and was proved to be an alternative to glycopeptides. It has some advantages, such as good oral availability and activity against glycopeptide-resistant *enterococci* (GRE). But it also has some disadvantages, such as a range of adverse effects and drug interactions.(Gould, 2011)

Daptomycin (Eisenstein et al., 2010) is a lipopeptide antibiotics used in the treatment of systemic and life-threatening infections caused by Gram-positive organisms. It was isolated from *Streptomyces roseosporus*, a soil organism found in Turkey. Although it has very serious adverse effects, it was approved by FDA in 2003. Tigecycline(Greer, 2006) was a new broad-spectrum antibiotics which was approved in 2005, after moxifloxacin was approved in 2000.

In the 2010s, there are some combination drugs of cephalosporin and beta-

lactamase inhibitors were developed to fight against resistant Pseudomonas and *Enterobacteriaceae*. There is a challenge for the treatment of infections caused by resistant bacterial strains, even pan-resistant strains, such as *Enterobacteriaceae* and *Pseudomonas*. During the golden era of antibiotic discovery, pharmaceutical companies generated a lot of new classes of antibiotics to treat resistant bacteria. But since the 1980s, there are few new drugs approved by FDA.(Spellberg and Gilbert, 2014) This is why some old drugs, such as colistin and fosfomycin, were reconsidered that whether they can be used alone or as combination drugs.

1.2. Causes of antibiotic resistance

1.2.1. Overuse of antibiotics

The overuse of antibiotics was warned by Fleming in as early as the 1940s, and he said to the public that as people demanded more and more antibiotics to treat infections, there would be a new age of antibiotics abuse.(Spellberg and Gilbert, 2014) This means antibiotic resistance can be facilitated by antibiotics abuse. There is an evidence in epidemiological research, which reported that the use of antibiotics was related to the emergence and spread of antibiotic resistance.(2013) There is a special gene transfer way called horizontal gene transfer (HGT) in bacteria. This means that genes in bacteria can not only be transferred to the next generation, but also to the same generation by plasmids.(Read and Woods, 2014) This means that if bacterial strains obtain resistant gene, antibiotic resistance can be disseminated very fast and widely. Although bacteria cannot obtain the resistant gene from their previous generation or other species of the same generation, this strain can still obtain the resistant gene by mutation. The wild type strain is sensitive to antibiotics, while the mutant strain is resistant to antibiotics. Another cause of antibiotic resistance is natural selection. Every round use of an antibiotic, sensitive bacterial

strains are killed by the antibiotic, but the resistant strains are left. After several rounds use of the antibiotic, only resistant bacterial strains are left, and same antibiotics is no longer effective for this infection.(Read and Woods, 2014)

Although awareness of the drawbacks of antibiotic abuse have increased, antibiotics are still overused worldwide, because of their convenient use.(2013) Data from the United States also indicate that antibiotics is overused and some actions should be taken to change this situation.(Gross, 2013) It was assessed in 2010, that there were 22.0 standard units of antibiotics sold per person in the United States. This data is from the IMS Health Midas database, which contains information on the antibiotic consumption and sold from hospital pharmacies and retail stores.(Van Boeckel et al., 2014) The antibiotics consumptions are different from state and state, and the number is larger in the eastern regions than it in the western regions.(Gross, 2013) What is more, the number of prescriptions with antibiotics is larger than the number of population in some states, which is a very terrible situation and can lead to antibiotic resistance.

1.2.2. Inappropriate antibiotics prescribing

Another reason for the emergence of antibiotic resistance is the inappropriate prescription of antibiotics. There are 30%-50% inappropriate prescription cases in hospital, including improper choice of antibiotics, wrong treatment method indication and the short period of antibiotics use.(Luyt et al., 2014) Another analysis showed that only less than 10% of patients' bacterial strains could be confirmed in the United States hospital, such as community-acquired *pneumonia* (CAP).(Bartlett et al., 2013) However, this pathogen has a higher identified rate in Sweden of as high as 89%, a study from Karolinska Institute,(Bartlett et al., 2013) where can improve the diagnostic technology by using polymerase chain reaction [PCR] and

semiquantitative PCR. Another improper prescription of antibiotics occurs in intensive care units (ICUs) in US, where 30%-60% of the prescription was inessential or incorrect.(Luyt et al., 2014)

This inappropriate prescription of antibiotics would cause potential complications in patients.(Lushniak, 2014) Sometimes people use lower concentration of antibiotics to treat infections, which we called subtherapeutic concentration. This lower concentration can facilitate the emergence of antibiotic resistance because it is easy to drive gene expression to alter, causing mutagenesis and horizontal gene transfer.(Viswanathan, 2014) The lower concentration of antibiotics can cause bacterial species diversity. For example, a lower concentration of piperacillin can lead to the proteomic change in *Bacteroides fragilis*.(Viswanathan, 2014) In most cases, the change in gene expression can increase bacterial virulence and the rate of mutagenesis, while horizontal gene transfer can facilitate the emergence of antibiotic resistance and accelerate the spread of resistant strains.(Viswanathan, 2014)

1.2.3. Extensive use of antibiotics in agriculture

Antibiotics are not only used in humans but also widely used in livestock. What is more, the consumption of antibiotics used in livestock to treat infections and facilitate their growth, is far more than the use in humans, which only accounts for 20% of antibiotics sold in the United States.(Spellberg and Gilbert, 2014) Although the antibiotics left in livestock can be eaten by people, the farmers still consider that it benefits for their incomes, because the antibiotics can keep their livestock healthy, and generate good quality and higher yield products.(Michael et al., 2014)

The resistant bacteria strains can be transferred to humans through human

consumption of the livestock. This problem first drew people's attention several decades ago when antibiotic resistance was found from both animals and farmers.(Bartlett et al., 2013) In recent years, with the development of molecular detection technology, researchers have found that this transfer of resistant bacteria from livestock to humans is through human consumption of meat.(Bartlett et al., 2013) There are a few possible ways for this transfer to happen. First, the antibiotics used in livestock kills the susceptible bacteria, but resistant bacteria strains are left and transferred to humans. Second, these resistant bacteria strains can transfer resistant genes to the original bacteria in humans through HGT. Third, some resistant bacteria strains can cause serious adverse effects on humans, but they are safe in livestock.

The antibiotics used in livestock not only affect human health but also influence the microbiome in the environment. Because most of the antibiotics used in livestock are excreted through urine and stool, the figure of this part is up to 90%, so these antibiotics can be disseminated by fertilizer and groundwater.(Bartlett et al., 2013) Other methods of dissemination of antibiotics, such as through wind, should also be considered. For example, fruit trees should be sprayed with tetracyclines to prevent the plant from being damaged by bacteria in the agricultural regions in the United States, although this case is just account for a very minor part of the total use of antibiotics.(Golkar et al., 2014) The long-term use of antibiotics in this way can also change the ecology of local environment because it increases the number of resistant strains and reduces the number of susceptible strains.(Golkar et al., 2014)

Some antibiotics that are used for disinfection can also lead to the spread of antibiotic resistance in the environment, although these antibiotics just used to clean the environment or protect the health of older people and children. These disinfectants can also reduce the ability of the immune system to fight against

virulent organisms, and lead to a high rate of morbidity and mortality.(Michael et al., 2014)

1.2.4. Lack of new antibiotics

Many good pharmaceutical companies have stopped their projects and research related to antibiotics due to different reasons, such as economic benefit and strict regulations in this field over the past decades, although these companies were good at this fields and developed some successful antibiotics in history.(Bartlett et al., 2013) An analysis data showed that 15 out of the top 18 pharmaceutical companies have given up antibiotics development. Another reason is a lot of mergers between these companies, which led to the disappearance of research team diversity and the number of research teams dropped dramatically.(Piddock, 2012) This financial tight also occurred in the academic field, where the funding for antibiotic research project have been cut off and lead to fewer researchers applying the projects related to this field.

The pharmaceutical industry is considered as time consuming and low benefit, which is not an economically wise investment for the capital venture or other financial groups. Due to some reasons, it is not worth to invest, for example, people think the antibiotics are often used with a short time, so the benefit for the drug companies is not continuous.(Piddock, 2012) A report by Office of Health Economics in the UK indicated that the net present value (NPV) of a new antibiotic is only approximately 50 million dollar in the world, while a new neuromuscular drug is about 1 billion dollar.(Piddock, 2012) This cost-benefit analysis showed that chronic drugs are more profitable, and that is why pharmaceutical companies like to invest on these fields.(Gould and Bal, 2013)

Another reason to lead to antibiotic pharmaceutical industry with low profit is that the price of a new antibiotic is very low, so fewer pharmaceutical companies are interested in this field. For example, the maximum price of a new antibiotic is approximately from 1000 dollars to 3000 dollars per course, while the price of a new drug for cancer chemotherapy will be far more than ten thousand dollars.(Gould and Bal, 2013, Wright, 2014, Bartlett et al., 2013) Antibiotics were considered that they only have low value, because they are used conveniently with low price, and the public perception also influences the consideration of pharmaceutical companies.

when a new antibiotic was approved by FDA, microbiological scientists and experts always suggest that hold on this new antibiotic and continue to use old drugs to treat infections, because public have improved the awareness of drawbacks of antibiotics abuse, which can facilitate the emergence of antibiotic resistance.(Golkar et al., 2014) A new antibiotic can only be used when the old drugs have lost their effect or to treat very serious infections. This is why new antibiotics always work as the last line for serious infections. This awareness leads to a drop in the use of antibiotics, which causes investment decrease by pharmaceutical companies.(Piddock, 2012)

Once bacteria have developed resistance to antibiotics, the profits of these antibiotics would drop, although the huge amount of money has already been invested in the research and development for these antibiotics.(Gould and Bal, 2013) When there is economic crisis, such as the Great Recession, consumers of antibiotics also can be affected by their financial situation. Nowadays, many pharmaceutical companies produce antibiotics without protection of patent. This action helps antibiotics go into the market at a very low price and benefit the public.(Wright, 2014)

As a result of above reasons, most top antibiotic pharmaceutical companies are worry about the profit drop when they plan to invest a new antibiotic project, and this profit drop will lose millions of dollars. This will lead to fewer and fewer pharmaceutical companies invest in this field and fewer new antibiotics can be promoted into the clinic and market. A report by The Infectious Diseases Society of America in 2013 indicated that there were very few chemical compounds for treating infections in phase 2 or 3 clinical trials.(Lushniak, 2014) The more serious situation is that there is a lack of therapy of resistant bacteria of Gram-negative, such as *Enterobacteriaceae* , *P. aeruginosa*, and *A. baumannii*, while there is an urgent demand for effective antibiotics against these high threat bacterial species.(Lushniak, 2014) Compared with Gram-negative resistant bacteria, the antibiotics for treating resistant Gram-positive bacteria have drawn more attention from pharmaceutical companies, which have invested more research teams and money in developing drugs for treating serious pathogens, such as MRSA. The reason for the pharmaceutical companies having more interest in the MRSA may be that the MRSA is still a major global problem for public health, while the resistant Gram-negative bacteria are still a regional problem and the property of Gram-negative pathogens is that they do not acquire resistance easily.(Gould and Bal, 2013)

1.2.5. Regulation

Although some pharmaceutical companies may have strong interest in discovering and developing new antibiotics, the process of application and other related regulations are often a barrier for these companies. From 1983 to 2007, the number of newly approved antibiotics dropped obviously.(Gould and Bal, 2013) There are some reasons why they are difficult to be approved according to the regulation. First, these applications must be approved by different bureaus. Second, the experimental data is not very clear to be approved. Third, the clinical trials should be conducted in different countries and should comply with the local regulation. (Piddock, 2012)

Over the past two decades, the clinical trials standards changed a lot by FDA, causing challenges for new antibiotics to apply for clinical trials. The changes include many aspects, such as the new definition of the new disease and endpoint, a new method for endpoint assessing, the requirement of guidance on patients, the evidence for support and the new judgment for noninferiority margins through statistical data.(Itani and Shorr, 2014)

The new rules said that it is not ethical to compare antibiotics with placebo, so new clinical trials should be planned to indicate that noninferiority of new antibiotics compares with existing drugs through a series of different statistical margin.(Wright, 2014) This process will cost more money and need to find a huge number of population for participating, and this will decrease the net profit. The gap of investment in antibiotic research and development can be filled by small pharmaceutical companies. However, the problem is if their projects go well and come in phase 3 clinical trials, the high cost and complexity would exceed their financial ability. For example, a small pharmaceutical company name Cubist cooperated with Merck in 2014, which can facilitate the project and drive new

antibiotic drugs to be approved quickly in the future.(Piddock, 2012)

The new regulatory methods should be adapted to the requirement of continued development and the antibiotic medication. A new limited-population antibiotic drug regulation was approved by The Infectious Diseases Society of America, and it obtained more people's positive comments from FDA official report.(Bartlett et al., 2013) This regulation will make the clinical trials approved faster with small-scale and lower cost, and it will let new antibiotics focus on high-risk patients.(Bartlett et al., 2013)

1.3. Threat of antibiotic resistance

1.3.1. Infections caused by antibiotic resistant bacteria

Infections caused by bacteria have been a major heathy problem in the United States and all over the world, especially pan-resistant bacteria, which cannot be effectively treated by antibiotics. A national investigation conducted by The Infectious Diseases Society of America emerging infections network in 2011, reported that more than 60% of pathogens are pan-resistant bacterial species.(Spellberg and Gilbert, 2014) The problem become more and more serious, declared by different public health organizations, and they said that the appearance of resistant bacteria is the crisis for human.(Viswanathan, 2014) We are living in the post-antibiotic era, as declared by The Centers for Disease Control and Prevention in 2013. The WHO indicated in 2014 that antibiotic resistance is becoming a global threat,(Michael et al., 2014) and multidrug-resistant bacteria are a big threat to American people's health, as declared by The Infectious Diseases Society of America, the Institute of Medicine and federal Interagency Task Force on Antimicrobial Resistance.(Golkar et al., 2014)

For the Gram-positive bacteria, the current biggest global threat was caused by pan-resistant *S. aureus* and *Enterococcus*.(Rossolini et al., 2014) Every year, the number of American people killed by Methicillin-resistant *Staphylococcus Aureus* was larger than the number killed by several combined diseases, such as the combination of AIDS, Parkinson's disease and emphysema.(Golkar et al., 2014) Recently, with the emergence of Vancomycin-resistant *enterococci*, more and more pathogens have developed resistance to so many antibiotics, and the more serious problem is that some respiratory bacteria, such as *S. pneumoniae* and *M. tuberculosis* have evolved resistance to drugs, these respiratory bacteria are easier epidemic and to spread than other pathogens.(Rossolini et al., 2014)

The antibiotic resistance problem is more serious in Gram-negative pathogens than Gram-positive bacteria, because these Gram-negative bacteria are developing resistance to nearly all existing antibiotic drugs.(Rossolini et al., 2014) Infections commonly caused by Gram-negative pathogens from health care settings, such as *K. pneumoniae*, *P. aeruginosa* and *Acinetobacter*, are always more serious.(Rossolini et al., 2014) In the community, multidrug-resistant Gram-negative bacteria are becoming more and more prevalent, including much species producing beta-lactamases, such as *E. coli* and *N. gonorrhoeae*.(Rossolini et al., 2014)

There was an analysis of antibiotic-resistant bacterial infections by The Centers for Disease Control and Prevention based on several aspects, such as economic impact, transmissibility, and difficulties to prevention. Levels of threat of bacteria are classified into three groups, urgent, serious and concerning, as shown in Table 1.

This summary of threat levels can provide us with the information, about which bacteria need more action to control and prevent the situation from becoming more

serious, such as the bacteria in the urgent level, while the bacteria in the concerning level considered to need fewer actions.

Table 1. Assessment of antibacterial resistance threats from CDC.

Urgent Threats
• *Clostridium difficile*
• Carbapenem-resistant Enterobacteriaceae (CRE)
• Drug-resistant *Neisseria gonorrhoeae*
Serious Threats
• Multidrug-resistant *Acinetobacter*
• Drug-resistant *Campylobacter*
• Fluconazole-resistant *Candida* (a fungus)
• Extended spectrum beta-lactamase-producing Enterobacteriaceae (ESBLs)
• Vancomycin-resistant Enterococci (VRE)
• Multidrug-resistant *Pseudomonas aeruginosa*
• Drug-resistant nontyphoidal *Salmonella*
• Drug-resistant *Salmonella* Typhimurium
• Drug-resistant *Shigella*
• Methicillin-resistant *Staphylococcus aureus* (MRSA)
• Drug-resistant *Streptococcus pneumoniae*
• Drug-resistant tuberculosis
Concerning Threats
• Vancomycin-resistant *Staphylococcus aureus* (VRSA)
• Erythromycin-resistant Group A *Streptococcus*
• Clindamycin-resistant Group B *Streptococcus*

Methicillin-Resistant *Staphylococcus Aureus*

Methicillin-Resistant *Staphylococcus Aureus* (MRSA), which was identified in the 1960s, is still a global public health problem.(Spellberg and Gilbert, 2014) MRSA infections can be found all over the world, in nearly all countries in Europe, Asia and America.(Rossolini et al., 2014) Infections caused by MRSA are the most dangerous threats, because these infections are always with a high rate of morbidity and mortality. There are more than ten thousands of deaths caused by MRSA every year in the United States.(Gross, 2013)

Nowadays, MRSA is not only resistant to methicillin, but also to other penicillin-

type beta-lactam antibiotics.(Sengupta et al., 2013) Fortunately, there are still some antibiotics, containing glycopeptides, daptomycin, and other new beta-lactam antibiotics, such as cephalosporins, that can be used to treat MRSA.(Rossolini et al., 2014) Over past decades, MRSA has been proved that it is good at spreading and appearing in various regions and health care settings.(Rossolini et al., 2014) These properties of MRSA make MRSA infections complicated and they challenge to the old infection control system, which focuses on infections from health care settings. The resistance can be spread by bacteria mutation and resistance gene transfer by plasmids, and some reports indicated that MRSA has developed resistance to anti-MRSA antibiotics, such as linezolid and glycopeptides, and become a public concern.(Rossolini et al., 2014)

The good news is the morbidity of healthcare-associated infections seems to have dropped in recent years, because a lot of actions have been taken, such as hygiene measures to prevent bacterial infections in some European countries to control MRSA infections.(Rossolini et al., 2014) The numbere of invasive MRSA cases dramatically declined by 31% from 2005 to 2011, and among all invasive MRSA infections, healthcare-associated infections have the largest drop by about 54%. The result shows that infection control plays an important role in reducing the spread speed of MRSA.(Rossolini et al., 2014) The bad news is that the incidence of community-acquired MRSA (CA-MRSA) infections has increased dramatically in the public over the past decades.

Vancomycin-Resistant *Enterococci*

Vancomycin-Resistant *Enterococci* (VRE) infections are also a big threat for public health, because they can cause serious illness among patients, who are in hospitals and using healthcare settings.(Sengupta et al., 2013) Vancomycin-Resistant

Enterococci infections are frequently caused by *E. faecium*, which is less prevalent and has a lower epidemiological influence worldwide.(Rossolini et al., 2014) Every year, there are approximately more than sixty thousands healthcare-associated infections caused by *Enterococci* in the United States. Among hospital-acquired *enterococcal* infections, 30% are the vancomycin-resistant *Enterococci* infections, and they cause more than one thousand death cases every year.

Vancomycin-resistant *Enterococci* infection is considered a big threat because there are few antibiotics that can effectively treat it. The antibiotics which can be used to treat VRE infections contain linezolid, daptomycin, and tigecycline, but the latter two antibiotics need more study to confirm their efficacy and adverse effects.(Rossolini et al., 2014) Fortunately, many pharmaceutical companies have made a lot of efforts on novel antibiotics research and development in order to develop effective drugs to fight against this infection, and oritavancin is a good example.(Rossolini et al., 2014)

Drug-Resistant *Streptococcus pneumoniae*

Streptococcus pneumoniae can also lead to a range of serious and life-threatening infections, such as bacterial pneumonia, meningitis and sinus infections.(Gross, 2013) Resistant *Streptococcus pneumoniae* can cause more serious infections and lead to complication of treatment. Every year, there are more than 1 million people infected by Resistant *S. pneumoniae* and lead to seven thousand deaths. Among these infected people, those who are over 50 have the highest mortality, and those who over 65 have the highest morbidity. Resistant *S. pneumoniae* has evolved resistance to penicillin-type antibiotics, such as amoxicillin. It is also resistant to erythromycin-type antibiotics, such as azithromycin. What is more, among the most serious infections caused by *S. pneumoniae*, 30% of these cases are resistant to at

least one relevant antibiotic used in clinic.

Fortunately, the incidence of resistant *S. pneumoniae* infections is dropping for several years, because a new version of vaccine named pneumococcal conjugate vaccine has been available to prevent the people from infections caused by most of resistant *pneumococcus* bacterial strains since 2010. This new vaccine (PCV13) expanded its spectrum of protection against 13 different *pneumococcal* strains, compared with the old version (PCV7) used between 2000 and 2009, which can only offer protection against 7 strains. The benefit of using this new version vaccine does not only help prevent people from infecting by *pneumococcal* strains, but also prevent the spread of antibiotic resistance by inhibiting the transmission of resistant *S. pneumococcal* bacterial strains.

Drug-Resistant *Mycobacterium Tuberculosis*

Infections caused by drug-resistant *Mycobacterium Tuberculosis* are also a big threat in the United States and worldwide.(Gross, 2013) The report from WHO said that there are more than one hundred thousand deaths caused by drug-resistant *tuberculosis* (TB) infections in 2012.(Gross, 2013) Another threat of *M. tuberculosis* for the public is that it can spread by air. The most common infections caused by *M. tuberculosis* are lung related diseases. The number of infections caused by antibiotic resistant *M. tuberculosis* account for about 10% of the total reported *tuberculosis* infections in 2011.

Most *tuberculosis* infections can be treated by the first line antibiotic drugs containing isoniazid, but in some cases, the first line antibiotic drugs are not effective, because these infections are resistant to at least one first line antibiotics. Infections caused by these drug-resistant *tuberculosis* strains should be treated with

longer time, and these will lead to increase in cost and adverse effects. The most terrible situation is that the infections caused by Extensively drug-resistant TB (XDR-TB) bacterial strains, which have developed resistance to most first line *tuberculosis* drugs, containing rifampicin and fluoroquinolones, and second line drugs, such as amikacin and kanamycin. If people are infected by these XDR-TB strains, there is few antibiotics available, so it is hard to treat the patients. Fortunately, infections caused by XDR-TB and XDR-TB strains are not prevalent in the United States. This result shows that the actions taken by the U. S. government have a positive effect on controlling and preventing these infections.

Carbapenem-Resistant *Enterobacteriaceae* (CRE)

Carbapenem-resistant *Enterobacteriaceae* (CRE) bacteria strains contain a group of bacteria, that have developed resistance to nearly all antibiotics, such as carbapenems, which is called the last resort to fight against drug-resistant bacteria.(Gross, 2013) There is an enzyme named New Delhi metallo-beta-lactamase (NDM-1) expressed in some Gram-negative *Enterobacteriaceae* bacteria strains, such as *E. coli* and *K. pneumoniae*, which lets these bacteria resistant to nearly all beta-lactam antibiotics that containing carbapenems.(Sengupta et al., 2013)

The infections caused by Carbapenem-resistant *Enterobacteriaceae* bacteria are difficult to treat, but the situation is becoming more and more serious because the number of these infections is increasing in the patient groups who accept health care in hospital. There are more than one hundred thousand infections caused by healthcare-associated *Enterobacteriaceae* in the United States every year, and more than nine thousandof these infections are caused by Carbapenem-resistant *Enterobacteriaceae*. The most prevalent strains of Carbapenem-resistant *Enterobacteriaceae* are carbapenem-resistant *Klebsiella* and carbapenem-resistant

E. coli, and the infections caused by these two strains lead to about 600 deaths every year.

MDR *Pseudomonas Aeruginosa*

Pseudomonas aeruginosa always leads to healthcare-associated infections, containing pneumonia and surgical-site infections. There are more than fifty thousand healthcare-associated infections caused by *P. aeruginosa* in the United States every year, and 13% of these *P. aeruginosa* infections are caused by MDR *P. aeruginosa* strains.(Rossolini et al., 2014) There are about 400 deaths caused by these infections every year, and some MDR *P. aeruginosa* strains have developed resistance to all drugs, such as aminoglycosides, cephalosporins, and carbapenems.

MDR *Acinetobacter*

Acinetobacter is a genus of Gram-negative bacteria belonging to the wider class of *Gammaproteobacteria*, and it can cause a range of infections, such as bacterial pneumonia and bloodstream infections. Some MDR *Acinetobacter* strains also have developed resistance to nearly all antibiotic drugs, such as carbapenems, the so-called the last resort antibiotics. There are more than ten thousand healthcare-associated infections caused by *Acinetobacter* in the United States every year, and 63% of these infections are caused by MDR *Acinetobacter* strains, which are resistant to at least 3 different types of antibiotics, and these infections lead to 500 deaths every year.

ESBL-Producing *Enterobacteriaceae*

The *Enterobacteriaceae* which carry a broad spectrum beta-lactamase enzyme is

called Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* , the ESBL can make these strains resistant to nearly all beta-lactam antibiotics, such as penicillin types and cephalosporin types.(Gross, 2013) There are 26 kinds of healthcare-associated infections caused by ESBL-producing *Enterobacteriaceae* strains and more than one thousand deaths every year. Although these bacteria have broad-spectrum resistance, the major treating method is still by using carbapenems. Another problem is that if these antibiotics are used inappropriately, this will lead to development of resistance.

Drug-resistant *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is the major cause of the sexual disease gonorrhea, and there has been Drug-resistant *Neisseria gonorrhoeae* in the United States recently.(Golkar et al., 2014) *Gonorrhea*-related diseases are diagnosed by the inflammation of cervix, pharynx and so on. These diseases can spread easily and lead to serious complications in the reproductive system.(Gross, 2013) It is the second most frequent infections in the United State and there are eight hundred thousand infections caused by *Gonorrhea* every year, reported by The Centers for Disease Control and Prevention. Drug-resistant *N. gonorrhoeae* has become widespread, and there are more than seventy thousand pelvic inflammation, fifteen thousand epididymitis, and more than two hundred HIV infections from 2005 to 2015 in US.(Ventola, 2015)

There are many different types of Drug-resistant *N. gonorrhoeae* strains, for example, cephalosporin-resistant *N. gonorrhoeae* strain. This resistant strain is not only resistant to cephalosporins, but also resistant to other classes of antibiotics, such as fluoroquinolones and penicillins.(Rossolini et al., 2014) In order to solve these problems, the treatment method has been changed by The Centers for Disease

Control and Prevention, and the guidelines suggest that the first line drugs are using ceftriaxone plus either azithromycin or doxycycline to treat gonorrhea.(Gross, 2013)

1.3.2. The economic burden of antibiotic resistance

The treatment for antibiotic resistant infections is a huge burden not only to the US health care system but also to individual.(Golkar et al., 2014) These huge economic burdens often happened in the hospitals because there are lots of patients, who always accept excess use of antibiotics and other treatment procedures.(Golkar et al., 2014) There are about 2 million healthcare-associated infections and about one hundred thousand deaths in the US every year, and the major cause of this situation is antibacterial-resistant bacteria. Among these healthcare-associated infections, sepsis and pneumonia are the two most common infections, which lead to about fifty thousand deaths in total in the US and cost at least 8 billion dollars from the health care system.(Golkar et al., 2014)

The health care system has been overburdened, and the treatment for antibiotic-resistant infections impose extra costs on the national health care system, which makes this system overload. When the first and second line antibiotics are not available for treatment of these infections, specialists have to use more expensive and even more toxic antibiotics to treat patients with higher frequency, leading to the economic pressure that is transferred to patients.(Lushniak, 2014) A report shows that the period of staying hospital for the patients with resistant bacterial infections was elongated from 6.4 to 12.7 days compared with other cases of infections.(Golkar et al., 2014)

The cost for patients with resistant bacterial infections is about from eighteen thousand dollars to thirty thousand dollars.(Bartlett et al., 2013) These resistant

bacterial infections cost 20 billion US dollars on the health care and 35 billion US dollars of productivity lost every year.(Golkar et al., 2014) These resistant bacterial infections also bring more pressure to individuals, because they need more money for their health care and lose their incomes for their sick leave or lose their workforce.(Michael et al., 2014)

The emergence of antibiotic-resistant bacteria has become a big threat for public health, and this threat has been spread worldwide over the past several decades. The major cause is the overuse of antibiotics and the lack of new classes of antibiotics.(Bartlett et al., 2013) Infections caused by antibiotic resistant bacteria also impose a huge economic burden on the national health care system and the individuals.(Golkar et al., 2014) Therefore, there is an urgent need for producing more new antibiotics to solve this problem.(Spellberg and Gilbert, 2014)

1.4. Bacterial cell division protein: FtsZ

1.4.1. Introduction of the new antibiotic target FtsZ

FtsZ is an essential bacterial cell-division protein, which can polymerize and assemble into a ring to initiate cell division. It is encoded by *fts* genes and called Fts for filamentous temperature-sensitive. FtsZ is a member of Fts protein group, and it can form a ring, called Z-ring, during the period of cell division. It is also a guanosine triphosphatase and the homolog of mammalian β-tubulin, because FtsZ and β-tubulin have similar amino acid sequence. This protein is widely conserved in all bacteria and there are not any antibiotics in the market that target this protein, so this kind of proteins are regarded as unexploited and attractive targets for antibacterial drug discovery. In the past few decades, there were a large number of studies on this target, including the research of its structure and function. These

studies provide enough knowledge for us to develop inhibitors for this target.

1.4.2. Visualizing the Z-ring

In 1991, Lutkenhaus and co-workers first demonstrated that FtsZ had a precise localization in bacterial cells.(Bi and Lutkenhaus, 1991) Immuno-gold electron microscopy showed when a cell began to divide, a ring would be formed near the membrane. During the division process, the FtsZ ring constricted at the tip of the membrane and made it invaginate.

In 1996, Addinall and co-workers suggested that Z-ring was localized in the middle of normal cells, except the smallest one. The term Z-ring was first used to describe this FtsZ ring structure.(Addinall and Lutkenhaus, 1996) In the same year, Levin and co-workers were the first to confirm the localization of FtsZ by immunofluorescence. They found Z-ring was in almost all *B. subtilis* and *E. coli* cells, and they also indicated that Z-ring was formed in early stage after the generation of a new cell.(Levin and Losick, 1996) In both *B. subtilis* and *E. coli* cells, the Z-ring appeared to disassemble during septation. Ma and Margolin showed the position of FtsZ in *E. coli* by using FtsZ-GFP chimera.(Ma et al., 1996)

1.4.3. The homologue of tubulin

In 1992, the possibility that FtsZ might be a homolog of tubulin was first suggested due to a short segment of its amino acid sequence, GGGTGTG, which is virtually identical to the tubulin signature motif, (G/A)GGTGSG, found in all three kinds of tubulins.(RayChaudhuri and Park, 1992, de Boer et al., 1992, Mukherjee et al., 1993a) In 1994, Mukherjee and Lutkenhaus used sequence alignment to confirm some conserved residues and many of them with only conservative substitutions

between tubulins and FtsZ.(Mukherjee and Lutkenhaus, 1994) This result is a piece of strong evidence that FtsZ and tubulin are homologs. Other analysis of the sequences of FtsZ and tubulin, with a focus on predicting secondary structures and solvent accessibility, provide deep understanding for the structure of this protein family.(de Pereda et al., 1996)

FtsZ is a homolog of tubulin, but do they have similar functions? At first, Z-rings were regarded as actin-myosin rings. However, recent studies indicated that FtsZ was a homolog of tubulin with the ability to hydrolyze GTP.(RayChaudhuri and Park, 1994, Mukherjee and Lutkenhaus, 1994, de Boer et al., 1992) Tubulin can form a cytoskeletal polymer, called the microtubule. FtsZ was first reported to have a similar cytoskeletal function in 1994.(Mukherjee and Lutkenhaus, 1994, Bramhill and Thompson, 1994) The evidence of this conclusion was from immunoelectron microscopy that showed FtsZ forming a ring and localizing the middle of *E. coli* cells in 1991.(Bi and Lutkenhaus, 1991) FtsZ and tubulin can form protofilaments, which their common function as a cytoskeletal filament. The following analysis of the FtsZ crystal structure showed that its structure was similar to the subunits of tubulin.(Löwe and Amos, 1998a)

Like tubulin, GTP binds to the interface of FtsZ between two monomers, which can form the active site for the hydrolysis of GTP.(RayChaudhuri and Park, 1992, de Boer et al., 1992, Mukherjee et al., 1993a) But unlike tubulin, the GTPase activity of FtsZ *in vitro* appears independent of its assembly into rings. FtsZ has the highest GTPase activity with a rate of 2-5 molecules per minute.

1.4.4. The phylogeny of FtsZ - archeabacteria and all prokaryotes

FtsZ has been known to be present in bacteria for years. But recently, it was found

in other four species of archaebacteria.(Wang and Lutkenhaus, 1996, Margolin et al., 1996, Baumann and Jackson, 1996, Bult et al., 1996) There is no known prokaryote that lacks FtsZ, and it seems safe to conclude that this is a universal and essential protein in both eubacteria and archaebacteria. In the conserved core region used for this alignment, the sequence of the *E. coli* FtsZ shares 60-70% identity with other Gram-negative bacteria, nearly 50% identity with the Gram-positive bacteria, and 40-45% identity with archaebacteria.(Margolin et al., 1996, Baumann and Jackson, 1996)

1.4.5. Models for FtsZ-dependent Cell Division

When a cell starts to divide, FtsZ polymerizes to form a dynamic ring in the middle of the cell in the presence of GTP.(Adams and Errington, 2009) There are twelve extra cell-division proteins needed to form a cell-division complex called a divisome.(Errington et al., 2003) The divisome constricts to form a septum, which plays an important role during cell division process (**Figure 1**).(Osawa et al., 2008b, Ma and Ma, 2012) During this process, FtsZ works as an essential skeleton for the Z-ring and works as the motor for constriction.(Lock and Harry, 2008, Vollmer, 2006)

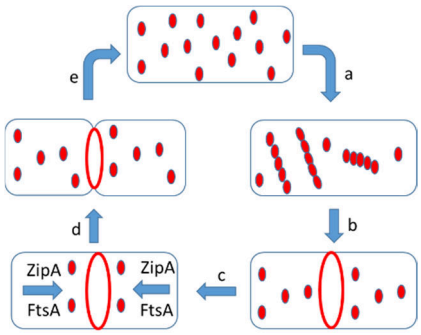


Figure 1. FtsZ assembly in the prokaryotic cell-division process. a) FtsZ polymerization into protofilaments; b) self-assembly of FtsZ at mid-cell into Zring; c) recruitment of other division proteins; d) constriction of Z-ring and septum formation; e) disassembly of Z-ring and cell division.

1.4.5.1. The dynamic behavior of FtsZ in the Z-ring

Generally, there are about fifteen thousand FtsZ molecules in a *E. coli* cell, but only one-third of them participate in Z-ring formation at a certain time.(Stricker et al., 2002) FtsZ proteins in the Z-ring exchange with those out of the Z-ring all the time and the interaction between overlapping protofilaments causes the formation of the dynamic Z-ring structure,(Erickson et al., 2010, Guck et al., 2005) while the overall morphology of the Z-ring seems to be static.(Weiss, 2004) In the beginning of the cell division process, GTP-FtsZ polymerizes into linear protofilaments.(Romberg et al., 2001, Caplan and Erickson, 2003, Guck et al., 2005) At this stage, the GTPase catalytic site is formed between two connected FtsZ monomers.(Lu et al., 2000) The GTP was hydrolyzed by GTPase, and then GDP-FtsZ polymers, which were unstable products, were formed with releasing phosphate. Finally, GDP that are bound to FtsZ exchanges with the free GTP in solution,(Mingorance et al., 2001, Mukherjee et al., 1993b) which may be caused by direct nucleotide exchange, or depolymerization.(Läppchen et al., 2005, Romberg and Mitchison, 2004) Generally, GTP binding facilitates FtsZ polymerization, whereas FtsZ depolymerization is caused by GTP hydrolysis proposed by Weiss.(Weiss, 2004)

1.4.5.2. Mechanism of the precise location of Z-ring

Levin and co-workers indicated Z-ring formation is regulated by a series of proteins to ensure that the dynamic ring forms at the precise time and position.(Romberg and Levin, 2003) There are two mechanisms: one is called “Min system”, which prevents the Z-ring from localizing near the cell poles by the inhibitory proteins MinC/MindD.(de Boer et al., 1989, Lan et al., 2009) The other is called “nucleoid occlusion”, which prevents the Z-ring formation close to the nucleoid by the DNA-binding protein Noc in *B. subtilis* cell or SlmA in *E. coli* cell.(Bernhardt and De Boer, 2005, Wu and Errington, 2004)

1.4.5.3. The force of the Z-ring constriction

Z ring is proposed to offer the force to cause membrane constriction during the process of cell division.(Li et al., 2013) Erickson imagined two possible mechanisms of constriction in 1997[19]. In my opinion, the possible one is that FtsZ is not only the scaffold but also the motor. The constriction is caused by changing the conformation from the straight protofilament attached to the membrane to a sharply curved mini-ring, and then achieve septation.[14]

In fact, there are no motor proteins in bacteria, but it is sufficient for the membrane to offer a pinching force by FtsZ polymerization/depolymerization dynamics. It should be remembered that there are many other proteins required for Z-ring regulation and constriction *in vivo* such as FtsA, FtsE, FtsN, FtsX, Zap, and ZipA. FtsZ polymers attach to liposomes by the interaction with FtsA when GTP binds to FtsZ, and pinch liposome inward.(Osawa et al., 2008a, Osawa and Erickson, 2013) To generate an inward constriction force, the FtsZ protofilament rafts need to coordinate inputs from the septal wall synthesis machinery. The mechanism of this process has been proposed: FtsE and FtsX transmit signals from the cell wall machinery to FtsZ by attaching to GTP.(Yang et al., 2011) At the same time, FtsA receives signals from the cell wall machinery via FtsN.(Busiek et al., 2012, Busiek Kimberly and Margolin, 2014) These signals from the divisome would ensure that force generation by the Z-ring is precisely coordinated and to control the progress of septum synthesis.

FtsZ GTPase activity occurs in FtsZ protofilaments which can form the binding site of GTP between adjacent FtsZ subunits. The GDP-containing linear protofilament is changed to curved GDP-FtsZ polymers by two subunits rotating, and the energy comes from hydrolysis of GTP, which is the rate-limiting step.(Erickson et al., 2010, Li et al., 2013) Moreover, subunits rapidly exchange within protofilaments, with a

timescale of fewer than ten seconds.(Anderson et al., 2004)

1.2.6 The structure of FtsZ

Jan and co-workers reported that the crystal structure of FtsZ consisted of residues 23-256, 116 molecules of water, and 1 GDP molecule. Residues 1-22 was visible with weak density as an extension from helix H0.(Löwe and Amos, 1998b)

FtsZ is a protein with relative a molecular mass 40K and contains two domains connected by helix H5 in a manner similar to tubulin.(Nogales et al., 1998) The N-terminal portion of the FtsZ molecule, which contains residues 38-227, is called the GTPase domain where GTP can bind. It contains a six-stranded parallel β-sheet and two and three helices on both sides. The C-terminal domain spans residues 228-356 and contains a parallel four-stranded central β-sheet with two helices on one side. The four-stranded β-sheet isntilted by 90º against the β-sheet of the GTPase domain.(Lowe and Amos, 1998) The three-dimensional structure of FtsZ is similar to the structure of α- and β-tubulin, which is consistent with the report that FtsZ is weak sequence homology to human tubulins.

Therefore, if the function of FtsZ is inhibited, cell division will fail. To date, a large number of FtsZ inhibitors have been synthesized, but none of them can enter into clinical use. There is a big gap between target FtsZ and effective inhibitors, although so many inhibitors have been published by other research groups.

1.5. FtsZ protein Inhibitors

1.5.1. Background of FtsZ inhibitors

With the widespread of antibiotic-resistant bacteria, there is much demand for new drugs with new mechanisms of action. FtsZ is an attractive target for antibacterial drug discovery because it has been well studied and it is essential for bacterial cell division. If the protein is inhibited, the divisome will lose the function and cause cell death.(Addinall et al., 1996, Slayden et al., 2006) FtsZ proteins are highly conserved in prokaryotic and archaea species, which offers a chance to develop broad spectrum-antibiotics.(Rajagopalan et al., 2005, Margolin, 2000) Currently, there is no drug in the market that targets this protein, although many researchers have made great effort on studying inhibitors of FtsZ.

1.5.2. Natural products as FtsZ inhibitor

Curcumin

Curcumin has been shown to inhibit the function of tubulin (Figure 2).(Gupta et al., 2006) Rai et al. reported that FtsZ can be inhibited by curcumin in a dose-dependent manner. GTPase assay indicated that FtsZ is inhibited by increasing the GTPase activity and then cause FtsZ polymers destabilization.(Rai et al., 2008) Kaur et al. reported possible binding sites by docking study in *E. coli*-FtsZ (*Ec*-FtsZ) and *B. subtilis*-FtsZ (*Bs*-FtsZ), which were confirmed by mutagenesis studies.(Kaur et al., 2010) The possible binding site overlapped with the GTP binding site and several amino acid residues were common to both binding sites.(Gupta et al., 2006) But this kind of structure has been criticized due to its PAINS's property.(Baell and Walters, 2014)

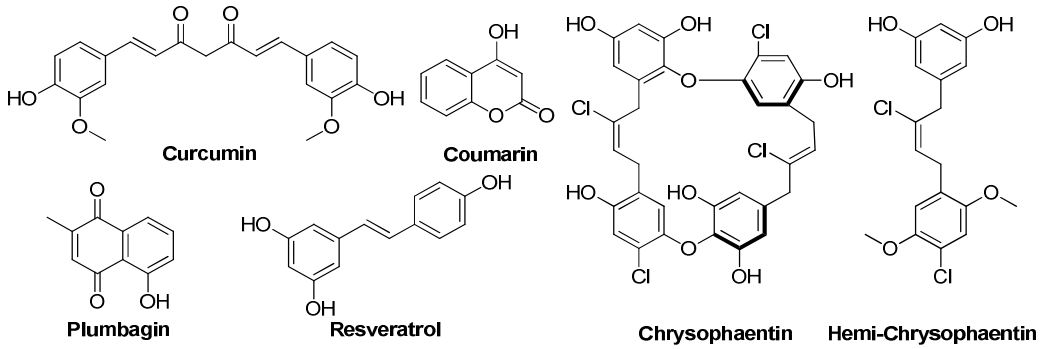


Figure 2. The structures of natural products.

Coumarins

Coumarins were reported by Duggirala to inhibit polymerization of *Bs*-FtsZ in a dose-dependent manner.(Duggirala et al., 2014) For example, its derivatives, Scopoletin and daphnetin can inhibit the GTPase activity. Docking proGram study showed that the binding site was located in T7 loop.

Plumbagin

Plumbagin was reported by Acharya to inhibit the activities of tubulin and FtsZ.(Acharya et al., 2008) Their results indicated that the FtsZ polymerization of *B. subtilis* 168 was inhibited in a dose-dependent manner. 10 μM of plumbagin reduced the assembly of the *Bs*-FtsZ by 45% and decreased FtsZ's GTPase activity. Docking studying showed that the binding site was near the C-terminal, and it was different from the GTP binding site. Although plumbagin can inhibit the polymerization of *Bs*-FtsZ, it cannot inhibit the *Ec*-FtsZ. This suggests that there is a difference in the structure of FtsZ among different bacteria.(Bhattacharya et al., 2013) In my opinion, this kind of structure is also very similar to the structure of PAINS.(Baell and Walters, 2014)

Resveratrol

Hwang et al. found that resveratrol affected Z-ring formation in *E. coli*.(Hwang and Lim, 2015) To confirm this result, an RNA silencer PNA-FtsZ, which has synergistic antibacterial effect with resveratrol was used. Their data showed that cells were led to elongation, and the mechanism of action was the inhibition of *Ec*-FtsZ gene expression.

Chrysophaentins

Chrysophaentins have the ability to fight against Gram-positive bacteria.(Plaza et al., 2010) Chrysophaentin A is a competitive inhibitor that can bind to *Ec*-FtsZ.(Plaza et al., 2010) Since both chrysophaentin A and hemi-chrysophaentin cannot show any antimicrobial activity against Gram-negative bacteria, while *Ec*-FtsZ can be inhibited *in vitro*, so researchers considered that these compounds cannot go into the membrane of Gram-negative bacteria. To test this hypothesis, the activities of these two compounds were tested against *E. coli envA1*, a strain with increased compound permeability, and found a good result, which proved this hypothesis.

Berberine and its derivatives

Berberine was reported to inhibit FtsZ assembly and GTPase activity in a dose-dependent manner (Figure 3). Docking studies in *Ec*-FtsZ indicated that its binding site overlapped with the GTP binding site.(Domadia et al., 2008)

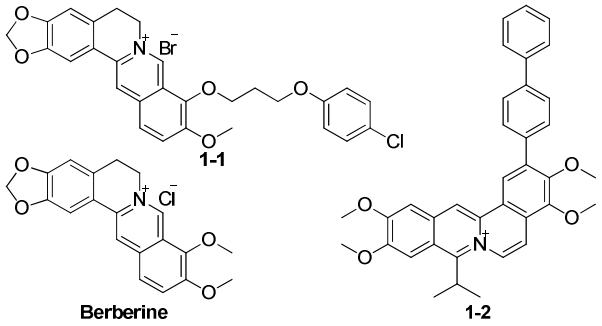


Figure 3. The structures of berberine and its analogues.

Based on the crystal structure of *S. aureus*-FtsZ (*Sa*-FtsZ), Sun et al. designed and synthesized a series of 9-phenoxyalkylberberine derivatives. This series of compounds showed MIC values as good as 2 μg/mL against *S. aureus*, and 4 μg/mL against *Enterococcus faecalis* (*E. faecalis*). These berberine analogs also had the effect on Gram-negative bacteria with MIC values as good as 32 μg/mL. The most potent compound in this series was compound **1-1**, which can inhibit *Sa*-FtsZ GTPase with IC_{50} 38 μg/mL and inhibited FtsZ polymerization in a dose-dependent manner. It can significantly reduce the thickness and size of FtsZ polymers, as well as the bundling of FtsZ protofilaments, which can be observed by transmission electron microscopy (TEM).(Sun et al., 2014)

Parhi et al. found another potential Compound **1-2** from other series of compounds, and compound **1-2** had good MIC value of 0.5 μg/mL against *S. aureus* and 2 μg/mL against *E. faecalis*. The mechanism of action behind compound **1-2** was promoting the FtsZ polymerization, which is similar to that of the compound PC190723.(Parhi et al., 2012)

Phenylpropanoids

Eight phenylpropanoids were selected and their antibacterial activity targeting FtsZ were determined(Figure 4).(Puupponen-Pimiä et al., 2001) The results showed that Chlorogenic acid was the most potential one with IC_{50} of 69.55 ± 3.6 μM. Others had a low IC_{50} of more than 250 μM, but most of them would inhibit GTPase activity.(Hemaiswarya et al., 2011)

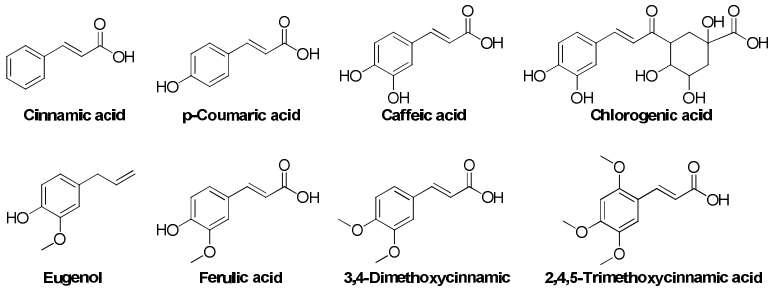


Figure 4. The structures of phenylpropanoid analogs.

Cinnamaldehyde and its derivatives

Cinnamaldehyde showed a very good the MIC values of 0.1-0.5 μg/mL against *E. coli*, *B. subtilis* and MRSA. (Figure 5).(Ali et al., 2005) It can inhibit the GTPase activity and inhibit the FtsZ protofilaments assembly.(Domadia et al., 2007)

A series of cinnamaldehyde analogs were synthesized by Li et al. (Figure 5) and determined for their antibacterial activity against a variety of bacteria *in vitro*.(Li, 2015) Several compounds showed as good as MIC values of 0.25 μg/mL against *S. aureus* ATCC25923. Compounds **1-3** and **1-4** exhibited the best activity among these compounds.

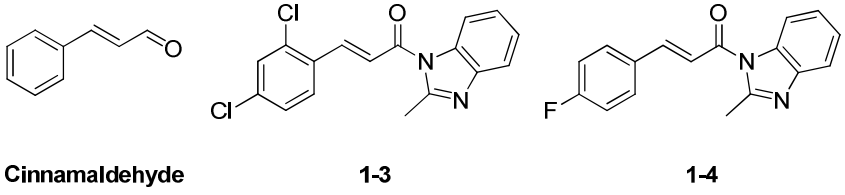


Figure 5. The structures of Cinnamaldehyde and its analogs.

1.5.3. Synthetic small molecules

Benzamides

The antibacterial activity of 3-methoxybenzamide (3-MBA) was tested on both *B. subtilis* and FtsZ mutant strains by Ohashi et al. The result showed that it could inhibit the growth of *B. subtilis* with the concentration is more than 5 mM, whereas the growth of mutant FtsZ strain was not inhibited even at 35 mM of 3-MBA. This indicates that FtsZ was a possible target of 3-MBA.(Ohashi et al., 1999) 3-MBA showed poor anti-bacterial activity,(Czaplewski et al., 2009) PC190723 had better potency than 3-MBA,(Haydon et al., 2008) and it showed antibacterial activity as good as 0.5 μg/mL and the good efficacy *in vivo* on *staphylococcal* infection.

Andreu et al. reported that PC190723 could reduce GTPase activity in *Bs*-FtsZ, which led to straight bundles and ribbons formation.(Andreu et al., 2010) Elsen et al. suggested that PC190723 could make the FtsZ polymer more stable than the monomer.(Elsen et al., 2012)

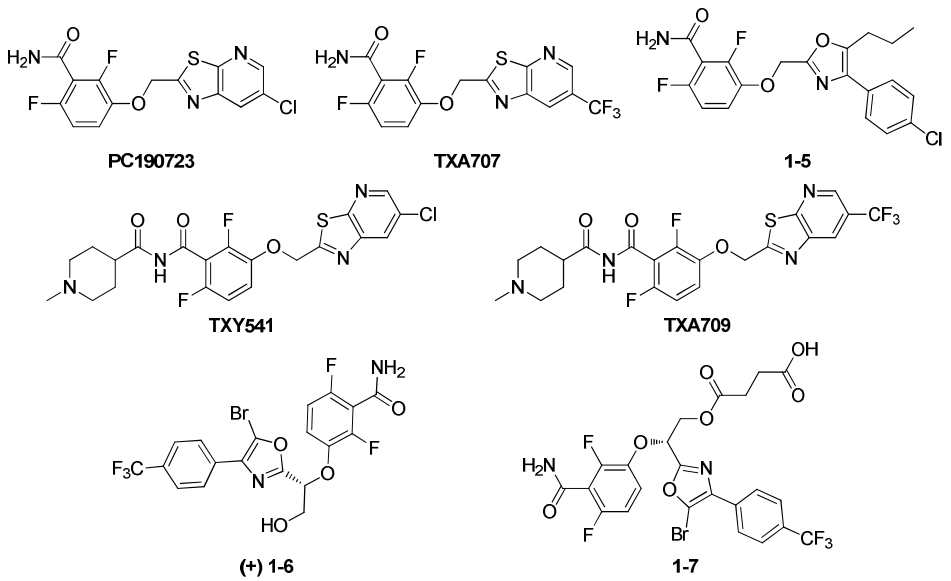


Figure 6. The structures of PC190723 and its analogs.

Stokes et al. synthesized a lot of PC190723 analogs by using a phenyloxazole group instead of a thiazolopyridine. Compound **1-5** has the best potency against the wild type *S. aureus* with the MIC of 0.03 μg/mL. To address the pharmacokinetic problems, the pseudo benzylic position was introduced some polar groups, which can improve solubility and metabolic stability. Two enantiomers of compound **1-6** were isolated and their antibacterial activities were examined, which revealed that (R)-(+)-enantiomer is 128 times more potent than the other one. To improve the solubility of compound **1-6**, they produced a prodrug **1-7** with better solubility than compound **1-6**.

To enhance the pharmacological properties of PC190723, TXY541, a prodrug of PC190723 was generated by Kaul et al..(Kaul et al., 2013) TXY541 has better solubility than PC190723 in an aqueous acidic vehicle and good efficacy *in vivo* experiment on mouse peritonitis model of systemic infection with *S. aureus*.(Kaul et al., 2013) TXA707 and its prodrug TXA709 were generated, which can address the problem that TXY541 is easily mediated by CYP.(Kaul et al., 2015) The introduction of a trifluoromethyl group can help improve metabolic stability of TXY541.

2-Nitro-vanillin-aniline Schiff bases

Vanillin derivatives were synthesized by Sun et al. and these compounds were tested for their antibacterial activities against Gram-negative and Gram-positive bacteria.(Sun et al., 2013) The SAR study showed that the aniline part with electron-donating groups helped increase the antibacterial potency, while electron-withdrawing groups led to weaked potency. Among these compounds, compound **1-8** showed the most potent activity against *E. coli* with a MIC of 0.28 μg/mL, which is even better than that of the kanamycin.

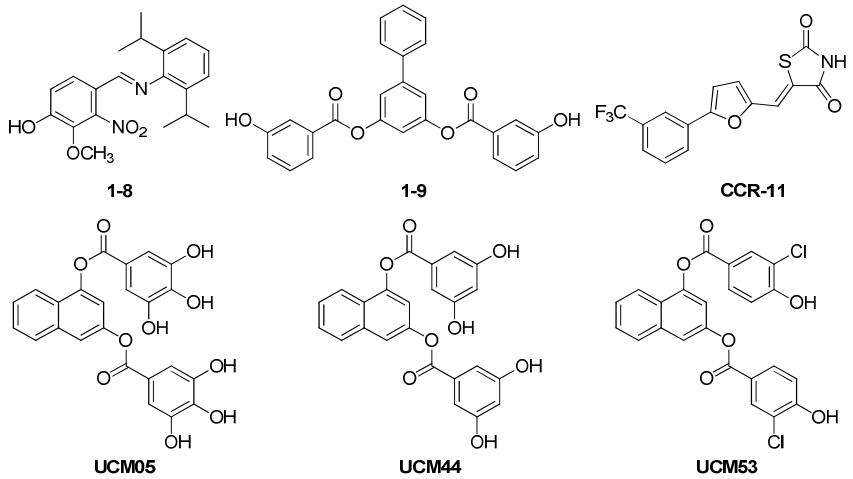


Figure 7. The structures of 2-nitro-vanillin-aniline Schiff bases, arene-diol digallates and a rhodanine derivative.

Arene-diol digallates

A docking study of *Bs*-FtsZ protein with over 4 million compounds by Andreu and collaborators screened out some hit compounds, such as UCM05, UCM44, and UCM53. But the drawback of these hit compounds was that they did not have antibacterial effect on *E. coli.* Then a series of analogs of UCM05 and UCM44 were designed and synthesized, and the biological data showed that the most potent compound was compound **1**-**9** with strong binding (K_d 0.5 μM) and MIC of 7 μM.

Rhodanine derivatives

A library of 151 rhodanine compounds were screened by Singh et al. and the antibiotic activity data showed that the target of these compounds was FtsZ.(Singh et al., 2012) Among these compounds, CCR11 bound to FtsZ with K_d 1.5 μM and could inhibit both FtsZ assembly and GTPase activity *in vitro*. Its MIC value for *B. subtilis* cells is 3 μM, and the Z-ring formation was inhibited.

Benzo[g]quinazolines, quinazolines, quinoxalines and 1,5-naphthyridines

Several zantrin **Z3(1)** (**ZZ3(1)**) derivatives were synthesized by Nepomuceno et al..(Nepomuceno et al., 2015) Among these compounds, compound **ZZ3(2)** was more potent than **ZZ3(1)** with an IC_{50} of 12 μM. The SAR study showed that benzo[g]quinazoline could be replaced by quinazoline, which is smaller in size, to produce the most active compound **1-10** with an IC_{50} of 9 μM. It was essential that 4-chlorostyryl should be kept in the structure for good activity.

A series of new compounds were synthesized by Parhi et al. and their antibacterial activities were tested on various bacteria strains. Among these compounds, compounds **1-11**, **1-11a** and compound **1-12** significantly increased the antibacterial activity, and compounds **1-13**, **1-13A**, **1-14** and **1-15** showed as good MIC values as 4 μg/mL for *S. aureus*.(Parhi et al., 2013)

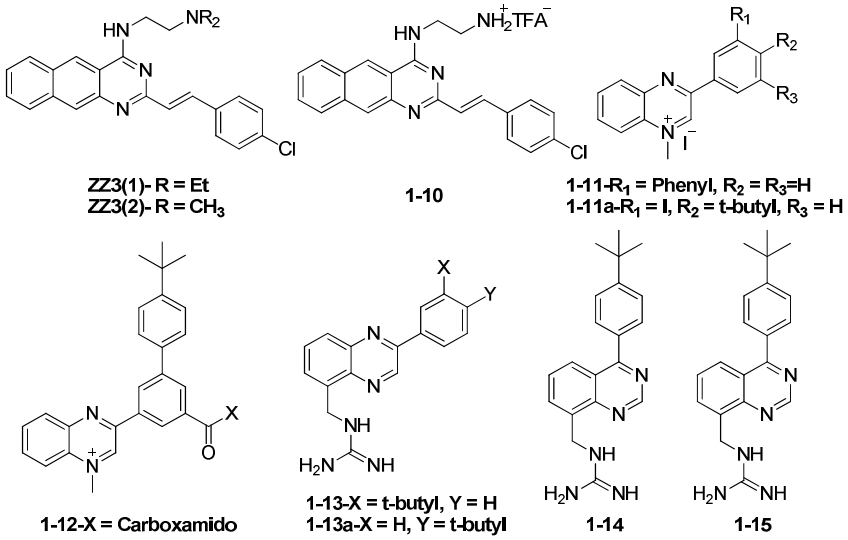


Figure 8. The structures of Quinoline, quinoxalines, quinazolines and 1,5-naphthyridine derivatives.

Pyrimidine-quinuclidine derivatives

The docking study of the crystal structure of *Methanococcus jannaschii*-FtsZ (*Mj*-FtsZ) helped Chan et al. to screen out some hit compounds from over 20,000

compounds, which interacted with FtsZ by binding to GTP binding site.(Chan et al., 2013) They tested 10 compounds with the best docking score for their GTPase inhibitory activity *in vitro* and MIC against bacteria. Among these compound libraries, compound **1-16** showed some extent GTPase inhibitory activity with IC_{50} of 317 μM and MIC for *E. coli* and *S. aureus* were 449 μM and 897 μM, respectively. Thus, compound **1-16** was considered as the lead compound for further studies, such as optimization *in silico*, library synthesis and SAR study. Compound **1-17** was the most potent compound with GTPase inhibitory activity of IC_{50} 37.5 μM and antibacterial activity against *S. aureus* of MIC 24.6 μM of and *E. coli* of MIC 49.6 μM.

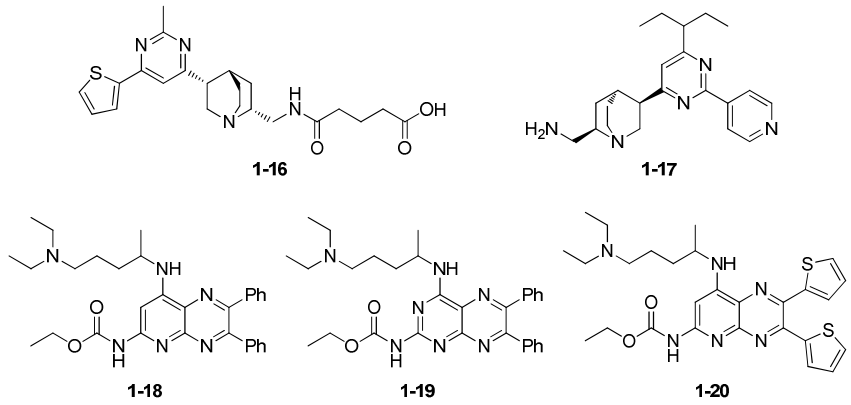


Figure 9. The structures of Pyrimidine-quinuclidine and pyridopyrazine analogs.

Pyridopyrazine and pyrimidothiazine analogs

Based on compounds **1-18** and **1-19** that were reported to inhibit *Mtb*-FtsZ, a series of pyridopyrazine and pyrimidothiazine analogs were synthesized by Mathew et al. and their biological activities were tested.(Mathew et al., 2011) The data showed that compounds **1-18** and **1-19** could inhibit *Mtb*-FtsZ, and compounds **1-18** and **1-20** showed good antibacterial activity against *M. tuberculosis* H37Rv with an IC_{90} of less than 0.19 μM, and a moderate inhibitory activity with IC_{50} ~34 μM for *Mtb*-FtsZ polymerization. Both compounds **1-18** and **1-20** were tested *in vivo* for their efficacy in mice.

Taxanes

Paclitaxel is a very important drug in cancer chemotherapy and it shows antibiotic activity against *M. tuberculosis* with MIC of 40 μM.(Huang et al., 2006) Due to the similar function of tubulin and FtsZ, it was suggested that taxanes could be the starting point for discovery of FtsZ inhibitors.

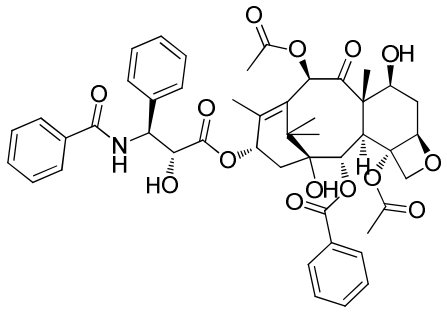


Figure 10. The structure of Paclitaxel.

Singh et al. reported that SB-RA-2001 (TRA) could inhibit the growth of *B. subtilis* and *Mycobacterium smegmatis* (*M. smegmatis*) cells, and its target was FtsZ.(Singh et al., 2014) It could increase the cell length of bacteria, inhibit the GTPase, and promote *Bs*-FtsZ protofilaments. Docking analysis showed that the binding site was similar to that of PC190723.

SB-RA-2001 also showed good antitubercular activity against *M. tuberculosis* H37Rv and *M. tuberculosis* IMCJ946.K2, but it had a high cytotoxic value, while antiangiogenic C-*seco*-taxane IDN5390 had a less cytotoxic value than paclitaxel.(Appendino et al., 1997, Taraboletti et al., 2002) Thus, C-*seco*-baccatin analogs of SB-RA-2001 were synthesized and evaluated, and the result showed that SB-RA-5001 and analogs had good antitubercular activity with MIC_{99} of 1.25-5 μM, and the cytotoxic value of IC_{50} was more than 80 μM.(Huang et al., 2007) SB-

RA-5001 could stabilize *Mtb*-FtsZ polymers, which was confirmed by Transition electron microscopy (TEM) analysis.(Ojima et al., 2014)

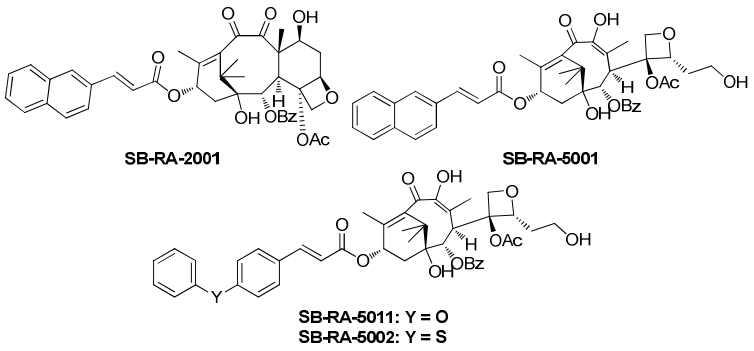


Figure 11. The structures of a lead taxane and C-*seco*-taxanes.

Benzimidazoles

Sarcina et al. proved that FtsZ was the target of albendazole and thiabendazole.(Sarcina and Mullineaux, 2000) Then Slayden et al. showed that the septation of *M. tuberculosis* cells could be inhibited by this kind of compounds and finally led to cell death.(Slayden et al., 2006)

204 hit compounds were screened from about 1,100 compounds and their antibacterial activities against *M. tuberculosis* H37Rv were as low as 5 μg/mL.(Awasthi et al., 2013, Kumar et al., 2011, Ojima et al., 2014) Then, 56 compounds from these hit compounds were tested for MIC and showed a range from 0.06 to 6.1 μg/mL.(Awasthi et al., 2013, Kumar et al., 2011, Ojima et al., 2014) SB-P17G-C2 was the most potent compound with MIC of 0.06 μg/mL.(Awasthi et al., 2013) It inhibited the *Mtb*-FtsZ polymerization in a dose-dependent manner and could increase the GTPase activity and decrease the stability of FtsZ polymers. Some lead compounds were tested for their biological activity against clinical strains of *M. tuberculosis*.(Knudson et al., 2014)

SB-P17G-A33, SB-P17G-A38 and SB-P17G-A42 with additional fluorine atoms were synthesized, and they could improve the metabolic stability.(Knudson et al., 2015) The most potent compound inhibited *M. tuberculosis* with MIC values as good as 0.18 μg/mL. SB-P17G-A38 and SB-P17G-A42 had efficacy equivalent to that of isoniazid (INH), and these two compounds could inhibit INH-resistant patient-derived *M. tuberculosis* strains. Thus, they are promising candidates for treating tuberculosis.

Kumar et al. screened out 23 hits that could inhibit more than 90% growth of *Francisella tularensis* (*F. tularensis*) LVS strain at a concentration of 10 μg/mL.(Kumar et al., 2013) The most potent one was Compound **1-21** with MIC_{90} of 0.35 μg/mL. The result of other compounds **1-22e**, **1-22g** and **1-22h** showed that they could reduce CFU by 2-3 log units at 10-50 μg/mL.

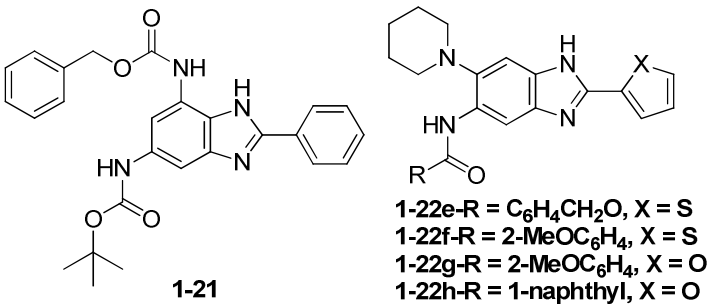


Figure 12. Trisubstituted benzimidazoles active against *F. tularensis*.

Ray et al. reported BT-benzo-29 could inhibit *B. subtilis* proliferation and *M. smegmatis* cells with IC_{50} of 1 and 1.6 μM, respectively.(Ray et al., 2015) It was proposed that the C-terminal domain of FtsZ near the T7 loop was its binding site.

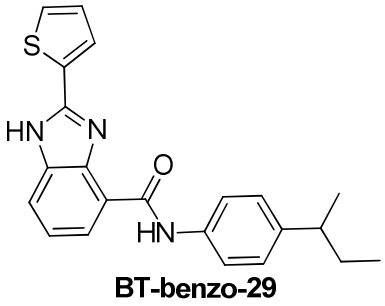


Figure 13. The structure of BT-benzo-29.

1.5.4. Polypeptides and Nucleic acids

Apart from natural products and synthesized small molecules, there are some other types of FtsZ inhibitors.

Cathelin-related antimicrobial peptide was reported to have some antibiotic activity.(Bergman et al., 2006) Recent studies showed CRAMP could inhibit FtsZ polymerization in a dose-dependent manner.(Ray et al., 2014) This peptide could decrease the GTPase activity of FtsZ, and make *B. subtilis* elongate and inhibit Z-ring formation. A docking study showed that there was a possible binding site near the T7 loop crevice.

Peptide nucleic acids (PNAs) are oligonucleotide analogs with a polypeptide-like backbone replaced by a ribose-phosphate backbone.(Paulasova and Pellestor, 2004, Nielsen and Egholm, 1999) PNAs can silence genes and prevent them from expression. Recently, cell viability assays showed that some of them were bactericidal.(Liang et al., 2014)

Locked nucleic acids (LNA) are nucleic acid analogues with the ribose ring.(Gruegelsiepe et al., 2006) Gene silencing with LNA can kill *E. coli* cells by

targeting RNase P expression. Recently, a peptide-LNA hybrid was synthesized and showed activity against MRSA-FtsZ.(Meng et al., 2015)

1.6. Objectives of this study

The rise of antibiotic resistance becomes a big concern for public health and unfortunately, most new antibiotics that have come into the market lack variety in their structure and target, as compared to older products. That means that bacteria can easily acquire resistance to these old drugs. Since the 1980s, there are only three new classes of antibacterial agents approved for clinical use: an oxazolidinone (linezolid/Zyvox) in 2000, a cyclic lipopeptide (daptomycin/Cubicin) in 2003, and more recently, a pleuromutilin derivative (retapamulin) in 2007. However, with the rapid spread of bacteria with antibiotic resistance, together with the emerging of novel high virulent pathogens, there is an urgent demand for generating new antibacterial agents with a novel mode of action and broad antibacterial spectrum.

From the literature review, we found that the bacterial cell division protein FtsZ plays an essential role during the cell division process and is an attractive drug target. However, there is no drug target this protein yet, so in this project, FtsZ was selected to be the target of our synthetic small molecular inhibitors. There have been a large number of FtsZ inhibitors discovered and synthesized by other researchers, but most of them only have antibacterial activity against Gram-positive bacteria, and do not have good antibacterial activity against Gram-negative bacteria.

To address the drawback of these FtsZ inhibitors, we planned to use FtsZ inhibitors **F332** together with beta-lactam antibiotics to form combination drugs, which was proposed to have a synergistic effect. The synergy effect can restore the efficacy of beta-lactam antibiotics to treat resistant bacteria. We also planned to develop broad

antibacterial spectrum FtsZ inhibitors through structural modification of the lead compound **F332**. The antibacterial spectrum can also be broadened by employing a double warhead strategy, which is using a linker to connect two scaffolds interacting with two different targets. We would connect the scaffolds of **F332** and triclosan by different linkers, and these new types of compounds are proposed to broaden the antibacterial spectrum of **F332** and to reduce the toxicity of triclosan.

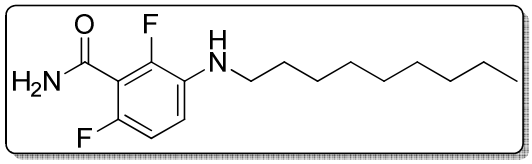
The biological activities of all these novel compounds will be evaluated and the purpose of this project is to develop some small molecules as FtsZ inhibitors with broad-spectrum antibacterial activity.

Chapter 2. Restore the efficacy of beta-lactam antibiotics by a FtsZ inhibitor

2.1. Introduction

Due to the strong antibiotic resistance of MRSA, most beta-lactam antibiotics have lost their ability to treat MRSA. How to restore their efficacy that have become a hot topic for research workers.

Antibiotic adjuvants, are compounds with little antibiotic activity themselves but can work together with antibiotics to enhance antibiotic action.(Wright, 2016) For example, clavulanic acid, a beta-lactam inhibitor, is used together with beta-lactam antibiotics to form a combination drug, which can restore the efficacy of beta-lactam antibiotics to treat resistant bacterial strains. Therefore, the use of antibiotic adjuvants can be an alternative strategy to help existing drugs treat resistant bacterial infections. Tan and co-workers have reported that FtsZ inhibitor can restore the antibacterial activity of beta-lactam antibiotics for treating MRSA.(Tan et al., 2012)



F332

Figure 14. The structure of compound **F332**.

Compound **F332** (Figure 14) as a potential FtsZ inhibitor has already been proven by our previous study.(Lui et al., 2019) In this chapter, we planned to use FtsZ inhibitor **F332** as antibiotic adjuvant together with beta-lactam antibiotics, to try to restore the efficacy of beta-lactam antibiotics to treat MRSA infections. The synthesis method and biological activity of compound **F332** were reported in our

previous study.(Lui et al., 2019)

2.2. Experimental section

2.2.1. Materials and bacterial strains

2.2.1.1. Materials

Nutrient agar was obtained from Oxoid Ltd. (Nepean, Ontario, Canada). Luria-Bertani (LB) medium was purchased from USB Corporation and used for preparation and transformation of competent cells for *E. coli*, *S. aureus* and other bacterial strains. Müller-Hinton broth (MHB) and Müller-Hinton agar and trypticase soy broth (TSB) for Minimum Inhibitory Concentration (MIC) determination were purchased from Becton, Dickinson, and Company (New Jersey, USA).

2.2.1.2. Bacterial strains

The bacterial strains used in minimum inhibitory concentration (MIC) determination such as *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and other bacterial strains, including clinical bacteria, were obtained from Prof. Chen Sheng' s research group.

2.2.2. Antibacterial susceptibility test

The minimum inhibitory concentrations (MICs) of the test compounds were determined using a broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution at a concentration of 16 mg/mL. The compound stock solution was then treated with a

series of 2-fold dilutions in Müller-Hinton broth (MHB) to prepare compound-containing media at concentrations ranging from 0.25 to 256 μg/mL in 150 μL solution. Four to five single colonies of the tested bacterial strain on a TSB agar plate were inoculated in 5 mL of MH broths. The cells were incubated at 37 ºC until the OD_{600} (absorbance of 600 nm) of the growing cells reached 1.0. The cells were then diluted to a final concentration of approximately OD_{600} of 0.08 to 0.1, in MH broth containing two-fold dilutions of the test compounds in a 96-well microtiter plate. After 18 h of incubation at 37 ºC, the OD_{600} values were measured to calculate the percentage inhibition of bacterial growth with respect to the control. The MIC value is defined as the lowest concentration of tested compounds that causes ≥ 90% inhibition of bacterial growth.

2.2.3. Cytotoxicity (IC_{50}) testing

Standard MTS assay was employed to determine the cytotoxicity of the compound towards the L929 cells as previously described. Briefly, 10,000 cells were mixed with compounds at different concentrations in a final volume of 100 μL in each well of a 96-well plate, followed by incubation at 37 °C for 3 days. DMSO at 1% was used as a solvent control. The half-maximal inhibition of the compounds was determined using a CellTiter 96 Aqueous assay (Promega). An aliquot of the freshly prepared MTS/phenazine methosulfate mixture at a ratio of 20:1 was added into each well, followed by incubation at 37 °C for 2 h. Optical absorbance at 490 nm was measured with a microplate reader. The IC_{50} values were determined from the dose-response curves of the MTS assay (Prism 4.0).

2.2.4. Isolation of F332R mutants

Cells of *Staphylococcus Aureus* ATCC 29213 were cultured in lysogeny broth (LB)

with constant shaking at 250 rpm at 37°C. We transferred bacteria by inoculating 50 µL of stationary-phase culture into 3 mL of LB. Cells were initially grown in medium without **F332**. At the second transfer, 50 µL of cell suspension was added to 3 ml of nutrient broth, with or without **F332** at a final concentration of half the MIC (MIC= 2µg/mL), for 20 h with shaking at 250 rpm, to obtain 2 transfers, T(0) and T(1). The regrown bacteria in T(1) were thereafter transferred to a broth containing a double concentration of **F332** compared with previous transfers and cultured using the above method. If the growth in T(1) is very weak, bacteria in T(0) should be transferred in another T(1) culture and this step should be repeated until the bacteria can grow well in T(1). The experiment was conducted several times with escalating concentration levels of **F332** and need to collect the strain with the concentration of **F332** at 32 µg/mL (T(32)), 64 µg/mL (T(64)) and 128 µg/mL (T(128)), repectively. The MIC value of **F332** for each induced bacterial was determined and all MIC >32 µg/mL.

2.2.5. Whole-genome sequencing and bioinformatics analysis

Total DNA was extracted from an overnight culture (3 ml) by using Genomic DNA Mini Kit according to the protocol provided by the manufacturer. Following extraction, DNA was quantified by quality ratios of DNA (A260/280) determined via Nanodrop (ThermoFisher Scientific). Genomic DNA libraries were prepared for whole-genome sequencing using the TruePrep Index Kit V3 and TruePrepTM DNA Library Prep Kit V2 as described by the manufacturer. Paired-end sequencing was performed using the Illumina NextSeq platform (NextSeq 500/550 Kits v2; 2 × 151 cycles). Raw reads were de novo-assembled into contigs using SPAdes (3.11.0) with pre-defined kmers set. Then analysis was conducted by using the assembled contigs. Reference FtsZ gene sequence was downloaded from NCBI GenBank. The genome was BLAST against the FtsZ gene using CLC workbench. Relative sequences were

extracted from the genome sequences. The extracted sequences were then aligned against reference FtsZ sequence to view the identity.

2.2.6. Docking study

CLC Drug Discovery Workbench (Version 2.5, QIAGEN) software was used for docking study. The 2D structure of compound **F332** was generated from SIMLES and imported into the docking study software. The X-ray crystal structure of *S. aureus* FtsZ (PDB ID: 4DXD) was downloaded from Protein Data Bank (https://www.rcsb.org/) and used directly for our docking without any changes. The software function of "Find Binding Pockets" was used to find the potential binding pocket. The identification of ligand binding modes was done iteratively by evaluating 10,000 ligand conformations and estimating the binding energy of their interactions with these binding pockets. The binding poses with the top 5% of highest scores were returned for further visual inspection. The highest scores that positioned compound **F332** into the binding site with potential binding pose was shown in the following result part.

2.3. Results and discussion

2.3.1. Evaluation of antibacterial and cytotoxic activities

The antibacterial activity of compound **F332** against *S. aureus* and *E. coli*, and the cytotoxicity of compound **F332** were evaluated and results were shown in Table 2. The IC_{50} of L929 was larger than 100 μM, indicating that this compound had very low cytotoxicity in normal cells.

Table 2. The MIC and cytotoxicity results of compound **F332**.

Compound	MIC (μg/mL)		IC_{50} (μM)
	S. aureus 29213	*E. coli* 25922	L929
F332	1	>64	>100

The MIC result showed that the compound **F332** had a pretty good antibacterial activity against *S. aureus* with the MIC of 1 μg/mL, but it had no effect on *E. coli*. A possible reason may be that it cannot enter the Gram-negative bacterial cell because the membrane of Gram-negative bacteria is hard to pass than the Gram-positive bacteria, or there are efflux pumps in the Gram-negative bacteria, which pump out the compound **F332**.

Collectively, compound **F332** had a good antibacterial activity against *S. aureus* and low cytotoxicity in normal cells. This result demonstrated that it was a good choice for us to further study its biological activity.

2.3.2. Validation of FtsZ protein as a drug target of compound F332

Our previous study has indicated that the FtsZ protein is a potential drug target of compound **F332**.(Lui et al., 2019) This hypothesis is supported by many experiment results, such as the effect of compound **F332** on FtsZ protein polymerization, microscopic study of bacterial cell morphology and localization of the Z-ring of *S. aureus* cell.

In this study, a genetic study was carried out to compare the *ftsz* gene sequence of the wild type *S. aureus* strain with the *ftsz* gene sequence of the mutant *S. aureus* strain, and computational docking study was used to explain the resistant mechanism of the mutant *S. aureus* strain.

2.3.3. Genetic study

We used compound **F332** to generate three **F332**-resistant *S. aureus* strains, mutant32, mutant64 and mutant128. The MIC of all three strains are larger than 32 μg/mL (Table 3), which means that these three strains are resistant to **F332**.

Table 3. Summary of MIC, nucleotide changes and amino acid substitutions of F332-resistant mutants.

Strains	MIC of **F332** (μg/mL)	Nucleotide at position 786	Amino acid at position 262
S. aureus 29213	1	G	Met
Mutant32	>32	A	Ile
Mutant64	>64	A	Ile
Mutant128	>128	A	Ile

Then the sequence of its *ftsz* gene of this resistant strain was tested (Figure 15), and we compared the *ftsz* gene sequence of mutant strain with the wild type strain. The result showed that there was a single mutant nucleotide on the position 786, which was changed from G to A. at the same time, the amino acid on the position 262 was changed from M (methionine) to I (isoleucine). This mutant protein FtsZ was caused by compound **F332**, which indicated that FtsZ is a drug target of compound **F332**.

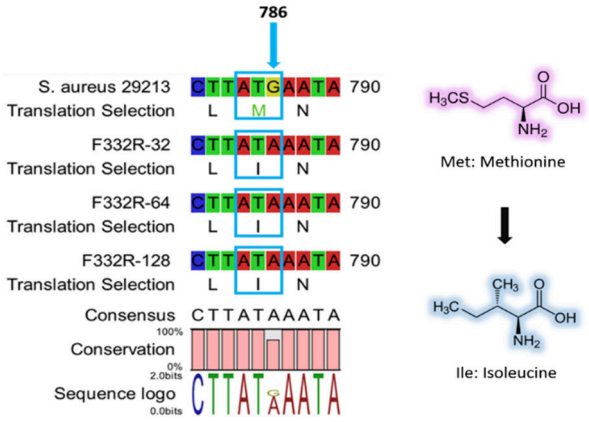


Figure 15. The gene sequence of *ftsz* in mutants and wild type *S. aureus*.

2.3.4. Docking study

Docking study showed that the mutant amino acid position is just on the surface of the binding pocket, we can see from Figure 16, the blue bond is compound **F332**, the cleft space is the binding pocket, the amino acid position 262 is just on the surface of amino acid. This means that if the binding pocket surface amino acid is mutant to another amino acid, it will change the structure of binding pocket, so that compound **F332** cannot bind to the binding pocket again. This is that why the **F332** lost their activity against mutant *S. aureus* strains. This result is also a piece of evidence to support that FtsZ protein is the target of **F332**.

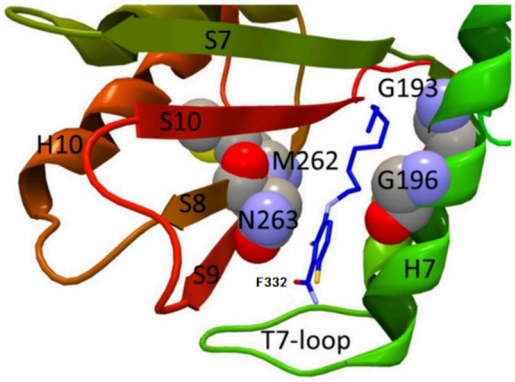


Figure 16. Docking model of compound **F332** with *S. aureus* FtsZ protein.

Collectively, **F332** is a FtsZ inhibitor and it can also be a lead compound for us to design out broad spectrum FtsZ inhibitors. This will be demonstrated in Chapter 3.

2.3.5. Beta-lactam antibiotics combination studies

FtsZ inhibitor **F332** was used as an antibiotic adjuvant together with beta-lactam antibiotics to restore the efficacy of beta-lactam antibiotics to treat MRSA infections. The property of antibiotic adjuvant is that this compound has little or no antibiotic activity. In order to prove that **F332** is suitable to work as antibiotic

adjuvants, its antibiotic activities against MRSA strains were evaluated and the MIC result was shown in Table 4.

Table 4. The MIC result of **F332** for clinical MRSA strains.

	MIC (μg/mL)													
MRSA Strain No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
F332	32	512	1024	32	8	1024	32	1024	128	16	512	128	16	16
MRSA Strain No.	15	16	17	18	19	20	21	22	23	24	25	26	27	28
F332	8	16	16	16	1024	16	128	16	1024	2	2	4	2	4

There were 28 clinical MRSA strains for testing the antibacterial activity of compound **F332** and the MIC result showed that it only had little or no effect on most of these clinical MRSA strains. This result indicates that compound **F332** was suitable to work as an antibiotic adjuvant.

Then, we planned to use compound **F332** and beta-lactam antibiotics to form combination drugs, and the synergistic effect of these combination drugs was evaluated through the MIC of 28 clinical MRSA strains. The MIC results and FIC values were shown in Table 5 and Table 6.

Combination drugs contain compound **F332** and different types of beta-lactam antibiotics, including ME, Methicillin; CL, cloxacillin; CX, cefuroxime; AM, amoxicillin. The MIC results showed that most of the combination drugs had better antibacterial activity against MRSA strains than the beta-lactam antibiotics used alone. Most combination drugs have synergistic effect and they were highlighted with blue color in Table 5.

When AM was used alone, 85% of strains had MIC of more than 64 μg/mL, but

when the strains were treated with a combination drug of compound **F332** and AM, nearly all strains had MIC of less than 16 μg/mL, except strain No. 10, which had MIC of 64 μg/mL. For ME, the MIs for all strains was larger than 8 μg/mL when ME was used alone but a combination drug could improve the antibacterial dactivity and led to the MIC reduction to less than 4 μg/mL for all strains. For CL, the MIC was larger than 2 μg/mL for 75% of strains when CL was used alone but when a combination drug was used, the MIC dropped to less than 1 μg/mL for 68% of strains. For CX, the MIC was more than 8 μg/mL in 25 out of 28 strains when CX was used alone, but when a combination drug was used, the MIC was less than 4 μg/mL for all strains.

Table 5. The MIC of antibiotic alone and their combination with **F332** (**ME**, Methicillin; **CL**, Cloxacillin; **CX**, Cefuroxime; **AM**, Amoxicillin).

	MIC (μg/mL)								
MRSA No.	F332	ME	ME+F	CX	CX+F	CL	CL+F	AM	AM+F
1	32	1024	2	1024	2	64	2	512	8
2	512	256	2	16	2	2	0.5	512	16
3	1024	16	2	8	2	8	2	512	64
4	32	16	2	1024	2	2	1	256	8
5	8	1024	4	1024	4	64	2	256	4
6	1024	128	4	256	2	8	1	256	16
7	32	64	4	512	2	16	2	64	8
8	1024	64	4	256	2	4	1	8	2
9	128	64	4	32	2	8	1	8	2
10	16	32	4	16	2	4	1	1024	8
11	512	16	4	1024	2	2	1	512	8
12	128	16	4	8	4	16	1	512	8
13	16	1024	4	1024	4	32	2	128	8
14	16	256	4	256	4	16	1	128	16
15	8	256	4	512	2	32	2	128	8
16	16	128	4	512	2	8	2	64	8
17	16	64	4	512	2	8	2	512	16
18	16	64	4	64	4	8	2	256	16
19	1024	8	4	8	2	0.5	0.5	256	8
20	16	1024	4	32	2	2	1	128	8
21	128	32	4	16	2	1	1	512	8
22	16	16	2	2	2	1	1	256	4
23	1024	16	4	2	2	1	1	512	8
24	2	128	2	1024	2	32	1	256	4
25	2	32	2	16	2	0.5	0.5	64	2
26	4	32	2	16	2	2	1	256	4
27	2	16	2	16	2	0.5	1	32	2
28	4	8	2	2	2	1	0.5	2	0.5

All these results showed that the FtsZ inhibitor compound **F332** could work as an antibiotic adjuvant and could be used together with beta-lactam antibiotics to form combination drugs, which could restore the efficacy of beta-lactam antibiotics to treat MRSA strains *in vitro*.

The FIC index is calculated by using FIC index = FIC (compound) + FIC (drug), where FIC (compound) is the (MIC of compound in combination with drug)/(MIC of compound alone), while FIC (drug) is the (MIC of compound in combination with drug)/(MIC of drug alone). Theoretically, the combination is considered as synergistic if the FIC Index ≤0.5.

Table 6. The FIC index of combination drugs.

	FIC Index			
MRSA No.	ME+F332	CX+F332	CL+F32	AM+F332
1	0.1	0.1	0.1	0.3
2	0.0	0.1	0.3	0.1
3	0.1	0.3	0.3	0.2
4	0.2	0.1	0.5	0.3
5	0.5	0.5	0.3	0.5
6	0.0	0.0	0.1	0.1
7	0.2	0.1	0.2	0.4
8	0.1	0.0	0.3	0.3
9	0.1	0.1	0.1	0.3
10	0.4	0.3	0.3	0.5
11	0.3	0.0	0.5	0.0
12	0.3	0.5	0.1	0.1
13	0.3	0.3	0.2	0.6
14	0.3	0.3	0.1	1.1
15	0.5	0.3	0.3	1.1
16	0.3	0.1	0.4	0.6
17	0.3	0.1	0.4	1.0
18	0.3	0.3	0.4	1.1
19	0.5	0.3	1.0	0.0
20	0.3	0.2	0.6	0.6
21	0.2	0.1	1.0	0.1
22	0.3	1.1	1.1	0.3
23	0.3	1.0	1.0	0.0
24	1.0	1.0	0.5	2.0
25	1.1	1.1	1.3	1.0
26	0.6	0.6	0.8	1.0
27	1.1	1.1	2.5	1.1
28	0.8	1.5	0.6	0.4

The synergistic effect was evaluated by calculating the FIC index according to the FIC index formulation and the results were shown in Table 6. According to the theoretic synergistic effect, if the FIC is not larger than 0.5, it is considered to have the synergistic effect. The FIC index showed that most of the combination drugs had synergistic effect, because their value was not larger than 0.5. Combination drugs with synergistic effect were highlighted in blue color. We summarized the synergy percentage of each combination drug in Figure 17.

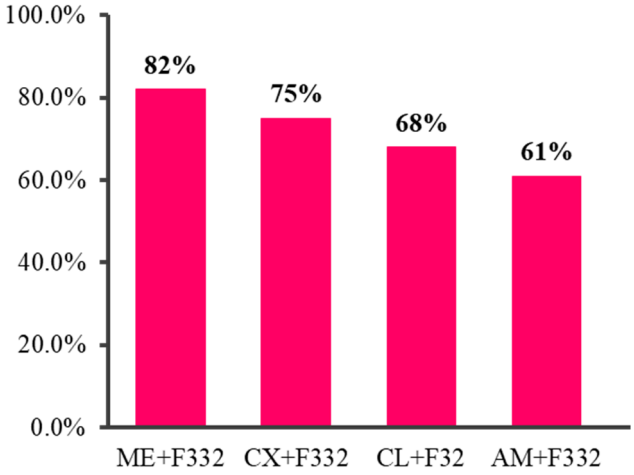


Figure 17. Percentage of number of strains with synergistic effect (FIC index ≤ 0.5). FIC index = FIC (compound) + FIC (drug). FIC = (MIC in combination)/(MIC alone).

Figure 17 showed that the combination of compound **F332** and ME had the highest percentage of the synergistic effect, with as high as 82%. This result indicated that compound **F332** could well restore the efficacy of ME to treat MRSA *in vitro*. The combination drugs of compound **F332** with other beta-lactam antibiotics, such as CX, CL and AM also showed high percentage of the synergistic effect, with more than 60%. This result indicated that compound **F332** could also restore the efficacy of other types of beta-lactam antibiotics to treat MRSA *in vitro*.

Overall, compound **F332** could restore the efficacy of beta-lactam antibiotics, to effectively treat MRSA *in vitro*.

2.4. Concluding remarks

In this chapter, a FtsZ inhibitor was used as antibiotic adjuvant to restore the efficacy of beta-lactam antibiotics to treat MRSA infections. **F332** was chosen as the antibiotic adjuvant and it had been considered as a selective FtsZ inhibitor because it had good antibacterial activity against *S. aureus* and no effect on *E. coli*.(Lui et al., 2019) FtsZ protein as a drug target of compound **F332** was further confirmed by genetic study and docking study in this study, and its antibacterial activity against MRSA strains was also evaluated. The MIC result showed that **F332** only had little or no antibacterial activity against these clinical MRSA strains, which suggested that compound **F332** was suitable to be an antibiotic adjuvant.

Then compound **F332** was used together with different types of beta-lactam antibiotics, including methicillin, cloxacillin, cefuroxime, and amoxicillin, to form combination drugs, in order to restore the efficacy of these beta-lactam antibiotics to treat MRSA infections. The MIC result of combination drugs showed that most of these combination drugs had better antibacterial activity against these MRSA strains than antibiotics used alone. The FIC index also indicated that most of these combination drugs had synergistic effect for treating MRSA strains in vitro, especially for methicillin, which had the highest percentage of synergistic effect among these beta-lactam antibiotics. These data suggested that compound **F332** could work as an antibiotic adjuvant to restore the efficacy of beta-lactam antibiotics to treat MRSA in vitro.

Collectively, combination drugs by using FtsZ inhibitors as antibiotic adjuvant can restore the efficacy of beta-lactam antibiotics *in vitro*, and this strategy can be an alternative method to treat the MRSA infections.

Chapter 3. Synthesis of a new class of FtsZ inhibitors with broad antimicrobial spectrum

3.1. Introduction

The threat from antibacterial resistance is more and more serious. However, there is a lack of effective strategies to solve this problem. There is nearly no method to treat the infections caused by resistant Gram-negative bacteria. Hence, there is an urgent need to develop some new types of antibiotics to treat Gram-negative bacterial strains.

Although many different classes of FtsZ inhibitors have been developed, most of them only have antibacterial activity against *S. aureus* or some Gram-positive bacterial strains, or they have weak antibacterial activity against Gram-negative bacterial strains, such as *E. coli*. In this study, we tried to develop some broad antibacterial spectrum FtsZ inhibitors through structural modification of **F332**, which has been approved as a FtsZ inhibitor.

3.2. Experimental section

3.2.1. Chemical synthesis

3.2.1.1. Materials

All the starting materials and chemical reagents for compound synthesis were purchased from commercial chemical companies, such as Aldrich and International Laboratory. All organic solvents were purchased from Oriental Chemicals. Thin-layer chromatography (TLC) analysis was performed on Merck silica gel plates, and the version is TLC silica gel 60 F_{254} (0.25 mm thickness) purchased from E.

Merck. Compounds were visualized under short and long UV light (254 and 365 nm) and/or immersed in a 10% phosphomolybdic acid solution in ethanol followed by gentle heating with a heat gun and/or stained with silica gel powder combined with I_2. Flash column chromatography for the compound purification was performed on silica gel and chromatography silica gel is MN silica gel 60 (230-400 mesh). All the chemicals and organic solvents mentioned above were reagent grade and they were directly used without any further purification unless otherwise stated.

3.2.1.2. Instrumentation

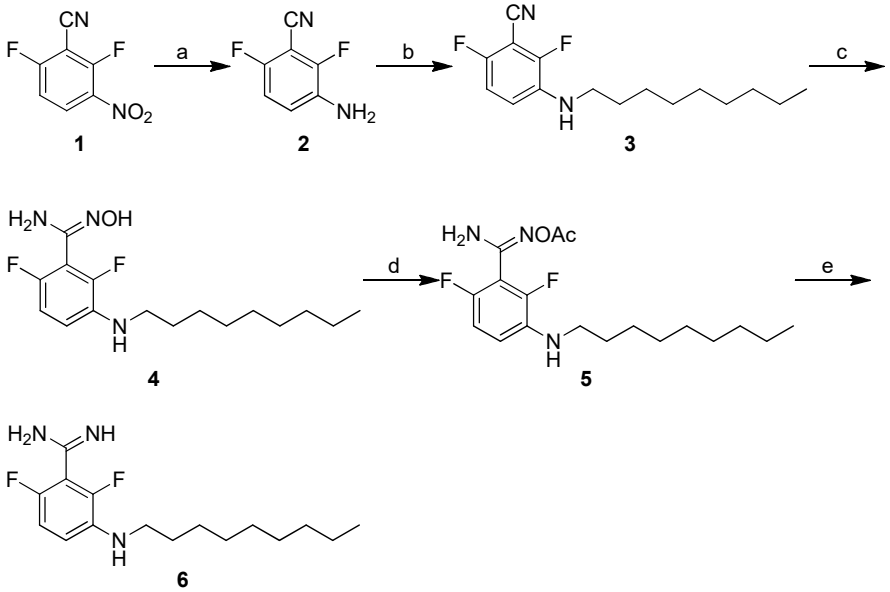
All compounds synthesized in our lab were confirmed by ^{1}H-NMR, ^{13}C-NMR and ESI-MS spectra. All NMR spectra were recorded at room temperature on a Bruker Advance-III spectrometer at 400.13 MHz for ^{1}H-NMR and 100.62 MHz for ^{13}C-NMR. All chemical shifts were reported as parts per million (ppm) in the unit relative to the resonance of $CDCl_3$, Methanol-d_6, DMSO-d_6. Low-resolution (LRMS) mass spectra were obtained on mass spectrometer by electron spray ionization (ESI) mode. High-resolution mass spectra (HRMS) were also obtained by ESI mode, a Micromass Q-TOF-2 mass spectrometer located in the The University Research Facility in Life Sciences (ULS) in Hong Kong Polytechnic University.

3.2.1.3. Synthesis and characterization

All compounds were synthesized according to the synthetic pathways as shown in Scheme 1-3. In Scheme 1, the starting material was 2,6-difluoro-3-nitrobenzonitrile, which could be purchased from the commercial chemical company at a very low price. The first step was the catalytic reduction reaction. The nitro group of the starting material was reduced to amine group through the hydrogenation by using palladium catalyst on the carbon and hydrogen gas with a high yield and it is easy for purification. The mixture was reacted in methanol at room temperature

overnight. The second step was the alkylation of amine group, using 1-bromononane to alkylate with the amine group to generate compound **3** with nine carbon-chain. Interestingly, no double alkylated product was obtained, in the presence of potassium iodide and potassium carbonate. The mixture was dissolved in dimethylformamide and stirred at 110 ^{o}C overnight with a good yield. The next step was using hydroxylamine hydrochloride to react with the cyano group. Then compound **4** with an amidoxime group was obtained. This reaction was conducted in methanol containing triethylamine at 80 ^{o}C overnight. The fourth step was the acetylation, acetic anhydride in acetic acid was used to react with amidoxime, and to acetylate with the hydroxyl group. In this reaction, the starting material could be completely consumed just by 5 min at room temperature. The final step was also a reduction, still using palladium on the carbon and hydrogen gas to react with compound **5** in methanol at room temperature overnight to obtain the final target compound.

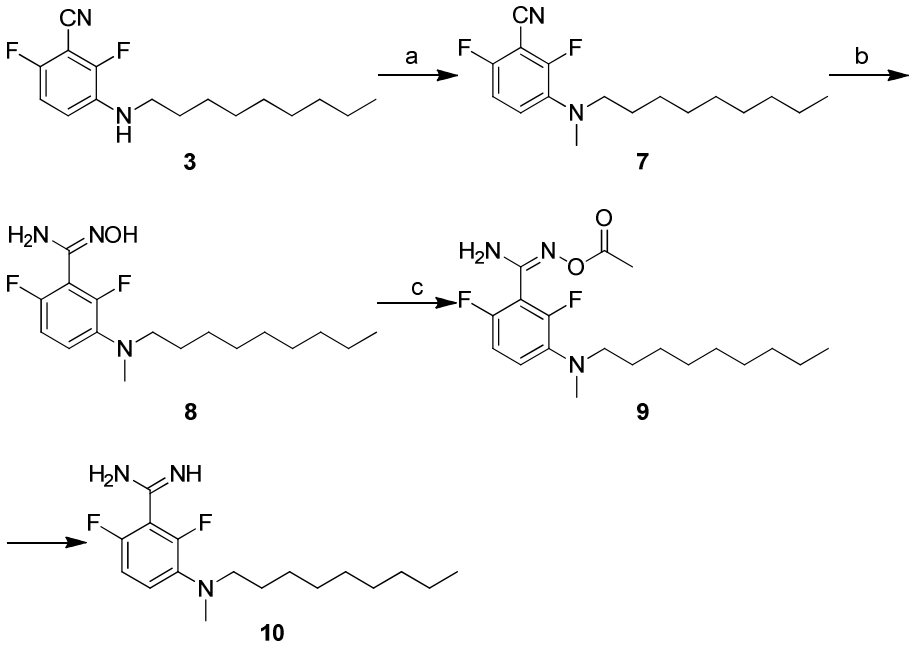
Scheme 1. Synthesis of compound 6 and its intermediates



Reagents and conditions: a) H_2, Pd/C, MeOH, room temperature (RT), overnight; b) 1-bromononane, KI, K_2CO_3, DMF, 110^{o}C, overnight; c) $NH_2OH.HCl$, Et_3N, MeOH, 80^{o}C, overnight; d) Ac_2O, HOAc, RT, 5 min; e) H_2, Pd/C, MeOH, RT, overnight.

For the synthesis of a derivative of compound **6** in Scheme 2, the starting material was compound **3**, which was synthesized according to the synthesis pathway shown in Scheme 1. The first step was also alkylation, iodomethane and potassium carbonate were stirred in the DMF at room temperature for 24 hours. The second step was the same method mentioned in Scheme 1, in which hydroxylamine hydrochloride was used to react with cyano group in the presence of triethylamine, and the mixture was stirred at 80 ^{o}C overnight to change cyano group to amidoxime. The next step was also the acetylation reaction, and acetic anhydride was used to react with amidoxime in acetic acid, to acetylate with a hydroxyl group. The final step is also a reduction reaction, and compound **10** was generated from compound **5**.

Scheme 2. Synthesis of compound 10 and its intermediates



Reagents and conditions: a) iodomethane, K_2CO_3, DMF, room temperature (RT), 24h; b) $NH_2OH.HCl$, Et_3N, MeOH, 80^0C, overnight; c) Ac_2O, HOAc, RT, 5 min; d) H_2, Pd/C, RT, overnight.

For the synthesis of derivatives of compounds with amidines, according to the general synthesis pathway shown in Scheme 3, the starting material was compound **3** with a cyano group. The first step was using compound **3** with fluorine to react

with different phenol groups to form different ethers. The reaction was conducted in the presence of strong base potassium hydroxide in DMF and the mixture was stirred at room temperature for 10 hours. For this reaction, there were two fluorine atoms on the aromatic ring (Figure 18), and the ^{13}C-NMR spectrum showed that the F on the C_5 was replaced.

C-F coupling constant is affected by the distance of C-F, and they are ^{1}JCF = 245 Hz, ^{2}JCF = 21, ^{3}JCF = 8 and ^{4}JCF = 3. Firstly, we analyzed the upper spectrum in Figure 18C and the compound in Figure 18A. Figure 18C showed that the coupling constant of peak 1 and 5 was about 245, so they were the C_1 and C_5 connected with F directly. C_6 was coupled with two F and both two C-F coupling constant are about 21. The chemical shift of C_4 connected with amine should be 136 ppm, which was consistent with the peak at 134 showed in Figure 18A. The C-F coupling constant of peak 2 was 19.2 Hz and 4.0 Hz, which indicated that this carbon was ortho to the F, so the peak 2 matched with C_2. The C-F coupling constant of peak 3 is 8 Hz and 6 Hz, which was consistent with ^{3}JCF = 8. Therefore, the peak 3 matched with C_3. Then, we analyzed the lower spectrum in Figure 18C and the compound with one fluorine in Figure 18B. The C-F coupling constant of C_4 was 3 Hz, indicating that the ortho fluorine was replaced. Because if the para fluorine was replaced, the C-F coupling constant of C_4 should be 10 Hz. The other evidence was that the C-F coupling constant of C_2 was 20 Hz, which indicated that C_2 was ortho to fluorine. Therefore, the fluorine on C_5 was replaced.

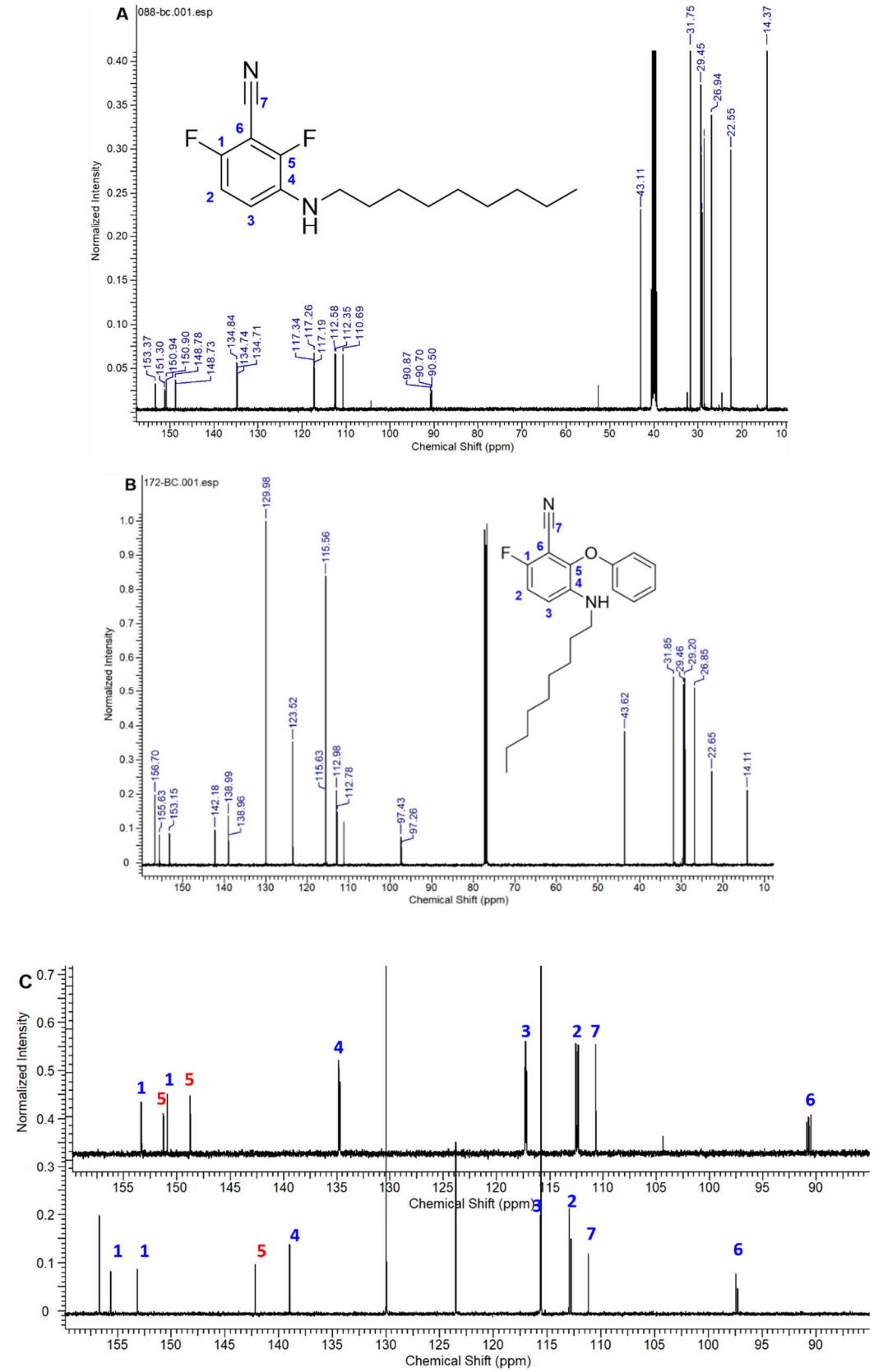
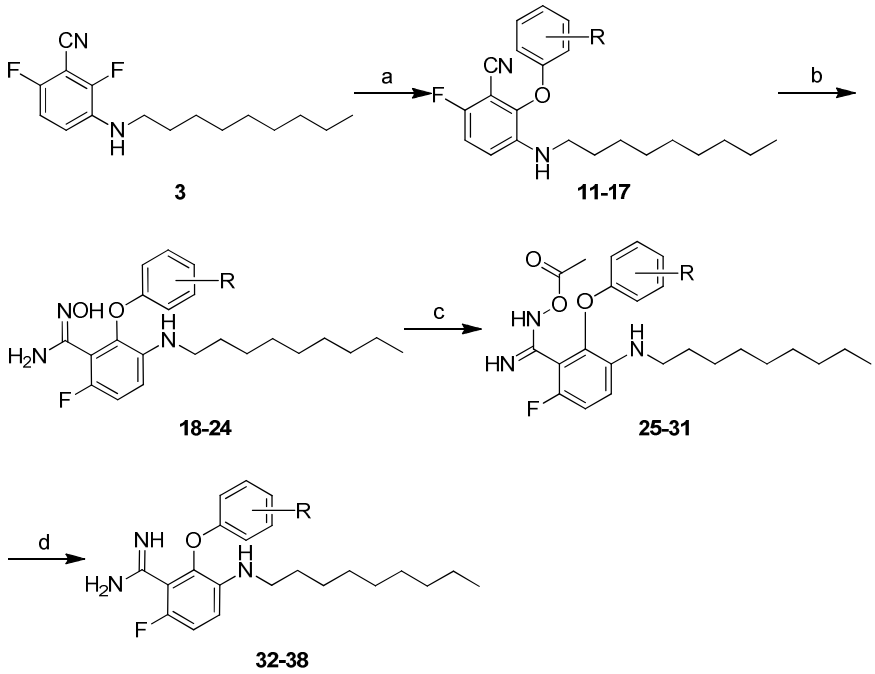


Figure 18. The ^{13}C NMR of (a) compound **3** and (b) the fluorine replaced of compound **3**, c) the merge of aromatic part of two compounds.

The next step was the same method mentioned in previous Scheme. hydroxylamine hydrochloride was used to react with cyano group in the presence of triethylamine, and the mixture was stirred at 80 ºC overnight to change cyano group to amidoxime. The following step is also the acetylation reaction, which is the same with the method for generating compound **9**. The final step is also the same with the method for generating compound **10**.

Scheme 3. Synthesis of compounds with amidine group and their intermediates



Reagents and conditions: a) phenol derivatives, KOH, DMF, room temperature (RT), 10h; b) NH2OH.HCl, Et3N, MeOH, reflux, 10h; c) Ac2O, HOAc, RT, 5 min; d) H2, Pd/C, RT, overnight.

Procedure for synthesis

3-amino-2,6-difluorobenzonitrile (2)

The mixture of compound **1** (0.50 g, 2.7 mmol) in MeOH (5 mL) was added Pd/C (10% Pd, 55% H_2O) (0.10 g) and stirred at room temperature with H_2 overnight. TLC showed that the starting material was consumed completely. The mixture was filtered, and the filtrate was concentrated in vacuum and purified by flash

chromatography on silica gel with gradient elution (hexane/ethyl acetate from 10:1 to 4:1) to obtain the product as dark yellow solid 0.38 g, yield in 91%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.09 - 7.12 (m, 2H), 5.58 (s, 2H);

^{13}C NMR (101 MHz, DMSO-d_6) δ 153.0 (dd, J = 247.5, 4.0 Hz), 149.3 (dd, J = 253.5, 5.1 Hz), 134.4 (dd, J = 11.1, 3.0 Hz), 121.9 (t, J = 8.1 Hz), 112.6 (dd, J = 20.2, 4.0 Hz), 110.8, 90.7 (d, J = 16.2 Hz);

MS (ESI$^+$) *m/z*: 155.40 (Calcd for [M+H]$^+$: 155.11);

HRMS (ESI$^+$) *m/z*: 155.0417 (Calcd for [M+H]$^+$: 155.0415).

2,6-difluoro-3-(nonylamino)benzonitrile (3)

The mixture of compound **2** (1.00 g, 6.4 mmol), 1-bromononane (1.61 g, 7.7 mmol), K_2CO_3 (1.35 g, 9.7 mmol) and KI (1.08 g, 6.4 mmol) in DMF (10 mL) was stirred at 110 °C overnight. Then cooled the mixture and water was added. The mixture was extracted with ethyl acetate for 3 times and combined the organic layer was washed by brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by flash chromatography on silica gel with gradient elution (hexane/ethyl acetate from 200:1 to 50:1) to obtain starting material 0.73 g and product as pale yellow oil 0.39 g, yield in 79%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.13 - 7.18 (m, 1H), 7.01 - 7.07 (m, 1H), 5.86 (t, J = 4.89 Hz, 1H), 3.06 (q, J = 6.85 Hz, 2H), 1.53 (quin, J = 7.09 Hz, 2H), 1.24 - 1.28 (m, 12H), 0.85 (t, J = 6.36 Hz, 3H);

^{13}C NMR (101 MHz, DMSO-d_6) δ 152.1 (dd, J = 245.4, 4.0 Hz), 150.0 (dd, J = 254.5, 4.0 Hz), 134.8 (dd, J = 10.1, 3.0 Hz), 117.3 (dd, J = 8.1, 6.1 Hz), 112.5 (dd, J = 19.2, 4.0 Hz), 110.7 (d, J = 2.0 Hz), 90.7 (dd, J = 20.2, 17.2 Hz), 43.1, 31.8, 29.4, 29.3, 29.1, 28.7, 26.9, 22.6, 14.4;

MS (ESI$^+$) *m/z*: 281.05 (Calcd for [M+H]$^+$: 281.36);

HRMS (ESI$^+$) *m/z*: 281.1833 (Calcd for [M+H]$^+$: 281.1824).

2,6-difluoro-N'-hydroxy-3-(nonylamino)benzimidamide (4)

The mixture of compound **3** (0.42 g, 1.5 mmol) and Et_3N (0.76 g, 7.5 mmol) in MeOH (4 mL) was added hydroxylamine hydrochloride (0.42 g, 6.0 mmol) and stirred at 80 °C overnight. TLC showed that the starting material was consumed completely. The mixture was cooled and solvent was removed in vacuum. Then water was added into the mixture and extracted with ethyl acetate for 3 times, the combined organic layer was washed with brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by flash chromatography on silica gel with gradient elution (DCM/MeOH from 100:1 to 10:1) to obtain product as pale yellow oil 0.24 g, yield in 51%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 6.81 (t, *J* = 9.29 Hz, 1H), 6.61 - 6.69 (m, 1H), 4.97 (br. s., 1H), 1.60 - 1.67 (m, 2H), 1.51 - 1.58 (m, 2H), 1.28 (br. s., 12H), 0.88 - 0.91 (m, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 163.45, 151.6 (dd, *J* = 241.4, 5.1 Hz), 148.5 (dd, *J* = 246.4, 8.1 Hz), 144.5 (d, *J* = 10.1 Hz), 133.9 (dd, *J* = 12.1, 3.0Hz), 112.3 (dd, *J* = 9.1, 5.1 Hz), 111.0 (dd, *J* = 23.2, 4.0 Hz), 62.9, 44.0, 32.7, 31.9, 31.8, 27.1, 25.7, 22.6, 14.1;

MS (ESI$^+$) *m/z*: 314.40 (Calcd for $[M+H]^+$: 314.39);

HRMS (ESI$^+$) *m/z*: 314.2045 (Calcd for $[M+H]^+$: 314.2038).

***N*'-acetoxy-2,6-difluoro-3-(nonylamino)benzimidamide (5)**

The mixture of compound **4** (0.12 g, 0.3 mmol) and Ac_2O (0.04 g, 0.3 mmol) in HOAc (1 mL) was stirred at RT for 5 minutes. TLC showed the starting material was consumed completely. Then water was added into the mixture and extracted with ethyl acetate for 3 times, the combined organic layer was washed with brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified

by flash chromatography on silica gel with gradient elution (Hexane/ethyl acetate from 5:1 to 1:1) to obtain product as pale yellow oil 0.11 g, yield in 81%. The obtained product was proceeded to next step without further characterization.

2,6-difluoro-3-(nonylamino)benzimidamide (6)

The mixture of compound **5** (0.12 g, 0.3 mmol) and Pd/C (10% Pd, 55% H_2O) (0.02 g) in MeOH (2 mL) and stirred under H_2 atmosphere for overnight. The mixture was filtered and the organic layer was concentrated in vacuum to obtain a crude product. Then the product was purified by flash chromatography on silica gel with gradient elution (DCM/MeOH from 100:1 to 10:1) to obtain the product as a pale yellow oil 0.08 g, yield in 80%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 6.90 (t, J = 8.0 Hz, 1H), 6.70 - 6.64 (m, 1H), 5.36 (s, 1H), 3.01 - 3.06 (m, 2H), 1.52 - 1.55 (m, 2H), 1.26 (br. s., 12H), 0.86 (t, J = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, DMSO-d_6) δ 156.82, 149.2 (dd, J = 236.3, 6.1 Hz), 146.7 (dd, J = 244.4, 7.1 Hz), 134.3 (dd, J = 13.1, 3.0 Hz), 115.1 (dd, J = 23.2, 19.2 Hz), 111.5 (dd, J = 9.1, 6.1 Hz), 111.2 (dd, J = 22.2, 3.0 Hz), 43.31, 31.8, 29.5, 29.4, 29.2, 28.9, 27.0, 22.6, 14.4;

MS (ESI$^+$) *m/z*: 298.67 (Calcd for $[M+H]^+$: 298.39);

HRMS (ESI$^+$) *m/z*: 314.2045 (Calcd for $[M+H]^+$: 314.2038).

2,6-difluoro-3-(methyl(nonyl)amino)benzonitrile (7)

The mixture of compound **3** (0.59 g, 2.1 mmol) and K_2CO_3 (0.59 g, 4.2 mmol) in DMF (5 mL) was added MeI (1.20 g, 8.4 mmol) and stirred in a seal tube at 60 °C for 24 h. TLC showed the starting material was consumed completely. Water was added into the mixture and extracted with ethyl acetate for 3 times, the combined organic layer was washed by brine and dried over Na_2SO_4. The organic layer was

concentrated in vacuum and purified flash chromatography on by silica gel with gradient elution (hexane/ethyl acetate from 200:1 to 100:1) to obtain the product as a brown oil 0.34 g, yield in 54%.

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 7.35 (dt, *J* = 5.87, 9.78 Hz, 1H), 7.23 - 7.28 (m, 1H), 3.08 (t, *J* = 7.34 Hz, 2H), 2.78 (s, 3H), 1.45 - 1.49 (m, 2H), 1.22 (br. s., 12H), 0.83 - 0.86 (m, 3H);

^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 155.5 (dd, *J* = 251.5, 4.0 Hz), 153.8 (dd, *J* = 258.6, 4.0 Hz), 137.4 (dd, *J* = 7.1, 3.0 Hz), 125.4 (dd, *J* = 9.1, 6.1 Hz), 112.5 (dd, *J* = 19.2, 4.0 Hz), 110.4 (d, *J* = 2.0 Hz), 92.0 (dd, *J* = 21.2, 19.2 Hz), 54.7, 54.7, 31.7, 29.4, 29.2, 29.1, 26.9, 26.7, 22.5, 14.3;

MS (ESI+) *m/z*: 295.67 (Calcd for [M+H]+: 295.38);

HRMS (ESI+) *m/z*: 295.1985 (Calcd for [M+H]+: 295.1980).

2,6-difluoro-N'-hydroxy-3-(methyl(nonyl)amino)benzimidamide (8)

The mixture of compound **7** (0.28 g, 0.9 mmol) and Et_3N (0.48 g, 4.7 mmol) in MeOH (4 mL) was added hydroxylamine hydrochloride (0.26 g, 3.8 mmol) and stirred at 80 °C overnight. The solvent was removed in vacuum. Then water was added into the mixture and extracted with ethyl acetate for 3 times, the combined organic layer was washed with brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by flash chromatography on silica gel with gradient elution (hexane/ethyl acetate from 20:1 to 5:1) to obtain the product as a pale yellow oil 0.24 g, yield in 77%.

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.51 (s, 1H), 6.95 - 7.06 (m, 2H), 5.91 (s, 1H), 2.97 - 3.02 (m, 2H), 2.71 (s, 3H), 1.47 (br. s., 2H), 1.25 (br. s., 12H), 0.86 (t, *J* = 6.85 Hz, 3H);

^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 162.4, 154.7 (dd, *J* = 242.4, 6.1 Hz), 153.1 (dd, *J* = 250.5, 6.1 Hz), 137.2 (dd, *J* = 9.1, 2.0 Hz), 120.3 (dd, *J* = 10.1, 5.1 Hz), 113.1

(dd, *J* = 22.2, 20.2 Hz), 110.9 (dd, *J* = 22.2, 4.0 Hz), 55.3, 55.2, 31.7, 29.5, 29.4, 29.1, 27.1, 27.0, 22.6, 14.4;
MS (ESI+) *m/z*: 328.27 (Calcd for [M+H]+: 328.41);
HRMS (ESI+) *m/z*: 328.2201 (Calcd for [M+H]+: 328.2195).

N'-acetoxy-2,6-difluoro-3-(methyl(nonyl)amino)benzimidamide (9)

The mixture of compound **8** (0.10 g, 0.3 mmol) and Ac_2O (0.03 g, 0.3 mmol) in HOAc (1 mL) was stirred at RT for 5 minutes. TLC showed the starting material was consumed completely. Then water was added into the mixture and extracted with ethyl acetate for 3 times, the combined organic layer was washed with brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with gradient elution (Hexane/ethyl acetate from 5:1 to 1:1) to obtain product as pale yellow oil 0.08 g, yield in 74%.

2,6-difluoro-3-(methyl(nonyl)amino)benzimidamide (10)

The mixture of compound **9** (0.10 g, 0.3 mmol) and Pd/C (10% Pd, 55% H_2O) (0.02 g) in MeOH (2 mL) was stirred with H_2 overnight. Then the mixture was filtered and the filtrate was concentrated in vacuum and the crude product was purified by silica gel with gradient elution (DCM/MeOH from 100:1 to 15:1) to obtain the final product as the pale yellow oil 0.18 g, yield in 19%.
^{1}H NMR (400 MHz, DMSO-d_6) δ 9.69 (br. s., 3H), 7.17 - 7.27 (m, 2H), 3.03 - 3.06 (m, 2H), 2.76 (s, 3H), 1.49 (br. s., 2H), 1.25 (br. s., 12H), 0.84 - 0.87 (m, 3H);
^{13}C NMR (101 MHz, DMSO-d_6) δ 158.49, 152.2 (dd, *J* = 245.4, 4.0 Hz), 150.7 (dd, *J* = 252.5, 6.1 Hz), 137.5 (dd, *J* = 8.1, 2.0 Hz), 122.8 (dd, *J* = 9.1, 6.1 Hz), 111.9 (dd, *J* = 21.2, 4.0 Hz), 109.6 (t, *J* = 19.1 Hz), 55.1, 55.0, 31.7, 29.5, 29.4, 29.1, 27.1, 26.9, 22.6, 14.4;

MS (ESI+) *m/z*: 312.33 (Calcd for [M+H]+: 312.41);
HRMS (ESI+) *m/z*: 312.2251 (Calcd for [M+H]+: 312.2246).

2-(3-bromophenoxy)-6-fluoro-3-(nonylamino)benzonitrile (11)

The mixture of compound **3** (0.88 g, 3.1 mmol), KOH (0.70 g, 12.6 mmol) and phenol (1.63 g, 9.4 mmol) in DMF (6 mL) was stirred at room temperature for 10 h. TLC showed the starting material was consumed completely. Then water was added into the mixture and extracted with ethyl acetate for 3 times, the combined organic layer was washed with brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with Hexane/ethyl acetate 100:1 to obtain product as pale yellow oil 1.10 g, yield in 79%.
^{1}H NMR (400 MHz, CHLOROFORM) δ 7.29 - 7.18 (m, 1H), 7.10 - 7.03 (m, 2H), 6.86 (t, *J* = 8.0 Hz, 1H), 6.82 - 6.78 (m, 1H), 6.72 - 6.68 (m, 1H), 4.79 (br. s., 1H), 3.09 (t, *J* = 8.0 Hz, 2H), 1.65 (t, *J* = 8.0 Hz, 2H), 1.30 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);
^{13}C NMR (101 MHz, CHLOROFORM) δ 156.8, 155.1 (d, *J* = 249.5 Hz), 153.9, 142.4, 140.9, 130.7, 123.6, 118.9, 114.3, 114.2 (d, *J* = 8.1 Hz), 112.3 (d, *J* = 21.2 Hz), 110.6 (d, *J* = 20.2 Hz), 97.9, 43.9, 31.9, 29.5, 29.4, 29.3, 29.3, 27.1, 22.7, 14.1;
MS (ESI^+) *m/z*: 433.45 MS (Calcd for $[M+H]^+$: 433.35);
HRMS (ESI+) *m/z*: 433.1282 (Calcd for [M+H]+: 433.1285).

2-(3-ethylphenoxy)-6-fluoro-3-(nonylamino)benzonitrile (12)

Following the experimental procedure for the preparation of compound **11** described above, but with compound **3** (0.85 g, 3.0 mmol), 3-ethylphenol (1.13 g, 9.1 mmol) and KOH (0.68 g, 12.1 mmol) as a starting material, the product was obtained as pale yellow oil 0.42 g, yield in 36%.

^{1}H NMR (400 MHz, CHLOROFORM): δ 7.23 (t, *J* = 8.0 Hz, 1H), 7.01 - 6.95 (m, 2H), 6.90 - 6.88 (m, 1H), 6.80 (br. s., 1H), 6.71 - 6.69 (m, 1H), 3.98 (t, *J* = 4.0 Hz, 1H), 3.12 - 3.07 (m, 2H), 2.68 - 2.62 (m, 2H), 1.54 (t, *J* = 4.0 Hz, 2H), 1.26 (br. s., 12H), 0.92 - 0.89 (m, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 156.8, 154.8 (d, *J* = 249.5 Hz), 146.6, 142.3 (d, *J* = 3.0 Hz), 139.0 (d, *J* = 2.0 Hz), 129.7, 123.1, 115.6 (d, *J* = 8.1 Hz), 115.2, 112.8 (d, *J* = 20.2 Hz), 112.53, 111.26, 97.3 (d, *J* = 17.2 Hz), 43.6, 42.5, 31.9, 29.5, 29.3, 29.2, 29.1, 26.9, 22.7, 15.3, 14.1

MS (ESI^{+}) *m/z*: 382.47 MS (Calcd for [M+H]$^{+}$: 382.51);

HRMS (ESI+) *m/z*: 383.2490 (Calcd for [M+H]+: 383.2493).

6-fluoro-2-(4-methoxyphenoxy)-3-(nonylamino)benzonitrile (13)

Following the experimental procedure for the preparation of compound **11** described above, but with compound **3** (1.00 g, 3.6 mmol), 4-methoxyphenol (1.33 g, 10.7 mmol) and KOH (0.80 g, 14.2 mmol) as a starting material, the product was obtained as pale yellow oil 0.70 g, yield in 51%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 6.98 - 6.94 (m, 1H), 6.89 - 6.84 (m, 5H), 4.05 (br. s., 1H), 3.78 (s, 3H), 3.09 (t, *J* = 4.0 Hz, 2H), 1.55 (t, *J* = 4.0 Hz, 2H), 1.27 (s, 12H), 0.91 (t, *J* = 4.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 155.7, 154.3 (d, *J* = 249.5 Hz), 150.7, 143.0 (d, *J* = 2.0 Hz), 139.0 (d, *J* = 2.0 Hz), 116.7, 115.6 (d, *J* = 8.1 Hz), 115.0, 112.6 (d, *J* = 19.2 Hz), 111.3, 97.0 (d, *J* = 18.2 Hz), 55.6, 43.6, 31.9, 29.5, 29.3, 29.2, 29.1, 26.9, 22.7, 14.1;

MS (ESI^{+}) *m/z*: 384.55 MS (Calcd for [M+H]$^{+}$: 384.49);

HRMS (ESI+) *m/z*: 385.2282 (Calcd for [M+H]+: 385.2286).

6-fluoro-3-(nonylamino)-2-(4-propylphenoxy)benzonitrile (14)

Following the experimental procedure for the preparation of compound **11** described above, but with compound **3** (0.95 g, 3.4 mmol), 4-propylphenol (1.38 g, 10.2 mmol) and KOH (0.76 g, 13.6 mmol) as a starting material, the product was obtained as pale yellow oil 0.80 g, yield in 59%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.13 (d, *J* = 8.0 Hz, 2H), 7.00 - 6.95 (m, 1H), 6.89 - 6.86 (m, 1H), 6.84 (d, *J* = 8.0 Hz, 2H), 4.01 - 3.98 (m, 1H), 3.09 (d, *J* = 8.0 Hz, 2H), 2.57 (t, *J* = 8.0 Hz, 2H), 1.64 (q, *J* = 8.0 Hz, 2H), 1.54 (t, *J* = 8.0 Hz, 2H), 1.26 (br. s., 12H), 0.96 (t, *J* = 8.0 Hz, 3H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 154.8, 154.4 (d, *J* = 249.5 Hz), 142.7 (d, *J* = 3.0 Hz), 139.0, 137.8, 129.8, 115.5 (d, *J* = 8.1 Hz), 115.4, 112.7, 111.2, 97.4 (d, *J* = 18.2 Hz), 43.6, 37.2, 31.8, 29.5, 29.3, 29.2, 29.1, 26.8, 24.6, 22.6, 14.1, 13.7.

MS (ESI^{+}) *m/z*: 396.54 MS (Calcd for [M+H]$^{+}$: 396.54);

HRMS (ESI+) *m/z*: 397.2653 (Calcd for [M+H]+: 397.265).

2-(3,5-dimethylphenoxy)-6-fluoro-3-(nonylamino)benzonitrile (15)

Following the experimental procedure for the preparation of compound **11** described above, but with compound **3** (0.90 g, 3.2 mmol), 3,5-dimethylphenol (1.18 g, 9.6 mmol) and KOH (0.72 g, 12.8 mmol) as a starting material, the product was obtained as pale yellow oil 0.80 g, yield in 65%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.01 - 6.96 (m, 1H), 6.89 - 6.85 (m, 1H), 6.75 (s, 1H), 6.52 (s, 2H), 3.96 (s, 1H), 3.09 (q, *J* = 8.0 Hz, 2H), 2.29 (s, 6H), 1.53 (t, *J* = 8.0 Hz, 2H), 1.26 (br. s., 12H), 0.91 (t, *J* = 3.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 156.7, 154.4 (d, *J* = 249.5 Hz), 142.4 (d, *J* = 3.0 Hz), 139.8, 139.0 (d, *J* = 2.0 Hz), 125.3, 115.5 (d, *J* = 9.0 Hz), 113.1, 112.7

(d, *J* = 20.2 Hz), 111.3, 97.5 (d, *J* = 3.0 Hz), 43.6, 31.8, 29.5, 29.3, 29.2, 29.1, 26.8, 22.6, 21.4, 14.1.

MS (ESI^+) *m/z*: 382.55 MS (Calcd for $[M+H]^+$: 382.51);

HRMS (ESI+) *m/z*: 383.2493 (Calcd for [M+H]+: 383.2493).

2-(4-bromophenoxy)-6-fluoro-3-(nonylamino)benzonitrile (16)

Following the experimental procedure for the preparation of compound **11** described above, but with compound **3** (0.90 g, 3.2 mmol), 4-bromophenol (1.67 g, 9.6 mmol) and KOH (0.72 g, 12.8 mmol) as a starting material, the product was obtained as pale yellow oil 1.00 g, yield in 71%.

^1H NMR (400 MHz, CHLOROFORM) δ: 7.44 (d, *J* = 8.0 Hz, 2H), 7.03 - 6.99 (m, 1H), 6.91 - 6.88 (m, 1H), 6.82 (d, *J* = 12.0 Hz, 2H), 3.93 (s, 1H), 3.09 (t, *J* = 8.0 Hz, 2H), 1.55 (t, *J* = 8.0 Hz, 2H), 1.26 (br. s., 12H), 0.90 (t, *J* = 3.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 155.8, 154.4 (d, *J* = 250.5 Hz), 142.4 (d, *J* = 3.0 Hz), 141.6 (d, *J* = 3.0 Hz), 138.9, 132.9, 117.3, 115.9 (d, *J* = 8.1 Hz), 112.2 (d, *J* = 21.2 Hz), 111.0, 97.3 (d, *J* = 18.2 Hz), 43.6, 31.8, 29.5, 29.3, 29.2, 29.1, 26.9, 22.7, 14.1.

MS (ESI^+) *m/z*: 433.35 MS (Calcd for $[M+H]^+$: 433.36);

HRMS (ESI+) *m/z*: 433.1286 (Calcd for [M+H]+: 433.1285).

2-(4-aminophenoxy)-6-fluoro-3-(nonylamino)benzonitrile (17)

Following the experimental procedure for the preparation of compound **11** described above, but with compound **3** (1.10 g, 3.9 mmol), 4-aminophenol (1.28 g, 11.8 mmol) and KOH (0.88 g, 15.7 mmol) as a starting material, the product was obtained as pale yellow oil 0.75 g, yield in 51%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 6.95 - 6.91 (m, 1H), 6.85 - 6.82 (m, 1H), 6.76 (d, *J* = 8.0 Hz, 2H), 6.63 (d, *J* = 8.0 Hz, 2H), 4.05 (s, 1H), 3.55 (s, 2H), 3.08 (t, *J* = 8.0 Hz, 2H), 1.55 (t, *J* = 8.0 Hz, 2H), 1.27 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 154.4 (d, *J* = 125.2 Hz), 149.6, 143.5 (d, *J* = 3.0 Hz), 142.6, 139.0 (d, *J* = 2.0 Hz), 116.9, 116.3, 115.3 (d, *J* = 8.1 Hz), 112.2 (d, *J* = 10.1 Hz), 111.4, 96.9 (d, *J* = 17.2 Hz), 43.7, 31.9, 29.5, 29.3, 29.2, 29.1, 26.9, 22.7, 14.1.

MS (ESI^{+}) *m/z*: 369.44 MS (Calcd for [M+H]$^{+}$: 369.48);

HRMS (ESI+) *m/z*: 370.2292 (Calcd for [M+H]+: 370.2289).

6-fluoro-N-hydroxy-2-(4-methoxyphenoxy)-3-(nonylamino)benzimidamide (18)

The mixture of compound **13** (0.70 g, 1.8 mmol), Et_3N (1.47 g, 14.6 mmol) and Hydroxylamine hydrochloride (0.51 g, 7.3 mmol) in MeOH (7 mL) was stirred at reflux for 10 h. TLC showed the starting material was consumed completely. Then cool down the mixture and water was added into the mixture. It was extracted with ethyl acetate for 3 times, and the combined organic layer was washed with brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel (Hexane/ethyl acetate from 100:1 to 2:1) to obtain product as pale yellow oil 0.65 g, yield in 85%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 6.93 - 6.88 (m, 1H), 6.84 - 6.76 (m, 4H), 6.72 - 6.67 (m, 1H), 3.86 (s, 1H), 3.74 (s, 3H), 3.05 (t, *J* = 8.0 Hz, 2H), 1.50 (t, *J* = 8.0 Hz, 2H), 1.24 (br. s., 12H), 0.89 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 155.0, 154.3 (d, *J* = 168.7 Hz), 151.5, 145.6 (d, *J* = 1.0 Hz), 139.0 (d, *J* = 5.1 Hz), 138.8 (d, *J* = 3.0 Hz), 116.3, 115.9 (d, *J* = 18.2 Hz), 114.7, 112.7 (d, *J* = 23.2 Hz), 112.2, (d, *J* = 9.1 Hz), 55.6, 43.9, 31.9,

29.5, 29.3, 29.2, 29.2, 26.9, 22.7, 14.1.

MS (ESI^+) *m/z*: 403.50 MS (Calcd for $[M+H]^+$: 403.49);

HRMS (ESI+) *m/z*: 418.2499 (Calcd for [M+H]+: 418.25).

2-(4-aminophenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (19)

Following the experimental procedure for the preparation of compound **18** described above, but with compound **17** (0.75 g, 2.0 mmol), Et_3N (1.64 g, 16.2 mmol) and Hydroxylamine hydrochloride (0.56 g, 8.1 mmol) as starting material to obtain product as pale yellow oil 0.65 g, yield in 79%.

^1H NMR (400 MHz, CHLOROFORM) δ: 6.90 - 6.86 (m, 1H), 6.67 - 6.65 (m, 3H), 6.50 (d, *J* = 8.0 Hz, 2H), 4.72 (s, 2H), 3.90 (s, 1H), 3.04 (t, *J* = 8.0 Hz, 2H), 1.51 (s, 2H), 1.26 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 152.3 (d, *J* = 241.4 Hz), 150.5, 145.6, 141.5, 140.2 (d, *J* = 5.1 Hz), 138.9 (d, *J* = 2.0 Hz), 116.6, 116.3, 116.0 (d, *J* = 18.2 Hz), 112.5 (d, *J* = 23.2 Hz), 112.1 (d, *J* = 9.1 Hz), 44.0, 31.9, 29.5, 29.4, 29.3, 29.2, 27.0, 22.7, 14.1.

MS (ESI^+) *m/z*: 402.50 MS (Calcd for $[M+H]^+$: 402.51);

HRMS (ESI+) *m/z*: 403.2505 (Calcd for [M+H]+: 403.2504).

2-(4-bromophenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (20)

Following the experimental procedure for the preparation of compound **18** described above, but with compound **16** (0.50 g, 1.2 mmol), Et_3N (0.93 g, 0.9 mmol) and Hydroxylamine hydrochloride (0.32 g, 4.6 mmol) as starting material to obtain product as pale yellow oil 0.20 g, yield in 36%.

^1H NMR (400 MHz, CHLOROFORM) δ: 7.36 (d, *J* = 12.0 Hz, 2H), 6.98 - 6.94 (m, 1H), 6.77 (d, *J* = 8.0 Hz, 2H), 6.74 - 6.70 (m, 1H), 4.71 (s, 2H), 3.77 (s, 1H), 3.07

(t, *J* = 8.0 Hz, 2H), 1.52 (t, *J* = 8.0 Hz, 2H), 1.25 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 156.6, 152.2 (d, *J* = 242.4 Hz), 145.3, 138.8 (d, *J* = 5.1 Hz), 138.6 (d, *J* = 2.0 Hz), 132.5, 117.2, 115.8 (d, *J* = 18.2 Hz), 114.9, (d, *J* = 23.2 Hz), 113.35, 112.5 (d, *J* = 9.1 Hz), 43.8, 31.9, 29.5, 29.3, 29.2, 29.2, 26.9, 22.7, 14.1.

MS (ESI$^+$) *m/z*: 466.40 MS (Calcd for [M+H]$^+$: 466.39);

HRMS (ESI+) *m/z*: 466.1500 (Calcd for [M+H]+: 466.1500).

6-fluoro-N-hydroxy-3-(nonylamino)-2-(4-propylphenoxy)benzimidamide (21)

Following the experimental procedure for the preparation of compound **18** described above, but with compound **14** (0.80 g, 2.0 mmol), Et_3N (1.63 g, 16.1 mmol) and Hydroxylamine hydrochloride (0.56 g, 8.1 mmol) as starting material to obtain product as pale yellow oil 0.55 g, yield in 63%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.06 (d, *J* = 8.0 Hz, 2H), 6.96 - 6.91 (m, 1H), 6.80 (d, *J* = 8.0 Hz, 2H), 6.72 - 6.69 (m, 1H), 4.72 (s, 2H), 3.83 (s, 1H), 3.06 - 3.05 (m, 2H), 2.53 (t, *J* = 8.0 Hz, 2H), 1.62 (t, *J* = 8.0 Hz, 2H), 1.50 (s, 2H), 1.24 (br. s., 12H), 0.95 - 0.89 (m, 6H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 155.5, 152.3 (d, *J* = 241.4 Hz), 145.6, 139.5 (d, *J* = 4.0 Hz), 138.8 (d, *J* = 2.0 Hz), 136.8, 129.5, 115.9 (d, *J* = 17.2 Hz), 115.11, 112.9, (d, *J* = 22.2 Hz), 112.2 (d, *J* = 8.1 Hz), 43.9, 37.2, 31.9, 29.5, 29.3, 29.2, 29.2, 26.9, 24.6, 22.7, 14.1, 13.8.

MS (ESI$^+$) *m/z*: 429.54 (Calcd for [M+H]$^+$: 429.57);

HRMS (ESI+) *m/z*: 430.2866 (Calcd for [M+H]+: 430.2864).

2-(3,5-dimethylphenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (22)

Following the experimental procedure for the preparation of compound **18** described above, but with compound **15** (0.80 g, 2.1 mmol), Et_3N (1.69 g, 16.7 mmol) and Hydroxylamine hydrochloride (0.58 g, 8.4 mmol) as starting material to obtain product as pale yellow oil 0.37 g, yield in 42%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 6.96 - 6.92 (m, 1H), 6.73 - 6.69 (m, 1H), 6.67 (s, 1H), 6.52 (m, 2H), 4.75 (s, 2H), 3.82 (s, 1H), 3.08 (t, *J* = 8.0 Hz, 2H), 2.26 (s, 6H), 1.51 (t, *J* = 8.0 Hz, 2H), 1.26 (br. s., 12H), 0.92 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 157.3, 152.3 (d, *J* = 241.4 Hz), 145.5, 139.5, 139.3 (d, *J* = 5.1 Hz), 138.7 (d, *J* = 2.0 Hz), 124.5, 116.0 (d, *J* = 18.2 Hz), 112.9, 112.9, (d, *J* = 23.2 Hz), 112.3 (d, *J* = 9.1 Hz), 43.9, 31.9, 29.5, 29.4, 29.3, 29.2, 26.9, 22.7, 21.3, 14.1.

MS (ESI$^+$) *m/z*: 415.55 (Calcd for $[M+H]^+$: 415.54);

HRMS (ESI+) *m/z*: 416.2710 (Calcd for [M+H]+: 416.2708).

2-(3-bromophenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (23)

Following the experimental procedure for the preparation of compound **18** described above, but with compound **11** (0.20 g, 0.4 mmol), Et_3N (0.37 g, 3.7 mmol) and Hydroxylamine hydrochloride (0.13 g, 1.9 mmol) as starting material to obtain product as pale yellow oil 0.07 g, yield in 32%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.18 - 7.11 (m, 2H), 7.06 - 7.05 (m, 1H), 7.00 - 6.95 (m, 1H), 6.83 - 6.81 (m, 1H), 6.75 - 6.71 (m, 1H), 4.71 (s, 1H), 3.76 (t, *J* = 4.0 Hz, 2H), 3.08 (t, *J* = 8.0 Hz, 2H), 1.52 (t, *J* = 8.0 Hz, 2H), 1.25 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 158.1, 152.2 (d, *J* = 241.4 Hz), 145.5, 138.5, 138.5 (d, *J* = 5.1 Hz), 130.8, 125.8, 122.9, 118.9, 115.8 (d, *J* = 18.2 Hz), 114.2, 113.5, 112.5 (d, *J* = 9.1 Hz), 43.8, 31.9, 29.5, 29.3, 29.2, 29.2, 26.9, 22.7, 14.1.

MS (ESI$^+$) *m/z*: 466.34 (Calcd for [M+H]$^+$: 466.39);

HRMS (ESI+) *m/z*: 466.1497 (Calcd for [M+H]+: 466.15).

2-(3-ethylphenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (24)

Following the experimental procedure for the preparation of compound **18** described above, but with compound **12** (0.10 g, 0.3 mmol), Et_3N (0.21 g, 2.1 mmol) and Hydroxylamine hydrochloride (0.07 g, 1.1 mmol) as starting material to obtain product as pale yellow oil 0.05 g, yield in 46%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.18 - 7.14 (m, 1H), 6.96 - 6.91 (m, 1H), 6.87 (d, *J* = 8.0 Hz, 1H), 6.77 (s, 1H), 6.73 - 6.66 (m, 2H), 4.72 (s, 2H), 3.82 (s, 1H), 3.06 (t, *J* = 8.0 Hz, 2H), 2.60 (q, *J* = 8.0 Hz, 2H), 1.50 (t, *J* = 8.0 Hz, 2H), 1.24 (br. s., 12H), 1.21 (t, *J* = 8.0 Hz, 3H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 157.5, 152.2 (d, *J* = 242.4 Hz), 146.3, 145.6 (d, *J* = 2.0 Hz), 139.3 (d, *J* = 5.1 Hz), 138.7 (d, *J* = 2.0 Hz), 129.5, 122.2, 115.9 (d, *J* = 18.2 Hz), 115.0, 112.9 (d, *J* = 23.2 Hz), 112.41, 112.3 (d, *J* = 9.1 Hz), 43.9, 31.9, 29.5, 29.3, 29.2, 29.2, 28.8, 26.9, 22.7, 15.4

MS (ESI$^+$) *m/z*: 415.55 (Calcd for [M+H]$^+$: 415.54);

HRMS (ESI+) *m/z*: 416.2707 (Calcd for [M+H]+: 416.2708).

N-acetoxy-6-fluoro-2-(4-methoxyphenoxy)-3-(nonylamino)benzimidamide (25)

The mixture of compound **18** (0.35 g, 0.8 mmol) and acetic anhydride (0.13 g, 1.3 mmol) in Acetic acid (2 mL) was stirred at RT for 5 minutes. TLC showed the

starting material was consumed completely. Then water was added into the mixture and extracted with ethyl acetate for 3 times, and the combined organic layer was washed with sodium carbonate solution and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with Dichloromethane to obtain product as pale yellow oil 0.15 g, yield in 38%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 6.96 - 6.91 (m, 1H), 6.85 (d, *J* = 8.0 Hz, 2H), 6.80 (d, *J* = 8.0 Hz, 2H), 6.75 - 6.71 (m, 1H), 4.92 (s, 2H), 3.85 (s, 1H), 3.77 (s, 3H), 3.06 (t, *J* = 8.0 Hz, 2H), 2.09 (s, 3H), 1.50 (t, *J* = 8.0 Hz, 2H), 1.24 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.2, 155.1, 152.1 (d, *J* = 241.4 Hz), 151.5, 149.4, 139.8 (d, *J* = 4.0 Hz), 138.8 (d, *J* = 2.0 Hz), 116.4, 114.7, 114.5, 113.0 (d, *J* = 8.1 Hz), 112.8 (d, *J* = 21.2 Hz), 55.6, 43.8, 31.8, 29.5, 29.3, 29.2, 29.2, 26.9, 22.6, 19.8, 14.1.

MS (ESI$^+$) *m/z*: 431.51 (Calcd for $[M+H]^+$: 431.50);

HRMS (ESI+) *m/z*: 460.2605 (Calcd for [M+H]+: 460.2606).

N-acetoxy-2-fluoro-5-(nonylamino)-6-(4-propylphenoxy)cyclohexa-2,4-dienecarboximidamide (26)

Following the experimental procedure for the preparation of compound **25** described above, but with compound **21** (0.55 g, 1.3 mmol) and acetic anhydride (0.14 g, 1.4 mmol) in Acetic acid (2 mL) as starting material to obtain product as pale yellow oil 0.36 g, yield in 58%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.04 (d, *J* = 8.0 Hz, 2H), 6.94 - 6.89 (m, 1H), 6.80 (d, *J* = 8.0 Hz, 2H), 6.73 - 6.70 (m, 1H), 4.98 (s, 2H), 3.81 (s, 1H), 3.03 (t, *J* = 8.0 Hz, 2H), 2.51 (t, *J* = 8.0 Hz, 2H), 2.01 (s, 3H), 1.58 (q, *J* = 8.0 Hz, 2H), 1.47 (t, *J* = 8.0 Hz, 2H), 1.22 (br. s., 12H), 0.93 - 0.86 (m, 6H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.5, 155.5, 152.0 (d, *J* = 242.4 Hz), 149.5, 139.4 (d, *J* = 4.0 Hz), 138.7 (d, *J* = 2.0 Hz), 137.0, 129.5, 115.2, 114.7 (d, *J* = 18.2 Hz), 113.0 (d, *J* = 4.0 Hz), 112.8 (d, *J* = 18.2 Hz), 43.8, 37.1, 31.8, 29.5, 29.3, 29.2, 29.1, 26.8, 24.6, 22.6, 19.7, 14.1, 13.7.

MS (ESI$^+$) *m/z*: 473.60 (Calcd for [M+H]$^+$: 473.62);

HRMS (ESI+) *m/z*: 472.2969 (Calcd for [M+H]+: 472.297).

N-acetoxy-2-(4-aminophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (27)

Following the experimental procedure for the preparation of compound **25** described above, but with compound **19** (0.65 g, 1.6 mmol) and acetic anhydride (0.16 g, 1.6 mmol) in Acetic acid (2 mL) as starting material to obtain product as pale yellow oil 0.34 g, yield in 46%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 6.94 - 6.90 (m, 1H), 6.75 - 6.70 (m, 3H), 6.59 (d, *J* = 8.0 Hz, 2H), 4.91 (s, 2H), 3.87 (s, 1H), 3.05 (t, *J* = 8.0 Hz, 2H), 2.11 (s, 3H), 1.50 (t, *J* = 8.0 Hz, 2H), 1.26 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.3, 152.1 (d, *J* = 241.4 Hz), 150.5, 149.6, 141.7, 140.1 (d, *J* = 4.0 Hz), 138.9 (d, *J* = 3.0 Hz), 116.5, 116.3, 114.6 (d, *J* = 17.2 Hz), 112.9 (d, *J* = 9.1 Hz), 112.6 (d, *J* = 22.2 Hz), 43.9, 31.9, 29.5, 29.3, 29.2, 29.2, 26.9, 22.7, 19.8, 14.1.

MS (ESI$^+$) *m/z*: 445.52 (Calcd for [M+H]$^+$: 445.54);

HRMS (ESI+) *m/z*: 445.2609 (Calcd for [M+H]+: 445.2609).

N-acetoxy-2-(3,5-dimethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (28)

Following the experimental procedure for the preparation of compound **25** described above, but with compound **22** (0.37 g, 0.9 mmol) and acetic anhydride

(0.09 g, 0.9 mmol) in Acetic acid (2 mL) as starting material to obtain product as pale yellow oil 0.22 g, yield in 54%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 6.95 - 6.90 (m, 1H), 6.74 - 6.70 (m, 1H), 6.65 (s, 1H), 6.51 (s, 2H), 4.99 (s, 2H), 3.82 (s, 1H), 3.04 (t, *J* = 8.0 Hz, 2H), 2.24 (s, 6H), 2.04 (s, 3H), 1.47 (t, *J* = 8.0 Hz, 2H), 1.22 (br. s., 12H), 0.89 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.5, 157.4, 152.0 (d, *J* = 242.4 Hz), 149.5, 139.6, 139.2 (d, *J* = 4.0 Hz), 138.7 (d, *J* = 3.0 Hz), 124.5, 114.7 (d, *J* = 18.2 Hz), 113.0, 113.0, 112.9 (d, *J* = 18.2 Hz), 43.8, 31.9, 29.5, 29.3, 29.2, 29.1, 26.8, 22.6, 21.3, 19.7, 14.1.

MS (ESI^{+}) *m/z*: 458.51 (Calcd for [M+H]$^{+}$: 458.58);

HRMS (ESI+) *m/z*: 458.2813 (Calcd for [M+H]+: 458.2813).

N-acetoxy-2-(4-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (29)

Following the experimental procedure for the preparation of compound **25** described above, but with compound **20** (0.10 g, 0.2 mmol) and acetic anhydride (0.02 g, 0.2 mmol) in Acetic acid (2 mL) as starting material to obtain product as pale yellow oil 0.06 g, yield in 54%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.36 (d, *J* = 8.0 Hz, 2H), 6.99 - 6.95 (m, 1H), 6.80 (d, *J* = 8.0 Hz, 2H), 6.77 - 6.73 (m, 1H), 4.94 (s, 2H), 3.77 (s, 1H), 3.06 (t, *J* = 8.0 Hz, 2H), 2.08 (s, 3H), 1.50 (t, *J* = 8.0 Hz, 2H), 1.24 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.1, 156.6, 152.0 (d, *J* = 242.4 Hz), 149.1, 140.4, 138.7 (d, *J* = 5.1 Hz), 138.6 (d, *J* = 3.0 Hz), 132.5, 117.3, 115.1, 113.4 (d, *J* = 22.2 Hz), 113.2 (d, *J* = 9.1 Hz), 43.7, 31.8, 29.5, 29.3, 29.2, 29.1, 26.9, 22.7, 19.7, 14.1

MS (ESI^{+}) *m/z*: 508.40 (Calcd for [M+H]$^{+}$: 508.42);

HRMS (ESI+) *m/z*: 508.1607 (Calcd for [M+H]+: 508.1606).

N-acetoxy-2-(3-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (30)

Following the experimental procedure for the preparation of compound **25** described above, but with compound **23** (0.07 g, 0.2 mmol) and acetic anhydride (0.02 g, 0.2 mmol) in Acetic acid (2 mL) as starting material to obtain product as pale yellow oil 0.04 g, yield in 52%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.19 - 7.12 (m, 1H), 7.09 (s, 1H), 7.01 - 6.96 (m, 1H), 6.85 - 6.84 (m, 1H), 6.78 - 6.75 (m, 1H), 4.93 (s, 2H), 3.83 (s, 1H), 3.08 (t, *J* = 8.0 Hz, 2H), 2.09 (s, 3H), 1.52 (t, *J* = 8.0 Hz, 2H), 1.26 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.2, 158.1, 152.0 (d, *J* = 242.4 Hz), 148.9, 138.6 (d, *J* = 2.0 Hz), 133.9, 130.8, 127.7, 125.9, 122.8, 119.1, 114.3, 113.5 (d, *J* = 22.2 Hz), 113.2 (d, *J* = 9.1 Hz), 43.7, 31.9, 29.5, 29.3, 29.2, 29.1, 26.9, 22.7, 19.7, 14.1.

N'-acetoxy-2-(3-ethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (31)

Following the experimental procedure for the preparation of compound **25** described above, but with compound **24** (0.05 g, 0.1 mmol) and acetic anhydride (0.01 g, 0.1 mmol) in Acetic acid (2 mL) as starting material to obtain product as pale yellow oil 0.03 g, yield in 45%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.17 (t, *J* = 8.0 Hz, 1H), 6.98 - 6.94 (m, 1H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.78 (s, 1H), 6.76 - 6.73 (m, 1H), 6.70 - 6.67 (m, 1H), 4.92 (s, 2H), 3.82 (s, 1H), 3.06 (t, *J* = 8.0 Hz, 2H), 2.64 - 2.58 (m, 2H), 2.06 (s, 3H), 1.49 (t, *J* = 8.0 Hz, 2H), 1.23 (br. s., 12H), 1.21 (t, *J* = 8.0 Hz, 3H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.4, 157.6, 152.0 (d, *J* = 242.4 Hz), 149.4, 146.4, 138.8 (d, *J* = 2.0 Hz), 138.6, 129.5, 122.3, 115.2, 115.1, 113.1, 112.9 (d, *J* = 11.1 Hz), 112.5, 43.8, 31.9, 29.5, 29.3, 29.2, 29.1, 28.8, 26.9, 22.6, 19.7, 15.5, 14.1.

MS (ESI$^+$) *m/z*: 458.54 (Calcd for [M+H]$^+$: 458.58);

HRMS (ESI+) *m/z*: 458.2812 (Calcd for [M+H]+: 458.2813).

6-fluoro-2-(4-methoxyphenoxy)-3-(nonylamino)benzimidamide (32)

The mixture of compound **25** (0.05 g) and Pd/C (10% Pd, 55% H_2O) (0.01 g) in MeOH (2 mL) was stirred with H_2 at room temperature overnight. TLC showed the starting material was consumed completely. Then the solvent was concentrated in vacuum and purified by silica gel (Dichloromethane/MeOH from 100:1 to 10:1) to obtain product as pale yellow oil 0.02 g, yield in 54%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.29 (s, 2H), 7.10 - 7.05 (m, 1H), 6.92 - 6.88 (m, 1H), 6.85 - 6.78 (m, 4H), 3.96 (s, 1H), 3.78 (s, 3H), 3.06 (t, *J* = 8.0 Hz, 2H), 1.50 (t, *J* = 4.0 Hz, 2H), 1.23 (br. s., 12H), 0.89 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 160.8, 156.0, 150.9 (d, *J* = 246.4 Hz), 149.7, 139.6, 138.1 (d, *J* = 3.0 Hz), 116.0 (d, *J* = 9.1 Hz), 115.7, 115.5, 114.3 (d, *J* = 23.2 Hz), 110.9 (d, *J* = 14.1 Hz), 55.7, 43.5, 31.8, 29.4, 29.2, 29.2, 29.0, 26.8, 22.6, 14.1.

MS (ESI$^+$) *m/z*: 402.51 (Calcd for [M+H]$^+$: 402.52);

HRMS (ESI+) *m/z*: 402.2558 (Calcd for [M+H]+: 402.2551).

6-fluoro-3-(nonylamino)-2-(4-propylphenoxy)benzimidamide (33)

Following the experimental procedure for the preparation of compound **32** described above, but with compound **26** (0.09 g, 0.2 mmol) and and Pd/C (10% Pd,

55% H_2O) (0.02 g) in MeOH (2 mL) as starting material to obtain product as pale yellow oil 0.06 g, yield in 75%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 8.02 (s, 3H), 7.04 (d, *J* = 8.0 Hz, 2H), 6.96 - 6.91 (m, 1H), 6.78 - 6.74 (m, 1H), 6.72 (d, *J* = 8.0 Hz, 2H), 3.85 (s, 1H), 2.98 (d, *J* = 8.0 Hz, 2H), 2.48 (d, *J* = 8.0 Hz, 2H), 1.56 (q, *J* = 8.0 Hz, 2H), 1.41 (t, *J* = 8.0 Hz, 2H), 1.20 (br. s., 12H), 0.90 - 0.83 (m, 6H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 160.5, 154.4, 150.6 (d, *J* = 245.4 Hz), 139.0 (d, *J* = 3.0 Hz), 137.8, 137.8 (d, *J* = 4.0 Hz), 129.9, 114.8, 114.7, 113.6 (d, *J* = 23.2 Hz), 113.2 (d, *J* = 15.2 Hz), 43.5, 37.1, 31.8, 29.4, 29.2, 29.2, 29.0, 26.8, 24.5, 22.6, 14.1, 13.7.

MS (ESI$^+$) *m/z*: 414.53 (Calcd for $[M+H]^+$: 414.57);

HRMS (ESI+) *m/z*: 414.2924 (Calcd for [M+H]+: 414.2915).

2-(3,5-dimethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (34)

Following the experimental procedure for the preparation of compound **32** described above, but with compound **28** (0.11 g, 0.2 mmol) and and Pd/C (10% Pd, 55% H_2O) (0.02 g) in MeOH (2 mL) as starting material to obtain product as pale yellow oil 0.06 g, yield in 57%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 8.81 (s, 2H), 6.99 - 6.95 (m, 1H), 6.80 - 6.77 (m, 1H), 6.67 (s, 1H), 6.42 (s, 2H), 3.85 (s, 1H), 3.03 (s, 2H), 2.22 (s, 6H), 1.45 (t, *J* = 8.0 Hz, 2H), 1.20 (br. s., 12H), 0.88 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 160.5, 156.4, 150.6 (d, *J* = 245.4 Hz), 140.1, 139.1 (d, *J* = 2.0 Hz), 137.6 (d, *J* = 4.0 Hz), 125.3, 114.4 (d, *J* = 9.1 Hz), 113.8 (d, *J* = 15.2 Hz), 113.6 (d, *J* = 23.2 Hz), 112.5, 43.5, 31.8, 29.5, 29.3, 29.2, 29.0, 26.8, 22.6, 21.3, 14.1.

MS (ESI$^+$) *m/z*: 400.55 (Calcd for $[M+H]^+$: 400.54);

HRMS (ESI+) *m/z*: 400.2765 (Calcd for [M+H]+: 400.2759).

2-(4-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (35)

Following the experimental procedure for the preparation of compound **32** described above, but with compound **29** (0.03 g, 0.1 mmol) and and Pd/C (10% Pd, 55% H_2O) (0.01 g) in MeOH (2 mL) as starting material to obtain product as pale yellow oil 0.02 g, yield in 56%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.34 - 7.29 (m, 2H), 7.12 - 7.06 (m, 2H), 6.88 (d, *J* = 8.0 Hz, 2H), 3.89 (s, 1H), 3.05 (t, *J* = 8.0 Hz, 2H), 1.47 (t, *J* = 8.0 Hz, 2H), 1.21 (br. s., 12H), 0.89 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 160.6, 156.0, 150.7 (d, *J* = 245.4 Hz), 139.4, 137.3 (d, *J* = 3.0 Hz), 130.4, 124.0, 115.9 (d, *J* = 9.1 Hz), 114.9, 114.5, 114.2, 43.5, 31.8, 29.4, 29.2, 29.2, 29.0, 26.8, 22.6, 14.1.

2-(3-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (36)

Following the experimental procedure for the preparation of compound **32** described above, but with compound **30** (0.04 g, 0.1 mmol) and and Pd/C (10% Pd, 55% H_2O) (0.01 g) in MeOH (2 mL) as starting material to obtain product as pale yellow oil 0.02 g, yield in 66%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.58 (s, 2H), 7.33 (s, 1H), 7.14 - 7.08 (m, 2H), 6.94 - 6.90 (m, 1H), 6.86 (d, *J* = 8.0 Hz, 2H), 3.92 (s, 1H), 3.07 (t, *J* = 8.0 Hz, 2H), 1.49 (t, *J* = 8.0 Hz, 2H), 1.22 (br. s., 12H), 0.90 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 160.7, 155.7, 150.9 (d, *J* = 246.4 Hz), 139.6, 137.4 (d, *J* = 3.0 Hz), 133.9, 130.5, 129.5, 127.7, 124.2, 116.2 (d, *J* = 10.1 Hz), 114.8, 114.6 (d, *J* = 23.2 Hz), 43.4, 31.8, 29.4, 29.2, 29.2, 29.0, 26.8, 22.6, 14.1

2-(3-ethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (37)

Following the experimental procedure for the preparation of compound **32** described above, but with compound **31** (0.03 g, 0.1 mmol) and and Pd/C (10% Pd, 55% H_2O) (0.01 g) in MeOH (2 mL) as starting material to obtain product as pale yellow oil 0.01 g, yield in 54%.

^{1}H NMR (METHANOL-d_4) δ: 7.18 - 7.14 (m, 1H), 7.05 - 7.00 (m, 1H), 6.87 (d, *J* = 8.0 Hz, 2H), 6.75 (s, 1H), 6.66 (d, *J* = 8.0 Hz, 1H), 3.34 (s, 1H), 3.08 (t, *J* = 8.0 Hz, 2H), 2.57 (t, *J* = 8.0 Hz, 2H), 1.88 (s, 3H), 1.46 (t, *J* = 8.0 Hz, 2H), 1.20 (br. s., 12H), 0.88 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, METHANOL-d_4) δ 160.4, 157.2, 150.2 (d, *J* = 246.4 Hz), 146.3, 139.1 (d, *J* = 2.0 Hz), 137.4 (d, *J* = 5.1 Hz), 129.3, 122.1, 114.8, 114.5, 113.5 (d, *J* = 8.1 Hz), 112.7 (d, *J* = 22.2 Hz), 112.1, 42.9, 31.6, 29.3, 29.1, 28.9, 28.7, 26.5, 22.8, 22.3, 14.6, 13.0.

MS (ESI$^+$) *m/z*: 400.55 (Calcd for $[M+H]^+$: 400.54);

HRMS (ESI+) *m/z*: 400.2764 (Calcd for [M+H]+: 400.2759).

6-fluoro-3-(nonylamino)-2-phenoxybenzimidamide (38)

Following the experimental procedure for the preparation of compound **32** described above, but with N'-acetoxy-6-fluoro-3-(nonylamino)-2-phenoxybenzimidamide (0.09 g, 0.2 mmol) and and Pd/C (10% Pd, 55% H_2O) (0.02 g) in MeOH (2 mL) as starting material to obtain product as pale yellow oil 0.06 g, yield in 75%.

^{1}H NMR (400 MHz, CHLOROFORM) δ: 7.88 (s, 3H), 7.29 (t, *J* = 8.0 Hz, 2H), 7.07 - 7.01 (m, 2H), 6.89 - 6.83 (m, 3H), 3.88 (s, 1H), 3.02 (t, *J* = 8.0 Hz, 2H), 1.46 (t, *J* = 8.0 Hz, 2H), 1.20 (br. s., 12H), 0.88 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 160.5, 156.1, 150.7 (d, *J* = 246.4 Hz), 139.2 (d, *J* = 2.0 Hz), 137.4 (d, *J* = 3.0 Hz), 130.3, 123.7, 115.7 (d, *J* = 9.1 Hz), 115.0, 114.2 (d, *J* = 23.2 Hz), 111.6 (d, *J* = 16.2 Hz), 43.5, 31.8, 29.4, 29.2, 29.2, 29.0, 26.8, 22.6, 14.1.

MS (ESI$^+$) *m/z*: 372.44 (Calcd for [M+H]$^+$: 372.49);

HRMS (ESI+) *m/z*: 372.2453 (Calcd for [M+H]+: 372.2446).

2,6-difluoro-3-(methylamino)benzonitrile (52)

The mixture of **3-amino-2,6-difluorobenzonitrile** (0.50 g, 3.2 mmol) and K_2CO_3 (0.89 g, 6.4 mmol) in DMF (5 mL) was added MeI (0.69, 4.8 mmol) and stirred in a seal tube at room temperature for 24 h. TLC showed the starting material was consumed completely. Water was added into the mixture and extracted with ethyl acetate for 3 times, the combined organic layer was washed by brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with gradient elution (hexane/ethyl acetate from 50:1 to 20:1) to obtain product as brown oil 0.04 g, yield in 7%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 6.96 - 6.91 (m, 1H), 6.87 - 6.81 (m, 1H), 4.00 (br. s., 1H), 2.91 (d, *J* = 4.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 153.5 (dd, *J* = 250.5, 3.0 Hz), 150.7 (dd, *J* = 255.5, 4.0 Hz), 134.7 (dd, *J* = 11.1, 3.0 Hz), 115.5 (dd, *J* = 9.1, 7.1 Hz), 111.5 (dd, *J* = 20.2, 5.1 Hz), 109.9 (d, *J* = 1.0 Hz), 91.5 (d, *J* = 38.4 Hz), 30.3;

N'-(3-cyano-2,4-difluorophenyl)-N,N-dimethylformimidamide (53)

The procedure was the same with preparation of compound **3**, and obtain product as with solid 0.14 g, yield in 14%

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 7.84 (s, 1H), 7.47 - 7.41 (m, 1H), 7.27 - 7.22 (m,

1H), 3.04 (s, 3H), 2.95 (s, 3H);

^{13}C NMR (101 MHz, DMSO-d_6) δ 156.8 (dd, J = 252.5, 4.0 Hz), 152.2 (dd, J = 258.6, 5.1 Hz), 156.2, 138.2 (dd, J = 8.1, 3.0 Hz), 128.3 (dd, J = 14.1, 5.1 Hz), 112.6 (dd, J = 20.2, 5.1 Hz), 110.7 (d, J = 1.0 Hz), 91.3 (d, dd, J = 19.2, 18.2 Hz), 34.4;

2,4-difluoro-3-(1H-tetrazol-5-yl)aniline (54)

The mixture of **3-amino-2,6-difluorobenzonitrile** (0.20 g, 1.3 mmol) and NaN_3 (0.19 g, 2.8 mmol), $ZnCl_2$ (0.21, 1.5 mmol) in DMF (2 mL) and water (2 mL) and stirred at reflux overnight. TLC showed the starting material was consumed completely. Water was added into the mixture and adjusted pH=2 and extracted with ethyl acetate for 3 times, the combined organic layer was washed by brine and dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with ethyl acetate to obtain product as brown oil 0.10 g, yield in 40%.

1H NMR (400 MHz, DMSO-d_6) δ 7.10 - 6.98 (m, 3H), 5.50 (br. s., 2H);

13C NMR (101 MHz, DMSO-d_6) δ 149.6 (dd, J = 240.4, 5.1 Hz), 148.0 (dd, J = 248.5, 5.1 Hz), 134.8 (dd, J = 11.1, 2.0 Hz), 134.21, 114.2 (dd, J = 9.1, 7.1 Hz), 112.0 (dd, J = 21.2, 4.0 Hz), 102.7;

MS (ESI$^+$) *m/z*: 198.16 (Calcd for $[M+H]^+$: 198.14);

HRMS (ESI+) *m/z*: 198.0589 (Calcd for [M+H]+: 198.0586).

2,4-difluoro-N-nonyl-3-(1H-tetrazol-5-yl)aniline (55)

The mixture of **2,6-difluoro-3-(nonylamino)benzonitrile** (0.18 g, 0.6 mmol) and NaN_3 (0.10 g, 1.6 mmol), $ZnCl_2$ (0.11, 0.7 mmol) in DMF (2 mL) and water (2 mL) and stirred at reflux overnight. TLC showed the starting material was consumed completely. Water was added into the mixture and adjusted pH=2 and extracted with ethyl acetate for 3 times, the combined organic layer was washed by brine and dried

over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with ethyl acetate to obtain product as brown oil 0.11 g, yield in 53%.

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 7.17 - 7.12 (m, 1H), 6.96 - 6.90 (m, 1H), 5.66 (br. s., 1H), 3.10 (t, *J* = 4.0 Hz, 2H), 1.56 (q, *J* = 8.0 Hz, 2H), 1.25 (s, 12H), 0.86 (t, *J* = 4.0 Hz, 3H);

^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 149.8 (dd, *J* = 240.4, 5.1 Hz), 147.4 (dd, *J* = 248.5, 5.1 Hz), 134.8 (dd, *J* = 11.1, 2.0 Hz), 114.3 (dd, *J* = 9.1, 7.1 Hz), 112.0 (dd, *J* = 21.2, 4.0 Hz), 43.2, 31.8, 29.5, 29.3, 29.1, 28.8, 27.0, 22.6, 14.4;

MS (ESI$^+$) *m/z*: 324.35 (Calcd for [M+H]$^+$: 324.38);

HRMS (ESI+) *m/z*: 324.2006 (Calcd for [M+H]+: 324.1994).

3.2.2. Biological assays

3.2.2.1. Materials

Nutrient agar was obtained from Oxoid Ltd. (Nepean, Ontario, Canada). Luria-Bertani (LB) medium was purchased from USB Corporation and used for preparation and transformation of competent cells for *E. coli*, *S. aureus* and other bacterial strains. Müller-Hinton broth (MHB), Müller-Hinton agar and trypticase soy broth (TSB) for Minimum Inhibitory Concentration (MIC) determination were purchased from Becton, Dickinson and Company (New Jersey, USA).

3.2.2.2. Bacterial strains

The bacterial strains used in minimum inhibitory concentration (MIC) determination such as *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and other bacterial strains, including clinical bacteria, were from Prof. Chen Sheng' s research group.

3.2.3. Antibacterial susceptibility test

The minimum inhibitory concentrations (MICs) of compounds were determined using a broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution at a concentration of 16 mg/mL. Stock solution of compounds was then treated with a series of 2-fold dilutions in Müller-Hinton broth (MHB) to prepare compound-containing media at concentrations ranging from 0.25 to 256 μg/mL in 150 μL solution. Four to five single colonies of the testing bacterial strain on a TSB agar plate were inoculated in 5 mL of MH broths. The cells were incubated at 37 ºC until the OD_{600} (absorbance of 600 nm) of the growing cells reached 1.0. The cells were then diluted to a final concentration of approximately OD_{600} of 0.08 to 0.1, in MH broth containing two-fold dilutions of the test compounds in a 96-well microtiter plate. After 18 h of incubation at 37 ºC, the OD_{600} values were measured to calculate the percentage inhibition of bacterial growth with respect to the control. The MIC value is defined as the lowest concentration of tested compounds that causes ≥ 90% inhibition of bacterial growth.

3.2.4. Isolation of F332R mutants

Cells of *Staphylococcus Aureus* ATCC 29213 were cultured in lysogeny broth (LB) with constant shaking at 250 rpm at 37°C. Bacteria were transferred by inoculating 50 μL of stationary-phase culture into 3 ml of LB. Cells were initially grown in a medium without **F332**. At transfer 2, 50 μL of cell suspension was added to 3 ml of nutrient broth, with or without **F332** at a final concentration of half the MIC (MIC= 2 μg/mL), for 20 h with shaking at 250 rpm to obtain 2 transfers, T(0) and T(1). The regrown bacteria in T(1) were thereafter transferred to a broth containing a double

concentration of **F332** compared with previous transfers and cultured as above method. If the growth in T(1) was very weak, bacteria in T(0) should be transferred in another T(1) culture and repeat this step until the bacteria could grow well in T(1). The experiment was conducted several times with escalating concentration levels of **F332** and need to retain the strain in the concentration of **F332** is 32 μg/mL (T(32)), 64 μg/mL (T(64)) and 128 μg/mL (T(128)). The MIC value of **F332** for each induced bacteria was determined and all MIC >32 μg/mL.

3.2.5. Whole-genome sequencing and bioinformatics analysis

Total DNA was extracted from an overnight culture (3 ml) by using Genomic DNA Mini Kit according to the protocol provided by the manufacturer. Then, DNA was quantified by quality ratios of DNA (A260/280) determined via Nanodrop (ThermoFisher Scientific). Genomic DNA libraries were prepared for whole-genome sequencing using the TruePrep Index Kit V3 and TruePrepTM DNA Library Prep Kit V2, as described by the manufacturer. Paired end sequencing was performed using the Illumina NextSeq platform (NextSeq 500/550 Kits v2; 2 × 151 cycles). Raw reads were de novo-assembled into contigs using SPAdes (3.11.0) with pre-defined kmers set. Then analysis was carried out by using the assembled contigs. Reference FtsZ gene sequence was downloaded from NCBI GenBank. The genome was BLAST against the FtsZ gene using CLC workbench. Relative sequences were extracted from the genome sequences. The extracted sequences were then aligned against reference FtsZ sequence to check the identity.

3.3. Results and discussion

3.3.1. F332 with selective activity against *S. aureus*

In 2008, David and co-workers reported a selective small molecule FtsZ inhibitor called PC190723 (Figure 19), which was published in *Science*.(Haydon et al., 2008)

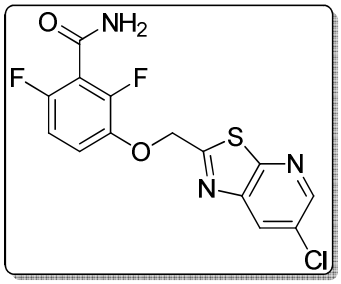


Figure 19. The structure of FtsZ inhibitor **PC190723**

The data from Table 7 suggested that this small molecule just exhibited potent antibacterial activity against Gram-positive bacteria, but they had no effect on Gram-negative bacteria.

Table 7. Microbiological profile of **PC190723.**(Haydon et al., 2008)

Organism and genotype	MIC (μg/mL)
B. subtilis	1
S. aureus	1
S. aureus (MRSA)	1
S. aureus (MDRSA)	1
S. epidermidis	1
S. haemolyticus	0.5
S. hominis	1
S. lugdunensis	1
S. saprophyticus	1
S. warneri	1
Enterococcus faecalis	>64
Escherichia coli	>64
Haemophilus influenzae	>64
Pseudomonas aeruginosa	>64
Streptococcus pneumoniae	>64
Saccharomyces cerevisiae	>64

In order to address this problem, we optimized the structure of **PC190723** and synthesized a series of compounds with different substituents on the benzyl moiety (Figure 20).(Lui et al., 2019) We looked for some small molecules as inhibitors for

broad-spectrum bacteria.

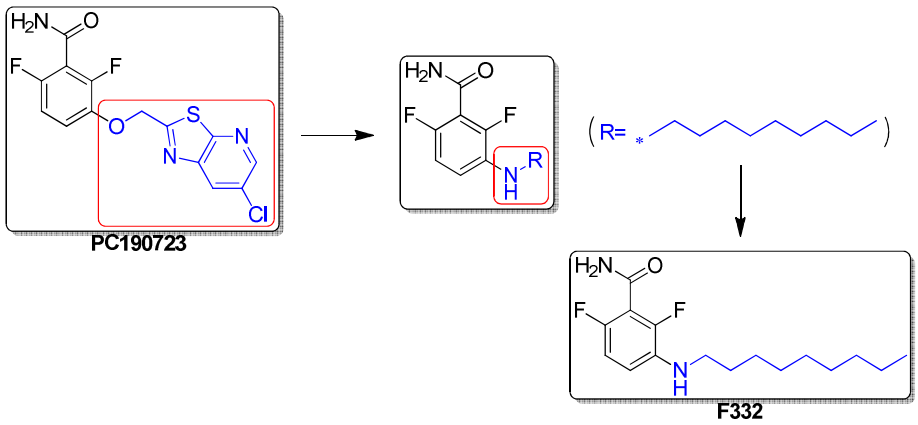
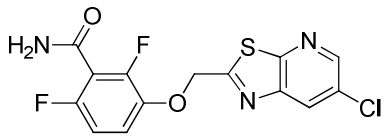
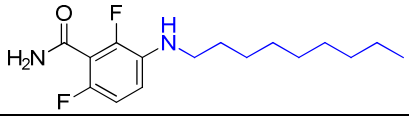


Figure 20. Optimization of the structure of PC190723.

Among the first series of compounds, the most potential compound was compound **F332**, which have a very similar antibacterial activity to **PC190723** (Table 8).

Table 8. The MIC of compound **F332** and **PC190723**.

Compound	Structure	MIC (μg/mL) *S. aureus* 29213	MIC (μg/mL) *E. coli* 25922
PC190723		1	>64
F332		1	>64

The result shows that **F332** was a selective antibiotic small molecule that could only inhibit the growth of *S. aureus*, but had no effect on *E. coli*. Then, we looked for more active compounds with broad spectrum through structure modification of compound **F332**.

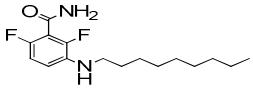
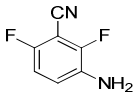
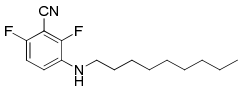
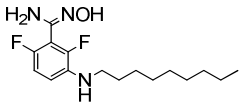
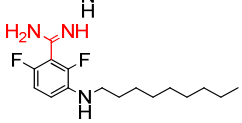
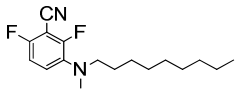
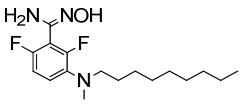
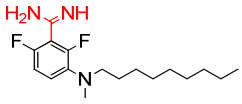
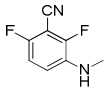
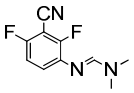
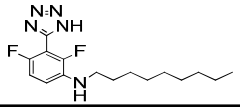
3.3.2. The compounds design and their antimicrobial activity

The next step was to design more active compounds based on the structure of **F332**. The data obtained in Chapter 1 clearly showed that compound **F332** could inhibit the function of protein FtsZ in *S. aureus*, but it had no effect on *E. coli*. A possible reason was that this compound was unable to go through the membrane of Gram-negative bacteria. In order to address this problem, we needed to increase the number of the nitrogen atoms, which showed positive charge and could help improve the permeability of the membrane, due to Gram-negative membrane with negative charge.(Richter et al., 2017)

At this stage, **F332** could be regarded as a lead compound and we tried to optimize the structure of **F332** in order to achieve broad-spectrum antibacterial activity. We designed several new compounds and tried to use amidine group and tetrazole group, which had more nitrogen atoms, to replace the amide group on the benzyl moiety. The MIC result of this series of compounds showed in Table 9.

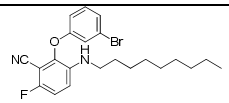
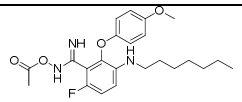
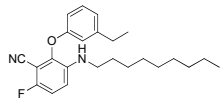
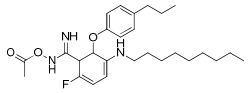
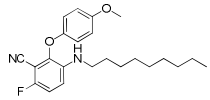
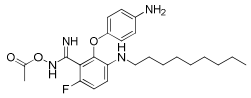
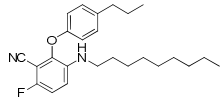
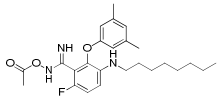
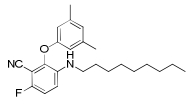
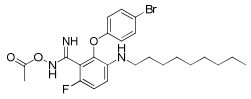
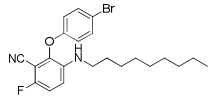
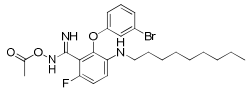
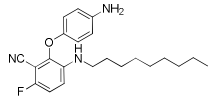
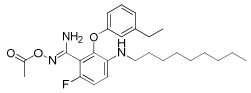
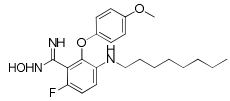
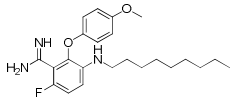
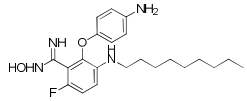
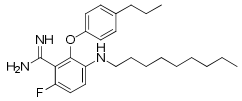
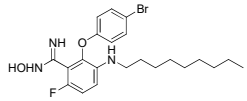
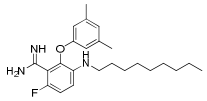
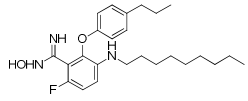
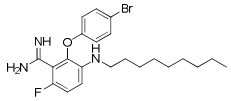
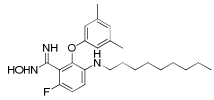
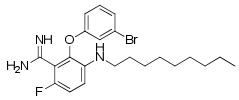
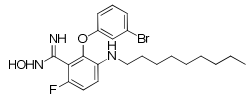
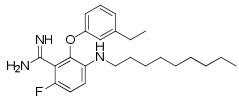
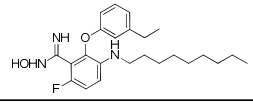
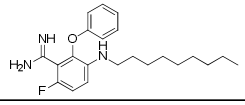
The data shown in Table 9 indicated that compounds **4**, **6**, **8**, **10** and **55** had some extent antibacterial activity against *S. aureus* with MIC ranging from 4 to 32 μg/mL. Moreover, both compounds **6** and **10** had moderate antibacterial activity against *E. coli* with MIC of 16 μg/mL.

Table 9. The MIC of **F332** analogs and intermediates.

Compound	Structure	MIC (μg/mL) *S. aureus* 29213	MIC (μg/mL) *E. coli* 25922
F332		1	>256
2		64	>256
3		>256	>256
4		16	>256
6		32	32
7		>256	>256
8		4	>256
10		16	32
52		256	>256
53		>256	>256
54		>256	>256
55		16	>256

These preliminary data indicated that the amidine group was a good functional group for antibacterial activity against *E. coli*. In the next step of structure optimization, the amidine group would be kept in the scaffold, and a new class of compounds was designed and synthesized. Their antibacterial activity was evaluated, and the MIC results are shown in Table 10.

Table 10. The MIC of amidine compounds and their intermediates.

		MIC (μg/mL)					
Compound	Structure	*S. aureus* 29213	*E. coli* 25922	Compound	Structure	*S. aureus* 29213	*E. coli* 25922
11		>256	>256	**25**		32	>256
12		>256	>256	**26**		>256	>256
13		>256	>256	**27**		16	>256
14		>256	>256	**28**		>256	>256
15		>256	>256	**29**		>256	>256
16		>256	>256	**30**		>256	>256
17		>256	>256	**31**		4	>256
18		4	>256	**32**		4	16
19		16	>256	**33**		4	64
20		4	>256	**34**		4	64
21		>256	>256	**35**		8	64
22		4	>256	**36**		8	64
23		16	>256	**37**		4	64
24		8	>256	**38**		4	16

The phenoxy group was used to replace the fluorine because the structure of target compounds is similar to the structure of triclosan, which has excellent antibacterial activity against both Gram-positive and Gram-negative bacteria.

Table 10 shows that compounds with cyano group did not have any antibacterial activity against either *S. aureus* or *E. coli*, but when the cyano group was changed to amidoxime group or acetyl amidoxime group, most compounds exhibited good antibacterial activity against *S. aureus* with MIC value ranging from 4 μg/mL to 32 μg/mL, while they did not have any effect on *E. coli*. Finally, when these functional groups were changed to amidine group, the antibacterial activity against *S. aureus* was improved with MIC value ranging from 4 μg/mL to 8 μg/mL, At the same time, these amidine compounds showed moderate antibacterial activity against *E. coli* with MIC value ranging from 16 μg/mL to 64 μg/mL. This meant that this kind of compounds could not only inhibit the growth of *S. aureus* (Gram-positive), but also had some extent antibacterial activity against *E. coli* (Gram-negative).

3.3.3. Structure-activity relationship of novel compounds

The MIC results shows that this series of compounds have better activity against *S. aureus* than against *E. coli*. For the beginning of compound design, if amide group of **F332** was change to cyano group, compounds lost their activity at all, such as compounds **3** and **7**, which have no effect on either *S. aureus* or *E. coli*. This means that cyano group was the worst choice for this series of compounds. But if the nine carbon-chain of **F332** was removed and only amine group was left, like compound **2**, they exhibited some extent activity against *S. aureus* with MIC of 64 μg/mL, indicating that the advantage of amine group could offset the disadvantage of the cyano group.

When one hydrogen atom on the amine group of compound **2** was replaced by other alkyl group, such as compounds **52** and **53**, they also lost their activity completely. This result also indicated that the amine group was a good choice for this series of compound to fight against *S. aureus*.

If the amide group of **F332** was changed to other groups containing more nitrogen atoms, such as amidoxime, amidine and tetrazole (compounds **4, 6, 8, 10, 55**) These compounds had some extent activity against *S. aureus* with MIC value ranging from 4 μg/mL to 32 μg/mL, especially for amidine group, such as compounds **6** and **10**, which had the same activity against *E. coli* with the MIC of 32 μg/mL. This was a very good clue for us to do further structure modification. Compound **54** had both tetrazole group and amine group, but it lost its activity at all, maybe because it had strong ability of interacting with other proteins in bacterial cells.

Structure-activity relationship showed that the nine-carbon chain on the amine group and using functional group (such as amidoxime and amidine groups) instead of amide group are essential for this series of compounds keeping activity. In particularly, amide group of **F332** should not be replaced by cyano group, because this would lead to lost activity at all. Therefore, we designed compounds with nine-carbon chain and change the amide group to other groups in the next stage.

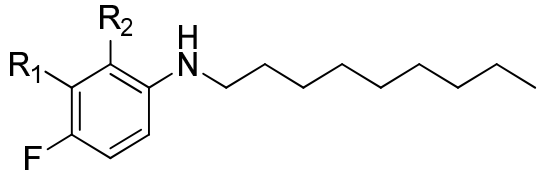


Figure 21. The structure of the designed compound.

The second round MIC results shows that this series of compounds had a nine-carbon chain connected to the amine group. The fluorine on R_2 position was replaced by different phenol groups (Figure 21), which showed that this fluorine was not important for the activity of compounds. When the R_1 group was cyano group, like compounds **11**-**17**, these compounds lost their activity at all with MIC value of more than 256 μg/mL. This result was consistent with the result of the first-round analysis of SAR, which proving that cyano group was a bad choice for this series of compounds.

When the R_1 position was replaced by amidoxime, nearly all compounds (compounds **18**-**24**) restored their activity against *S. aureus* with the MIC ranging from 4 μg/mL to 16 μg/mL, except for compound **21**, which exhibited the MIC of more than 256 μg/mL. The reason of compound **21** lost its activity might be due to the bulky volume of the molecule that lead to its failing to bind to the binding pocket.

But when the hydroxyl group on the amidoxime was acetylated by acetic anhydrate, only compound **31** increased its activity against *S. aureus*, from 8 μg/mL to 4 μg/mL. For other compounds, such as compounds **25** and **27**, their antibacterial activity against *S. aureus* were not better than that of compounds **18** and **19**. Even more, the rest compounds, such as compound **26** and compounds **28**-**30**, lost their activity completely. At the same time, they were not effective in inhibiting the growth of *E. coli*. This result shows that the acetyl amidoxime is worse than amidoxime itself for compounds against *S. aureus*.

The next couple of compounds had amidine group, and the MIC result showed that compared with the compound with amidoxime group, the antibacterial activity of nearly all compounds against *S. aureus* was improved, especially for compound **33**, whose MIC changed from more than 256 μg/mL to 4 μg/mL. This result indicated that the amidine group are a better group for binding to the target, perhaps because it had more nitrogen, which can form hydrogen bond with protein. The more exciting discovery was that all these compounds had extent antibacterial activity against *E. coli* with the MIC ranging from 16 μg/mL to 64 μg/mL. These compounds had activity against *E. coli* might because amidine group had more positive charges, which enabled compounds to enter into the membrane of Gram-negative bacteria, like *E. coli*. This result indicated that the amidine group was a good start point for structure modification to develop broad spectrum FtsZ

inhibitors.

3.4. Concluding remarks

In this chapter, a series of compounds were synthesized and their antibacterial activities against *S. aureus* and *E. coli* were evaluated. Compounds were designed from the structure modification of the lead compound **F332**. From the SAR, we found that nine-carbon chain was essential for this series of compounds keeping antibacterial activity, while the cyano group was a bad choice. Among these compounds, most of them exhibited some extent antibacterial activity against *S. aureus*, especially for the compounds with amidoxime group and amidine group with MIC as good as ranging from 4 μg/mL to 32 μg/mL.

In the other hand, it was not easy to find some compounds with antibacterial activity against *E. coli*, and most of our compounds had no effect on *E. coli*. Fortunately, our compounds with amidine group had some extent antibacterial activity against *E. coli* with MIC between 16 μg/mL and 64 μg/mL. Collectively, compounds **6** and **10** exhibited moderate broad antibacterial activity against *S. aureus* and *E. coli*. Furthermore, compounds **32** and **38** exhibited stronger antibacterial activity against both *S. aureus* and *E. coli*. Their antibacterial activity against *S. aureus* increased 8-fold, compared with compound **6**, while the antibacterial activity against *E. coli* increased 2-fold.

Chapter 4. Double warhead with FtsZ inhibitors to fight against Gram-negative bacteria

4.1. Introduction

The global health problem caused by resistant bacteria, which have been defeated once, calls for rethinking of the principle of antibiotic drug discovery.(Brown, 2015) FDA also encourages adopting alternative strategy that uses current drugs rather than developing entirely new drugs to solve the antibiotic resistance problem.(Woodford et al., 2013) The double warhead strategy connects two drugs or scaffolds, which can interact with two different drug targets. This strategy can better utilize current drugs, and their antibacterial activity could be improved by their synergistic effect. Furthermore, resistance often emerges when a single antibiotic is used for therapy.(Prabhudesai et al., 2011) Schweizer and co-worker have reported that this strategy can successfully treat resistant bacteria.(Lyu et al., 2017)

FtsZ inhibitors are hard to inhibit the growth of Gram-negative bacteria strains. For example, the compound **F332** had strong antibacterial activity against *S. aureus*, but no effect on *E. coli*. Hence, we proposed that double warhead small molecules with the scaffold of FtsZ inhibitors can interact with two targets at the time, and these inhibitors may lead to broaden the antibacterial spectrum.

Triclosan is a broad-spectrum antibacterial agent that inhibits bacterial fatty acid synthesis (FAS) at the enoyl-acyl carrier protein reductase (FabI) step, which is a key enzyme of the type II FAS system, but the risk of triclosan is antimicrobial resistance, and its possible role in disrupting hormonal development remains controversial. To address these drawbacks of triclosan, we proposed combining

triclosan and the functional group of FtsZ inhibitor to broaden the antibacterial activity of compound **F332** and reduce the cytotoxicity of triclosan.

4.2. Experimental section

4.2.1. Chemical synthesis

4.2.1.1. Materials

All the starting materials and chemical reagents for compound synthesis were purchased from commercial chemical companies, such as Aldrich and International Laboratory. All the organic solvents were purchased from Oriental Chemicals. All the chemicals and organic solvents mentioned above were of reagent grade and used directly without any further purification unless otherwise stated. Thin-layer chromatography (TLC) analysis was performed on Merck silica gel plates, and the version was TLC silica gel 60 F254 (0.25 mm thickness) purchased from E. Merck. Compounds were visualized under short and long UV light (254 and 365 nm) and/or immersed in 10% phosphomolybdic acid solution in ethanol followed by gentle heating with a heat gun and/or staining with silica gel powder combined with I_2. Flash column chromatography for purification of compounds was performed on silica gel, and the chromatography silica gel was MN silica gel 60 (230-400 mesh).

4.2.1.2. Instruments

The structure of compounds was confirmed by ^{1}H-NMR and ^{13}C-NMR spectrum, and the molecular weight of compounds was confirmed by ESI-MS spectrum. All NMR spectra were recorded at room temperature on a Bruker Advance-III spectrometer at 400.13 MHz for ^{1}H-NMR and 100.60 MHz for ^{13}C-NMR. All chemical shifts were reported as parts per million (ppm) in the unit relative to the resonance of $CDCl_3$, Methanol-d_4, DMSO-d_6. Low-resolution mass spectra (LRMS)

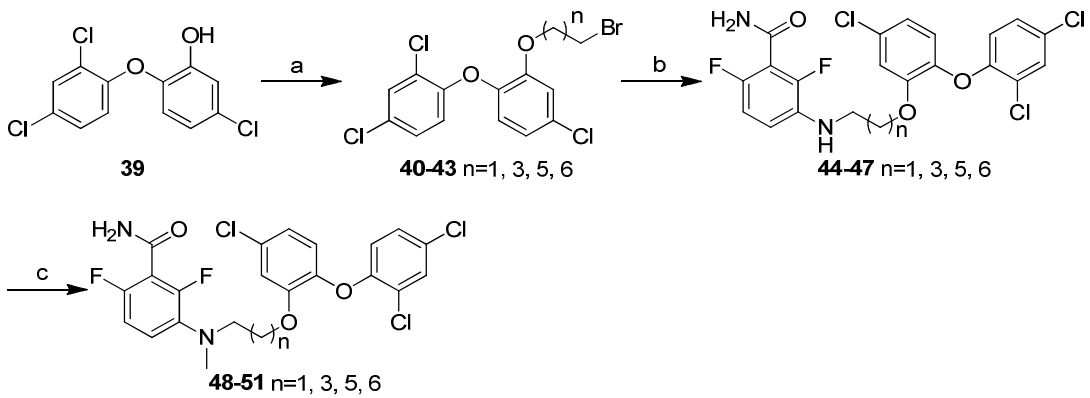
were obtained on a mass spectrometer in electron spray ionization (ESI) mode. High-resolution mass spectra (HRMS) were also obtained in an ESI mode Micromass Q-TOF-2 mass spectrometer located in the The University Research Facility in Life Sciences (ULS) at The Hong Kong Polytechnic University.

4.2.1.3. Synthesis and characterization

The general procedure of synthesis of double warhead compounds with both scaffolds of the FtsZ inhibitor **F332** and FabI inhibitor triclosan was according to the synthesis pathway with only three steps shown in Scheme 4.

The starting material triclosan was purchased from a commercial chemical company at a very low price. The first step was alkylation reaction, and the hydroxyl group was alkylated with dibromo alkane with different carbons in acetone at room temperature overnight to generate the bromide compounds **40**-**43** with a high yield. Products produced in this step didn't need purification and could be used in the next step directly. The second step was also alkylation reaction, which used bromide compounds to alkylate with aniline. This reaction was conducted in the presence of potassium iodide and potassium carbonate, and the mixture was dissolved in dimethylformamide and stirred at 120 ºC overnight to produce compounds **44**-**47**. Finally, the compounds obtained in the last step were alkylated with iodomethane in the presence of potassium carbonate, and the mixture was dissolved in dimethylformamide and stirred at room temperature overnight. The final compound was purified by flash chromatography for the further biological activity test.

Scheme 4. Synthesis of compounds of double warhead inhibitors



Reagents and conditions: a) Dibromo alkane, K_2CO_3, acetone, room temperatrue (RT), overnight; b) 3-amino-2,6-difluorobenzamide, K_2CO_3, KI, DMF, 120^{0}C, overnight; c) MeI, K_2CO_3, DMF, RT, overnight.

Procedure for synthesis

2-(2-bromoethoxy)-4-chloro-1-(2,4-dichlorophenoxy)benzene (40)

The mixture of **triclosan** (0.50 g, 1.7 mmol), K_2CO_3 (0.36 g, 1.6 mmol) and **1,2-dibromoethane** (0.31 g, 1.6 mmol) in acetone (10 mL) was stirred at room temperature overnight. TLC showed the starting material was consumed completely. Then filter the solid and remove the solvent in vacuum to obtain crude product as pale yellow oil, without any purification for next step use.

2-(4-bromobutoxy)-4-chloro-1-(2,4-dichlorophenoxy)benzene (41)

Following the experimental procedure for the preparation of compound **40** described above, but with **triclosan** (1.00 g, 3.5 mmol), K_2CO_3 (0.60 g, 4.5 mmol) and **1,4-dibromobutane** (0.75 g, 3.5 mmol) as a starting material to obtain crude product as pale yellow oil, without any purification for next step use.

2-((6-bromohexyl)oxy)-4-chloro-1-(2,4-dichlorophenoxy)benzene (42)

Following the experimental procedure for the preparation of compound **40** described above, but with **triclosan** (1.00 g, 3.5 mmol), K_2CO_3 (0.57 g, 4.2 mmol) and **1,6-dibromohexane** (1.69 g, 6.9 mmol) as a starting material to obtain crude product as pale yellow oil, without any purification for next step use.

2-((7-bromoheptyl)oxy)-4-chloro-1-(2,4-dichlorophenoxy)benzene (43)

Following the experimental procedure for the preparation of compound **40** described above, but with **triclosan** (0.80 g, 2.8 mmol), K_2CO_3 (0.46 g, 3.3 mmol) and **1,7-dibromoheptane** (0.64 g, 2.5 mmol) as a starting material to obtain crude product as pale yellow oil, without any purification for next step use.

3-((2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)ethyl)amino)-2,6-difluorobenzamide (44)

The mixture of compound **40** (0.52 g, 1.3 mmol), **2-(2-bromoethoxy)-4-chloro-1-(2,4-dichlorobenzyl)benzene** (0.15 g, 0.9 mmol), K_2CO_3 (0.18 g, 1.3 mmol) and KI (0.29 g, 1.7 mmol) in ACN (2 mL) was stirred at 120 ºC overnight. TLC showed the starting material was consumed completely. Then water was added into the mixture and extracted with ethyl acetate for 3 times, and the combined organic layer was washed with brine and dried over Na_2SO_4. Then the solvent was removed in vacuum and purified by silica gel (Hexane/ethyl acetate from 10:1 to 2:1) to obtain the product as pale yellow oil 0.10 g, yield in 23%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 7.47 - 7.43 (m, 1H), 7.14 - 7.12 (m, 1H), 7.00 - 6.96 (m, 3H), 6.82 - 6.77 (m, 1H), 6.71 - 6.69 (m, 2H), 5.98 (br. s., 1H), 5.85 (br. s., 1H), 4.18 - 4.14 (m, 2H), 4.08 (br. s., 1H), 3.47 - 3.43 (m, 2H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 162.7, 152.3, 151.8 (dd, *J* = 243.4, 6.1 Hz), 150.6, 150.3, 148.1 (dd, *J* = 247.5, 6.1 Hz), 143.5, 133.1 (dd, *J* = 12.1, 3.0 Hz), 130.8, 130.3, 128.3, 127.8, 124.7, 122.1, 121.9, 118.3, 115.5, 113.6 (dd, *J* = 9.1, 5.1 Hz), 112.8 (dd, *J* = 20.2, 17.2 Hz), 111.6 (dd, *J* = 24.2, 4.0 Hz), 68.3, 43.1.

MS (ESI$^+$) *m*/*z*: 487.76 (Calcd for [M+H]$^+$: 487.71);

HRMS (ESI$^+$) *m*/*z*: 487.0191 (Calcd for [M+H]$^+$: 487.0189).

3-((4-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)butyl)amino)-2,6-difluorobenzamide (45)

Following the experimental procedure for the preparation of compound **44** described above, but with compound **41** (0.52 g, 1.2 mmol), **2-(2-bromoethoxy)-4-chloro-1-(2,4-dichlorobenzyl)benzene** (0.14 g, 0.8 mmol), K_2CO_3 (0.17 g, 1.2 mmol) and KI (0.11 g, 0.7 mmol) in ACN (2 mL) as a starting material to obtain the product as pale yellow oil 0.08 g, yield in 42%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 7.42 (d, *J* = 4.0 Hz, 1H), 7.11 - 7.09 (m, 1H), 7.00 - 6.94 (m, 3H), 6.85 - 6.81 (m, 1H), 6.67 - 6.61 (m, 2H), 6.08 (s, 1H), 6.04 (s, 1H), 3.99 (t, *J* = 8.0 Hz, 2H), 3.78 (br. s., 1H), 3.12 - 3.07 (m, 2H), 1.81 - 1.74 (m, 2H), 1.62 - 1.55 (m, 2H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 162.7, 152.5, 150.8 (dd, *J* = 242.4, 6.1 Hz), 150.8, 147.9 (dd, *J* = 248.5, 7.1 Hz), 143.0, 133.8 (dd, *J* = 12.1, 2.0 Hz), 130.7, 130.1, 127.9, 127.6, 124.3, 122.2, 121.2, 117.8, 114.9, 113.0 (dd, *J* = 9.1, 5.1 Hz), 112.5 (dd, *J* = 21.2, 16.2 Hz), 111.4 (dd, *J* = 23.2, 4.0 Hz), 68.7, 43.3, 26.5, 25.8.

MS (ESI$^+$) *m*/*z*: 515.75 (Calcd for [M+H]$^+$: 515.76);

HRMS (ESI$^+$) *m*/*z*: 515.0496 (Calcd for [M+H]$^+$: 515.0502).

3-((6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexyl)amino)-2,6-difluorobenzamide6(46)

Following the experimental procedure for the preparation of compound **44** described above, but with compound **42** (0.32 g, 0.7 mmol), **2-(2-bromoethoxy)-4-chloro-1-(2,4-dichlorobenzyl)benzene** (0.06 g, 0.3 mmol), K_2CO_3 (0.07 g, 0.5 mmol) and KI (0.12 g, 0.7 mmol) in ACN (2 mL) as a starting material to obtain the product as pale yellow oil 0.04 g, yield in 21%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 7.42 (d, *J* = 4.0 Hz, 1H), 7.10 - 7.07 (m, 1H), 7.01 - 6.93 (m, 3H), 6.84 (t, *J* = 8.0 Hz, 1H), 6.70 - 6.63 (m, 2H), 6.30 (s, 1H), 6.08 (s, 1H), 3.93 (t, *J* = 8.0 Hz, 2H), 3.81 (br. s., 1H), 3.09 (br. s., 2H), 1.68 - 1.62 (m, 2H), 1.61 - 1.53 (m, 2H), 1.38 - 1.31 (m, 2H), 1.27 - 1.21 (m, 2H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 162.9, 152.7, 151.0 150.7 (dd, *J* = 241.4, 8.1 Hz), 147.9 (dd, *J* = 248.5, 7.1 Hz), 142.8, 134.0 (dd, *J* = 11.1, 2.0 Hz), 130.7, 130.1, 127.6, 127.5, 124.2, 122.3, 120.9, 117.6, 114.7, 113.0 (dd, *J* = 10.1, 6.1 Hz), 112.5 (dd, *J* = 21.2, 16.2 Hz), 111.6 (dd, *J* = 22.2, 3.0 Hz), 68.8, 43.7, 29.2, 28.9, 26.6, 25.6.

MS (ESI$^+$) *m*/*z*: 543.84 (Calcd for $[M+H]^+$: 543.82);

HRMS (ESI$^+$) *m*/*z*: 543.081 (Calcd for $[M+H]^+$: 543.0815).

3-((7-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)heptyl)amino)-2,6-difluorobenzamide (47)

Following the experimental procedure for the preparation of compound **44** described above, but with compound **43** (0.24 g, 0.5 mmol), **2-(2-bromoethoxy)-4-chloro-1-(2,4-dichlorobenzyl)benzene** (0.06 g, 0.3 mmol), K_2CO_3 (0.06 g, 0.4 mmol) and KI (0.06 g, 0.3 mmol) in ACN (2 mL) as a starting material to obtain the product as pale yellow oil 0.07 g, yield in 36%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 7.43 (d, *J* = 4.0 Hz, 1H), 7.11 - 7.08 (m, 1H), 7.01 - 6.93 (m, 3H), 6.87 - 6.83 (m, 1H), 6.72 - 6.63 (m, 1H), 6.04 (s, 2H), 3.92 (t, *J* = 8.0 Hz, 2H), 3.82 (br. s., 1H), 3.12 (t, *J* = 8.0 Hz, 2H), 1.65 - 1.61 (m, 4H), 1.34 - 1.23 (m, 6H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 160.7, 150.8, 149.2, 148.9 (dd, *J* = 241.4, 5.1 Hz), 146.1 (dd, *J* = 244.4, 5.1 Hz), 141.1, 132.3 (dd, *J* = 11.1, 1.0 Hz), 128.8, 128.2, 125.7, 125.6, 122.4, 120.4, 119.0, 115.8, 113.0, 111.2 (dd, *J* = 9.1, 5.1 Hz), 109.4 (dd, *J* = 22.2, 2.0 Hz), 67.2, 42.0, 27.3, 27.0, 27.0, 24.9, 23.7.

MS (ESI^{+}) *m*/*z*: 557.87 (Calcd for [M+H]$^{+}$: 557.84);

HRMS (ESI^{+}) *m*/*z*: 557.0974 (Calcd for [M+H]$^{+}$: 557.0972).

3-((2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)ethyl)(methyl)amino)-2,6-difluorobenzamide (48)

The mixture of compound **44** (0.05 g, 0.1 mmol), MeI (0.02 g, 0.2 mmol) and K_2CO_3 (0.02 g, 0.2 mmol) in DMF (2 mL) was stirred at room temperature overnight. TLC showed the starting material was consumed completely. Then water was added into the mixture and extracted with ethyl acetate for 3 times, and the combined organic layer was washed with brine and dried over Na_2SO_4. Then the solvent was removed in vacuum and purified by silica gel (Hexane/ethyl acetate from 10:1 to 3:1) to obtain the product as pale yellow oil 0.02 g, yield in 34%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 7.45 (d, *J* = 4.0 Hz, 1H), 7.13 - 7.10 (m, 1H), 6.97 - 6.94 (m, 3H), 6.90 - 6.80 (m, 2H), 6.62 (d, *J* = 8.0 Hz, 1H), 6.02 (s, 1H), 5.97 (s, 1H), 4.12 (t, *J* = 4.0 Hz, 2H), 3.45 (t, *J* = 4.0 Hz, 2H), 2.79 (s, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 162.5, 152.3, 150.7, 142.7, 136.5, 130.8, 130.2, 127.8, 127.7, 124.2, 122.2, 121.5, 120.8 (dd, *J* = 10.1, 6.1 Hz), 117.5, 114.9, 111.5 (dd, *J* = 23.2, 4.0 Hz), 68.3, 54.1, 40.8.

MS (ESI^{+}) *m*/*z*: 501.77 (Calcd for [M+H]$^{+}$: 501.74);

HRMS (ESI^+) *m*/*z*: 501.0351 (Calcd for $[M+H]^+$: 501.0346).

3-((4-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)butyl)(methyl)amino)-2,6-difluorobenzamide (49)

Following the experimental procedure for the preparation of compound **48** described above, but with compound **45** (0.04 g, 0.1 mmol), MeI (0.04 g, 0.3 mmol) and K_2CO_3 (0.02 g, 0.2 mmol) in DMF (2 mL) as a starting material to obtain the product as pale yellow oil 0.02 g, yield in 51%.

^1H NMR (400 MHz, CHLOROFORM) δ 7.42 (d, *J* = 4.0 Hz, 1H), 7.11 - 7.08 (m, 1H), 7.00 - 6.84 (m, 5H), 6.63 (d, *J* = 8.0 Hz, 1H), 5.99 (s, 2H), 3.95 (t, *J* = 8.0 Hz, 2H), 3.01 (t, *J* = 8.0 Hz, 2H), 3.20 (s, 3H), 1.72 - 1.65 (m, 2H), 1.54 - 1.47 (m, 2H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 162.6, 152.6, 151.0, 142.8, 137.4 (dd, *J* = 10.1, 3.0 Hz), 130.8, 130.1, 127.6, 127.6, 124.1, 122.3, 121.3 (dd, *J* = 10.1, 5.1 Hz), 121.2, 121.0, 117.6, 114.8, 111.2 (dd, *J* = 22.2, 3.0 Hz), 68.9, 54.8, 39.7, 30.9, 26.4.

MS (ESI^+) *m*/*z*: 529.75 (Calcd for $[M+H]^+$: 529.79);

HRMS (ESI^+) *m*/*z*: 529.0660 (Calcd for $[M+H]^+$: 529.0659).

3-((6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexyl)(methyl)amino)-2,6-difluorobenzamide (50)

Following the experimental procedure for the preparation of compound **48** described above, but with compound **46** (0.04 g, 0.1 mmol), MeI (0.04 g, 0.3 mmol) and K_2CO_3 (0.02 g, 0.2 mmol) in DMF (2 mL) as a starting material to obtain the product as pale yellow oil 0.02 g, yield in 41%.

^1H NMR (400 MHz, CHLOROFORM) δ ppm 7.41 (s, 1H), 7.10 - 7.07 (m, 1H), 7.00 - 6.85 (m, 5H), 6.64 (d, *J* = 8.0 Hz, 1H), 6.04 (s, 1H), 6.00 (s, 1H), 3.91 (t, *J* =

8.0 Hz, 2H), 3.03 (t, *J* = 8.0 Hz, 2H), 2.80 (s, 3H), 1.64 - 1.61 (m, 4H), 1.35 - 1.28 (m, 4H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 162.7, 153.7 (dd, *J* = 246.4, 6.1 Hz), 152.7, 152.3 (dd, *J* = 263.6, 6.1 Hz), 151.0, 142.9, 137.5 (dd, *J* = 5.1, 1.0 Hz), 130.7, 130.0, 127.6, 127.5, 124.2, 122.3, 121.2 (dd, *J* = 9.1, 5.1 Hz), 120.9, 117.7, 114.7, 111.2 (dd, *J* = 22.2, 4.0 Hz), 68.9, 55.4, 40.0, 29.7, 28.9, 26.5, 25.6.

MS (ESI$^+$) *m*/*z*: 557.85 (Calcd for [M+H]$^+$: 557.84);

HRMS (ESI$^+$) *m*/*z*: 557.0976 (Calcd for [M+H]$^+$: 557.0972).

3-((7-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)heptyl)(methyl)amino)-2,6-difluorobenzamide (51)

Following the experimental procedure for the preparation of compound **48** described above, but with compound **47** (0.03 g, 0.1 mmol), MeI (0.08 g, 0.5 mmol) and K_2CO_3 (0.01 g, 0.1 mmol) in DMF (2 mL) as a starting material to obtain the product as pale yellow oil 0.01 g, yield in 39%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 7.43 (d, *J* = 4.0 Hz, 1H), 7.10 - 7.07 (m, 1H), 7.00 - 6.92 (m, 4H), 6.89 - 6.85 (m, 1H), 6.64 (d, *J* = 8.0 Hz, 1H), 6.12 (s, 1H), 6.01 (s, 1H), 3.91 (t, *J* = 8.0 Hz, 2H), 3.05 (t, *J* = 8.0 Hz, 2H), 2.80 (s, 3H), 1.65 - 1.58 (m, 2H), 1.53 - 1.49 (m, 2H), 1.23 - 1.18 (m, 6H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 162.8, 152.7, 151.1, 142.9, 137.5 (dd, *J* = 10.1, 3.0 Hz), 130.7, 130.1, 127.6, 127.5, 124.2, 122.3, 121.2 (dd, *J* = 10.1, 6.1 Hz), 120.8, 117.7, 114.7, 111.1 (dd, *J* = 22.2, 4.0 Hz), 69.0, 55.5, 40.1, 29.1, 28.8, 27.1, 26.8, 25.7.

MS (ESI$^+$) *m*/*z*: 571.85 (Calcd for [M+H]$^+$: 571.87);

HRMS (ESI$^+$) *m*/*z*: 571.1130 (Calcd for [M+H]$^+$: 571.1128).

tert-butyl 2-((3-carbamoyl-2,4-difluorophenyl)amino)acetate (56)

Following the experimental procedure for the preparation of compound **44** described above, but with compound **3-amino-2,6-difluorobenzamide** (0.50 g, 2.9 mmol), tert-butyl 2-bromoacetate (0.85 g, 4.3 mmol), K_2CO_3 (0.80 g, 5.8 mmol) and KI (0.39 g, 2.3 mmol) in DMF (5 mL) as a starting material to obtain the product as pale yellow oil 0.45 g, yield in 54%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 6.87 - 6.82 (m, 1H), 6.62 - 6.56 (m, 1H), 6.20 (br. s., 1H), 6.09 (br. s., 1H), 4.49 (br. s., 1H), 3.83 (d, *J* = 4.0 Hz, 1H), 1.50 (s, 9H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 169.4, 162.6, 151.5 (dd, *J* = 243.4, 6.1 Hz), 148.2 (dd, *J* = 249.5, 7.1 Hz), 132.9 (dd, *J* = 12.1, 2.0 Hz), 113.4 (dd, *J* = 10.1, 6.1 Hz), 112.8 (dd, *J* = 21.2, 16.2 Hz), 111.4 (dd, *J* = 24.2, 4.0 Hz), 82.5, 46.3, 28.1

MS (ESI$^+$) *m*/*z*: 286.29 (Calcd for $[M+H]^+$: 287.27);

HRMS (ESI$^+$) *m*/*z*: 309.1023 (Calcd for $[M+Na]^+$: 309.1021).

2-((3-carbamoyl-2,4-difluorophenyl)amino)acetic acid (57)

The mixture of compound **56** (0.45 g, 1.6 mmol) was dissolved in DCM (5 mL), and TFA (2 mL) was added in the mixture at 0 ºC and stirred overnight. TLC showed that the starting material was consumed completely. Water was added into the mixture and the mixture was extracted with DCM 3 times. Then combined the organic layers and the organic layer was dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with ethyl acetate to obtain the product as dark yellow oil 0.18 g, yield in 47%.

^{1}H NMR (400 MHz, METHANOL-*d*$_4$) δ 6.90 - 6.85 (m, 1H), 6.74 - 6.68 (m, 1H), 3.97 (s, 2H);

^{13}C NMR (101 MHz, METHANOL-*d*$_4$) δ 173.3, 164.7, 150.6 (dd, *J* = 241.4, 7.1

Hz), 147.2 (dd, *J* = 246.4, 7.1 Hz), 133.2 (dd, *J* = 11.1, 2.0 Hz), 114.0 (dd, *J* = 23.2, 19.2 Hz), 112.8 (dd, *J* = 9.1, 4.0 Hz), 110.6 (dd, *J* = 22.2, 4.0 Hz), 44.6;

MS (ESI^+) *m*/*z*: 231.15 (Calcd for $[M+H]^+$: 231.17);

HRMS (ESI^+) *m*/*z*: 231.0577 (Calcd for $[M+H]^+$: 231.0576).

tert-butyl 2-((3-carbamoyl-2,4-difluorophenyl)(nonyl)amino)acetate (58)

Following the experimental procedure for the preparation of compound **44** described above, but with compound **F332** (0.15 g, 0.5 mmol), tert-butyl 2-bromoacetate (1.47 g, 0.8 mmol), K_2CO_3 (0.14 g, 1.0 mmol) and KI (0.67 g, 0.4 mmol) in DMF (5 mL) as a starting material to obtain the product as pale yellow oil 0.03 g, yield in 14%.

^1H NMR (400 MHz, CHLOROFORM) δ 7.09 - 7.04 (m, 1H), 6.88 - 6.84 (m, 1H), 6.25 (br. s., 1H), 6.06 (br. s., 1H), 3.86 (s, 1H), 3.24 - 3.21 (m, 1H), 1.52 - 1.48 (m, 2H), 1.43 (s, 9H), 1.27 (br. s., 12H), 0.89 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 170.2, 162.8, 153.8 (dd, *J* = 247.5, 7.1 Hz), 152.2 (dd, *J* = 252.5, 6.1 Hz), 135.0 (dd, *J* = 9.1, 3.0 Hz), 123.0 (dd, *J* = 9.1, 5.1 Hz), 111.1 (dd, *J* = 22.2, 4.0 Hz), 81.5, 54.8, 52.6, 31.8, 29.5, 29.4, 29.2, 28.1, 27.6, 26.9, 22.6, 14.1.

MS (ESI^+) *m*/*z*: 413.54 (Calcd for $[M+H]^+$: 413.51);

HRMS (ESI^+) *m*/*z*: 413.2609 (Calcd for $[M+H]^+$: 413.261).

2-((3-carbamoyl-2,4-difluorophenyl)(nonyl)amino)acetic acid (59)

The mixture of compound **58** (0.03 g, 0.1 mmol) was dissolved in DCM (2 mL), and TFA (2 mL) was added in the mixture at 0 ^oC and stirred overnight. TLC showed that the starting material was consumed completely. Water was added into the mixture and the mixture was extracted with DCM 3 times. Then combined the organic layers and the organic layer was dried over Na_2SO_4. The organic layer was

concentrated in vacuum and purified by silica gel with ethyl acetate to obtain the product as pale yellow oil 13 mg, yield in 50%.

^{1}H NMR (400 MHz, METHANOL-*d*$_4$) δ 7.19 - 7.13 (m, 1H), 6.95 - 6.91 (m, 1H), 3.98 (s, 1H), 3.30 - 3.26 (m, 2H), 1.57 - 1.52 (m, 2H), 1.31 (s, 12H), 0.91 (t, *J* = 8.0 Hz, 3H);

^{13}C NMR (101 MHz, METHANOL-*d*$_4$) δ 173.1, 164.6, 153.2 (dd, *J* = 251.4, 7.1 Hz), 151.5 (dd, *J* = 250.5, 7.1 Hz), 134.6 (dd, *J* = 10.1, 4.0 Hz), 122.3 (dd, *J* = 9.1, 5.1 Hz), 115.2 (dd, *J* = 23.2, 22.2 Hz), 110.5 (dd, *J* = 23.2, 4.0 Hz), 53.5, 52.4, 31.6, 29.3, 29.1, 29.0, 27.2, 26.6, 22.3, 13.0;

5-chloro-2-(2,4-dichlorophenoxy)phenyl 2-((3-carbamoyl-2,4-difluorophenyl)-amino)acetate (60)

The mixture of compound **57** (0.30 g, 1.3 mmol) and triclosan (0.38 g, 1.3 mmol) was dissolved in DMF (10 mL), and DCC (0.30 g, 1.4 mmol) was added in the mixture at 0 ºC. The mixture was stirred overnight at room temperature and then the TLC showed that the starting material was consumed completely. Water was added into the mixture and the mixture was extracted with DCM 3 times. Then combined the organic layers and the organic layer was dried over Na_2SO_4. The organic layer was concentrated in vacuum and purified by silica gel with gradient elution (hexane/ethyl acetate from 10:1 to 1:1) to obtain the product as yellow solid 0.13 g, yield in 19%.

^{1}H NMR (400 MHz, CHLOROFORM) δ 7.49 (d, *J* = 4.0 Hz, 1H), 7.22 - 7.19 (m, 3H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.81 - 6.72 (m, 2H), 6.65 - 6. 59 (m, 1H), 6.04 (s, 2H), 4.50 (s, 1H), 4.21 (t, *J* = 8.0 Hz, 2H);

^{13}C NMR (101 MHz, CHLOROFORM) δ 168.3, 162.3, 151.8 (dd, *J* = 244.4, 6.1 Hz), 150.5, 148.3 (dd, *J* = 249.5, 6.1 Hz), 146.7, 141.0, 130.5, 130.1, 129.2, 128.4, 127.5, 126.2, 124.0, 121.0, 119.6, 113.5 (dd, *J* = 9.1, 5.1 Hz), 111.4 (dd, *J* = 23.2, 4.0 Hz), 45.3;

MS (ESI^+) *m*/*z*: 500.71 (Calcd for $[M+H]^+$: 500.70);
HRMS (ESI^+) *m*/*z*: 500.9981 (Calcd for $[M+H]^+$: 500.9982).

4.2.2. Biological assays

4.2.2.1. Materials

Nutrient agar was obtained from Oxoid Ltd. (Nepean, Ontario, Canada). Luria-Bertani (LB) medium was purchased from USB Corporation and used for preparation and transformation of competent cells for *E. coli*, *S. aureus* and other bacterial strains. Müller-Hinton broth (MHB) and Müller-Hinton agar and trypticase soy broth (TSB) for Minimum Inhibitory Concentration (MIC) determination were purchased from Becton, Dickinson and Company (New Jersey, USA).

4.2.2.2. Bacterial strains

The bacterial strains used in minimum inhibitory concentration (MIC) determination such as *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and other bacterial strains, including clinical bacteria, were from Prof. Chen Sheng' s research group.

4.2.2.3. Antibacterial susceptibility test

The minimum inhibitory concentrations (MICs) of the test compounds were determined using a broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution at a concentration of 16 mg/mL. The compound stock solution was then treated with a series of 2-fold dilutions in Müller-Hinton broth (MHB) to prepare compound-

containing media at concentrations ranging from 0.25 to 256 μg/mL in 150 μL solution. Four to five single colonies of the testing bacterial strain on a TSB agar plate were inoculated in 5 mL of MH broths. The cells were incubated at 37 ºC until the OD_{600} (absorbance of 600 nm) of the growing cells reached 1.0. The cells were then diluted to a final concentration of approximately OD_{600} of 0.08 to 0.1, in MH broth containing two-fold dilutions of the test compound in a 96-well microtiter plate. After 18 h of incubation at 37 ºC, the OD_{600} values were measured to calculate the percentage inhibition of bacterial growth with respect to the control. The MIC value was defined as the lowest concentration of tested compounds that caused ≥ 90% inhibition of bacterial growth.

4.3. Results and discussion

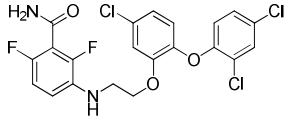
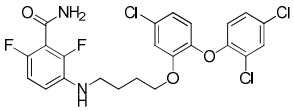
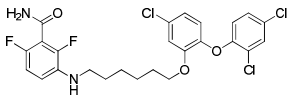
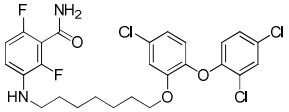
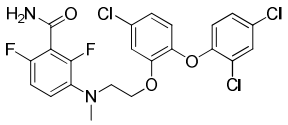
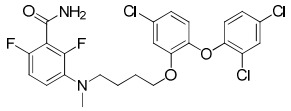
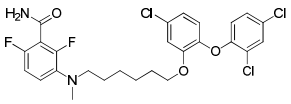
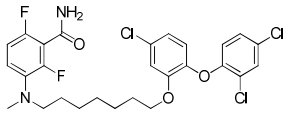
4.3.1. The design of compounds and their antimicrobial activity

Our previous study showed that compound **F332** has the best antibacterial activity against *S. aureus* with MIC values of 2 μg/mL. The drawback is that it has no effect on *E. coli*. On the other hand, triclosan is a fatty acid synthesis pathway inhibitor, and it is a very good antibacterial agent against both *S. aureus* and *E. coli*. However, there is concern over its toxicity, which would be harmful to the environment and people's health. In order to overcome these disadvantages, we combined the scaffolds of **F332** and triclosan, in the hope that the triclosan could help the combination molecule improve the antibacterial spectrum, while the scaffold of **F332** connected with triclosan by a linker could help reduce the toxicity of triclosan. We used carbon chain as a linker to connect the 3-aminobenzamide with triclosan by the ether bond to generate a series of new double warhead molecules and the MIC results of these compounds are shown in Table 11.

The MIC results showed that most compounds had no antibacterial activity and

their MIC values were all more than 64 μg/mL, except for compound **44** which had some extent antibacterial activity against *S. aureus* with MIC values of 64 μg/mL. The length of linker with different carbons was from 2 carbons to 7 carbons, and the amino and triclosan were connected by these carbon chains. In the next stage, we would use linker with no longer than two carbons and do further structure modification.

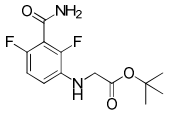
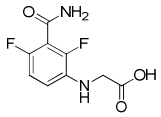
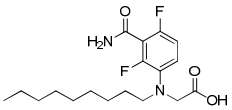
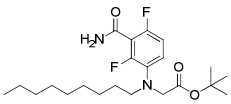
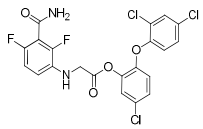
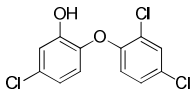
Table 11. The MIC values of double warhead compounds with ether bond.

		MIC (μg/mL)	
Compound	**Structure**	***S. aureus* 29213**	***E. coli* 25922**
44		64	>64
45		>64	>64
46		>64	>64
47		>64	>64
48		>64	>64
49		>64	>64
50		>64	>64
51		>64	>64

We planned to use the linker with two carbons to connect the 3-aminobenzamide moiety and triclosan by ester bond, then generate compound **60**. At the same time,

some intermediates were generated during the synthesizing process of compound **60**. The antibacterial activity of these compounds was evaluated and MIC values were shown in Table 12.

Table 12. The MIC values of double warhead compounds with ester bond.

		MIC (μg/mL)	
Compound	Structure	*S. aureus* 29213	*E. coli* 25922
56		>256	>256
57		128	>256
58		32	>256
59		4	>256
60		<0.03	<0.25
Ttriclosan		<0.03	<0.03

The MIC result shows that the nine-carbon chain could apparently improve the antibacterial activity against *S. aureus*. The antibacterial activity of compound **58** against *S. aureus* was improved 4-fold, from 128 μg/mL (compound **57**) to 32 μg/mL, while the antibacterial activity of compound **59** increased substantially, from more than 256 μg/mL (compound **56**) to 4 μg/mL. This result indicated the important role of nine-carbon chain for this series of compounds. However, the problem of this series of compounds was that they were not effective for *E. coli* or other Gram-negative bacteria. which is the reason why we needed to connect the

scaffold of **F332** with triclosan for the active compounds with broad antibacterial spectrum.

For this purpose, we synthesized compound **60** with 3-aminobenzamide and triclosan and connected by a linker with two carbons. The reason for using two carbons chain as linker was that according to a previous study shown in Table 12, the two carbons chain linker was better that longer chain linkers. The MIC result of compound **60** showed that the antibacterial activity was substantially improved from 64 μg/mL (compound **44**) to less than 0.03 μg/mL for *S. aureus*, and more than 64 μg/mL (compound **44**) to 0.25 μg/mL for *E. coli*. The reason may be that the linker of compound **60** was ester bond, which was easier hydrolyzed *in vivo* than ether bond used in the linker of compound **44**. This led to these two scaffolds work on their targets respectively and exhibit extremely good antibacterial activity against *S. aureus* and *E. coli*.

The purpose of this double warhead strategy was to fight against Gram-negative bacterial strains, which was more difficult to treat than Gram-positive bacterial strains. In the next stage, the antibacterial activity of compound **60** against Gram-negative bacterial strains was further evaluated, and the MIC result was shown in Table 13.

Table 13. The MIC values of compound **60** on Gram-negative clinical isolates.

Strains	MICs (μg/mL)									
	Triclosan	60	IPM	MEM	AMC	CAZ	CTX	ATM	TZP	SCF
KP-04	<0.125	0.5	8	16	128	>256	>256	>256	>256	>256
EC-33	<0.125	0.125	4	4	64	>256	>256	>256	>256	>256
KO-03	<0.125	0.5	8	16	128	>256	>256	>256	>256	>256
ECL-18	<0.125	0.25	4	32	256	256	>256	256	256	>256
KP-14	<0.125	0.125	16	16	128	256	>256	>256	>256	>256

IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; TZP, piperacillin-tazobactam; CPS, cefoperazone/sulbactam; AMC,

amoxicillin-clavulanic acid;KP, K. pneumoniae; EC, E. coli; KO, K. oxytoca; ECL, E. cloacae.

There were five Gram-negative bacterial strains, which are resistant to different types of antibiotics, including meropenem.(Wang et al., 2015) However, compound **60** exhibited very good antibacterial activity against these resistant strains. This result indicated that this double warhead strategy could an alternative strategy for treating Gram-negative bacterial strains.

4.3.2. Structure-activity relationship of novel compounds

The nine-carbon chain was essential for this series of compounds keeping good activity, which is consistent with our previous study in Chapter 3. If the nine-carbon chain was replaced by tert-butyl ester (compound **56**), the compound lost their activity completely, and if the nine-carbon chain was replaced by carboxyl acid (compound **57**), the compound just had weak antibacterial activity against *S. aureus* with the MIC of 128 μg/mL. Then compounds **58** and **59** were synthesized, and both compounds were with nine-carbon chain. The MIC result showed that the nine-carbon chain could apparently improve the antibacterial activity against *S. aureus*.

The linker with 2 carbons enabled compounds to have some extent of antibacterial activity, such as compound **44** with MIC values of 64 μg/mL against *S. aureus*. However, the length of the linker should not be longer than 2 carbons, otherwise the compound would lose its activity. Compound **60** was connected by a linker with two carbons, and it exhibited excellent antibacterial activity against *S. aureus* and *E. coli*.

It also exhibited very good antibacterial activity against other Gram-negative strains,

including *K. pneumoniae*, *K. oxytoca* and *E. cloacae,* and their MIC values were as good as less than 0.5 μg/mL. This result indicated that the linker type was also important for the compounds to maintain their antibacterial activity, and the ester bond is better than the ether bond.

4.4. Concluding remarks

In this chapter, we tried to use double warhead strategy to fight against Gram-negative bacteria, which are more difficult than Gram-positive bacterial strains to treat. We used the scaffold of FtsZ inhibitor **F332** to connect with triclosan by different linkers. On the one hand, double warhead molecule could broaden the antibacterial spectrum of **F332**. On the other hand, this double warhead molecule could reduce the cytotoxicity of triclosan. Considering these advantages of double warhead compounds, a series of double warhead molecules with different length linkers were synthesized.

The MIC result showed that if the linker was too long, the compound would lose their activity, and only compounds with a linker of two carbons had some antibacterial activity against *S. aureus*, such as compound **44** with MIC values of 64 μg/mL. Next, we designed compound **60** the same length of linker, but a linker with different bond types, from ether bond to ester bond.

The intermediates of compound **60** also exhibited weak antibacterial activity against *S. aureus*, for example, compound **57** with MIC values of 128 μg/mL. When the nine-carbon chain was added on the amino group, the antibacterial activity against *S. aureus* was substantially improved. For example, the antibacterial activity of compound **58** against *S. aureus* was increased by 4-fold, from 128 μg/mL to 32 μg/mL, while the antibacterial activity of compound **59** against *S. aureus* was

improved substantially, from more 256 μg/mL to 4 μg/mL. This result was consistent with the results obtained in chapter 3, indicating that nine-carbon chain was essential for this series of compounds to maintain good antibacterial activity.

Compound **60** exhibited extremely good antibacterial activity with an MIC value of less than 0.03 μg/mL for *S. aureus* and an MIC value of less than 0.25 μg/mL for *E. coli*. The bond type of the linker played a very important role in improving the antibacterial activity of the compound. For example, when the bond type of the linker was changed from ether bond to ester bond, the antibacterial activity against *S. aureus* was increased substantially from 64 μg/mL to less than 0.03 μg/mL, an increase of more than 2000-fold. Furthermore, the antibacterial activity against *E. coli* was as good as its antibacterial activity against *S. aureus*.

Due to the very good antibacterial activity against *E. coli* of compound **60**, it was further evaluated for its antibacterial activity against other Gram-negative bacterial strains. The MIC result showed that it could inhibit most of these strains with MIC values of as low as less than 0.5 μg/mL. This result indicated that compound **60** could not only fight against *S. aureus*, but also have good antibacterial activity against Gram-negative bacterial strains, including *E. coli*.

Collectively, the double warhead strategy provides a possible method for antibiotics development for treating infections caused by Gram-negative bacterial strains.

Chapter 5. Conclusion

Antibiotic resistance is still a global threat for public heath, because there is lacking of effective approaches to tackle with bacterial resistance. From the discovery of the first modern antibiotic penicillin in the 1930s, the development of the antibiotic drug had undergone a gold era for about 40 years. During this period, many different new classes of antibiotics were discovered and used in clinic, such as beta-lactams, sulfonamides, aminoglycosides, tetracyclines, macrolides, quinolones. Unfortunately, after 1980, only 3 three new types of antibiotic drugs have been brought to the market. Today we are still using very old drugs in clinics to treat the bacterial infections, to which bacteria may have already developed resistance.

Methicillin-Resistant *Staphylococcus Aureus* (MRSA) is the most frequently concerned bacterial for public, because infections caused by MRSA are always with high rates of morbidity and mortality. What is worse, nowadays MRSA is not only resistant to methicillin, but also resistant to other types of beta-lactam antibiotics, such as cephalosporins. It means existing antibiotic drugs are no longer effective to treat these infections caused by resistant bacteria. Fortunately, the use of antibiotic adjuvant together with antibiotics to form a combination drug can restore the efficacy of these antibiotic drugs, providing an alternative strategy to treat infections caused by MRSA, and helping protect the exiting antibiotic drugs. In this study, FtsZ inhibitor **F332** was used as antibiotic adjuvant together with different types of beta-lactam antibiotics to form combination drugs, in order to restore the efficacy of these beta-lactam antibiotics to treat the infections caused by MRSA.

The potential of **F332** as FtsZ inhibitor has been confirmed through different biological tests in our previous study. The MIC result of **F332** suggested that it is suitable to work as an antibiotic adjuvant due to its weak antibacterial activity

against MRSA. The efficacy of the combination drugs treating 28 clinical MRSA strains was evaluated, and the MIC results showed that most of combination drugs had better antibacterial activity against MRSA strains than the beta-lactam antibiotics used alone.

Furthermore, most of these combination drugs had synergistic effect, which was calculated by FIC index less than 0.5. The combination drug of compound **F332** and methicillin had the highest percentage (82%) of synergistic effect among all these combination drugs. This result indicated that compound **F332** could restore the efficacy of methicillin to treat MRSA *in vitro*. The other combination drugs of compound **F332** and different types of beta-lactam antibiotics also had high percentage of synergy, which was more than 60% for cefuroxime, cloxacillin and amoxicillin. All these results indicated that the FtsZ inhibitor **F332** could be used as antibiotic adjuvant together with beta-lactam antibiotics to form a combination drug, which can restore the efficacy of beta-lactam antibiotics to treat MRSA strains *in vitro*.

The lack of new classes of antibiotics in recent years is one reason of causes of antibiotic resistance. Therefore, there is an urgent demand to develop new types of antibiotic drugs to fight against infections caused by bacteria. FtsZ has been considered as a new drug target for antibiotic drug discovery and so many FtsZ inhibitors discovered by other research groups. However, the problem is that most of these FtsZ inhibitors have no antibacterial activity against Gram-negative bacterial strains, such as *E. coli*. A new class of FtsZ inhibitors has been developed in this study, which not only has antibacterial activity against *S. aureus*, but also has antibacterial activity against *E. coli*.

We designed and synthesized a series of compounds and their antibacterial activity

were evaluated. The design of compounds was based on the structure of compound **F332**, and a compound library was synthesized by structure modification. The MIC results showed that most of these compounds exhibited good antibacterial activity against *S. aureus* with MIC values of 4 μg/mL, such as compound **8**, some of compounds with amidoxime and most of compounds with amidine. Furthermore, compounds **32-38** were found to have antibacterial activity against both *S. aureus* and *E. coli*, especially for compounds **32** and **38**, of which the antibacterial activity against *S. aureus* and *E. coli* with MIC values of 4 μg/mL and 16 μg/mL, respectively.

The SAR analysis suggested that maintain the nine-carbon chain on amino group was essential for this series of compounds to keep good antibacterial activity. For the R_1 group of **F332**, the amide group could be replaced by amidoxime and amidine, with which the compound still had good antibacterial activity against *S. aureus*. Moreover, when the amidoxime of compounds was acetylated by acetic anhydride, some of these compounds still had some extent antibacterial activity against *S. aureus*, such as compounds **25**, **27** and **31**. Excitedly, when the amide group of compounds was replaced by the amidine group, the antibacterial spectrum of these compounds was broadened.

Overall, we broadened the antibacterial spectrum of FtsZ inhibitors through structure modification of compound **F332**, and the broad-spectrum FtsZ inhibitors with amidine group were synthesized. This result indicated that the structure of compound **F332** was a potential scaffold to develop a new type of broad-spectrum antibiotic drugs.

We also tried to use double warhead strategy to fight against Gram-negative bacteria, which are harder to treat than Gram-positive bacteria. The scaffold of compound

F332 was chosen to be part of double warhead molecules. Another part of the double warhead molecule was triclosan, which is a FabI inhibitor. It can inhibit fatty acid synthesis and has a broad antibacterial spectrum, including *S. aureus* and *E. coli*. Its drawback is its health and environmental concerns due to its relative high toxicity and disposed problem. The double warhead molecule was hypothesized that such a strategy could not only broaden the antibacterial spectrum of FtsZ inhibitors, but also reduce the toxicity of triclosan.

We synthesized a series of double warhead compounds by using a linker to connect two scaffolds, and the antibacterial activity of these compounds and intermediates was evaluated by MIC test. We found that compound **60** had excellent antibacterial activity against both *S. aureus* and *E. coli*, with MIC values of less than 0.03 μg/mL. Furthermore, its antibacterial activity against other Gram-negative bacterial strains was also evaluated, and the result showed that MIC values of most Gram-negative bacterial strains was less than 0.5 μg/mL.

The SAR analysis showed that the length of the linker should not be more than two carbons, otherwise these compounds would lose their antibacterial activity. Overall, compound **60** not only exhibited excellent antibacterial activity against *S. aureus*, but also performed pretty good antibacterial activity against Gram-negative bacterial strains. This result indicated that the double warhead strategy with the scaffold of FtsZ inhibitor could be a potential method for discovering new types of antibacterial drugs to fight against Gram-negative bacterial strains.

Although the findings reported in this thesis are encouraging, the further structure modification of compound **F332** is required in order to find more new types of compounds with better broad-spectrum antibacterial activity. The amidine group was confirmed as a good starting point for the structure modification, because the

compounds with amidine exhibited very good antibacterial activity against both *S. aureus* and *E. coli*. In the next stage, we should keep this functional group on the structure and modified other position on the phenyl ring. The role of other position whether is also important should be studied in future work. Moreover, the effect of the number of fluorine atom on the antibacterial activity should be investigated, because the previous study showed that two fluorine atoms were essential for the compound to maintain antibacterial activity.

For the double warhead strategy, the compound **60** should be further investigated the mechanism of the effective action, whether the double warhead molecule can interact with two targets or it can interact with a completely new target. Furthermore, although it exhibited excellent antibacterial activity against *S. aureus* and many Gram-negative bacterial strains, its cytotoxicity is unknown. Its cytotoxicity should be evaluated on human cells to determine whether it is too high for normal cell. Another problem is whether this double warhead molecule has the good antibacterial activity against Gram-negative bacterial strains *in vivo*. The animal study should be conducted to confirm its efficacy *in vivo*, because we hope this double warhead strategy can provide an effective approach to treat infections caused by Gram-negative bacterial strains.

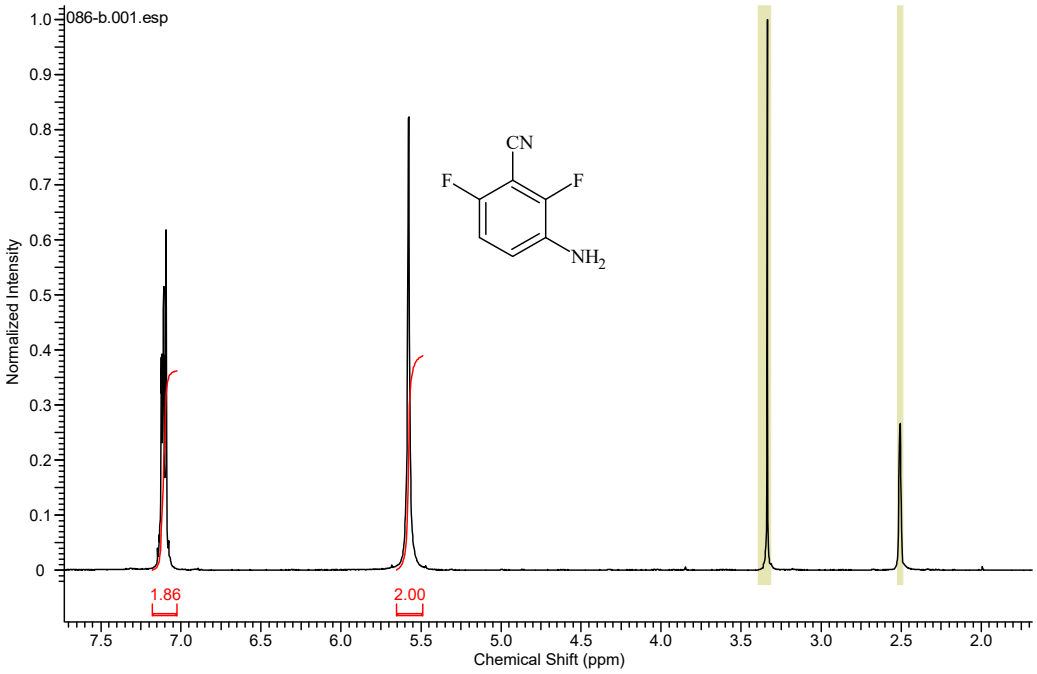
The lead compound **F332** has been confirmed as a good selective FtsZ inhibitor with very good antibacterial activity against *S. aureus*. It has been approved to work as antibiotic adjuvant to restore the efficacy of beta-lactam antibiotics to treat MRSA *in vitro*. We propose that it can also be used with other types of antibiotics to treat infections caused by resistant bacterial strains. The synergistic effect of compound **F332** used together with other antibiotics should be investigated widely. We hope this compound can be a good antibiotic adjuvant and can protect many types of existing antibiotic drugs, which can reduce the spread and emergence of

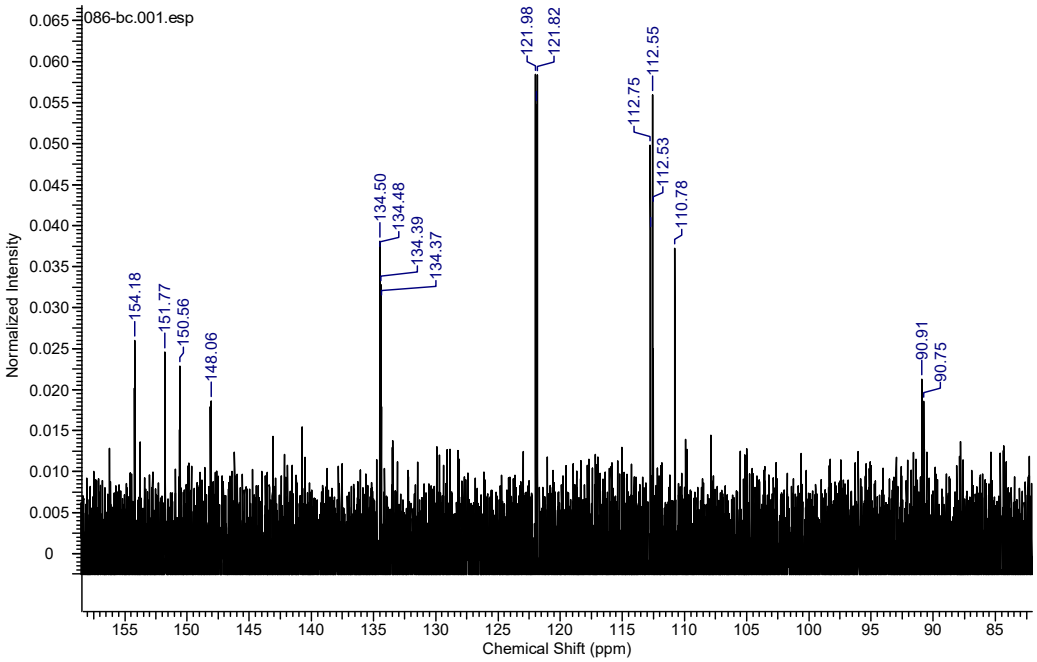
antibiotic resistance.

Collectively, bacterial cell division FtsZ protein as a new drug target has been well studied previously and its inhibitors play an important role in antibiotic drug discovery. Hopefully, these new hypothesis and proposal can be conducted in near future.

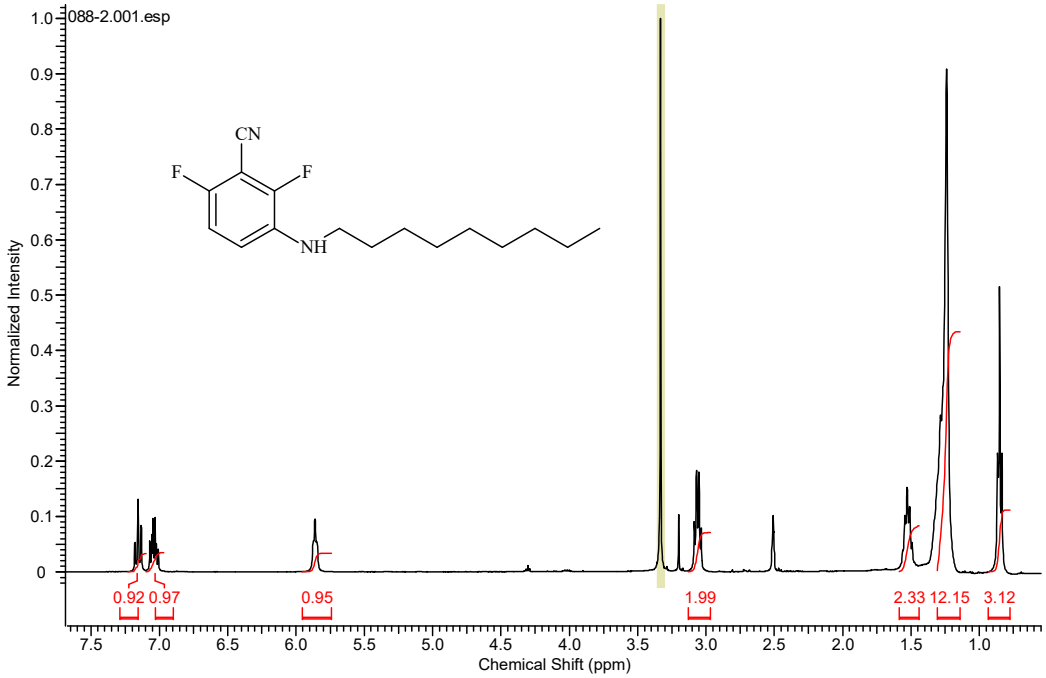
Appendix

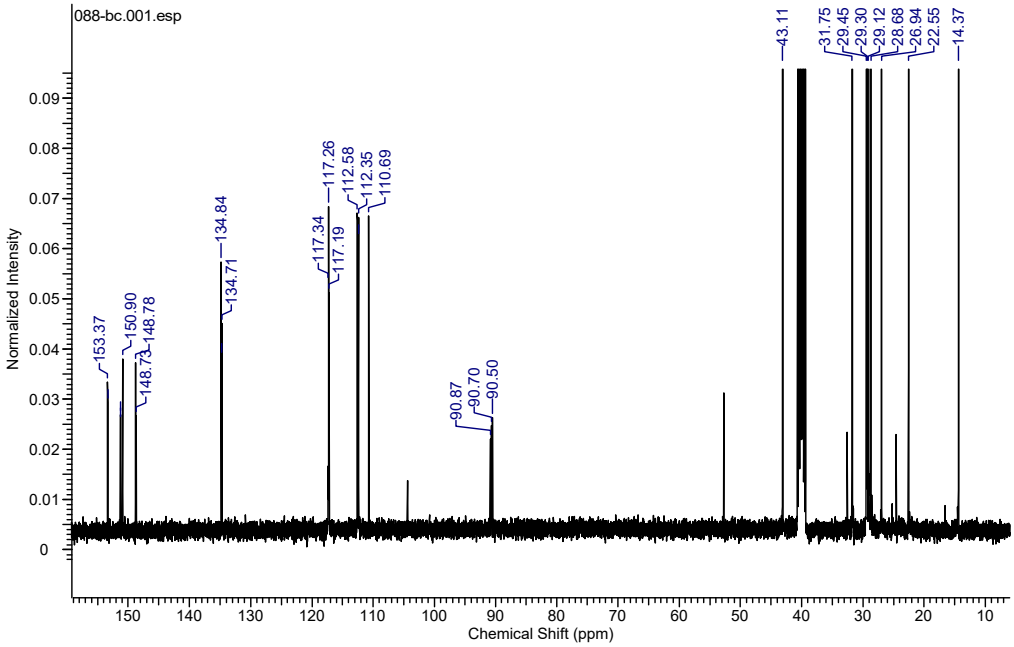
3-amino-2,6-difluorobenzonitrile (2)



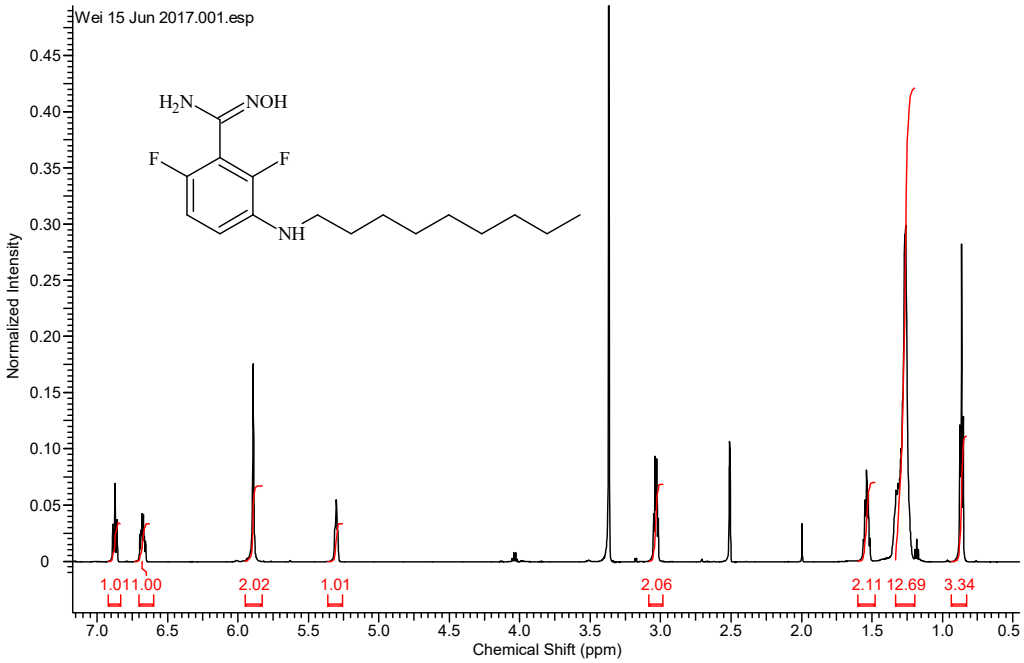


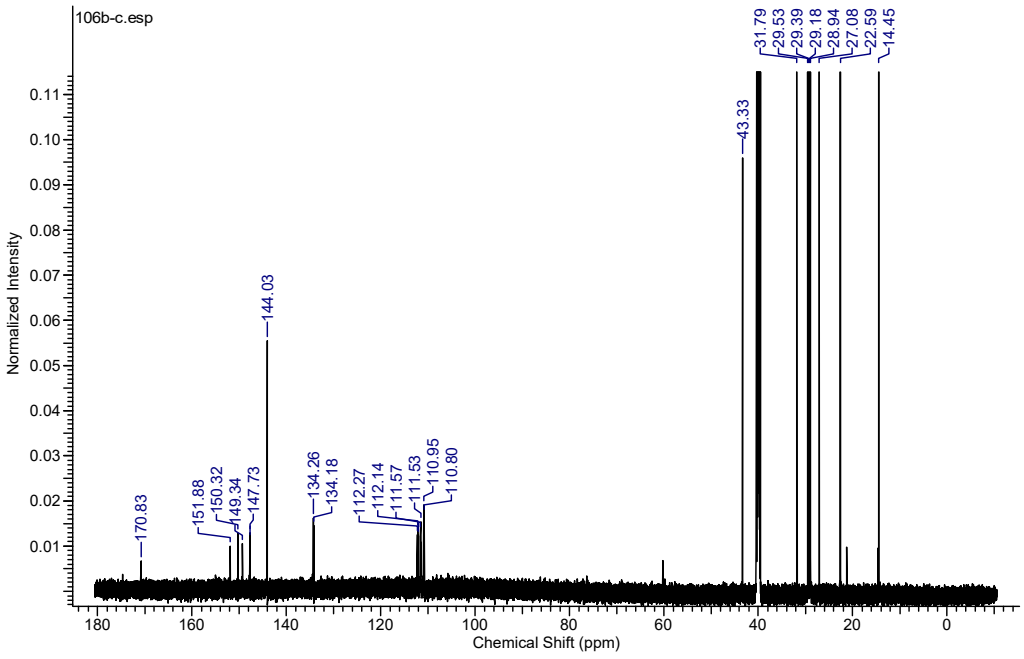
2,6-difluoro-3-(nonylamino)benzonitrile (3)



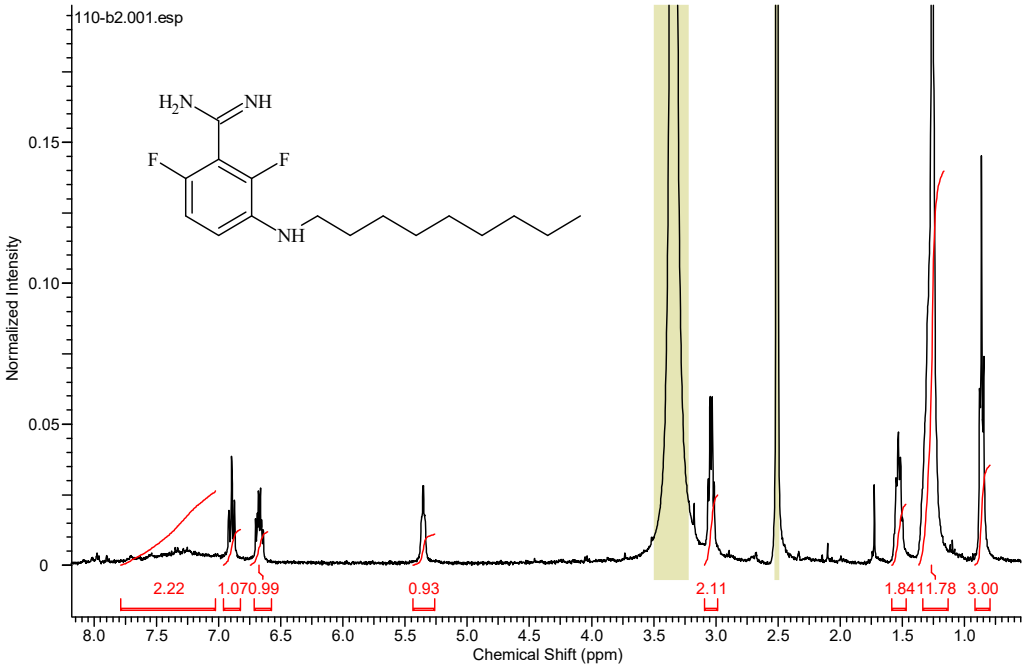


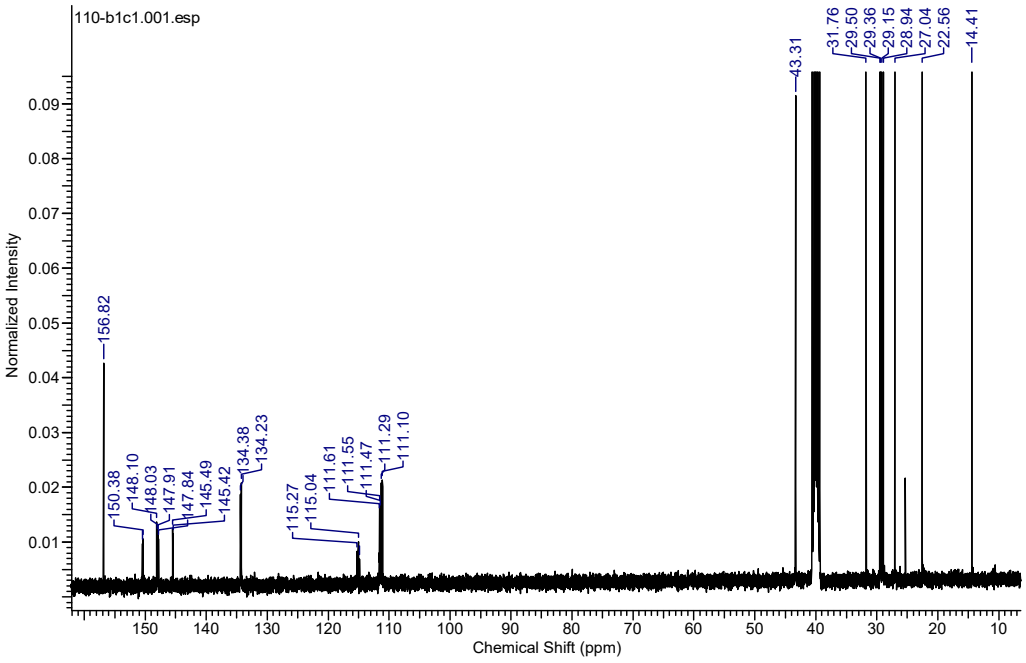
2,6-difluoro-N'-hydroxy-3-(nonylamino)benzimidamide (4)



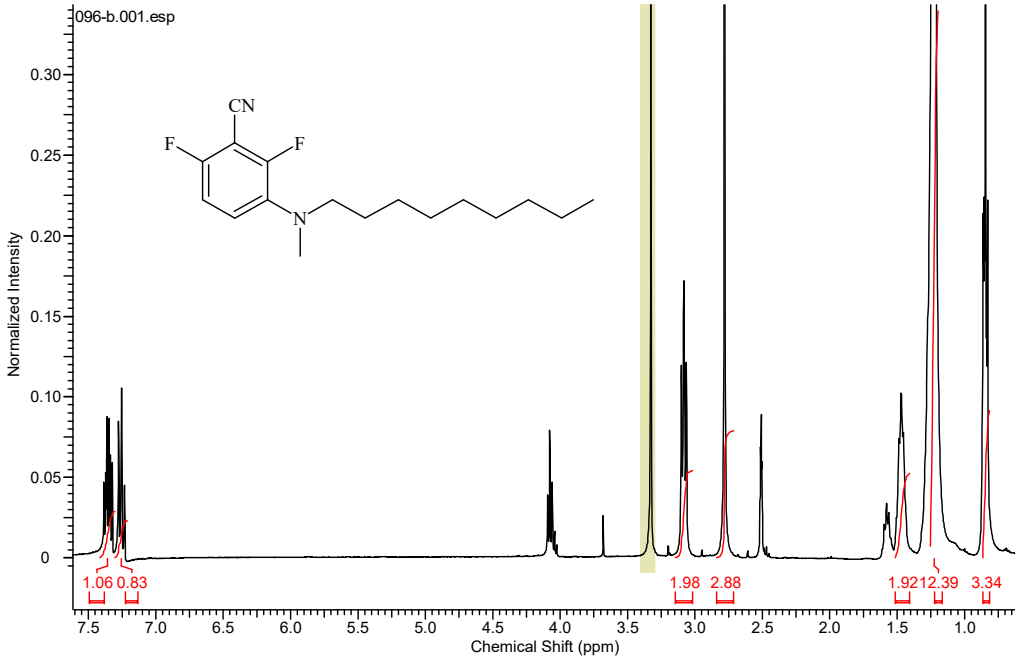


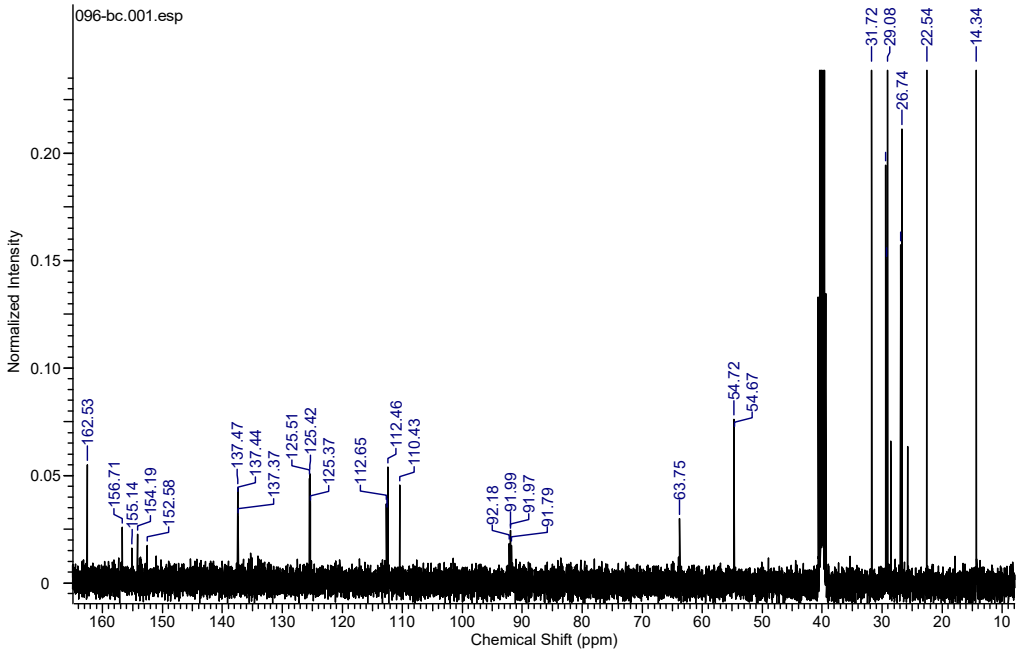
2,6-difluoro-3-(nonylamino)benzimidamide (6)



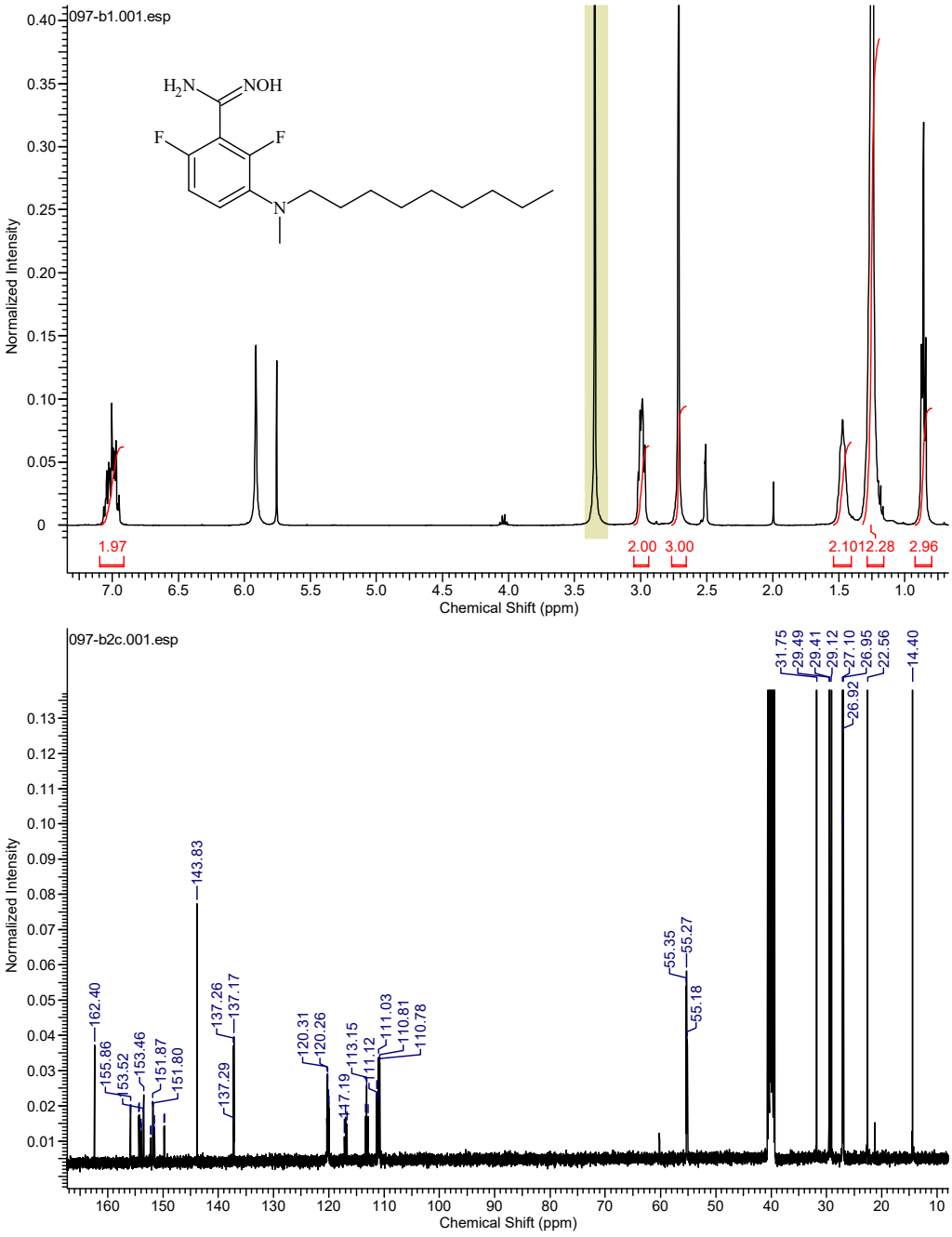


2,6-difluoro-3-(methyl(nonyl)amino)benzonitrile (7)

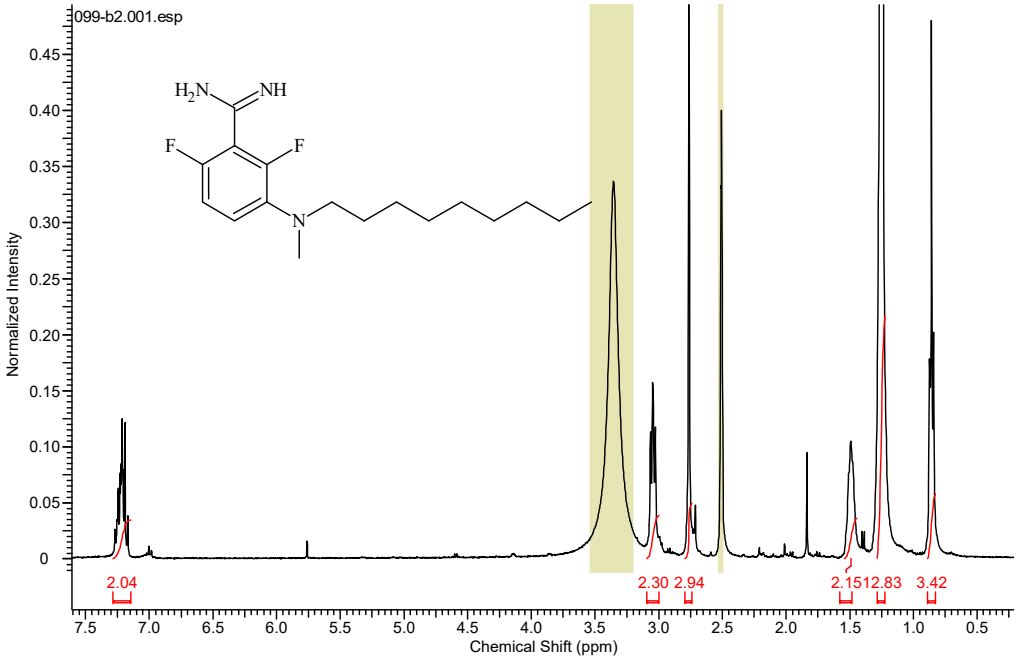


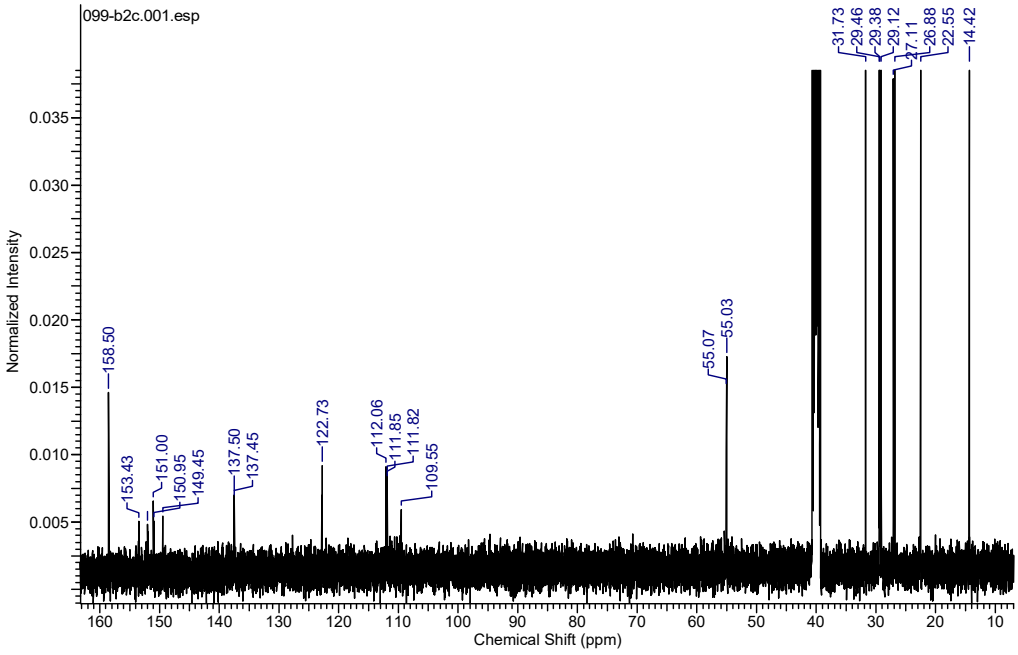


2,6-difluoro-N'-hydroxy-3-(methyl(nonyl)amino)benzimidamide (8)

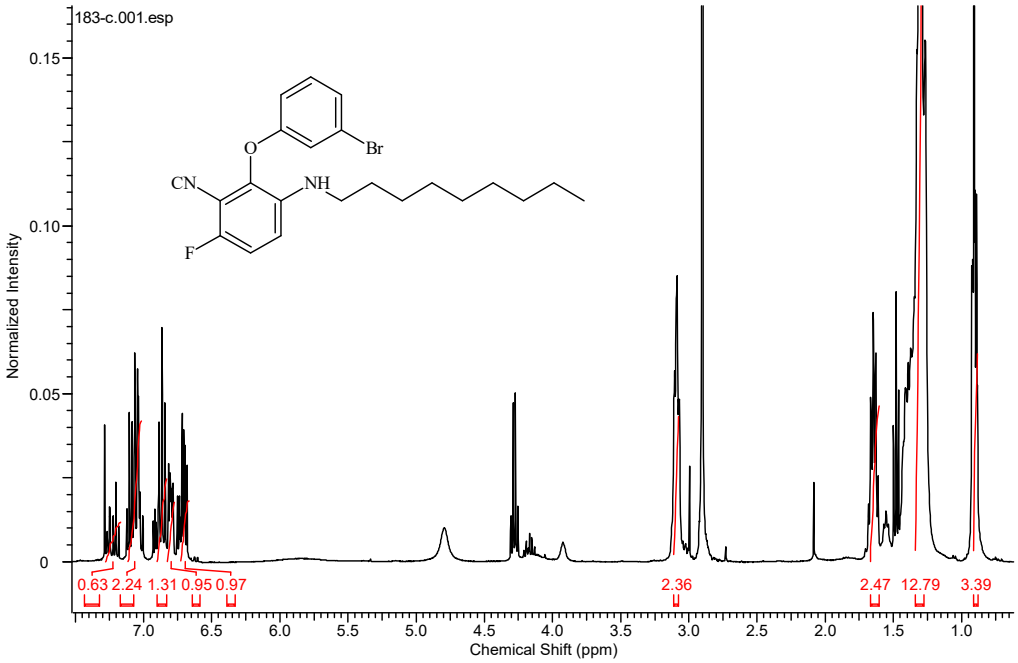


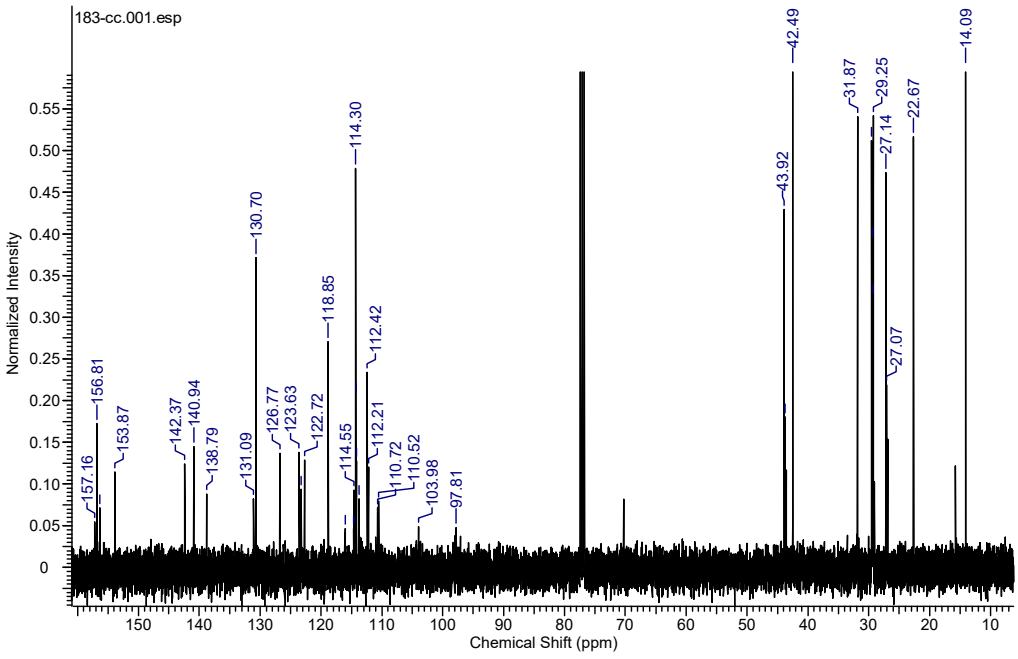
2,6-difluoro-3-(methyl(nonyl)amino)benzimidamide (10)



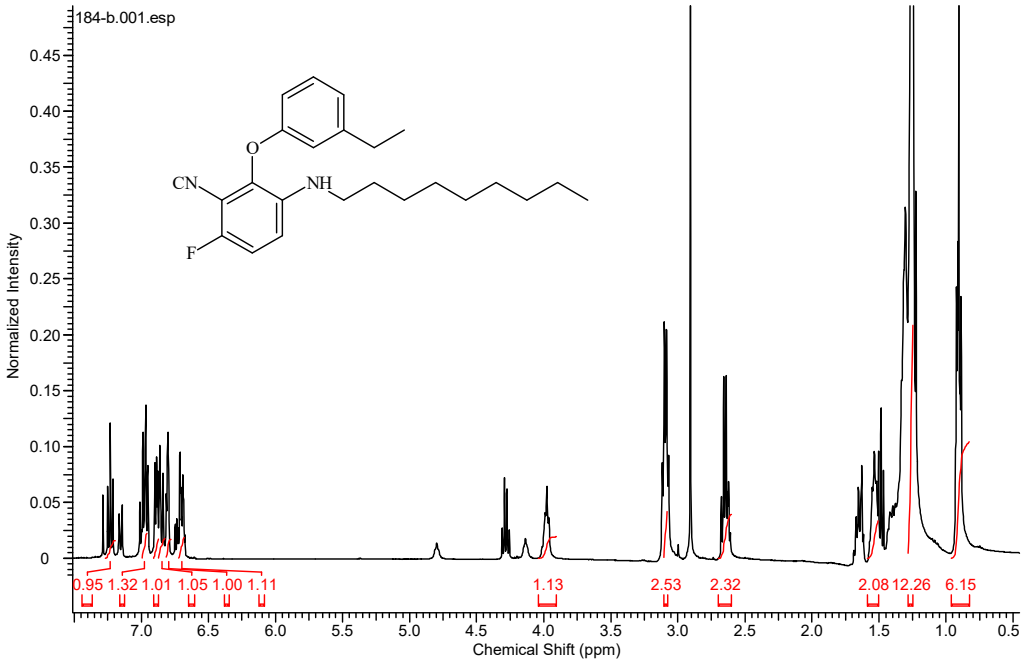


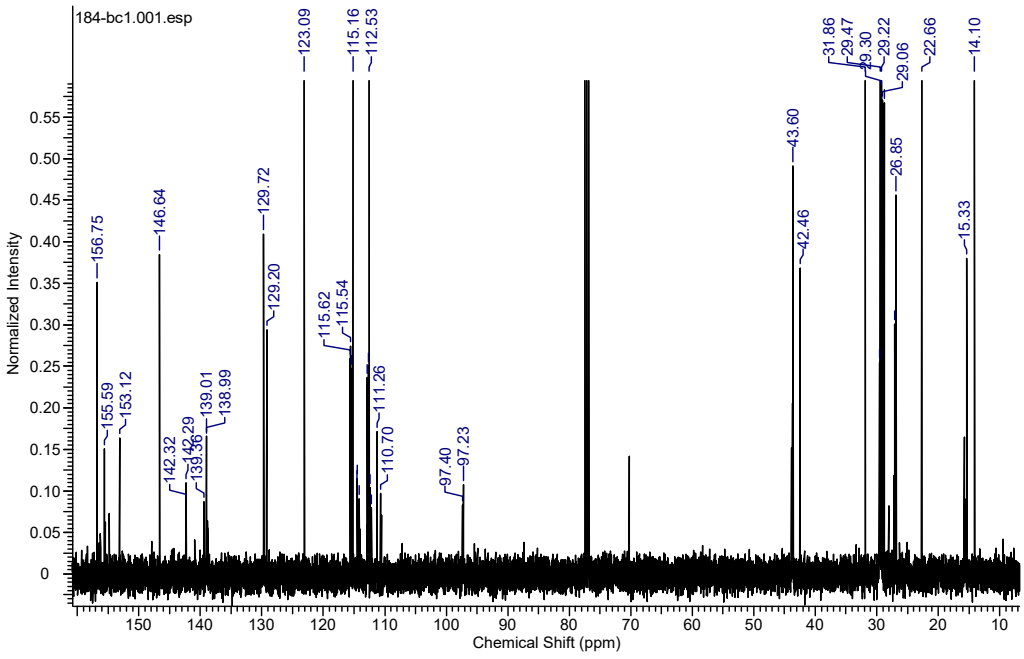
2-(3-bromophenoxy)-6-fluoro-3-(nonylamino)benzonitrile (11)



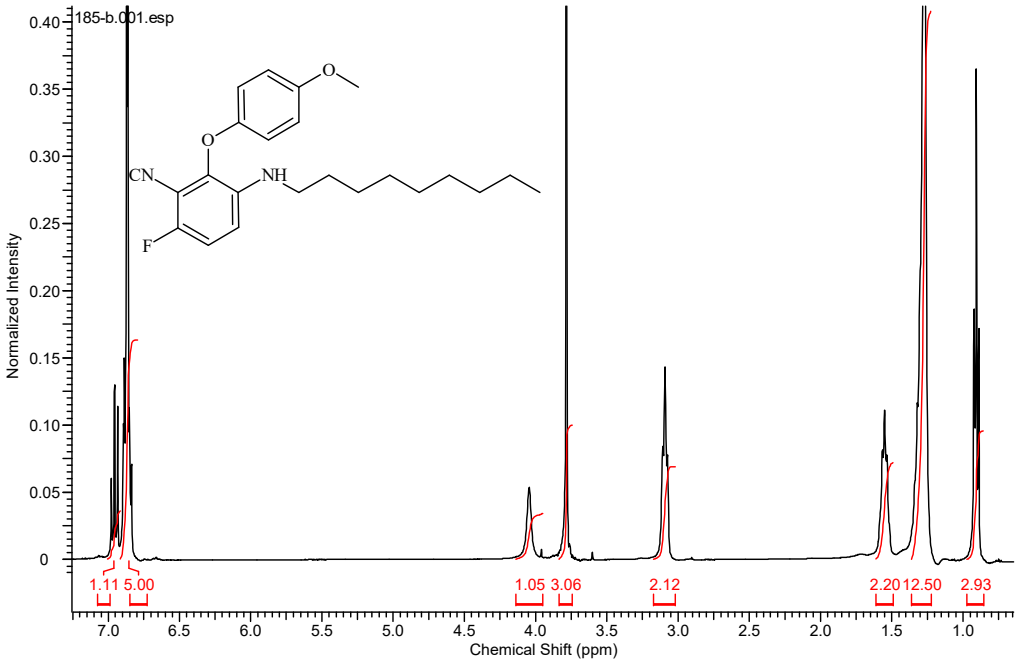


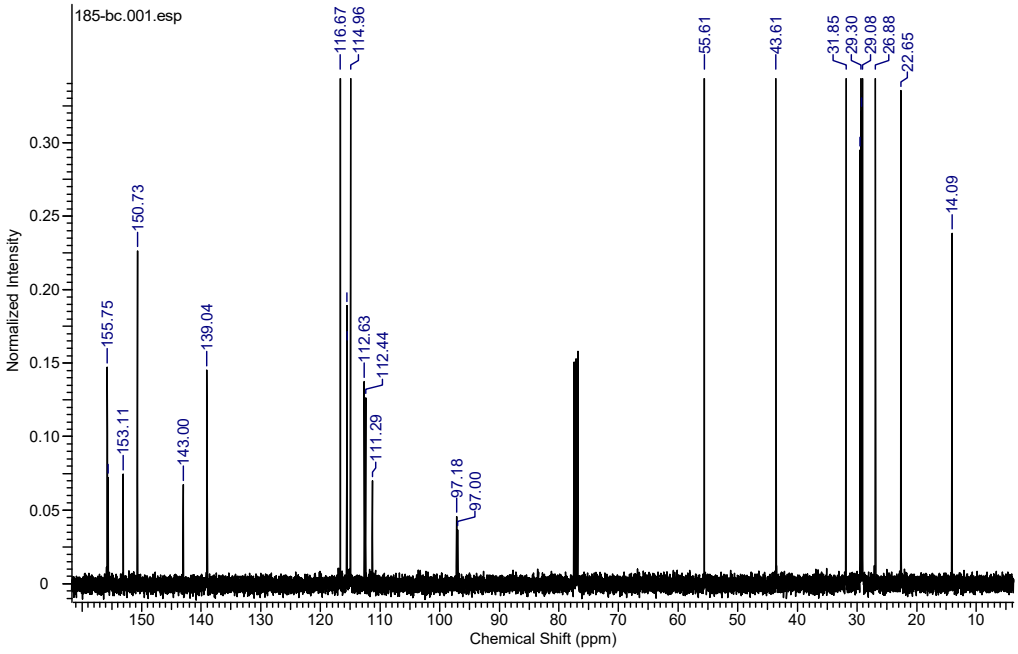
2-(3-ethylphenoxy)-6-fluoro-3-(nonylamino)benzonitrile (12)



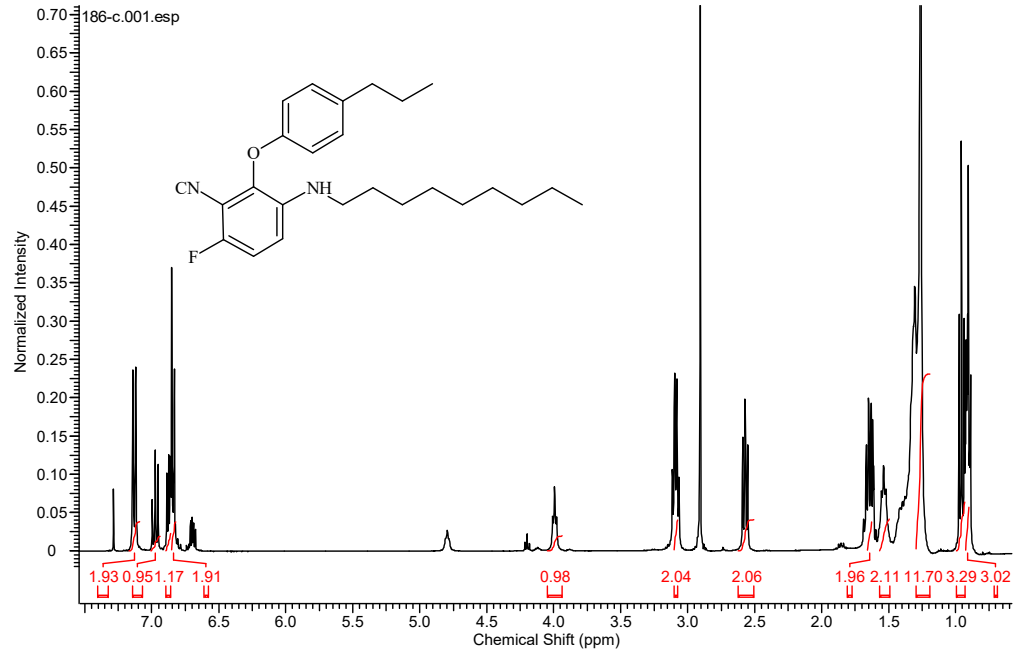


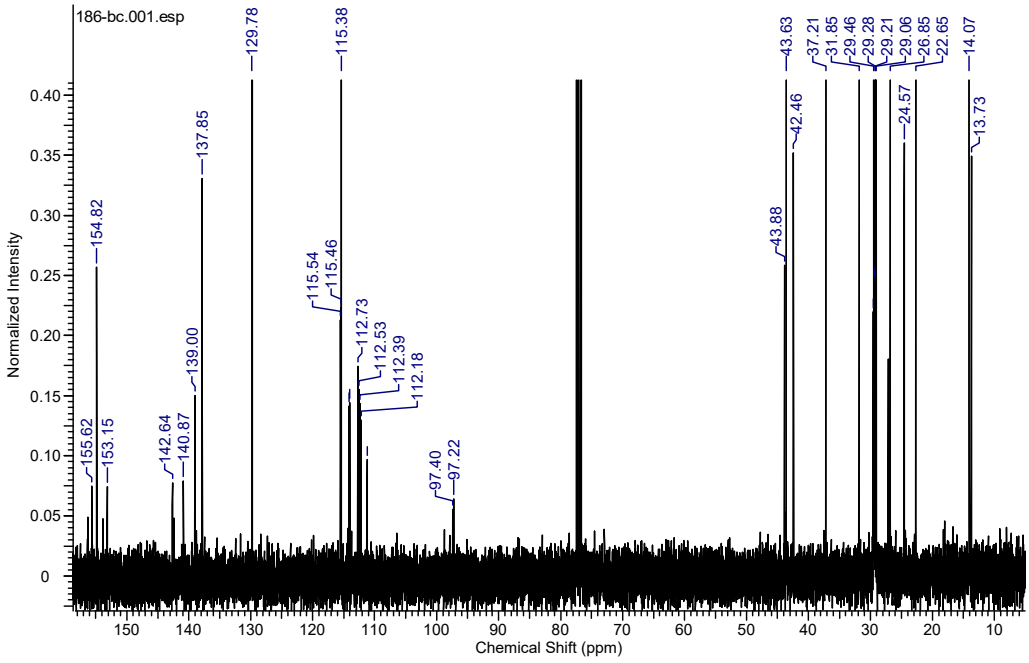
6-fluoro-2-(4-methoxyphenoxy)-3-(nonylamino)benzonitrile (13)



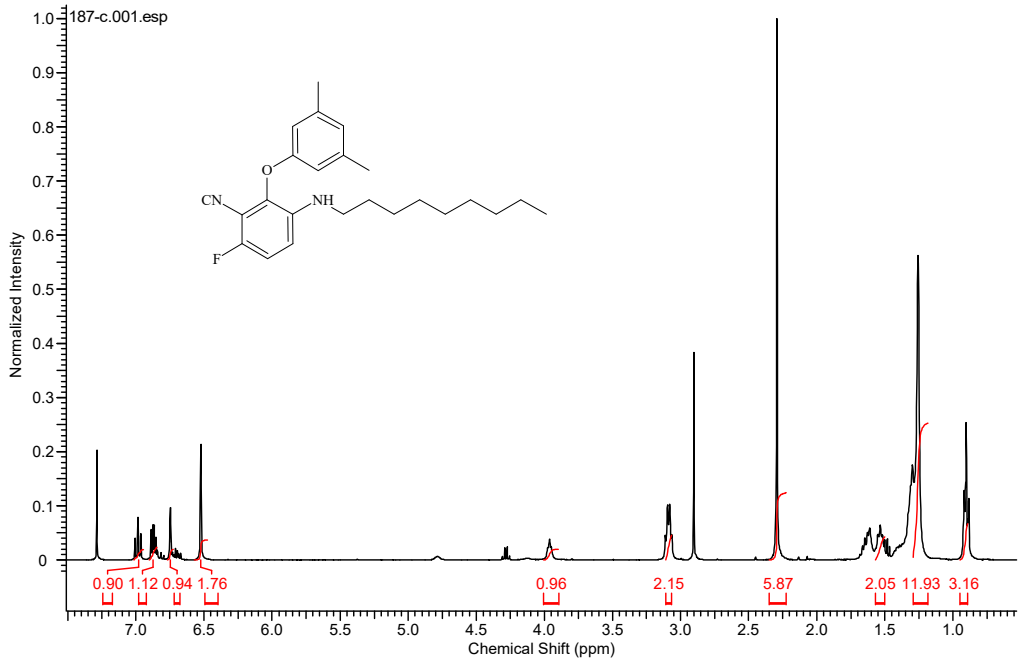


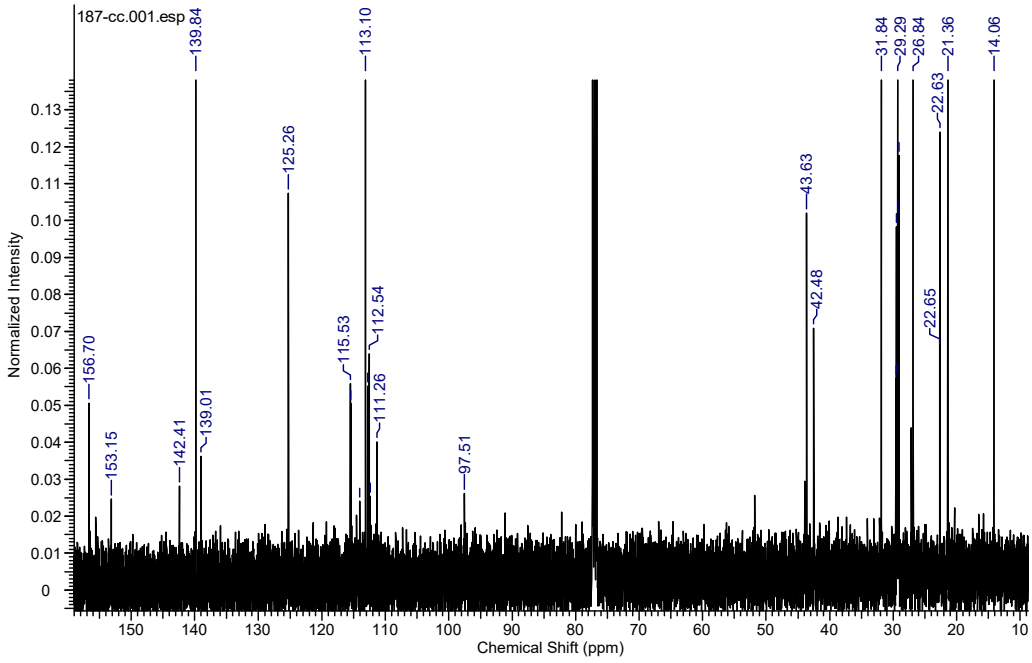
6-fluoro-3-(nonylamino)-2-(4-propylphenoxy)benzonitrile (14)



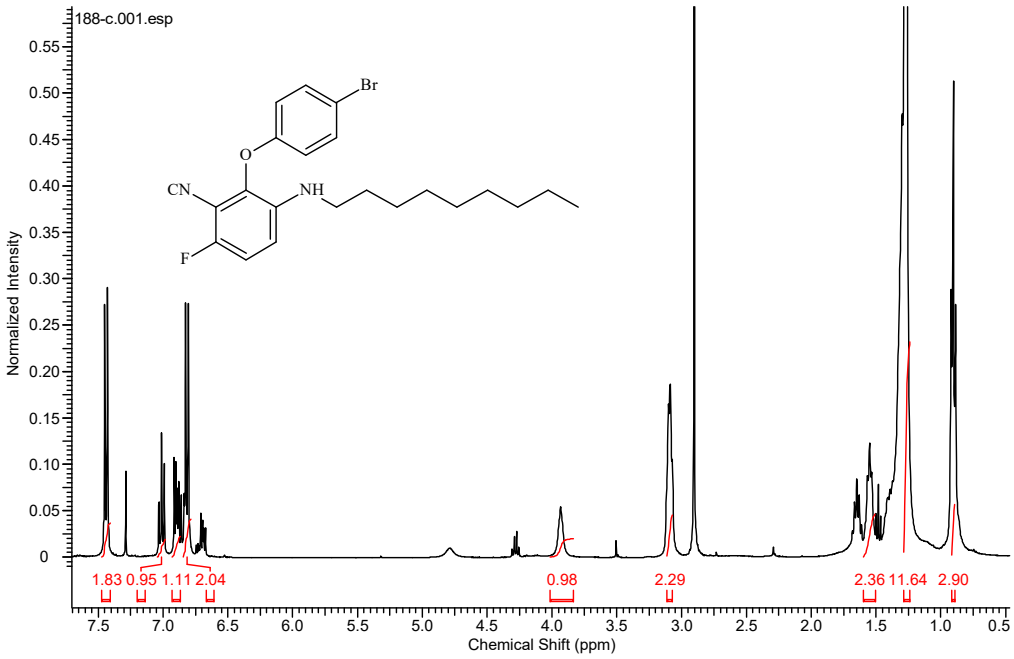


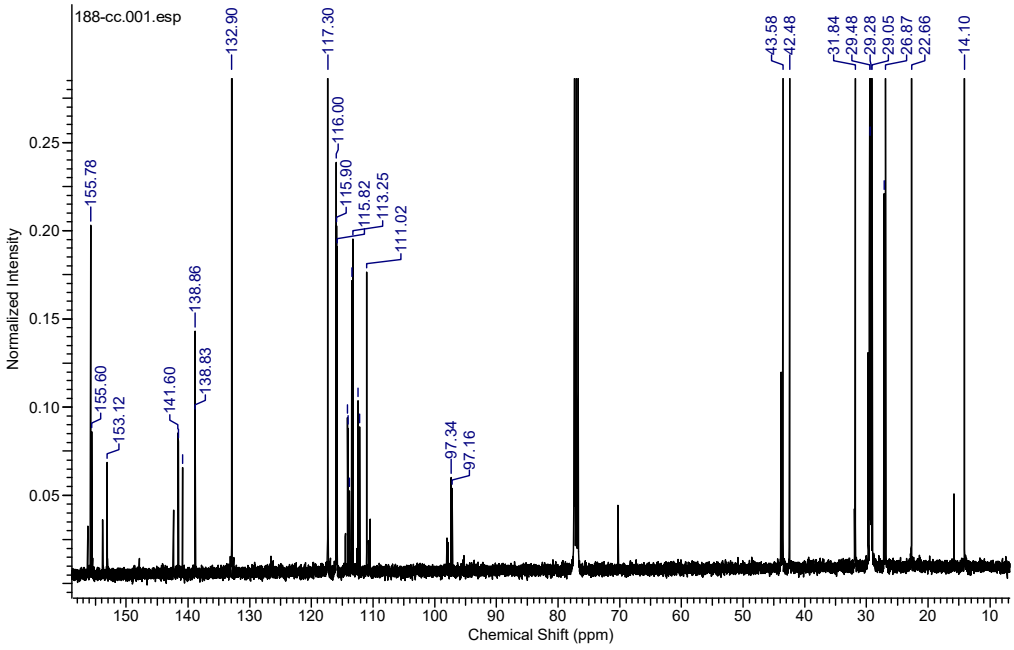
2-(3,5-dimethylphenoxy)-6-fluoro-3-(nonylamino)benzonitrile (15)



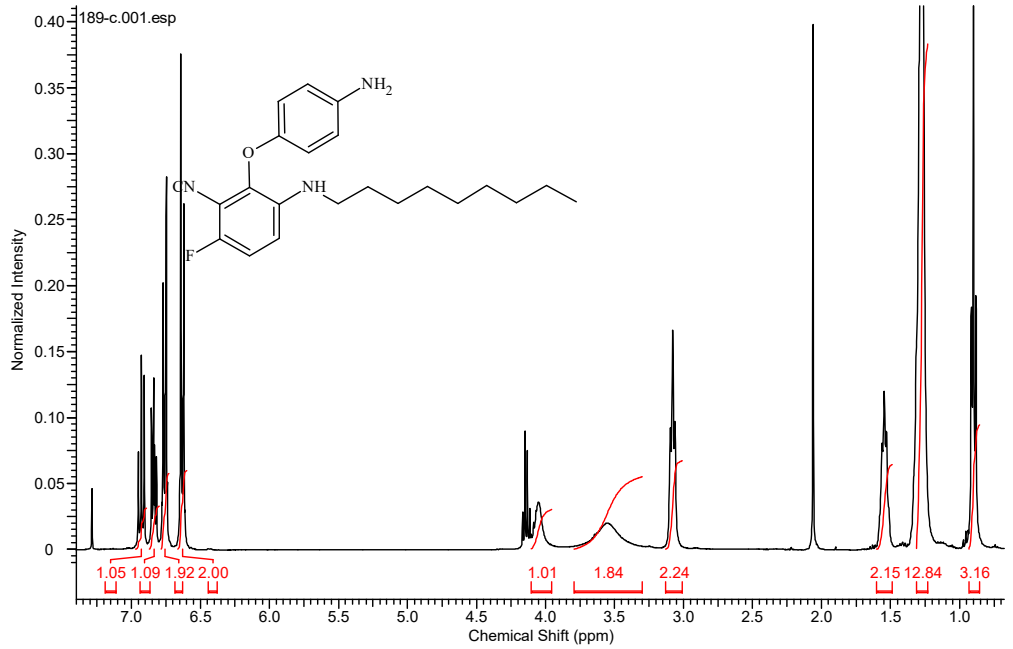


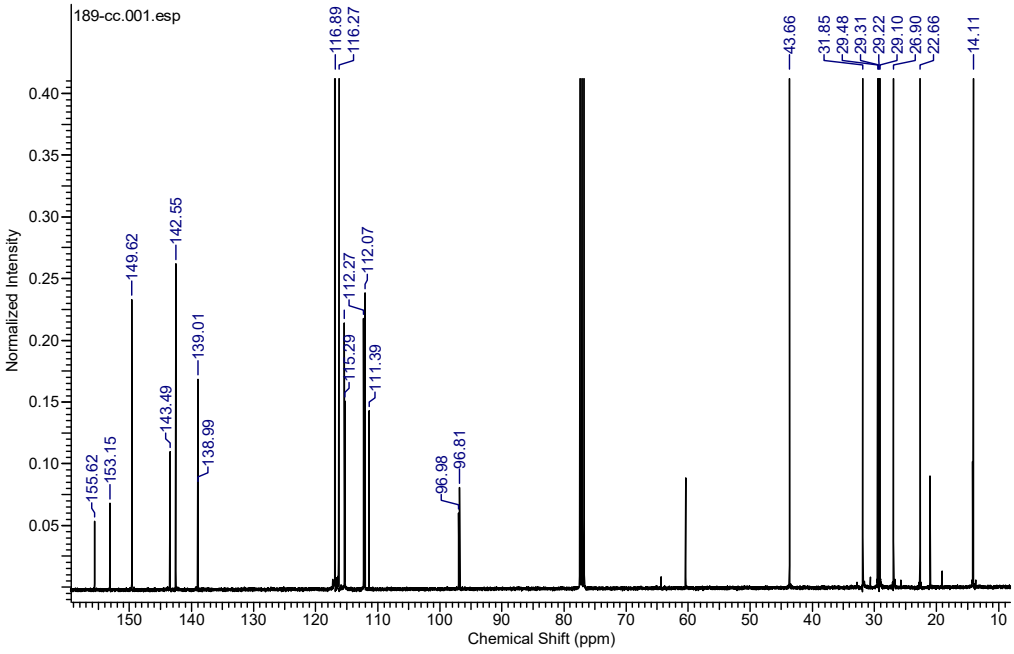
2-(4-bromophenoxy)-6-fluoro-3-(nonylamino)benzonitrile (16)



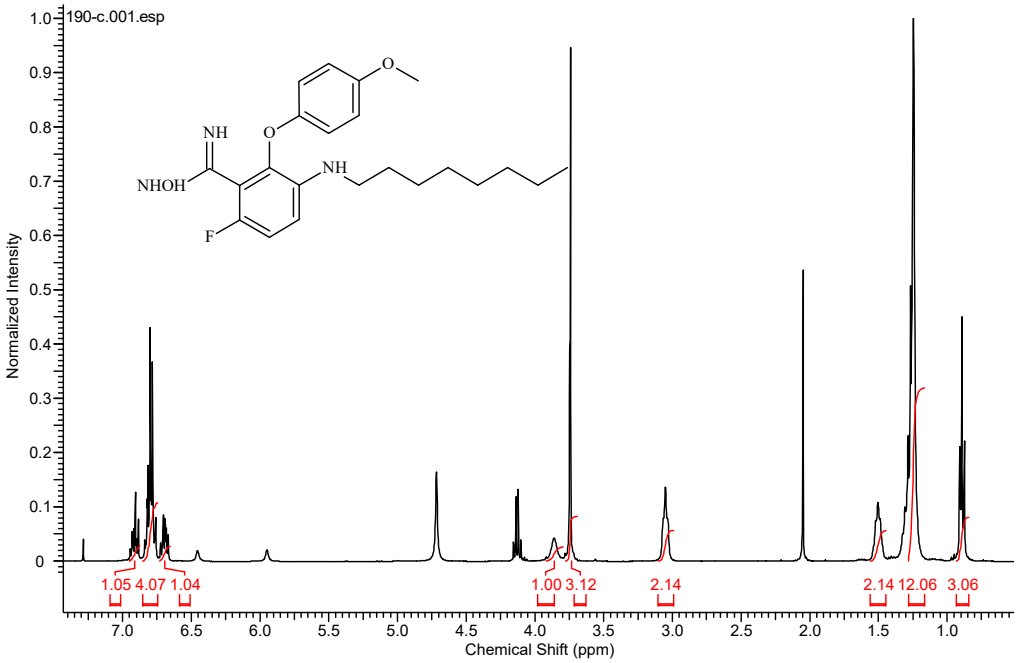


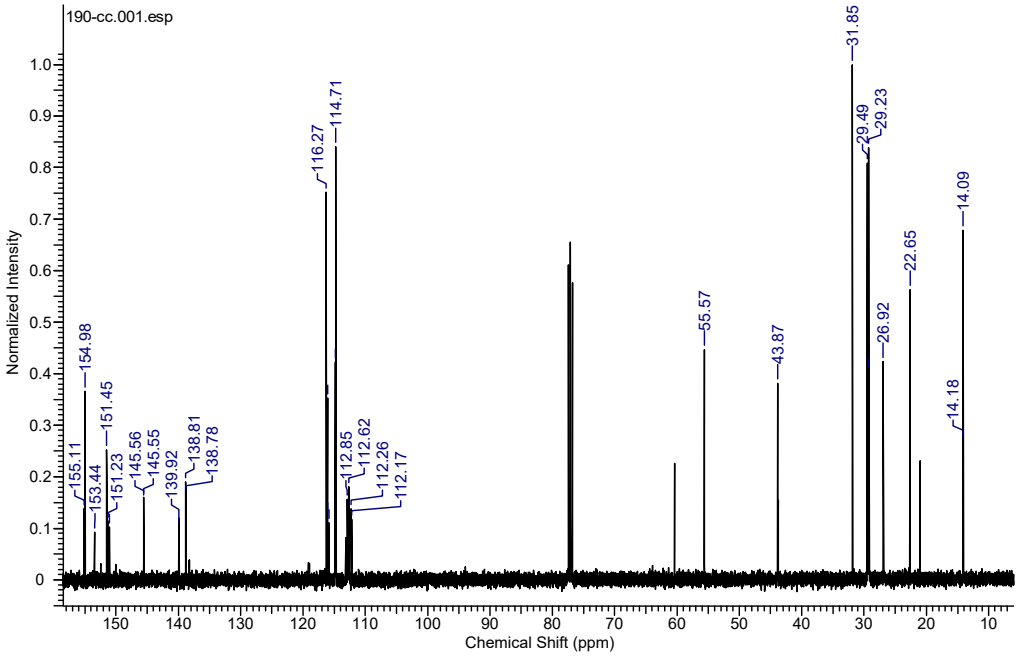
2-(4-aminophenoxy)-6-fluoro-3-(nonylamino)benzonitrile (17)



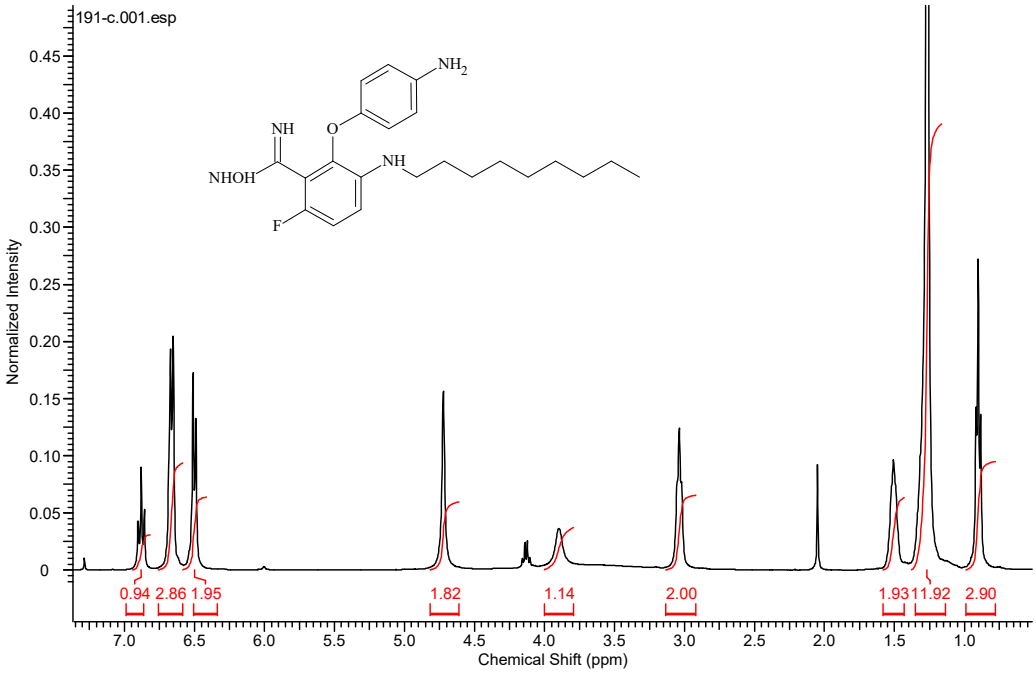


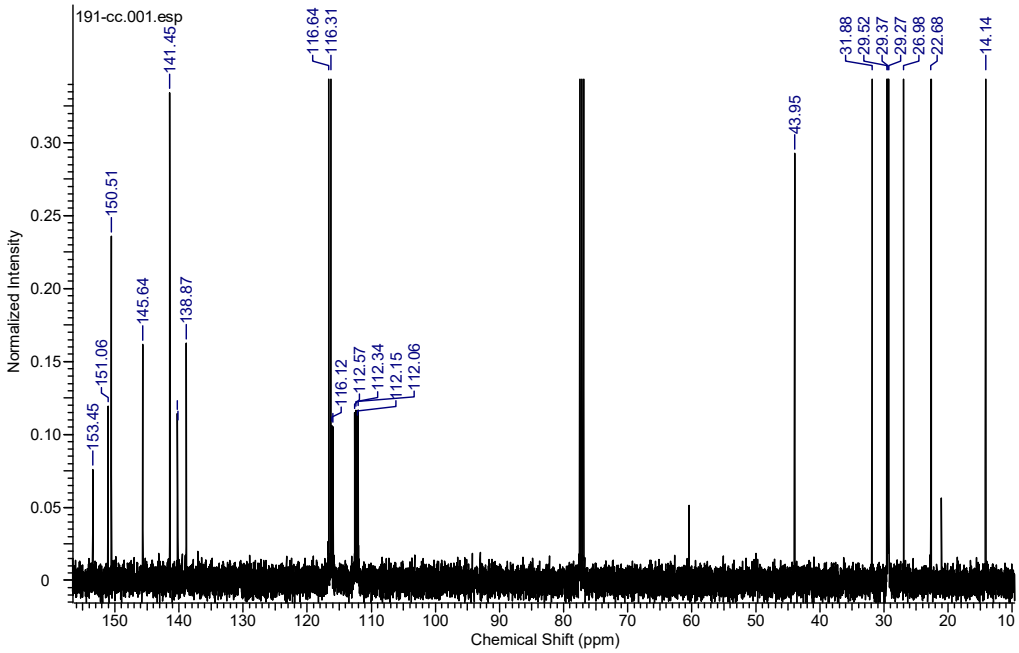
6-fluoro-N-hydroxy-2-(4-methoxyphenoxy)-3-(nonylamino)benzimidamide (18)



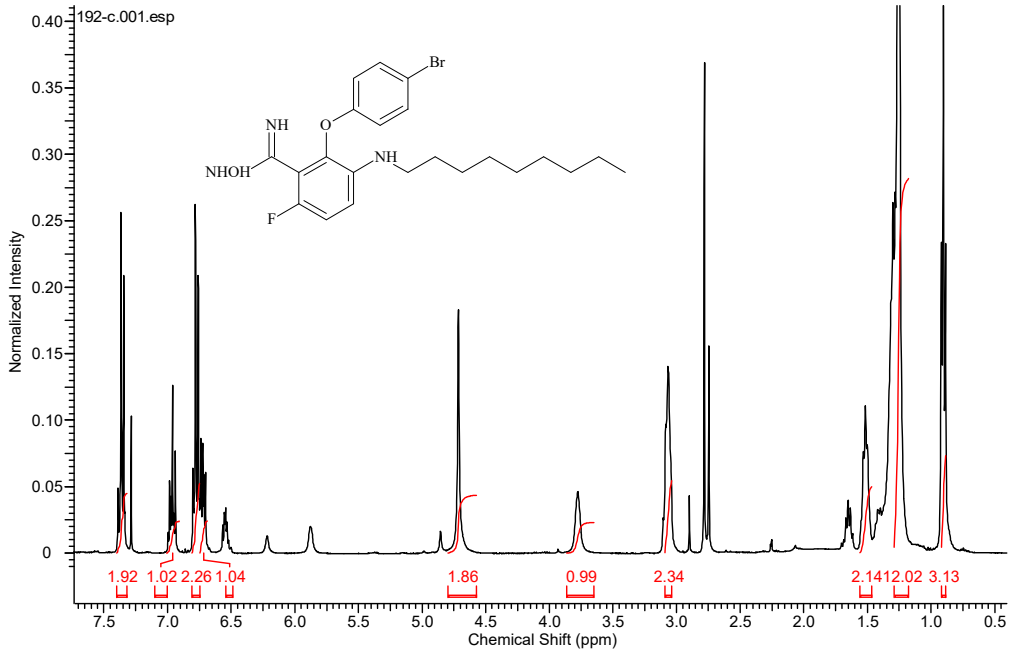


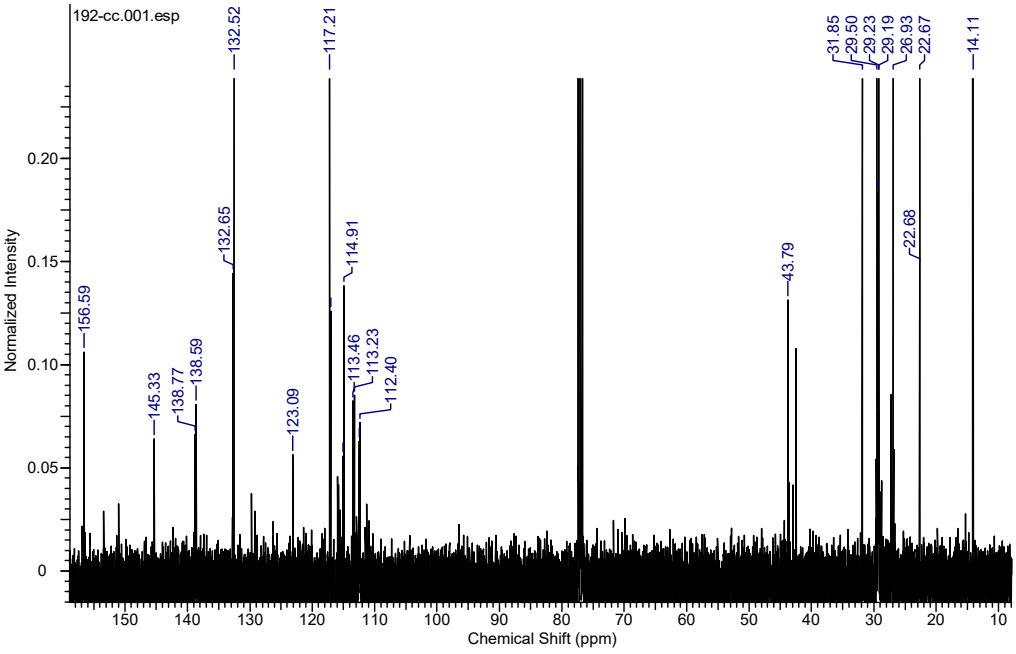
2-(4-aminophenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (19)



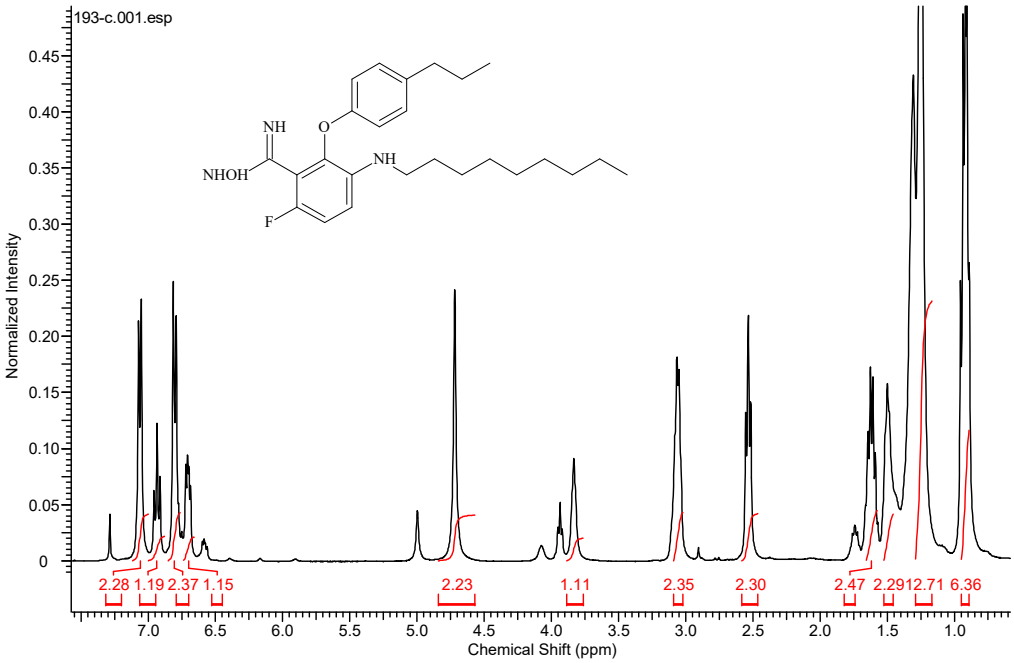


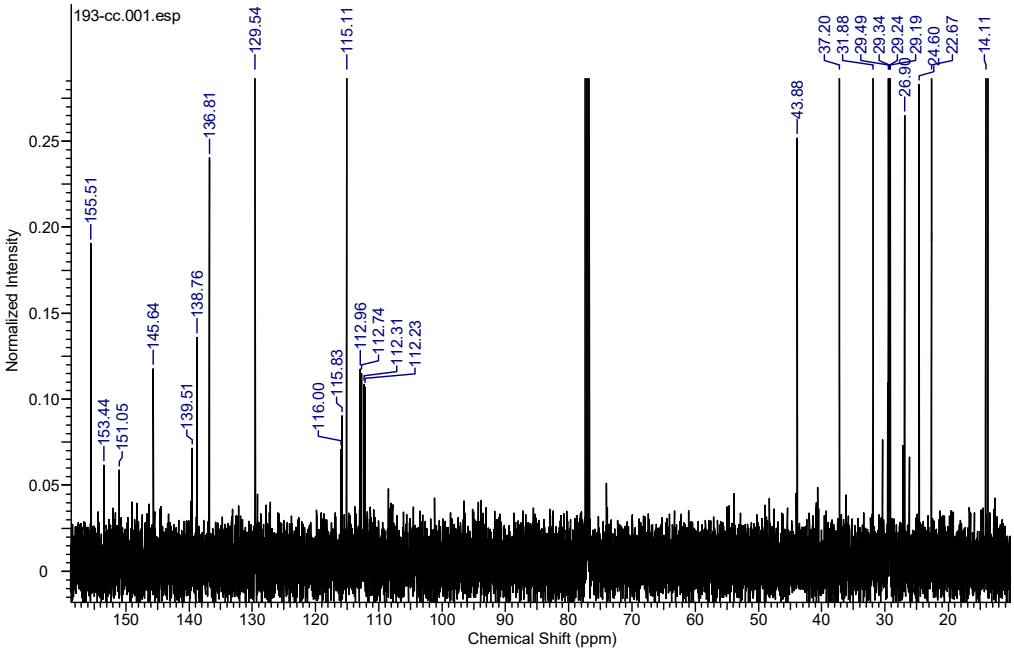
2-(4-bromophenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (20)



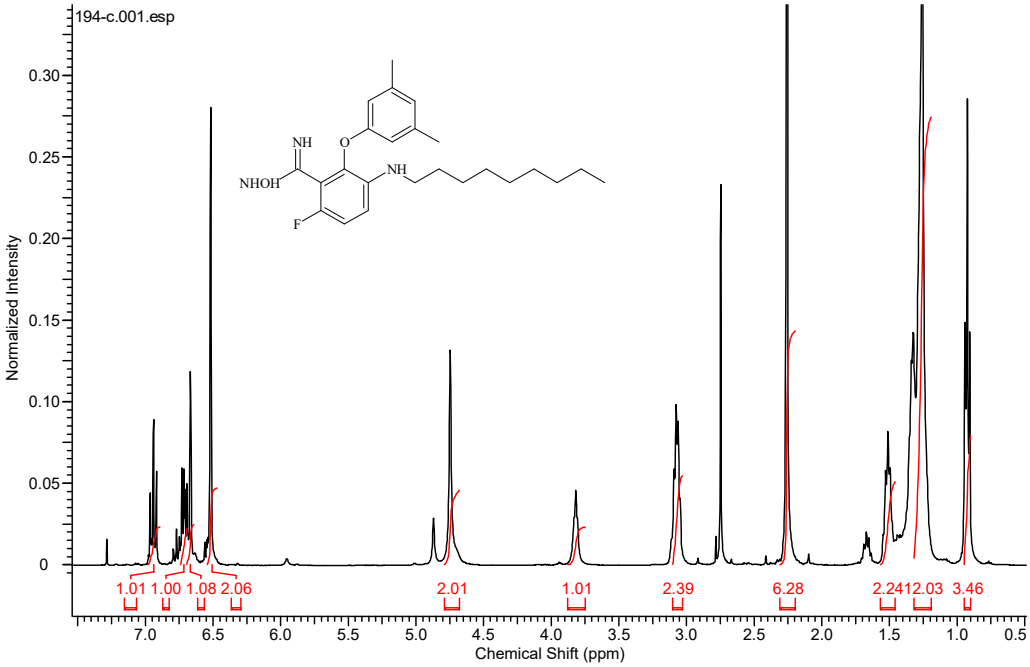


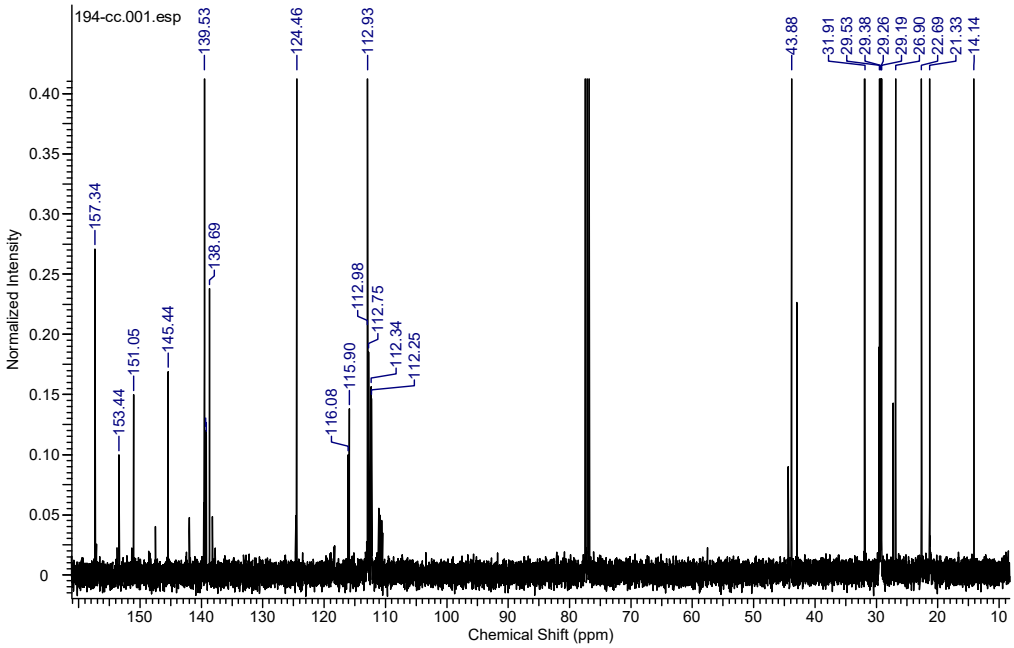
6-fluoro-N-hydroxy-3-(nonylamino)-2-(4-propylphenoxy)benzimidamide (21)



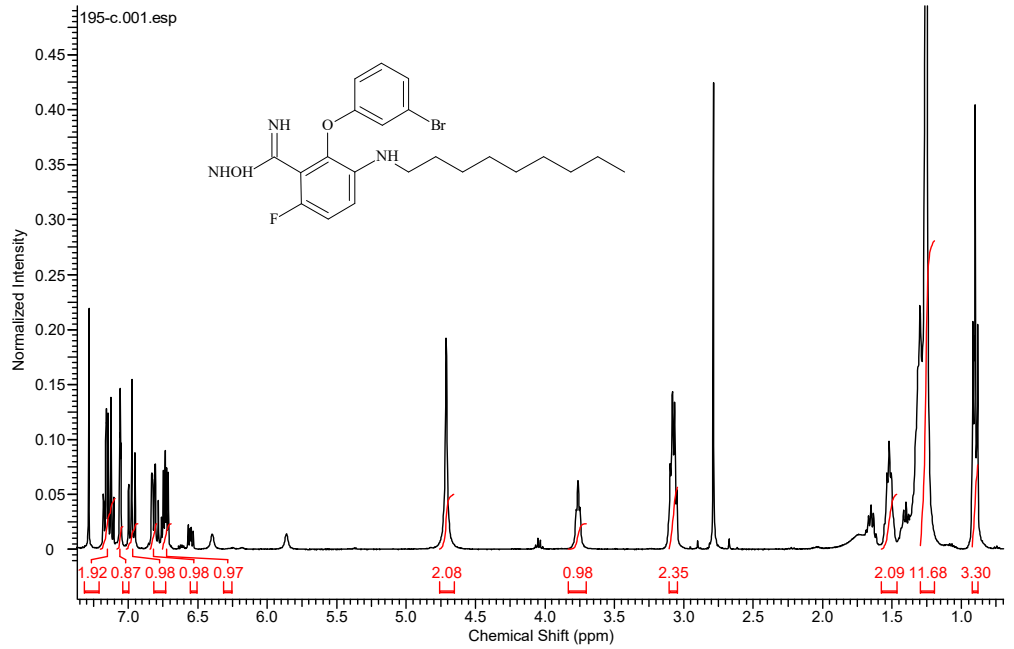


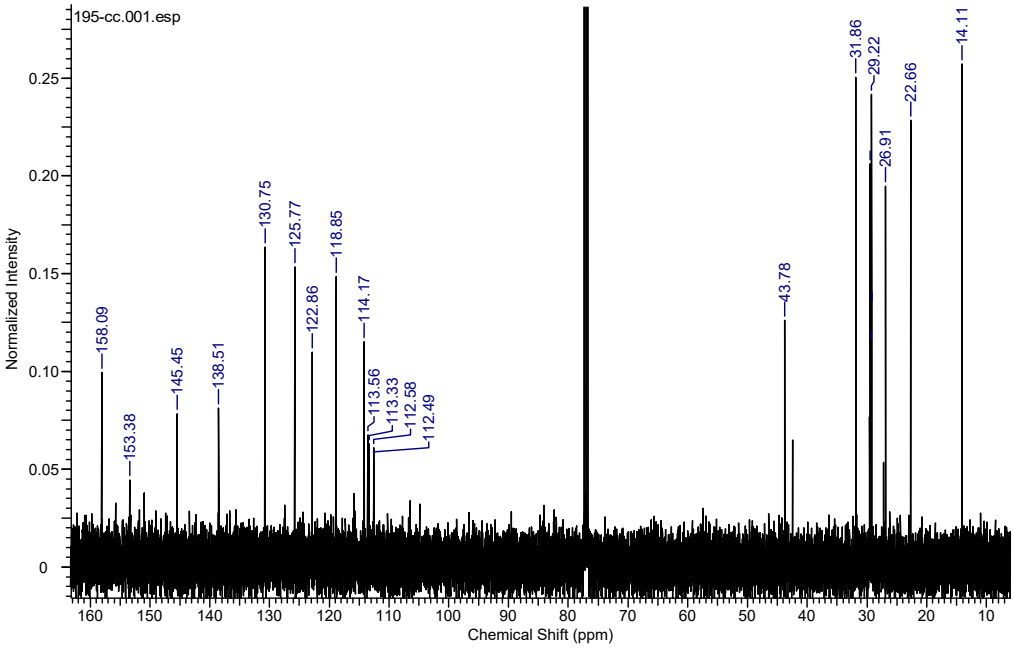
2-(3,5-dimethylphenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (22)



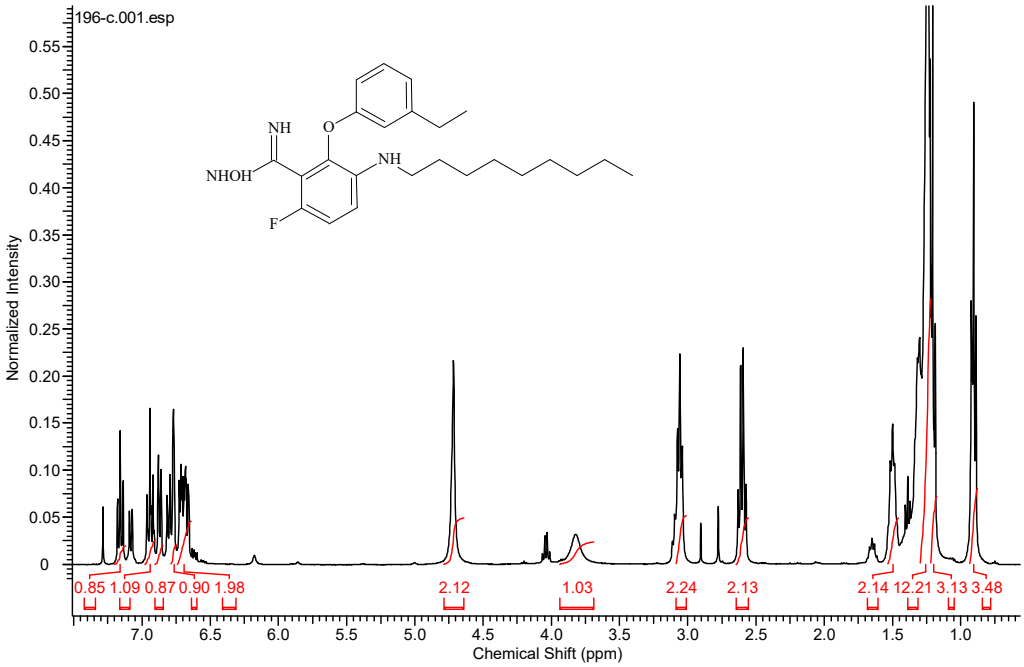


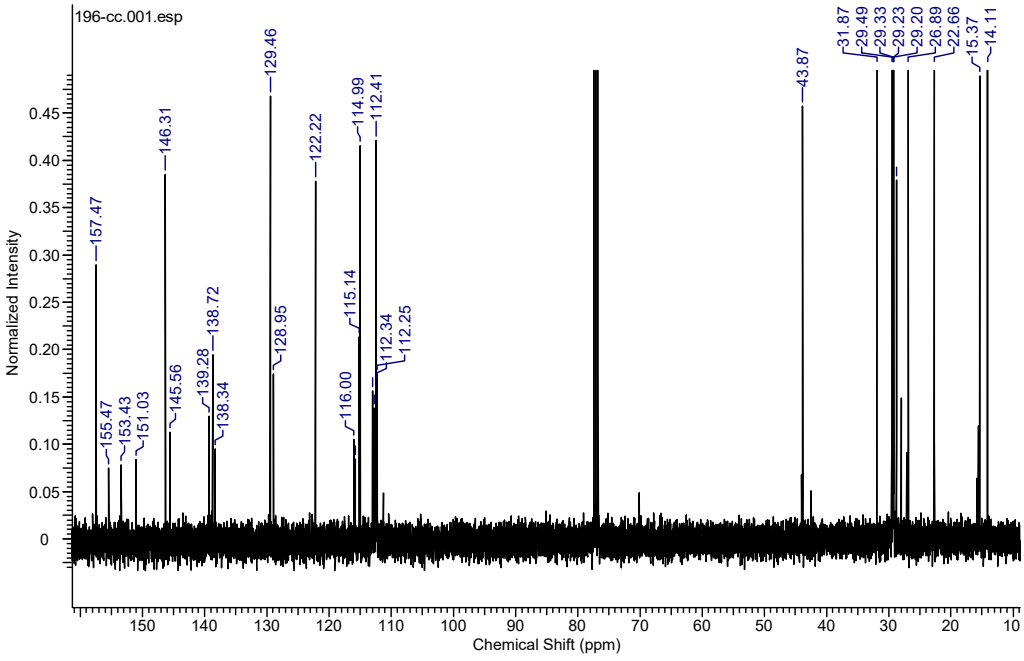
2-(3-bromophenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (23)



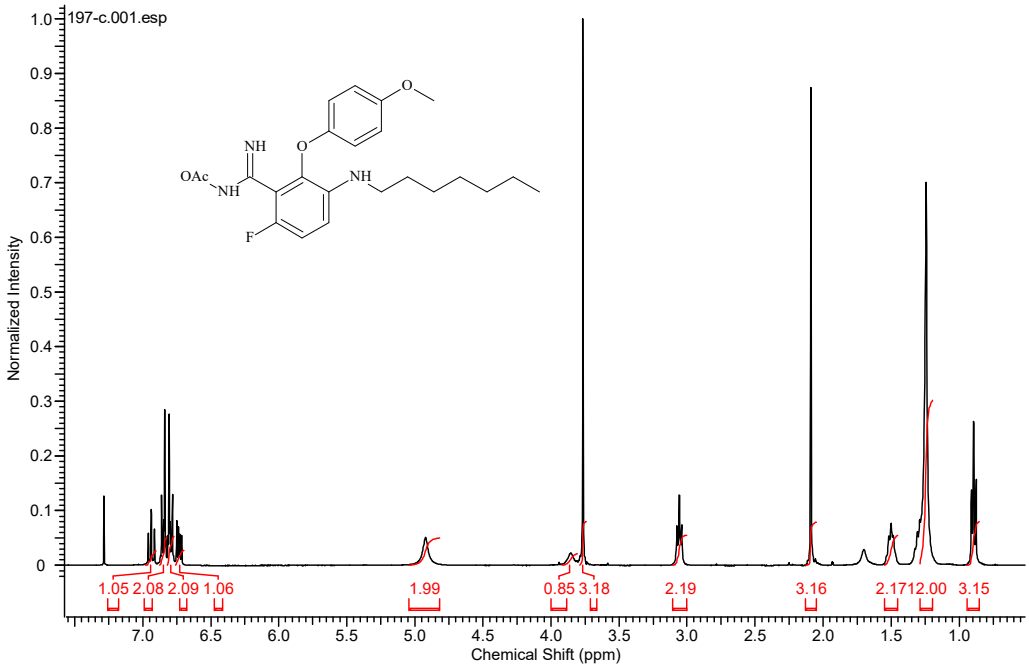


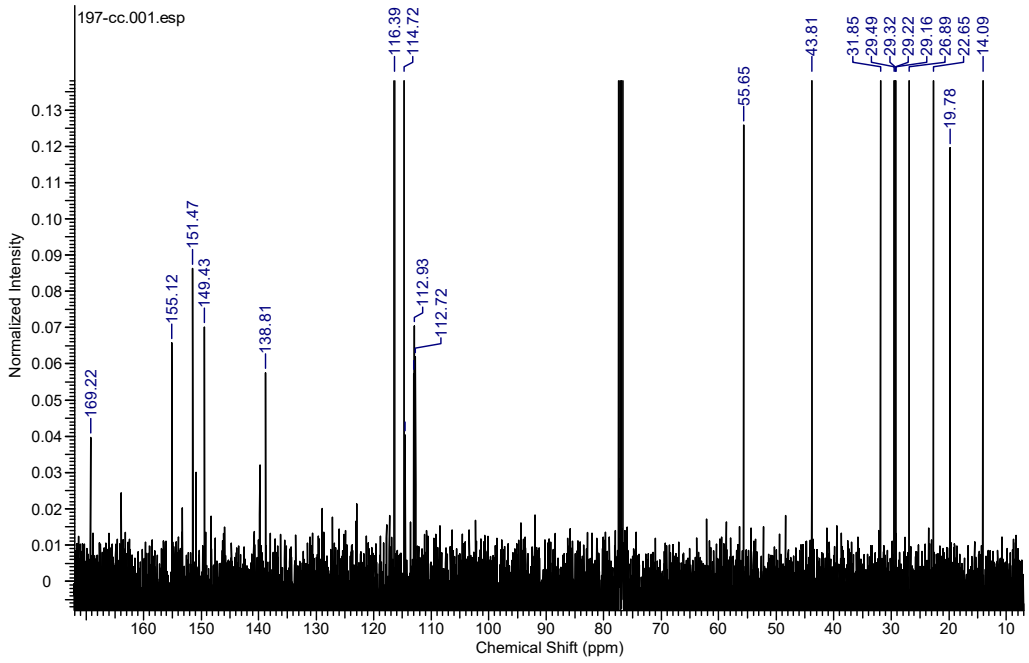
2-(3-ethylphenoxy)-6-fluoro-N-hydroxy-3-(nonylamino)benzimidamide (24)



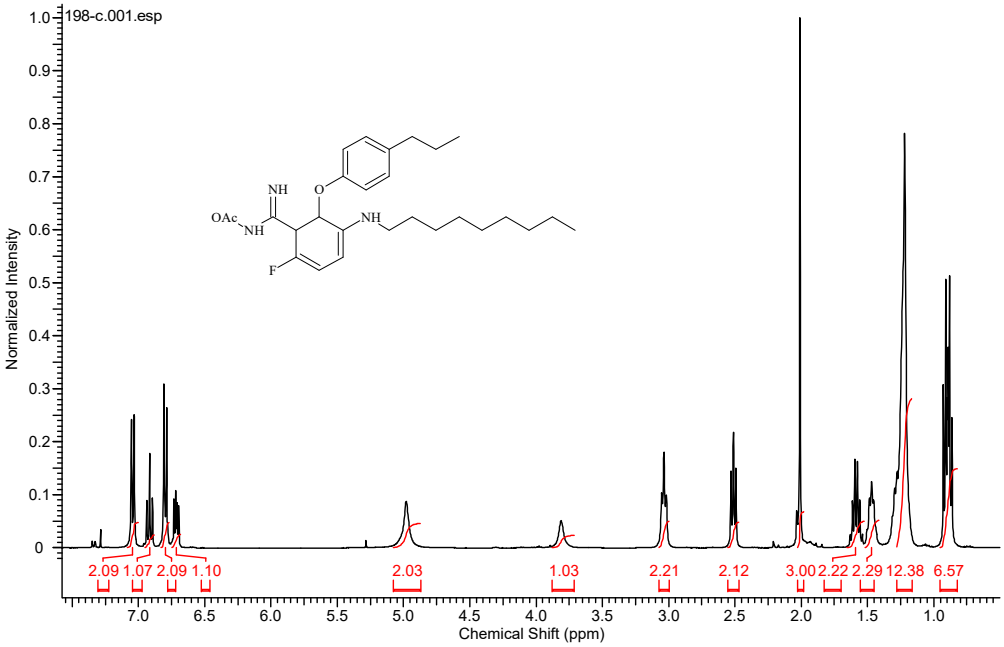


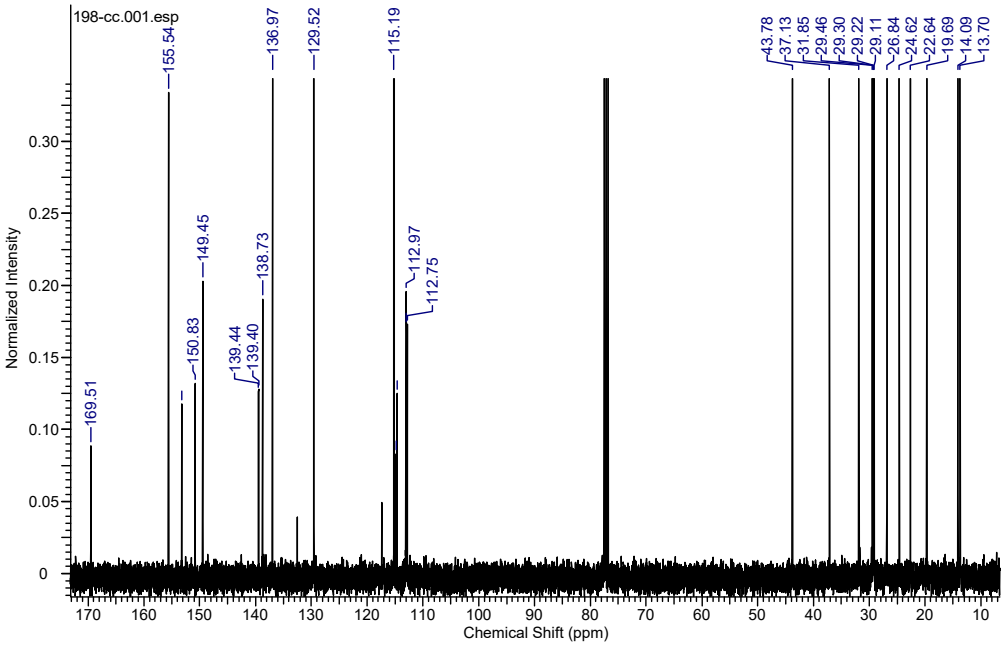
N-acetoxy-6-fluoro-2-(4-methoxyphenoxy)-3-(nonylamino)benzimidamide (25)



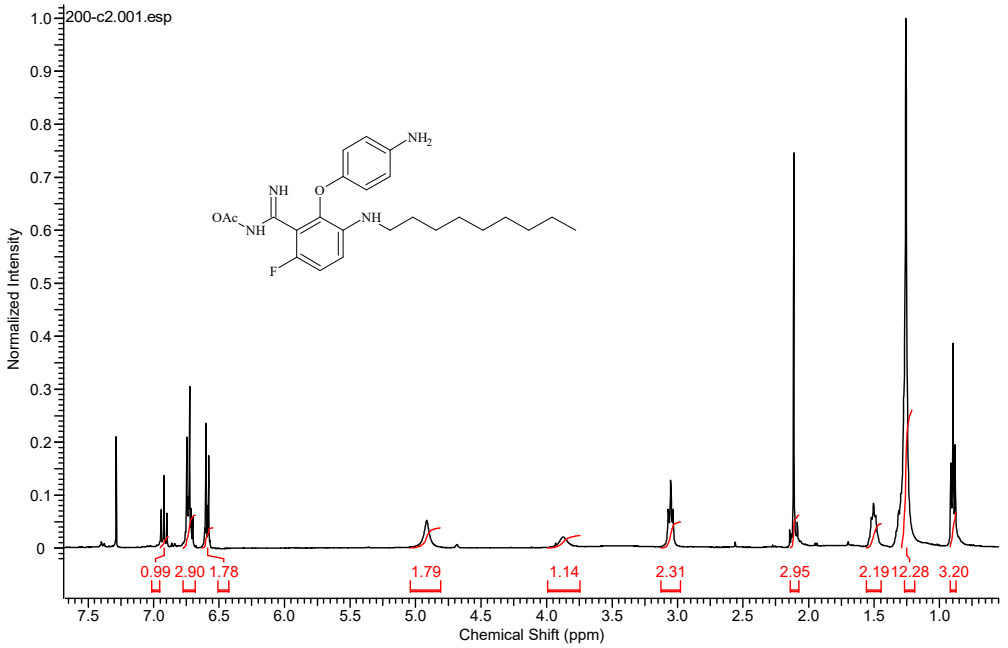


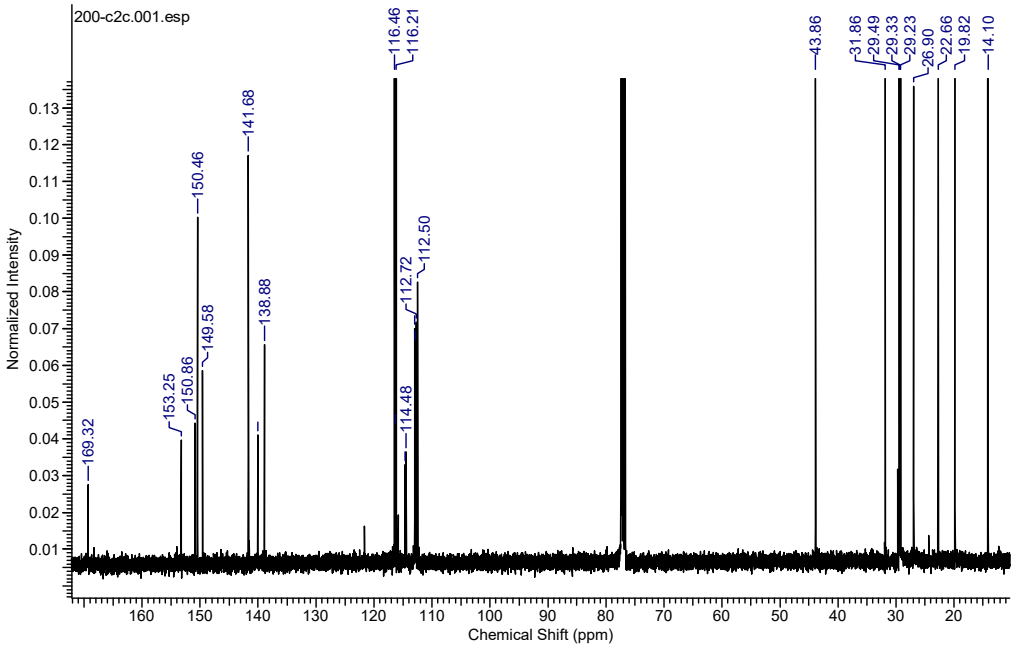
N-acetoxy-2-fluoro-5-(nonylamino)-6-(4-propylphenoxy)cyclohexa-2,4-dienecarboximidamide (26)



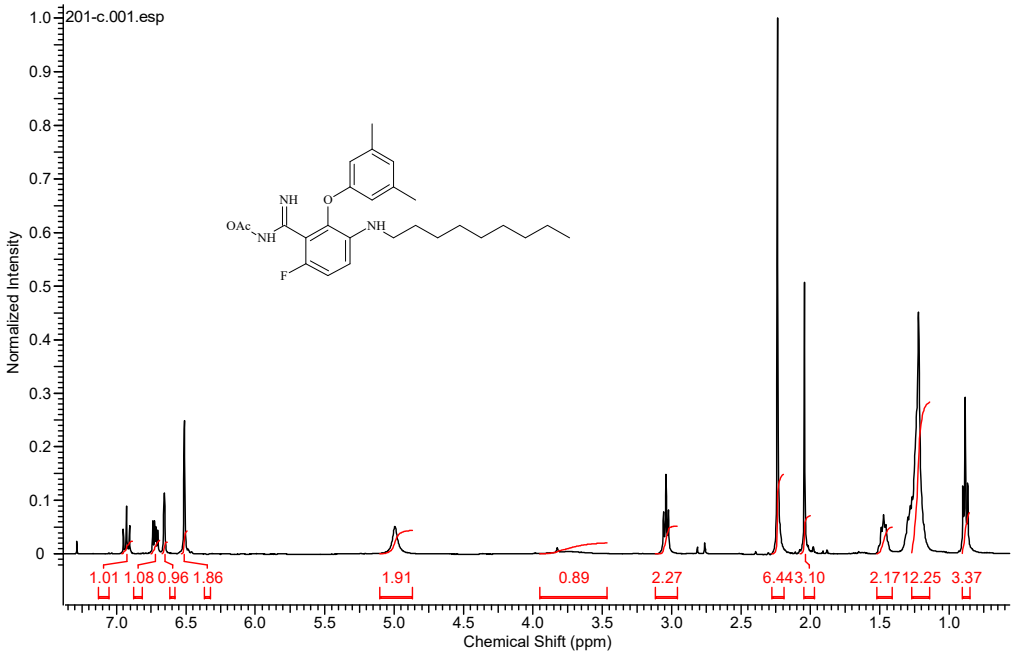


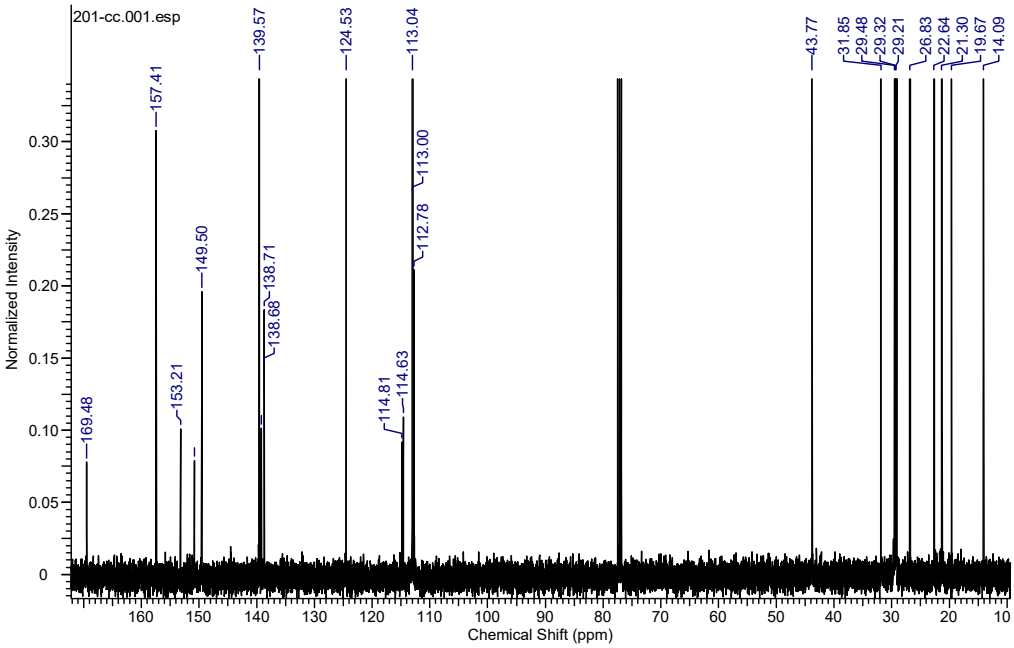
N-acetoxy-2-(4-aminophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (27)



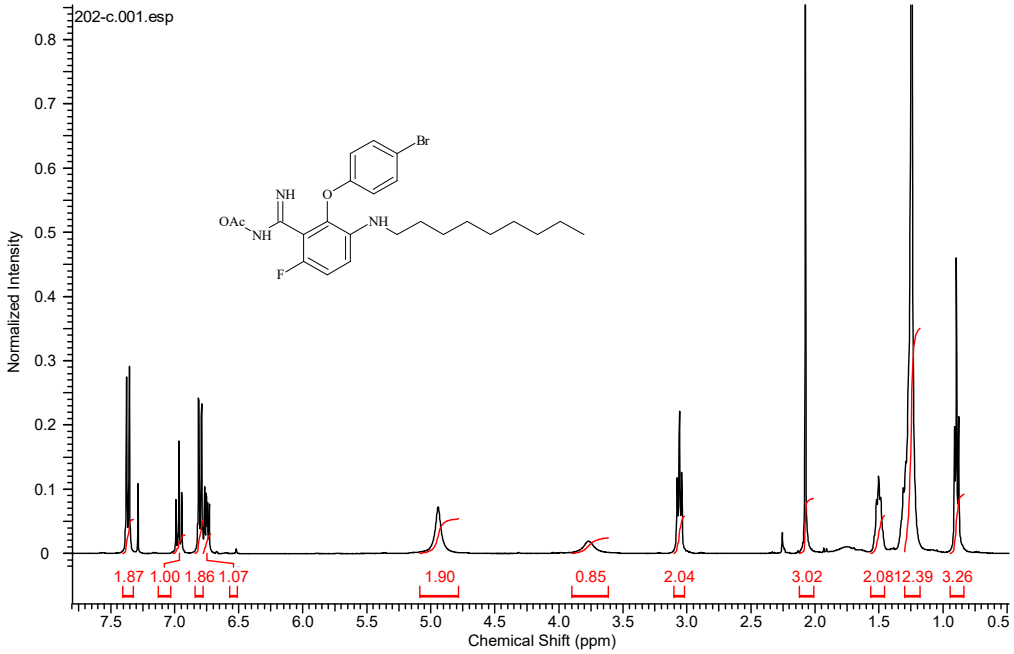


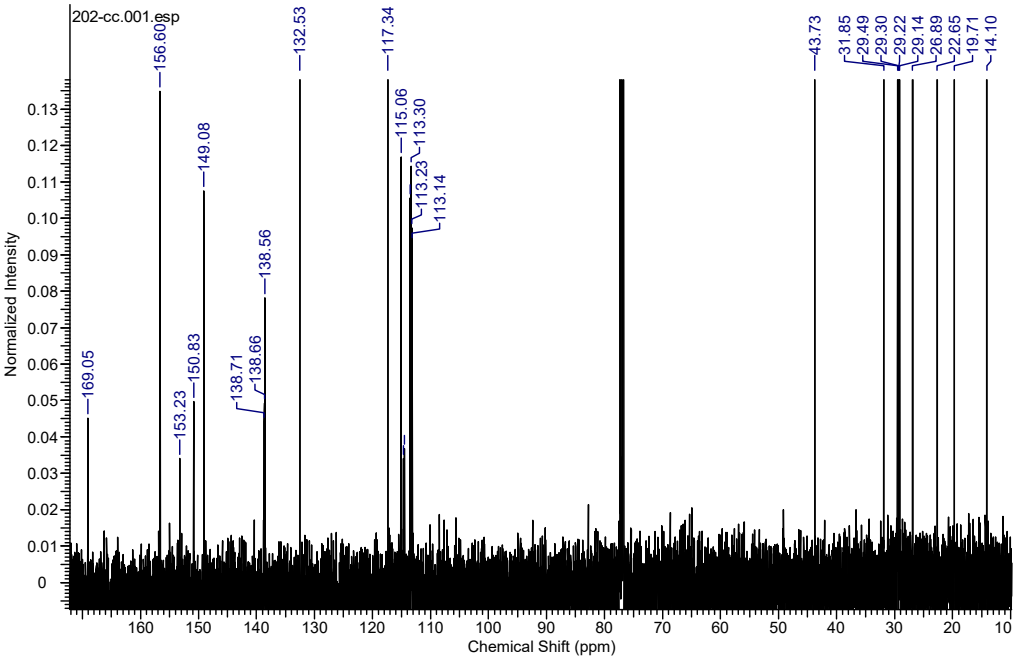
N-acetoxy-2-(3,5-dimethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (28)



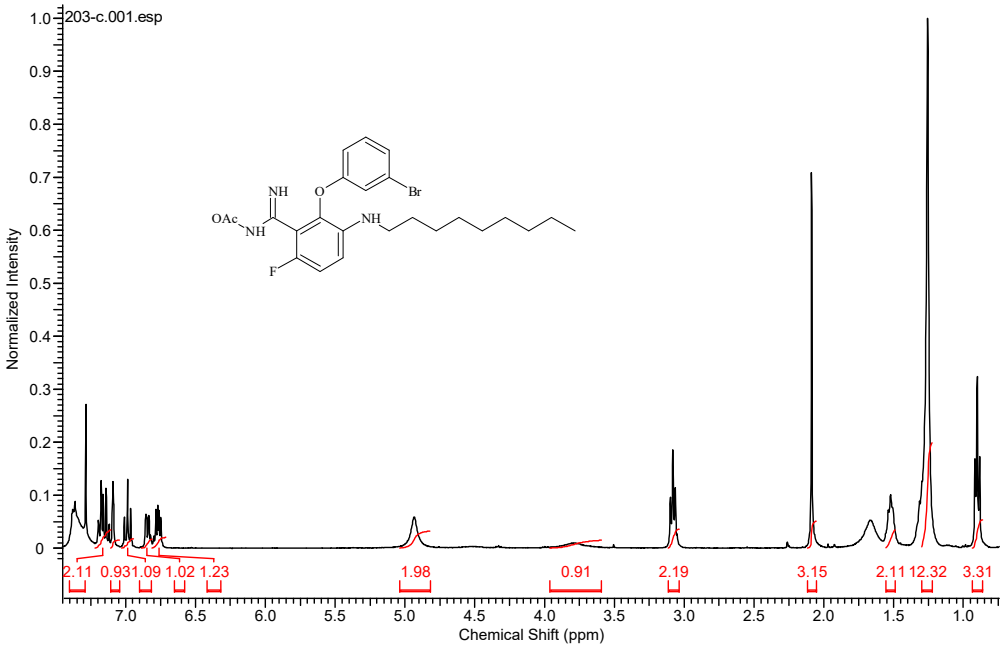


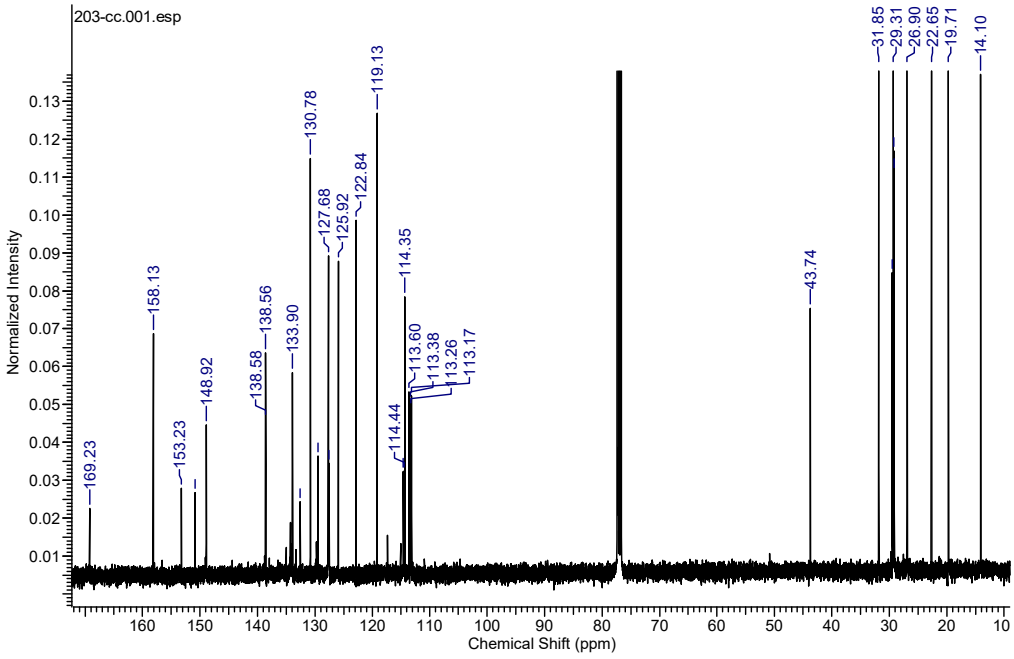
N-acetoxy-2-(4-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (29)



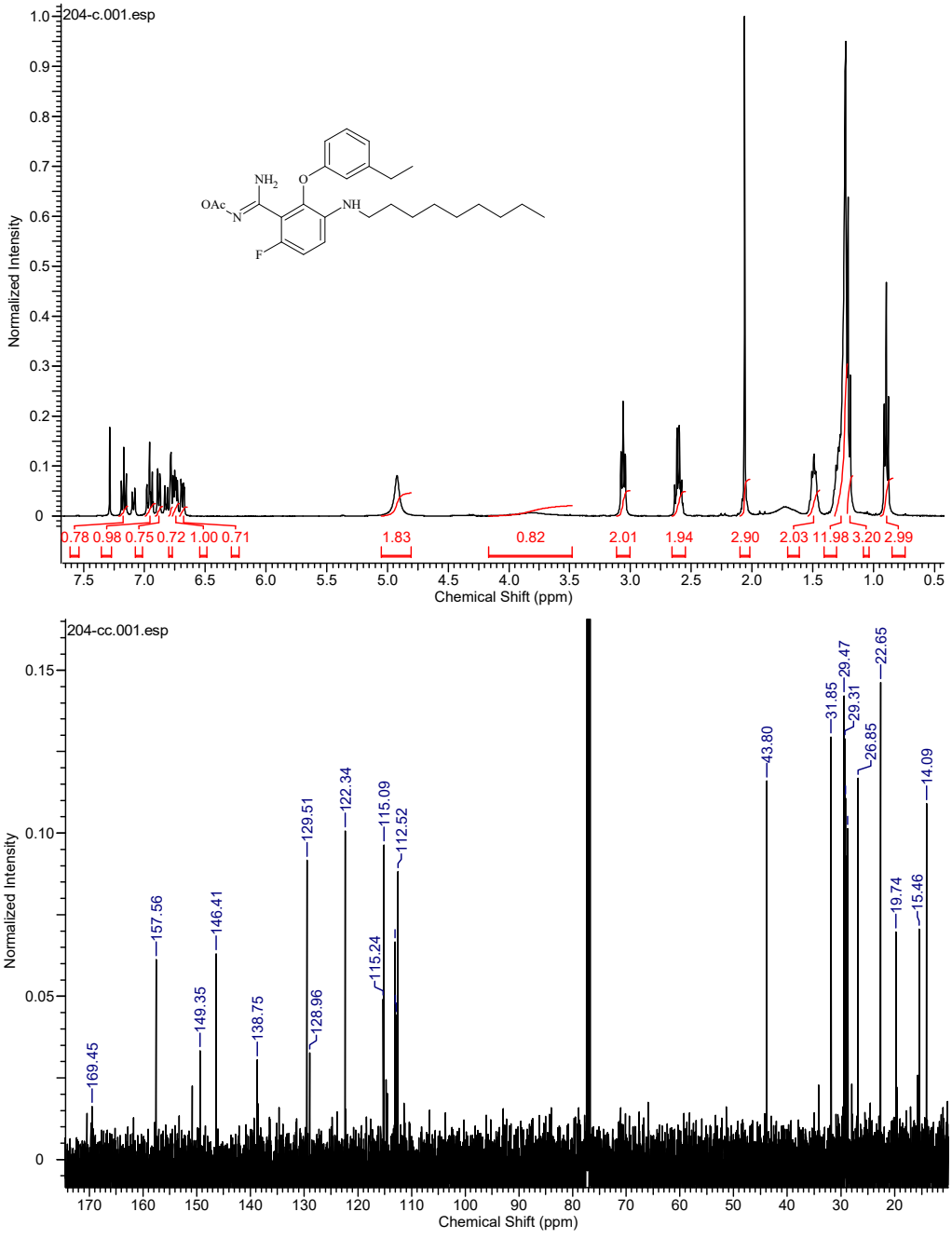


N-acetoxy-2-(3-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (30)

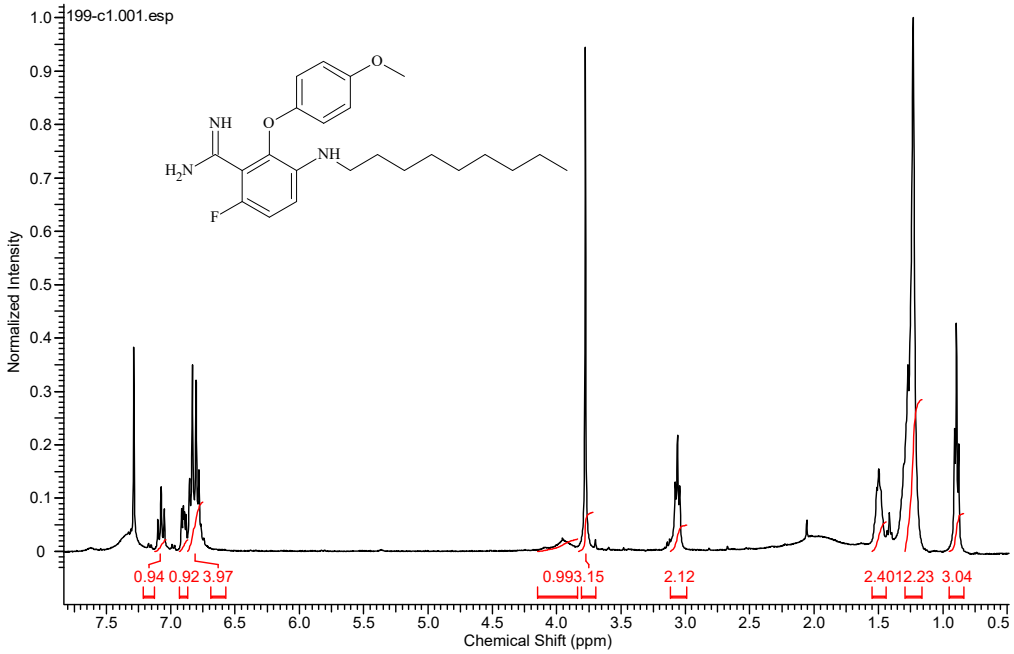


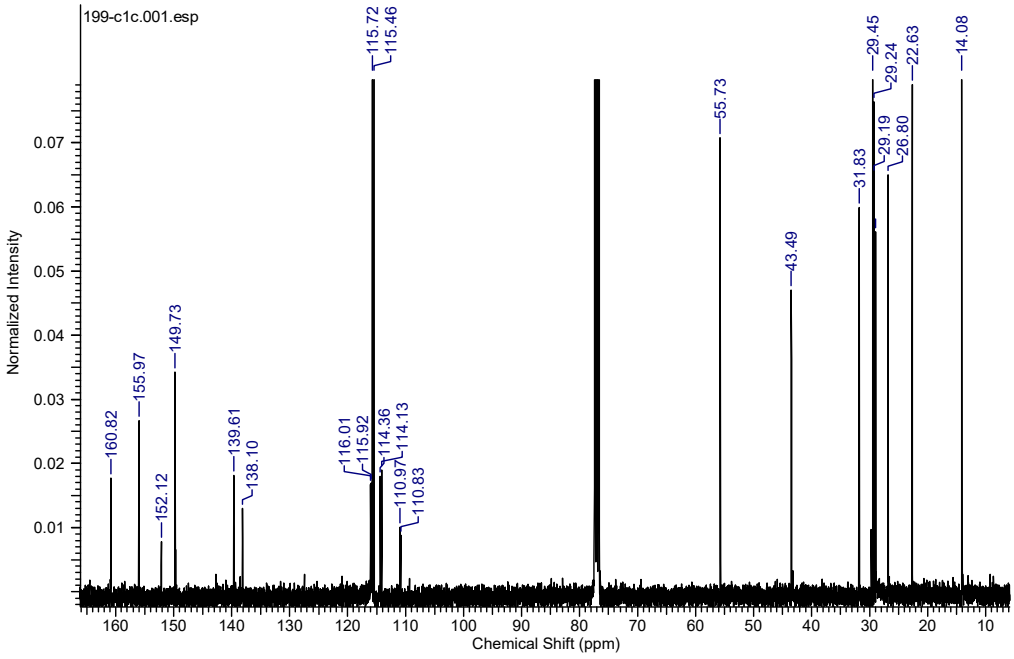


N-acetoxy-2-(3-ethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (31)

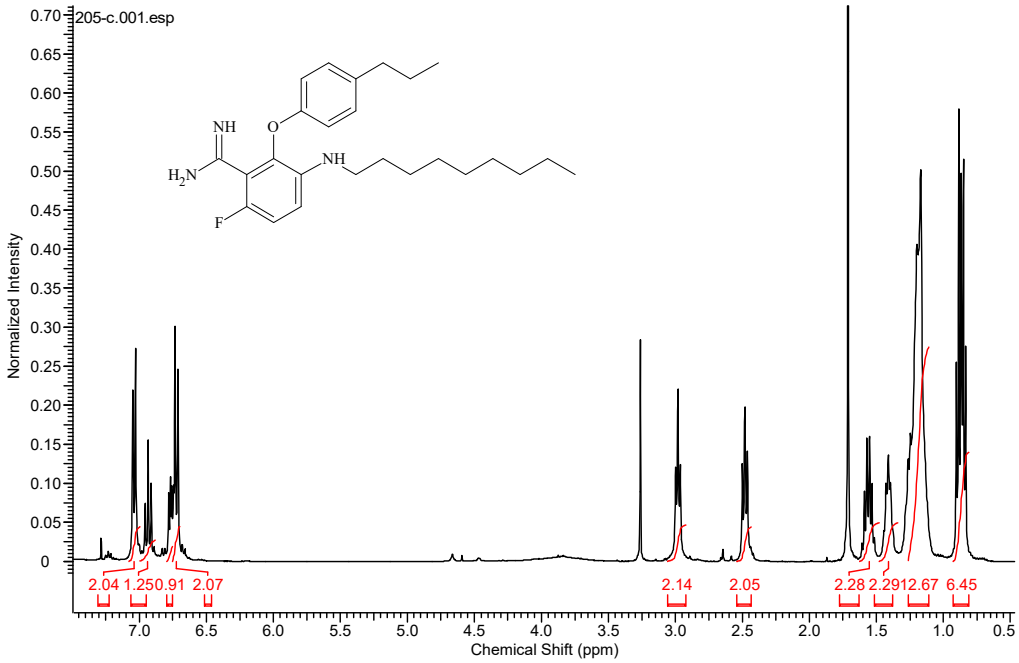


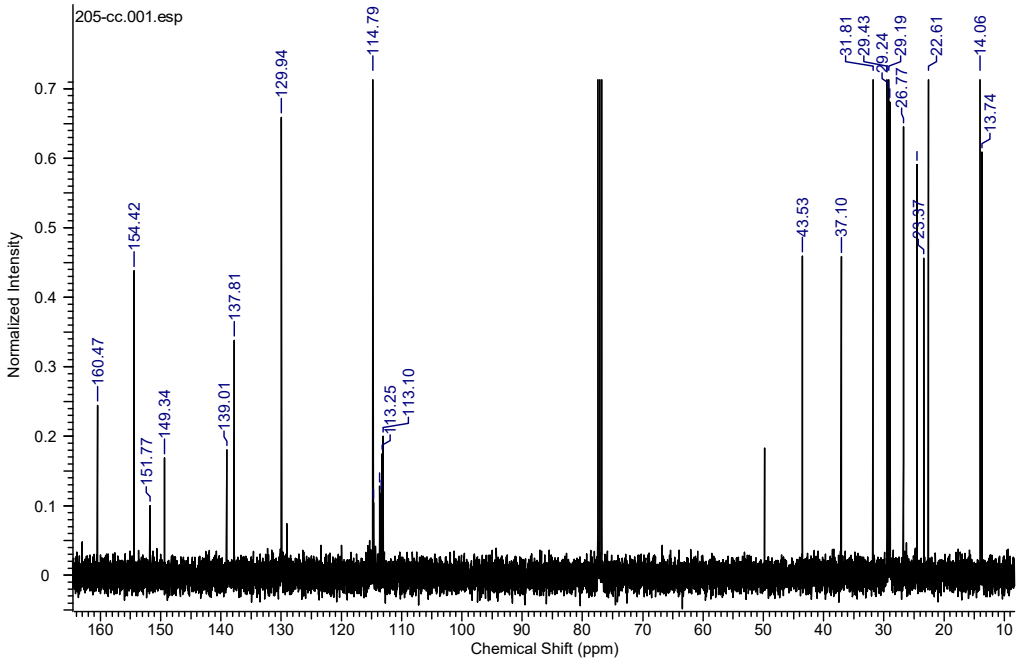
6-fluoro-2-(4-methoxyphenoxy)-3-(nonylamino)benzimidamide (32)



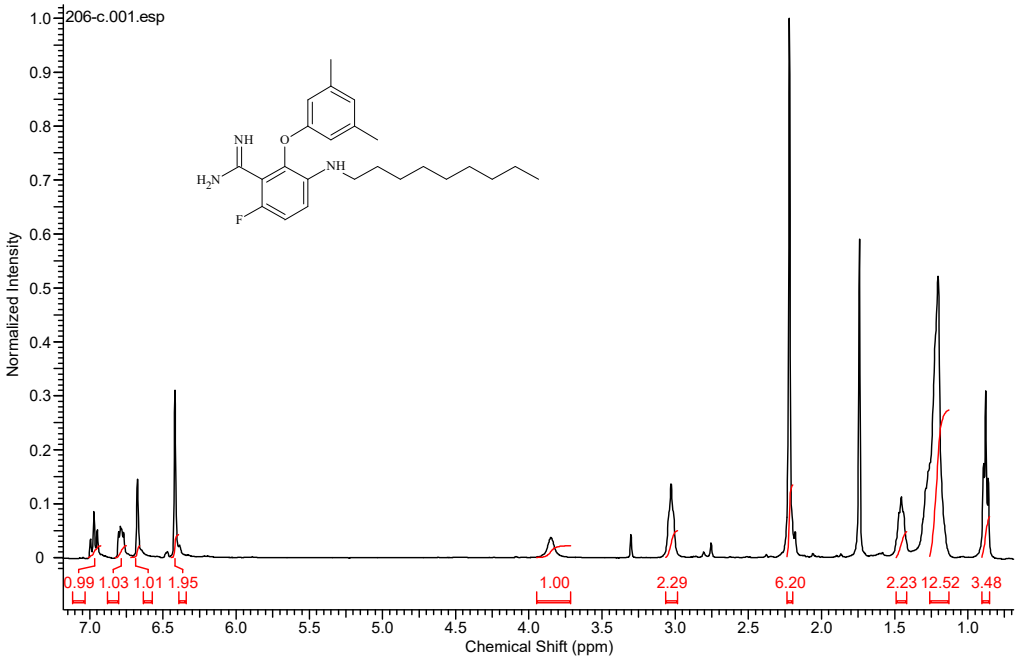


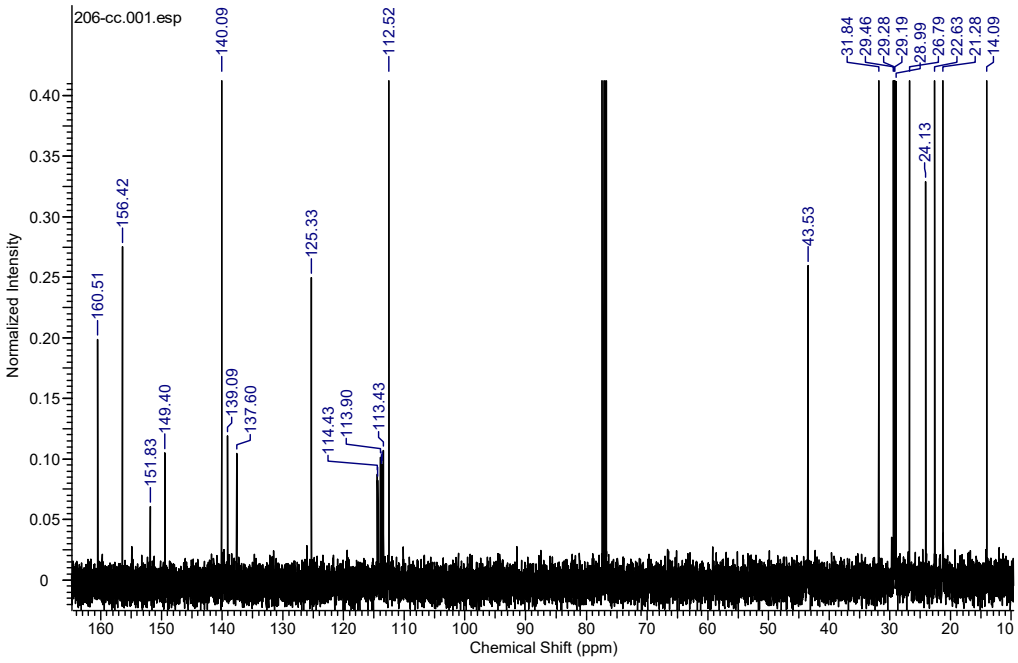
6-fluoro-3-(nonylamino)-2-(4-propylphenoxy)benzimidamide (33)



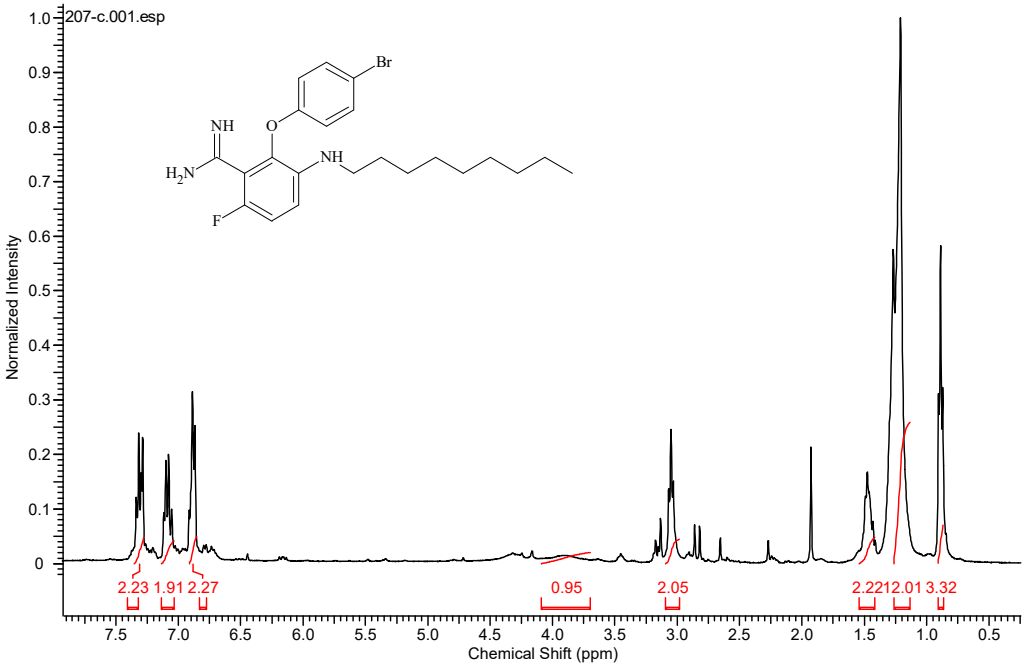


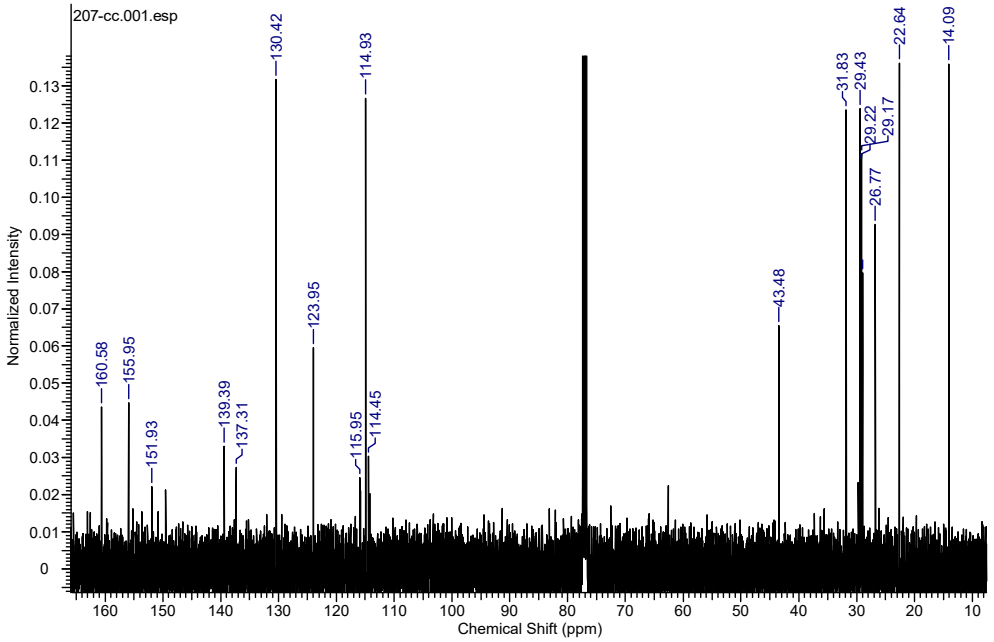
2-(3,5-dimethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (34)



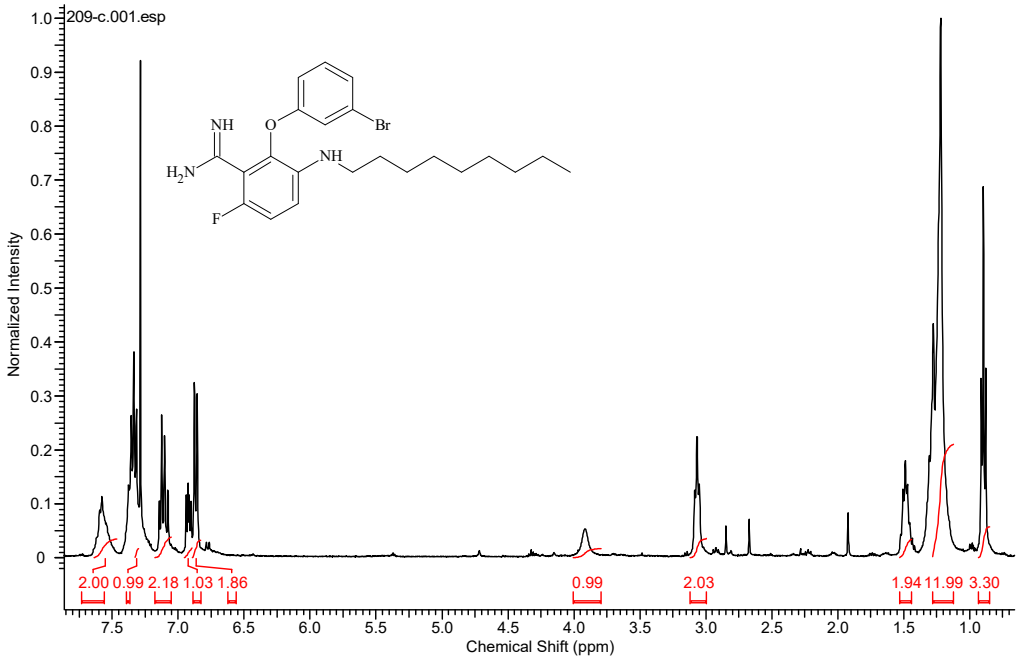


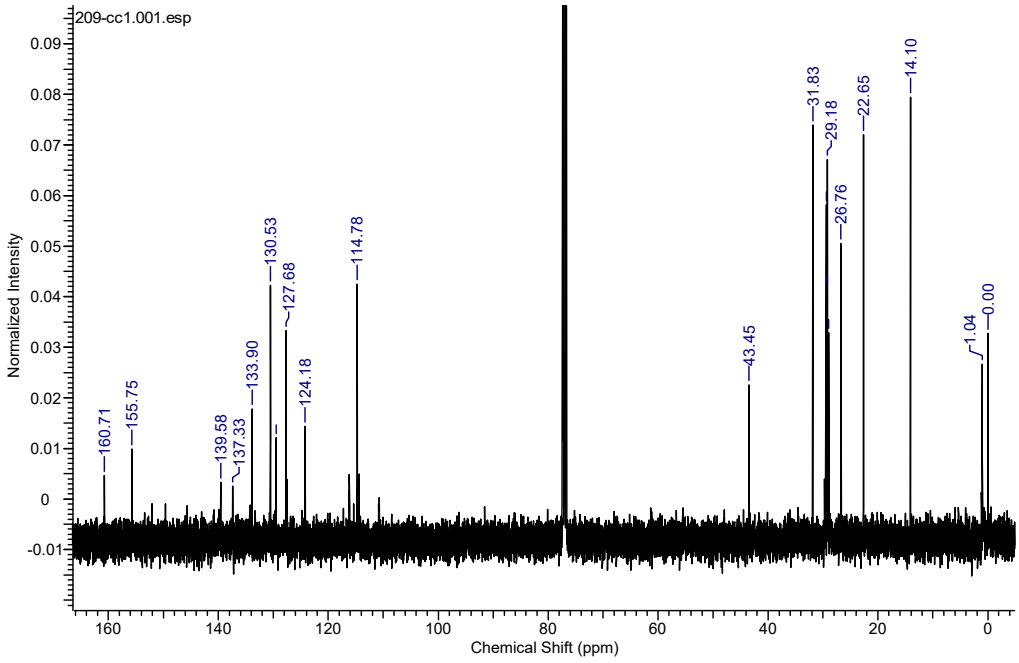
2-(4-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (35)



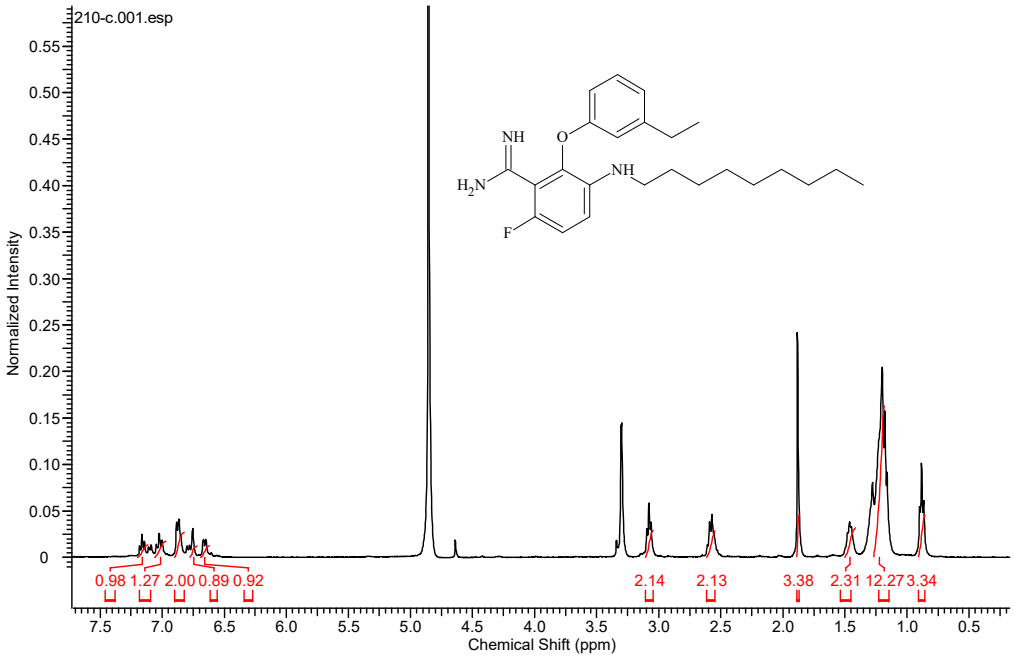


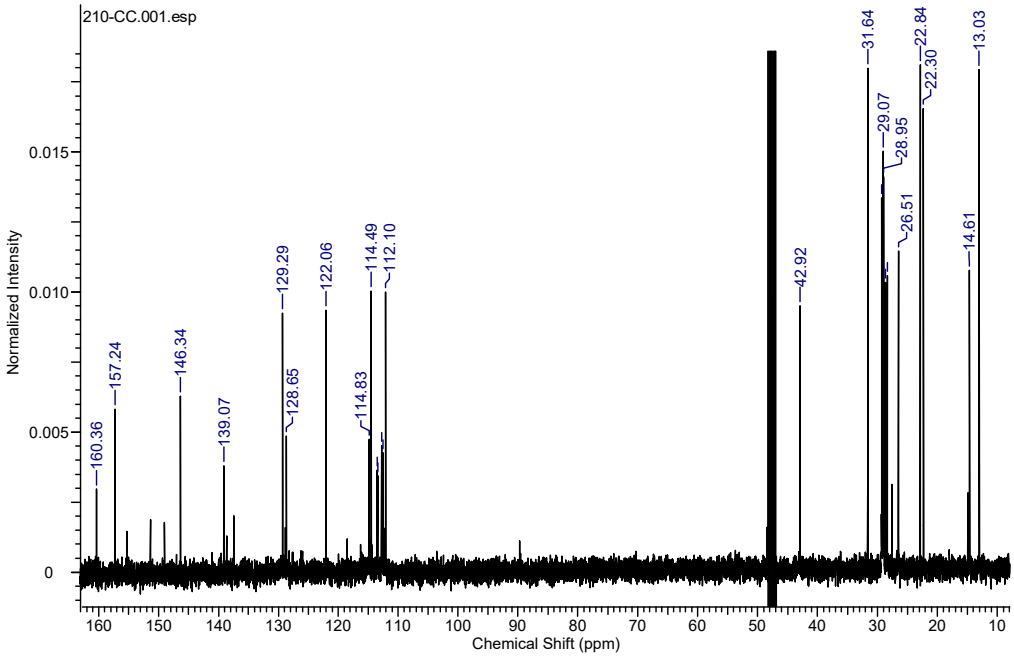
2-(3-bromophenoxy)-6-fluoro-3-(nonylamino)benzimidamide (36)



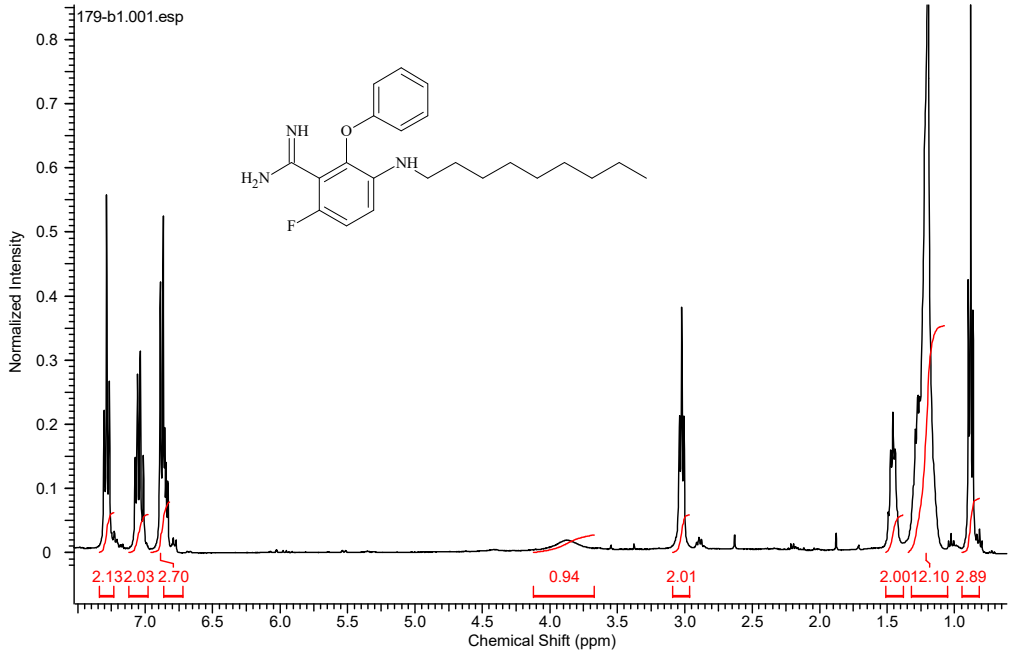


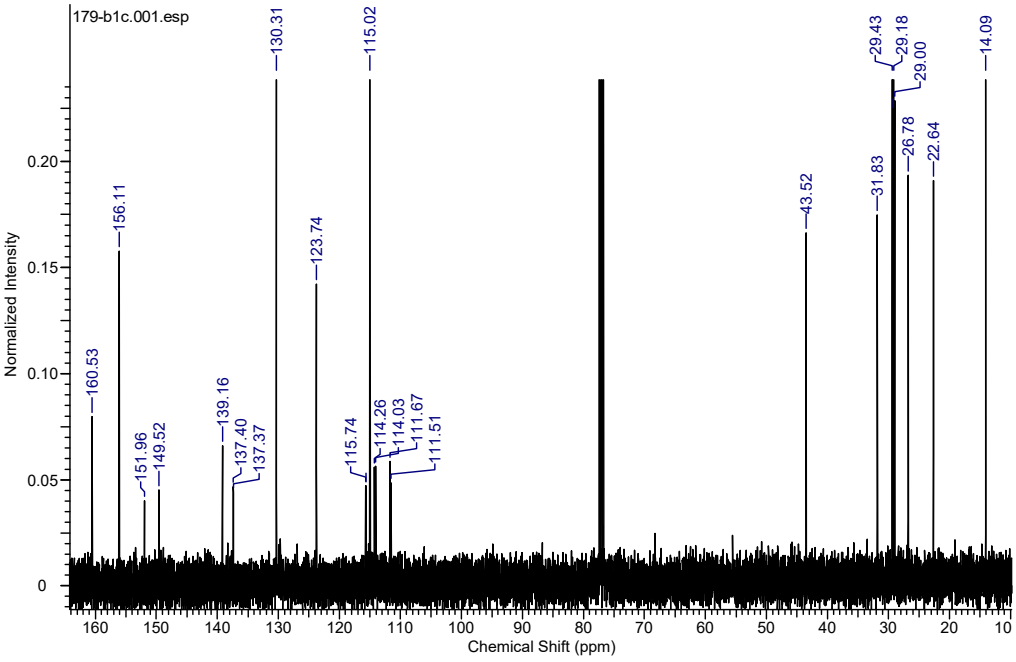
2-(3-ethylphenoxy)-6-fluoro-3-(nonylamino)benzimidamide (37)



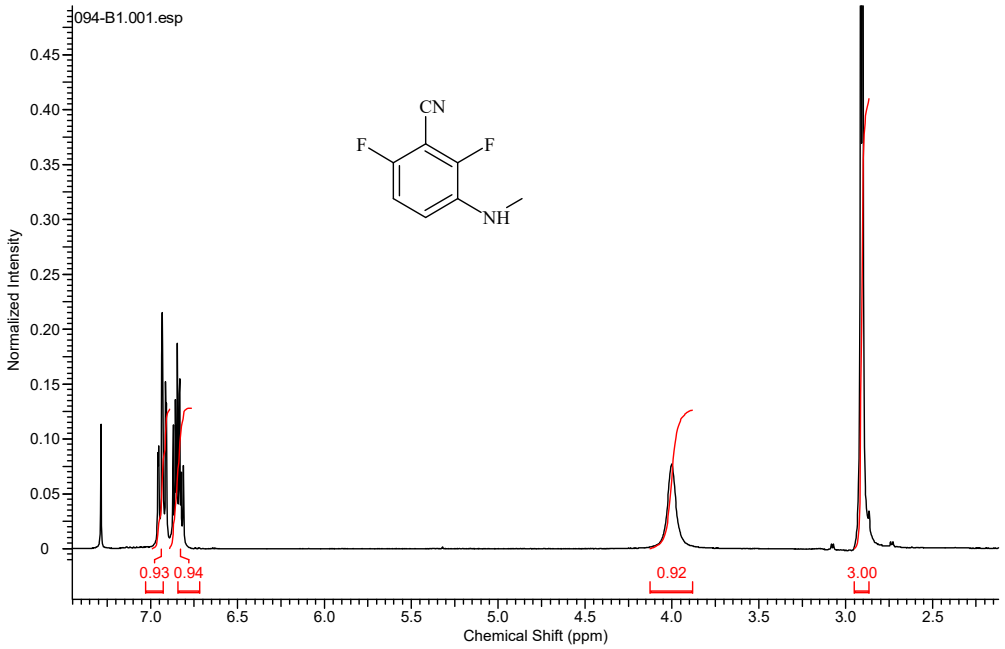


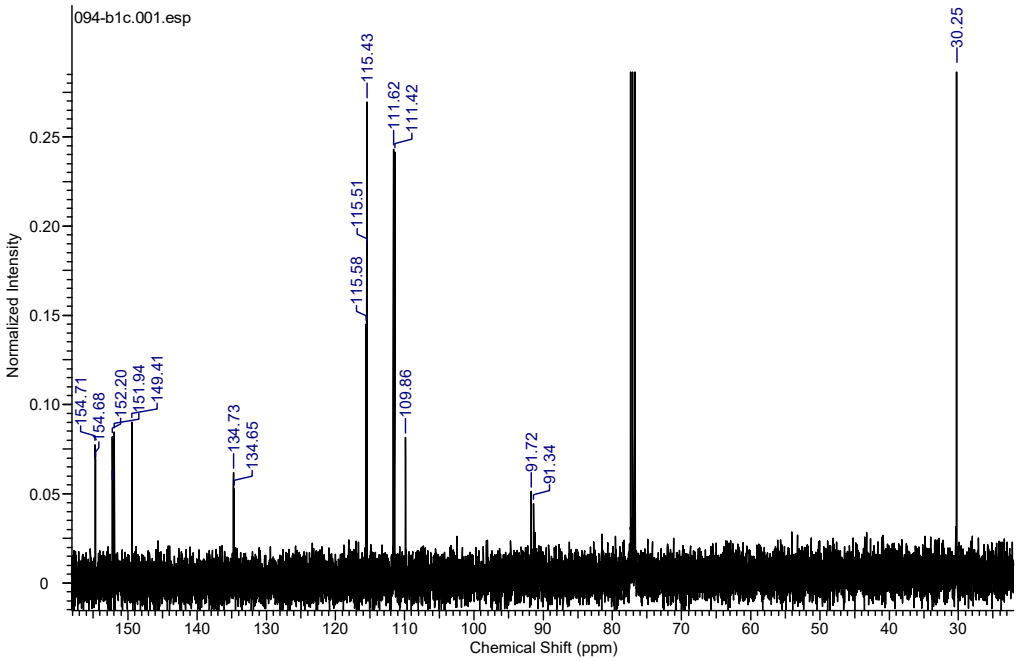
6-fluoro-3-(nonylamino)-2-phenoxybenzimidamide (38)



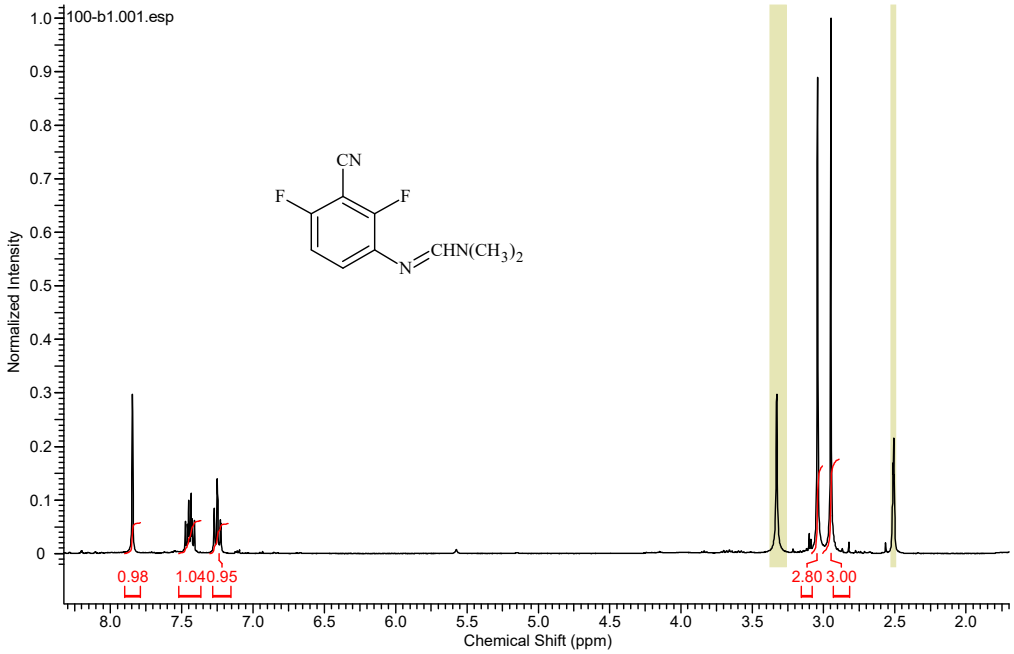


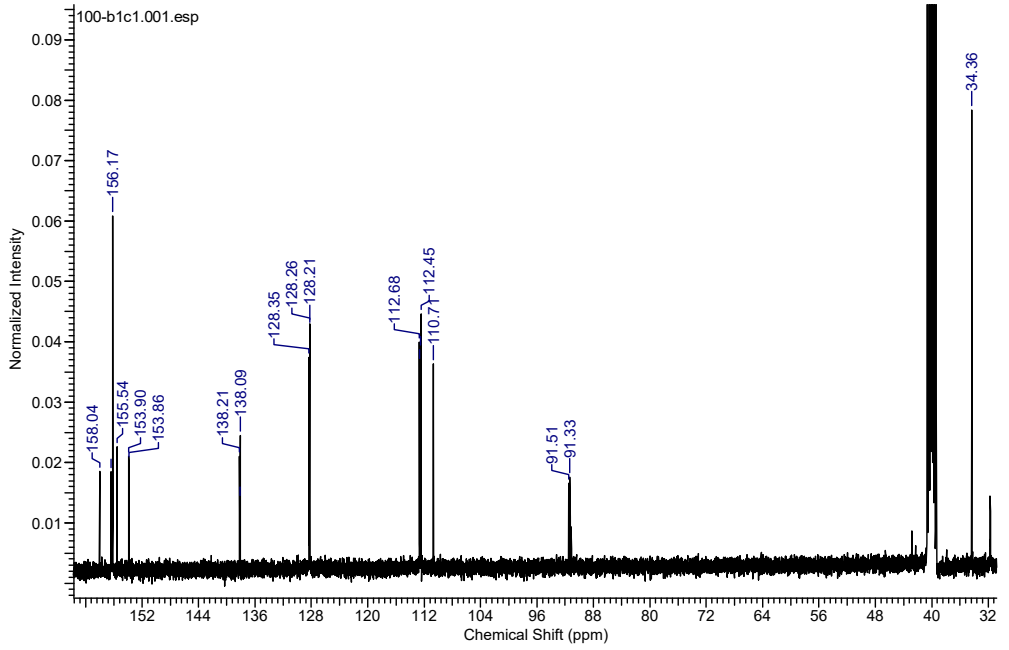
2,6-difluoro-3-(methylamino)benzonitrile (52)



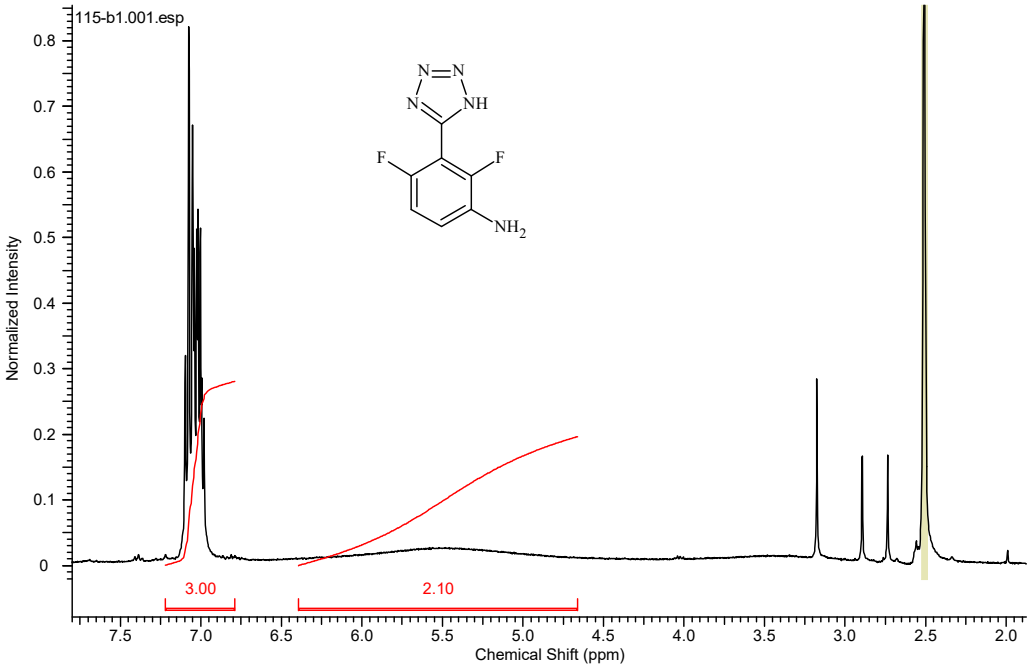


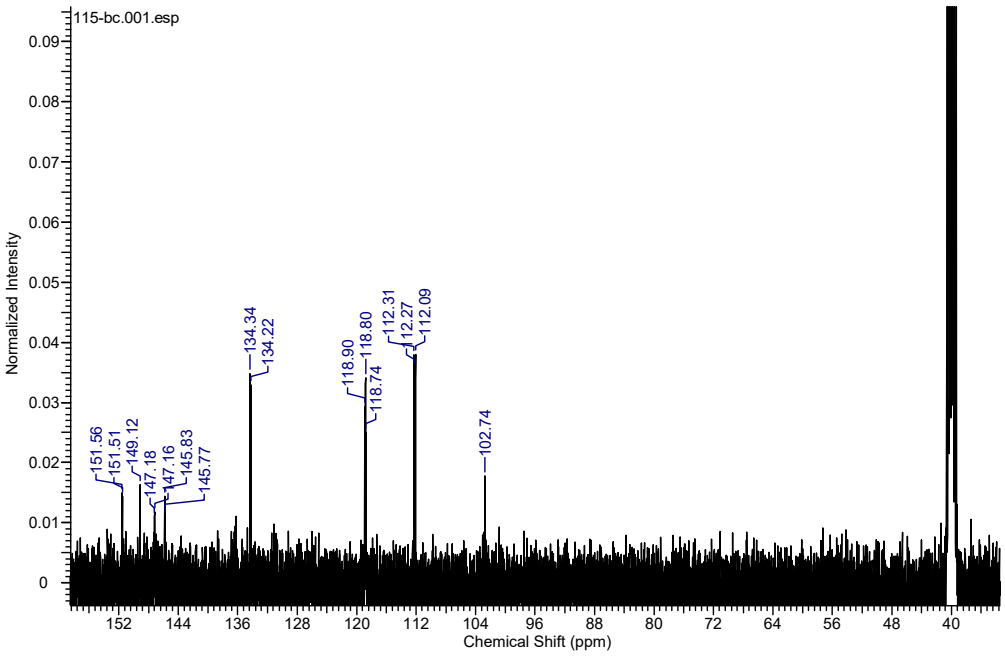
(E)-N'-(3-cyano-2,4-difluorophenyl)-N,N-dimethylformimidamide (53)



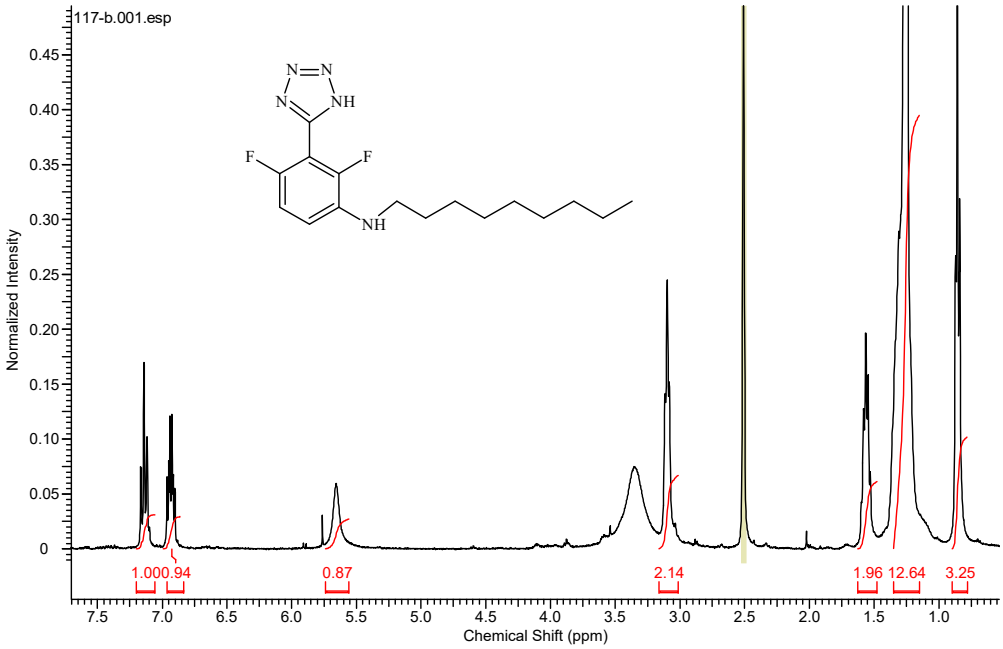


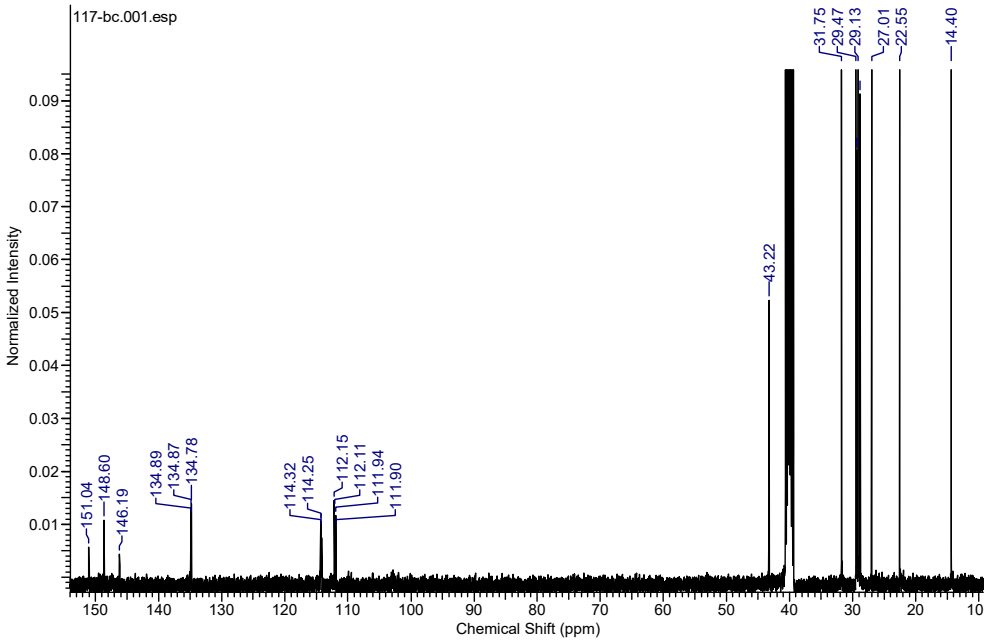
2,4-difluoro-3-(1H-tetrazol-5-yl)aniline (54)



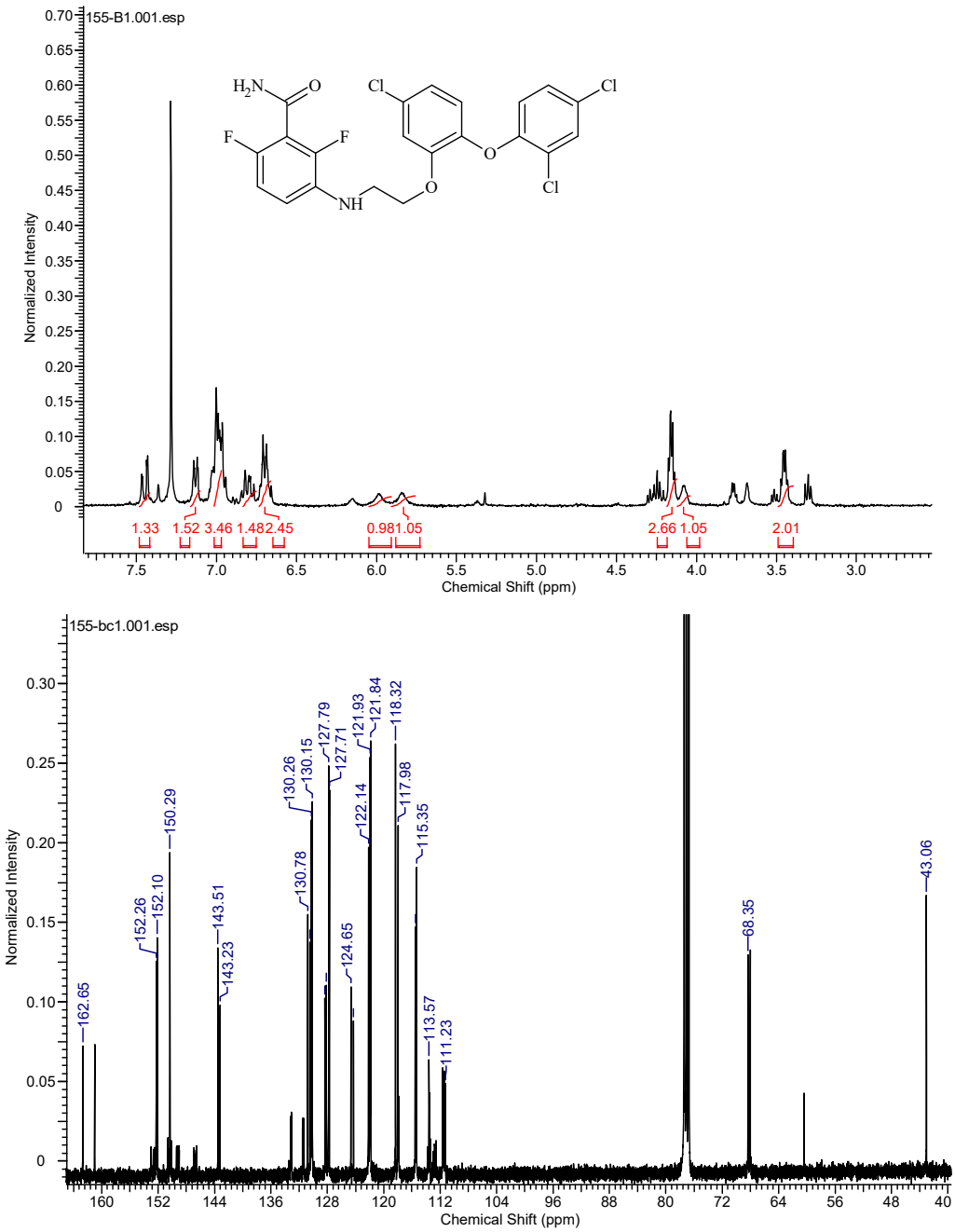


2,4-difluoro-N-nonyl-3-(1H-tetrazol-5-yl)aniline (55)

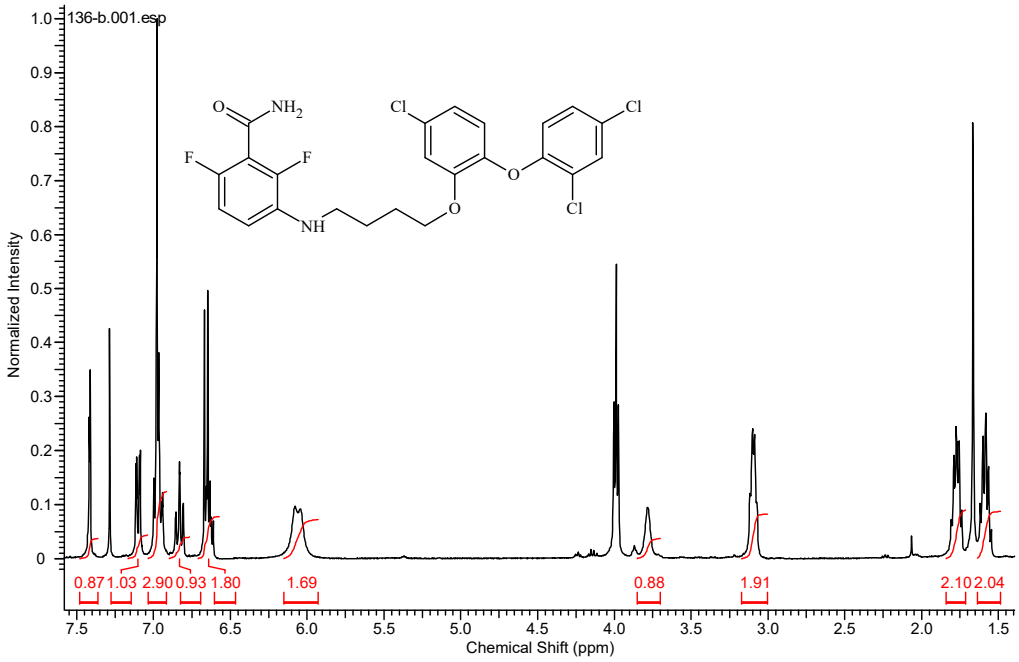


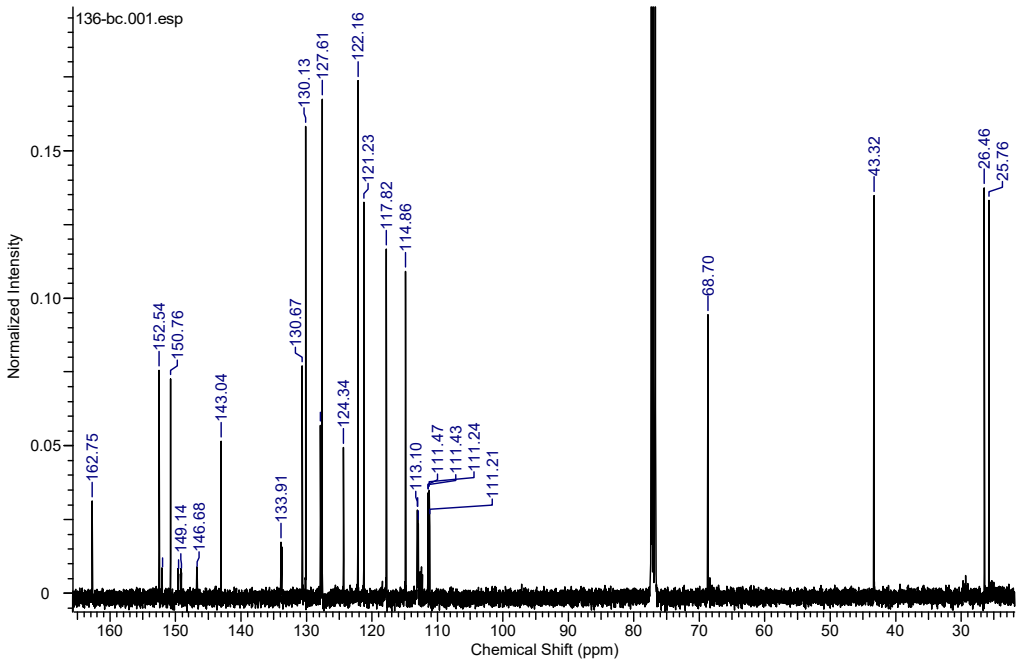


3-((2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)ethyl)amino)-2,6-difluorobenzamide (44)

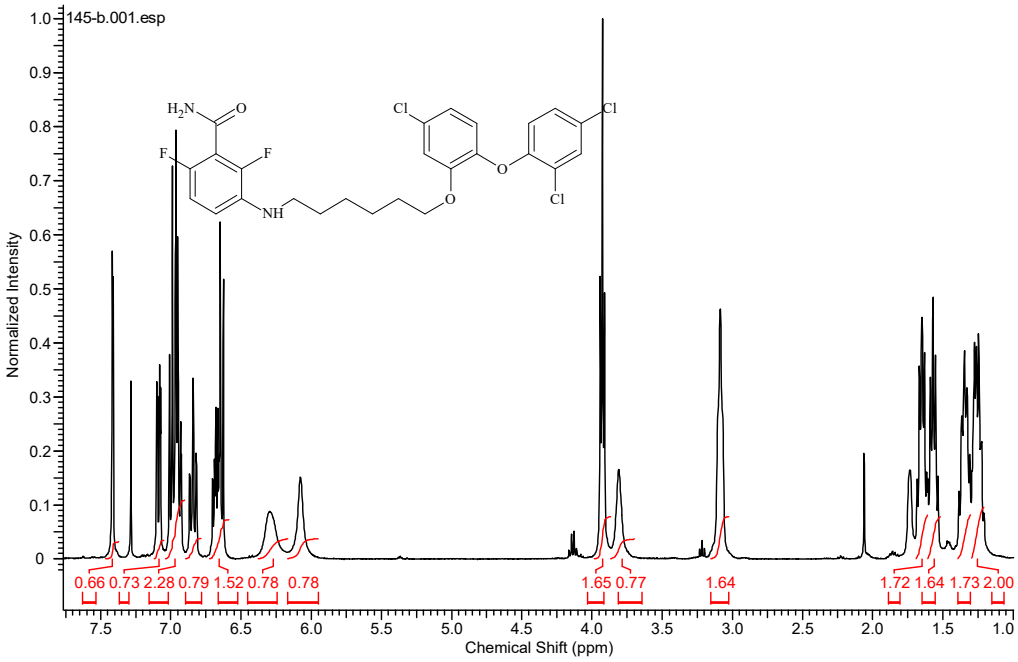


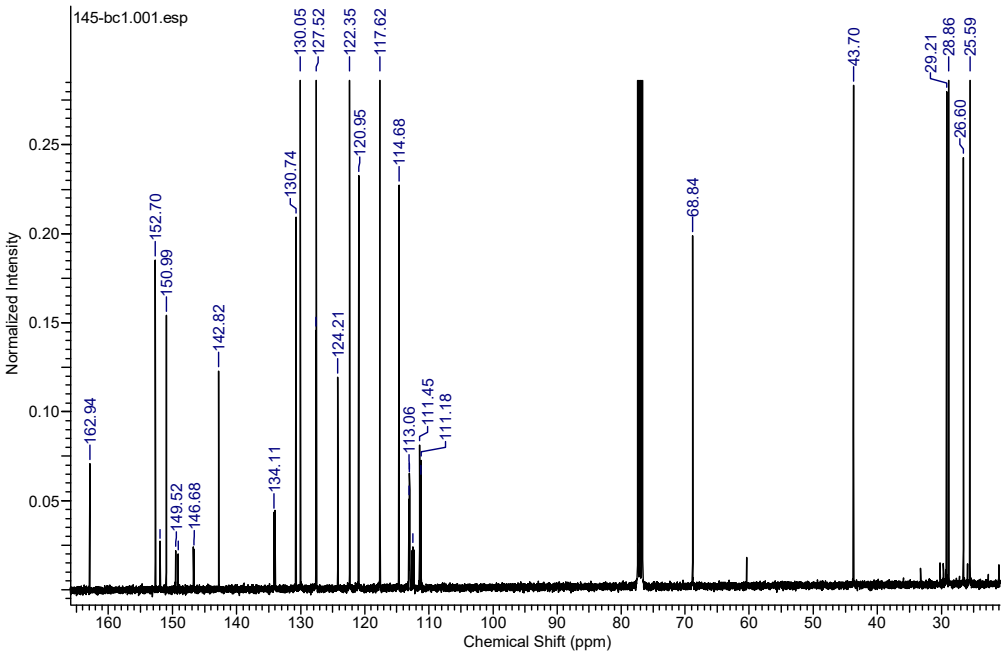
3-((4-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)butyl)amino)-2,6-difluorobenzamide (45)



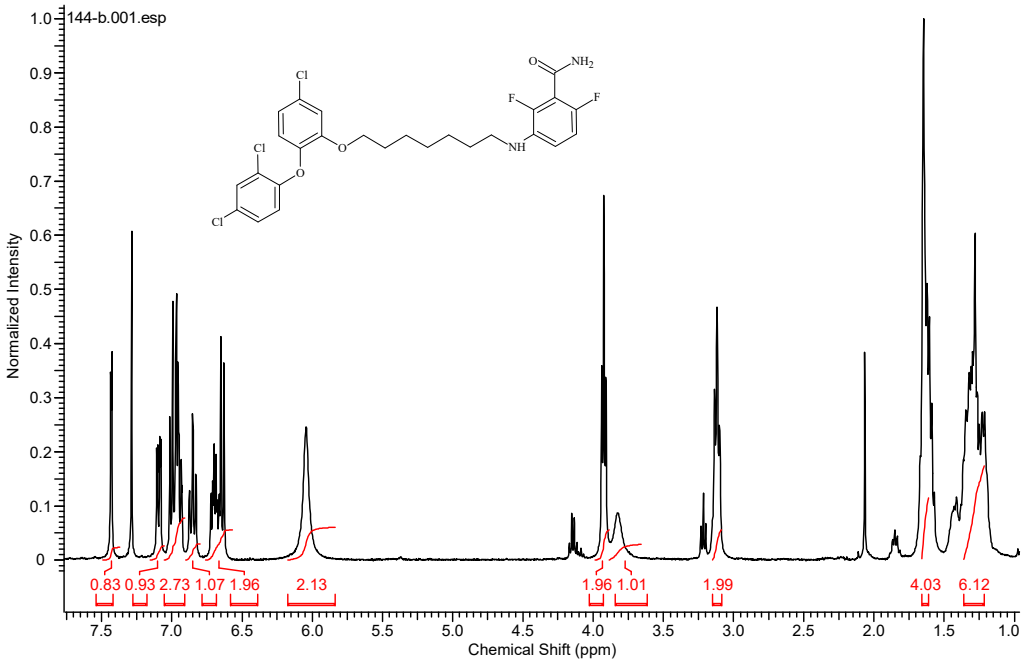


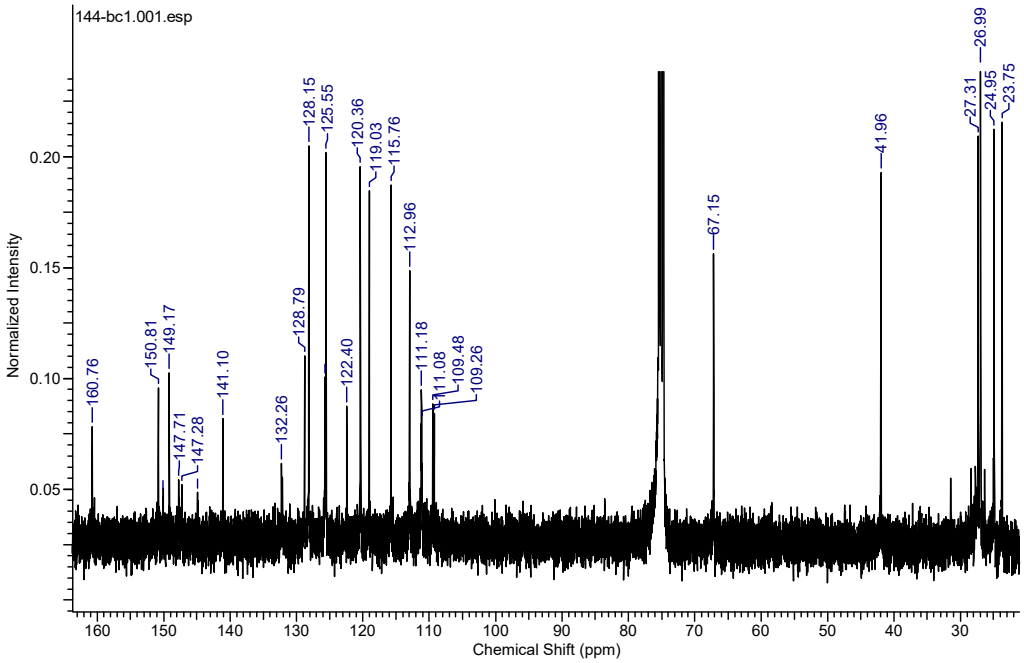
3-((6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexyl)amino)-2,6-difluorobenzamide6(46)



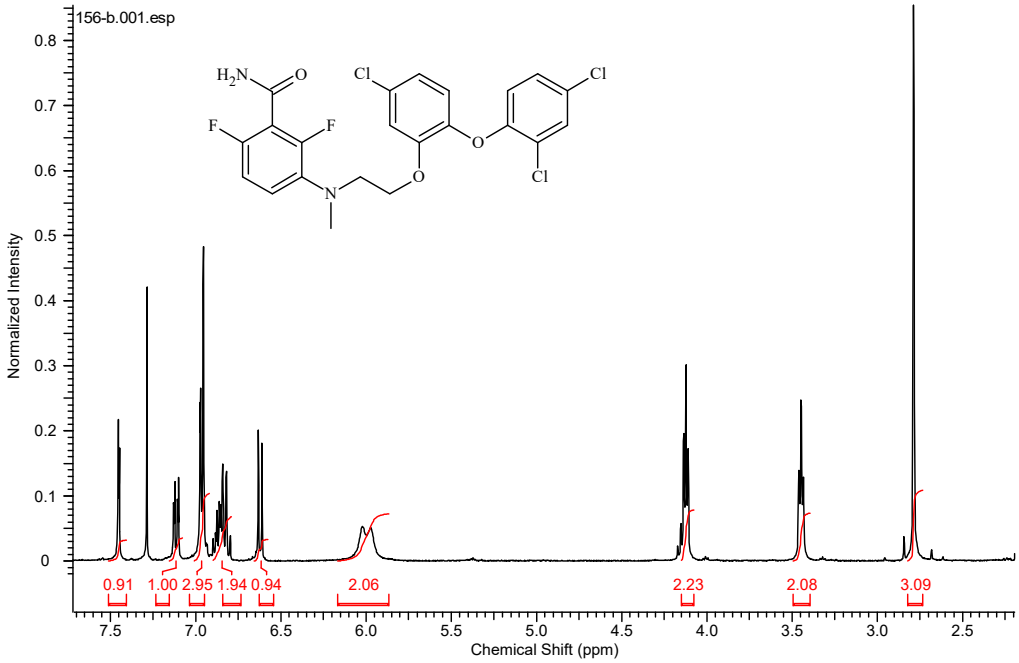


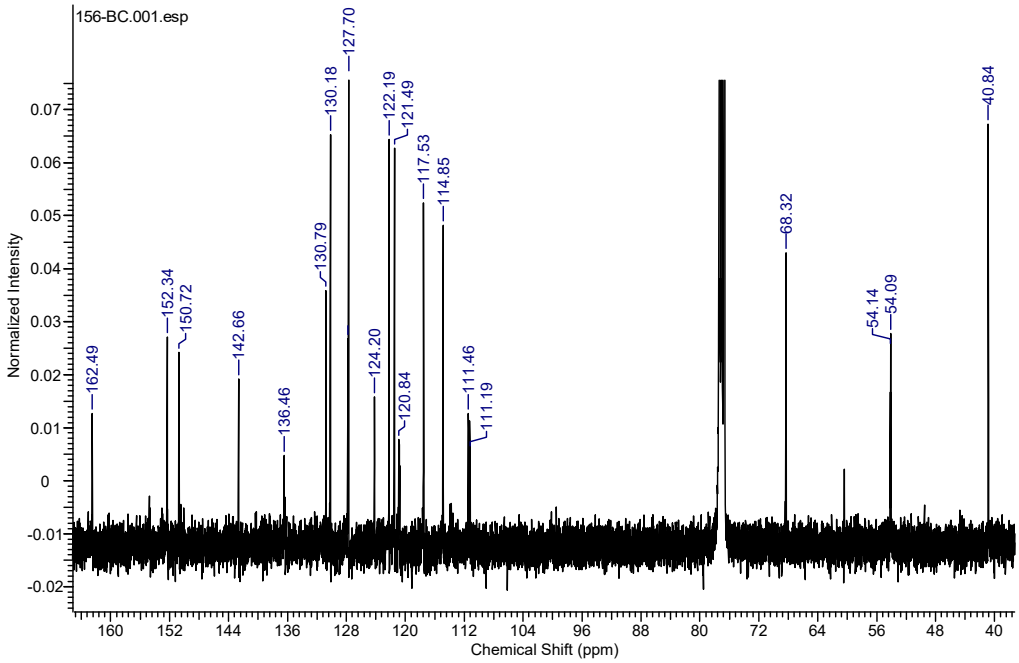
3-((7-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)heptyl)amino)-2,6-difluorobenzamide (47)



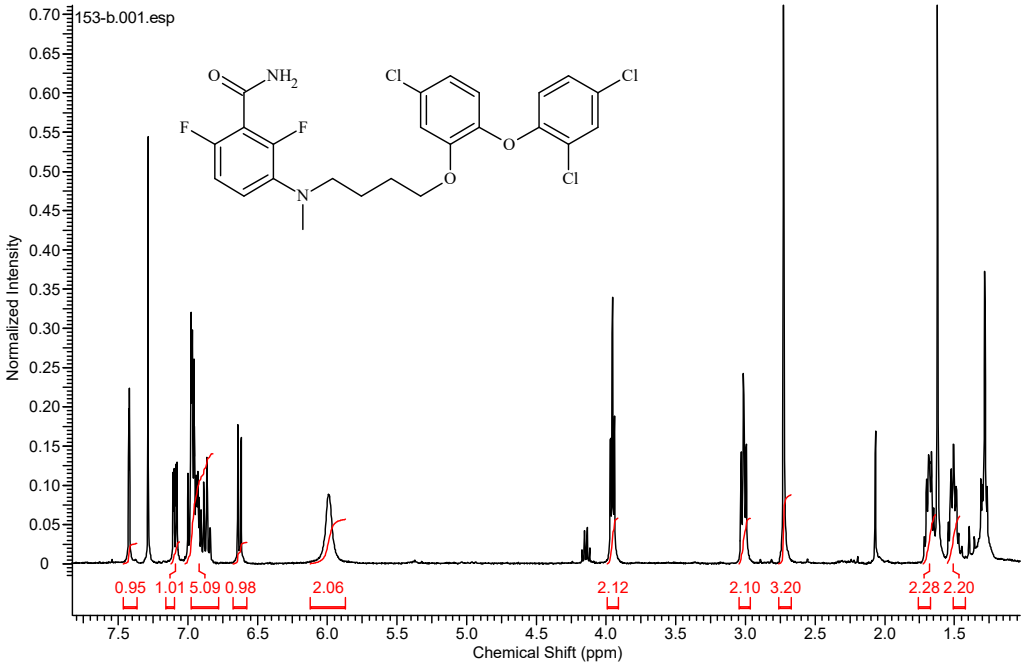


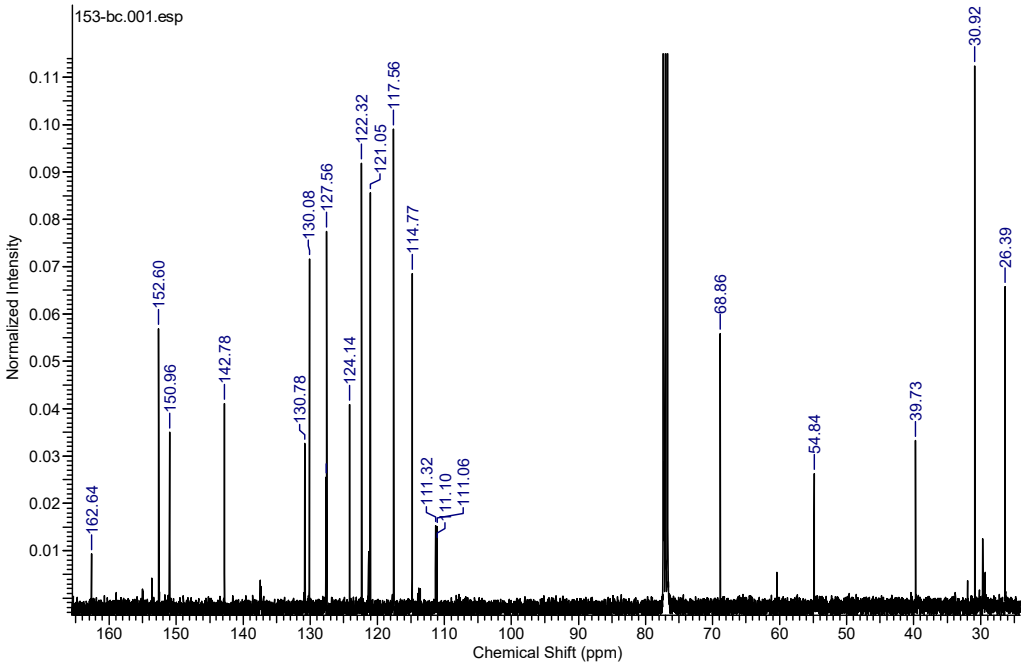
3-((2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)ethyl)(methyl)amino)-2,6-difluorobenzamide (48)



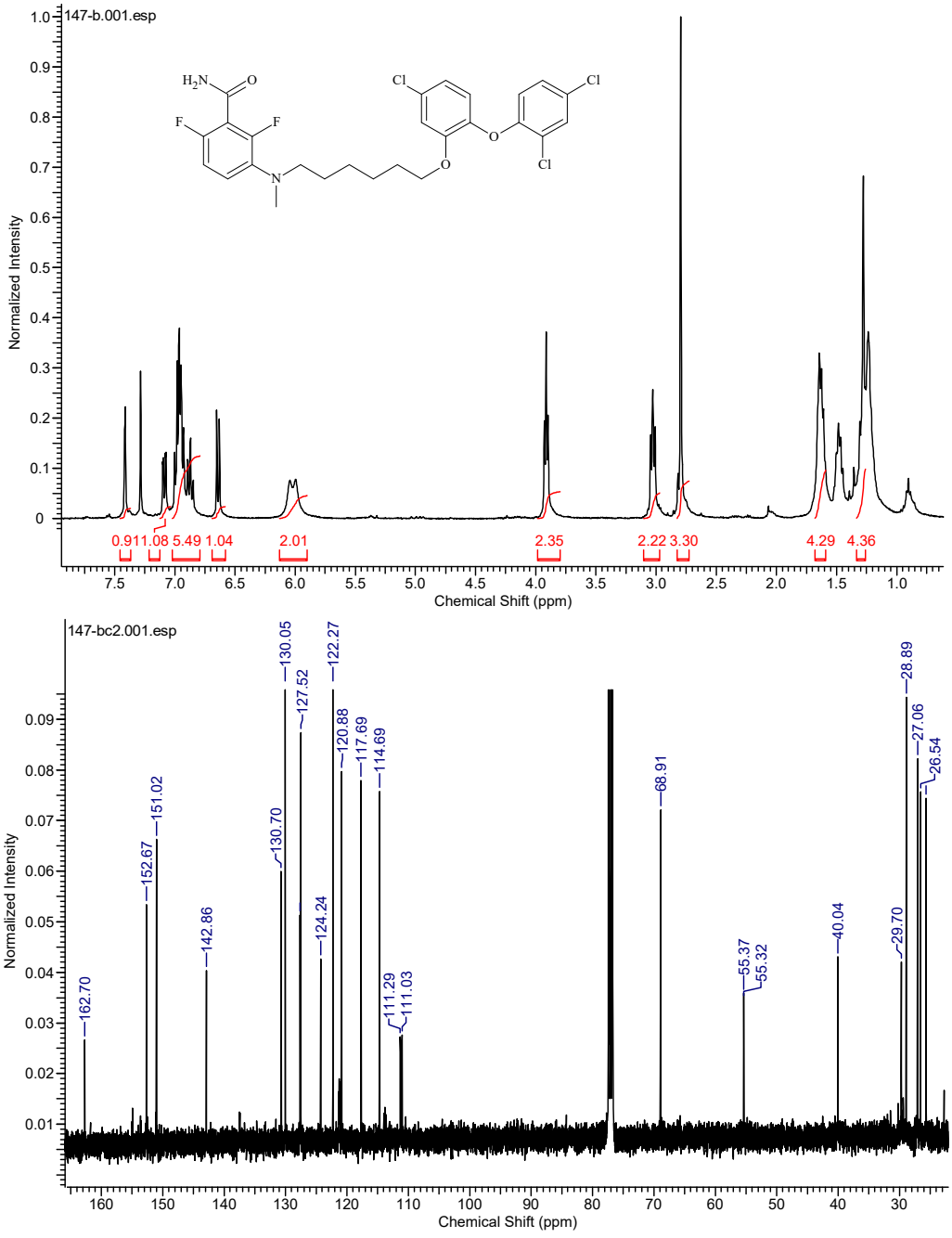


3-((4-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)butyl)(methyl)amino)-2,6-difluorobenzamide (49)

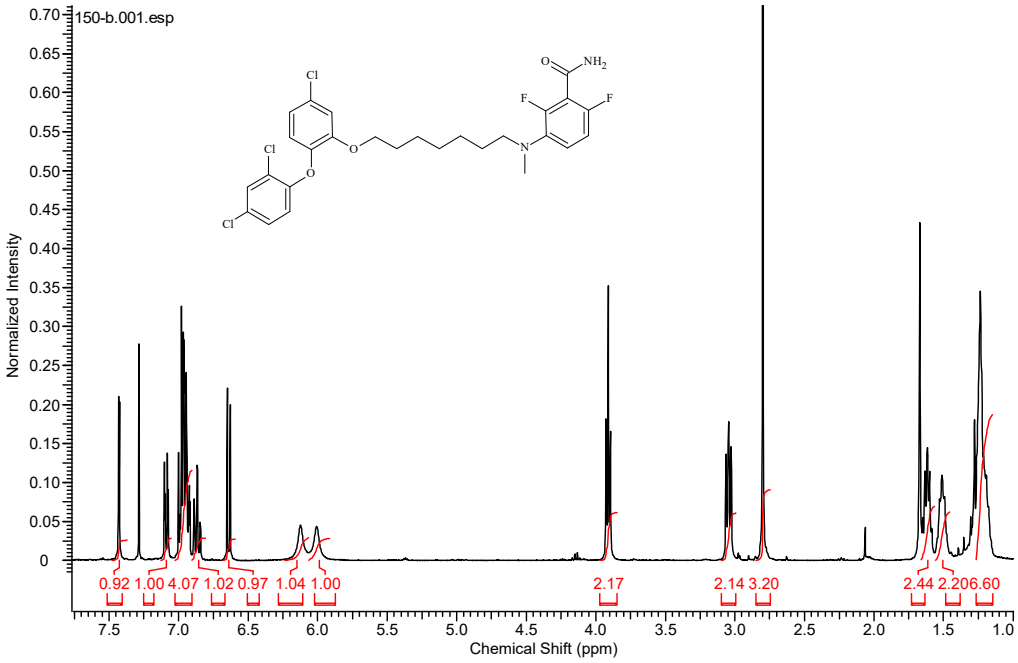


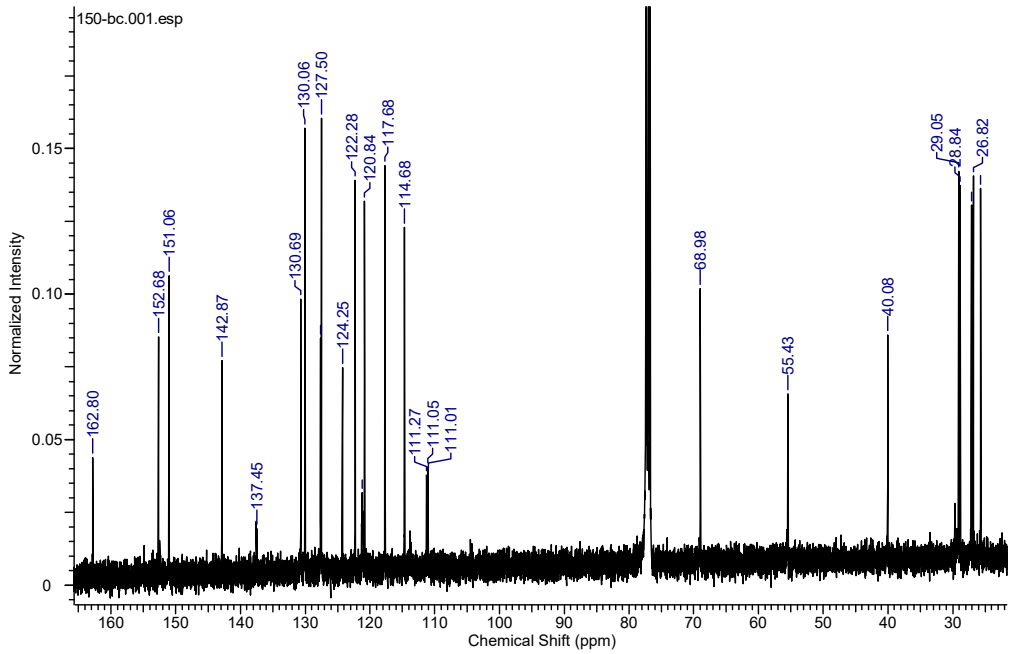


3-((6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexyl)(methyl)amino)-2,6-difluorobenzamide (50)

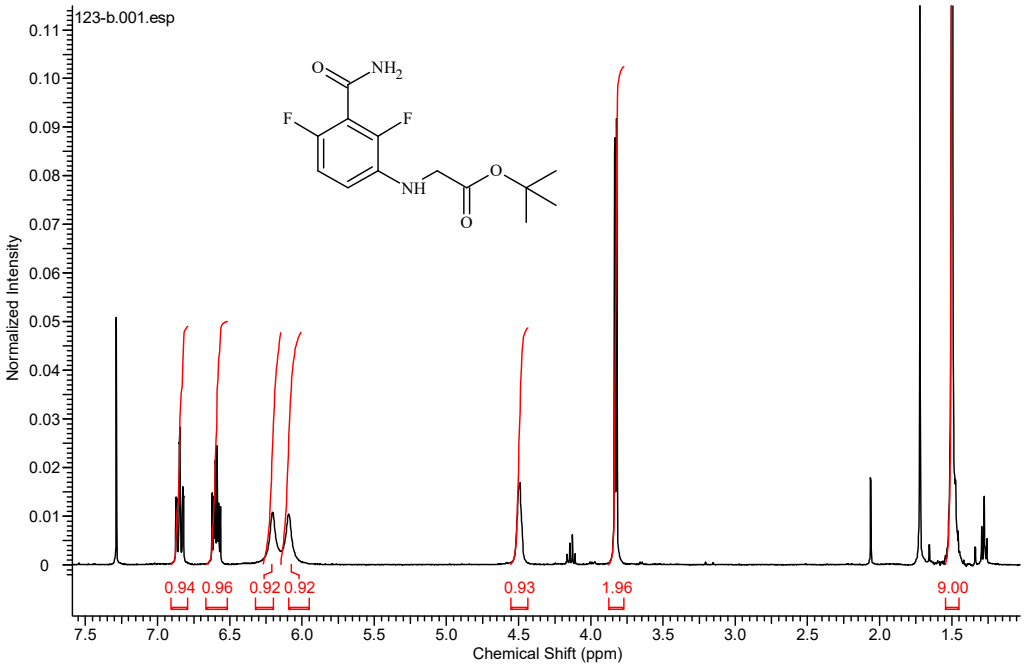


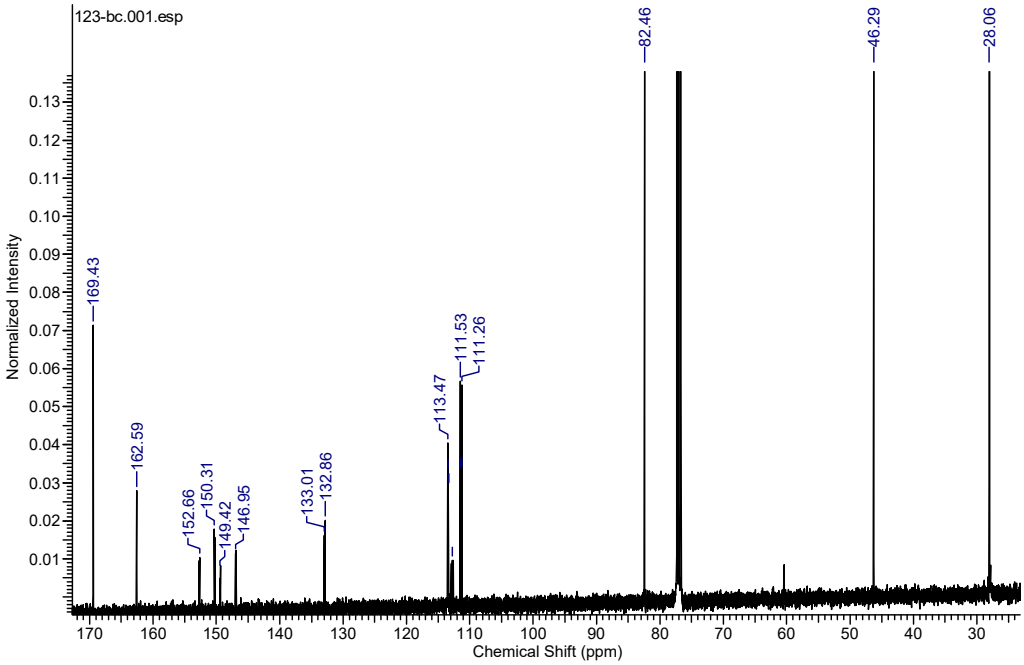
3-((7-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)heptyl)(methyl)amino)-2,6-difluorobenzamide (51)



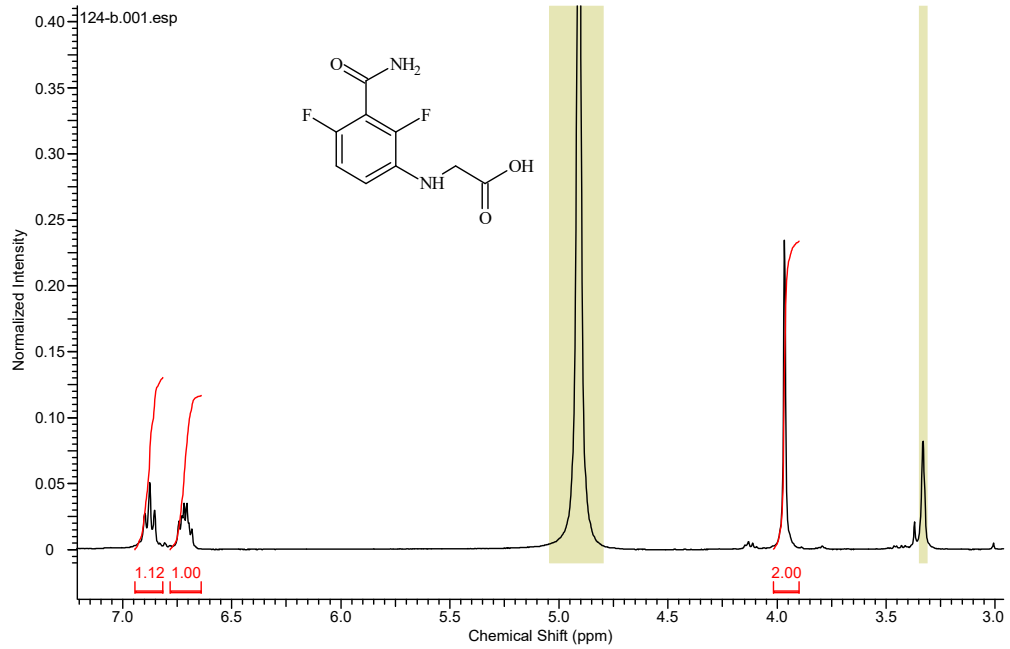


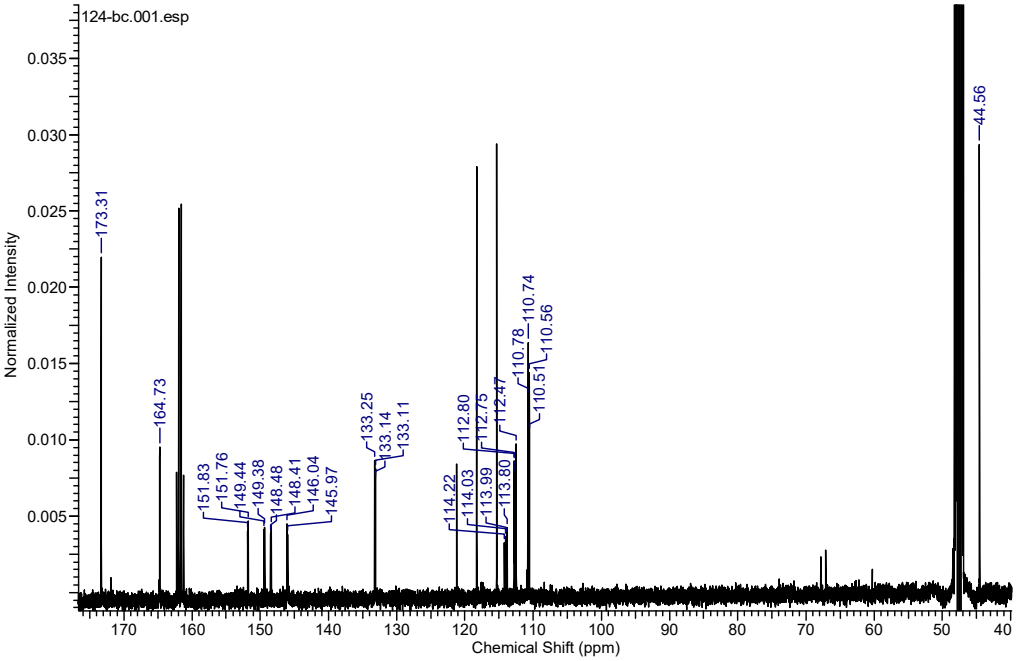
tert-butyl 2-((3-carbamoyl-2,4-difluorophenyl)amino)acetate (56)



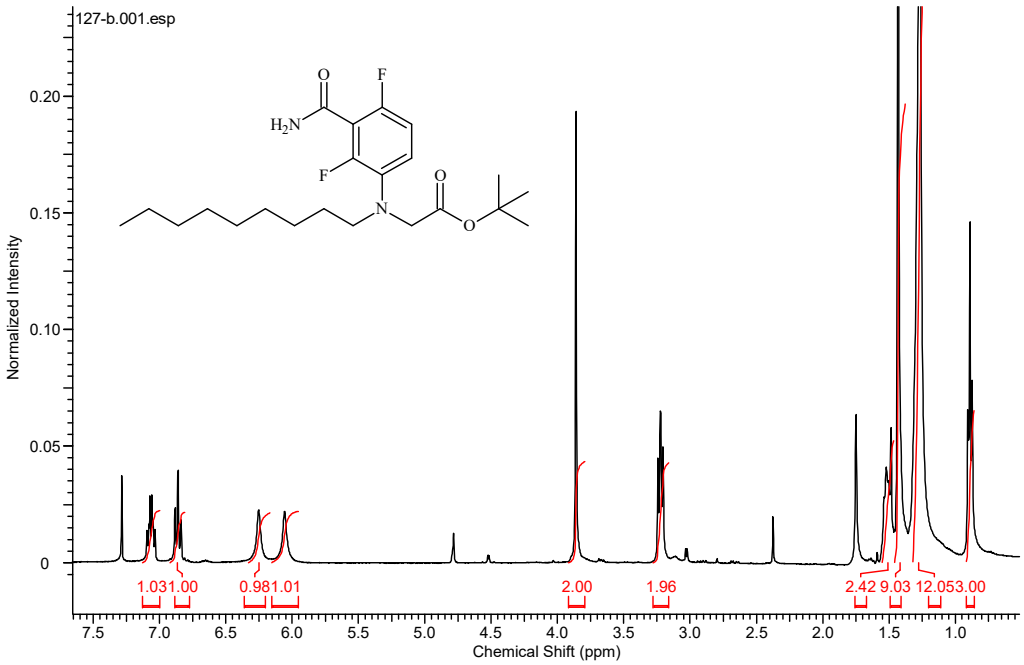


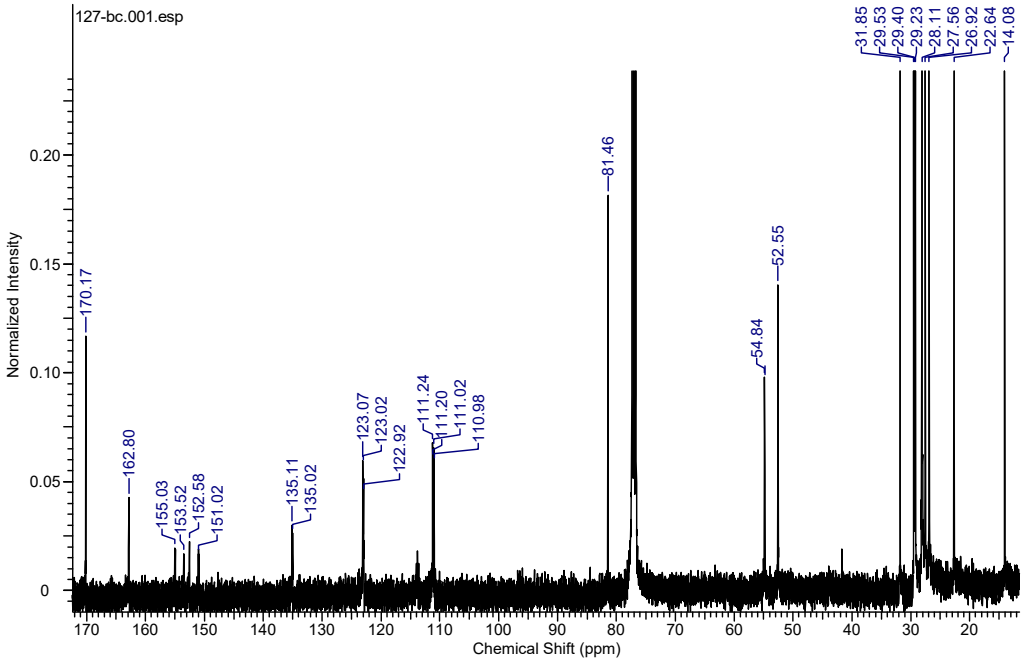
2-((3-carbamoyl-2,4-difluorophenyl)amino)acetic acid (57)



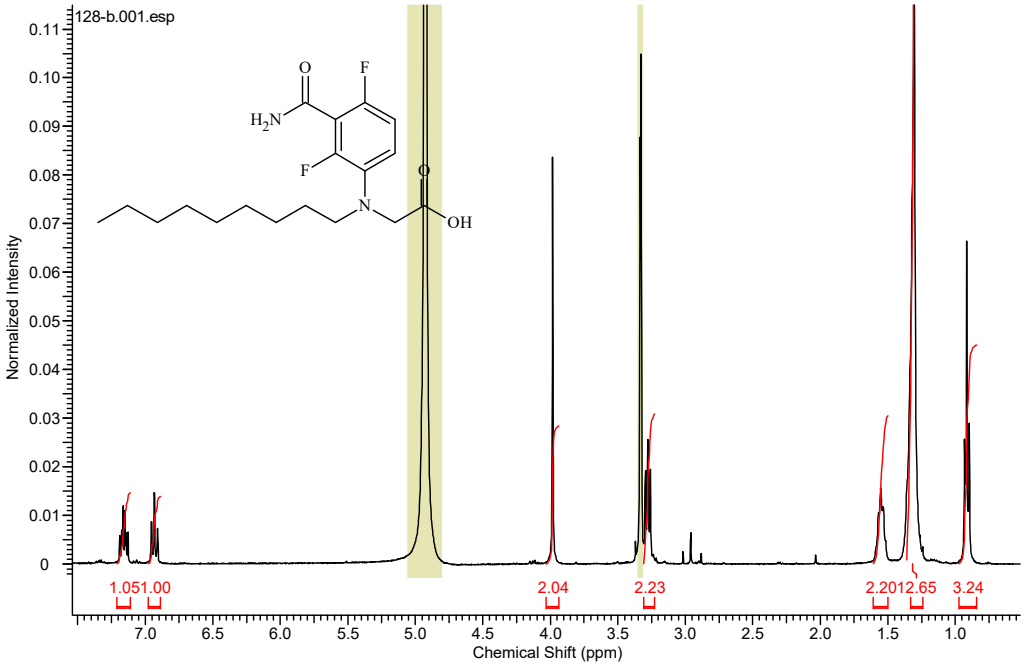


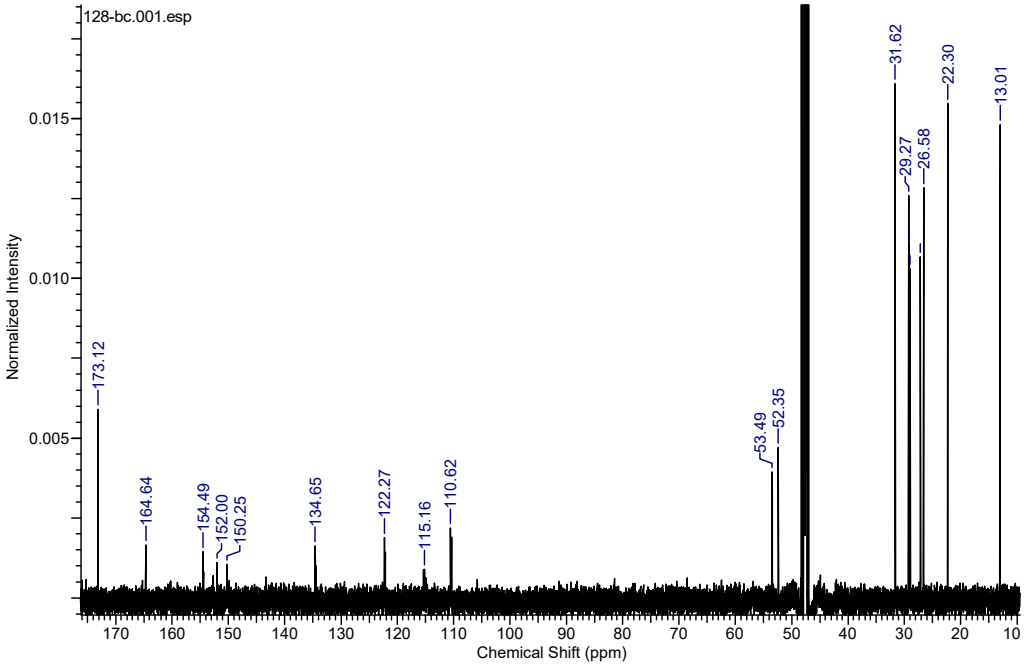
tert-butyl 2-((3-carbamoyl-2,4-difluorophenyl)(nonyl)amino)acetate (58)



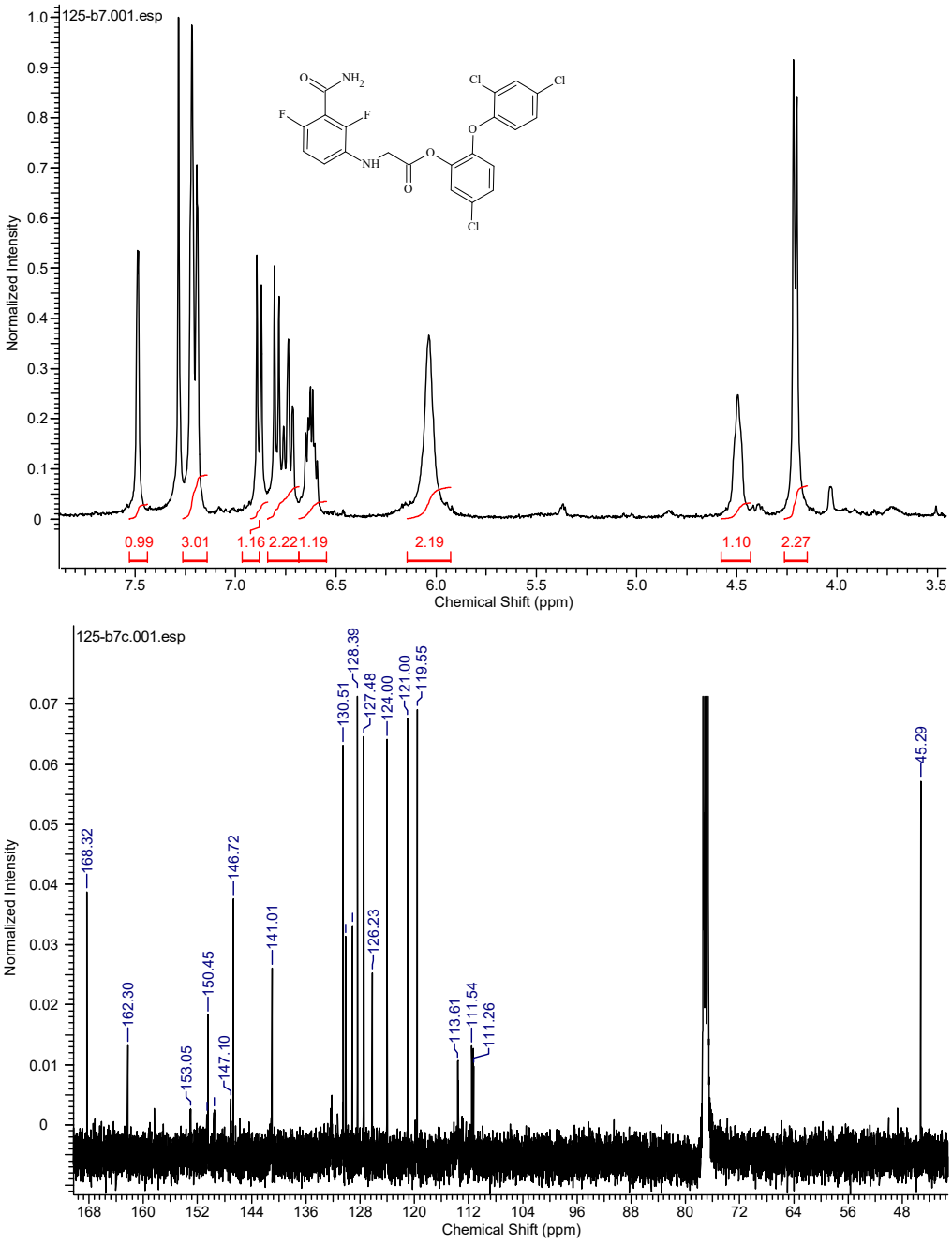


2-((3-carbamoyl-2,4-difluorophenyl)(nonyl)amino)acetic acid (59)





5-chloro-2-(2,4-dichlorophenoxy)phenyl 2-((3-carbamoyl-2,4-difluorophenyl)-amino)acetate (60)



References:

2013. The antibiotic alarm. *Nature,* 495**,** 141-141.

ACHARYA, B. R., BHATTACHARYYA, B. & CHAKRABARTI, G. 2008. The Natural Naphthoquinone Plumbagin Exhibits Antiproliferative Activity and Disrupts the Microtubule Network through Tubulin Binding. *Biochemistry,* 47**,** 7838-7845.

ADAMS, D. W. & ERRINGTON, J. 2009. Bacterial cell division: assembly, maintenance and disassembly of the Z ring. *Nature Reviews Microbiology,* 7**,** 642.

ADDINALL, S. G., ERFEI, B. & LUTKENHAUS, J. 1996. FtsZ ring formation in fts mutants. *Journal of Bacteriology,* 178**,** 3877-3884.

ADDINALL, S. G. & LUTKENHAUS, J. 1996. FtsA is localized to the septum in an FtsZ-dependent manner. *Journal of Bacteriology,* 178**,** 7167-72.

ALI, S. M., KHAN, A. A., AHMED, I., MUSADDIQ, M., AHMED, K. S., POLASA, H., RAO, L. V., HABIBULLAH, C. M., SECHI, L. A. & AHMED, N. 2005. Antimicrobial activities of Eugenol and Cinnamaldehyde against the human gastric pathogen Helicobacter pylori. *Annals of Clinical Microbiology and Antimicrobials,* 4**,** 20.

AMINOV, R. I. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in microbiology,* 1**,** 134.

ANDERSON, D. E., GUEIROS-FILHO, F. J. & ERICKSON, H. P. 2004. Assembly Dynamics of FtsZ Rings in Bacillus subtilis and Escherichia coli and Effects of FtsZ-Regulating Proteins. *Journal of Bacteriology,* 186**,** 5775-5781.

ANDREU, J. M., SCHAFFNER-BARBERO, C., HUECAS, S., ALONSO, D., LOPEZ-RODRIGUEZ, M. L., RUIZ-AVILA, L. B., NúñEZ-RAMíREZ, R., LLORCA, O. & MARTíN-GALIANO, A. J. 2010. The antibacterial cell division inhibitor PC190723 is an FtsZ polymer-stabilizing agent that induces filament assembly and condensation. *Journal of Biological Chemistry,* 285**,** 14239-14246.

APPENDINO, G., DANIELI, B., JAKUPOVIC, J., BELLORO, E., SCAMBIA, G. & BOMBARDELLI, E. 1997. Synthesis and evaluation of C-seco paclitaxel analogues. *Tetrahedron Letters,* 38**,** 4273-4276.

AWASTHI, D., KUMAR, K., KNUDSON, S. E., SLAYDEN, R. A. & OJIMA, I. 2013. SAR studies on trisubstituted benzimidazoles as inhibitors of Mtb FtsZ for the development of novel antitubercular agents. *Journal of Medicinal Chemistry,* 56**,** 9756-9770.

BAELL, J. & WALTERS, M. A. 2014. Chemical con artists foil drug discovery. *Nature,* 513**,** 481.

BARTLETT, J. G., GILBERT, D. N. & SPELLBERG, B. 2013. Seven Ways to Preserve the Miracle of Antibiotics. *Clinical Infectious Diseases,* 56**,** 1445-1450.

BASSETT, E. J., KEITH, M. S., ARMELAGOS, G. J., MARTIN, D. L. & VILLANUEVA, A. R. 1980. Tetracycline-labeled human bone from ancient Sudanese Nubia (AD 350). *Science,* 209**,** 1532-1534.

BAUMANN, P. & JACKSON, S. P. 1996. An archaebacterial homologue of the essential eubacterial cell division protein FtsZ. *Proceedings of the National Academy of Sciences,* 93**,** 6726.

BERGMAN, P., JOHANSSON, L., WAN, H., JONES, A., GALLO, R. L., GUDMUNDSSON, G. H., HöKFELT, T., JONSSON, A. B. & AGERBERTH, B. 2006. Induction of the antimicrobial peptide CRAMP

in the blood-brain barrier and meninges after meningococcal infection. *Infection and Immunity,* 74**,** 6982-6991.

BERNHARDT, T. G. & DE BOER, P. A. 2005. SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in E. coli. *Molecular cell,* 18**,** 555-564.

BHATTACHARYA, A., JINDAL, B., SINGH, P., DATTA, A. & PANDA, D. 2013. Plumbagin inhibits cytokinesis in Bacillus subtilis by inhibiting FtsZ assembly - A mechanistic study of its antibacterial activity. *FEBS Journal,* 280**,** 4585-4599.

BI, E. & LUTKENHAUS, J. 1991. FtsZ ring structure associated with division in Escherichia coli. *Nature,* 354**,** 161.

BRAMHILL, D. & THOMPSON, C. M. 1994. GTP-dependent polymerization of Escherichia coli FtsZ protein to form tubules. *Proceedings of the National Academy of Sciences,* 91**,** 5813.

BROWN, D. 2015. Antibiotic resistance breakers: can repurposed drugs fill the antibiotic discovery void? *Nature Reviews Drug Discovery,* 14**,** 821.

BULT, C. J., WHITE, O., OLSEN, G. J., ZHOU, L., FLEISCHMANN, R. D., SUTTON, G. G., BLAKE, J. A., FITZGERALD, L. M., CLAYTON, R. A., GOCAYNE, J. D., KERLAVAGE, A. R., DOUGHERTY, B. A., TOMB, J. F., ADAMS, M. D., REICH, C. I., OVERBEEK, R., KIRKNESS, E. F., WEINSTOCK, K. G., MERRICK, J. M., GLODEK, A., SCOTT, J. L., GEOGHAGEN, N. S. M., WEIDMAN, J. F., FUHRMANN, J. L., NGUYEN, D., UTTERBACK, T. R., KELLEY, J. M., PETERSON, J. D., SADOW, P. W., HANNA, M. C., COTTON, M. D., ROBERTS, K. M., HURST, M. A., KAINE, B. P., BORODOVSKY, M., KLENK, H. P., FRASER, C. M., SMITH, H. O., WOESE, C. R. & VENTER, J. C. 1996. Complete genome sequence of the Methanogenic archaeon, Methanococcus jannaschii. *Science,* 273**,** 1058-1073.

BUSIEK KIMBERLY, K. & MARGOLIN, W. 2014. A role for FtsA in SPOR‐independent localization of the essential Escherichia coli cell division protein FtsN. *Molecular Microbiology,* 92**,** 1212-1226.

BUSIEK, K. K., ERASO, J. M., WANG, Y. & MARGOLIN, W. 2012. The Early Divisome Protein FtsA Interacts Directly through Its 1c Subdomain with the Cytoplasmic Domain of the Late Divisome Protein FtsN. *Journal of Bacteriology,* 194**,** 1989-2000.

CAPLAN, M. R. & ERICKSON, H. P. 2003. Apparent cooperative assembly of the bacterial cell division protein FtsZ demonstrated by isothermal titration calorimetry. *Journal of Biological Chemistry,* 278**,** 13784-13788.

CHAN, F. Y., SUN, N., NEVES, M. A. C., LAM, P. C. H., CHUNG, W. H., WONG, L. K., CHOW, H. Y., MA, D. L., CHAN, P. H., LEUNG, Y. C., CHAN, T. H., ABAGYAN, R. & WONG, K. Y. 2013. Identification of a new class of FtsZ inhibitors by structure-based design and in vitro screening. *Journal of Chemical Information and Modeling,* 53**,** 2131-2140.

CZAPLEWSKI, L. G., COLLINS, I., BOYD, E. A., BROWN, D., EAST, S. P., GARDINER, M., FLETCHER, R., HAYDON, D. J., HENSTOCK, V., INGRAM, P., JONES, C., NOULA, C., KENNISON, L., ROCKLEY, C., ROSE, V., THOMAIDES-BREARS, H. B., URE, R., WHITTAKER, M. & STOKES, N. R. 2009. Antibacterial alkoxybenzamide inhibitors of the essential bacterial cell division protein FtsZ. *Bioorganic and Medicinal Chemistry Letters,* 19**,** 524-527.

DE BOER, P., CROSSLEY, R. & ROTHFIELD, L. 1992. The essential bacterial cell-division protein FtsZ is a GTPase. *Nature,* 359**,** 254.

DE BOER, P. A., CROSSLEY, R. E. & ROTHFIELD, L. I. 1989. A division inhibitor and a topological

specificity factor coded for by the minicell locus determine proper placement of the division septum in E. coli. *Cell,* 56**,** 641-649.

DE PEREDA, J. M., LEYNADIER, D., EVANGELIO, J. A., CHACóN, P. & ANDREU, J. M. 1996. Tubulin Secondary Structure Analysis, Limited Proteolysis Sites, and Homology to FtsZ. *Biochemistry,* 35**,** 14203-14215.

DOMADIA, P., SWARUP, S., BHUNIA, A., SIVARAMAN, J. & DASGUPTA, D. 2007. Inhibition of bacterial cell division protein FtsZ by cinnamaldehyde. *Biochemical Pharmacology,* 74**,** 831-840.

DOMADIA, P. N., BHUNIA, A., SIVARAMAN, J., SWARUP, S. & DASGUPTA, D. 2008. Berberine targets assembly of Escherichia coli cell division protein FtsZ. *Biochemistry,* 47**,** 3225-3234.

DRAWZ, S. M. & BONOMO, R. A. 2010. Three decades of β-lactamase inhibitors. *Clinical microbiology reviews,* 23**,** 160-201.

DUGGIRALA, S., NANKAR, R. P., RAJENDRAN, S. & DOBLE, M. 2014. Phytochemicals as Inhibitors of Bacterial Cell Division Protein FtsZ: Coumarins Are Promising Candidates. *Applied Biochemistry and Biotechnology,* 174**,** 283-296.

EISENSTEIN, B. I., OLESON JR, F. B. & BALTZ, R. H. 2010. Daptomycin: from the mountain to the clinic, with essential help from Francis Tally, MD. *Clinical Infectious Diseases,* 50**,** S10-S15.

ELSEN, N. L., LU, J., PARTHASARATHY, G., REID, J. C., SHARMA, S., SOISSON, S. M. & LUMB, K. J. 2012. Mechanism of action of the cell-division inhibitor PC190723: Modulation of FtsZ assembly cooperativity. *Journal of the American Chemical Society,* 134**,** 12342-12345.

EMMERSON, A. & JONES, A. 2003. The quinolones: decades of development and use. *Journal of Antimicrobial Chemotherapy,* 51**,** 13-20.

ERICKSON, H. P., ANDERSON, D. E. & OSAWA, M. 2010. FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one. *Microbiology and Molecular Biology Reviews,* 74**,** 504-528.

ERRINGTON, J., DANIEL, R. A. & SCHEFFERS, D.-J. 2003. Cytokinesis in bacteria. *Microbiology and Molecular Biology Reviews,* 67**,** 52-65.

GOLKAR, Z., BAGASRA, O. & PACE, D. G. 2014. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *J Infect Dev Ctries,* 8**,** 129-36.

GOULD, F. K. 2011. Linezolid: safety and efficacy in special populations. *Journal of Antimicrobial Chemotherapy,* 66**,** iv3-iv6.

GOULD, I. M. & BAL, A. M. 2013. New antibiotic agents in the pipeline and how they can help overcome microbial resistance. *Virulence,* 4**,** 185-191.

GOULD, K. 2016. Antibiotics: from prehistory to the present day. *Journal of Antimicrobial Chemotherapy,* 71**,** 572-575.

GREENWOOD, D. 1988. Microbiological properties of teicoplanin. *Journal of Antimicrobial Chemotherapy,* 21**,** 1-13.

GREER, N. D. Tigecycline (Tygacil): the first in the glycylcycline class of antibiotics. Baylor University Medical Center Proceedings, 2006. Taylor & Francis, 155-161.

GROSS, M. 2013. Antibiotics in crisis. *Current Biology,* 23**,** R1063-R1065.

GRUEGELSIEPE, H., BRANDT, O. & HARTMANN, R. K. 2006. Antisense inhibition of RNase P: Mechanistic aspects and application to live bacteria. *Journal of Biological Chemistry,* 281**,** 30613-30620.

GUCK, J., SCHINKINGER, S., LINCOLN, B., WOTTAWAH, F., EBERT, S., ROMEYKE, M., LENZ, D., ERICKSON, H. M., ANANTHAKRISHNAN, R. & MITCHELL, D. 2005. Optical deformability as

an inherent cell marker for testing malignant transformation and metastatic competence. *Biophysical journal,* 88**,** 3689-3698.

GUPTA, K. K., BHARNE, S. S., RATHINASAMY, K., NAIK, N. R. & PANDA, D. 2006. Dietary antioxidant curcumin inhibits microtubule assembly through tubulin binding. *FEBS Journal,* 273**,** 5320-5332.

HAYDON, D. J., STOKES, N. R., URE, R., GALBRAITH, G., BENNETT, J. M., BROWN, D. R., BAKER, P. J., BARYNIN, V. V., RICE, D. W., SEDELNIKOVA, S. E., HEAL, J. R., SHERIDAN, J. M., AIWALE, S. T., CHAUHAN, P. K., SRIVASTAVA, A., TANEJA, A., COLLINS, I., ERRINGTON, J. & CZAPLEWSKI, L. G. 2008. An Inhibitor of FtsZ with Potent and Selective Anti-Staphylococcal Activity. *Science,* 321**,** 1673-1675.

HEMAISWARYA, S., SOUDAMINIKKUTTY, R., NARASUMANI, M. L. & DOBLE, M. 2011. Phenylpropanoids inhibit protofilament formation of Escherichia coli cell division protein FtsZ. *Journal of Medical Microbiology,* 60**,** 1317-1325.

HUANG, Q., KIRIKAE, F., KIRIKAE, T., PEPE, A., AMIN, A., RESPICIO, L., SLAYDEN, R. A., TONGE, P. J. & OJIMA, I. 2006. Targeting FtsZ for antituberculosis drug discovery: Noncytotoxic taxanes as novel antituberculosis agents. *Journal of Medicinal Chemistry,* 49**,** 463-466.

HUANG, Q., TONGE, P. J., SLAYDEN, R. A., KIRIKAE, T. & OJIMA, I. 2007. FtsZ: A novel target for tuberculosis drug discovery. *Current Topics in Medicinal Chemistry,* 7**,** 527-543.

HWANG, D. & LIM, Y.-H. 2015. Resveratrol antibacterial activity against Escherichia coli is mediated by Z-ring formation inhibition via suppression of FtsZ expression. *Scientific reports,* 5**,** 10029.

ITANI, K. M. F. & SHORR, A. F. 2014. FDA Guidance for ABSSSI Trials: Implications for Conducting and Interpreting Clinical Trials. *Clinical Infectious Diseases,* 58**,** S4-S9.

KAUL, M., MARK, L., ZHANG, Y., PARHI, A. K., LAVOIE, E. J. & PILCH, D. S. 2013. Pharmacokinetics and in vivo antistaphylococcal efficacy of TXY541, a 1-methylpiperidine-4-carboxamide prodrug of PC190723. *Biochemical Pharmacology,* 86**,** 1699-1707.

KAUL, M., MARK, L., ZHANG, Y., PARHI, A. K., LYU, Y. L., PAWLAK, J., SARAVOLATZ, S., SARAVOLATZ, L. D., WEINSTEIN, M. P., LAVOIE, E. J. & PILCHA, D. S. 2015. TXA709, an FtsZ-targeting benzamide prodrug with improved pharmacokinetics and enhanced in vivo efficacy against methicillin-resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 59**,** 4845-4855.

KAUR, S., MODI, N. H., PANDA, D. & ROY, N. 2010. Probing the binding site of curcumin in Escherichia coli and Bacillus subtilis FtsZ - A structural insight to unveil antibacterial activity of curcumin. *European Journal of Medicinal Chemistry,* 45**,** 4209-4214.

KNUDSON, S. E., AWASTHI, D., KUMAR, K., CARREAU, A., GOULLIEUX, L., LAGRANGE, S., VERMET, H., OJIMA, I. & SLAYDEN, R. A. 2014. A trisubstituted benzimidazole cell division inhibitor with efficacy against Mycobacterium tuberculosis. *PloS one,* 9**,** e93953.

KNUDSON, S. E., AWASTHI, D., KUMAR, K., CARREAU, A., GOULLIEUX, L., LAGRANGE, S., VERMET, H., OJIMA, I. & SLAYDEN, R. A. 2015. Cell division inhibitors with efficacy equivalent to isoniazid in the acute murine Mycobacterium tuberculosis infection model. *Journal of Antimicrobial Chemotherapy,* 70**,** 3070-3073.

KUMAR, K., AWASTHI, D., LEE, S. Y., CUMMINGS, J. E., KNUDSON, S. E., SLAYDEN, R. A. & OJIMA, I. 2013. Benzimidazole-based antibacterial agents against Francisella tularensis. *Bioorganic*

and Medicinal Chemistry, 21**,** 3318-3326.

KUMAR, K., AWASTHI, D., LEE, S. Y., ZANARDI, I., RUZSICSKA, B., KNUDSON, S., TONGE, P. J., SLAYDEN, R. A. & OJIMA, I. 2011. Novel trisubstituted benzimidazoles, targeting Mtb FtsZ, as a new class of antitubercular agents. *Journal of Medicinal Chemistry,* 54**,** 374-381.

LAN, G., DANIELS, B. R., DOBROWSKY, T. M., WIRTZ, D. & SUN, S. X. 2009. Condensation of FtsZ filaments can drive bacterial cell division. *Proceedings of the National Academy of Sciences,* 106**,** 121.

LäPPCHEN, T., HARTOG, A. F., PINAS, V. A., KOOMEN, G.-J. & DEN BLAAUWEN, T. 2005. GTP analogue inhibits polymerization and GTPase activity of the bacterial protein FtsZ without affecting its eukaryotic homologue tubulin. *Biochemistry,* 44**,** 7879-7884.

LEVIN, P. A. & LOSICK, R. 1996. Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in Bacillus subtilis. *Genes & Development,* 10**,** 478-488.

LEVINE, D. P. 2006. Vancomycin: a history. *Clinical Infectious Diseases,* 42**,** S5-S12.

LI, X. 2015. Nutritional requirements dietary demand research among sports coaches. *Advance Journal of Food Science and Technology,* 7**,** 32-34.

LI, Y., HSIN, J., ZHAO, L., CHENG, Y., SHANG, W., HUANG, K. C., WANG, H.-W. & YE, S. 2013. FtsZ Protofilaments Use a Hinge-Opening Mechanism for Constrictive Force Generation. *Science,* 341**,** 392.

LIANG, S., LIANG, K., LUO, L., ZHANG, Q. & WANG, C. 2014. Study on low-velocity impact of embedded and co-cured composite damping panels with numerical simulation method. *Composite Structures,* 107**,** 1-10.

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., J. RYBAK, M., TALAN, D. A. & CHAMBERS, H. F. 2011. Clinical Practice Guidelines by the Infectious Diseases Society of America for the Treatment of Methicillin-Resistant Staphylococcus aureus Infections in Adults and Children: Executive Summary. *Clinical Infectious Diseases,* 52**,** 285-292.

LOCK, R. L. & HARRY, E. J. 2008. Cell-division inhibitors: new insights for future antibiotics. *Nature Reviews Drug Discovery,* 7**,** 324.

LOWE, J. & AMOS, L. A. 1998. Crystal structure of the bacterial cell-division protein FtsZ. *Nature,* 391**,** 203-6.

LöWE, J. & AMOS, L. A. 1998a. Crystal structure of the bacterial cell-division protein FtsZ. *Nature,* 391**,** 203.

LöWE, J. & AMOS, L. A. 1998b. Crystal structure of the bacterial cell-division protein FtsZ. *Nature,* 391**,** 203.

LU, C., REEDY, M. & ERICKSON, H. P. 2000. Straight and curved conformations of FtsZ are regulated by GTP hydrolysis. *Journal of bacteriology,* 182**,** 164-170.

LUI, H. K., GAO, W., CHEUNG, K. C., JIN, W. B., SUN, N., KAN, J. W. Y., WONG, I. L. K., CHIOU, J., LIN, D., CHAN, E. W. C., LEUNG, Y. C., CHAN, T. H., CHEN, S., CHAN, K. F. & WONG, K. Y. 2019. Boosting the efficacy of anti-MRSA beta-lactam antibiotics via an easily accessible, non-cytotoxic and orally bioavailable FtsZ inhibitor. *Eur J Med Chem,* 163**,** 95-115.

LUSHNIAK, B. D. 2014. Antibiotic resistance: a public health crisis. *Public health reports (Washington, D.C. : 1974),* 129**,** 314-316.

LUYT, C.-E., BRéCHOT, N., TROUILLET, J.-L. & CHASTRE, J. 2014. Antibiotic stewardship in the intensive care unit. *Critical care (London, England),* 18**,** 480-480.

LYU, Y., YANG, X., GOSWAMI, S., GORITYALA, B. K., IDOWU, T., DOMALAON, R., ZHANEL, G. G., SHAN, A. & SCHWEIZER, F. 2017. Amphiphilic Tobramycin-Lysine Conjugates Sensitize Multidrug Resistant Gram-Negative Bacteria to Rifampicin and Minocycline. *J Med Chem,* 60**,** 3684-3702.

MA, S. & MA, S. 2012. The development of FtsZ inhibitors as potential antibacterial agents. *ChemMedChem,* 7**,** 1161-72.

MA, X., EHRHARDT, D. W. & MARGOLIN, W. 1996. Colocalization of cell division proteins FtsZ and FtsA to cytoskeletal structures in living Escherichia coli cells by using green fluorescent protein. *Proceedings of the National Academy of Sciences,* 93**,** 12998-13003.

MARGOLIN, W. 2000. Themes and variations in prokaryotic cell division. *FEMS Microbiology Reviews,* 24**,** 531-548.

MARGOLIN, W., WANG, R. & KUMAR, M. 1996. Isolation of an ftsZ homolog from the archaebacterium Halobacterium salinarium: implications for the evolution of FtsZ and tubulin. *Journal of Bacteriology,* 178**,** 1320-7.

MATHEW, B., SRIVASTAVA, S., ROSS, L. J., SULING, W. J., WHITE, E. L., WOOLHISER, L. K., LENAERTS, A. J. & REYNOLDS, R. C. 2011. Novel pyridopyrazine and pyrimidothiazine derivatives as FtsZ inhibitors. *Bioorganic and Medicinal Chemistry,* 19**,** 7120-7128.

MENG, J., DA, F., MA, X., WANG, N., WANG, Y., ZHANG, H., LI, M., ZHOU, Y., XUE, X., HOU, Z., JIA, M. & LUO, X. 2015. Antisense Growth Inhibition of Methicillin-Resistant Staphylococcus aureus by Locked Nucleic Acid Conjugated with Cell-Penetrating Peptide as a Novel FtsZ Inhibitor. *Antimicrobial Agents and Chemotherapy,* 59**,** 914-922.

MICHAEL, C. A., DOMINEY-HOWES, D. & LABBATE, M. 2014. The antimicrobial resistance crisis: causes, consequences, and management. *Frontiers in public health,* 2**,** 145-145.

MINGORANCE, J., RUEDA, S., GóMEZ‐PUERTAS, P., VALENCIA, A. & VICENTE, M. 2001. Escherichia coli FtsZ polymers contain mostly GTP and have a high nucleotide turnover. *Molecular microbiology,* 41**,** 83-91.

MUKHERJEE, A., DAI, K. & LUTKENHAUS, J. 1993a. Escherichia coli cell division protein FtsZ is a guanine nucleotide binding protein. *Proceedings of the National Academy of Sciences,* 90**,** 1053.

MUKHERJEE, A., DAI, K. & LUTKENHAUS, J. 1993b. Escherichia coli cell division protein FtsZ is a guanine nucleotide binding protein. *Proceedings of the National Academy of Sciences,* 90**,** 1053-1057.

MUKHERJEE, A. & LUTKENHAUS, J. 1994. Guanine nucleotide-dependent assembly of FtsZ into filaments. *Journal of Bacteriology,* 176**,** 2754-2758.

NEPOMUCENO, G. M., CHAN, K. M., HUYNH, V., MARTIN, K. S., MOORE, J. T., OBRIEN, T. E., POLLO, L. A. E., SARABIA, F. J., TADEUS, C., YAO, Z., ANDERSON, D. E., AMES, J. B. & SHAW, J. T. 2015. Synthesis and evaluation of quinazolines as inhibitors of the bacterial cell division protein FtsZ. *ACS Medicinal Chemistry Letters,* 6**,** 308-312.

NIELSEN, P. E. & EGHOLM, M. 1999. An introduction to peptide nucleic acid. *Current issues in molecular biology,* 1**,** 89-104.

NOGALES, E., DOWNING, K. H., AMOS, L. A. & LöWE, J. 1998. Tubulin and FtsZ form a distinct family of GTPases. *Nature Structural and Molecular Biology,* 5**,** 451.

OHASHI, Y., CHIJIIWA, Y., SUZUKI, K., TAKAHASHI, K., NANAMIYA, H., SATO, T., HOSOYA, Y., OCHI, K. & KAWAMURA, F. 1999. The Lethal Effect of a Benzamide Derivative, 3-Methoxybenzamide, Can Be Suppressed by Mutations within a Cell Division Gene, ftsZ, in Bacillus subtilis. *Journal of Bacteriology,* 181**,** 1348-1351.

OJIMA, I., KUMAR, K., AWASTHI, D. & VINEBERG, J. G. 2014. Drug discovery targeting cell division proteins, microtubules and FtsZ. *Bioorganic & Medicinal Chemistry,* 22**,** 5060-5077.

OSAWA, M., ANDERSON, D. E. & ERICKSON, H. P. 2008a. Reconstitution of Contractile FtsZ Rings in Liposomes. *Science,* 320**,** 792.

OSAWA, M., ANDERSON, D. E. & ERICKSON, H. P. 2008b. Reconstitution of contractile FtsZ rings in liposomes. *Science,* 320**,** 792-794.

OSAWA, M. & ERICKSON, H. P. 2013. Liposome division by a simple bacterial division machinery. *Proceedings of the National Academy of Sciences,* 110**,** 11000.

PAPP-WALLACE, K. M., ENDIMIANI, A., TARACILA, M. A. & BONOMO, R. A. 2011. Carbapenems: past, present, and future. *Antimicrobial agents and chemotherapy***,** AAC. 00296-11.

PARHI, A., LU, S., KELLEY, C., KAUL, M., PILCH, D. S. & LAVOIE, E. J. 2012. Antibacterial activity of substituted dibenzo[a,g]quinolizin-7-ium derivatives. *Bioorganic and Medicinal Chemistry Letters,* 22**,** 6962-6966.

PARHI, A. K., ZHANG, Y., SAIONZ, K. W., PRADHAN, P., KAUL, M., TRIVEDI, K., PILCH, D. S. & LAVOIE, E. J. 2013. Antibacterial activity of quinoxalines, quinazolines, and 1,5-naphthyridines. *Bioorganic and Medicinal Chemistry Letters,* 23**,** 4968-4974.

PAULASOVA, P. & PELLESTOR, F. 2004. The peptide nucleic acids (PNAs): A new generation of probes for genetic and cytogenetic analyses. *Annales de Genetique,* 47**,** 349-358.

PIDDOCK, L. J. V. 2012. The crisis of no new antibiotics—what is the way forward? *The Lancet Infectious Diseases,* 12**,** 249-253.

PLAZA, A., KEFFER, J. L., BIFULCO, G., LLOYD, J. R. & BEWLEY, C. A. 2010. Chrysophaentins A-H, antibacterial bisdiarylbutene macrocycles that inhibit the bacterial cell division protein FtsZ. *Journal of the American Chemical Society,* 132**,** 9069-9077.

PRABHUDESAI, P., JAIN, S., KESHVANI, A. & KULKARNI, K. 2011. The efficacy and safety of amoxicillin-clavulanic acid 1000/125mg twice daily extended release (XR) tablet for the treatment of bacterial community-acquired pneumonia in adults. *J. Indian Med. Assoc,* 109**,** 124-127.

PRUITT, R. William Osler and his Gulstonian Lectures on malignant endocarditis. Mayo Clinic Proceedings, 1982. 4-9.

PUUPPONEN-PIMIä, R., NOHYNEK, L. & MEIER, C. 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology,* 90**,** 494-507.

RAI, D., SINGH, J. K., ROY, N. & PANDA, D. 2008. Curcumin inhibits FtsZ assembly: An attractive mechanism for its antibacterial activity. *Biochemical Journal,* 410**,** 147-155.

RAJAGOPALAN, M., ATKINSON, M. A. L., LOFTON, H., CHAUHAN, A. & MADIRAJU, M. V. 2005. Mutations in the GTP-binding and synergy loop domains of Mycobacterium tuberculosis ftsZ compromise its function in vitro and in vivo. *Biochemical and Biophysical Research Communications,* 331**,** 1171-1177.

RAY, S., DHAKED, H. P. S. & PANDA, D. 2014. Antimicrobial peptide CRAMP (16-33) stalls bacterial cytokinesis by inhibiting FtsZ assembly. *Biochemistry,* 53**,** 6426-6429.

RAY, S., JINDAL, B., KUNAL, K., SUROLIA, A. & PANDA, D. 2015. BT-benzo-29 inhibits bacterial cell proliferation by perturbing FtsZ assembly. *FEBS Journal,* 282**,** 4015-4033.

RAYCHAUDHURI, D. & PARK, J. T. 1992. Escherichia coli cell-division gene ftsZ encodes a novel GTP-binding protein. *Nature,* 359**,** 251.

RAYCHAUDHURI, D. & PARK, J. T. 1994. A point mutation converts Escherichia coli FtsZ septation GTPase to an ATPase. *Journal of Biological Chemistry,* 269**,** 22941-22944.

READ, A. F. & WOODS, R. J. 2014. Antibiotic resistance management. *Evolution, medicine, and public health,* 2014**,** 147-147.

RICHTER, M. F., DROWN, B. S., RILEY, A. P., GARCIA, A., SHIRAI, T., SVEC, R. L. & HERGENROTHER, P. J. 2017. Predictive compound accumulation rules yield a broad-spectrum antibiotic. *Nature,* 545**,** 299-304.

ROMBERG, L. & LEVIN, P. A. 2003. Assembly dynamics of the bacterial cell division protein FtsZ: poised at the edge of stability. *Annual Reviews in Microbiology,* 57**,** 125-154.

ROMBERG, L. & MITCHISON, T. J. 2004. Rate-limiting guanosine 5 '-triphosphate hydrolysis during nucleotide turnover by FtsZ, a prokaryotic tubulin homologue involved in bacterial cell division. *Biochemistry,* 43**,** 282-288.

ROMBERG, L., SIMON, M. & ERICKSON, H. P. 2001. Polymerization of ftsz, a bacterial homolog of tubulin is assembly cooperative? *Journal of Biological Chemistry,* 276**,** 11743-11753.

ROSSOLINI, G. M., ARENA, F., PECILE, P. & POLLINI, S. 2014. Update on the antibiotic resistance crisis. *Current Opinion in Pharmacology,* 18**,** 56-60.

RUSSELL, A. D. 1975. The antibacterial activity of a new cephalosporin, cefamandole. *Journal of Antimicrobial Chemotherapy,* 1**,** 97-101.

SARCINA, M. & MULLINEAUX, C. W. 2000. Effects of tubulin assembly inhibitors on cell division in prokaryotes in vivo. *FEMS Microbiology Letters,* 191**,** 25-29.

SCHWARTZ, R. S. 2004. Paul Ehrlich's magic bullets. *New England Journal of Medicine,* 350**,** 1079-1080.

SENGUPTA, S., CHATTOPADHYAY, M. K. & GROSSART, H.-P. 2013. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in microbiology,* 4**,** 47-47.

SHAMBAUGH, G. E. 1966. History of sulfonamides. *Archives of Otolaryngology,* 83**,** 1-2.

SINGH, D., BHATTACHARYA, A., RAI, A., DHAKED, H. P. S., AWASTHI, D., OJIMA, I. & PANDA, D. 2014. SB-RA-2001 inhibits bacterial proliferation by targeting FtsZ assembly. *Biochemistry,* 53**,** 2979-2992.

SINGH, P., JINDAL, B., SUROLIA, A. & PANDA, D. 2012. A rhodanine derivative CCR-11 inhibits bacterial proliferation by inhibiting the assembly and GTPase activity of FtsZ. *Biochemistry,* 51**,** 5434-5442.

SLAYDEN, R. A., KNUDSON, D. L. & BELISLE, J. T. 2006. Identification of cell cycle regulators in Mycobacterium tuberculosis by inhibition of septum formation and global transcriptional analysis. *Microbiology,* 152**,** 1789-1797.

SPELLBERG, B. & GILBERT, D. N. 2014. The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 59 Suppl 2**,** S71-S75.

STRICKER, J., MADDOX, P., SALMON, E. & ERICKSON, H. P. 2002. Rapid assembly dynamics of the Escherichia coli FtsZ-ring demonstrated by fluorescence recovery after photobleaching. *Proceedings of the National Academy of Sciences,* 99**,** 3171-3175.

SUN, N., CHAN, F.-Y., LU, Y.-J., NEVES, M. A. C., LUI, H.-K., WANG, Y., CHOW, K.-Y., CHAN, K.-F., YAN, S.-C., LEUNG, Y.-C., ABAGYAN, R., CHAN, T.-H. & WONG, K.-Y. 2014. Rational Design of Berberine-Based FtsZ Inhibitors with Broad-Spectrum Antibacterial Activity. *PLOS ONE,* 9**,** e97514.

SUN, W., WANG, C. & ZHANG, Y. 2013. A power grid planning method considering transmission line load rate constraint and flexible cost evaluation within full planning cycle. *Dianwang Jishu/Power System Technology,* 37**,** 2985-2990.

TAN, C. M., THERIEN, A. G., LU, J., LEE, S. H., CARON, A., GILL, C. J., LEBEAU-JACOB, C., BENTON-PERDOMO, L., MONTEIRO, J. M., PEREIRA, P. M., ELSEN, N. L., WU, J., DESCHAMPS, K., PETCU, M., WONG, S., DAIGNEAULT, E., KRAMER, S., LIANG, L., MAXWELL, E., CLAVEAU, D., VAILLANCOURT, J., SKOREY, K., TAM, J., WANG, H., MEREDITH, T. C., SILLAOTS, S., WANG-JARANTOW, L., RAMTOHUL, Y., LANGLOIS, E., LANDRY, F., REID, J. C., PARTHASARATHY, G., SHARMA, S., BARYSHNIKOVA, A., LUMB, K. J., PINHO, M. G., SOISSON, S. M. & ROEMER, T. 2012. Restoring methicillin-resistant Staphylococcus aureus susceptibility to beta-lactam antibiotics. *Sci Transl Med,* 4**,** 126ra35.

TARABOLETTI, G., MICHELETTI, G., RIEPPI, M., POLI, M., TURATTO, M., ROSSI, C., BORSOTTI, P., ROCCABIANCA, P., SCANZIANI, E., NICOLETTI, M. I., BOMBARDELLI, E., MORAZZONI, P., RIVA, A. & GIAVAZZI, R. 2002. Antiangiogenic and antitumor activity of IDN 5390, a new taxane derivative. *Clinical Cancer Research,* 8**,** 1182-1188.

VAN BOECKEL, T. P., GANDRA, S., ASHOK, A., CAUDRON, Q., GRENFELL, B. T., LEVIN, S. A. & LAXMINARAYAN, R. 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases,* 14**,** 742-750.

VENTOLA, C. L. 2015. The antibiotic resistance crisis: part 1: causes and threats. *P & T : a peer-reviewed journal for formulary management,* 40**,** 277-283.

VISWANATHAN, V. K. 2014. Off-label abuse of antibiotics by bacteria. *Gut microbes,* 5**,** 3-4.

VOLLMER, W. 2006. The prokaryotic cytoskeleton: a putative target for inhibitors and antibiotics? *Applied microbiology and biotechnology,* 73**,** 37-47.

WANG, X., CHEN, G., WU, X., WANG, L., CAI, J., CHAN, E. W., CHEN, S. & ZHANG, R. 2015. Increased prevalence of carbapenem resistant Enterobacteriaceae in hospital setting due to cross-species transmission of the bla NDM-1 element and clonal spread of progenitor resistant strains. *Front Microbiol,* 6**,** 595.

WANG, X. & LUTKENHAUS, J. 1996. FtsZ ring: The eubacterial division apparatus conserved in archaebacteria. *Molecular Microbiology,* 21**,** 313-319.

WEISS, D. S. 2004. Bacterial cell division and the septal ring. *Molecular microbiology,* 54**,** 588-597.

WOODFORD, N., WAREHAM, D. W., GUERRA, B. & TEALE, C. 2013. Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae from animals and the environment: an emerging public health risk of our own making? *Journal of Antimicrobial Chemotherapy,* 69**,** 287-291.

WRIGHT, G. D. 2014. Something old, something new: revisiting natural products in antibiotic drug discovery. *Canadian Journal of Microbiology,* 60**,** 147-154.

WRIGHT, G. D. 2016. Antibiotic Adjuvants: Rescuing Antibiotics from Resistance. *Trends in Microbiology,* 24**,** 862-871.

WU, L. J. & ERRINGTON, J. 2004. Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in Bacillus subtilis. *Cell,* 117**,** 915-925.

YANG, D. C., PETERS, N. T., PARZYCH, K. R., UEHARA, T., MARKOVSKI, M. & BERNHARDT, T. G. 2011. An ATP-binding cassette transporter-like complex governs cell-wall hydrolysis at the bacterial cytokinetic ring. *Proceedings of the National Academy of Sciences,* 108**,** E1052.