Synthesis and Characterization of Caffeic Acid and Dihydrocaffeic Acid Derivatives as Antifungal Agents

by

Yuxi Chen

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

School of Pharmacy
Memorial University of Newfoundland

Abstract

Invasive fungal infection, a major cause of morbidity and mortality in immuno-compromised hosts, is of importance and is significantly increasing in incidence in recent years. Although there are a number of antifungal agents currently available, they are associated with various limitations. Being inhibitors of fungal cell wall biosynthesis, the class of echinocandins is characterized by outstanding safety profiles and great potential in combination antifungal therapy, but they fall short in oral bioavailability. A discovery of peptidomimetic analogues of echinocandin B, with potential inhibitory activity against 1,3-β-D-glucan synthase (an enzyme essential for the biosynthesis of fungal cell walls), was initiated by our research group. A series of structurally related derivatives (chlorogenic, quinic, caffeic and dihydrocaffeic acid derivatives) were later designed to mimic the backbone of echinocandins.

Further studies have been carried out on optimization of synthesis and modifications of a caffeic acid derivative. Due to the instability problems of previously reported compounds, a new molecule, a dihydrocaffeic acid derivative, has been designed and synthesized. It was found that adopting a few optimized procedures can not only improve the yield and purity of the caffeic acid derivatives, but also save a lot of time in synthesis.

Acknowledgments

Foremost, I would like to express my sincere gratitude to the late Dr. Mohsen Daneshtalab, who was my supervisor when I started my MSc program. I feel very grateful to have had him in my life for all the support and guidance he gave to me, and all the time we spent together in the lab. The promise I made to him drove me to overcome difficulties in finishing my thesis. I will always miss him and cherish the memory.

I would never have been able to finish dissertation without the help and guidance of my supervisor Dr. Lili Wang and my committee member Dr. Sunil V. Pansare. I would like to offer my deepest gratitude to Dr. Lili Wang for giving me all the help when I needed them the most and also for her continuous caring and patience. I would like to thank Dr. Sunil Pansare, who patiently answered every question I had and kindly offered me so many valuable advices. I also feel so blessed of my friendship with many students and colleagues during my graduate study. I gratefully acknowledge the support from School of Graduate Studies at Memorial University and the A. G. Hatcher Memorial Scholarship.

Most of all, I would like to thank my parents for their non-stop love and support through all these years. I would also like to thank my husband, Runlin Shi, for his love, understanding and encouragement. He was always there for me when I felt lost and stood by me through ups and downs.

Table of Contents

List of Tables

List of Figures

List of Schemes

List of Abbreviations

Ac_2O	Acetic anhydride
ACS	American Chemical Society
AmB	Amphotericin B
AUC-SROC	Area under the curve summary receiver operating characteristic
BAL	Bronchoalveolar lavage
BDG	1,3-β-D-glucan
Boc	Butyloxycarbonyl
Bp	Boiling point
C. albicans	*Candida albicans*
$CaCl_2$	Calcium chloride
$CDCl_3$	Deuterated chloroform (Chloroform-*d*)
cDNA	Complementary deoxyribonucleic acid
CD_3OD	Deuterated methanol (Methanol-d_4)
CDR	*Candida* drug resistance
CGA	Chlorogenic acid
CH_2Cl_2	Dichloromethane
CMC	1-Cyclohexyl-3-(2-morpholinoethyl) carbo-diimidemetho-p-toluenesulfonate
$(COCl)_2$	Oxalyl chloride

CO	Carbon monoxide
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
CT	Computed tomography
DMAP	4-Dimethylamino pyridine
DMF	*N,N*-Dimethylformamide
DNA	Deoxyribonucleic acid
DOR	Diagnostic odd ratio
EAT	Empirical antifungal treatment
ECIL	European Conference of Infections in Leukaemia
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EORTC/MSG	The European Organization for Research and Treatment of Cancer / Mycoses Study Group
ESI	Electrospray
FDA	Food and Drug Administration
Fmoc	Fluorenylmethyloxycarbonyl
GC	Gas chromatography
GM	Galactomannan
^{1}H NMR	Proton nuclear magnetic resonance
HCl	Hydrogen chloride
HOBt	Hydroxybenzotriazole

HPLC	High-performance liquid chromatography
HRCT	High resolution computed tomography
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IA	Invasive aspergillosis
IC	Invasive candidiasis
IC_{50}	Inhibitory concentration 50%
ICU	Intensive care unit
IDSA	Infectious Disease Society of America
IFI	Invasive fungal infection
IR	Infrared
ITS	Internally transcribed spacer
KOH	Potassium hydroxide
L-AmB	Liposomal amphotericin B
LC-MS	Liquid chromatography-mass spectrometry
MDR	Multidrug resistance
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$NHEt_2$	Diethylamine
NMR	Nuclear magnetic resonance
OD	Optical density

ODS	Octadecyl-functionalized silica
OH^-	Hydroxide anion
PCR	Polymerase chain reaction
PT	Piperacillin-tazobactam
RCT	Randomized controlled trial
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SBECD	Sulfobutyl ether β-cyclodextrin sodium
SOT	Solid organ transplant
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UDP	Uridine diphosphate
UGT	Uridine diphosphate-glucuronosyltransferase
UPLC-APCI-MS/MS	Ultra-performance liquid chromatography-atmospheric-pressure chemical ionization-tandem mass spectrometry
UV	Ultraviolet

Chapter 1. Introduction

1.1 Fungi and fungal infections

1.1.1 Fungi

Fungi are actually primitive plants on Earth and can be found in air, in soil, on plants, and in water. [1] Although there is still no body fossil evidence for the earliest organisms like fungi, biologists believe that fungi are an ancient group that must have evolved relatively early in geologic time, perhaps more than 1.5 billion years ago. [2] They are a large group of eukaryotic organisms including microorganisms such as mushrooms, yeast, mold, and mildew. Despite their completely different appearance, fungi share many features in common with human beings, which creates a unique opportunity for them to cause severe infections. Eukaryotic cells, membrane-bound organelles and a nucleus, are key components for both humans and fungi. [3] Due to their similarity to humans, certain opportunistic fungi can cause severe fungal infections that are characterized by high morbidity and mortality. Moreover, cell walls of fungi, as major components of these organisms, are different from plants, protists and bacteria. Instead of cellulose, which constitutes the cell walls of plants and some protists, chitin is the critical element of the cell walls of fungal cells.

1.1.2 Fungal infections

Among pathogenic microbes, eukaryotic pathogens, which include fungi and parasites, are receiving more and more attention from all over the world. This is due to the fact that drug resistance has been clinically observed more often than before, and fewer drugs or vaccines are available, compared to antibacterials and antivirals. [4] Fungal infections of animals, including humans, are known as mycoses, with many different environmental and physiological factors contributing to the development of fungal diseases. Some fungi reproduce by spreading microscopic spores (Figure 1.1). These spores are often present in the air, where they can be inhaled or come into contact with the surfaces of the body, primarily the skin. As a result, fungal infections usually begin in the lungs or on the skin.

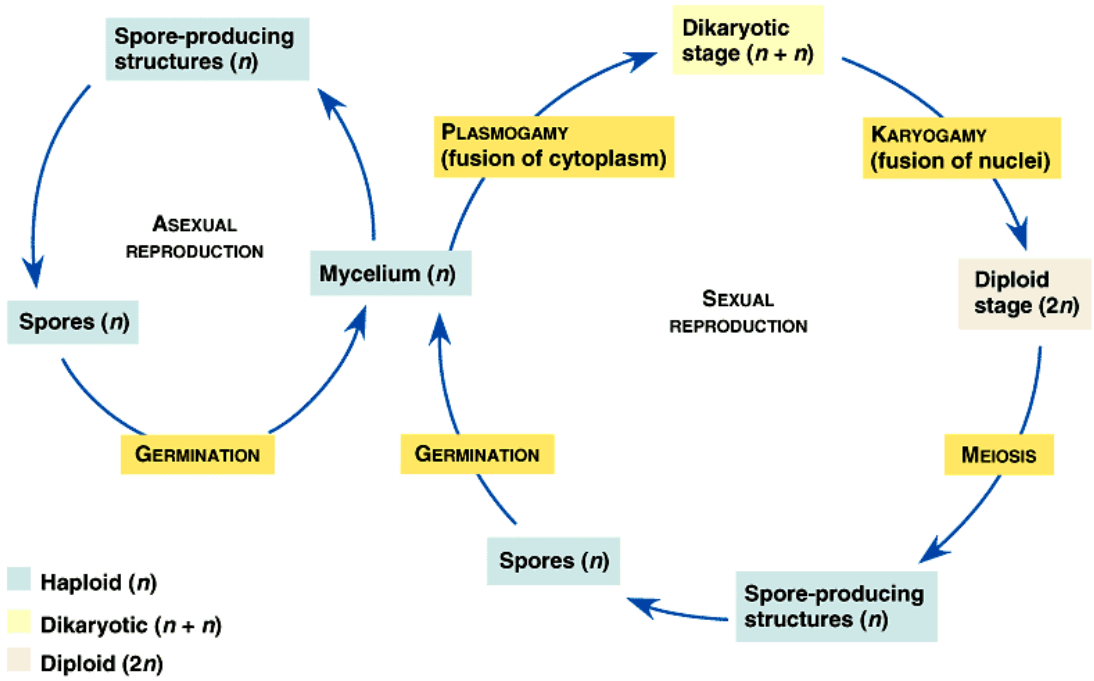


Figure 1.1. Fungal reproduction

External factors, mainly environmental conditions, can trigger genetically-determined developmental states that lead to the emergence of specialized structures for sexual or asexual reproduction. As a critical element in fungal reproduction, the specialized structures are able to disperse spores or spore-containing propagules to facilitate the process. Different species of fungi have their own characteristics in reproduction. By comparing yeasts with molds, yeasts grow as solitary cells, while molds grow in the form of multicellular filaments called hyphae. As for reproduction, yeasts usually reproduce asexually through mitosis, also known as budding. On the other hand, molds are characterized by the development of hyphae through dispersing small spores, which can be either sexual or asexual (Figure 1.1). [5] However, it is common that many fungi reproduce by using more than one method of propagation. In addition to budding yeast cells, yeasts such as *Candida albicans* (*C. albicans*) may also form pseudohyphae or even true hyphae as well.

Yet, of the wide variety of spores that land on the skin or are inhaled into the lungs, most do not cause infection, i.e., not all species of fungi would cause disease in human. Many different kinds of fungi live inside the human body in a benign equilibrium with it. When the body's immune system is weakened due to an illness or treatment such as with patients who have had hematopoietic stem cell (HSC) and solid organ transplants (SOT) or chemotherapy, the balance can be disturbed, allowing fungi to

overgrow causing disease. [6] In fact, many fungi that cause mycoses may be part of the normal body flora (e.g., *Candida*), and opportunists (e.g., *Aspergillus*), existing in the environment, infect mainly immuno-compromised people. Moreover, dimorphic organisms that are spore-forming molds in nature are able to convert into yeasts after entry into the human body, often by inhalation (e.g., *Histoplasma*). [7] It is noteworthy that dimorphic fungi can cause infections even in immunocompetent people. Statistically, the most frequent aetiological fungi are *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. The estimated annual incidence of invasive mycoses due to these pathogens is 72-228 infections per million population for *Candida* species, 30-66 infections per million population for *Cryptococcus neoformans*, and 12-34 infections per million population for *Aspergillus* species. [8,9,10,11]

Symptoms of fungal infections vary depending on the area of the body infected, which normally includes eyes, nails, skin, mouth, lung, genitals, and digestive tract. When the infection happens on the skin, itch, red rash, scaling and flaking of the skin are general symptoms. The most common symptom of onychomycose is the nail becoming thickened, discolored and maybe even bulged out or separated from the finger or toe completely. In addition, fungal infections in the lung and systemic fungal infections are characterized by cough, loss of appetite, and persistent fever of unknown cause.

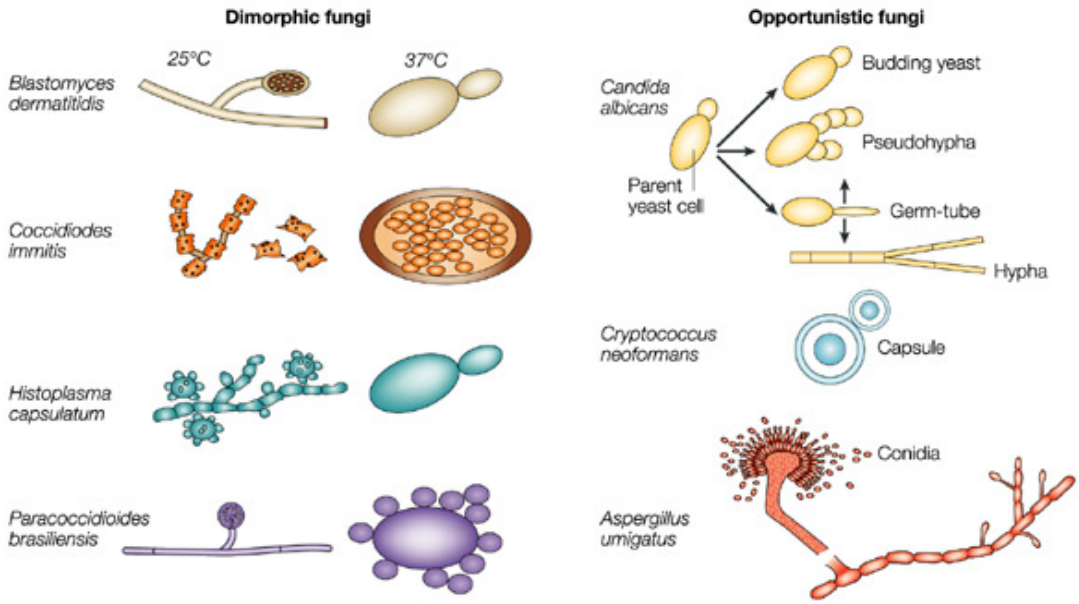


Figure 1.2. Morphogenesis in different fungal species, adapted from Romani L. [12]

As mentioned earlier, fungi do not always mean pathogenicity, since there are other factors contributing to actual fungal infections. With dimorphic fungi and some other fungal species, the transformation in growing forms plays a key role in the development of fungal infections. Due to thermotolerance, dimorphic fungi have the ability to grow at 37°C by transforming from filamentous moulds to unicellular yeasts in the host. Some opportunistic fungi can also grow in different forms depending on the infection sites, which has shown the flexibility in morphogenesis and metabolism of fungi (Figure 1.2). [12] This may have been the result of fungi evolvement in order to adapt to various environments.

However, in recent years, patient characteristics, antifungal prophylaxis, and other factors appear to have contributed to a change in the spectrum of invasive fungal pathogens. [13] Non-*albicans Candida*, Non-*fumigatus Aspergillus*, and molds other than *Aspergillus* have become more common pathogens causing invasive fungal infections (IFI). Many of these fungi were previously thought to be nonpathogenic and are now recognized as causes of invasive mycoses in immunocompromised patients. And most of these emerging fungi are resistant to or less susceptible than others to standard antifungal treatments, which makes the invasive infections caused by these previously rare fungi more difficult to diagnose and treat.

IFI is a fungal infection that can occur when fungi enter the bloodstream. For example, when *Candida* yeasts enter the bloodstream, it can cause invasive candidiasis (IC). IFI, as a major cause of morbidity and mortality in immunocompromised hosts, is of importance and has significantly increased in incidence in recent years. [14] A few reasons have been proposed for the increase in IFIs, including the use of antineoplastic and immunosuppressive agents, broad-spectrum antibiotics, and prosthetic devices and grafts, and more aggressive surgery. [15] The rate of sepsis due to fungal organisms in the United States alone increased by 207% during the period from 1979 through 2000. [16] In order to have efficient management of established IFI, the critical step is assessing the

clinical situation carefully and evaluating the risk factors that might have contributed to its emergence. [13] Based on the current situation, some approaches have been developed to manage these infections, including prevention, antifungal prophylaxis, and empirical antifungal treatment (EAT). Lately, due to the recognition of risk factors that contribute to the development of IFIs, a few alternative management strategies, such as preemptive therapy and targeted prophylaxis, have been proposed to protect those patients who are at a very high risk. The main aim of management strategies is to prevent, or promptly treat, patients at risk. [23] However, the management of IFIs currently has two major challenges: (1) In addition to difficulties in treatment, IFIs are also difficult to diagnose. The limited number and impact of current diagnostic techniques is largely responsible for the fact that many IFIs are only detected at quite a late stage. (2) The lack of reliability in diagnosis of IFIs has led to the categorization of patients according to the certainty of the diagnosis on their fungal infection, such as “possible”, “probable” or “proven” infection. The uncertainty of diagnosis together with poor prognosis have driven many researchers to come up with better antifungal strategies. [13] Several studies have investigated the risk factors associated with IFI. Some of the common risk factors for developing IFI are summarized in Figure 1.3.

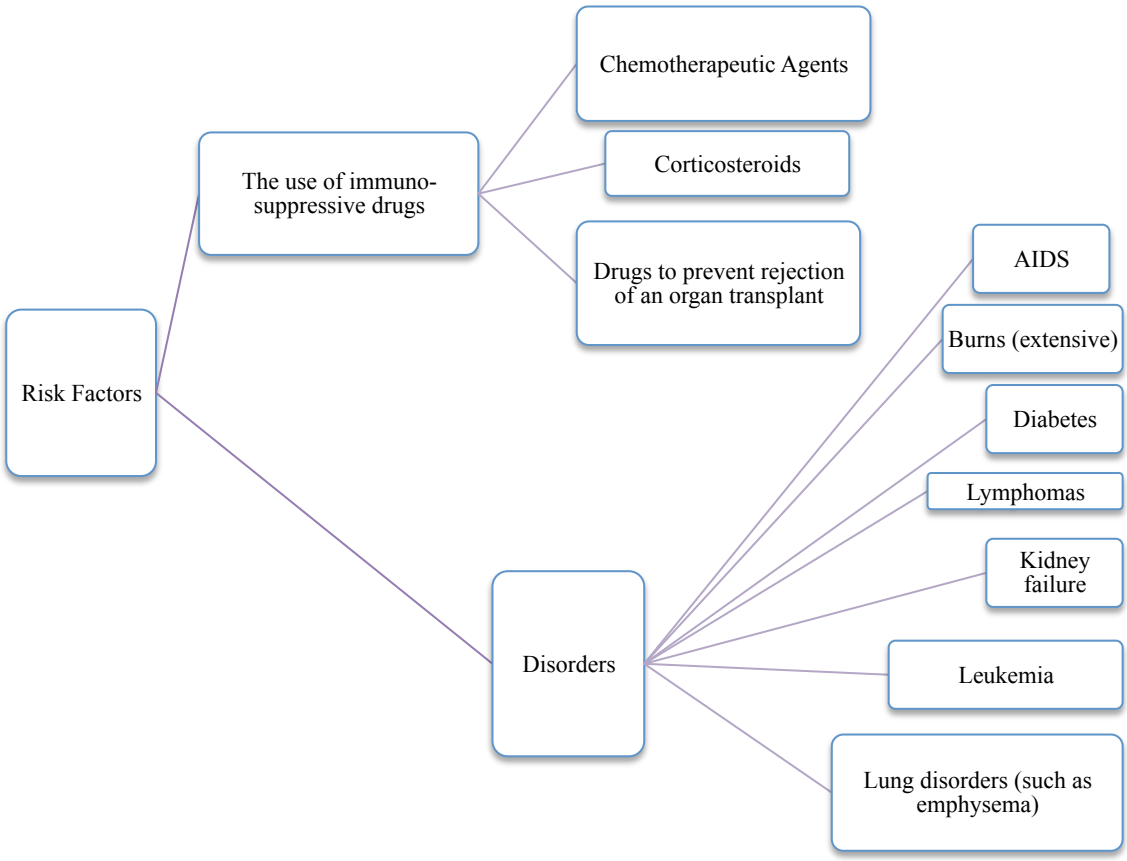


Figure 1.3. Risk factors for developing IFI

Due to the absence of standardization in the statistical analysis used in studies of risk factors for developing IFI, the results may be different from study to study. However, many studies of risk factors have revealed the trend for certain individuals to be at higher risk for acquiring IFI as a result of their underlying medical conditions. [17,18]

With regard to risk factors and patients at high risk, patients in intensive care units (ICUs) are definitely on the top of the list that needs attention. Since *Candida* species are the most common cause of IFIs in humans, IC also has long been established as a major problem in ICUs, with a ten times higher incidence in this setting than in general medical

and surgical wards. [19] The Infectious Disease Society of America (IDSA) guidelines of 2009 outlined the therapeutic response to Candidemia and also highlighted the main risk factors including the use of broad-spectrum antimicrobials, the presence of central venous catheters, use of total parenteral nutrition, neutropenia, immunosuppression and renal replacement therapy in the ICU setting. [20]

1.2 Diagnostic methods for fungal infections

Despite the emergence of new advanced techniques, traditional diagnostic tests for IFIs such as histology, microscopy and microbiological culture-based tests remain the foundation and routine methods to determine whether a subject is infected. For many superficial skin, nail and yeast infections, a clinical examination of the potentially infected person and microscopic examination of the samples (e.g. scrapings of scale, nail clippings, skin biopsy or swab from a mucosal surface) might be sufficient to determine if a fungal infection is present. In the case of IFIs, examinations of cultures of tissue, blood or other fluids are conducted to identify the presence of specific fungi. The sample collected needs to be transferred onto a medium such as Sabouraud's dextrose agar containing cycloheximide and chloramphenicol. If it is a mold that requires identification, cycloheximide is left out. Furthermore, susceptibility testing performed on fungi isolated from a culture is used to

determine which antifungal agent is the best to use for treatment. However, the conventional methods are associated with problems, such as low-yield and time-delay, which has significantly limited their impact on the management of potential fungal infection in clinical settings. [21] Growing fungi, incubated in cultures at 25-30°C, may take several weeks for results to become available.

Due to the presence of severe thrombocytopenia, aggressive procedures such as biopsy of the infected site may have to be precluded. Although the culture-based tests are inexpensive and provide valuable material upon which to perform drug-sensitivity testing, cultures only become positive at a late stage of infection and organisms may take weeks to grow in culture, which would lead to the delay of diagnosis and therapy. [22] Insisting on irrefutable evidence of deep tissue infection before any therapeutic intervention not only exposes many patients to invasive, potentially life-threatening tests but also results in an unacceptable delay of potentially life-saving therapy.

New diagnostic approaches have been developed with the knowledge of non-culture based methods, which are generally less invasive and usually provide an earlier diagnosis than morphologic evaluation by culturing. Recent advances in detecting fungal cell-wall components and genomic deoxyribonucleic acid (DNA) allow earlier diagnosis than conventional tests. [23] Among all the new advanced

diagnostic technologies, the use of biomarkers has become prevalent in recent years, since it can provide critical information in a short period of time by relatively noninvasive specimen sources beyond the routine traditional methods. Two fungal biomarkers have been extensively researched and published over the last 20 years: galactomannan (GM) and 1,3-β-D-glucan (BDG).

1.2.1 GM antigen test

GM is a polysaccharide cell-wall component that is released by the fungus during its growth, especially for *Aspergillus spp*., in serum, bronchoalveolar lavage (BAL) fluid, and other body fluids. [24] It is considered specific for aspergillosis because it contains higher levels of GM than other fungi species. The GM antigen test, which is now commercially available as a kit and marketed as the Platelia™ Aspergillus Ag (Bio-Rad), is a noninvasive technique used for the early identification of invasive aspergillosis (IA). [25] The test is an enzyme-linked immunosorbent assay (ELISA), which employs the rat monoclonal antibody EB-A2 and recognizes the (1→5)-β-D-galactofuranosyl side chains of the GM molecule, and serial tests are recommended to attain the greatest yield. Results are expressed as the ratio (index) of the optical density (OD) obtained from the patient serum sample to that of the control serum containing 1 mg of GM per mL. Compared to conventional

approaches, an obvious superiority of the GM test is time-saving, since results can be available within 3 hours. [24] A single result of GM index ≥0.8 or two continuous results of ≥0.5 should be treated as indication of IA and a full diagnostic process should be started. Furthermore, quantitative GM testing is a useful diagnostic tool, which is mainly used in high-risk haemato-oncological patients when it is combined with computed tomography (CT) imaging, in the detection of early IA. [26]

However, limitations of this test include relatively low sensitivity (50–80%) and cross-reaction with other fungi (e.g., *Penicillium* species, *Histoplasma* species, and *Blastomyces* species). [27] Due to the species-specificity of the assay, some emerging non-*Aspergillus* mycoses cannot be detected by this method. It is also suggested in some studies that the GM assay has a limited impact on SOT recipients, due to relatively poor sensitivity and specificity, compared to hematology-oncology patients. [28] However, it does not seem to make sense by comparing these two groups of patient populations in clinical settings. In addition, certain penicillin antibiotics have been reported to produce a false-positive GM test, likely because they are derived from *Penicillium* species. [29] Among these antibiotics, piperacillin-tazobactam (PT) (a widely prescribed drug combination in patients with cancer who have febrile neutropenia) was one of the antibiotic combinations identified as causing false positive results, which has been reported by several authors. [30,31,32,33] Since the rat

monoclonal antibody has also been shown to recognize constituents of molds from other genera, such as *Penicillium* species, the antibiotics that are semisynthetic penicillins, including piperacillin, tazobactam, and amoxicillin, may be able to cross-react with the Platelia *Aspergillus* GM test. [34] As a result, it has been indicated that maybe GM is carried through the production process of this antibiotic into batches designed for therapeutic use. Another study indicates that a positive Platelia *Aspergillus* test with PT is likely due to the presence of GM in PT rather than to a cross-reaction with the antibiotic. [35] Some food products, including hospital meals, canned vegetables, pasta, and cereals, which may be contaminated with GM, can also be one of the sources of false-positive results. Also, damage to the intestinal mucosa could allow ingested GM to enter the bloodstream. [36] The occurrence of false-positive GM assay results due to treatment with PT is a matter of concern because it may lead to unnecessary CT scans, undesirable (semi)-invasive investigations, over-treatment with expensive antifungal drugs that may produce toxicity and undesirable side effects. However, for all these years, opinions are divided on this point. Some studies indicated that they did not find any association between PT and GM positivity. [37,38,39] In a previous study from Turkey, 28 of 43 sera (65.1%) were positive in patients receiving concomitant PT in the absence of IA. [34] While another institution from Turkey analyzed the false positivity of GM due to PT treatment with the

same method during the same period, surprisingly only 5 of 40 non-IA febrile episodes tested positive (false positive rate 12.5%). [40] The conflicting results appeared to undermine the previous hypothesis regarding PT to be concerned as one of the major causes of false positivity. It was also reported in one study that they tested several PT batches and found that their GM content was reduced over the time of study. As a result, the authors speculated about the possibility of a modification in the manufacturing process to have occurred over time, resulting in better purity and less GM contamination. [41] In the same study, [41] besides the variable GM contamination in PT batches, their results also showed that the risk of false-positive GM tests can be minimized by performing blood sampling before PT administration and by using separate tubes of the central venous line for blood sampling and for administration of antibiotics. Some studies also come to the conclusion that the false positivity rate of GM antigen testing may be decreased by serial sampling by taking into account at least two consecutively positive values and by using a higher cut-off level (0.7 instead of 0.5). [34,38,42]

1.2.2 BDG detection assay

BDG is a major cell wall polysaccharide that can be found in most fungi, with the exception of the *Cryptococci*, the *zygomycetes*, and *Blastomyces dermatitidis*, which either lack the glucan entirely or produce

it at minimal levels. [22] BDGs are 1,3-β-D-glucopyranosyl polymers with randomly dispersed single β-D-glucopyranosyl units held by 1,6-β linkages, giving a comb-like structure. Although being common in fungi, yeasts, algae and bacteria, BDG cannot be found in prokaryotes and viruses, as well as human cells. [43] Many studies indicated that glucans are released from fungal cell walls into the systemic circulation of patients with proven or probable IFIs, no matter if they were receiving antifungal medications. Therefore, the BDG's presence in blood or other normally sterile body fluids may be a good serological marker of systemic fungal infection. [21] Many BDG detection assays have been developed in the past two decades and some of them are commercially available: Fungitell (Associates of Cape Code, Inc., East Falmouth, MA, USA), Wako (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), Fungitec-G (Seikagaku, Kogyo, Tokyo, Japan), and Maruha (Maruha-Nichiro, Foods Inc., Tokyo, Japan). However, the Fungitell assay is the only one that has been approved by the Food and Drug Administration (FDA) in 2003 for the diagnosis of invasive mycoses in serum in the United States. [44] This assay is a chromogenic, quantitative enzyme immunoassay (EIA) designed to detect BDG by using purified, lysed horseshoe crab (*Limulus polyphemus*) amebocytes. These cells contain components of the *Limulus* clotting cascade, including Factors C and G, which initiate coagulation in the presence of bacterial liposaccharide and BDG, respectively. By

eliminating Factor C from the lysate, the activation of the cascade has been limited to BDG alone, which assures the specificity of the assay. [45]

The use of BDG assay is included in the revised IFI diagnosis criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/ MSG) criteria. According to the new edition, a positive BDG result can be treated as mycological evidence of infection meeting their criteria. [46] However, no guideline has been provided to standardize the use of BDG assays and also its analysis. As a consequence, inconsistency was found in results from various studies while comparing data, because different patient populations, cutoff values, and sites of infections (e.g. deep-seated colonization vs. fungal superficial colonization) [47] have been taken into account in studies of BDG and other fungal biomarkers. [48]

It has been reported in a meta-analysis of 15 studies assessing the overall accuracy of BDG in the diagnosis of IFI that BDG assays have a high specificity in patients with hematologic disorders but relatively low performance in SOT recipients. [49] Although the number of measurements required for defining a positive BDG test result has not been standardized yet, compared to a single positive result, according to the same study, [49] requiring two consecutive BDG results reduced the overall sensitivity value, but enhanced the overall specificity value.

In a study about the kinetics of BDG in persistently neutropenic rabbits with IA, the results suggested that both cell wall components (GM and BDG) appeared to be comparable and provided similar information regarding the extent and stage of infection. For the models with invasive pulmonary aspergillosis, concentrations of both biomarkers begin to rise within the first 24 h of infection, well before pulmonary infarction is evident, supporting the concept that these molecules, when circulating, signal invasive disease almost immediately after host defenses are breached. [50] Another intriguing meta-analysis, which included six cohort studies, reported a diagnostic odds ratio (DOR) of 111.8 versus 16.3 for the presence of IFIs in neutropenic hemato-oncological patients following two consecutively positive BDG assays compared to a single positive BDG assay. [51] Both the analysis of the kinetics of BDG levels and DOR results in the meta-analysis suggest that at least two sequential positive BDG results are required for indirect mycological criteria to allow possible invasive fungal diseases to be upgraded to probable invasive fungal diseases and also for higher diagnostic performance and better homogeneity of test results.

Recently, a meta-analysis, which covered 28 individual studies, provided a new direction in this area, i.e., assessing the diagnostic accuracy of serum BDG for IFIs on different cutoff values. The cutoff value of 60 pg/mL has the best diagnostic accuracy according to the

EORTC/MSG criteria, and it also shows the best diagnostic accuracy with the Fungitell assay compared to other BDG detection assays. [44] The area under the curve of the summary receiver operating characteristic (AUC-SROC) analysis has been recommended to represent the performance of a diagnostic test, based on data from a meta-analysis. Therefore, sensitivity, specificity, DOR and AUC-SROC were estimated in this study, based on different cutoff values. [44] When the AUC-SROC is between 0.80 and 0.90, it is usually considered as a good diagnostic test accuracy, and a value >0.90 indicates a very high overall diagnostic accuracy of the test. In their general analysis, patients who had proven or probable IFIs were compared with patients who did not have IFIs for serum BDG measurement with the AUC-SROC being 0.8855. It suggested that the BDG assay had a good diagnostic accuracy for the diagnosis of IFIs. When it comes to the diagnostic accuracy of different cutoff values of BDG, the analysis of cohort studies indicated that the cutoff value of BDG at 80 pg/mL had the best diagnostic accuracy (with an AUC-SROC of 0.9284), whereas the case-control studies indicated that the cutoff value of 20 pg/mL had the best diagnostic accuracy (with an AUC-SROC of 0.9943). When using the EORTC/MSG criteria as a reference standard, the cutoff value of 60 pg/mL has the best diagnostic accuracy, with an AUC-SROC of 0.8804. [44]

Being one of the useful noninvasive diagnostic tools, a key advantage of the BDG assay is the required specimen source being serum, which is readily available and easily accessible, regardless of patient conditions. However, similar to GM assays, BDG assays also suffer from false positivity, which may result from: glucan-contaminated blood-collection tubes and surgical gauze dressings; dialysis with cellulose membranes; bacteremia due to several gram-positive organisms, particularly *Streptococcus pneumoniae*; use of products with cellulose depth filters such as albumin; gut inflammation; and use of antibiotics such as amoxicillin-clavulanate. [52,53,54]

Although it did not improve the sensitivity of each test, the combination of BDG and GM detection was very useful in confirming the existence of IA. Furthermore, the use of both assays could be useful to identify the false positive results as well, due to discrepancies in positivity of each test. [49] A number of groups have reported that among critically ill patients with proven or probable IFIs, many developed detectable BDG antigenemia prior to the onset of clinical symptoms or radiologic signs or the return of positive culture results. The percentages of patients in whom this occurs vary between studies (64 to 87%), as do the numbers of days between BDG and culture (blood, biopsy, or BAL fluid) positivity (1 to 10 days). [43,55,56,57,58] However, positive results from the tests still need verification in the course of systematic monitoring of consecutive serum

samples to exclude false-positive results and warrant the use of other diagnostic methods, for example radiological studies, including high resolution CT (HRCT). In patients with proven IA, the BDG antigen level corresponded with the response to the antifungal therapy. In patients with regression of the illness, a decrease in serum BDG was noted but continuously high levels of antigen were observed in patients not responding to therapy, so tracking quantitative values following initiation of antifungal therapy may be used as a prognostic marker for patient response. [43]

Although biomarkers are prevalent clinically in the care of high-risk patients with suspected IFI, biomarkers fall short in many respects. As mentioned earlier, GM assays have a high sensitivity among patients with hematologic malignancies (72%) and among bone marrow transplant recipients (82%), but it is decreased among SOT recipients (22%). [28] It also suffers from false-positive or false-negative results, because certain antibiotics, fungal infection due to *Histoplasma* species, and even the ingestion of certain foods may increase GM levels, whereas antifungal therapy may rapidly decrease GM levels. Meanwhile, BDG is a helpful serological marker of systemic fungal infection, but it is nonspecific, since it can be found in many different fungal cell walls, such as those of *Fusarium* species, *Acremonium* species, or *Pneumocystis jirovecii*, and even certain foods. In addition, biomarkers can neither detect all fungal

pathogens nor identify to the species level and sometimes cannot even reliably identify organisms to the genus level. Therefore, biomarker assays (both GM and BDG) are recommended to be used together with other diagnostic tools as corroborative evidence.

1.2.3 Polymerase chain reaction (PCR) assay

The PCR method for DNA amplification was developed by Kary Mullis and colleagues in 1984 and was rapidly adapted to detect a variety of infectious agents, particularly viruses. [59] Along with the progress in PCR in recent years, it has become more popular in detecting fungal pathogens in human infections. Clinical samples such as blood and BAL fluid often contain only a small number of fungal cells, which may lead to a low sensitivity in other routine assays. However, PCR can be used to amplify the number of fungal DNA in samples providing better performance in other diagnostic tests. In order to achieve the amount for PCR-based fungal diagnostic assays, extraction methods need to be applied to lyse all available fungal cells to maximize DNA quantity. [60] Appropriate selection of the target gene is of great importance because of the opportunity it can provide to increase the sensitivity of a PCR assay. The detection limits of PCR assays can be a few gene copies per reaction, providing the ability to detect a fraction of an organism when targeting genes present in multiple copies per fungal genome. [61] In fact, targeting

multicopy genes has the advantage of increasing their sensitivity, compared with single-copy genes. The common multicopy targets are the 18S ribosomal ribonucleic acid (rRNA) and 28S rRNA genes, the internally transcribed spacer (ITS) regions, and mitochondrial gene regions.

The key for the PCR process is a uniquely synthesized pair of oligonucleotide primers that flank and define the DNA segment of interest. The highly conserved regions of the fungal rRNA gene are used to "prime" the synthesis of the remainder of the gene. With the fungal DNA extracted from the fungi or clinical sample, a DNA polymerase is added to react with the target DNA strand to which the primer has bound as a template to synthesize a complementary strand of DNA (cDNA). [62] The source or product of amplification or replication is also known as an amplicon. There are several different approaches for detection and analysis of the targeted amplicon. First of all, the most classical approach for amplicon detection is agarose gel electrophoresis, which is capable of separating amplicons according to their specific length. However, due to the inability to differentiate amplicons with identical or similar lengths, this procedure has a deficiency in specificity. [62] In order to get better interpretation of data, oligonucleotide probes are specifically designed to bind complementary sequences in the target DNA, showing characteristic dye chemistries, including fluorescence resonance energy transfer,

hydrolysis, molecular beacon, and Scorpion systems. The amplified products are hybridized to DNA probes specific for each organism, allowing for a greater degree of specificity than the use of primers alone. [63] Another method to identify fungal species is amplicon sequencing, when amplicon contains species-specific base pairs. Even if only minor differences exist, one base pair between different species are often sufficient for identification. After sequencing, comparative analysis of the nucleotide sequence is performed to find a match in public databases. [64]

Among all these approaches, real-time PCR has become a research hot topic in recent years. Real-time PCR technology combines nucleic acid target amplification using standard PCR chemistry and detection using fluorescent probes in a simultaneous, single-well reaction. [65] Compared to conventional PCR testing, real-time PCR assay can decrease time for testing by eliminating the necessity to perform post-amplification processing and detection. Since the real-time PCR procedure can be completed in a single, closed-reaction vessel, it can reduce the possibility of environmental contamination with amplified nucleic acids, which is particularly important for fungal diagnostics. [66] It has been reported that *Aspergillus* species DNA can be detected by PCR-based assays of blood for a mean duration of 14 days before the clinical diagnosis of IA. [67] PCR positivity can also precede positive GM assay results (median, 17 days; range, 4–63 days) or positive CT findings (median, 8 days; range,

1–84 days), which shows the outstanding performance of PCR-based assays in the ability of providing earlier diagnosis. [68]

Due to many different reasons and risk factors, the epidemiology of fungal infections is rapidly evolving, and non-*Aspergillus*, non-*Candida* species and other rare fungi have emerged as major opportunistic pathogens with even more difficulties in diagnosis and treatment. In a recent study with 51 patients involved, a considerable number of fungi (8 of 18, 44.4%) other than major ones such as *Aspergillus* and *Candida* species were identified by PCR, when reliable serological diagnostic methods are generally absent for these organisms. [69] Therefore, DNA analysis might be the only diagnostic tool when no information can be gained from the culture study, which not only indicates the importance of PCR technology but also attracts our attention to the further development of DNA analysis.

Nevertheless, there are still some major shortcomings in PCR-based assays that might hinder the implementation of its clinical utility. First, there is lack of standardization for the use of PCR-based assays and the EORTC/MSG did not include PCR tests in the diagnostic criteria of probable IFI. As a result, different laboratories use different primer pairs, various interpretations of positive results and a wide range of amplification protocols. [61,64,70] Second, the impact of PCR-based assays on clinical decisions is limited by the non-negligible percentage of false-

positive results due to colonization or contamination of the sample. [59] The factors contributing towards false-positivity can be divided into procedural false positivity and clinical false positivity. Procedural false positivity can be caused by contamination from the environment, previously amplified PCR products, or cross-reactivity of PCR primers and probes with non-target fungi or other organisms. On the other hand, clinical false positivity could be caused by colonization of human tissue surfaces with fungi. Whereas it is not a reflection of inadequacies in assay design or protocol, since the assay has detected something that it was designed to do. [65,71] To avoid or at least reduce the possibility of false positivity, more attention should be paid on adopting rigorous quality control measures, optimizing DNA extraction methods and PCR platforms.

1.3 Antifungal agents

Although the number of agents available to treat fungal diseases has increased by 30% since 2000, the medications that have been approved for clinical use are still less than 20. Depending on the modes of action, current antifungal agents fall into the following three categories, polyenes, azoles and echinocandins.

1.3.1 Polyenes

As the principal member of the polyene class of antifungals, amphotericin B (AmB), **1**, deoxycholate is a broad-spectrum antifungal agent (the structure of AmB is shown in Figure 1.4). AmB has been used as the standard treatment for IFIs for more than 30 years [13] by acting on ergosterol of the fungal cell membrane. Since ergosterol is the main sterol of the fungal cell, by forming a complex with ergosterol, polyenes can thereby perturb the fungal plasma membrane function, which can result in increased membrane permeability, leakage of the cytoplasmic contents and ultimately death of the fungal cell. [24] The antifungal selectivity of AmB is based on the conformational difference between the fungal ergosterol molecule and the mammalian cholesterol. Ergosterol has a cylindrical three-dimensional structure, while cholesterol has a sigmoidal shape. [72,73] However, the selectivity is low, which leads to toxicities for mammalian cells, particularly causing nephrotoxicity. Moreover, AmB has also been associated with other adverse effects including low blood potassium, infusion-related acute reactions (such as chills, fever, hypoxaemia, and hypotension), especially when administered over a short period of time. [74]

In an effort to reduce AmB toxicity, a variety of its lipid preparations have been introduced, including AmB lipid complex, AmB colloidal dispersion and liposomal AmB (L-AmB). [75] After enclosing the

amphotericin molecule within a liposome or other lipid carrier, to release free amphotericin, it needs to be dissociated from the AmB-lipid complex within the body. Therefore, it may reduce the time of exposure of amphotericin resulting in diversion from potentially vulnerable tissues, most importantly, the kidney. [76] In a systematic review of the literature, studies showed that lipid-based formulations significantly reduced all-cause mortality risk by about 28 %, compared with conventional AmB. [77] It also has been reported that the use of lipid AmB (versus AmB deoxycholate) can provide better outcomes and clinical benefits in SOT recipients with cryptococcal meningitis. [78] So far, despite the higher costs, for the treatment of many IFIs, an AmB lipid formulation seems to provide a safer alternative than AmB deoxycholate, with at least equivalent efficacy. However, due to the limited data available regarding therapeutic equivalency between lipid AmB and conventional AmB, more well designed, randomized trials are needed to compare these formulations in different clinical settings. [79]

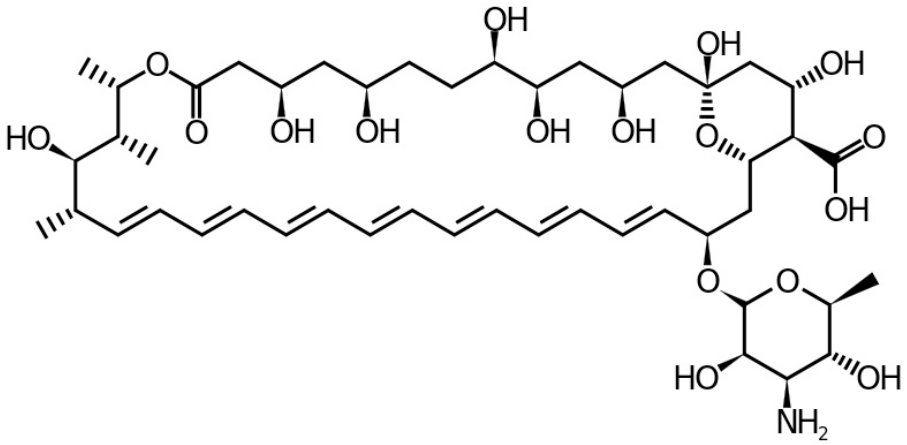


Figure 1.4. Structure of amphotericin B (AmB) **1**.

1.3.2 Azoles

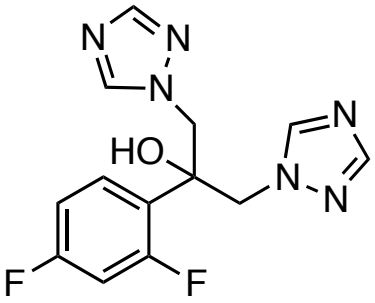
While the use of AmB has been limited by its side effects, the arrival of azoles such as fluconazole and itraconazole brought a major advance for the treatment of fungal infections. Since it was first introduced for clinical use in the early 1980s, the azole class of antifungal agents has rapidly become the most widely used in antifungal therapy and prophylaxis. [80] Similar to the polyenes, azoles also take effect on ergosterol, although via a different mechanism. In addition to being the major component of fungal cell membrane, ergosterol participates in the regulation of membrane fluidity, asymmetry and integrity, as well as stimulating fungal growth and proliferation. [81] The primary molecular targets of azoles are the P450 enzymes that catalyze the oxidative removal of the 14α-methyl group of lanosterol. [82] Consequently, the main effect of azoles is to inhibit 14α-demethylation of lanosterol in the ergosterol biosynthetic pathway, resulting in a decreased ergosterol synthesis and a concomitant accumulation of 14-methylated sterols, which changes the permeability and fluidity of the fungal membrane. [83] Meanwhile, due to the fact that the same P450 enzymes are responsible for production of cholesterol in mammalian liver cells, azoles are also able to block this biosynthesis, which is directly associated with their adverse effects. [84] On the other hand, since one of the 14-methylated intermediates of lanosterol, 14-methyl-ergosta-8,24(28)-dien-3,6-diol, is toxic, some

authors have suggested that the accumulation of toxic intermediates may have played the key role for azoles to inhibit fungal growth. [85]

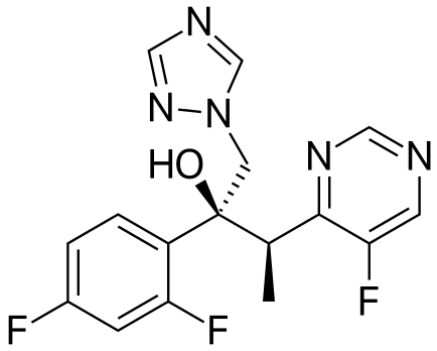
The first generation of antifungals in this class was the N-substituted imidazoles introduced in the late 1960s. Over the last few decades, the imidazole has been replaced by a triazole nucleus as the active pharmacophore, to enhance the specificity of coupling with fungal P450 enzymes, which leads to the introduction of triazole antifungals like fluconazole, itraconazole and the recent triazole derivatives, such as voriconazole and posaconazole. [83] After being metabolized by CYP450 enzymes in the liver, the inactive metabolites of azoles are eliminated via the urinary and gastro-intestinal tracts. Due to this metabolic pathway, there is a long list of drug-drug interactions, including the use of benzodiazepines, prednisolone, methyl-prednisolone, digoxin, phenytoin, carbamazepine, cyclosporine, rifampin, and other drugs in common use, which can cause problems for physicians in treating critically ill patients who receive multiple medications. [81,86,87,88]

Resistance to azoles has already been observed and well documented. [24] A large number of studies have been performed on the mechanisms of antifungal resistance. As for *Candida* species, there are four principal mechanisms suggested with respect to azole resistance. [89,90] Firstly, many of the studies indicated the induction of efflux pumps, which leads to decreased drug concentrations at the target within the fungal cell. [89]

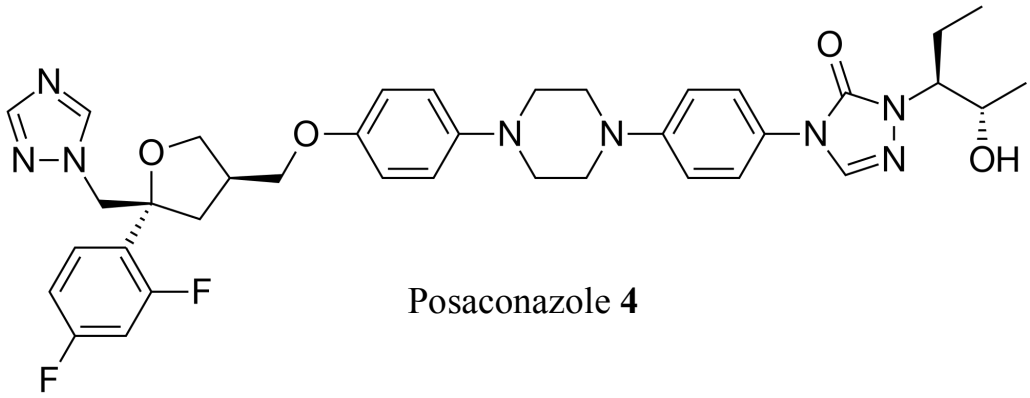
Upregulation of efflux pumps encoded by either multidrug resistance (*MDR*) or *Candida* drug resistance (*CDR*) genes is usually associated with drug resistance with some differences according to species. [91] In addition, point mutations in the gene (*ERG11*) encoding for the target enzyme lanosterol C14α-demethylase lead to an altered target with decreased affinity or incapacity for azoles to bind to the enzymatic site. [92] Thus, overexpression of the mutated gene or upregulation of the altered target enzyme can also become a cause of resistance, which results in higher levels of azole cellular targets that cannot be bound by therapeutic concentrations of azole antifungals. [89] As the last potential mechanism of azole resistance, the development of an alternative pathway of sterol synthesis can be caused by another mutation on the *ERG3* gene, which leads to replacement of ergosterol with 14α-methylfecosterol. [93] The unusual accumulation of ergosta-7,22-dienol-3β-ol in azole-resistant *C. albicans* clinical isolates allows the cells to bypass the synthesis of the toxic sterol and thereby makes the effects of azoles on the ergosterol biosynthetic pathway negated. [94] Due to the *ERG3* mutation, the fungal cell membrane that cannot be affected by azole does not contain ergosterol, and this absence of ergosterol provides a mechanism for cross-resistance to polyene antifungals. [95] Fluconazole **2**, voriconazole **3** and posaconazole **4** are important members in the azole class and their structures are illustrated in Figure 1.5.



Fluconazole **2**



Voriconazole **3**



Posaconazole **4**

Figure 1.5. Structures of Fluconazole **2**, Voriconazole **3** and Posaconazole **4**.

Fluconazole (Diflucan®)

Although some newer antifungal agents in the azole class have been introduced to clinical use, fluconazole still remains one of the most frequently prescribed triazole antifungal agents because of its great bioavailability and low toxicity, combined with low cost and availability as both intravenous and oral formulations. [96] Among azole antifungals, fluconazole is the most frequently used agent for prophylaxis and EAT, which is appropriate for patients with low risk for mold pathogens (e.g. *Aspergillus* species). [97]

The oral bioavailability of fluconazole is generally good with more than 80% of ingested drug being found in the circulation. Moreover, fluconazole is also characterized as the only azole to achieve therapeutic levels in urine, with 60% to 70% of the drug being excreted unchanged in the urine. [98] For many antifungal agents, it is difficult for them to penetrate the blood-brain barrier and achieve therapeutic cerebrospinal fluid (CSF) concentrations, since they are relatively large molecules. Noteworthily, fluconazole presents a relatively large volume of distribution with good penetration into various tissues including penetration into the CSF, which enables it to achieve good levels in the brain and CSF. [99,100] However, although fluconazole is active against most *Candida* spp., it is inactive against *Candida krusei* and *Candida glabrata* isolates. [101] Fluconazole also shows no activity against *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., or the Zygomycetes, which greatly limits its clinical use. [102]

Voriconazole (Pfizer®)

Voriconazole is a second-generation azole antifungal agent and a synthetic derivative of fluconazole with broadened antifungal spectrum. Among all of the second-generation triazoles, voriconazole was the first to be approved by the FDA for the treatment of IA, serious infections caused by *Fusarium* and *Scedosporium apiospermum*, and fluconazole-resistant invasive *Candida* infections. [103] By adding an α-methyl group

and replacing one of the triazole moieties with fluorinated pyrimidine, voriconazole exhibits a broader spectrum of activity when compared to fluconazole. [104] Voriconazole is active against *Aspergillus* spp., *Fusarium* spp. and all *Candida* species (including the fluconazole resistant or less susceptible spp. of *C. glabrata* and *C. krusei*), except Zygomycetes. [105]

Similar to fluconazole, voriconazole is available as oral and intravenous forms and the latter form depends on sulfobutyl ether β–cyclodextrin sodium (SBECD) for solubility. Since SBECD is excreted via the kidneys, where accumulation of SBECD could occur, dose adaptation is required in cases of renal impairment. [106] The oral bioavailability of voriconazole is more than 90% when the stomach is empty, but it decreases when food is present. Fatty foods have been found to reduce bioavailability by 80%. [107,108] Voriconazole is distributed widely in humans with a reasonable penetration of the blood-brain barrier and CSF levels being around 50% of plasma levels. [109] Due to the fact that the efficiency and distribution of a drug may be affected by the degree to which it binds to the proteins within blood plasma, it is a very important factor in determining the amount of active drug present at a given site of infection. As for voriconazole, it is 58% protein bound and has a large volume of distribution, while polyene agents and many other azole antifungals are highly protein bound (>90%). [104]

Voriconazole undergoes extensive hepatic metabolism via the CYP450 enzymes, primarily by CYP2C19 and to a lesser extent by CYP2C9 and CYP3A4. [110,111] Several metabolites are excreted in urine, with <2% being unchanged. Unfortunately, none of the voriconazole metabolites exhibit antifungal activity. [112] Due to the metabolic pathway by CYP450 isoenzymes, the potential for drug-drug interactions with voriconazole is high. Specifically, inducers of CYP450, such as rifampin, long-acting barbiturates, and carbamazepine, can cause decreased voriconazole concentrations, so that concurrent use of these drugs with voriconazole should be avoided. On the other hand, voriconazole can also interfere with the metabolism of several other drugs through inhibition of either the CYP3A4 or the CYP2C9 pathway, and coadministration can lead to toxic levels of those common drugs. [113]

Although voriconazole is generally well tolerated, there is a common side effect associated with the use of voriconazole, being not previously noted with other azoles, a reversible disturbance of vision (photopsia). This side effect occurs in about 30% of patients but rarely leads to discontinuation of the drug, since the visual effect is transient and no permanent damage to the retina has been noted. [114,115] Other well-known adverse effects of voriconazole include skin rash (phototoxicity-related rash sometimes) and transaminase elevation, which are all reversible after discontinuation of the therapy. [116,117] Despite the side effects it may

suffer from, voriconazole has become the first choice of antifungal agents with regard to IA in most of the cases. [80,81] According to a multicenter, randomized, comparative study of IA with great significance, voriconazole was superior to AmB in efficacy (53% vs. 32% response) and resulted in improved 12-week survival (71% vs. 58%), which makes voriconazole the primary treatment choice for IA and oral voriconazole is preferred for 'step-down' or maintenance therapy. [114]

Posaconazole (Noxafil®)

Being approved by the FDA in 2006, posaconazole is characterized as a lipophilic second-generation of the antifungal triazole with a broad spectrum of activity. [118] Since posaconazole has a low solubility in aqueous and acidic media, no intravenous formulation is available. To determine what oral formulation would provide better bioavailability, a suspension of posaconazole was compared with the tablet formulation. [119] It turned out that being administered as a cherry-flavored 40-mg/mL suspension using polysorbate 80 as an excipient helps lower sedimentation of solid particles because of the greater surface area and enhanced gastric mixing of the drug. [120] Its oral bioavailability also varies depending on dosage regimen and food intake, which is potentially problematic for clinical use. Unlike the high bioavailability of voriconazole with an empty stomach, food intake significantly increases the oral bioavailability

of posaconazole, with either a nonfat or high-fat meal (168% and 290%, respectively) or with a nutritional supplement. [121,122]

Posaconazole has a large volume of distribution after oral administration suggesting extensive extravascular distribution and penetration into intracellular spaces. [123] In general, posaconazole primarily circulates in plasma and then is widely distributed to the tissues and is slowly eliminated. However, posaconazole is highly protein bound (>98%), predominantly to albumin and binding is concentration independent. As a result, the penetration of posaconazole into CSF was found to be poor. [124] Although posaconazole is eliminated primarily unchanged, its main metabolites come from glucuronidation produced through Phase II biotransformations via uridine diphosphate (UDP) - glucuronosyltransferase (UGT) enzyme pathways. [125] According to a study of eight healthy male subjects, elimination of posaconazole occurred primarily through fecal excretion (79.6%), mainly as unchanged, and to a lesser extent through the urinary route (14%). [126] Since posaconazole is metabolized by the liver and mainly excreted in the faeces, the limited renal elimination allows for the use of posaconazole without dosage adjustment in patients with renal impairment, which makes it an appropriate alternative to AmB for patients with renal dysfunction. [124,127] Unlike voriconazole, which is a substrate and an inhibitor of CYP3A4, CYP2C9 and CYP2C19, posaconazole is not

significantly metabolized through the CYP450 enzyme system. [113,128] However, posaconazole has been found to greatly decrease the hepatic activity of CYP3A4, which can inhibit the clearance of drugs metabolized by CYP3A4 resulting in increased plasma concentrations of these drugs. [129] Depending on the chemical structure of the substrate and the CYP isoforms involved, the degree of inhibition varies. [130] For instance, coadministration of posaconazole with some of the CYP3A4 substrates is contraindicated due to the risk of corrected QT interval (the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle) prolongation, while for some other CYP3A4 substrates, the doses should be reduced to counteract the increased exposure and the whole-blood concentrations of these agents should be carefully monitored. [131]

Overall, posaconazole appears to be well tolerated in clinical trials so far and rarely requires discontinuation because of adverse effects. The most frequently reported side effects have been associated with gastrointestinal disturbances (14-18%, nausea, vomiting, abdominal pain, diarrhoea, etc.) and hepatic toxicities (3%, transaminase elevation and hyperbilirubinemia). [81,123,132] In a long-term safety study, 428 patients with refractory IFIs (n = 362) or febrile neutropenia (n = 66) who received posaconazole, 38% of the patients reported treatment-related adverse effects. It is noteworthy that among all of the 428 patients, 109 of them received posaconazole therapy for more than six months and no severe

side effect was observed, suggesting that posaconazole is well tolerated for long-term use. [133]

As the first-line choice for prophylaxis of invasive *Aspergillus* and *Candida* infections in severely immunocompromised patients, posaconazole has been proven to be effective by several studies. In a double-blind multicenter study, 600 allogeneic HSC recipients with graft-versus-host disease were enrolled. Based on the situation that patients had received intensive immunosuppressive therapy, posaconazole was compared with fluconazole for prophylaxis of invasive mycoses. [134] It was shown that posaconazole was as efficacious as fluconazole in preventing all IFIs (5.3% and 9.0%, respectively; P=0.07), and was superior to fluconazole in preventing IA with significantly lower incidence in the posaconazole group (2.3% vs. 7.0%; P=0.006) at the end of the 112-day treatment period. Posaconazole was also found to be superior to fluconazole in preventing breakthrough IFIs (2.4% vs. 7.6%, P=0.004), particularly in cases of IA (1.0% vs. 5.9%, P = 0.001). Although there was no significant difference in overall mortality, a significant difference was observed in the number of deaths from IFIs, which was much lower in the posaconazole arm than fluconazole arm (1% vs. 4%, P=0.046). Another multicenter, open-label study evaluated posaconazole oral suspension as salvage therapy for IA, which included 107 posaconazole recipients and 86 control subjects (control subjects

could not receive any antifungal agents as standard therapy that were investigational at the time of their treatment, i.e., voriconazole or an echinocandin). [135] Patients were enrolled to receive posaconazole for salvage treatment if they were confirmed to have a diagnosis of IA, and if they were refractory to at least seven days of antifungal therapy or were intolerant of conventional therapy. The overall success rate was 42% (45 of 107) for posaconazole recipients and 26% (22 of 86) for control subjects (P=0.006). The results also showed that posaconazole appeared to provide better survival rates, since in the arm of posaconazole the cumulative rates of survival at 30 days and at the end of therapy were 74% and 38%, respectively; for control subjects, those survival rates were 49% and 22%, respectively. Based on the favorable results in both efficacy and safety, the current dosage regimen for posaconazole is 200 mg three times daily for prophylaxis, and 800 mg divided in two or four doses in the salvage setting.

1.3.3 Echinocandins

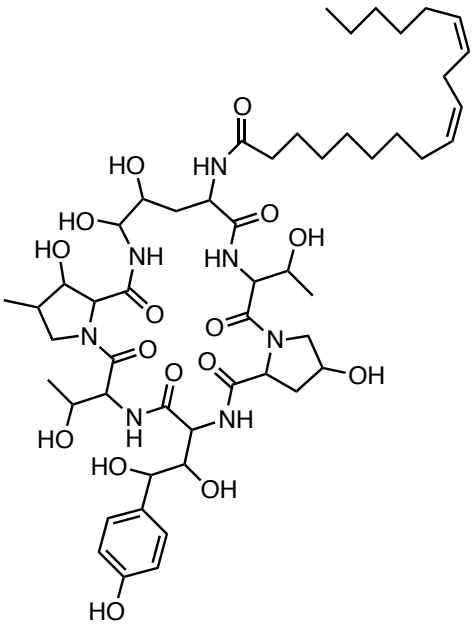
The changing pattern of fungal infections and increasing incidence of azole-resistance have led to the necessity of finding a new drug target for antifungal therapy. Notably, as a new drug class, the echinocandins, which aim at a drug target different from polyenes and azoles, have been approved for clinical use. [136] Being rigid structures, fungal cell walls are

composed of large polysaccharides BDG, 1,4-β-D-glucan, 1,6-β-D-glucan, chitin, mannan or GM, and α-glucans and various glycoproteins. [137,138] The target for echinocandins is the complex of proteins responsible for synthesis of BDG. By inhibiting this glucan synthase, it can impair cell wall integrity and ultimately lead to osmotic lysis and death of susceptible fungal cells. [139] Therefore, the activity spectrum of echinocandins is limited to fungi that rely on these types of glucan polymers, which makes them less broad than the activity spectra of polyene or azole agents. Still, the echinocandins have shown fungicidal activity against many pathogenic fungi such as *Candida* spp., *Aspergillus* spp. and *Pneumocystis jirovecii*. [72,139] Owing to their completely different drug target and mechanism of action, echinocandins are broadly active against azole-resistant *Candida* spp. [140]

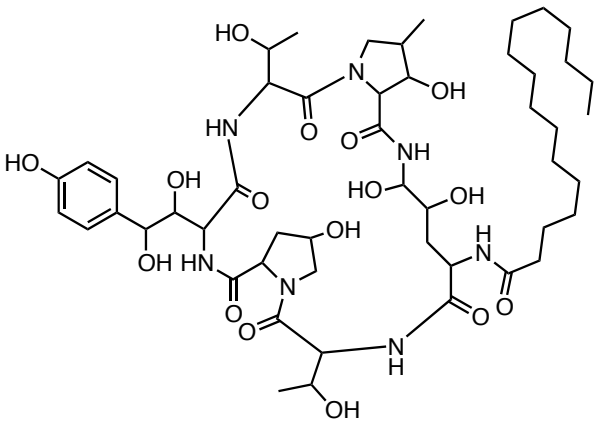
The echinocandins are semisynthetic amphiphilic lipopeptides being composed of a cyclic hexapeptide core with an alkyl side chain (see Figure 1.7). As a result of the structural characteristics, the echinocandins are usually large molecular weight compounds (around 1200). [72,141]

The antifungal activity of echinocandins was discovered from prototypes, echinocandin B **5** and aculeacin A **6**, by random screening in the 1970s. [73] The structures are shown in Figure 1.6. Both prototypes were obtained from products of fermentation of some fungi such as *Zalerion arboricola* or *Aspergillus nidulans* var. *echinulatus*. [142] With the

knowledge that both echinocandin B and aculeacin A showed some antifungal activity, further studies regarding antifungal efficacy and mechanism of action were performed. Later, studies found that besides the direct fungicidal and fungistatic activities, echinocandins also have immunomodulatory effects. [143] Inhibition of the biosynthesis of BDG synthase disrupts the equilibrium required to maintain cell wall structure, resulting in increased β-glucan exposure, which exhibits immunostimulatory properties. Due to the additional exposed antigens, antibody deposition will be triggered, which will cause further recognition by the host immune system. [81,143]



Echinocandin B **5**

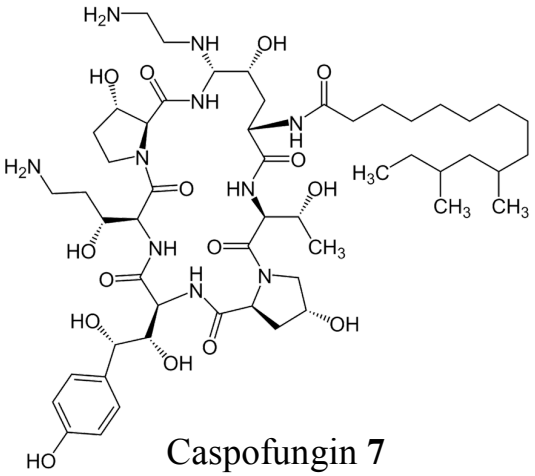


Aculeacin A **6**

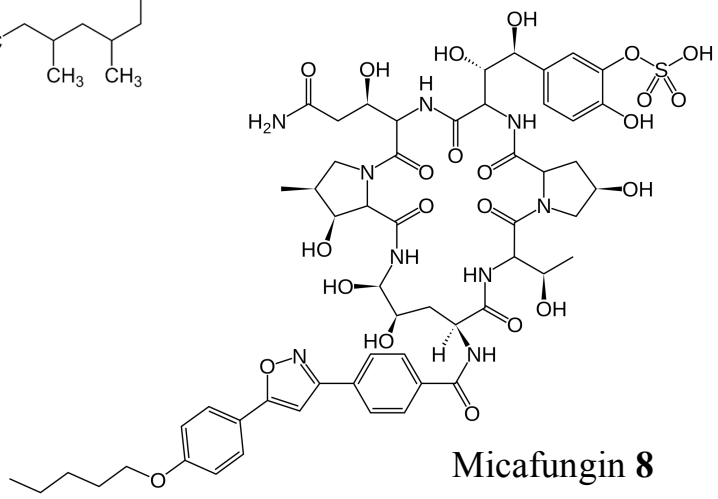
Figure 1.6. Structures of echinocandin B **5** and aculeacin A **6**.

At present, there are three members in use in the class of echinocandins: caspofungin **7**, micafungin **8** and anidulafungin **9**. [103] The structures are shown in Figure 1.7. All echinocandin preparations currently in use are for intravenous use only because of the poor oral bioavailability (only around 3%). [72,81,139] In addition, echinocandins are highly protein bound (from 85% to 99%), which limits the amount of free drug available for activity. [121,144] Echinocandins undergo degradation mainly in the liver by hydrolysis and *N*-acetylation rather than through CYP450 (to a lesser extent). [145] For caspofungin, it is metabolized very slowly, because of the hepatic uptake after initial distribution, and it also undergoes degradation producing an inactive open ring compound in the liver. [121,146] Two inactive metabolites are produced during the metabolism, which are excreted in the bile and feces. [146,147] Owing to the metabolic pathway of caspofungin, dosage reduction is recommended for patients with severe hepatic dysfunction. [148] Micafungin undergoes non-oxidative metabolism to produce two metabolites. One of the metabolites is formed as the side chain of micafungin is hydrolyzed by CYP isoenzymes (mainly CYP3A). [149] Although micafungin is a substrate for, and a weak inhibitor of, CYP3A4 *in vitro*, hydrolysis plays only a minor role in the metabolism of this drug. [150] The main metabolic pathway of micafungin is facilitated by arylsulfatase with further decomposition by catechol-*O*-methyltransferase. Unlike the two members in this class discussed above,

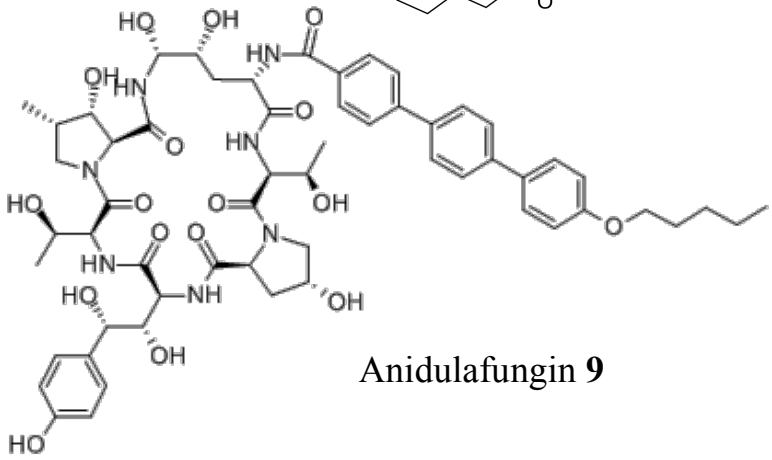
more than 90% of anidulafungin goes through non-enzymatic degradation resulting an inactive open-ring peptide that appears to be further degraded by plasma peptidases. [151] There is no hepatic metabolism mediated by CYP450 observed. As for elimination, anidulafungin is eliminated by fecal excretion, mainly as degradation products via biliary system, rather than passing into urine. [144] In addition, none of the members in the class of echinocandins can be dialyzed, and so no dosage adjustment is necessary for patients having renal replacement treatment. [139,145]



Caspofungin **7**



Micafungin **8**



Anidulafungin **9**

Figure 1.7. Structures of Caspofungin **7**, Micafungin **8** and Anidulafungin **9**.

Although fungi are eukaryotes like human beings, cell walls and targets of echinocandins do not exist in mammalian cells, which attributes to the minimal toxicity of this class of antifungal agents. [103,145] Therefore, compared with AmB, whether in complex with lipid or not, the echinocandins exhibit a better safety profile. [121]

However, histamine-mediated infusion-related reactions, such as facial swelling, rash, and vasodilatation, have been observed when rapid administration of echinocandins is given. Such reactions seem to be easily relieved by slowing the rate of infusion or premedicating with an antihistamine. [152] Since the echinocandins are poor substrates for the CYP450 enzymes, and are not substrates for intestinal or tissue P-glycoprotein, fewer drug interactions have been reported for these compounds than for the azole antifungals. [139] Nevertheless, there are still some drug interactions that have been documented. The concentration of caspofungin can be diminished when administrated with CYP450 inducers, such as rifampin and phenytoin, which merits a slight increase in dose. [153] Micafungin has shown weak inhibitory effects against CYP3A4 causing increased serum concentrations of substrates of this enzyme, like sirolimus and nifedipine. [121,139] Yet, anidulafungin does not seem to exhibit these CYP450-related interactions. [151]

Since being approved for clinical use, the echinocandins are considered to be the choice of treatment for IC due to their broad

antifungal spectrum and fungicidal activity against various species of *Candida*. Along with the findings of many clinical studies and trials with regard to echinocandins, indications appropriate for oesophageal candidiasis, IC and candidemia, IA, and prophylaxis of IFIs have been explored as an intravenous drug. [145]

In 2001, caspofungin became the first member in the class of echinocandins approved by the FDA for treatment of patients with IA who cannot tolerate or who are refractory to other antifungal therapies. It was also approved for the EAT of fungal infections in febrile neutropenia, candidemia and the following *Candida* infections: oesophageal candidiasis, intra-abdominal abscesses, peritonitis and pleural space infections. [103,154,155,156] In a randomized double-blind multicenter trial of caspofungin versus AmB for treatment of IC (mainly candidemia), caspofungin was found to be as effective as AmB but less toxic. [155] Among patients who received intravenous antifungal therapy for 10 days, the rate of favorable response was 73.4% in the caspofungin group and 61.7% in the AmB group suggesting that caspofungin is at least equally efficacious as AmB. [155] On the other hand, the number of discontinuation due to adverse events was significantly higher in the AmB arm than that in the caspofungin arm (23.2% vs. 2.6%, respectively), which reveals the superiority of caspofungin on toxicity profiles compared to AmB. [155] Among three members of echinocandins, caspofungin is the only one that

has been studied in a large randomized double-blind controlled trial (RCT) for febrile neutropenia in which over 1000 patients were enrolled. [157] Both efficacy and safety of caspofungin have been assessed and compared with L-AmB for EAT in the study. The overall success rates were 33.9% for caspofungin and 33.7% for L-AmB, showing similar efficacy, while discontinuation occurred less often in the caspofungin group than in the L-AmB group (10.3% vs. 14.5%, P=0.03). [157] Caspofungin has also been studied as salvage therapy for IA in a noncomparative, multicenter clinical trial in which 83 patients were involved, who were either refractory to (71, 86%) or intolerant of (12, 14%) previous antifungal therapy. [156] Among the 83 patients who received at least one dose of caspofungin, a favorable response was observed in 37 (45%) patients, which actually led to the first approval of the use of an echinocandin for treatment of IA in patients intolerant of or refractory to conventional antifungal treatment by North America and the European Union. [156]

The second echinocandin, micafungin, was approved in 2005 initially for the treatment of esophageal candidiasis and prophylaxis of invasive *Candida* infections in patients undergoing HSC transplantation (HSCT). [103] Of echinocandin class, only micafungin has been formally evaluated, in two randomized trials for antifungal prophylaxis, and found to be effective in preventing invasive *Candida* infections and also be a

potential alternative to fluconazole as prophylaxis for IA. [158] Since it is common to use fluconazole for antifungal prophylaxis, two randomized, double-blind, multicenter, comparative trials of micafungin and fluconazole were designed to compare their efficacy and safety profile with different dosages of micafungin (a daily dose of 50 mg, 100 mg or 150 mg) in the two studies. Both studies defined success as absence of suspected, proven, or probable IFIs through the end of prophylaxis therapy and no proven or probable IFI through the end of the 4-week period following treatment. [159,160] In the first study, either 50 mg of micafungin or 400 mg of fluconazole was administered once daily as a one-hour infusion. [159] A higher overall success rate was observed in the micafungin group (80%) than that of the fluconazole group (73.5%). [159] In the other study, patients were randomly assigned with either micafungin 150 mg or fluconazole 400 mg once daily. [160] Although the molar amount of micafungin and fluconazole (molecules, mole as unit) were not mentioned in the literature, it would have been better to compare their molar amount of substance, instead of their physical weights, due to a significant difference in their molar weights. Therefore, the comparison of 150 mg micafungin (1270.28 g/mol) with 400 mg fluconazole (306.27 g/mol) is equal to 0.039 mmol micafungin and 1.306 mmol fluconazole. The results of the study showed that the overall success rate in the micafungin arm was comparable to that of the fluconazole arm (94% vs.

88%, respectively), which seems to suggest that micafungin could be treated as a new valuable option for antifungal prophylaxis. [160] Although 50 mg of micafungin has been approved for IFI prophylaxis, a higher dose of micafungin (150 mg daily; 0.118 mmol) has been found to be significantly more effective in several trials (for the treatment of esophageal candidiasis; endoscopic cure rate: 89.8% vs. 68.8%, respectively). [161,162] In addition to the antifungal prophylaxis, the efficacy of micafungin for the treatment of severe IC has been studied in a Phase III randomized, double-blind, multinational trial of over 500 adult patients to compare micafungin (100 mg/day) with L-AmB (3 mg/kg per day). [163] Of 202 patients in the micafungin group, overall treatment success was seen in 181 patients (89.6%), which is comparable to the results in the L-AmB group with an overall success observed in 170 of 190 patients (89.5%). [163] Meanwhile, fewer treatment-related adverse effects were found to be attributed to the use of micafungin than with L-AmB, which was consistent with reports about safety profiles from other reports. [163] However, a different unit system (mg/day vs. mg/kg per day) was used for dosage in the study, which may lead to untenable conclusions due to the difference in molar weight between micafungin and AmB. Still, several comparative studies have indicated micafungin being equally efficacious, yet less toxic than AmB, despite AmB and L-AmB being considered as the standard EAT for fungal infections. Therefore, a recent

study was designed and performed to evaluate micafungin for EAT of febrile neutropenia in patients with hematological malignancies. [164] Of 53 patients who received chemotherapy for hematological malignancies, and more than 72 hours of systemic antibacterial therapy while fever and neutropenia persisted, resolution of fever during neutropenia was achieved in 37 patients (70%) by taking micafungin (150 mg/day). All patients who were positive for BDG or *Aspergillus* became negative. [164] According to the results, micafungin appears to be safe and effective as used in EAT for treating febrile neutropenia in hematologic malignancies.

Anidulafungin is the newest echinocandin approved in 2006 by the FDA to use in the treatment of oesophageal candidiasis, candidemia, peritonitis and intra-abdominal abscesses due to *Candida* spp. in this class. [103] As mentioned earlier, anidulafungin is unique because it undergoes a process of non-enzymatic biotransformation for degradation rather than being metabolized via CYP450 enzymes. Consequently, anidulafungin is endowed with an outstanding advantage and it is not affected by concomitant treatment with substrates, inhibitors, or inducers of the CYP450. [151,165] In addition, there is no need for dose adjustments for patients with any degree of renal or hepatic insufficiency for the use of anidulafungin. [166,167] In a randomized double-blind trial comparing anidulafungin with fluconazole for treatment of IC, eligible patients (245 out of 261) were randomized to receive either intravenous anidulafungin

or intravenous fluconazole for 14-42 days. [168] At the end of intravenous therapy, there was a significantly better global response rate with anidulafungin (75.6%) than with fluconazole (60.2%; P=0.01). [168] Since *C. albicans* were isolated in 62% of the 245 patients as the major causative pathogen, it is noteworthy that the difference in the global response rates against *C. albicans* infections was evident between the two treatments (81.1% for anidulafungin vs. 62.3% for fluconazole, P=0.02). [168] It is well known that critically ill patients are the vulnerable population against nosocomial infections frequently caused by *Candida spp.* An echinocandin is recommended by the IDSA 2009 candidiasis guideline for the treatment of IC in patients who are considered to be "moderately severe or severely" ill. [20] Therefore, a *post hoc* analysis was conducted to compare the responses to anidulafungin with fluconazole in this population. [169] One hundred and sixty-three patients were identified from the original study [168] that met at least one criterion for critical illness (89 in anidulafungin group, 74 in fluconazole group). Global responses at the end of therapy were more favorable with anidulafungin (70.8%) than with fluconazole (54.1%, P=0.03), which supported the 2009 IDSA guidelines for anidulafungin being more effective than fluconazole in treating critically ill patients with candidemia. [169] Since anidulafungin has a broad spectrum of activity against *Candida* species, including strains resistant to azoles and AmB, an open-label, non-

comparative small-scale trial was performed to evaluate efficacy of anidulafungin in patients with azole-refractory oropharyngeal and esophageal candidiasis. [170] Nineteen patients, who had failed treatment of 14 days with either fluconazole or voriconazole, were enrolled in the study. At the end of the therapy, clinical success was observed in 18 of 19 patients (95%) and 11 of 12 patients (92%) were defined as endoscopic success. [170] For the follow-up visit, the success rates were evaluated again, and 14 patients (47%) were found to remain successful, 10-14 days after end of therapy. Although this study was limited by the small sample size, it still provided some valuable information and suggested that anidulafungin could be an effective, well-tolerated treatment option for azole-refractory mucosal candidiasis. [170]

Yet, there are not many studies that have been performed to evaluate the safety profiles of anidulafungin in the use of clinical settings, and adverse events were rarely reported so far. A recent study was conducted to evaluate the safety profile of anidulafungin in adult SOT recipients. [171] Eighty-six SOT recipients from 14 centers were enrolled in this study over 14 months who received anidulafungin for at least 48 hours as the treatment of IFIs (24, 28%) or as prophylaxis (62, 72%). [171] The results showed that anidulafungin was generally safe and well tolerated in SOT recipients and there was no patient discontinued from anidulafungin due to severe adverse effects. In particular, there was no case of hepatotoxicity

or renal failure directly attributed to the use of anidulafungin and no need for modification of immunosuppressive drug doses due to anidulafungin therapy, which is important to SOT recipients. [171]

1.4 Antifungal strategies

Due to the limited diagnostic techniques that are currently available and the fact that many IFIs can only be detected at a relatively late stage, developing more effective antifungal strategies has been very important for the management of IFIs. To be a successful antifungal strategy, it should not only match the individual case with proper treatment and regimen, but also be able to balance efficacy and safety (to avoid overuse and underuse). There are four general strategies available now for the management of IFIs, including prophylaxis, EAT, preemptive antifungal therapy and targeted therapy (treatment of established IFI). The definition of each strategy is described in Table 1.1.

1.4.1 Antifungal prophylaxis

Before antifungal prophylaxis became widely used, about 20% of febrile neutropenic patients treated for acute leukemia or those undergoing HSCT had developed an IFI with *Candida* or *Aspergillus* species by day 20 of neutropenia. [172]

Strategy	Definition
Prophylaxis	Antifungal agents are provided to patients at high risk of infection to prevent potential fungal infections
Empirical treatment	Treatment is initiated on an empirical basis in persistently febrile patients with neutropenia, which is without a known source and is not responding to adequate antibacterial therapy
Preemptive therapy	Although similar to empirical treatment to treat a suspected early IFI, preemptive therapy is usually based on clinical and/or laboratory findings suggesting IFI without microbiology identification
Targeted therapy	Targeted therapy is only used in patients with proven IFI based on pathogen identification and should be modified according to patient's clinical presentation and previous antifungal therapy and prophylaxis

Table 1.1. Strategies for management of IFIs, modified from Segal B.H., et al. [13,173]

Due to the high mortality associated with IFIs in immunocompromised patients, antifungal prophylaxis has been recommended in selected high-risk patients. According to the recommendations of the 'First European Conference of Infections in Leukaemia' (ECIL 1) and the 'Infectious Diseases Working Party of the German Society of Haematology and Oncology', antifungal prophylaxis should be provided to patients with granulocytopenia (<500 cells μl^{-1}) for more than seven days and also in allogeneic HSCT recipients. [174,175] For prophylaxis to get so much attention, one of the clinical trials played a very important role in the process. Back in 1992, a randomized, placebo-controlled trial demonstrated the utility and benefits of using fluconazole

for prophylaxis. In the study, unexplained fever was observed in many of the patients in both fluconazole (101 of 179) and placebo (116 of 177) arms and empirical AmB therapy was provided to patients with persistent fever. [176] By analyzing the causes of fever in patients who received AmB therapy, IFIs occurred in 21 of 116 patients in placebo group, but in only 1 of 101 in fluconazole group (18.1% vs. 1%, P<0.001). [176] In total, proven IFIs were present in 28 of 177 patients receiving placebo (15.8%), but in only 5 of the 179 patients receiving fluconazole (2.8%, P<0.001). [176] The positive results not only placed the foundation of using fluconazole as antifungal prophylaxis, but also prompted intensive evaluation on fluconazole and other antifungal agents for prophylaxis in the following decades.

However, whether an antifungal should be recommended and how to select the correct population still remain controversial in practical use, because it may increase the medical expenses and also cause possible exposure to unnecessary drug-related toxicity and induction of drug-resistant fungal species. [158] Due to all of these concerns, the target groups for prophylaxis should be defined strictly. Primary prophylaxis, which aims at truly preventing fungal infection in patients with no history of IFIs, should be clearly separated from secondary prophylaxis, which targets prevention of recurrence of IFIs for patients at high risk. [158] Although fluconazole remains the most frequently prescribed agent for

prophylaxis, it lacks activity against moulds (e.g. *Aspergillus spp.*), and also many different fungal species have shown drug-resistance (e.g. *C. krusei*) or poor susceptibility (e.g. *C. glabrata*) towards fluconazole, which significantly limits its use. [177] Along with the development of newer antifungal agents and formulations with broader spectrum of activity, and better pharmacokinetic and safety profiles, there are more and more antifungal agents available for prophylaxis, such as L-AmB, posaconazole, voriconazole or echinocandins. In order to find better alternatives to fluconazole for antifungal prophylaxis, a large number of studies were initiated that focused on comparison of different antifungal agents used for prophylaxis. [158] The recommendations of antifungal prophylaxis using a grading system were included in 2009 IDSA guidelines on the management of candidiasis [20] and aspergillosis, [53] which includes fluconazole, itraconazole, posaconazole, caspofungin and micafungin for different populations.

1.4.2 EAT

Although not proven with enough evidence, EAT remains the standard of care in patients with granulocytopenia and persistent or recurrent fever of unknown causes despite treatment with broad-spectrum antibiotics. [172] Two studies, which were conducted in the 1980s, showed that a lower incidence of IFIs in antibiotic-treated neutropenic patients

with persistent fever was attributed to EAT with AmB. [178,179] In the study with a larger sample size, the number of deaths from IFI was much lower in patients receiving empirical AmB compared with the control group (0 vs. 4; *P*=0.05). [178] In the controlled study published in 1989, initiation of AmB after four days if antibiotics seemed ineffective was compared with continuation of antibiotics. The result suggested a decrease in breakthrough fungal infections during and after therapy and also an overall better response (defervescence). [179] Since then, 40-50% of high-risk neutropenic patients received EAT without requiring any microbiological or radiological documentation of IFIs. [180] The rationale for the use of EAT is also supported by the evidence from previous studies indicating that withholding antifungal treatment until getting a conclusive diagnosis frequently leads to disseminated infection with high mortality. [177] Due to the lack of pathogen identification, the clinician has to select an EAT regimen covering the most likely causative pathogens based on local epidemiology and clinical presentations and symptoms. [181] A study in clinical practices showed that the choice of EAT regimen is also influenced by the previously used antifungal prophylaxis, in which case the clinician will want to avoid an antifungal agent from the same class as prophylaxis, due to the concern of cross-resistance. [182]

In recent years, EAT has been increasingly questioned, because the two historical studies supporting this approach are not convincing due to

the small sample size and questionable methodology that only compared AmB with placebo without showing significant effectiveness in reducing IFI incidence or mortality. [178,179] Moreover, when it comes to the entry point for initiation of EAT, it is also not well accepted. Although fever in neutropenic patients should be given attention and trigger further tests and evaluation, the appropriateness of using fever alone as a specific criterion for initiation of EAT has been widely debated, since there are many origins of fever in neutropenic patients. [173,182,183] In addition, at the time the trials were conducted, there were few diagnostic techniques available to provide early diagnosis for IFIs. [184] The development in antifungal agents and improvements in the treatment and clinical management of IFIs are significant over the years, which makes it difficult and untenable to draw comparison between the results of historic trials and current practices. [184] On the other hand, the cost of antifungals has a significant impact on the overall antimicrobial budget. Although the published data about antifungal consumption and cost-effectiveness are limited, it can still be inferred from the studies that the majority of antifungal budget was spent on antifungal prophylaxis and even more on EAT. [185] In a seven-year antimicrobial stewardship program conducted by a large tertiary hospital, data indicated that the average antifungal cost represented 29.5% of the overall antimicrobial expenditure ranging from 47.7% ($3.7 million) to 18.8% ($1.2 million) before and after the program

implementation, which lays the emphasis on management and appropriate indications for EAT. [186]

Following the two historic studies of EAT, in which there was a control group who received no antifungal treatment, all subsequent trials have used different classes of antifungal agents as comparators, including various doses or formulations of AmB, azoles and also echinocandins to identify the most appropriate agent for EAT. [80,172] Nevertheless, all clinical trials of EAT performed during the last two decades have neither revealed a distinct superior antifungal agent with regard to efficacy, even with substantially larger sample sizes, nor indicated expected reduction in IFI incidence or IFI related mortality associated with EAT. [172,185] Moreover, as IFI symptoms are usually unspecific, EAT brings similar risks as antifungal prophylaxis, namely exposure of the patients without IFI to unnecessary drug-related toxicity and increased costs and also possible induction of antifungal resistance.

Although EAT of suspected fungal infections may not be the ideal approach to deal with the increasing incidence of IFIs, it has been well-accepted as a standard therapy, since delayed treatment will clearly lead to increased morbidity and mortality. Without better diagnostic methods and novel antifungal agents, EAT will remain as a very important antifungal therapy, especially for patients at high risk. Overall, EAT remains the standard approach for management of presumed IFIs

allowing early exposure of fungal pathogens to adequate proper treatments and also helps to avoid the high mortality associated with various fungal species, limited early diagnostic methods and undetected infections. [172]

1.4.3 Preemptive therapy

It is known that establishing a definitive diagnosis of IFI is difficult and time-consuming, because the symptoms are usually nonspecific and may not be present until very late stages of diseases. [72] With improved non-invasive diagnostic methods, advanced radiological tests and better understanding of the pathogenesis of fungal infections, new approaches should be proposed to restrict the administration of antifungal agents to patients without any evidence from diagnostic tests. The preemptive approach is based on the combination of clinical and laboratory findings suggesting early IFIs before the development of clinically overt disease, without pathogen identification. The original intentions of proposing preemptive approach included avoiding the unnecessary use of antifungal agents, as well as drug-related toxicities in patients with non-fungal caused fever. [173] Preemptive therapy is a newer and evolving approach for the management of IFIs that has been receiving increasing attention, but it is still at an exploratory level and does not have standardized criteria. [181] Due to the lack of standardization, various diagnostic

techniques are adopted in different approaches, mainly focused on high-incidence infections, namely IA and IC. By using GM screening, BDG assay and CT scans, the common goal is to be more selective towards patients at risk of IFIs and decreased toxicity, costs and risk of emerging resistance. [177,185] However, use of different diagnostic methods, criteria used for initiating antifungal treatment and enrolling patients have caused tremendous difficulties in comparing the limited existing trials regarding preemptive therapy. [187]

Amongst all of the techniques adopted in the diagnosis of IFIs, radiology, particularly CT, plays a very important role. The typical radiological 'halo' or 'air-crescent' sign of an IA, which can be better observed by HRCT, is defined and allowed to support the diagnosis of probable or possible fungal infections according to EORTC/MSG criteria, even though the sign is not entirely specific for fungal infections. [13,187] Moreover, preemptive therapy is also guided by biomarker detection, including GM ELISA and BDG assays. Additionally, direct microscopy and culture-based tests are suggested on BAL fluid in patients with pulmonary infiltrates. [13,72,187] The combination of sequential monitoring of GM with HRCT was reported to reduce antifungal use by 78% (from 35% to 7.7%) when compared with fever-driven EAT in a prospective feasibility non-randomized study. This study has also opened up new ways forward for similar algorithm incorporating non-culture-based

microbiological techniques with the use of HRCT to become well-accepted by many other clinical trials. [188] However, there is no consensus on recommendations for variables in GM assay that can have a great impact on the performance, including GM index cutoff level (varying from 0.5 to 1.0), test interval and required number of positive samples. [13,177,188] Generally, a GM assay should be carried out at least twice per week when the cutoff for positivity is 0.5 and two consecutive positive samples are required, while HRCT of the chest is suggested to be performed at least every 7-10 days. [187,188]

Although only limited data have been published comparing a preemptive with an empirical strategy, an open-label, randomized, controlled trial of empirical versus preemptive therapy in high-risk patients due to chemotherapy or HSCT has reported similar survival rates with both arms. [184] All patients were screened twice weekly by GM ELISA with the GM index ≥1.5 adopted as a cutoff level and chest radiograph together with CT as further confirmation in the group of preemptive therapy. [184] AmB or L-AmB, depending on patient's renal function, was used as antifungal treatment in both groups. It was shown that there was no significant difference in overall survival between the two strategies (preemptive 95.1% vs. EAT 97.3%). [184] However, the incidence of IFI was significantly higher in the preemptive group than that in the EAT group (9.1% vs. 2.7%, respectively), so that the use of

antifungal agents was much lower in the preemptive arm (1 out of 56 treated patients, 1.8%) compared with EAT arm (55 out of 92 treated patients, 59.8%, P<0.001). [184] Instead of using the combination of chest CT and non-culture laboratory findings as diagnostic methods, clinical symptoms were adopted in this study as criteria for preemptive therapy. Still, the results have shown that preemptive therapy was more selective towards patients at-risk, so that antifungal consumption decreased without increasing mortality. [184] With all of the uncertainty and questions, more research is needed to get preemptive therapy standardized and to compare preemptive with EAT strategies. Further studies are also required to investigate which diagnostic method, biomarker or clinical finding or both, should be dominant to guide preemptive therapy.

1.4.4 Targeted therapy

As mentioned earlier, the classification of IFIs is based on the degree of diagnostic certainty, including proven, probable and possible IFIs. The reason for setting these strict diagnostic criteria of IFIs is to help standardize the target groups of patients in the clinical routine and studies. Yet, these criteria may not intend to guide therapeutic decisions in individual patients due to their limited flexibility and applicability. Although an increasing number of new technologies have become available as diagnostic methods, the definite diagnosis of IFI still can only

be obtained by sterile conditions (e.g. blood-cultures) and/or tissue specimens for histological tests, according to the criteria. However, the microbiological and histological detection of fungi is often associated with many problems, such as being time-consuming and relatively insensitive, also being too invasive to conduct, particularly on the deep-seated infection sites. [13,21,24] The term "targeted therapy", also known as treatment of established IFIs, is used for the treatment of patients with proven IFIs based on the pathogen identification achieved via microbiological and histopathological examinations. [13,173] Due to the known limitations of conventional diagnostic techniques, especially the risk coming along with invasive diagnostic procedures, targeted therapy is not always achievable or suitable for patients in different conditions. [177] Therefore, the first step before providing targeted therapy is elaborate evaluation of clinical presentations, risk factors that may lead to the emergence of IFI and previous antifungal treatment and prophylaxis. [177]

There have been many studies and guidelines with regard to targeted therapy in IFIs, especially in IC and IA, which aim at studying not only the effectiveness of the strategy itself, but also comparing various antifungal agents for different indications. [20,189,190,191,192] With regard to targeted therapy in IC, international guidelines recommend several agents for primary and second-line treatment for IC depending on the patient's conditions, previous use of antifungal agents and local epidemiology. [185]

For non-neutropenic patients, fluconazole or an echinocandin is recommended as primary therapy in most international guidelines, and AmB is suggested as an alternative in case of intolerance. [20,189] Recently, the use of an echinocandin as first-line treatment of IC in non-neutropenic patients has become increasingly popular considering the risk of azole-resistant *Candida spp.* strains, while more clinical trials are still needed to support this recommendation. [185] For neutropenic patients, caspofungin or micafungin is preferred as primary antifungal therapy and L-AmB remains a second-line treatment. [193] On the other hand, voriconazole is considered the agent of choice for first-line therapy in IA and L-AmB may be used as an alternative therapy with similar efficacy but higher toxicity. [177] Regardless of which drug is selected for targeted therapy, the regimen should be adjusted to fit the patient's situation according to clinical presentation, concomitant medication and risk of resistance.

1.4.5 Combination therapy

In order to fight against the high mortality and poor prognosis associated with IFIs, IA in particular, combination therapy tends to draw attention from clinicians as a salvage therapy based on the clinical situation. [13] Echinocandins have contributed to combination therapy and become even more attractive, since the new class of antifungal agents exhibits a distinctive mechanism of action. However, the benefits of

combination therapy are still controversial as a result of insufficient clinical trials, even though combination therapy is already recommended in the IDSA guidelines for second-line (salvage) therapy. [53] The basis of using combination therapy are the improvements in outcomes observed from a few *in vitro* and animal studies that indicated synergistic, or at least additive, effects when an echinocandin was added in the use of a mold-active triazole or AmB. [194,195] In the most recent review, a meta-analysis was performed to compare mold-active triazoles or L-AmB plus an echinocandin to non-echinocandin monotherapy for acute IA. [196] The results did confirm that certain themes of combination therapy could lead to significantly improved 12-week survival and composite success over monotherapy in salvage setting of IA. [196] Nevertheless, routine use of combination therapy for initial target IA treatment is not well-supported. [196] Both the recent meta-analysis and a previous critical review [197] have come to the same conclusion in which the cumulative evidence is not strong enough to support combination therapy as a routine primary treatment for IA, while some favorable effects were reported as salvage therapy. By combining antifungal agents with different modes of action, combination therapy may be useful before the IA species is specified. [196] Given the heterogeneity of the regimens, patient populations, primary and secondary endpoints in the limited number of existing studies, more well-

designed controlled, randomized, multicenter clinical trials are required to allow well-grounded conclusions on the usefulness of this approach. [196,197]

1.5 Rationale of the project

Since the late 1970s, inhibitors of fungal cell wall biosynthesis have gained great attention all over the world as a new direction of drug design for the treatment of IFIs because of their distinct mode of action. So far, glucan synthesis remains the only component of cell wall synthesis for the development of antifungal agents with BDG as the drug target in particular. Echinocandin B **5** (Figure 1.6), one of the cyclic lipopeptides in this class, was reported in 1974. [142] Later on, efforts in natural product screening led to the discovery of the pneumocandin series. In 1987, pneumocandin A_0 **5a** was isolated and its structure was then determined. Its antifungal activity was shown to be achieved by inhibiting the synthesis of the fungal cell wall, via inhibition of BDG synthase. [198] Right after the study of pneumocandin A_0, pneumocandin B_0 **5b** was isolated and characterized as the desmethyl congener. [198] Pneumocandin B_0 became the key for further development of BDG inhibitors. [141,199] Pneumocandins that mainly include pneumocandin A_0 **5a**, pneumocandin B_0 **5b** and pneumocandin C_0 **5c**, are derived from fermentation of fungus *Glarea lozoyensis* (originally called *Zalerion arboricola*). [200,201] The

structures of echinocandin B **5** and the three pneumocandins are illustrated in Figure 1.8.

Nevertheless, the research on pneumocandin A_0 and B_0 was not completed due to a shortage of material and the fact that the pneumocandin A_0 lead had been lost owing to its poor oral bioavailability, water solubility and a lack of broad antifungal spectrum, as well as discovery of its semisynthetic analog, cilofungin **5d** (Figure 1.8). The clinical trial for cilofungin was actually halted in 1988, owing to the toxicity of a co-solvent used for oral administration, which led the focus of research back to pneumocandin A_0 and B_0. Later that year, based on findings of DNA studies, it was suggested that *Pneumocystis* was a fungus rather than a protozoan (the pathogen in humans is *P. jiroveci*). [198] It is worth noting that the wall of the cyst form of *P. jiroveci* was reported to contain high levels of BDG. With all the information together, the program of pneumocandin A_0 and B_0 was reinvigorated in 1989 with a test in an immunosuppressed rat model of *P. carinii* pneumonia using pneumocandin A_0. [198] Subsequently, pneumocandin B_0 became the lead compound in a medicinal chemistry program for further development. [198] In order to improve the solubility, stability, antifungal spectrum and pharmacokinetic profiles, semisynthetic optimization of pneumocandin B_0 has been explored.

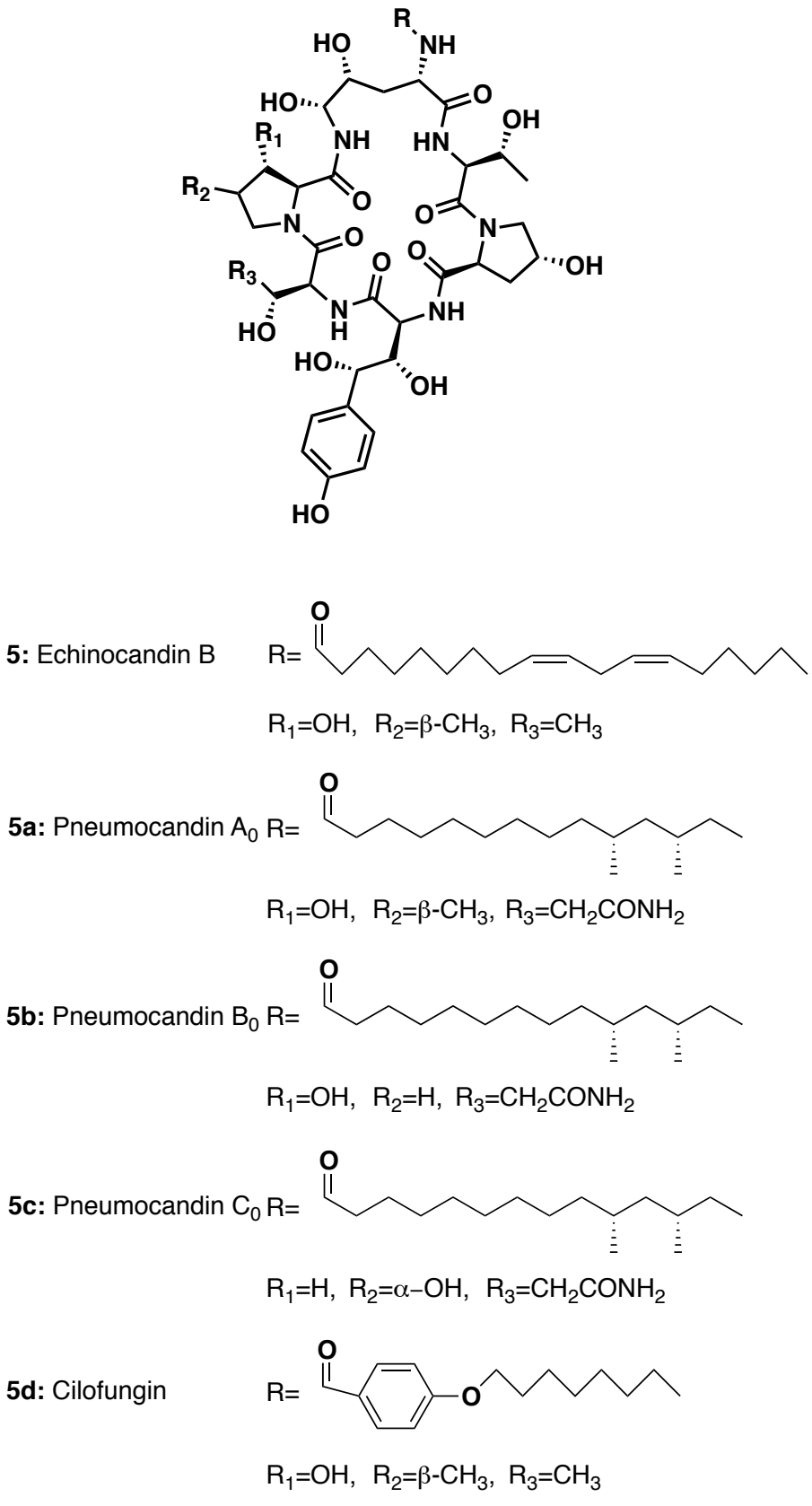


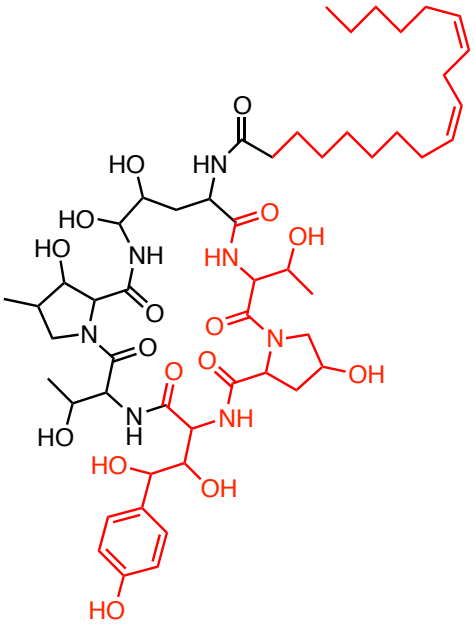
Figure 1.8. Structures of echinocandin B **5**, pneumocandin A_0 **5a**, B_0 **5b** and C_0 **5c**, and cilofungin **5d**.

Among the three members in the class of echinocandins described earlier, caspofungin (MK-0991) and micafungin (FK-463) are pneumocandin B_0 analogues and anidulafungin was derived from echinocandin B. [198] The semisynthetic nature of echinocandins not only demands procedures like fermentation, isolation and purification, but also brings additional cost, making echinocandins more expensive than other classes of antifungal agents. [202] Another limitation that comes along with echinocandins is their poor oral bioavailability, which makes them only available for intravenous use. [202] Considering the broad antifungal spectrum, outstanding safety properties, potential in combination therapy and also the limitations of the current echinocandins, efforts were made to design peptidomimetic analogues of echinocandin B that can be fully synthesized and are smaller molecules.

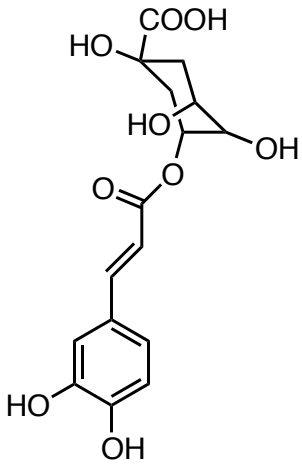
The target for echinocandins is the protein complex responsible for the synthesis of one of the essential fungal cell wall components, BDG polysaccharides. [203] The enzyme complex has been extensively studied in *S. cerevisiae* and Fks1p, Fks2p and Rho1p are indicated to be subunits of the complex. Among the three fractions, Fks1p is thought to be the target and binding site of echinocandins and may also be the catalytic center of BDG synthase. [203] Meanwhile, both Fks1p and Fks2p are regulated by elements of the calcineurin pathway, and by Rho1p, a GTP-binding peptide, which also interacts with protein kinase C. [203] Given the

possible interaction of echinocandins with the catalytic component of BDG synthase to achieve the inhibition of enzymatic activity, it was intended to use smaller linear or cyclic peptidomimetic molecules to mimic the backbone of echinocandins. [202] However, it turned out that the lipophilic side chain and the homotyrosine moiety of echinocandin are essential for their antifungal activity. [202,204,205] Even the relative orientation of the homotyrosine ring with the lipophilic side chain may have a significant impact on the activity of echinocandins as well. [202]

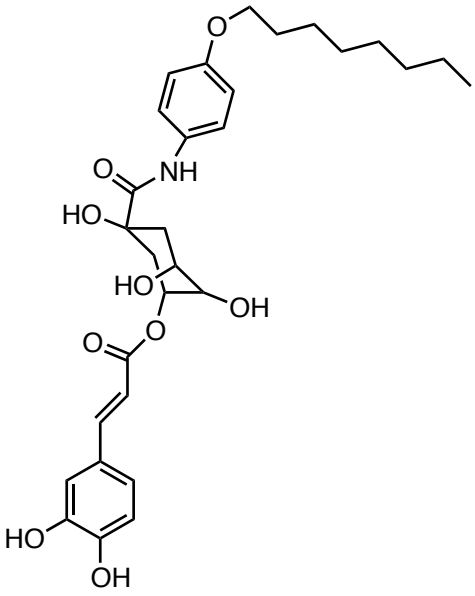
By adopting computer-aided drug design, a series of structurally related natural and synthetic analogues of homotyrosine were examined by Daneshtalab [202] by overlapping with echinocandin B. It was found that chlorogenic acid (CGA) **10** with a *para*-alkoxyphenylamido side-chain (CGA derivative **11**) was the best match and it has the most favorable spatial relationship of the essential groups with those of echinocandin B. [206] As a natural product, CGA exists widely in many vegetables and plants, and is known as an antioxidant. [202,206] Being an ester of caffeic acid and quinic acid, CGA can be considered as a bioisostere of homotyrosine-hydroxyproline, which is very similar to a part of echinocandin B, as shown in Figure 1.9. Hence, with lipophilic side chains, CGA derivatives could be potentially useful bioisosteric replacements for the particular part of echinocandin B, and could possess potential useful antifungal activity. [202,206]



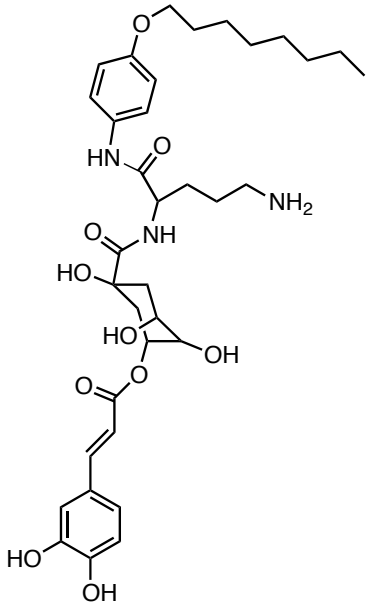
Echinocandin B **5**



Chlorogenic acid (CGA) **10**



CGA derivative **11**



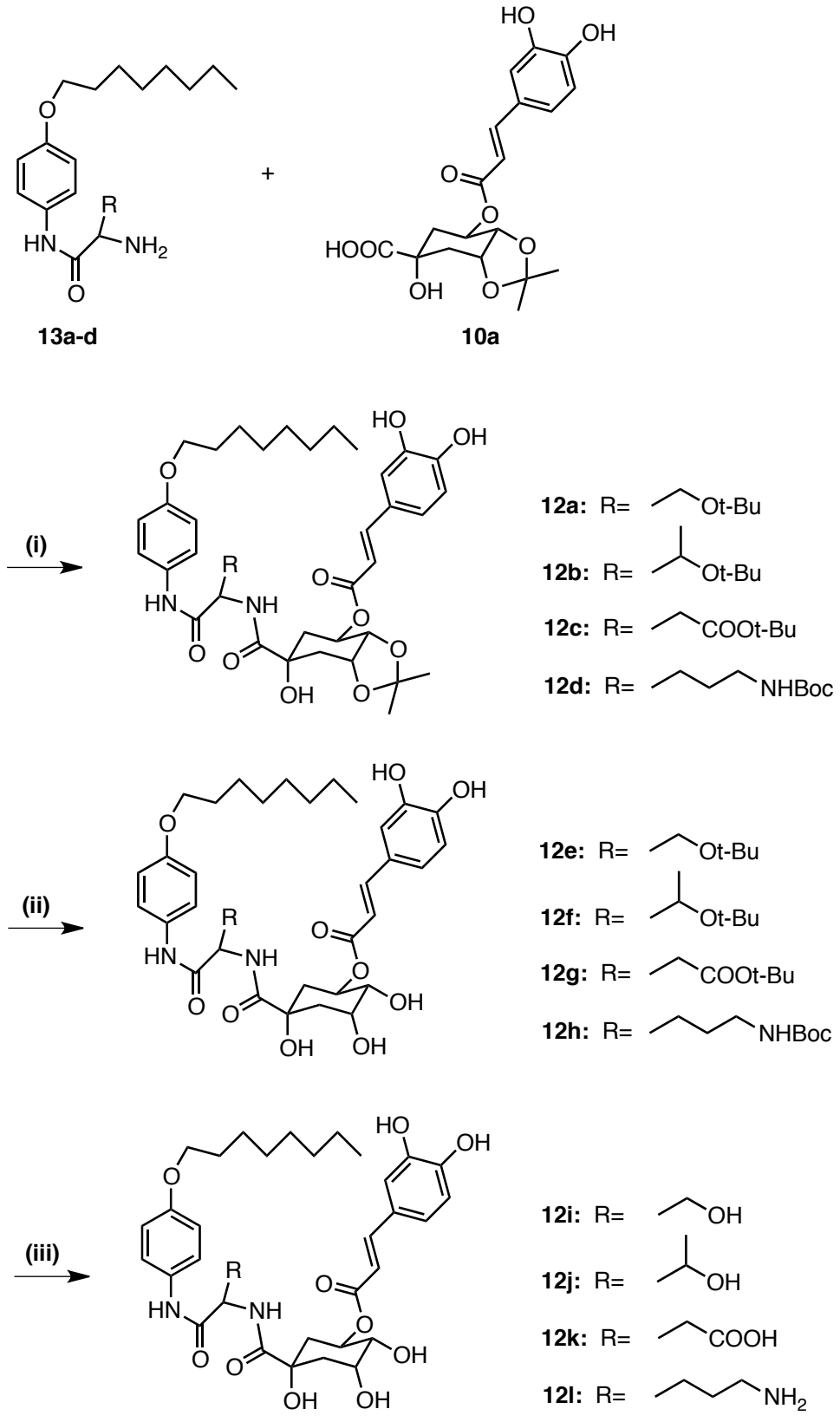
CGA derivative **12l**

Figure 1.9. Structures of echinocandin B **5**, CGA **10** and its derivatives **11** and **12l** (see Scheme 1.1).

Therefore, a series of CGA derivatives (Scheme 1.1) were synthesized and their activities were assessed. Their *in vitro* BDG synthase inhibitory activities were compared with that of aculeacin A **6** (Figure 1.6), which is also, like echinocandin B, a prototype inhibitor of BDG synthase. [206,207] Results expressed as IC_{50} values represent the concentrations that inhibit 50% of the enzyme activity. [207] When CGA was modified to form the octyloxyanilido derivative having a long alkyl chain to afford CGA derivative **11** (Figure 1.9), its IC_{50} was 23 μg/ml, which is better than that of CGA (>32 μg/ml), but still much higher than that of aculeacin A (9 μg/ml). [207]

In order to improve the enzymatic inhibitory activity, further structural modifications were conducted with the incorporation of different amino acids into the structure of CGA. [207] The synthetic routes for the preparation of these CGA derivatives **12a-l** are shown in Scheme 1.1, in which the *p*-octyloxyaniline was modified with the various protected amino acids.

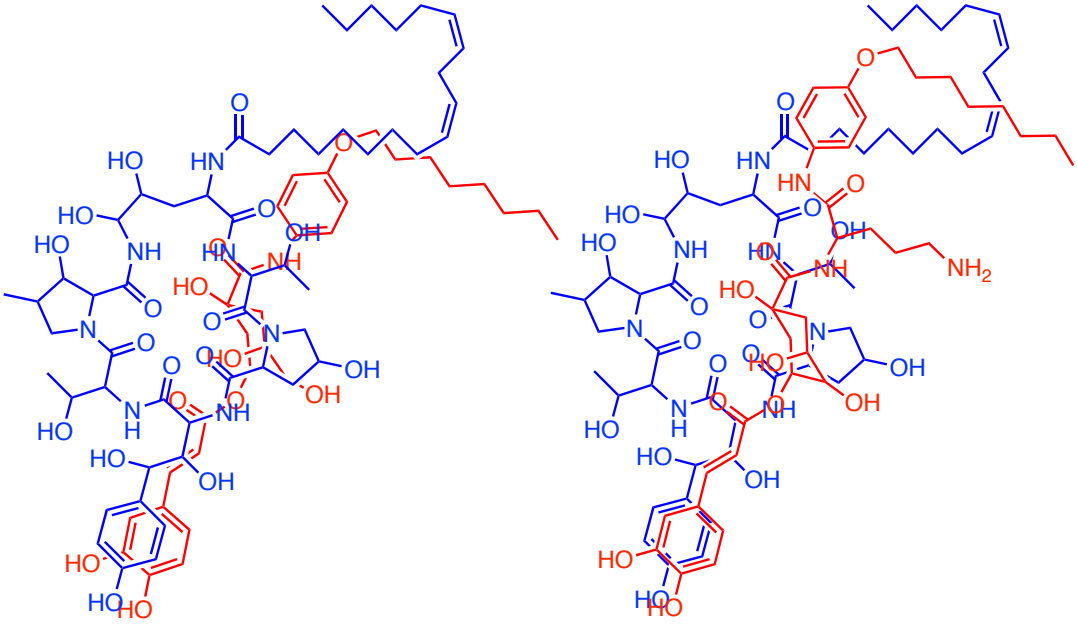
Among these compounds, CGA derivative **12l** showed the best activity with an IC_{50} value of 10 μg/ml, which is very close to that of the positive control compound (aculeacin A). [206,207] The specific structure of CGA derivative **12l** is illustrated in Figure 1.9. It was found that the incorporation of ornithine in CGA derivative **12l** resulted in greater overlap with echinocandin B than the previous molecules studied.



Scheme 1.1. Synthesis of CGA derivatives **12a-l**

Reagents and conditions: (i) CMC, HOBt; (ii) acetone-H_2O, 0.67 M HCl, rt, 1h; (iii) 90% TFA, rt, 30 min. [206]

The comparison between the overlap of **11** and **5** and that of **12l** and **5** is shown in Figure 1.10.



CGA derivative **11** vs. echinocandin B **5**

CGA derivative **12l** vs. echinocandin B **5**

Figure 1.10. Overlap of CGA derivatives **11**, **12l** with echinocandin B **5**.

As illustrated in Figure 1.11 (a), CGA derivative **12l** can be divided into various sections, including the octyloxyaniline grouping with a lipophilic side chain, the ornithine, the quinic acid and caffeic acid. Although proven to have a good enzymatic inhibitory activity *in vitro*, subsequent findings regarding CGA derivative **12l** showed that the ester bond could be easily broken *in vivo* during metabolism and the quinic acid derivative part would be released into the blood as shown in Figure 1.11 (b). [207]

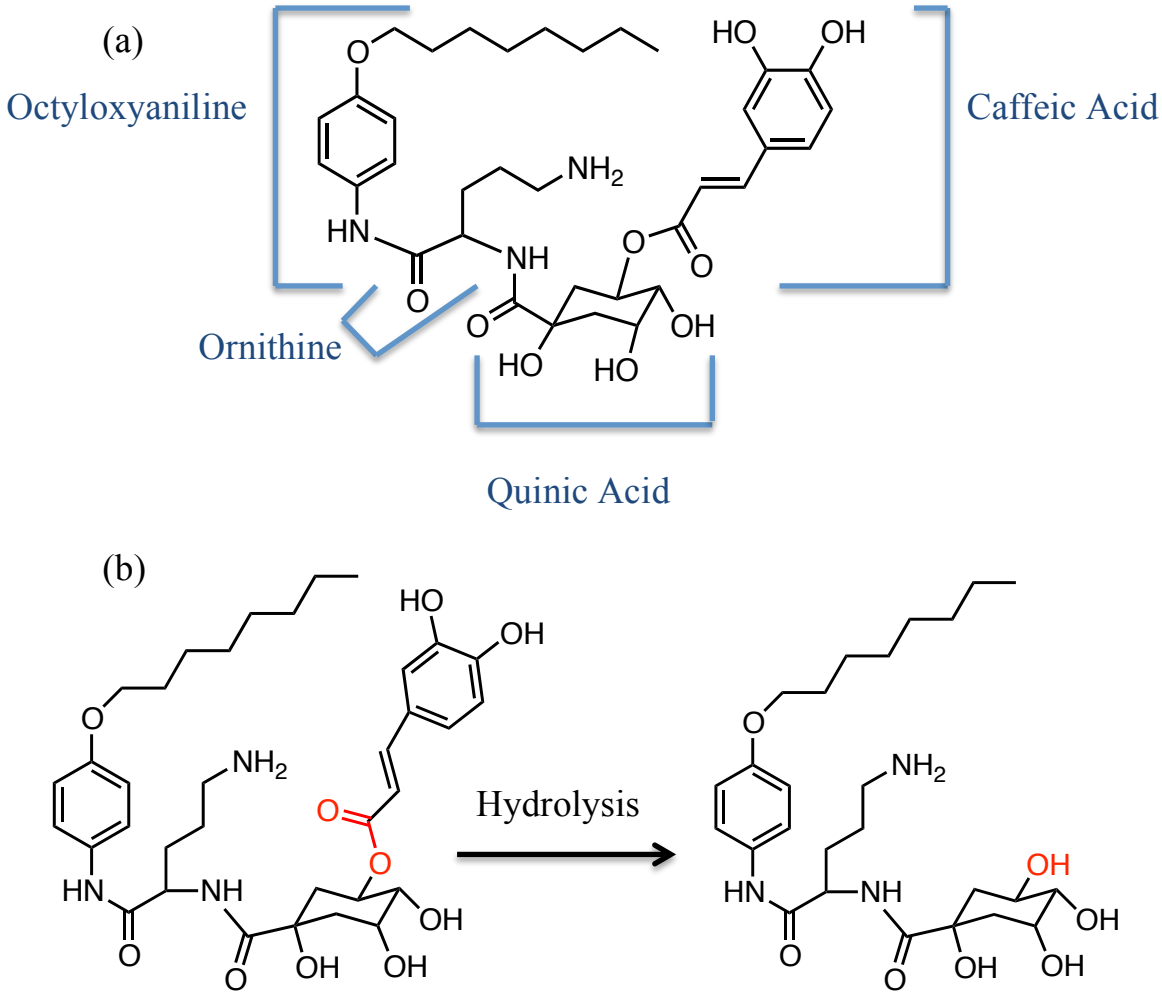
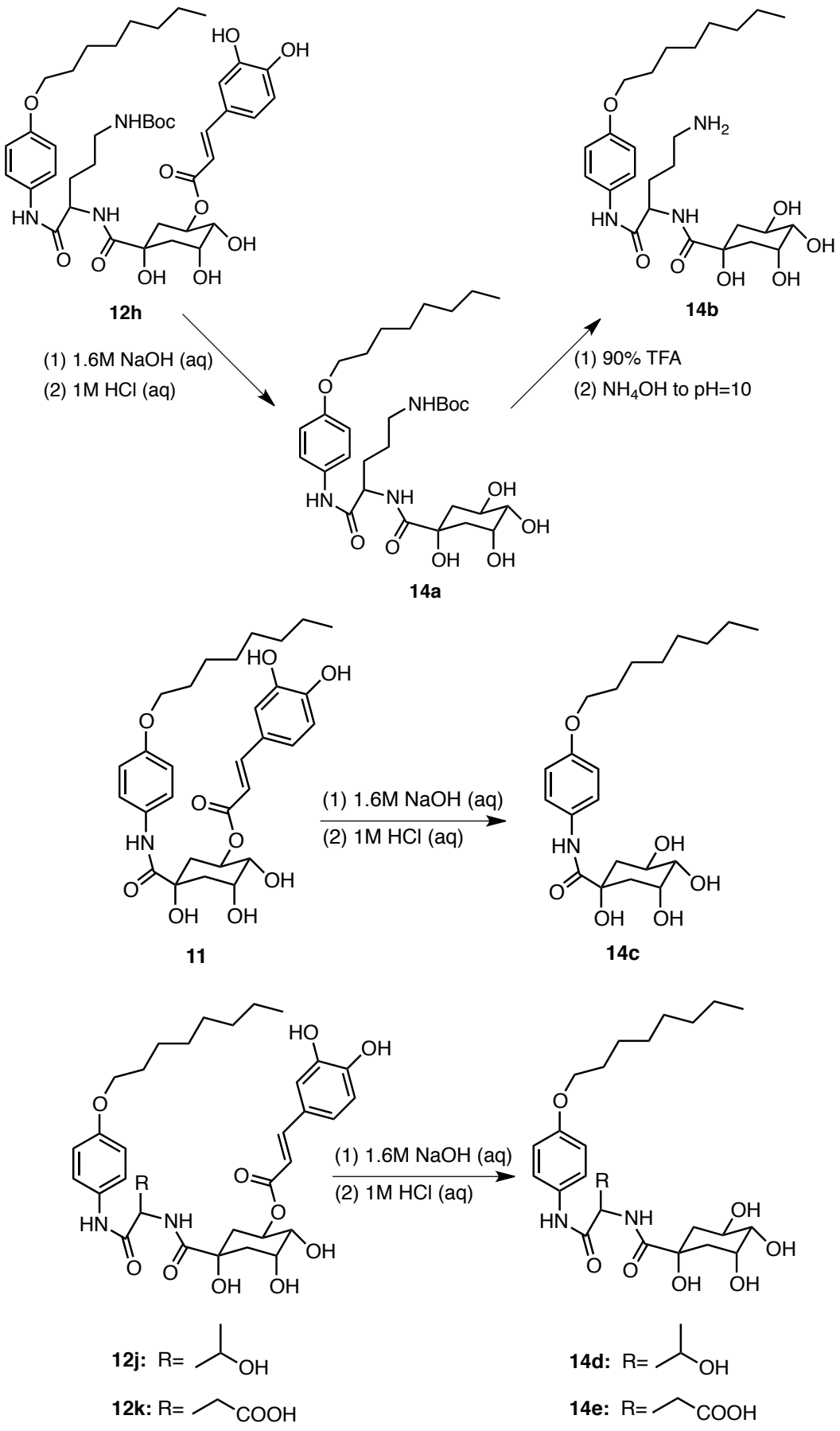


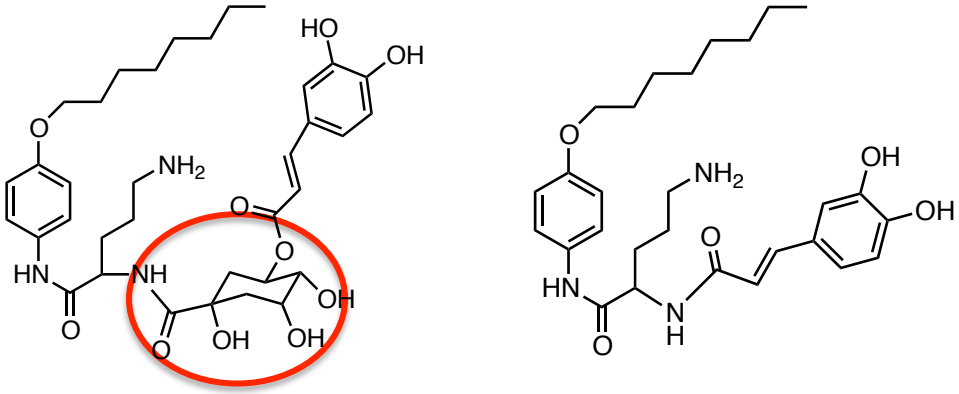
Figure 1.11. (a) Structure of the different sections in CGA derivative **12l** (b) Hydrolysis of CGA derivative **12l**

Consequently, it became important to investigate if the quinic acid derivative **14b** was active. If so, the CGA derivatives could still be developed as parent drugs. Subsequently, quinic acid derivatives **14a-e** were synthesized as shown in Scheme 1.2 and their *in vitro* activity was evaluated. It was found that quinic acid derivative **14b** was the most active of the quinic acid derivatives, with an IC_{50} of 27 µg/ml. However, it is not as active as CGA derivative **12l** (10 µg/ml). [207]



Scheme 1.2. Synthesis of quinic acid derivatives **14a-e** [207].

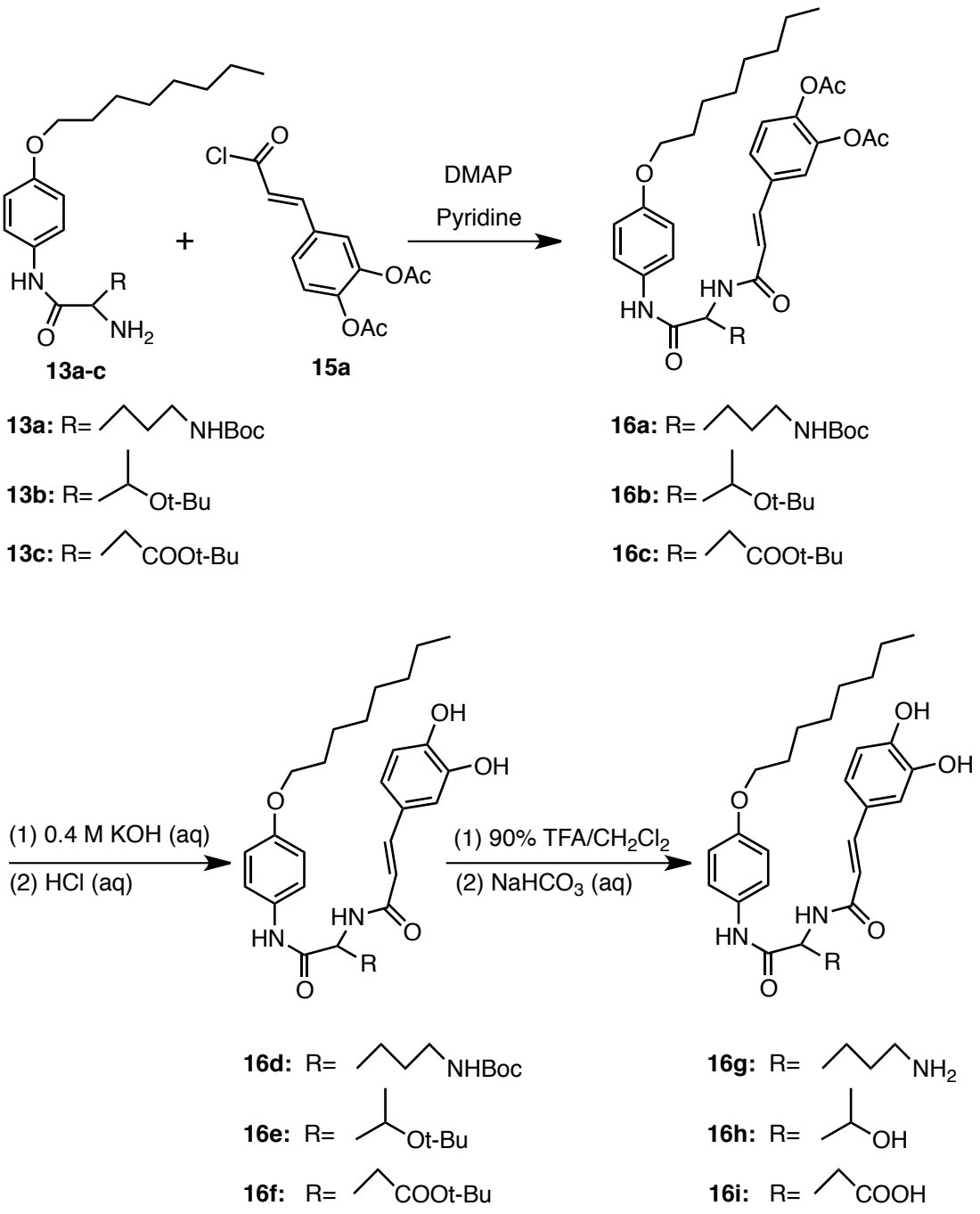
Due to the reactivity of the ester bond in CGA derivative **12l** and the hydrolytic product, quinic acid derivative **14b**, is not as active as CGA derivative **12l**. It was therefore considered necessary to remove the quinic acid section from the CGA derivatives in order to improve their stability and activity. In accordance with the above hypothesis, synthesis of the derivative **16g** of caffeic acid **15** as shown in Figure 1.12 was then pursued.



CGA derivative **12l** Caffeic acid derivative **16g**

Figure 1.12. Comparison of CGA derivative **12l** with caffeic acid derivative **16g**.

Since there is no ester bond in the structure of the targeted caffeic acid derivatives, a better stability was expected. [207] Hence, a series of caffeic acid derivatives (**16a-i**) were synthesized as shown in Scheme 1.4, and their BDG synthase inhibitory activities and stabilities were assessed. It was found that the activity of caffeic acid derivative **16g** (11 μg/ml) was similar to that of CGA derivative **12l**, yet better than that of the quinic acid derivative **14b**. [207]



Scheme 1.3. Synthesis of caffeic acid derivatives **16a-i** [207]

	BDG synthase inhibitory activity		*In vitro* antifungal activity	
Compound	IC_{50}* (µg/ml)	MIC** (µg/ml)	IC_{50} (µM)	MIC (µM)
Caffeic acid derivative **16d**	31	16	52	27
Caffeic acid derivative **16g**	11	2	22	4
Quinic acid derivative **14b**	27	8	53	16
Chlorogenic acid derivative **12l**	10	4	15	6
Chlorogenic acid **10**	>32	>64	>90	>181
Aculeacin A **6**	9	0.25	8	0.23

Table 1.2. BDG synthase inhibitory and antifungal activities

**IC_{50}: the concentration needed to inhibit a biological or biochemical function by half.*

***MIC: the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.*

Table 1.2 is a summary of the bioassay results of CGA and its derivative **12l**, caffeic and quinic acid derivatives. A more recent follow-up study investigated the metabolism of CGA derivative **12l**, quinic acid

derivative **14b**, and caffeic acid derivative **16g** conjugated with an H_2N-Orn-4-octyloxyaniline in rats after oral and intravenous administration using ultra-performance liquid chromatography–atmospheric-pressure-chemical ionization–tandem mass spectrometry (UPLC-APCI-MS/MS) with negative ion polarity mode. [208] The metabolic pathway in rats for chlorogenic, quinic and caffeic acid derivatives inferred from molecular fragments detected by MS is believed to be as shown in Scheme 1.4. The results in that study, not only revealed the metabolic pathway of those compounds in a rat model, but also pointed out a critical problem. After CGA derivative **12l** was administered orally or intravenously to rats, caffeic acid was detected in the plasma samples, which verified that the ester bond linked to caffeic acid in the CGA derivative **12l** did break to release quinic acid derivative and caffeic acid into the systemic circulation. [208] In the case of caffeic acid derivative **16g**, the parent compound was detected in the plasma sample after intravenous administration. However, from 15min to 4h following oral administration, both the substrate (the molecule after a loss of proton, *m/z* 496 $[M-H]^-$) of caffeic acid derivative **16g** (M_w=497.29) and caffeic acid were detected in the plasma samples. Therefore, it was found that the caffeic acid fraction in the caffeic acid derivative also had a tendency to dissociate. [208] Specifically, no caffeic acid or the substrate was found in the plasma samples collected at 30 min, 1 h and 1.5 h after oral administration of

caffeic acid derivative **16g**. However, 2 h after oral dose, both caffeic and isomerized caffeic acid derivative **16g** were detected in plasma sample, which indicated that caffeic acid derivative **16g** could take part in the systemic circulation while only for a limited time and amount. [208] Comparing the metabolic profiles of CGA derivative **12l** with caffeic acid derivative **16g**, the results are consistent with the well-accepted theory that amides are more stable than esters, though still less than ideal. [208]

As the metabolites of caffeic acid derivative **16g** were found to be inactive *in vivo*, it was necessary to re-examine the structure of caffeic acid derivative **16g** seeking ideas to improve the stability. In the structure of caffeic acid derivative **16g**, there is a double bond located next to the amide bond, which constitutes a conjugation system that might result in increasing reactivity of the amide bond. Due to keto-enol tautomerization, the amide bonds are endowed with partial-double bond character, which is stabilizing. With tautomerization, the nitrogen is enabled to donate its lone pair of electrons to the carbonyl carbon and push electrons from the carbonyl double bond towards the oxygen, forming the oxygen anion. By delocalizing the electrons over several atoms, the double bond resonance form of the peptide bond helps to increase stability and decrease rotation about that bond as shown in Figure 1.13.

On the other hand, either resonance form of the amide bond in caffeic acid derivative **16g** (-C=N or -C=O double bond) could form a conjugated

system with the double bond beside it, which has a region of overlapping *p*-orbitals, bridging the interjacent single bond that allows electrons delocalized across all of the adjacent aligned *p*-orbitals. Although a conjugated system originally may lower the overall energy of the molecule and increase stability, conjugation with a resonance form of amide bond could actually weaken the resonance that was playing the most important role to stabilize the amide bond resulting in increased amide reactivity. Therefore, further structural modification on caffeic acid derivative **16g** to remove the double bond next to the amide bond should be conducted to prepare a more stable molecule. As a result, a new molecule, a dihydrocaffeic acid (i.e. 3,4-dihydroxyhydrocinnamic acid) derivative, became one of the target compounds in this project, aiming at better stability. Its synthesis is described in section 3.2.4.

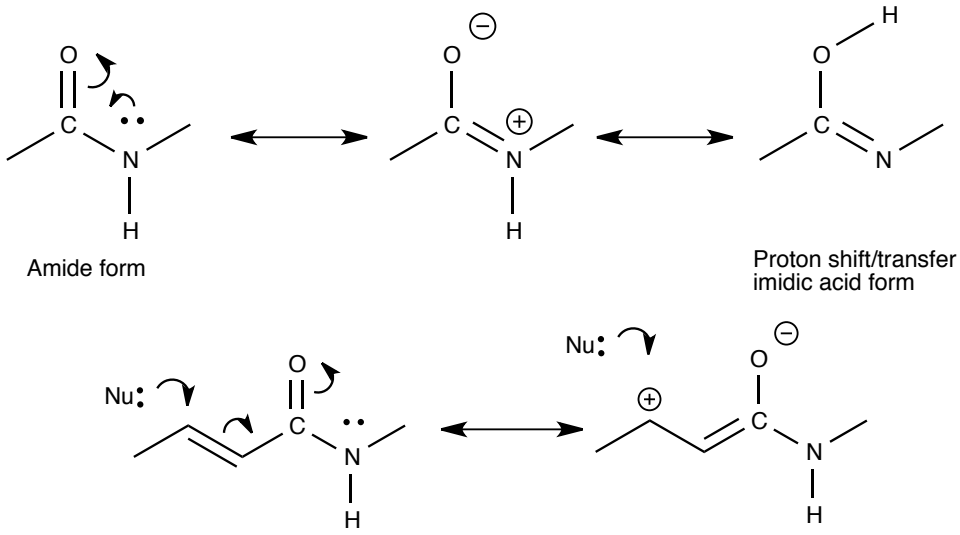
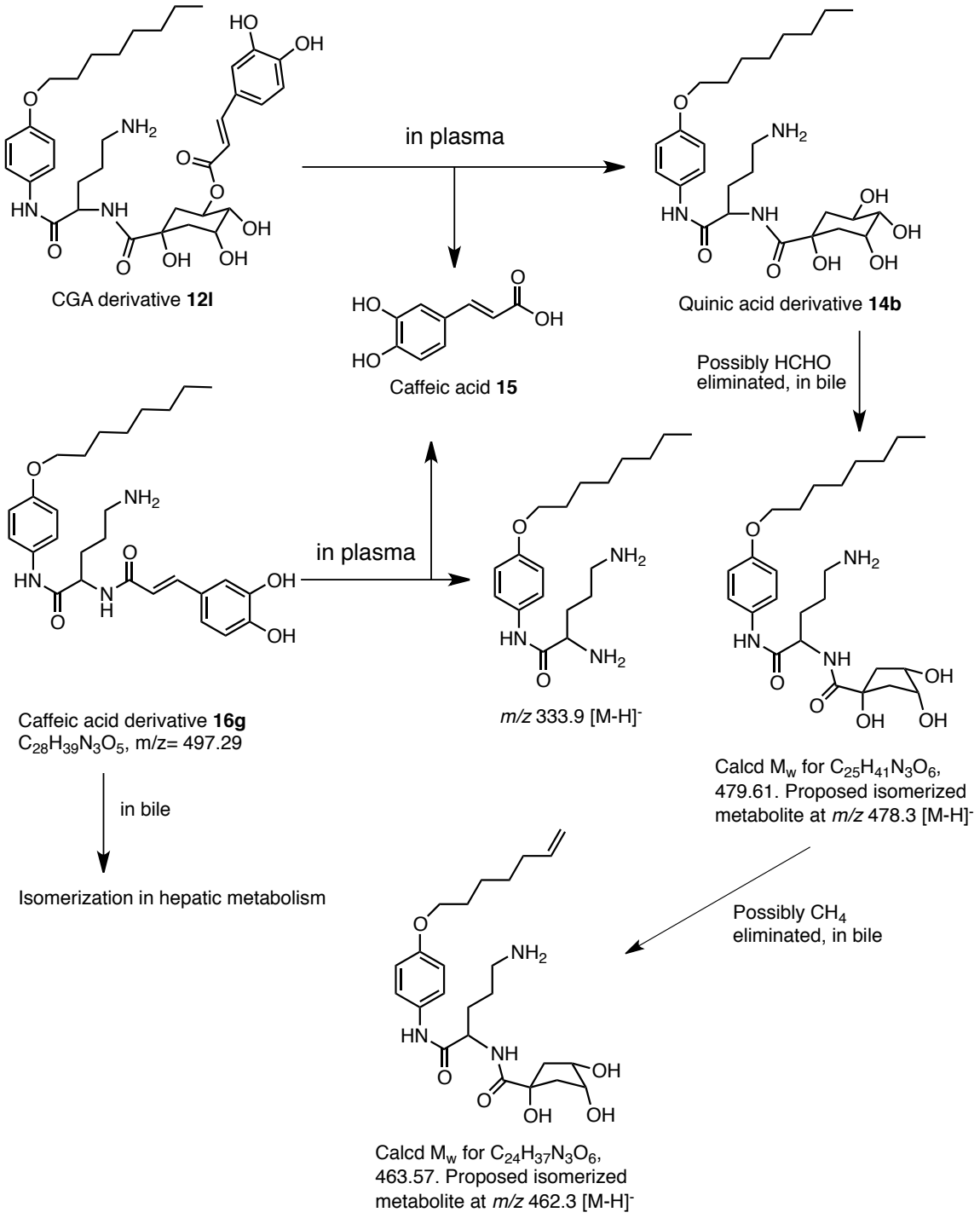


Figure 1.13. Resonance structures of amide bond (with and without double bond)



Scheme 1.4. The deduced metabolic scheme for CGA derivative **12l**, quinic acid derivative **14b**, and caffeic acid derivative **16g**, in rats. [208]

Chapter 2. Objectives

The objectives of this research project conducted by this author were as follows:

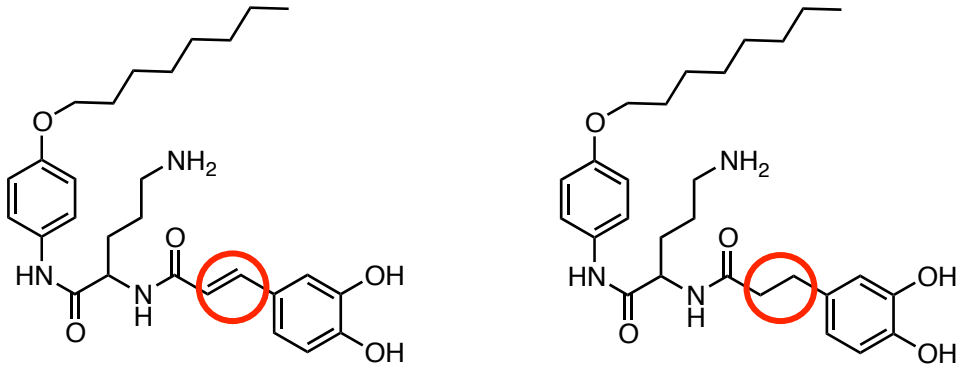
1. To repeat and refine previously synthesized caffeic acid derivative **16g**.
2. To synthesize and characterize novel caffeic acid derivatives of improved stability.
3. To evaluate the novel caffeic acid derivative synthesized.

The work described in this thesis is a part of a larger overall project and was based on previous findings, which has been conducted in our research group. The main compounds of this project were caffeic acid derivative **16g** and dihydrocaffeic acid derivative **20c** as shown in Figure 2.1.

Although the synthesis and metabolism of caffeic acid derivative **16g** had been previously reported, it was still necessary to repeat and optimize some of the synthetic details and to design better compounds. The aims of optimization were not only to improve the yield and purity of the product, but also to achieve higher efficiency and simplification.

Caffeic acid derivative **16g** was synthesized according to the method that was previously reported, [207] but with efforts made to improve it. After several attempts of using different reaction conditions and methods

to substitute the original operations, some techniques were verified to be effective in improving yield, purity and efficiency. Including inert gas (argon, nitrogen) protection to reactions, adopting longer reaction times and performing purification steps for final products were included in the optimizations undertaken.



Caffeic acid derivative **16g** Dihydrocaffeic acid derivative **20c**

Figure 2.1. Structures of caffeic acid derivative **16g** and dihydrocaffeic acid derivative **20c**

In order to characterize both caffeic acid derivative **16g** and dihydrocaffeic acid derivative **20c**, infrared (IR) vibrational spectroscopy, liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy were used to identify molecules by analysis of their constituent bonds, functional groups, determining elemental composition and providing structural and dynamic information to elucidate the chemical structures of these products.

Chapter 3. Materials and Methods

3.1 Materials

The glassware used for all of the experiments were dried overnight in an oven (100°C). Most of the reactions were carried out under argon atmosphere unless aqueous solutions were involved. The low temperature (below 0°C) required in some of the reactions was obtained by using an ice-salt bath, where sodium chloride (NaCl) or calcium chloride ($CaCl_2$) was used. A thermometer was used to check the inner temperature of the reaction mixture, making sure that it reached the desired temperature. The reaction flask was usually stirred and heated on an adjustable dual-purpose magnetic stirrer-hot plate.

Extractions of products were conducted with organic solvents followed by a drying process using anhydrous sodium sulfate (Na_2SO_4), filtering through a glass funnel with a filter paper (qualitative filter paper for technical use, medium speed) and concentrated to dryness under reduced pressure with a rotary evaporator.

Thin layer chromatography (TLC) was performed using pre-coated aluminium sheets with 0.20 mm silica gel 60 with fluorescent indicator UV_{254} (Fisher Chemical Company) to monitor the reaction progress, identify compounds present in a given mixture, and determine the purity of a substance. Caffeic and dihydrocaffeic derivatives with protecting

groups on the functional groups were examined under ultraviolet (UV) light, and unprotected derivatives were located by exposure to iodine vapor. Silica gel (60-200μm, 70-230 mesh) was used for column chromatography for isolation and purification. Due to the relatively high polarity of the compounds in this project, reversed-phase chromatography was necessary, where an octadecyl-functionalized silica gel (ODS) (200-400 mesh, Sigma-Aldrich, Inc.) was used.

Reagents:

Caffeic acid, 4-octyloxyaniline, 4-dimethylamino pyridine (DMAP), *N,N*-dimethylformamide (DMF) and all other reagents used were purchased from Sigma-Aldrich, Inc. and Tokyo Chemical Industry (TCI) Co., Ltd. The reagents that were involved in the experiments were either of American Chemical Society (ACS) reagents grade, high-performance liquid chromatography (HPLC) grade or anhydrous (99.8%). All reagents were used without further purification unless otherwise specified.

Solvents:

All solvents used in this research (except LC-MS and NMR analyses) were purchased from Caledon Laboratories Ltd., which were all reagent grade and tested by gas chromatography (GC) to be at least 99.5% in purity to meet the ACS specifications. All solvents used for LC-MS

analyses were of HPLC grade. The solvents used for NMR analyses included chloroform-*d* ($CDCl_3$) and methanol-d_4 (CD_3OD), which were all deuterated solvents with the isotopic purity of 99.96 atom% D containing 0.03% (v/v) tetramethylsilane (TMS) as internal standard for calibrating chemical shifts.

NMR Spectroscopy:

Proton nuclear magnetic resonance spectra (^1H NMR) were determined at 300 MHz on a Bruker AVANCE III 300 instrument and $CDCl_3$ and/or CD_3OD were used as solvent to dissolve the sample. ^1H shifts were referenced to residual $CDCl_3$ (δ=7.26). The signal splitting patterns were represented as follows: “s” for singlet, “d” for doublet, “t” for triplet, “q” for quartet and “m” for multiplet.

IR Spectroscopy:

The IR spectra of solid substances were conducted on a Bruker Tensor 27 FT-IR spectrometer, which allows for direct analysis of solids, liquids and powders. The results were measured in transmittance mode that is suitable even for very thin specimens. The IR spectra are very useful in identification of functional groups of molecules, which can be particularly used in verification of whether the deprotection step was successful or not.

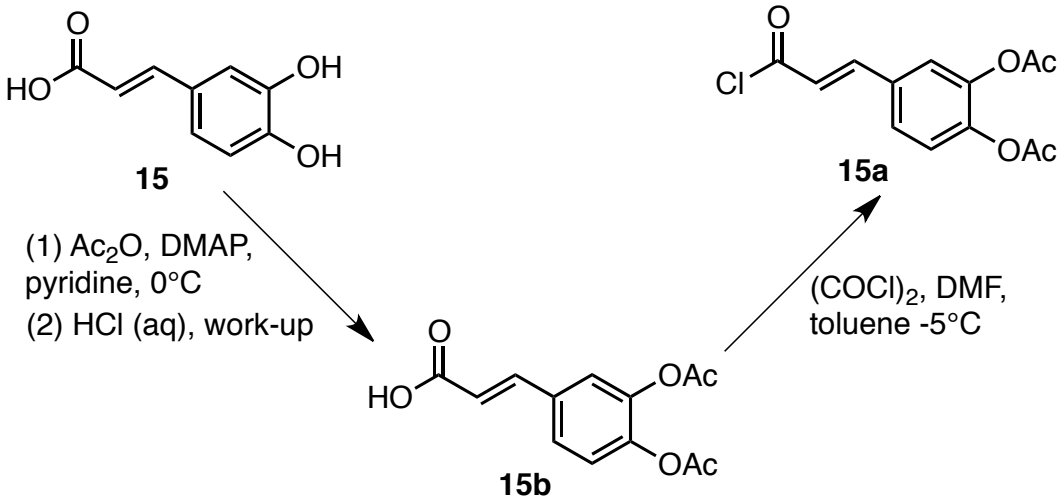
LC-MS:

The mass spectra were obtained using a LC/MSD (Trap) Agilent 1100 series SL with HPLC (1100 series) interfaced to an ion trap mass analyzer (model G2445D SL). Specifically, the HPLC is equipped with a vacuum degasser, quaternary pump, thermostatted auto sampler and UV-VIS diode array detector (wavelength range 190-900 nm), so that the data of UV absorption could be collected simultaneously. In addition, the mass spectrometry has two methods of ionization, electrospray (ESI) and atmospheric pressure chemical ionization (APCI), which can produce both monocharged and multiply-charged ions owing to the distinct mechanisms of ionization. Besides the MS mode, the LC/MSD (Trap) also has scan and multiple ion monitoring modes. Any one of the modes listed can be run in positive, negative or alternating polarity for ion detection.

3.2 Chemical synthesis

3.2.1 Synthesis of 3,4-diacetoxycinnamoyl chloride **15a**

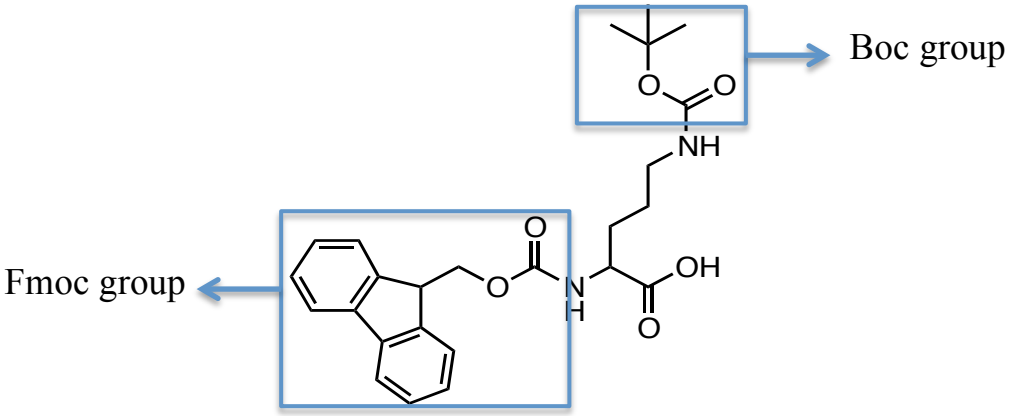
The acylation of caffeic acid **15** with acetic anhydride (≥99.5%) (Ac_2O) in pyridine to form **15b**, followed by chlorination with two equivalents of oxalyl chloride provided 3,4-diacetoxycinnamoyl chloride **15a** as shown in Scheme 3.1.



Scheme 3.1. Synthesis of 3,4-diacetoxycinnamoyl chloride **15a**.

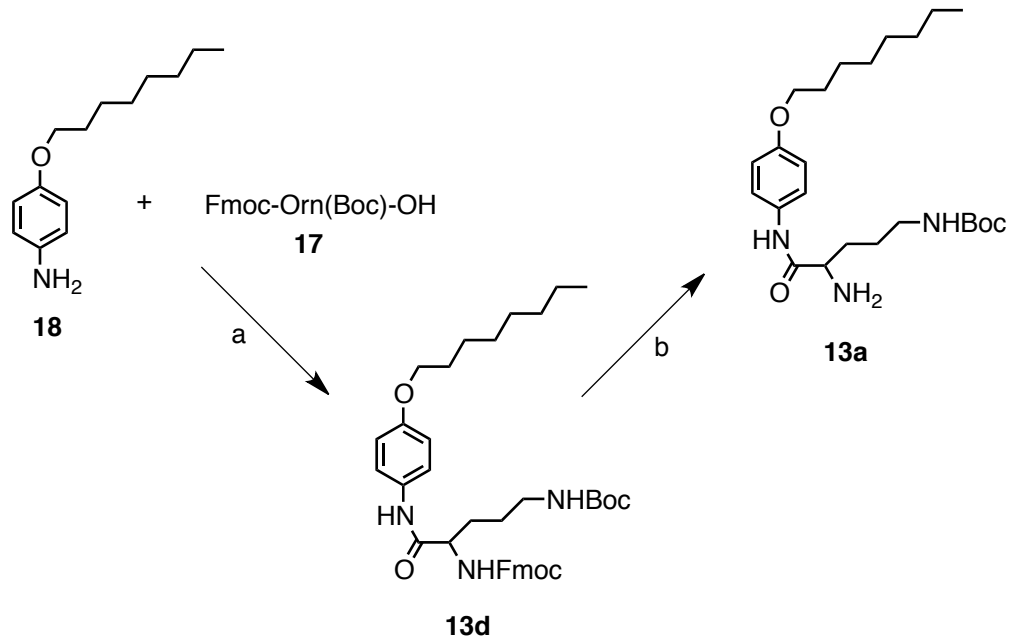
Following acetylation, extraction using ethyl acetate was performed to separate **15b** from the aqueous reaction solution, followed by drying over anhydrous Na_2SO_4, filtering and evaporating to dryness on a rotary evaporator. The subsequent reaction involved using the moisture sensitive oxalyl chloride ($(COCl)_2$) to form **15a**, which was used as shown in Scheme 1.3.

3.2.2 Synthesis of H_2N-Orn(Boc)-octyloxyaniline **13a**



17

Figure 3.1. Structure of 2-((((9H-fluoren-9-yl)methoxy)carboyl)amino)-5((*tert*-butoxycarbonyl)amino)pentanoic acid (Fmoc-Orn(Boc)-OH) **17**.



Reagents: (a) CMC, HOBt; (b) 10% Et_2NH, DMF

Scheme 3.2. Synthesis of H_2N-Orn(Boc)-octyloxyaniline **13a**

The Fmoc-protected amino acid ornithine, Fmoc-Orn(Boc)-OH **17** (Figure 3.1), was condensed with 4-octyloxyaniline **18** in the presence of 1-cyclohexyl-3-(2-morpholinoethyl) carbo-diimidemetho-*p*-toluenesulfonate (CMC, Figure 3.2) and hydroxybenzotriazole (HOBt) to obtain the amino acid derivative **13d** with the *O*-octyl lipophilic chain (Scheme 3.2). [206] The fluorenylmethyloxycarbonyl (Fmoc) and *tert*-butyloxycarbonyl (Boc) groups were used as the protecting groups for the two amino groups in ornithine (Figure 3.1). CMC, used in this reaction, is a water-soluble condensing reagent for peptide synthesis.

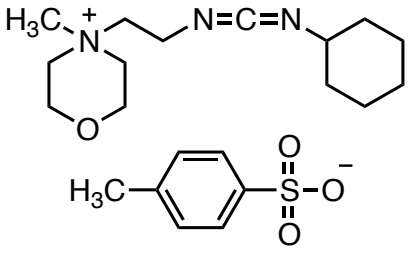
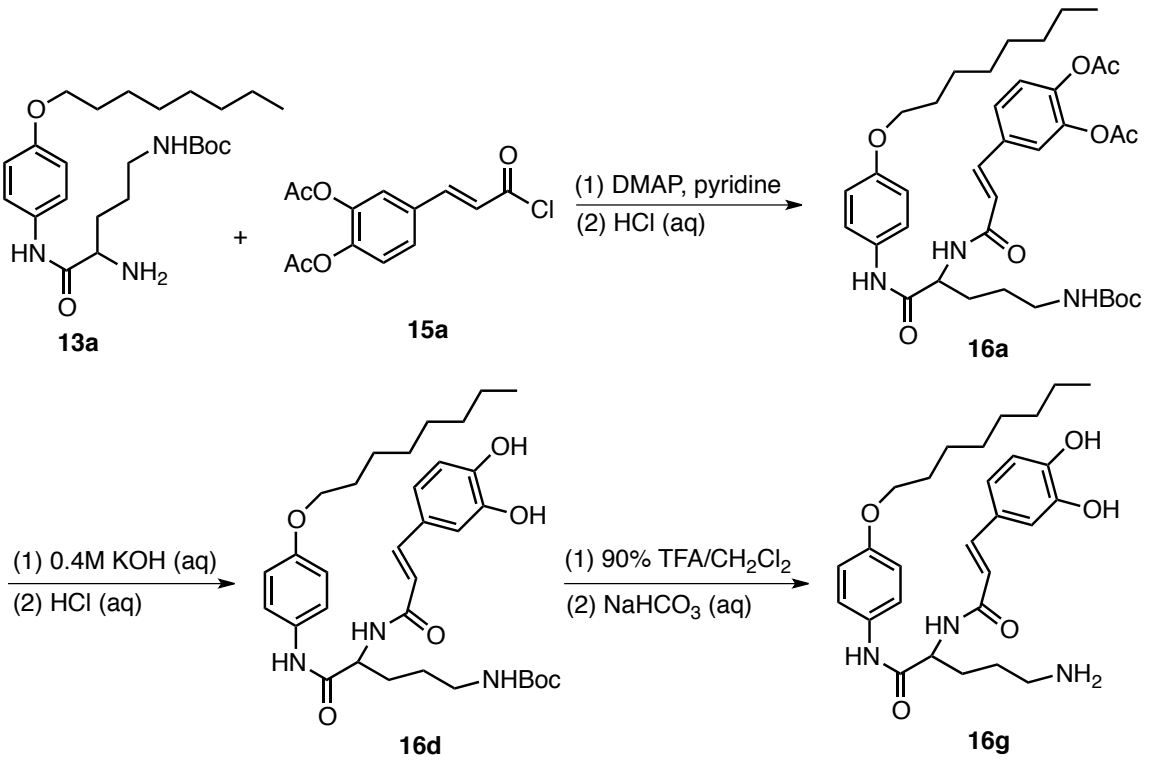


Figure 3.2. Structure of CMC

Before conducting the subsequent reactions, the product **13d** was purified from the reaction mixture by using regular silica gel column chromatography and reversed-phase column chromatography with ODS gel according to the previous findings. [206] Alternatively, another method can be used to replace the ODS column chromatography by adopting a series of washing steps to eliminate polar impurities. The compound was then treated with diethylamine (Et_2NH) for removal of the Fmoc group, to produce the product **13a** with the free secondary amino group.

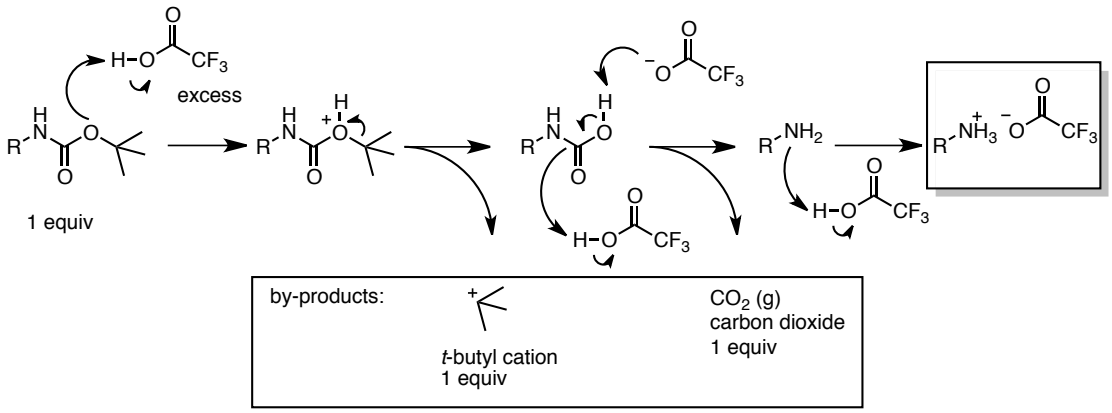
3.2.3 Synthesis of caffeic acid derivative **16g**



Scheme 3.3. Synthesis of caffeic acid derivative **16g**.

With the two compounds (3,4-diacetoxycinnamoyl chloride **15a** and H_2N-Orn(Boc)-octyloxyaniline **13a**) generated above, caffeic acid derivative **16g** with protecting groups was obtained by coupling the two reactants with DMAP in pyridine (Scheme 3.3). [207] The addition of pyridine not only provides basic conditions for the coupling reaction to occur and for the removal of generated HCl, but also helps to activate the carboxylic acid halides. The product of the coupling procedure was separated from the reaction mixture through extraction with dichloromethane (CH_2Cl_2) followed by purification using regular silica gel column chromatography before conducting the deprotection steps.

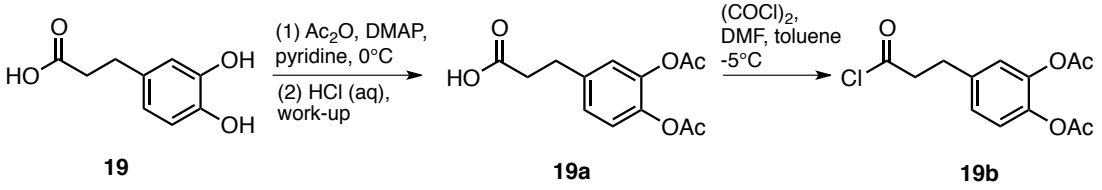
The deacetylation of **16a** to form **16d** was achieved with aqueous potassium hydroxide (KOH). The removal of the Boc group from **16d** was accomplished with trifluoroacetic acid (TFA) in CH_2Cl_2 to afford the product **16g** as the TFA salt. The mechanism of Boc deprotection with TFA is demonstrated in Scheme 3.4.



Scheme 3.4. Mechanism of Boc deprotection

In order to obtain caffeic acid derivative **16g** with a free primary amino group, the TFA salt prepared above was extracted using ethyl acetate following treatment with aqueous saturated sodium bicarbonate ($NaHCO_3$).

3.2.4 Synthesis of 3,4-diacetoxydihydro-caffeoyl chloride **19b**



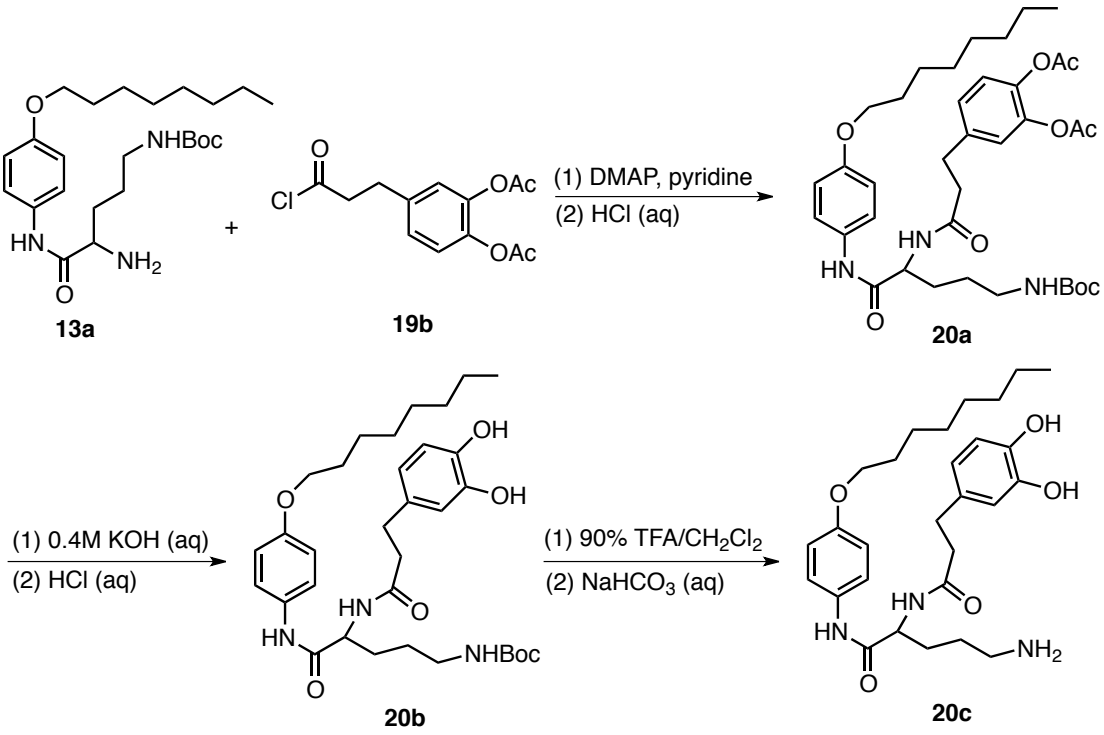
Scheme 3.5. Synthesis of 3,4-diacetoxydihydro-caffeoyl chloride **19b**

Instead of caffeic acid, 3,4-dihydroxyhydrocinnamic acid **19** is used as the starting material in this reaction. It was dissolved in pyridine, and then treated with Ac_2O at 0°C in the presence of DMAP to afford the 3,4-diacetoxydihydro-caffeoyl acid **19a**. Oxalyl chloride was then added to the solution of the product of acetylation in toluene with DMF to form product **19b**.

3.2.5 Synthesis of dihydrocaffeic acid derivative **20c**

To prepare the dihydrocaffeic acid derivative **20c**, 3,4-diacetoxydihydro-caffeoyl chloride **19b** was allowed to react with previously synthesized H_2N-Orn(Boc)-octyloxyaniline **13a** in the presence of pyridine and DMAP to afford the dihydrocaffeic acid derivative **20a**

with protecting groups, as shown in Scheme 3.6. The aqueous phase was separated from the reaction mixture and re-extracted with CH_2Cl_2.



Scheme 3.6. Synthesis of dihydrocaffeic acid derivative **20c**

The combined organic extracts were purified by regular silica gel column chromatography to yield the desired product **20c**. The polarity of this dihydrocaffeic acid derivative **20c** is different to that of caffeic acid derivative **16g**. Consequently, the difference in polarity resulted in changes in the elution solvent system needed for both TLC and column chromatography. With the same deprotection procedures as was applied for caffeic acid derivative **16g**, aqueous KOH and TFA were sequentially used to remove the acetyl and Boc groups, respectively. The TFA salt was subsequently neutralized by aqueous saturated $NaHCO_3$ to obtain dihydrocaffeic acid derivative **20c** with a free primary amino group.

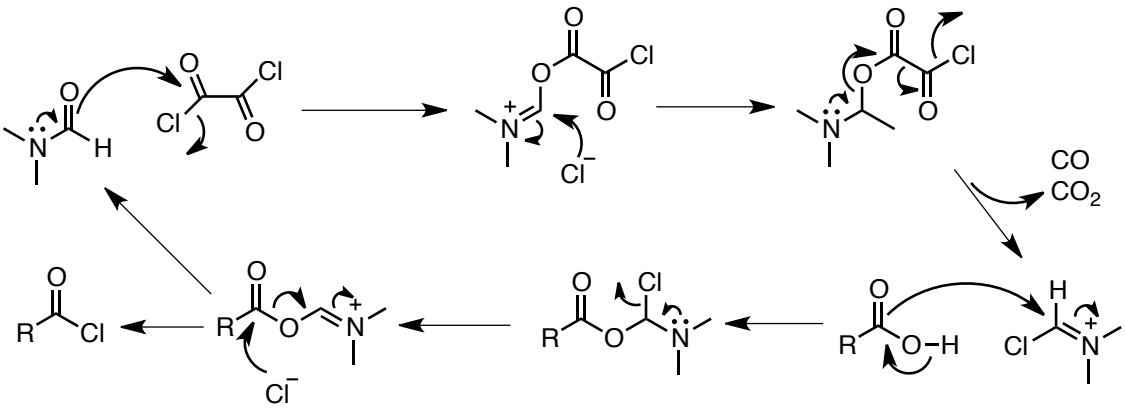
Chapter 4. Results and Discussion

4.1 Synthesis of 3,4-diacetoxycinnamoyl chloride **15a**

The attempts to reproduce procedures for acetylation went smoothly without any complications, regardless of the scale of the synthesis (from 0.2 to 5g). The chemical yield of the first several attempts was around 80%, likely due to inadequate control of temperature and rate of addition. Subsequently, the reaction was maintained at -5°C using an ice-salt bath and checking the temperature inside the reaction mixture during the dropwise addition of the acetic anhydride to the mixture, while being mixed with a magnetic stirrer. Owing to these measures, the isolated yield of **15b** was increased to 90-96%.

In order to achieve better chlorination to form **15a**, it is important to ensure the dryness of the acetylated product, since oxalyl chloride itself can react with water giving off hydrogen chloride (HCl), carbon dioxide (CO_2) and carbon monoxide (CO). As a result, the acetylated product was triturated with hexane and filtered from the mixture to afford 3,4-diacetoxycinnamoyl acid **15b** as a colorless powder, and was used immediately for the next reaction without further purification.

For the chlorination step to form **15a**, oxalyl chloride was used together with a catalytic amount of DMF. The mechanism of this reaction is illustrated in Scheme 4.1.

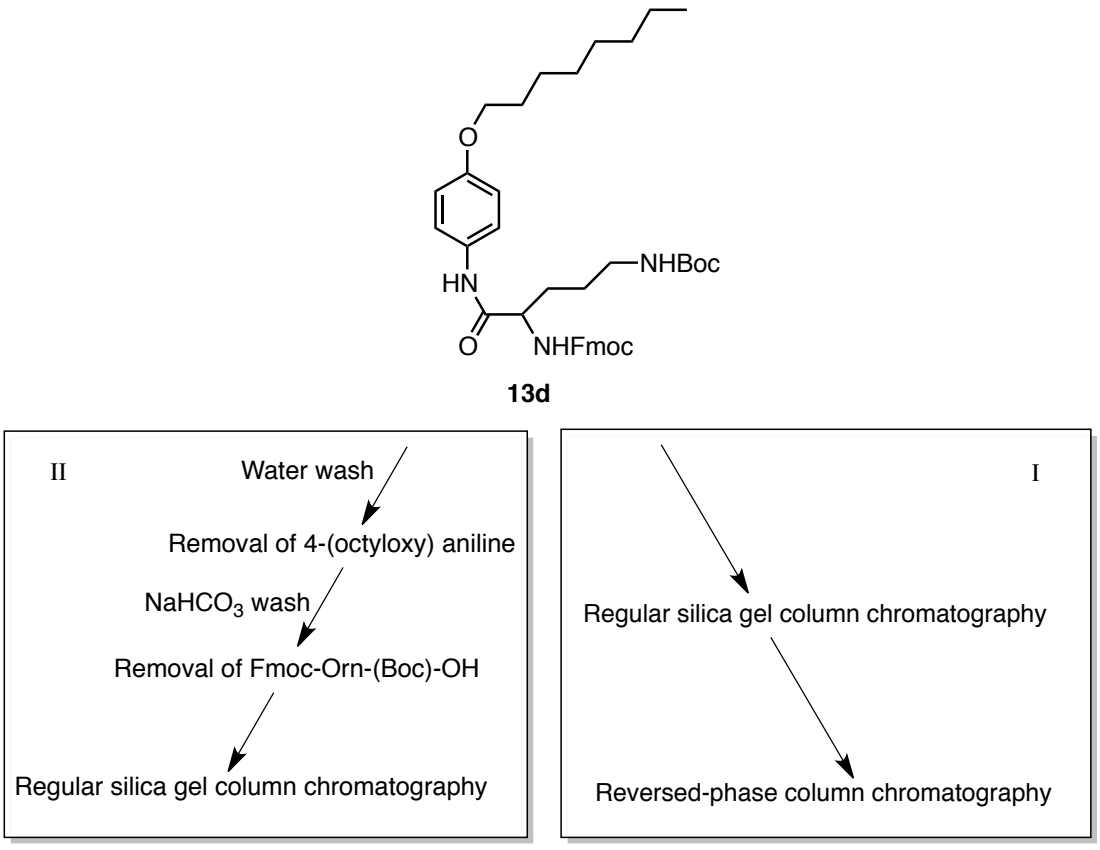


Scheme 4.1. Mechanism of acyl chloride formation with oxalyl chloride.

As above, by-products from this reaction are gaseous (CO, CO_2), which simplifies the purification of the product. Meanwhile, attention should be paid to keep the reaction from getting violent due to the generation of by-products. Thus, oxalyl chloride needs to be added dropwise at a low temperature (-5°C, ice-salt bath with NaCl or $CaCl_2$) to produce 3,4-diacetoxycinnamoyl chloride **15a** as a pale yellow powder. Toluene was used as a solvent in this reaction. In order to remove the solvent thoroughly (boiling point (Bp) being 111°C), it was necessary to triturate the product with hexane. The yield of **15a** was found to be 85%.

4.2 Synthesis of H_2N-Orn(Boc)-octyloxyaniline **13a**

The synthesis of **13a** was initially conducted according to previous findings. [206] Before the removal of the Fmoc group from **13d** to form **13a**, purification of **13d** was required. For the initial attempts, both regular silica gel column chromatography and reversed-phase column chromatography were used to separate polar and nonpolar impurities from **13d**, based on the procedures described in the literature. [206] The advantage of this technique is that a dependable purity of the product can be achieved via the use of both regular and reversed-phase chromatography. At the end of the purification, a large amount of colorless crystalline precipitate of product appeared in the collected component from the column chromatography. However, despite the good separation obtained with relatively polar impurities, the reversed-phase chromatography using ODS gel was associated with problems, such as slow elution and relatively low yields. The high mesh value (200-400) of ODS may have caused the separation to be so slow that it usually took a few days to collect all of the components. The yield of Fmoc-Orn(Boc)-octyloxyaniline **13d** was found to be 53% (after the use of both regular and reversed-phase chromatography).



Scheme 4.2. Preparation of Fmoc-Orn(Boc)-octyloxyaniline **13d** with the previously reported method and newly developed technique.

Being aware of the possibility of improvements to the synthetic procedure used, several techniques were tested to remove the polar impurities in this reaction. Eventually, one of the methods was verified to be effective by providing satisfactory separation results in a shorter period of time. In this method, the reversed-phase chromatography was substituted with two washing procedures "II" as shown in Scheme 4.2.

Due to the difference between the product and impurities in their reactivity with $NaHCO_3$ and their solubility in aqueous solution, the washing procedures were able to separate polar impurities from the

product. In addition, the process only took less than an hour, which makes it much more time-saving than the ODS column chromatography. After the optimization, the yield of Fmoc-Orn(Boc)-octyloxyaniline **13d** was increased to 76% (after purification).

Generally, the Fmoc group is susceptible to removal using weak bases, such as piperidine or piperazine. The removal also occurs to be more rapid in a relatively polar medium like DMF, compared to a relatively nonpolar one, such as CH_2Cl_2. The subsequent development of the procedures for the cleavage of Fmoc group has led to milder alternatives, by replacing piperidine with the use of Et_2NH in DMF. Although the polarity of DMF is helpful for deprotection of the Fmoc group, its high Bp (153°C) was an obstacle to obtain a dry product. As a result, DMF, together with the cleavage reagent, were washed out very carefully after Fmoc removal using water to afford the product as a pale yellow solid. The yield for the deprotection step to form product **13a** was found to be 87%.

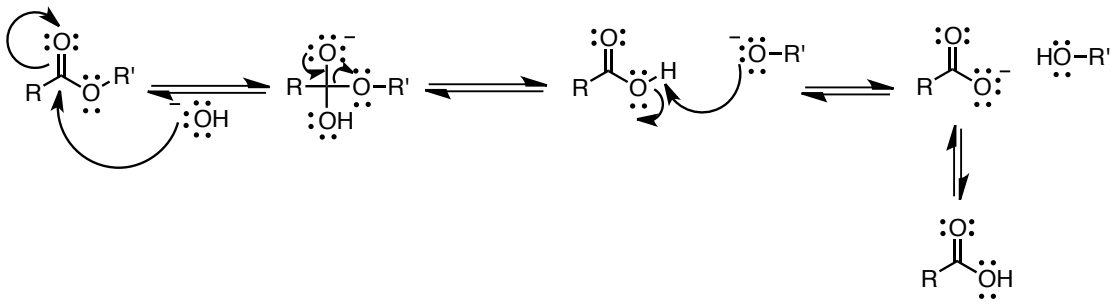
4.3 Synthesis of caffeic acid derivative **16g**

Based upon the procedures described in the literature, [207] a few details have been optimized to carry out the coupling reaction in this study. The reaction of 3,4-diacetoxycinnamoyl chloride **15a** with H_2N-

Orn(Boc)-octyloxyaniline **13a**, in the presence of DMAP and pyridine in CH_2Cl_2 was conducted under Argon atmosphere.

After the complete consumption of H_2N-Orn(Boc)-octyloxyaniline **13a** as monitored by TLC, the reaction mixture was acidified and subjected to extraction with CH_2Cl_2. The concentrated residue, which was a yellow oil, was then purified by silica gel column chromatography. By using gradient elution (hexane-ethyl acetate), the eluent was collected in a series of fractions, so that different compounds can be separated effectively. Along with the increase in the proportions of ethyl acetate to hexane, colorless precipitates started to appear in some of the fractions. TLC was used to identify the compounds that were collected in the different fractions. It turned out that all of the fractions with precipitation contained the same compound, namely the desired product **16a**, the Boc-acetyl caffeic acid derivative **16g**. The yield of product **16a** obtained was determined to be 93%.

To remove the acetyl protecting groups on the phenolic hydroxy groups, aqueous KOH was used and the mechanism for this reaction is shown in Scheme 4.3.



Scheme 4.3. Mechanism for the base-catalyzed hydrolysis of acetoxy group.

As a protecting group, the acetyl group is stable to acid and mild base, but is not compatible with strong nucleophiles. In this study, compound **16a** was treated with aqueous KOH solution in a mixture of methanol and tetrahydrofuran (THF) to remove the protecting groups, followed by acidification to form **16d**, the mono-Boc protected compound.

In the initial attempts, the reaction mixture was stirred at room temperature for one hour followed by acidification with aqueous HCl. However, the reaction time was found to be too short for the deprotection process to complete. Consequently, after several experiments, the reaction time was extended, so that the reaction mixture was stirred overnight, to afford the complete removal of acetyl groups. The results were verified by IR and LC-MS. The yield of product **16d** was 70%.

When it comes to the removal of the Boc group, it could be accomplished by using neat TFA as described in the literature. [207] However, it was found to be difficult, even with sodium hydroxide, to neutralize the excess of TFA to obtain product **16g** with free amino group. The removal of the Boc group with neat TFA was compared with using 55% TFA in CH_2Cl_2, and was found to obtain a better purity of product. [209] Therefore, the procedures for removal of the Boc group in compound **16d** were modified to allow the product to be dissolved in a minimum amount of CH_2Cl_2, followed by addition of TFA. The excess of TFA was then neutralized with aqueous saturated $NaHCO_3$ solution. After

extraction from the aqueous solution, and the organic solution was concentrated to dryness, the yield of caffeic acid derivative **16g** was found to be 89%. Due to the high polarity of the product, it could be completely dissolved in a mixture of $CDCl_3$ and CD_3OD, or CD_3OD alone, for NMR spectroscopy.

4.4 Synthesis of 3,4-diacetoxydihydro-caffeoyl chloride **19b**

By using a different starting material, 3,4-dihydroxyhydrocinnamic acid **19**, instead of caffeic acid, a novel compound without a double bond beside the carbonyl group was able to be obtained. The difference in structure also resulted in a change in the polarity of the molecule, which further led to changes in some of the experimental phenomena.

For the acetylation of 3,4-dihydroxyhydrocinnamic acid **19**, acetic anhydride was added into the reaction mixture at 0°C that was achieved with the use of an ice-salt bath (NaCl) for cooling, and the monitoring inner temperature of the reaction mixture was conducted with a thermometer. After extraction of the reaction mixture with ethyl acetate and THF, the organic solvent was removed by a rotary evaporator, to produce 3,4-diacetoxydihydro-caffeoyl acid **19a**. The isolated product **19a** was a pale yellow solid. Its yield after drying in a vacuum oven was determined to be 95%.

To prepare the corresponding di-*O*-acetylated dihydrocaffeoyl chloride, compound **19a** was suspended in toluene with a few drops of DMF. Oxalyl chloride was added dropwise into the reaction mixture. Owing to the generation of gaseous by-products, this reaction should not be performed in a closed system and the temperature should be carefully kept constant at -5°C. The resulting solution was clear and pale yellow when all the materials dissolved and reacted as monitored by TLC. After being concentrated under reduced pressure, a colorless crystalline precipitate appeared in the flask. After drying overnight in the vacuum oven, the yield of product **19b** was found to be 83%.

4.5 Synthesis of dihydrocaffeic acid derivative **20c**

The preparation of Boc-acetyl dihydrocaffeic acid derivative **20a** was carried out by allowing di-*O*-acetylated dihydrocaffeoyl chloride **19b** generated previously to react with H_2N-Orn(Boc)-octyloxyaniline **13a** in CH_2Cl_2 with DMAP in pyridine under argon atmosphere. After stirring overnight at ambient temperature, aqueous HCl was added into the mixture to remove the remaining reactants (pyridine, DMAP). The acidification turned the mixture cloudy, due to the poor solubility of the product in water. After extraction of the reaction mixture with CH_2Cl_2 and drying the extract in the usual way with anhydrous Na_2SO_4 and filtering, the solution turned clear. Then the clear yellow solution was

subjected to rotary evaporation to remove the solvent and afford the residue as light brown oily and viscous crude product.

This residue was dissolved with a minimum amount of CH_2Cl_2 and was purified by a regular silica gel chromatography using gradient elution (from hexanes to hexanes-ethyl acetate) to afford Boc-acetyl dihydrocaffeic acid derivative **20a** as a pale brown viscous solid. The yield of compound **20a** was determined to be 57%, after purification. This yield was not very impressive, which could be due to inadequate reaction time of the coupling step and/or unsatisfactory separation results from the silica column chromatography.

For the removal of the acetyl groups in the compound **20a**, several attempts were made to determine the most favorable reaction time for the deprotection process. With the compound **20a** dissolved in a mixture of methanol and THF, aqueous KOH solution was still used to restore the hydroxy groups. According to the literature procedure, [207] the reaction mixture was stirred for only one hour at room temperature, which was found to be too short in the present work.

Therefore, in the initial attempt, the reaction mixture of Boc-acetyl dihydrocaffeic acid derivative **20a** with the base was stirred for two hours at room temperature, followed by acidification with aqueous HCl and extraction with chloroform. However, the resulting yield of **20b** was only 34%, indicating insufficient time to complete the deprotection.

Consequently, the reaction time was extended to three hours, but the yield was still not good enough as 57%. Eventually, after the addition of aqueous KOH, the mixture was stirred for overnight to ensure adequate time for deprotection. The resulting yield of **20b** was found to be improved to 74%. During the experimental development, it was critical to have the correct analytic method to determine the completion of deprotection process. In this case, IR spectroscopy was the most efficient technique to verify the removal of the acetyl groups, which was indicated by the reappearance of an absorption peak of hydroxy groups at ~3330 cm^{-1} (broad, s) in IR spectrum (see A-16 and A-20).

The dihydrocaffeic acid derivative with Boc group **20b** was then dissolved in CH_2Cl_2 and treated with TFA for 1 hour to remove the Boc group. Due to the exothermic nature of this reaction, an ice bath should be applied during the addition of TFA to control the temperature. In order to reduce the consumption of the aqueous $NaHCO_3$ solution to neutralize the excessive TFA, the reaction mixture was directly subjected to rotary evaporation to remove some of the TFA (Bp: 72.4°C). By adding aqueous saturated $NaHCO_3$ solution, the pH of the mixture was changed to 8 as measured with pH test strips.

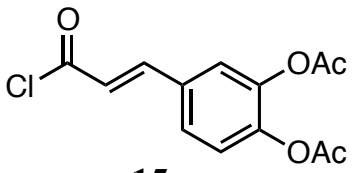
Owing to the high polarity of the resulting compound, ethyl acetate was used to extract the product from the aqueous phase. After concentration of the solvent to dryness, the yield of dihydrocaffeic acid

derivative **20c** was found to be 93%. For the compound's characterization, CD_3OD and methanol were used respectively to dissolve the product for NMR and LC-MS tests.

While the current work has indicated the synthesis of caffeic acid and dihydrocaffeic acid derivatives and their potential as antifungal agents, there are many directions for further development of this work. Further chemical modifications of caffeic acid and dihydrocaffeic acid derivatives may be carried out to achieve better activity and stability. More importantly, proper stability tests should be conducted to determine if the dihydrocaffeic acid derivative has better stability than caffeic acid derivatives. For biological tests, the evaluation of the products should be conducted with both the BDG synthase inhibition assay (with fungal membrane enzyme) and antifungal assay (*in vitro* cell-based).

Chapter 5. Experimental Procedures and Characterization

5.1 Synthesis of 3,4-diacetoxycinnamoyl chloride **15a**

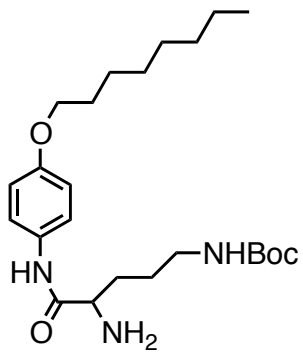


15a

To a solution of caffeic acid **15** (900 mg≅5.00 mmol) and DMAP (15.8 mg≅0.125 mmol) in pyridine (2.5 mL) was added acetic anhydride (1.2 mL≅13 mmol) at 0 °C. The reaction mixture was stirred at room temperature under argon atmosphere overnight. The resultant mixture (clear, almost colorless) was then poured onto crushed ice (turned cloudy right away). The aqueous phase was acidified with aqueous 2.0 M HCl to change the pH to about 2 (even more cloudy). The mixture was extracted with ethyl acetate/THF (3:1; 3 × 30 mL) and organic parts were collected (clear, colorless). The combined extracts were dried over anhydrous Na_2SO_4 and filtered. The solution was then subjected to evaporation on a rotary evaporator (Bp: 50-60 °C) and the residue was triturated with small amount of hexane to afford 3,4-diacetoxycinnamic acid **15b** (1.28 g ≅4.84 mmol; 96%) as a colorless powder. This product was used directly for the next reaction without further purification.

The di-*O*-acetylated caffeic acid **15b** was suspended in toluene (25 mL) with 3 drops of DMF (cloudy, milky white). Being kept in an ice-salt bath (NaCl and/or $CaCl_2$), oxalyl chloride (1.0 mL≅12 mmol) was added dropwise at -5 °C to the mixture. After stirring for 3 h at room temperature, all materials were dissolved resulting in a clear and pale yellow solution. Solvent and unreacted oxalyl chloride were remove with rotary evaporator under reduced pressure (Bp: 70-75 °C). The residual yellowish product was triturated with hexane containing a few drops of ethyl acetate to yield 3,4-diacetoxycinnamoyl chloride **15a** as a pale yellow powder (1.16 g≅4.10 mmol; 85%). ^{1}H NMR ($CDCl_3$) data (see A-2 for spectrum): δ = 7.72 (d, *J* = 15.8 Hz, 1H), 7.45 (d, *J* = 2.1 Hz, 1H), 7.43 (d, *J* = 2.1 Hz, 1H), 7.25 (d, *J* = 8.2 Hz, 1H), 6.43 (d, *J* = 16.0 Hz, 1H), 2.32 (s, 3H), 2.31 (s, 3H). APCI-MS (positive) *m/z* (see A-1): 284 (M+H), 282, 247, 205, 163.

5.2 Synthesis of H_2N-Orn(Boc)-octyloxyaniline **13a**



13a

A mixture of Fmoc-Orn(Boc)-OH **17** (1.82 g≅4.00 mmol), 4-octyloxyaniline (0.890 g≅4.00 mmol), HOBt (0.820 g≅6.00 mmol) and CMC (2.54 g≅6.00 mmol) in 30.0 mL DMF was stirred at room temperature overnight.

Previous method:

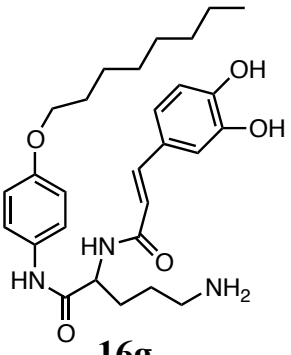
The resultant mixture was directly chromatographed on a silica gel column. After running the column with hexanes for several times to remove nonpolar impurities (checked by TLC), the column was eluted with chloroform to collect all components remaining. The chloroform eluted part was then re-chromatographed on a ODS column using gradient elution (from water neat to water : methanol = 1:9) to afford Fmoc-Orn(Boc)-octyloxyaniline **13d** as a colorless powder (1.42 g≅2.15 mmol, 53.8%).

Optimized method:

After stirring overnight, the mixture was diluted with 160 mL ethyl acetate, then washed by water (4 × 40 mL), saturated $NaHCO_3$ solution (4 × 40 mL), and dried with anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (continuous gradient from hexanes : ethyl acetate = 9:1 to ethyl acetate) to yield Fmoc-Orn(Boc)-octyloxyaniline **13d** (2.01 g ≅3.05 mmol, 76.3%).

The resulting product **13d** (2.01 g≅3.05 mmol) was then treated with 3.0 mL Et_2NH in 15 mL DMF at ambient temperature for 2 hours. The resultant mixture was diluted with 80 mL ethyl acetate, then washed with water (6 × 20 mL) and dried over anhydrous Na_2SO_4. After concentration of solvent to dryness, the H_2N-Orn(Boc)-octyloxyaniline **13a** (1.16 g ≅2.66 mmol, 87%) was used for further reaction without purification.

5.3 Synthesis of caffeic acid derivative **16g**



16g

To a solution of H_2N-Orn(Boc)-octyloxyaniline **13a** (0.770 g≅1.75 mmol) and DMAP (0.0800 g≅0.650 mmol) in CH_2Cl_2 (12 mL) and pyridine (4.5 mL), 3,4-diacetoxycinnamoyl chloride **15a** (1.13 g≅4.00 mmol) was added at room temperature. The reaction mixture (pale yellow solution) was stirred overnight under argon atmosphere. After checking the reaction progress with TLC, the pH value of the reaction mixture was changed to 3 by adding aqueous 1.0 M HCl. The aqueous phase was then extracted with more CH_2Cl_2 (3-4 times). The combined organic extracts were dried with anhydrous Na_2SO_4, filtered, and concentrated on a rotary

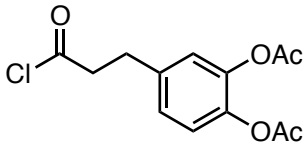
evaporator (Bp: 35-40°C). The residue was chromatographed on a silica gel column using gradient elution (10-90% ethyl acetate in hexanes) to afford caffeic acid derivative **16a** (1.12 g≅1.64 mmol, 93%) as a colorless powder. ^{1}H NMR ($CDCl_3$) data (see A-3 for spectrum): δ = 7.59 (m, 1H), 7.40 (m, 3H), 7.26 (d, *J* = 1.6 Hz, 1H), 7.17 (d, *J* = 8.4 Hz, 1H), 6.83 (d, *J* = 9.0 Hz, 2H), 6.37 (d, *J* = 15.9 Hz, 2H), 4.83 (s, 1H), 3.91 (t, *J* = 6.6 Hz, 2H), 3.49 (m, 1H), 3.15 (m, 1H), 2.31 (m, 2H), 2.29 (s, 3H), 2.28 (s, 3H), 1.59-1.82 (m, 6H), 1.43 (m, 9H), 1.28 (m, 8H), 0.87 (m, 3H), and NH signal. APCI-MS (positive) *m/z* (rel. intensity) (A-4): 682 (85), 664(100); APCI-MS (negative) (A-5): 680 (100).

The caffeic acid derivative **16a** (0.620 g≅0.900 mmol) was dissolved in THF (2.6 mL) and methanol (2.6 mL). To the mixture was added aqueous-methanolic 0.2 M KOH (water : methanol = 9:1, 3.0 mL). The reaction mixture was stirred overnight at ambient temperature. The mixture was then acidified with aqueous 1.0 M HCl (2.0 mL), followed by extraction with chloroform (3 times). The combined chloroform extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to yield caffeic acid derivative **16d** (0.370g≅0.620 mmol; 70%). ^{1}H NMR (CD_3OD) data (see A-6 for spectrum): δ = 7.61 (s, 1H), 7.55 (d, *J* = 4.8 Hz, 2H), 7.44 (d, *J* = 4.2 Hz, 1H), 7.06 (d, *J* = 2.1 Hz, 1H), 6.92 (s, 2H), 6.81 (d, *J* = 8.2 Hz, 2H), 6.22 (d, *J* = 15.9 Hz, 1H), 4.61 (s, 1H), 3.95 (t, *J* = 6.5 Hz, 2H), 3.38 (m, 1H),

3.13 (m, 1H), 2.32 (m, 2H), 1.94-1.61 (m, 6H), 1.43 (m, 10H), 1.30 (m, 9H), 0.88 (t, *J* = 6.5 Hz, 3H). APCI-MS (positive) *m/z* (rel. intensity) (A-7): 598.3 (100, $[M+H]^-$); APCI-MS (negative) (A-8): 596 (100, $[M-H]^-$).

The caffeic acid derivative **16d** with Boc group (0.370 g≅0.620 mmol) was dissolved in 15 mL CH_2Cl_2. While stirring with an ice bath, 2.0 mL TFA was added to the solution. The reaction mixture was then stirred at room temperature for 1 hour, followed by removal of unreacted TFA under reduced pressure (Temp: room temperature). To the residue was added 3.0 mL saturated $NaHCO_3$ solution to change the pH to around 8. The aqueous phase was extracted with ethyl acetate (3 times). The combined organic extracts were concentrated to dryness to obtain caffeic acid derivative **16g** (0.270 g≅0.550 mmol; 89%). ^{1}H NMR (CD_3OD) data (see A-9 for spectrum): δ = 7.42 (d, *J* = 3.0 Hz, 2H), 7.28 (d, *J* = 4.6 Hz, 1H), 6.94 (s, 1H), 6.77 (d, *J* = 8.2 Hz, 2H), 6.68 (s, 1H), 6.30 (d, *J* = 15.9 Hz, 1H), 4.62 (s, 1H), 3.94 (t, *J* = 6.4 Hz, 2H), 3.75 (d, *J* = 1.5Hz, 2H), 2.99 (s, 2H), 1.93 (m, 1H), 1.75 (m, 5H), 1.43 (m, 2H), 1.23-1.37 (m, 8H), 0.89 (t, *J* = 6.7 Hz, 3H), and some OH and NH signals. APCI-MS (positive) *m/z* (rel. intensity) (A-10): 498.3 (100, $[M+H]^-$); APCI-MS (negative) (A-11): 496.2 (100, $[M-H]^-$).

5.4 Synthesis of 3,4-diacetoxydihydro-caffeoyl chloride **19b**



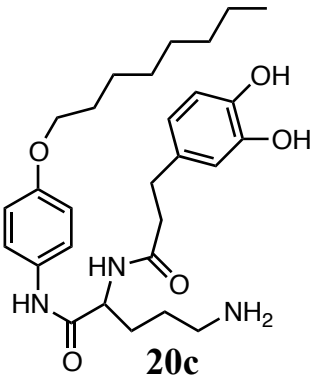
19b

To a solution of 3,4-dihydroxyhydrocinnamic acid **19** (1.82 g≅10.0 mmol) and DMAP (0.0300 g≅0.250 mmol) in pyridine (5.0 mL) was added acetic anhydride (2.4 mL≅25 mmol) dropwise at 0 °C. The reaction mixture was stirred overnight at room temperature under argon atmosphere. The resultant mixture (pale yellow, not clear) was then poured onto crushed ice (turned cloudy quickly). The aqueous phase was acidified with aqueous 2.0 M HCl to change the pH to about 2 (even more cloudy). The mixture was then extracted with ethyl acetate/THF (3:1; 3 × 30 mL) and organic parts were collected (clear, pale yellow). The combined extracts were dried over Na_2SO_4 and filtered. After the removal of the solvent on a rotary evaporator, 3,4-diacetoxydihydro-caffeoyl acid **19a** (2.38 g≅9.51 mmol; 95%) was obtained and immediately used for subsequent reaction.

The 3,4-diacetoxydihydro-caffeoyl acid **19a** (2.38 g≅9.51 mmol) was suspended in toluene (50 mL) with 5 drops of DMF (cloudy, milky white). Being kept in an ice-salt bath (NaCl and/or $CaCl_2$), oxalyl chloride (1.8 mL≅20 mmol) was added dropwise at -5 °C to the mixture. The reaction mixture was stirred for overnight at room temperature. The

resultant mixture was then subjected to rotary evaporation to remove the solvent and unreacted oxalyl chloride (Bp: 75-80 °C). The product was triturated with hexane containing a few drops of ethyl acetate to yield 3,4-diacetoxydihydro-caffeoyl chloride **19b** (2.27 g≅7.98 mmol; 83%). ^{1}H NMR ($CDCl_3$) data (see A-12 for spectrum): δ = 7.17 (dtd, *J* = 7.2, 1.5, 0.7 Hz, 1H), 7.04 (d, *J* = 1.2 Hz, 1H), 6.96 (t, *J* = 1.2 Hz, 1H), 2.99 (d, *J* = 7.4 Hz, 2H), 2.66 (dd, *J* = 4.8, 1.0 Hz, 2H), 2.28 (t, *J* = 1.4 Hz, 6H).

5.5 Synthesis of dihydrocaffeic acid derivative **20c**



20c

To a solution of H_2N-Orn(Boc)-octyloxyaniline (1.49 g≅3.42 mmol) and DMAP (0.140 g≅1.13 mmol) in CH_2Cl_2 (20 mL) and pyridine (8.0 mL), 3,4-diacetoxydihydro-caffeoyl chloride (2.27 g≅7.98 mmol) was added at room temperature. The reaction mixture (dark yellow solution) was stirred overnight under argon atmosphere. The resultant mixture was acidified with aqueous 1.0 M HCl to change the pH to about 3 (more cloudy). The aqueous phase was extracted with more CH_2Cl_2. The combined organic extracts were dried over anhydrous Na_2SO_4, filtered,

and concentrated on a rotary evaporator (Bp: 35-40°C). Regular silica gel column chromatography (continuous gradient from hexanes to hexanes : ethyl acetate = 1:9) was carried out to purify the residue and afford dihydrocaffeic acid derivative **20a** (1.32 g≅1.93 mmol; 57%). ^{1}H NMR ($CDCl_3$) data (see A-13 for spectrum): δ = 7.41 (d, *J* = 8.8 Hz, 1H), 7.14 (d, *J* = 1.6 Hz, 1H), 7.10 (d, *J* = 1.1 Hz, 2H), 7.04 (d, *J* = 4.2 Hz, 2H), 6.94 (m, 2H), 6.83 (m, 2H), 4.76 (d, *J* = 29.9 Hz, 2H), 3.92 (t, *J* = 6.6 Hz, 2H), 3.30 (m, 1H), 3.04 (m, 1H), 2.95 (m, 2H), 2.88 (d, *J* = 7.2 Hz, 1H), 2.70 (d, *J* = 4.8 Hz, 1H), 2.28 (s, 6H), 1.79-1.49 (m, 3H), 1.43 (d, *J* = 4.0 Hz, 9H), 1.16 (m, 8H), 0.87 (t, *J* = 6.9 Hz, 3H), and NH signals. APCI-MS (positive) *m/z* (rel. intensity) (A-14): 684 (43, $[M+H]^-$), 584 (100); APCI-MS (negative) (A-15): 682 (57, $[M+H]^-$), 640 (69), 598 (100).

The dihydrocaffeic acid derivative **20a** (0.200 g≅0.290 mmol) was dissolved in 1.0 mL THF and 1.0 mL methanol (clear, yellow solution). Aqueous-methanolic 0.2 M KOH (water : methanol = 9:1, 1.4 mL) was added to the mixture. The reaction mixture was then stirred overnight at room temperature. The resultant mixture was acidified with aqueous 1.0 M HCl (1.0 mL), followed by extraction with chloroform (3 times). The combined chloroform extracts were dried with anhydrous Na_2SO_4 (clear, dark yellow). After filtration and removal of the solvent, dihydrocaffeic acid derivative **20b** was obtained as brown solid (0.130 g≅0.220 mmol; 74%). ^{1}H NMR ($CDCl_3$) data (see A-17 for spectrum): δ = 7.11-6.77 (m,

1H), 6.73 (dd, *J* = 9.7, 3.0 Hz, 2H), 6.60 (dd, *J* = 8.1, 2.0 Hz, 1H), 4.72 (m, 1H), 3.96 (m, 1H), 3.89 (t, *J* = 6.4 Hz, 1H), 3.26 (m, 1H), 2.94 (m, 2H), 2.82 (t, *J* = 7.7 Hz, 1H), 2.57 (m, 1H), 1.82-1.64 (m, 3H), 1.43 (m, 9H), 1.35-1.20 (m, 5H), 1.09 (td, *J* = 7.1, 3.1 Hz, 1H), 0.88 (m, 3H), and NH signals. APCI-MS (positive) *m/z* (rel. intensity) (A-18): 600 (48, $[M+H]^-$), 610 (100); APCI-MS (negative) (A-19): 598 (100, $[M+H]^-$), 608 (48).

The dihydrocaffeic acid derivative **20b** (0.0500 g≅0.0800 mmol) was dissolved in 2.0 mL CH_2Cl_2. To the solution, 0.3 mL TFA was added in the presence of an ice bath. After stirring at room temperature for 1 hour, the reaction mixture was then subjected to rotary evaporation to remove unreacted TFA and solvent under reduced pressure (Temp: room temperature). To the residue was added 0.3 mL aqueous saturated $NaHCO_3$ solution to change the pH to around 8. The aqueous phase was extracted with ethyl acetate (3 times). The combined organic extracts were concentrated to dryness to afford dihydrocaffeic derivative **20c** (0.0400 g≅0.0700 mmol; 88%). ^1H NMR (CD_3OD) data (see A-21 for spectrum): δ = 6.93 (m, 2H), 6.67 (m, 2H), 6.65 (d, *J* = 1.3 Hz, 1H), 6.53 (d, *J* = 2.1 Hz, 1H), 6.50 (m, 1H), 3.99 (m, 1H), 3.93 (t, *J* = 6.4 Hz, 2H), 3.35 (s, 1H), 2.93 (m, 1H), 2.57 (m, 5H), 1.89 (m, 1H), 1.78-1.53 (m, 2H), 1.48-1.26 (m, 10H), 1.23-1.01 (m, 2H), 0.89 (m, 3H) and some OH, NH

signals. APCI-MS (positive) *m/z* (rel. intensity) (A-22): 500 (82, $[M+H]^-$), 510 (100); APCI-MS (negative) (A-23): 498 (100, $[M+H]^-$), 508 (30).

References

[1] **U.S. National Institutes of Health**. Understanding microbes in sickness and in health. *NIH Publication,* **2009**, No. 09-4914

[2] **Taylor T., Krings M., Taylor E.** How old are the fungi. *Fossil Fungi*, **2015**, 27-39

[3] **Hall L.** Keeping up with emerging fungal infections. *Clinical Microbiology Newsletter*, **2013**, 35:22

[4] **Heitman P.** Microbial pathogens in the fungal kingdom. *Fungal Biology Reviews*, **2011**, 25(1): 48-60

[5] **Barron S.** Medical microbiology (4th edition). *University of Texas Medical Branch at Galveston*, **1996**

[6] **Low C., Rotstein C.** Emerging fungal infections in immunocompromised patients. *F1000 Medicine Reports*, **2011**, 3:14

[7] **Walker D.H., McGinnis M.R.** Diseases caused by fungi. *Pathobiology of Human Disease*, **2014**, 217-221

[8] **Rees J.R., Pinner R.W., Hajjeh R.A., Brandt M.E., Reingold A.L.** The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992–1993: results of population-based laboratory active surveillance. *Clinical Infectious Diseases*, **1998**, 27(5): 1138-1147

[9] **Hajjeh R.A., Sofair A.N., Harrison L.H., Lyon G.M., Arthington-Skaggs B.A., Mirza S.A., Phelan M., Morgan J., Lee-Yang W., Ciblak M.A., Benjamin L.E., Sanza L.T., Huie S., Yeo S.F., Brandt M.E., Warnock D.W.** Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *Journal of Clinical Microbiology*, **2004**, 42(4): 1519-1527

[10] **Zaoutis T.E., Argon J., Chu J., Berlin J.A., Walsh T.J., Feudtner**

C. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. *Clinical Infectious Diseases*, **2005**, 41:1232-1239

[11] **Wilson L.S., Reyes C.M., Stolpman M., Speckman J., Allen K., Beney J.** The direct cost and incidence of systemic fungal infections. *Value in Health*, **2002**, 5(1): 26-34

[12] **Romani L.** Immunity to fungal infections. *Nature Reviews Immunology*, **2004**, 4: 11-24

[13] **Pauw B.E., Picazo J.J.** Present situation in the treatment of invasive fungal infection. *International Journal of Antimicrobial Agents*, **2008**, 32(S2): 167-171

[14] **Viscoli C., Castagnola E.** Emerging fungal pathogens, drug resistance and the role of lipid formulations of amphotericin B in the treatment of fungal infections in cancer patients: A review. *International Journal of Infectious Diseases*, **1998-1999**, 3(2): 109-118

[15] **Enoch D.A., Ludlam H.A., Brown N.M.** Invasive fungal infections: a review of epidemiology and management options. *Journal of Medical Microbiology*, **2006**, 55: 809-818

[16] **Martin G.S., Mannino D.M., Eaton S., Moss M.** The epidemiology of sepsis in the United States from 1979 through 2000. *The New England Journal of Medicine*, **2003**, 348:1546-1554

[17] **Neofytos D., Lu K., Hatfield-Seung A., Blackford A., Marr K.A., Treadway S., Ostrander D., Nussenblatt V., Karp J.** Epidemiology, outcomes, and risk factors of invasive fungal infections in adult patients with acute myelogenous leukemia after induction chemotherapy. *Diagnostic Microbiology and Infectious Disease*, **2013**, 75(2): 144-149

[18] **Bodro M., Sabé N., Gomila A., Ayats J., Baliellas C., Roca J., Melilli E., Carratalà J.** Risk factors, clinical characteristics, and outcomes of invasive fungal infections in solid organ transplant recipients. *Transplantation Proceedings*, **2012**, 44(9): 2682-2685

[19] **Rangel-Frausto M.S., Wiblin T., Blumberg H.M., Saiman L., Patterson J., Rinaldi M., Pfaller M., Edwards J.E. Jr., Jarvis W., Dawson J., Wenzel R.P.** National epidemiology of mycoses survey (NEMIS): variations in rates of bloodstream infections due to Candida species in seven surgical intensive care units and six neonatal intensive care units. *Clinical Infectious Diseases*, **1999**, 29(2): 253-258

[20] **Pappas P.G., Kauffman C.A., Andes D., Benjamin D.K. Jr., Calandra T.F., Edwards J.E. Jr., Filler S.G., Fisher J.F., Kullberg B., Zeichner L.O., Reboli A.C., Rex J.H., Walsh T.J., Sobe J.D.** Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases*, **2009**, 48(5): 503-535

[21] **Erjavec Z., Verweij P.E.** Recent progress in the diagnosis of fungal infections in the immunocompromised host. *Drug Resistance Updates*, **2002**, 5(1): 3-10

[22] **Schuetz A.N.** Invasive fungal infection: biomarkers and molecular approaches to diagnosis. *Clinics in Laboratory Medicine*, **2013**, 33(3): 505-525

[23] **Lai C.C., Tan C.K., Huang Y.T., Shao P.L., Hsueh P.R.** Current challenges in the management of invasive fungal infections. *Journal of Infection and Chemotherapy*, **2008**, 14 (2): 77-85

[24] **Dhillon R.H., Clark J.** Fungal infections in the critically ill. *Trends in Anaesthesia and Critical Care*, **2011**, 1: 210-218

[25] **Otting K.A., Stover K.R., Cleary J.D.** Drug-laboratory interaction between beta-lactam antibiotics and the galactomannan antigen test used to detect mould infections. *The Brazilian Journal of Infectious Diseases*, **2014**, 18(5): 544-547

[26] **Hadrich I., Makni F., Cheikhrouhou F., Neji S., Amouri I., Sellami H., Trabelsi H., Elloumi M., Ayadi A.** Clinical utility and prognostic value of galactomannan in neutropenic patients with invasive aspergillosis. *Patholgie Biologie (Paris)*, **2012**, 60: 357-361

[27] **Van Der Veer J., Lewis R.J., Emtiazjoo A.M., Allen S.D., Wheat L.J., Hage C.A.** Cross-reactivity in the Platelia™ Aspergillus enzyme immunoassay caused by blastomycosis. *Medical Mycology*, **2012**, 50(4): 396-398

[28] **Pfeiffer C.D., Fine J.P., Safdar N.** Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. *Clinical Infectious Diseases*, **2006**, 42(10): 1417-1427

[29] **Swanink C.M., Meis J.F., Rijs A.J., Donnelly J.P., Verweij P.E.** Specificity of a sandwich enzyme-linked immunosorbent assay for detecting Aspergillus galactomannan. *Journal Clinical Microbiology*, **1997**, 35(1): 257-260

[30] **Adam O., Auperin A., Wilquin F., Bourhis J.H., Gachot B., Chachaty E.** Treatment with piperacillin-tazobactam and false-positive Aspergillus galactomannan antigen test results for patients with hematological malignancies. *Clinical Infectious Diseases*, **2004**, 38(6): 917-920

[31] **Viscoli C., Machetti M., Cappellano P., Bucci B., Bruzzi P., Van Lint M.T., Bacigalupo A.** False-positive galactomannan platelia Aspergillus test results for patients receiving piperacillin-tazobactam. *Clinical Infectious Diseases*, **2004**, 38(6): 913-916

[32] **Alhambra A., Cuétara M.S., Ortiz M.C., Moreno J.M., del Palacio A., Pontón J.** False positive galactomannan results in adult hematological patients treated with piperacillin-tazobactam. *Revista Iberoamericana Micología*, **2007**, 24(2): 106-112

[33] **Zandijk E., Mewis A., Magerman K., Cartuyvels R.** False-Positive results by the platelia Aspergillus galactomannan antigen test for patients treated with amoxicillin-clavulanate. *Clinical and Vaccine Immunology*, **2008**, 15(7): 1132-1133

[34] **Tanriover M.D., Metan G., Altun B., Hascelik G., Uzun O.** False positivity for Aspergillus antigenemia related to the administration of piperacillin-tazobactam. *European Journal of Internal Medicine*, **2005**, 16(7): 489-491

[35] **Mennink-Kersten M.A.S., Klont R.R., Ruegebrink D., Dencamp H., Verweij P.E.** Amoxicillin-clavulanic acid (AMC) and piperacillin-tazobactam (PTZ) contain high molecular weight cross-reacting aspergillus antigen. In: Late-Breaker Abstract of the Forty-forth Interscience Conference on Antimicrobial Agents and Chemotherapy, Abstract M-1684a, p. 17. *American Society for Microbiology*, **2004**, Washington, DC, USA

[36] **Ansorg R., Van den Boom R., Rath P.M.** Detection of Aspergillus galactomannan antigen in foods and antibiotics. *Mycoses*, **1997**, 40: 353-357

[37] **Penack O., Schwartz S., Thiel E., Blau I.W.** Lack of evidence that false-positive Aspergillus galactomannan antigen test results are due to treatment with piperacillin-tazobactam. *Clinical Infectious Diseases*, **2004**, 39(9): 1401–1402

[38] **Metan G., Ağkuş C., Buldu H., Koç A.N.** The interaction between piperacillin-tazobactam and assays for Aspergillus galactomannan

and 1,3-beta-D-glucan in patients without risk factors for invasive fungal infections. *Infection*, **2010**, 38(3): 217-221

[39] **Xavier M.O., Pasqualotto A.C., Aquino V.R., Sukiennik T.C., Severo L.C.** Galactomannan detection from piperacillin-tazobactam brands available in the Brazilian market. *The Brazilian Journal of Infectious Diseases*, **2009**,13(5): 353-355

[40] **Ozkalemkas F., Ozcelik T., Ozkocaman V.** Treatment with piperacillin-tazobactam and Aspergillus galactomannan test results for patients with hematological malignancies. *European Journal of Internal Medicine*, **2007**, 18(1): 79

[41] **Penack O., Rempf P., Graf B., Thiel E., Blau I.W.** False-positive Aspergillus antigen testing due to application of piperacillin-tazobactam — is it still an issue? *Diagnostic Microbiology and Infectious Disease*, **2008**, 60(1): 117-120

[42] **Maerten J., Verhaegen J., Lagrou K., Van Eldere J., Boogaerts M.** Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. *The American Society of Hematology*, **2001**, 97(6): 1604-1610

[43] **Pazos C., Pontón J., Del Palacio A.** Contribution of (1→3)-β-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. *Journal of Clinical Microbiology*, **2005**, 43: 299-305

[44] **He S., Hang J.P., Zhang L., Wang F., Zhang D.C., Gong F.H.** A systematic review and meta-analysis of diagnostic accuracy of serum 1,3-β-d-glucan for invasive fungal infection: Focus on cutoff levels.

Journal of Microbiology, Immunology and Infection, **2014**, doi:10.1016/j.jmii.2014.06.009

[45] **Kedzierska A., Kochan P., Pietrzyk A., Kędzierska J.** Current status of fungal cell wall components in the immunodiagnostics of invasive fungal infections in humans: galactomannan, mannan and (1→3)-beta-d-glucan antigens. *European Journal of Clinical Microbiology and Infectious Diseases*, **2007**, 26(11): 755-766

[46] **De Pauw B., Walsh T.J., Donnelly J.P., Stevens D.A., Edwards J.E., Calandra T., Pappas P.G., Maertens J., Lortholary O., Kauffman C.A., Denning D.W., Patterson T.F., Maschmeyer G., Bille J., Dismukes W.E., Herbrecht R., Hope W.W., Kibbler C.C., Kullberg B.J., Marr K.A., Muñoz P., Odds F.C., Perfect J.R., Restrepo A., Ruhnke M., Segal B.H., Sobel J.D., Sorrell T.C., Viscoli C., Wingard J.R., Zaoutis T., Bennett J.E.; European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group.** Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clinical Infectious Diseases*, **2008**, 46(12): 1813-1821

[47] **Obayashi T., Yoshida M., Mori T., Goto H., Yasuoka A., Iwasaki H., Teshima H., Kohno S., Horiuchi A., Ito A.** Plasma (1→3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet*, **1995**, 345:17-20

[48] **Onishi A., Sugiyama D., Kogata Y., Saegusa J., Sugimoto T., Kawano S., Morinobu A., Nishimura K., Kumagai S.** Diagnostic accuracy of serum 1,3-β-D-glucan for *pneumocystis jiroveci* pneumonia, invasive candidiasis, and invasive aspergillosis: systematic review and meta-analysis. *Journal of Clinical Microbiology*, **2012**, 50(1): 7-15

[49] **Lu Y., Chen Y., Guo Y.L., Qin S.M., Wu C., Wang K.** Diagnosis of invasive fungal disease using serum (1→3)-β-D-glucan: a bivariate meta-analysis. *Internal Medicine*, **2011**, 50(22): 2783-2791

[50] **Hope W.W., Petraitis V., Petraitiene R., Aghamolla T., Bacher J., Walsh T.J.** The initial 96 hours of invasive pulmonary aspergillosis: histopathology, comparative kinetics of galactomannan and (1→3) beta-d-glucan and consequences of delayed antifungal therapy. *Antimicrobial Agents and Chemotherapy*, **2010**, 54(11): 4879-4886

[51] **Lamoth F., Cruciani M., Mengoli C., Castagnola E., Lortholary O., Richardson M., Marchetti O.; Third European Conference on Infectious in Leukemia (ECIL-3).** β-Glucan antigenemia assay for the diagnosis of invasive fungal infections in patients with hematological malignancies: a systematic review and meta-analysis of cohort studies from the Third European Conference on Infections in Leukemia (ECIL-3). *Clinical Infectious Diseases*, **2012**, 54(5): 633-643

[52] **Marty F.M., Lowry C.M., Lempitski S.J., Kubiak D.W., Finkelman M.A., Baden L.R.** Reactivity of (1→3)-beta-D-glucan assay with commonly used intravenous antimicrobials. *Antimicrobial Agents and Chemotherapy*, **2006**, 50(10): 3450-3453

[53] **Walsh T.J., Anaissie E.J., Denning D.W., Herbrecht R., Kontoyiannis D.P., Marr K.A., Morrison V.A., Segal B.H.,**

Steinbach W.J., Stevens D.A., van Burlk J.A., Wingard J.R., Patterson T.F.; Infectious Diseases Society of America. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clinical Infectious Diseases*, **2008**, 46(3): 327-360

[54] **Wingard J.R.** Have novel serum markers supplanted tissue diagnosis for invasive fungal infections in acute leukemia and transplantation? *Best Practice & Research Clinical Haematology*, **2012**, 25: 487-491

[55] **Odabasi Z., Mattiuzzi G., Estey E., Kantarjian H., Saeki F., Ridge R.J., Ketchum P.A., Finkelman M.A., Rex J.H., Ostrosky-Zeichner L.** β-D-Glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. *Clinical Infectious Diseases*, **2004**, 39(2): 199-205

[56] **Ellis M., Al-Ramadi B., Finkelman M., Hedstrom U., Kristensen J., Ali-Zadeh H., Klingspor L.** Assessment of the clinical utility of serial beta-D-glucan concentrations in patients with persistent neutropenic fever. *Journal of Medical Microbiology*, **2008**, 57:287-295

[57] **Senn L., Robinson J.O., Schmidt S., Knaup M., Asahi N., Satomura S., Matsuura S., Duvoisin B., Bille J., Calandra T., Marchetti O.** 1,3-β-D-glucan antigenemia for early diagnosis of invasive fungal infections in neutropenic patients with acute leukemia. *Clinical Infectious Diseases*, **2008**, 46(6): 878-885

[58] **Del Bono V., Delfino E., Furfaro E., Mikulska M., Nicco E., Bruzzi P., Mularoni A., Bassetti M., Viscoli C.** Clinical performance of the (1,3)-beta-D-glucan assay in early diagnosis of

nosocomial Candida bloodstream infections. *Clinical Vaccine Immunology*, **2011**, 18(12): 2113-2117

[59] **Khot D.P., Fredricks N.D.** PCR-based diagnosis of human fungal infection. *Expert Review of Anti-infective Therapy*, **2009**, 7(10): 1201-1221

[60] **Flahaut M., Sanglard D., Monod M., Bille J., Rossier M.** Rapid detection of Candida albicans in clinical samples by DNA amplification of common regions from C. albicans-secreted aspartic proteinase genes. *Journal of Clinical Microbiology*, **1998**, 36(2): 395-401

[61] **Springer J., Einsele H., Loeffler J.** Molecular techniques in diagnosis of deep and systemic mycosis. *Clinics in Dermatology*, **2012**, 30: 651-656

[62] **Melchers W.J., Verweij P.E., van den Hurk P., van Belkum A., De Pauw B.E., Hoogkamp-Korstanje J.A., Meis J.F.** General primer-mediated PCR for detection of Aspergillus species. *Journal of Clinical Microbiology*, **1994**, 32(7): 1710-1717

[63] **de Aguirre L., Hurst S.F., Choi J.S., Shin J.H., Hinrikson H.P., Morrison C.J.** Rapid differentiation of Aspergillus species from other medically important opportunistic molds and yeasts by PCR—enzyme immunoassay. *Journal of Clinical Microbiology*, **2004**, 42(8): 3495-3504

[64] **Kadmon G., Nahum E., Sprecher H., Stein J., Levy I., Schiller O., Schonfeld T.** Polymerase-chain-reaction-based diagnosis of invasive fungal pulmonary infections in immunocompromised children. *Pediatric Pulmonology*, **2012**, 47(10): 994-1000

[65] **Kourkoumpetis K.T., Fuchs B.B., Coleman J.J., Desalermos A., Mylonakis E.** Polymerase chain reaction-based assays for the

diagnosis of invasive fungal infections. *Clinical Infectious Diseases*, **2012**, 54(9): 1322-1331

[66] **Wengenack L.N., Binnicker J.M.** Fungal molecular diagnostics. *Clinics in Chest Medicine*, **2009**, 30(2): 391-408

[67] **Halliday C., Hoile R., Sorrell T., James G., Yadav S., Shaw P., Bleakley M., Bradstock K., Chen S.** Role of prospective screening of blood for invasive aspergillosis by polymerase chain reaction in febrile neutropenic recipients of haematopoietic stem cell transplants and patients with acute leukaemia. *British Journal of Haematology*, **2006**, 132(4): 478-486

[68] **Florent M., Katsahian S., Vekhoff A., Levy V., Rio B., Marie J.P., Bouvet A., Cornet M.** Prospective evaluation of a polymerase chain reaction-ELISA targeted to *Aspergillus fumigatus* and *Aspergillus flavus* for the early diagnosis of invasive aspergillosis in patients with hematological malignancies. *Journal of Infectious Diseases*, **2006**, 193(5): 741-747

[69] **Sugawara Y., Nakase K., Nakamura A., Ohishi K., Sugimoto Y., Fujieda A., Monma F., Suzuki K., Masuya M., Matsushima Y., Wada H., Nobori T., Katayama N.** Clinical utility of a panfungal polymerase chain reaction assay for invasive fungal diseases in patients with haematologic disorders. *European Journal of Haematology*, **2013**, 90(4): 331-339

[70] **White P.L., Bretagne S., Klingspor L., Melchers W.J., McCulloch E., Schulz B., Finnstorm N., Mengoli C., Barnes R.A., Donnelly J.P., Loeffler J., European Aspergillus PCR Initiative.** Aspergillus PCR: one step closer to standardization. *Journal of Clinical Microbiology*, **2010**, 48(4): 1231-1240

[71] **Cuchacovich R.** Clinical applications of the polymerase chain

reaction: An update, *Infectious Disease Clinics of North America*, **2006**, 20(4): 735-758

[72] **Metcalf S.C., Dockrell D.H.** Improved outcomes associated with advances in therapy for invasive fungal infections in immuno-compromised hosts. *Journal of Infection*, **2007**, 55(4): 287-299

[73] **Odds F.C., Brown A.J., Gow N.A.** Antifungal agents: mechanisms of action. *Trends in Microbiology*, **2003**, 11(6): 272-279

[74] **Brajtburg J., Powderly W.G., Kobayashi G.S., Medoff G.** Amphotericin B: Current understanding of mechanisms of action. *Antimicrobial agents and Chemotherapy*, **1990**, 34(2): 183-188

[75] **Dupont B.** Overview of the lipid formulations of amphotericin B. *Journal of Antimicrobial Chemotherapy*, **2002**, 49(Suppl. 1): 31-36

[76] **Slain D.** Lipid-based amphotericin B for the treatment of fungal infections. *Pharmacotherapy*, **1999**, 19(3): 306-323

[77] **Barrett J.P., Vardulaki K.A., Conlon C., Cooke J., Daza-Ramirez P., Evans E.G., Hawkey P.M., Herbrecht R., Marks D.I., Moraleda J.M., Park G.R., Senn S.J., Viscoli C.; Amphotericin B Systematic Review Study Group.** A systematic review of the antifungal effectiveness and tolerability of amphotericin B formulations. *Clinical Therapeutics*, **2003**, 25(5): 1295-1320

[78] **Sun H.Y., Alexander B.D., Lortholary O., Dromer F., Forrest G.N., Lyon G.M., Somani J., Gupta K.L., del Busto R., Pruett T.L., Sifri C.D., Limaye A.P., John G.T., Klintmalm G.B., Pursell K., Stosor V., Morris M.I., Dowdy L.A., Munoz P., Kalil A.C., Garcia-Diaz J., Orloff S., Houston S., Wray D., Huprikar S., Johnson L.B., Humar A., Razonable R.R., Husain S., Singh N.** Lipid formulations of amphotericin B significantly improve outcome

in solid organ transplant recipients with central nervous system cryptococcosis. *Clinical Infectious Diseases*, **2009**, 49(11): 1721-1728

[79] **Hamill J.R.** Amphotericin B formulations: A comparative review of efficacy and toxicity. *Drugs*, **2013**, 73(9): 919-934

[80] **Flückiger U., Marcbetti O., Bille J., Eggimann P., Zimmerli S., Imhof A., Garbino J., Ruef C., Pittet D., Täuber M., Glauser M., Calandra T.; Fungal Infection Network of Switzerland (FUNGINOS).** Treatment options of invasive fungal infections in adults. *Swiss Medical Weekly*, **2006**, 136(29-30): 447-463

[81] **Thompson R.G., Cadena J., Patterson F.T.** Overview of antifungal agents. *Clinics in Chest Medicine*, **2009**, 30(2): 203-215

[82] **Bossche H.V., Koymans L., Moereels H.** P450 inhibitors of use in medical treatment: Focus on mechanisms of action. *Pharmacology &Therapeutics*, **1995**, 67(1): 79-100

[83] **Carrillo-Muñoz A.J., Giusiano G., Ezkurra P.A., Quindós G.** Antifungal agents: Mode of action in yeast cells. *Revista Española de Quimioterapia*, **2006**, 19(2): 130-139

[84] **Sheehan J.D., Hitchcock A.C., Sibley M.C.** Current and emerging azole antifungal agents. *Clinical Microbiology Reviews*, **1999**, 12(1): 40-79

[85] **Akins R.A.** An update on antifungal targets and mechanisms of resistance in Candida albicans. *Medical Mycology*, **2005**, 43(4): 285-318

[86] **Kramer M.R., Marshall S.E., Denning D.W., Keogh A.M., Tucker R.M., Galgiani J.N., Lewiston N.J., Stevens D.A., Theodore J.** Cyclosporine and itraconazole interaction in heart and lung transplant recipients. *Annals of Internal Medicine*, **1990**,

113(4): 327-329

[87] **Varis T., Kaukonen K.M., Kivistö K.T., Neuvonen P.J.** Plasma concentrations and effects of oral methylprednisolone are considerably increased by itraconazole. *Clinical Pharmacology and Therapeutics*, **1998**,64(4): 363-368

[88] **Tucker R.M., Denning D.W., Hanson L.H., Rinaldi M.G., Graybill J.R., Sharkey P.K., Pappagianis D., Stevens D.A.** Interaction of azoles with rifampin, phenytoin, and carbamazepine: in vitro and clinical observations. *Clinical Infectious Diseases*, **1992**, 14(1): 165-174

[89] **Kanafani Z.A., Perfect J.R.** Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. *Clinical Infectious Diseases*, **2008**, 46(1): 120-128

[90] **Pemán J., Cantón E., Espinel-Ingroff A.** Antifungal drug resistance mechanisms. *Expert Review of Anti-infective Therapy*, **2009**, 7(4): 453-460

[91] **Lupetti A., Danesi R., Campa M., Del Tacca M., Kelly S.** Molecular basis of resistance to azole antifungals. *Trends in Molecular Medicine*, **2002**, 8(2): 76-81

[92] **Löffler J., Kelly S.L., Hebart H., Schumacher U., Lass-Flörl C., Einsele H.** Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. *FEMS Microbiology Letters*, **1997**, 151(2): 263-268

[93] **Lopez-Ribot, J.L., McAtee R.K., Lee L.N., Kirkpatrick W.R., White T.C., Sanglard D., Patterson T.F.** Distinct patterns of gene expression associated with development of fluconazole resistance in serial Candida albicans isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrobial*

Agents and Chemotherapy, **1998**, 42(11): 2932-2937

[94] **Pfaller A.M.** Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. *The American Journal of Medicine*, **2012**, 125(Suppl. 1): S3-13

[95] **Kelly S.L., Lamb D.C., Kelly D.E., Loeffler J., Einsele H.** Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. *Lancet*, **1996**, 348(9040): 1523-1524

[96] **DeMuria D., Forrest A., Rich J., Scavone J.M., Cohen L.G., Kazanjian P.H.** Pharmacokinetics and bioavailability of fluconazole in patients with AIDS. *Antimicrobial Agents and Chemotherapy*, **1993**, 37(10): 2187-2192

[97] **Lass-Flörl C.** Triazole antifungal agents in invasive fungal infections: a comparative review. *Drugs*, **2011**, 71(18): 2405-2419

[98] **Andriole V.T.** Current and future antifungal therapy: new targets for antifungal therapy. *International Journal of Antimicrobial Agents*, **2000**, 16(3): 317-321

[99] **Arndt C.A., Walsh T.J., McCully C.L., Balis F.M., Pizzo P.A., Poplack D.G.** Fluconazole penetration into cerebrospinal fluid: implications for treating fungal infections of the central nervous system. *The Journal of Infectious Diseases*, **1988**, 157(1): 178-180

[100] **Foulds G., Brennan D.R., Wajszczuk C., Catanzaro A., Garg D.C., Knopf W., Rinaldi M., Weidler D.J.** Fluconazole penetration into cerebrospinal fluid in humans. *Journal of Clinical Pharmacology*, **1988**, 28(4): 363-366

[101] **Pfaller M.A., Diekema D.J., Messer S.A., Boyken L., Hollis R.J., Jones R.N.; International Fungal Surveillance Participant Group.** In vitro activities of voriconazole, posaconazole, and four licensed systemic antifungal agents against Candida species

infrequently isolated from blood. *Journal of Clinical Microbiology*, **2003**, 41(1): 78-83

[102] **Sabatelli F., Patel R., Mann P.A., Mendrick C.A., Norris C.C., Hare R., Loebenberg D., Black T.A., McNicholas P.M.** In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. *Antimicrobial Agents and Chemotherapy*, **2006**, 50(6): 2009-2015

[103] **Petrikkos G., Skiada A.** Recent advances in antifungal chemotherapy. *International Journal of Antimicrobial Agents*, **2007**, 30(2): 108-117

[104] **Johnson L.B., Kauffman C.A.** Voriconazole: a new triazole anti-fungal agent. *Clinical Infectious Diseases*, **2003**, 36(5): 630-637

[105] **Sabo J.A., Abdel-Rahman S.M.** Voriconazole: a new triazole antifungal. *Annals of Pharmacotherapy*, **2000**, 34(9): 1032-1043

[106] **Theuretzbacher U., Ihle F., Derendorf H.** Pharmacokinetic/ pharmacodynamic profile of voriconazole. *Clinical Pharmacokinetics*, **2006**, 45(7): 649-663

[107] **Purkins L., Wood N., Kleinermans D., Greenhalgh K., Nichols D.** Effect of food on the pharmacokinetics of multiple-dose oral voriconazole. *British Journal of Clinical Pharmacology*, **2003**, 56(Suppl. 1): 17-23

[108] **Lazarus H.M., Blumer J.L., Yanovich S., Schlamm H., Romero A.** Safety and pharmacokinetics of oral voriconazole in patients at risk of fungal infection: a dose escalation study. *Journal of Clinical Pharmacology*, **2002**, 42(4): 395-402

[109] **Ghannoum M.A., Kuhn D.M.** Voriconazole better chances for patients with invasive mycoses. *European Journal of Medical*

Research, **2002**, 7(5): 242-256

[110] **Groll A.H., Gea-Banacloche J.C., Glasmacher A., Just-Nuebling G., Maschmeyer G., Walsh T.J.** Clinical pharmacology of antifungal compounds. *Infectious Diseases Clinics of North America*, **2003**, 17(1): 159-191

[111] **Dodds-Ashley E.** Management of drug and food interactions with azole antifungal agents in transplant recipients. *Pharmacotherapy*, **2010**, 30(8): 842-854

[112] **Patterson B., Roffey S., Jezequel S.G., Jones B.** UK-109, 496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: disposition in man. Presented at the 35th *Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Francisco, CA, September 17–20, **1995**, Abstract F79, 126

[113] **Hoffman H.L., Rathbun R.C.** Review of the safety and efficacy of voriconazole. *Expert Opinion on Investigational Drugs*, **2002**, 11(3): 409-429

[114] **Herbrecht R., Denning D.W., Patterson T.F., Bennett J.E., Greene R.E., Oestmann J.W., Kern W.V., Marr K.A., Ribaud P., Lortholary O., Sylvester R., Rubin R.H., Wingard J.R., Stark P., Durand C., Caillot D., Thiel E., Chandrasekar P.H., Hodges M.R., Schlamm H.T., Troke P.F., de Pauw B.; Invasive Fungal Infections Group of the European Organization for Research and Treatment of Cancer and the Global Aspergillus Study Group.** Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *The New England Journal of Medicine*, **2002**, 347(6): 408-415

[115] **Purkins L., Wood N., Ghahramani P., Greenhalgh K., Allen M.J., Kleinermans D.** Pharmacokinetics and safety of voriconazole

following intravenous-to oral-dose escalation regimens. *Antimicrobial Agents and Chemotherapy*, **2002**, 46(8): 2546-2553

[116] **Rubenstein M., Levy M.L., Metry D.** Voriconazole-induced retinoid-like photosensitivity in children. *Pediatric Dermatology*, **2004**, 21(6): 675-678

[117] **Denning D.W., Ribaud P., Milpied N., Caillot D., Herbrecht R., Thiel E., Haas A., Ruhnke M., Lode H.** Efficacy and safety of voriconazole in the treatment of acute invasive aspergillosis. *Clinical Infectious Diseases*, **2002**, 34(5): 563-571

[118] Kenilworth, NJ: **Schering-Plough**, Schering-Plough announces FDA approval of NOXAFIL® (posaconazole) for prevention of invasive fungal infections, *Schering-Plough News Release*, September **2006**

[119] **Courtney R., Wexler D., Radwanski E., Lim J., Laughlin M.** Effect of food on the relative bioavailability of two oral formulations of posaconazole in healthy adults. *British Journal of Clinical Pharmacology*, **2003**, 57(2): 218-222

[120] **Nagappan V., Deresinski S.** Reviews of anti-infective agents: posaconazole: a broad-spectrum triazole antifungal agent. *Clinical Infectious Diseases*, **2007**, 45(12): 1610-1617

[121] **Dodds-Ashley E.S., Lewis R., Lewis J.S., Martin C., Andes D.** Pharmacology of systemic antifungal agents. *Clinical Infectious Diseases*, **2006**, 43(Suppl. 1): S28-39

[122] **Courtney R., Wexler D., Radwanski E., Lim J., Laughlin M.** Effect of food on the relative bioavailability of two oral formulations of posaconazole in healthy adults. *British Journal of Clinical Pharmacology*, **2004**, 57(2): 218-222

[123] **Schiller D.S., Fung H.B.** Posaconazole: an extended-spectrum

triazole antifungal agent. *Clinical Therapeutics*, **2007**, 29(9): 1862-1886

[124] **Courtney R., Sansone A., Smith W., Marbury T., Statkevich P., Martinho M., Laughlin M., Swan S.** Posaconazole pharmacokinetics, safety, and tolerability in subjects with varying degrees of chronic renal disease. *Journal of Clinical Pharmacology*, **2005**,45(2): 185-192

[125] **Ghosal A., Hapangama N., Yuan Y., Achanfuo-Yeboah J., Iannucci R., Chowdhury S., Alton K., Patrick J.E., Zbaida S.** Identification of human UDP-glucuronosyltransferase enzyme(s) responsible for the glucuronidation of posaconazole (Noxafil). *Drug Metabolism and Disposition*, **2004**, 32(2): 267-271

[126] **Krieter P., Flannery B., Musick T., Gohdes M., Martinho M., Courtney R.** Disposition of posaconazole following single-dose oral administration in healthy subjects. *Antimicrobial Agents and Chemotherapy*, **2004**, 48(9): 3543-3551

[127] **Hachem R.Y., Langston A.A., Graybill J.R., Perfect J.R., Pedicone L.D., Patino H., Raad I.I.** Posaconazole as salvage treatment of invasive fungal infections in patients with underlying renal impairment. *Journal of Antimicrobial Chemotherapy*, **2008**, 62(6): 1386-1391

[128] **Groll A.H., Walsh T.J.** Posaconazole: clinical pharmacology and potential for management of fungal infections. *Expert Review of Anti-infective Therapy*, **2005**, 3(4): 467-487

[129] **Wexler D., Courtney R., Richards W., Banfield C., Lim J., Laughlin M.** Effect of posaconazole on cytochrome P450 enzymes: a randomized, open-label, two-way crossover study. *European Journal of Pharmaceutical Sciences*, **2004**, 21(5): 645-653

[130] **Worth L.J., Blyth C.C., Booth D.L., Kong D.C., Marriott D., Cassumbhoy M., Ray J., Slavin M.A., Wilkes J.R.** Optimizing antifungal drug dosing and monitoring to avoid toxicity and improve outcomes in patients with haematological disorders. *Internal Medicine Journal*, **2008**, 38(6b): 521-537

[131] Noxafil (posaconazole) oral suspension *package insert*], Summit, NJ: Schering-Plough, **2006**

[132] **Torres H.A., Hachem R.Y., Chemaly R.F., Kontoyiannis D.P., Raad I.I.,** Posaconazole: a broad-spectrum triazole antifungal. *Lancet Infectious Diseases*, **2005**, 5(12): 775-785

[133] **Raad I.I., Graybill J.R., Bustamante A.B., Cornely O.A., Gaona-Flores V., Afif C., Graham D.R., Greenberg R.N., Hadley S., Langston A., Negroni R., Perfect J.R., Pitisuttithum P., Restrepo A., Schiller G., Pedicone L., Ullmann A.J.** Safety of long-term oral posaconazole use in the treatment of refractory invasive fungal infections. *Clinical Infectious Diseases*, **2006**, 42(12): 1726-1734

[134] **Ullmann A.J., Lipton J.H., Vesole D.H., Chandrasekar P., Langston A., Tarantolo S.R., Greinix H., Morais de Azevedo W., Reddy V., Boparai N., Pedicone L., Patino H., Durrant S.** Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. *The New England Journal of Medicine*, **2007**, 356(4): 335-347

[135] **Walsh T.J., Raad I., Patterson T.F., Chandrasekar P., Donowitz G.R., Graybill R., Greene R.E., Hachem R., Hadley S., Herbrecht R., Langston A., Louie A., Ribaud P., Segal B.H., Stevens D.A., van Burik J.A., White C.S., Corcoran G., Gogate J., Krishna G., Pedicone L., Hardalo C., Perfect J.R.** Treatment

of invasive aspergillosis with posaconazole in patients who are refractory to or intolerant of conventional therapy: an externally controlled trial. *Clinical Infectious Diseases*, **2007**, 44(1): 2-12

[136] **Chen S.C., Playford E.G., Sorrell T.C.** Antifungal therapy in invasive fungal infections. *Current Opinion in Pharmacology*, **2010**, 10(5): 522-530

[137] **Klis F.M., de Groot P., Hellingwerf K.** Molecular organization of the cell wall of *Candida albicans*. *Medical Mycology*, **2001**, 39(Suppl. 1): 1-8

[138] **Bernard M., Latgé J.P.** Aspergillus fumigatus cell wall: composition and biosynthesis. *Medical Mycology*, **2001**, 39(Suppl. 1): 9-17

[139] **Cappelletty D., Eiselstein-McKitrick K.** The echinocandins. *Pharmacotherapy*, **2007**, 27(3): 369-388

[140] **Bachmann S.P., Patterson T.F., Lopez-Ribot J.L.** In vitro activity of caspofungin (MK-0991) against Candida albicans clinical isolates displaying different mechanisms of azole resistance. *Journal of Clinical Microbiology*, **2002**, 40(6): 2228-2230

[141] **Kurtz M.B., Rex J.H.** Glucan synthase inhibitors as antifungal agents. *Advances in Protein Chemistry*, **2001**, 56: 423-475

[142] **Nyfeler R., Keller-Schierlein W.** Metabolites of microorganisms. 143. Echinocandin B, a novel polypeptide-antibiotic from Aspergillus nidulans var. echinulatus: isolation and structural components. *Helvetica Chimica Actaca*, **1974**, 57(8): 2459-2477

[143] **Lamaris G.A., Lewis R.E., Chamilos G., May G.S., Safdar A., Walsh T.J., Raad I.I., Kontoyiannis D.P.** Caspofungin-mediated beta-glucan unmasking and enhancement of human

polymorphonuclear neutrophil activity against Aspergillus and non-Aspergillus hyphae. *Journal of Infectious Diseases*, **2008**, 198(2): 186-192

[144] **Theuretzbacher U.** Pharmacokinetics/pharmacodynamics of echinocandins. *European Journal of Clinical Microbiology and Infectious Diseases*, **2004**, 23(11): 805-812

[145] **Denning D.W.** Echinocandin antifungal drugs. *Lancet*, **2003**, 362(9390): 1142-1151

[146] **Balani S.K., Xu X., Arison B.H., Silva M.V., Gries A., DeLuna F.A., Cui D., Kari P.H., Ly T., Hop C.E., Singh R., Wallace M.A., Dean D.C., Lin J.H., Pearson P.G., Baillie T.A.** Metabolites of caspofungin acetate, a potent antifungal agent, in human plasma and urine. *Drug Metabolism and Disposition*, **2000**, 28(11): 1274-1278

[147] **Stone J.A., Holland S.D., Wickersham P.J., Sterrett A., Schwartz M., Bonfiglio C., Hesney M., Winchell G.A., Deutsch P.J., Greenberg H., Hunt T.L., Waldman S.A.** Single- and multiple-dose pharmacokinetics of caspofungin in healthy men. *Antimicrobial Agents and Chemotherapy*, **2002**, 46(3):739-745

[148] **Holland S., Stone J.A., Li S., Wickersham P., Deutsch P., Winchell G., Hesney M., Bi S., Friel M., Mistry G., Dilzer S.** Effect of hepatic insufficiency on the pharmacokinetics of caspofungin. Program and abstracts of the 41st *Interscience Conference on Antimicrobial Agents and Chemotherapy*, **2001**; Chicago, USA: abstract A-14

[149] **Hebert M.F., Smith H.E., Marbury T.C., Swan S.K., Smith W.B., Townsend R.W., Buell D., Keirns J., Bekersky I.** Pharmacokinetics of micafungin in healthy volunteers, volunteers

with moderate liver disease, and volunteers with renal dysfunction. *Journal of Clinical Pharmacology*, **2005**, 45(10): 1145-1152

[150] **Hiemenz J., Cagnoni P., Simpson D., Devine S., Chao N., Keirns J., Lau W., Facklam D., Buell D.** Pharmacokinetic and maximum tolerated dose study of micafungin in combination with fluconazole versus fluconazole alone for prophylaxis of fungal infections in adult patients undergoing a bone marrow or peripheral stem cell transplant. *Antimicrobial Agents and Chemotherapy*, **2005**, 49(4): 1331-1336

[151] **Stogniew M., Pu F., Henkel T., Dowell J.** Anidulafungin biotransformation in humans is by degradation not metabolism. *European Society of Clinical Microbiology and Infectious Diseases*, **2003**; 9(Suppl. 1): 291, abstract P-1223

[152] **Cleary J.D., Schwartz M., Rogers P.D., de Mestral J., Chapman S.W.** Effects of amphotericin B and caspofungin on histamine expression. *Pharmacotherapy*, **2003**, 23(8): 966-973

[153] **Stone J.A., Migoya E.M., Hickey L., Winchell G.A., Deutsch P.J., Ghosh K., Freeman A., Bi S., Desai R., Dilzer S.C., Lasseter K.C., Kraft W.K., Greenberg H., Waldman S.A.** Potential for interactions between caspofungin and nelfinavir or rifampin. *Antimicrobial Agents and Chemotherapy*, **2004**, 48(11): 4306-4314

[154] **Petrovic J., Ngai A., Bradshaw S., William-Diaz A., Taylor A., Sable C., Vuocolo S., Kartsonis N.** Efficacy and safety of caspofungin in solid organ transplant recipients. *Transplantation Proceedings*, **2007**, 39(10): 3117-3120

[155] **Mora-Duarte J., Betts R., Rotstein C., Colombo A.L., Thompson-Moya L., Smietana J., Lupinacci R., Sable C.,**

Kartsonis N., Perfect J.; Caspofungin Invasive Candidiasis Study Group. Comparison of caspofungin and amphotericin B for invasive candidiasis. *The New England Journal of Medicine*, **2002**, 347(25): 2020-2029

[156] **Maertens J., Raad I., Pettrikkos G., Boogaerts M., Selleslag D., Petersen F.B., Sable C.A., Kartsonis N.A., Ngai A., Taylor A., Patterson T.F., Denning D.W., Walsh T.J.; Caspofungin Salvage Aspergillosis Study Group.** Efficacy and safety of caspofungin for the treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clinical Infectious Diseases*, **2004**, 39(11): 1563-1571

[157] **Walsh T.J., Teppler H., Donowitz G.R., Maertens J.A., Baden L.R., Dmoszynska A., Cornely O.A., Bourque M.R., Lupinacci R.J., Sable C.A., de Pauw B.E.** Caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia. *The New England Journal of Medicine*, **2004**, 351(14): 1391-1402

[158] **Rogers T.R., Slavin M.A., Donnelly J.P.** Antifungal prophylaxis during treatment for haematological malignancies: are we there yet? *British Journal of Haematology*, **2011**, 153(6): 681-697

[159] **van Burik J.A., Ratanatharathorn V., Stepan D.E., Miller C.B., Lipton J.H., Vesole D.H., Bunin N., Wall D.A., Hiemenz J.W., Satoi Y., Lee J.M., Walsh T.J.; National Institute of Allergy and Infectious Diseases Mycoses Study Group.** Micafungin versus fluconazole for prophylaxis against invasive fungal infections during neutropenia in patients undergoing hematopoietic stem cell transplantation. *Clinical Infectious Diseases*, **2004**, 39(10): 1407-1416

[160] **Hiramatsu, Y., Maeda, Y., Fujii, N., Saito T., Nawa Y., Hara M., Yano T., Asakura S., Sunami K., Tabayashi T., Miyata A., Matsuoka K., Shinagawa K., Ikeda K., Matsuo K., Tanimoto M.; West-Japan Hematology and Oncology Group.** Use of micafungin versus fluconazole for antifungal prophylaxis in neutropenic patients receiving hematopoietic stem cell transplantation. *International Journal of Hematology*, **2008**, 88(5): 588-595

[161] **de Wet N., Lianos-Cuentas A., Suleiman J., Baraldi E., Krantz E.F., Della Negra M., Diekmann-Berndt H.** A randomized, double-blind, parallel-group, dose-response study of micafungin compared with fluconazole for the treatment of esophageal candidiasis in HIV-positive patients. *Clinical Infectious Diseases*, **2004**, 39(6): 842-849

[162] **de Wet N., Bester A.J., Viljoen J.J., Filho F., Suleiman J.M., Ticona E., Llanos E.A., Fisco C., Lau W., Buell D.** A randomized, double-blind, comparative trial of micafungin (FK463) vs. fluconazole for the treatment of oesophageal candidiasis. *Alimentary Pharmacology & Therapeutics*, **2005**, 21(7): 899-907

[163] **Kuse E.R., Chetchotisakd P., da Cunha C.A., Ruhnke M., Barrios C., Raghunadharao D., Sekhon J.S., Freire A., Ramasubramanian V., Demeyer I., Nucci M., Leelarasamee A., Jacobs F., Decruyenaere J., Pittet D., Ullmann A.J., Ostrosky-Zeichner L., Lortholary O., Koblinger S., Diekmann-Berndt H., Cornely O.A.; Micafungin Invasive Candidiasis Working Group.** Micafungin Invasive Candidiasis Working Group. Micafungin versus liposomal amphotericin B for candidemia and invasive candidiasis: a phase III randomized double-blind trial.

Lancet, **2007**, 369(9572): 1519-1527

[164] **Goto N., Hara T., Tsurumi H., Ogawa K., Kitagawa J., Kanemura N., Kasahara S., Yamada T., Shimizu M., Nakamura M., Matsuura K., Moriwaki H.** Efficacy and safety of micafungin for treating febrile neutropenia in hematological malignancies. *American Journal of Hematology*, **2010**, 85(11): 872-876

[165] **Dowell J.A., Knebel W., Ludden T., Stogniew M., Krause D., Henkel T.** Population pharmacokinetic analysis of anidulafungin, an echinocandin antifungal. *Journal of Clinical Pharmacology*, **2004**, 44(6): 590-598

[166] **Damle B.D., Dowell J.A., Walsky R.L., Weber G.L., Stogniew M., Inskeep P.B.** In vitro and in vivo studies to characterize the clearance mechanism and potential cytochrome P450 interactions of anidulafungin. *Antimicrobial Agents and Chemotherapy*, **2009**, 53(3): 1149-1156

[167] **Dowell J.A., Stogniew M., Krause D., Damle B.** Anidulafungin does not require dosage adjustment in subjects with varying degrees of hepatic or renal impairment. *Journal of Clinical Pharmacology*, **2007**, 47(4): 461-470

[168] **Reboli A.C., Rotstein C., Pappas P.G., Chapman S.W., Kett D.H., Kumar D., Betts R., Wible M., Goldstein B.P., Schranz J., Krause D.S., Walsh T.J.; Anidulafungin Study Group.** Anidulafungin versus fluconazole for invasive candidiasis. *The New England Journal of Medicine*, **2007**, 356(24): 2472-2482

[169] **Kett D.H., Shorr A.F., Reboli A.C., Reisman A.L., Biswas P., Schlamm H.T.** Anidulafungin compared with fluconazole in severely ill patients with candidemia and other forms of invasive

candidiasis: support for the 2009 IDSA treatment guideline for candidiasis. *Critical Care*, **2011**, 15(5): R253

[170] **Vazquez J.A., Schranz J.A., Clark K., Goldstein B.P., Reboli A., Fichtenbaum C.** A phase 2, open-label study of the safety and efficacy of intravenous anidulafungin as a treatment for azole-refractory mucosal candidiasis. *Journal of Acquired Immune Deficiency Syndromes*, **2008**, 48(3): 304-309

[171] **Aguado J.M., Varo E., Usetti P., Pozo J.C., Moreno A., Catalán M., Len O., Blanes M., Solé A., Muñoz P., Montejo M.; TOSCANA Study Group.** Safety of anidulafungin in solid organ transplant recipients. *Liver Transplantation*, **2012**, 18(6): 680-685

[172] **Wingard J.R.** Empirical antifungal therapy in treating febrile neutropenic patients. *Clinical Infectious Diseases*, **2004**, 39(Suppl. 1): S38-43

[173] **Segal B.H., Almyroudis N.G., Battiwalla M., Herbrecht R., Perfect J.R., Walsh T.J., Wingard J.R.** Prevention and early treatment of invasive fungal infection in patients with cancer and neutropenia and in stem cell transplant recipients in the era of newer broad-spectrum antifungal agents and diagnostic adjuncts. *Clinical Infectious Diseases*, **2007**, 44(3): 402-409

[174] **Maertens J.A., Frère P., Lass-Flörl C., Heinz W., Cornely O.A.** Primary antifungal prophylaxis in leukaemia patients. *European Journal of Cancer Supplements*, **2007**, 5: 43-48

[175] **Cornely O.A., Böhme A., Buchheidt D., Einsele H., Heinz W.J., Karthaus M., Krause S.W., Krüger W., Maschmeyer G., Penack O., Ritter J., Ruhnke M., Sandgerr M., Sieniawski M., Vehreschild J.J., Wolf H.H., Ullmann A.J.** Primary prophylaxis of invasive fungal infections in patients with hematologic

malignancies. Recommendations of the Infectious Diseases Working Party of the German Society for Haematology and Oncology. *Haematologica*, **2009**, 94(1): 113-122

[176] **Goodman J.L., Winston D.J., Greenfield R.A., Chandrasekar P.H., Fox B., Kaizer H., Shadduck R.K., Shea T.C., Stiff P., Friedman D.J., Powderly W.G., Silber J.L., Horowitz H., Lichtin A., Wolff S.N., Mangan K.F., Silver S.M., Weisdorf D., Ho W.G., Gilbert G., Buell D.** A controlled trial of fluconazole to prevent fungal infections in patients undergoing bone marrow transplantation. *The New England Journal of Medicine*, **1992**, 326(13): 845-851

[177] **Mikolajewska A., Schwartz S., Ruhnke M.** Antifungal treatment strategies in patients with haematological diseases or cancer: from prophylaxis to empirical, pre-emptive and targeted therapy. *Mycoses*, **2012**, 55(1): 2-16

[178] **Pizzo P.A., Robichaud K.J., Gill F.A., Witebsky F.G.** Empiric antibiotic and antifungal therapy for cancer patients with prolonged fever and granulocytopenia. *The American Journal of Medicine*, **1982**, 72(1): 101-111

[179] **EORTC International Antimicrobial Therapy Cooperative Group**. Empiric antifungal therapy in febrile granulocytopenic patients. *The American Journal of Medicine*, **1989**, 86(6 Pt 1): 668-672

[180] **Mahfouz T., Anaissie E.** Prevention of fungal infections in the immunocompromised host. *Current Opinion in Investigational Drugs*, **2003**, 4(8): 974-990

[181] **Kontoyiannis D.P.** Invasive mycoses: strategies for effective management. *The American Journal of Medicine*, **2012**, 125(Suppl.

1): S25-S38

[182] **Marchetti O., Cordonnier C., Calandra T.** Empirical antifungal therapy in neutropaenic cancer patients with persistent fever. *European Journal of Cancer Supplements*, **2007**, 5: 32-42

[183] **Bennet J.E., Powers J., Walsh T.J., Viscoli C., de Pauw B., Dismukes W., Galgiani J., Glauser M., Herbrecht R., Kauffman C., Lee J., Pappas P., Rex J., Verweij P.** Forum reports: issues in clinical trials of empirical antifungal therapy in treating febrile neutropenic patients. *Clinical Infectious Diseases*, **2003**, 36(Suppl. 3): S117–122

[184] **Cordonnier C., Pautas C., Maury S., Vekhoff A., Farhat H., Suarez F., Dhédin N., Isnard F., Ades L., Kuhnowski F., Foulet F., Kuentz M., Maison P., Bretagne S., Schwarzinger M.** Empirical versus preemptive antifungal therapy for high-risk, febrile, neutropenic patients: a randomized, controlled trial. *Clinical Infectious Diseases*, **2009**, 48(8): 1042-1051

[185] **Martín-Peña A., Aguilar-Guisado M., Cisneros J.M.** Does the current treatment of invasive fungal infection need to be reviewed? *Enfermedades Infecciosas y Microbiología Clínica*, **2014**, 32(8): 523-528

[186] **Standiford H.C., Chan S., Tripoli M., Weekes E., Forrest G.N.** Antimicrobial stewardship at a large tertiary care academic medical center: cost analysis before, during, and after a 7-year program. *Infection Control and Hospital Epidemiology*, **2012**, 33(4): 338-345

[187] **Schneider T., Halter J., Heim D., Passweg J., Stern M., Tichelli A., Weisser M., Gerull S.** Pre-emptive diagnosis and treatment of fungal infections-evaluation of a single-center policy. *Clinical Microbiology and Infection*, **2012**, 18(2): 189-194

[188] **Maertens J., Theunissen K., Verhoef G., Verschakelen J., Lagrou K., Verbeken E., Wilmer A., Verhaegen J., Boogaerts M., van Eldere J.** Galactomannan and computed tomography-based preemptive antifungal therapy in neutropenic patients at high risk for invasive fungal infection: a prospective feasibility study. *Clinical Infectious Diseases*, **2005**, 41(9): 1242-1250

[189] **Aguado J.M., Ruiz-Camps I., Muñoz P., Mensa J., Almirante B., Vázquez L., Rovira M., Martín-Dávila P., Moreno A., Alvarez-Lerma F., León C., Madero L., Ruiz-Contreras J., Fortún J., Cuenca-Estrella M.; Grupo de Estudio de Micología Médica de la SEIMC (GEMICOMED).** Guidelines for the treatment of Invasive Candidiasis and other yeasts, Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC). 2010 Update. *Enfermedades Infecciosas Microbiología Clínica*, **2011**, 29(5): 345-361

[190] **Fortún J., Carratalá J., Gavaldá J., et al.**, Guidelines for the treatment of invasive fungal disease by Aspergillus spp. and other fungi issued by the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC), 2011 Update, *Enfermedades Infecciosas Microbiología Clínica*, **2011**, 29:435-454

[191] **Maertens J., Marchetti O., Herbrecht R., Cornely O.A., Flückiger U., Frêre P., Gachot B., Heinz W.J., Lass-Flörl C., Ribaud P., Thiebaut A., Cordonnier C.; Third European Conference on Infections in Leukemia.** European guidelines for antifungal management in leukemia and hematopoietic stem cell transplant recipients: summary of the ECIL 3—2009 update. *Bone Marrow Transplant*, **2011**, 46(5): 709-718

[192] **Freifeld A.G., Bow E.J., Sepkowitz K.A., Boeckh M.J., Ito J.I.,**

Mullen C.A., Raad I.I., Rolston K.V., Young J.A., Wingard J.R.; Infectious Diseases Society of America. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the Infectious Diseases Society of America (IDSA). *Clinical Infectious Diseases*, **2011**, 52(4): 56-93

[193] **Rüping M.J., Vehreschild J.J., Cornely O.A.** Patients at high risk of invasive fungal infections: when and how to treat. *Drugs*, **2008**, 68(14): 1941-1962

[194] **Kirkpatrick W.R., Perea S., Coco B.J., Patterson T.F.** Efficacy of caspofungin alone and in combination with voriconazole in a guinea pig model of invasive aspergillosis. *Antimicrobial Agents and Chemotherapy*, **2002**, 46(8): 2564-2568

[195] **Nagasaki Y., Eriguchi Y., Uchida Y., Miyake N., Maehara Y., Kadowaki M., Harada M., Akashi K., Shimono N.** Combination therapy with micafungin and amphotericin B for invasive pulmonary aspergillosis in an immunocompromised mouse model. *The Journal of Antimicrobial Chemotherapy*, **2009**, 64(2): 379-382

[196] **Panackal A.A., Parisini E., Proschan M.** Salvage combination antifungal therapy for acute invasive aspergillosis may improve outcomes: a systematic review and meta-analysis. *International Journal of Infectious Diseases*, **2014**, 28: 80-94

[197] **Ostrosky-Zeichner L.** Combination antifungal therapy: a critical review of the evidence. *Clinical Microbiology and Infection*, **2008**, 14(Suppl. 4): 65-70

[198] **Balkovec J.M., Hughes D.L., Masurekar P.S., Sable C.A., Schwartz R.E., Singh S.B.** Discovery and development of first in class antifungal caspofungin (CANCIDAS®)--a case study. *Natural Product Reports*, **2014**, 31(1): 15-34

[199] **Schwartz R.E., Glacobbe R.A., Bland J.A., Monaghan R.L.** L-671,329, a new antifungal agent. I. Fermentation and isolation. *The Journal of Antibiotics (Tokyo)*, **1989**, 42(2): 163-167

[200] **Schwartz R.E., Sesin D.F., Joshua H. Wilson K.E., Kempf A.J., Goklen K.A., Kuehner D., Gailliot P., Gleason C., White R.** Pneumocandins from Zalerion arboricola. I. Discovery and isolation. *The Journal of Antibiotics (Tokyo)*, **1992**, 45(12): 1853-1866

[201] **Bills G.F., Platas G., Peláez F., Masurekar P.** Reclassification of a pneumocandin-producing anamorph, Glarea lozoyensis gen. et sp. nov., previously identified as Zalerion arboricola. *Mycological Research*, **1999**, 103(2): 179-192

[202] **Daneshtalab M.** Discovery of chlorogenic acid-based peptidomimetics as a novel class of antifungals. A success story in rational drug design. *Journal of Pharmacy & Pharmaceutical Sciences*, **2008**, 11(2): 44S-55S

[203] **Douglas C.M.** Fungal beta(1,3)-D-glucan synthesis. *Medical Mycology*, **2001**, 39(Suppl. 1): 55-66

[204] **Zambias R.A., Hammond M.L., Heck J.V., Bartizal K., Trainor C., Abruzzo G., Schmatz D.M., Nollstadt K.M.** Preparation and structure-activity relationships of simplified analogs of the antifungal agent cilofungin: a total synthesis approach. *Journal of Medicinal Chemistry*, **1992**, 35(15): 2843-2855

[205] **Taft C.S., Selitrennikoff C.P.** Cilofungin inhibition of (1-3)-beta-glucan synthase: the lipophilic side chain is essential for inhibition of enzyme activity. *The Journal of Antibiotics (Tokyo)*, **1990**, 43(4): 433-437

[206] **Ma C.M., Kully M., Khan J.K., Hattori M., Daneshtalab M.**

Synthesis of chlorogenic acid derivatives with promising antifungal activity. *Bioorganic & Medicinal Chemistry*, **2007**, 15(21): 6830-6833

[207] **Ma C.M., Abe T., Komiyama T., Wang W., Hattori M., Daneshtalab M.** Synthesis, anti-fungal and 1,3-β-D-glucan synthase inhibitory activities of caffeic and quinic acid derivatives. *Bioorganic & Medicinal Chemistry*, **2010**, 18(19): 7009-7014

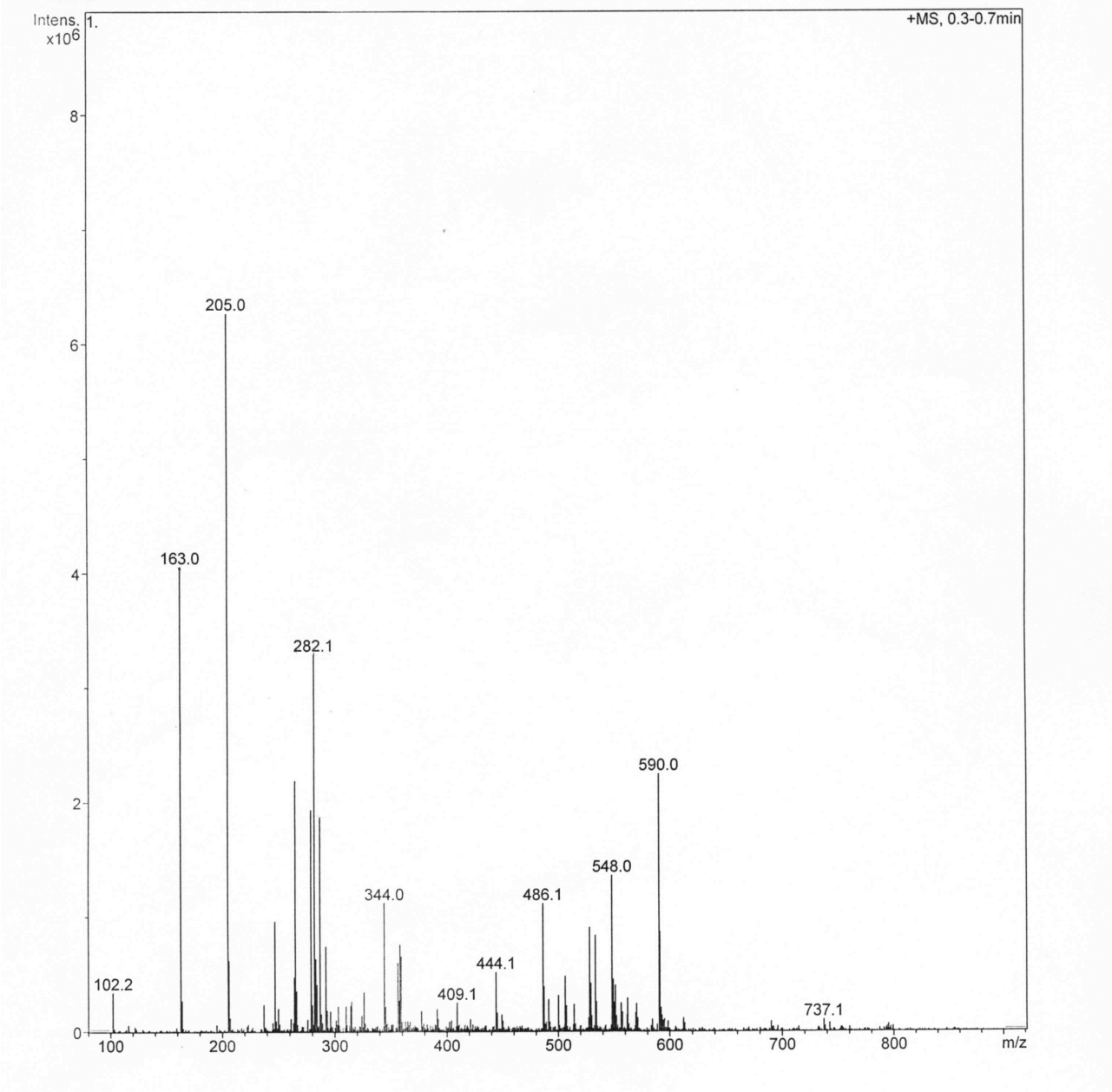
[208] **Wang W., He X.L., Ma C.M.** Metabolism schemes of chlorogenic, quinic and caffeic acid derivatives in rats deduced by molecular fragments detected with optimized UPLC-APCI-MS/MS conditions. *International Journal of Mass Spectrometry*, **2013**, 337: 34-42

[209] **Blondelle S.E., Houghten R.A.** Comparison of 55% TFA/CH2Cl2 and 100% TFA for Boc group removal during solid-phase peptide synthesis. *International Journal of Peptide and Protein Research*, **1993**, 41(6): 522-527

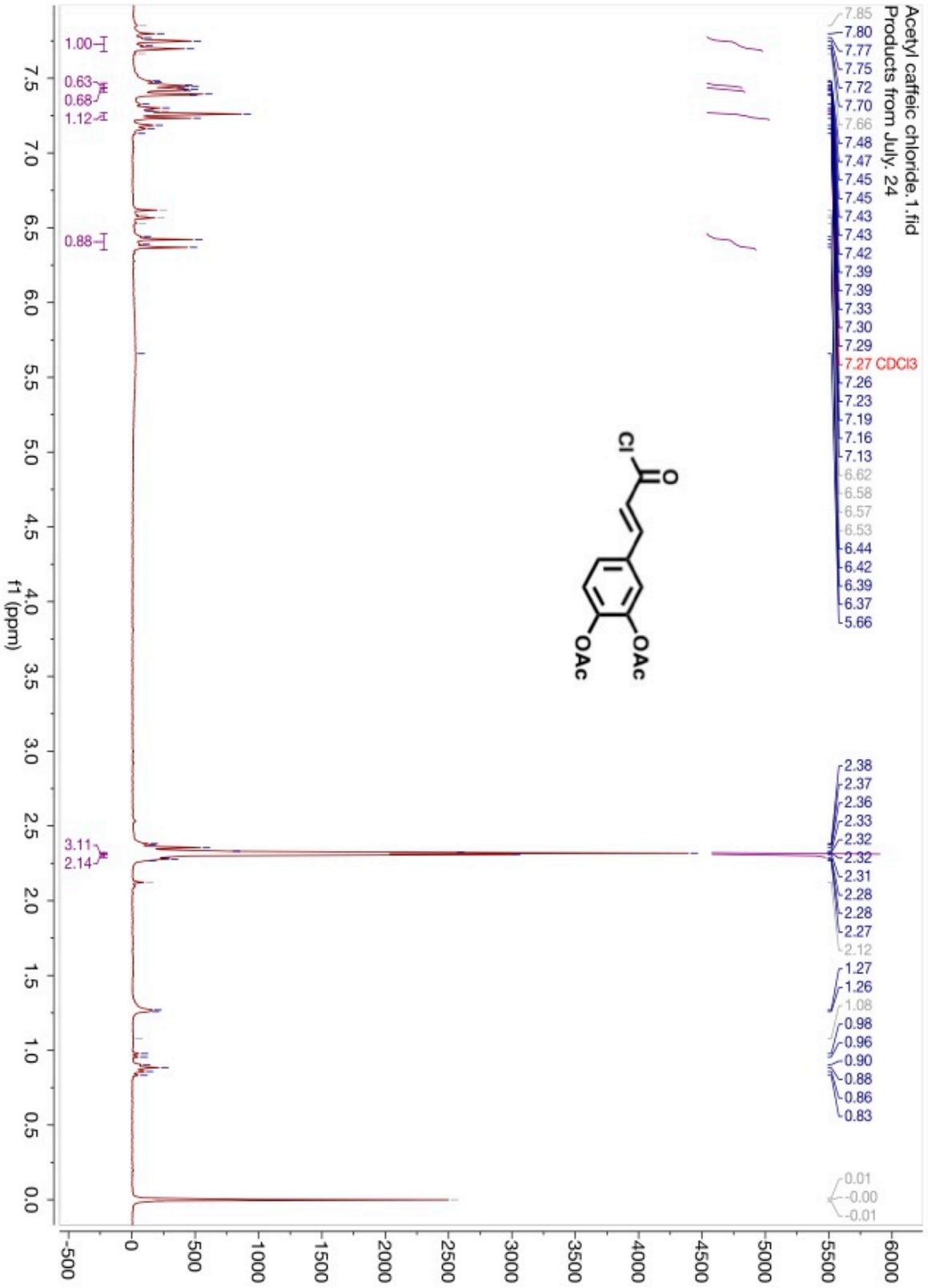
Appendices

^{1}H NMR, LC-MS and IR spectra of synthesized compounds:

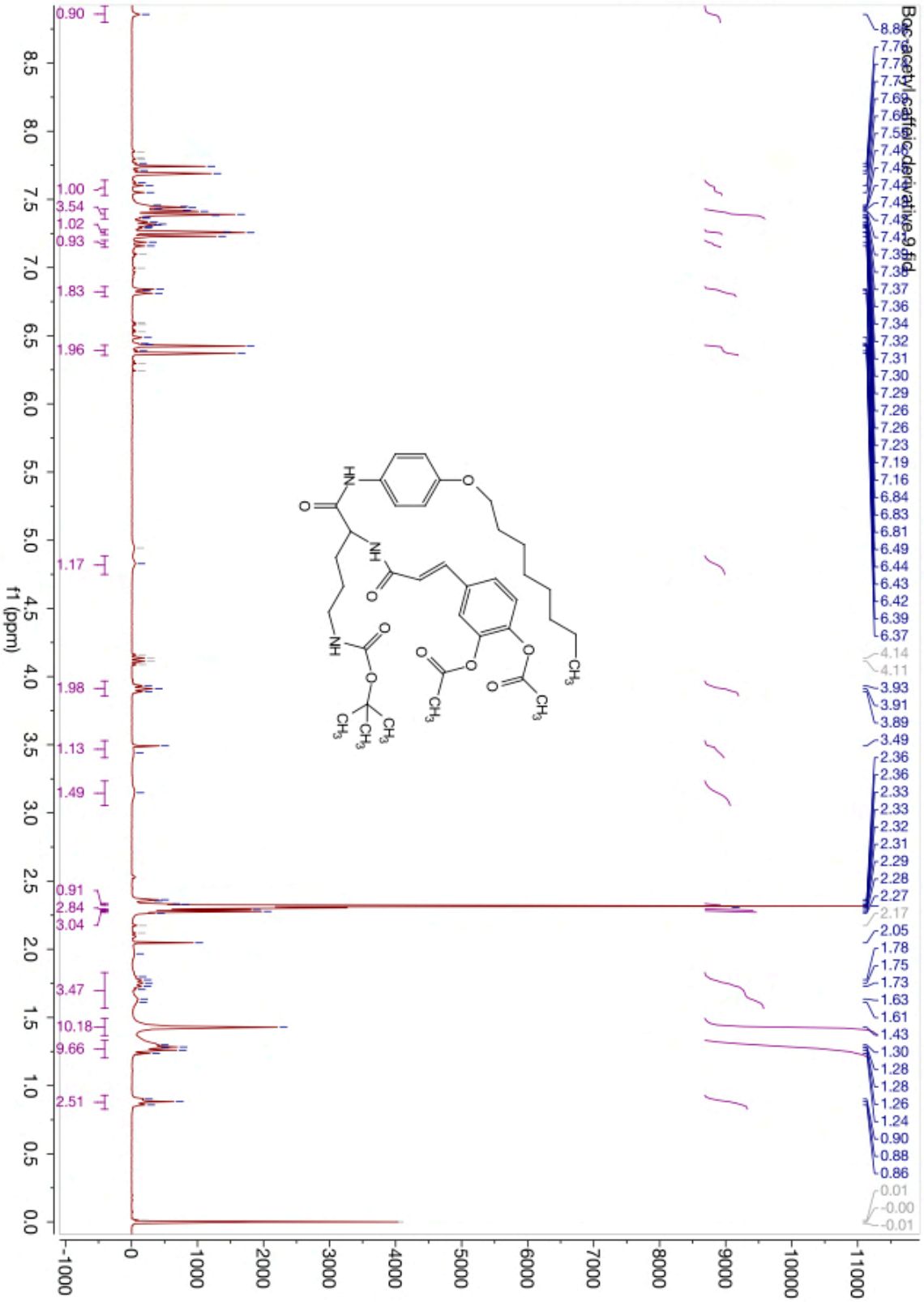
3,4-diacetoxycinnamoyl chloride **15a**: LC-MS



3,4-diacetoxycinnamoyl chloride **15a**: ^{1}H NMR



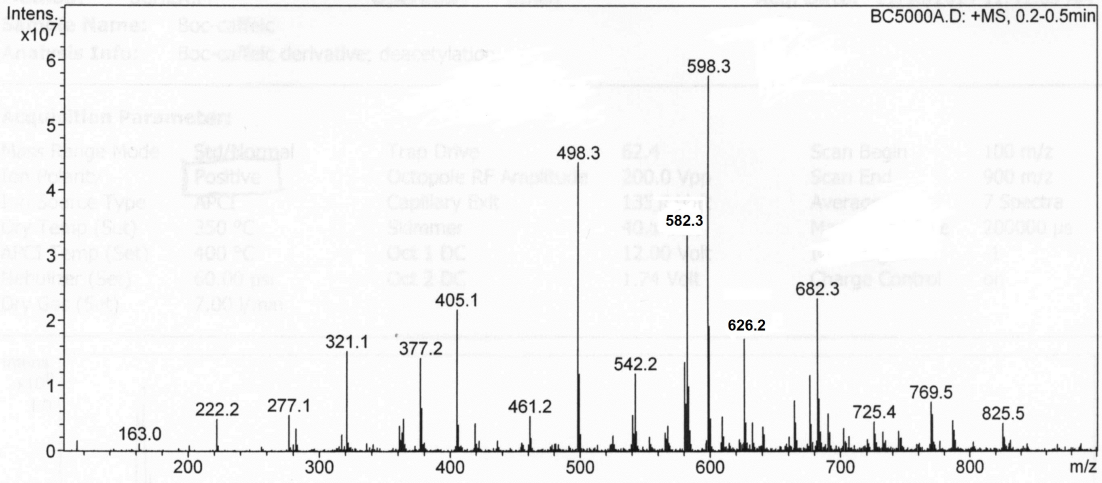
Caffeic acid derivative **16a**: ^{1}H NMR



Caffeic acid derivative **16a**: LC-MS (APCI-positive)

Compound Mass Spectrum List Report - MS

Cmpd 1, 0.3 min



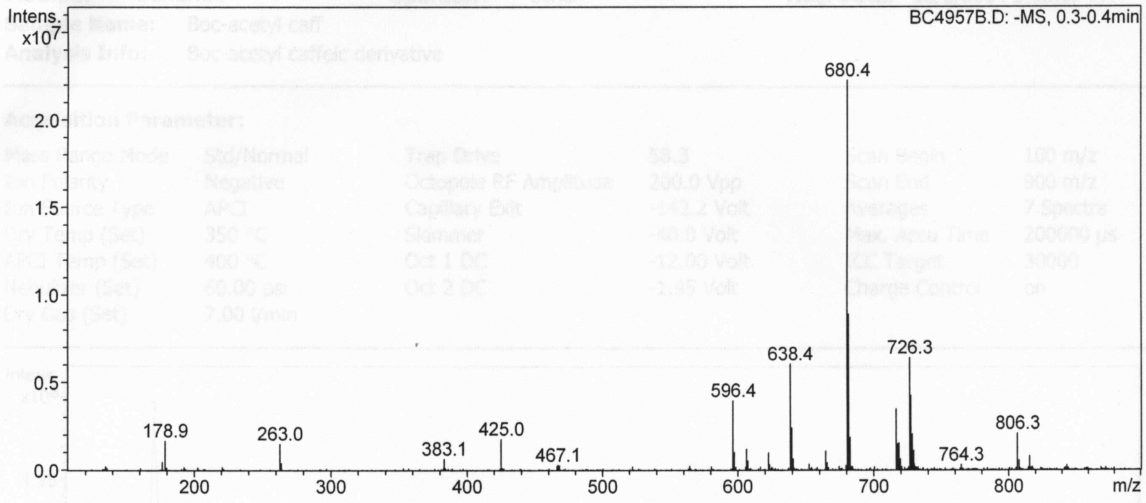
Mass List:

#	m/z	I
1	222.2	4848682
2	277.1	5458884
3	321.1	15318606
4	364.3	4937501
5	377.2	14251927
6	378.2	6566091
7	405.1	21645744
8	461.2	5318938
9	498.3	44224208
10	499.3	11825210
11	540.3	5510736
12	542.2	11869920
13	580.3	13612307
14	581.3	7270374
15	582.3	33022002
16	583.3	9872347
17	598.3	57612800
18	599.3	19141770
19	600.3	4950628
20	609.2	5232653
21	626.2	17210120
22	664.3	7712951
23	676.3	11598283
24	682.3	23399132
25	683.3	7996911
26	690.3	5698061
27	725.4	4482048
28	769.5	7490362
29	770.4	5585413
30	786.3	4621619

Caffeic acid derivative **16a**: LC-MS (APCI-negative)

Compound Mass Spectrum List Report - MS

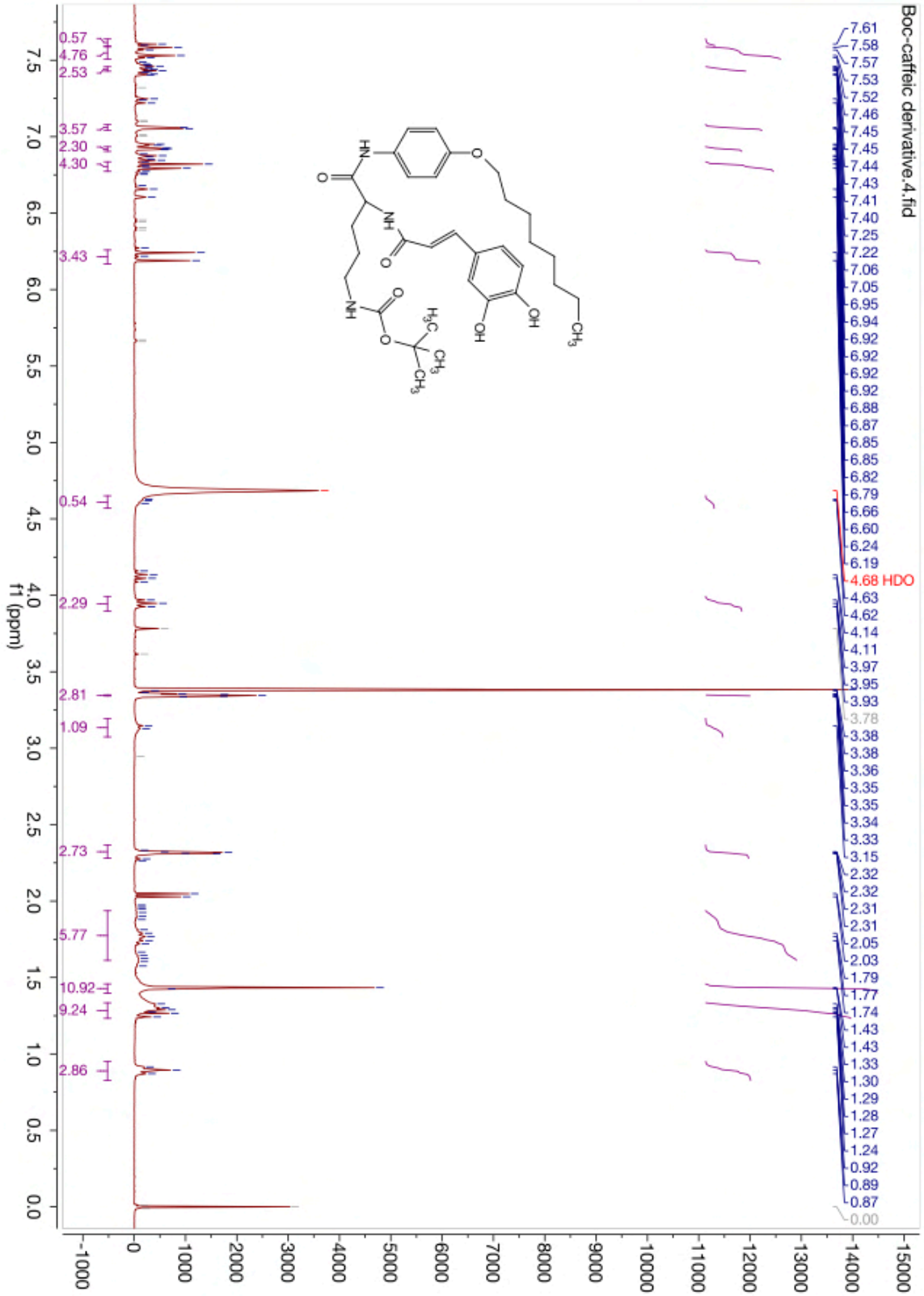
Cmpd 1, 0.3 min



Mass List:

#	m/z	I
1	178.9	1666021
2	263.0	1461452
3	263.0	1491688
4	383.1	639509
5	425.0	1753781
6	596.4	3947853
7	597.4	1012449
8	606.3	1191600
9	607.3	522759
10	622.4	976645
11	638.4	6034431
12	639.4	2386640
13	640.4	635548
14	664.4	1079461
15	680.4	22237438
16	681.3	8875094
17	682.3	1847777
18	716.3	3483388
19	717.3	1483637
20	718.3	1535745
21	719.4	638784
22	726.3	6405033
23	727.2	4237989
24	727.9	1129286
25	728.2	2048019
26	728.7	578957
27	729.2	1107414
28	806.3	2082123
29	807.3	554058
30	815.3	785907

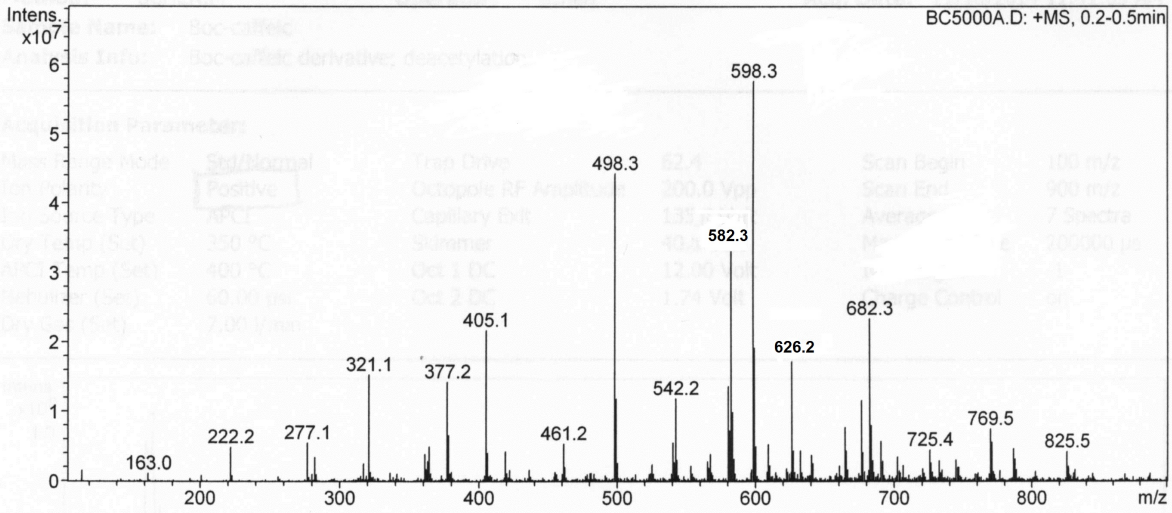
Caffeic acid derivative **16d**: ^{1}H NMR



Caffeic acid derivative **16d**: LC-MS (APCI-positive)

Compound Mass Spectrum List Report - MS

Cmpd 1, 0.3 min



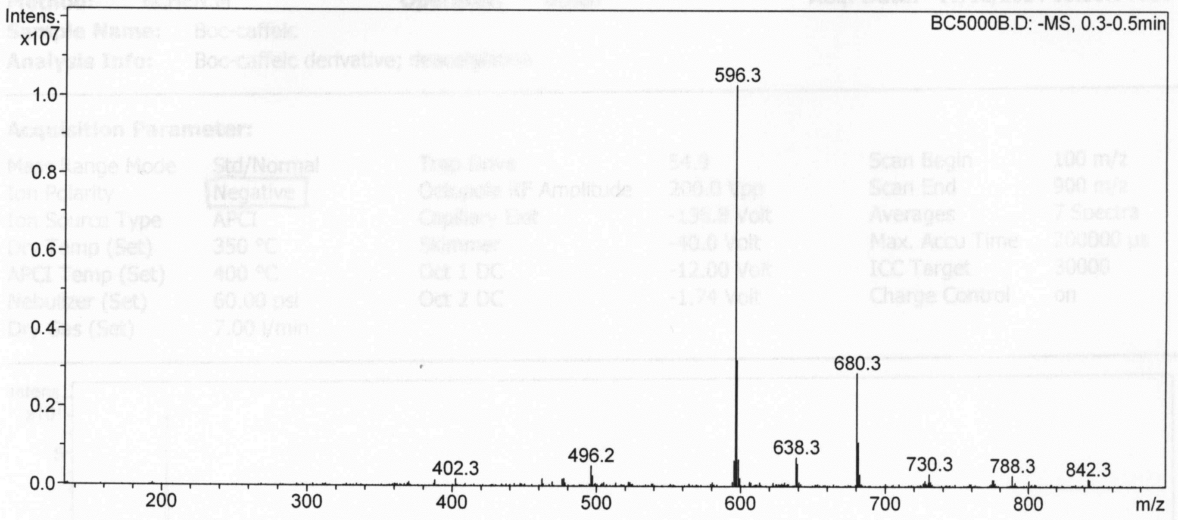
Mass List:

#	m/z	I
1	222.2	4848682
2	277.1	5458884
3	321.1	15318606
4	364.3	4937501
5	377.2	14251927
6	378.2	6566091
7	405.1	21645744
8	461.2	5318938
9	498.3	44224208
10	499.3	11825210
11	540.3	5510736
12	542.2	11869920
13	580.3	13612307
14	581.3	7270374
15	582.3	33022002
16	583.3	9872347
17	598.3	57612800
18	599.3	19141770
19	600.3	4950628
20	609.2	5232653
21	626.2	17210120
22	664.3	7712951
23	676.3	11598283
24	682.3	23399132
25	683.3	7996911
26	690.3	5698061
27	725.4	4482048
28	769.5	7490362
29	770.4	5585413
30	786.3	4621619

Caffeic acid derivative **16d**: LC-MS (APCI-negative)

Compound Mass Spectrum List Report - MS

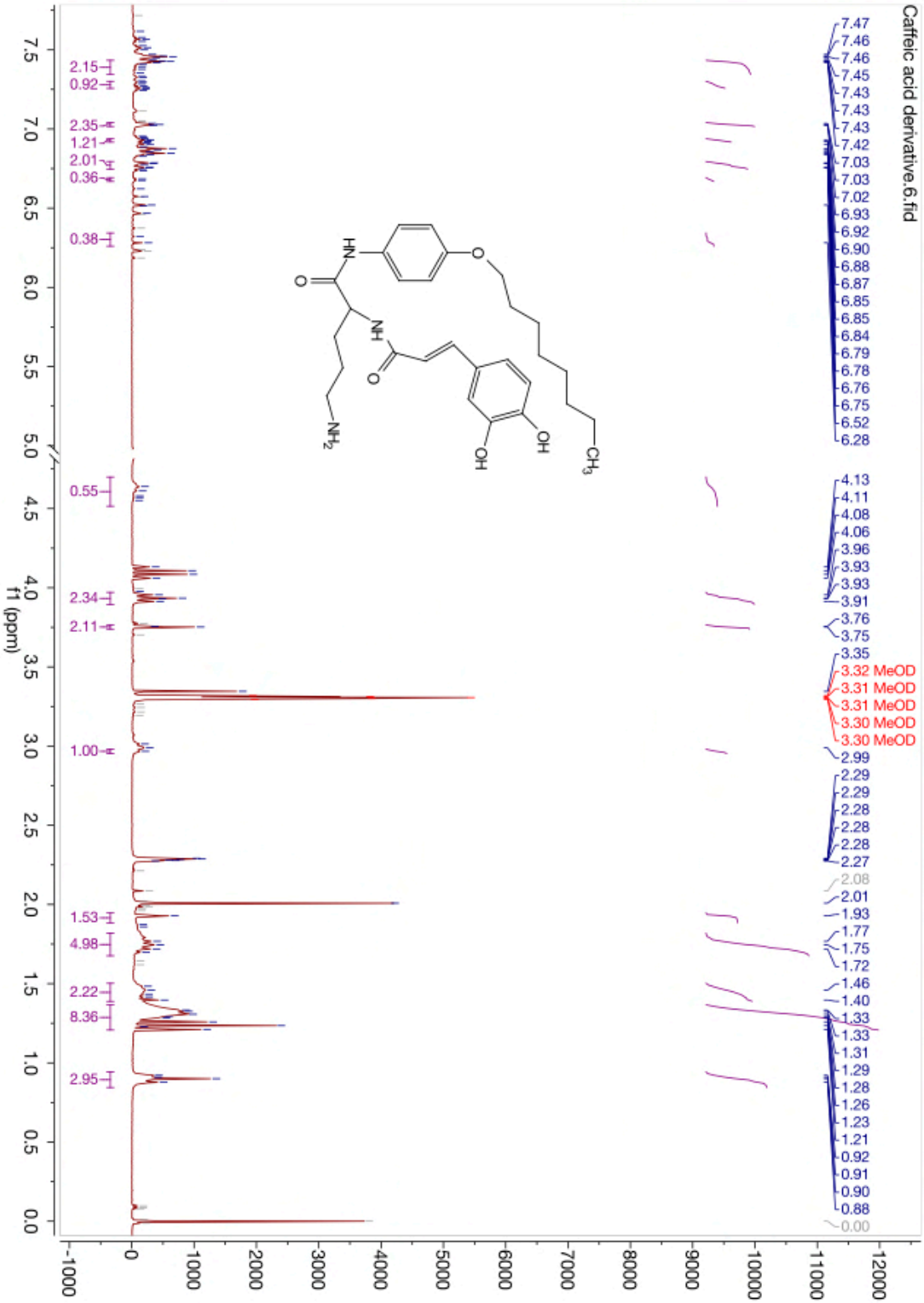
Cmpd 1, 0.3 min



Mass List:

#	m/z	I
1	388.1	134189
2	402.3	171915
3	462.3	173136
4	476.2	165453
5	477.3	184660
6	496.2	511783
7	497.3	253717
8	594.3	105035
9	595.3	655660
10	596.3	10300465
11	597.2	3220779
12	598.3	682938
13	598.5	106959
14	599.2	200130
15	606.2	102338
16	638.3	736102
17	639.2	571333
18	680.3	2899825
19	681.3	1118683
20	682.2	297278
21	727.4	136947
22	729.6	102836
23	730.3	315283
24	731.3	109158
25	774.2	167647
26	775.2	157983
27	788.3	267584
28	800.2	142683
29	842.3	182584
30	843.3	162953

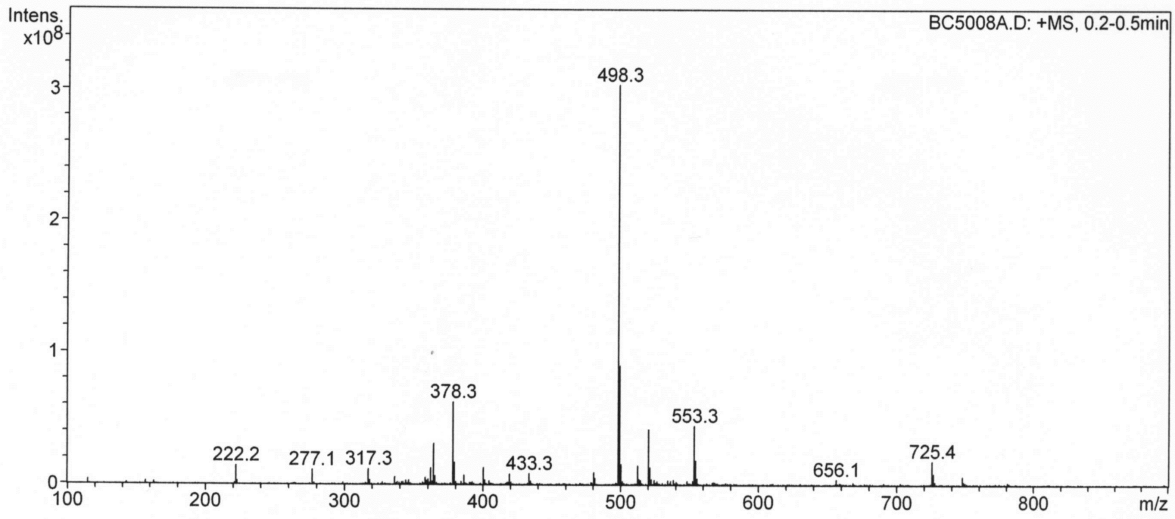
Caffeic acid derivative **16g**: ^{1}H NMR



Caffeic acid derivative **16g**: LC-MS (APCI-positive)

Compound Mass Spectrum List Report - MS

Cmpd 1, 0.3 min



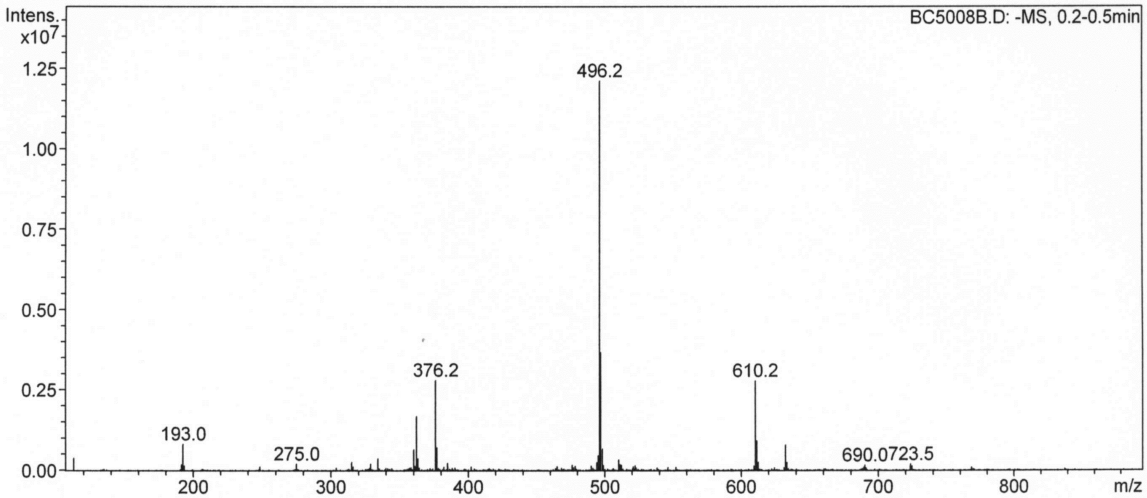
Mass List:

#	m/z	I
1	222.2	14153879
2	277.1	11003185
3	317.3	11593977
4	336.3	5678566
5	358.2	5176760
6	362.2	12639204
7	364.3	30863834
8	365.2	6761619
9	378.3	62233312
10	379.3	16848156
11	386.2	7167539
12	400.3	12845885
13	419.3	7951169
14	433.3	8537771
15	480.3	9225176
16	481.3	4936975
17	498.3	303199264
18	499.2	90249480
19	500.3	15528652
20	512.3	14438689
21	513.3	4173266
22	520.2	43738236
23	521.3	13111618
24	553.3	44647952
25	554.3	18421292
26	555.3	4783623
27	656.1	4214391
28	725.4	18020594
29	726.4	8437433
30	747.4	6220928

Caffeic acid derivative **16g**: LC-MS (APCI-negative)

Compound Mass Spectrum List Report - MS

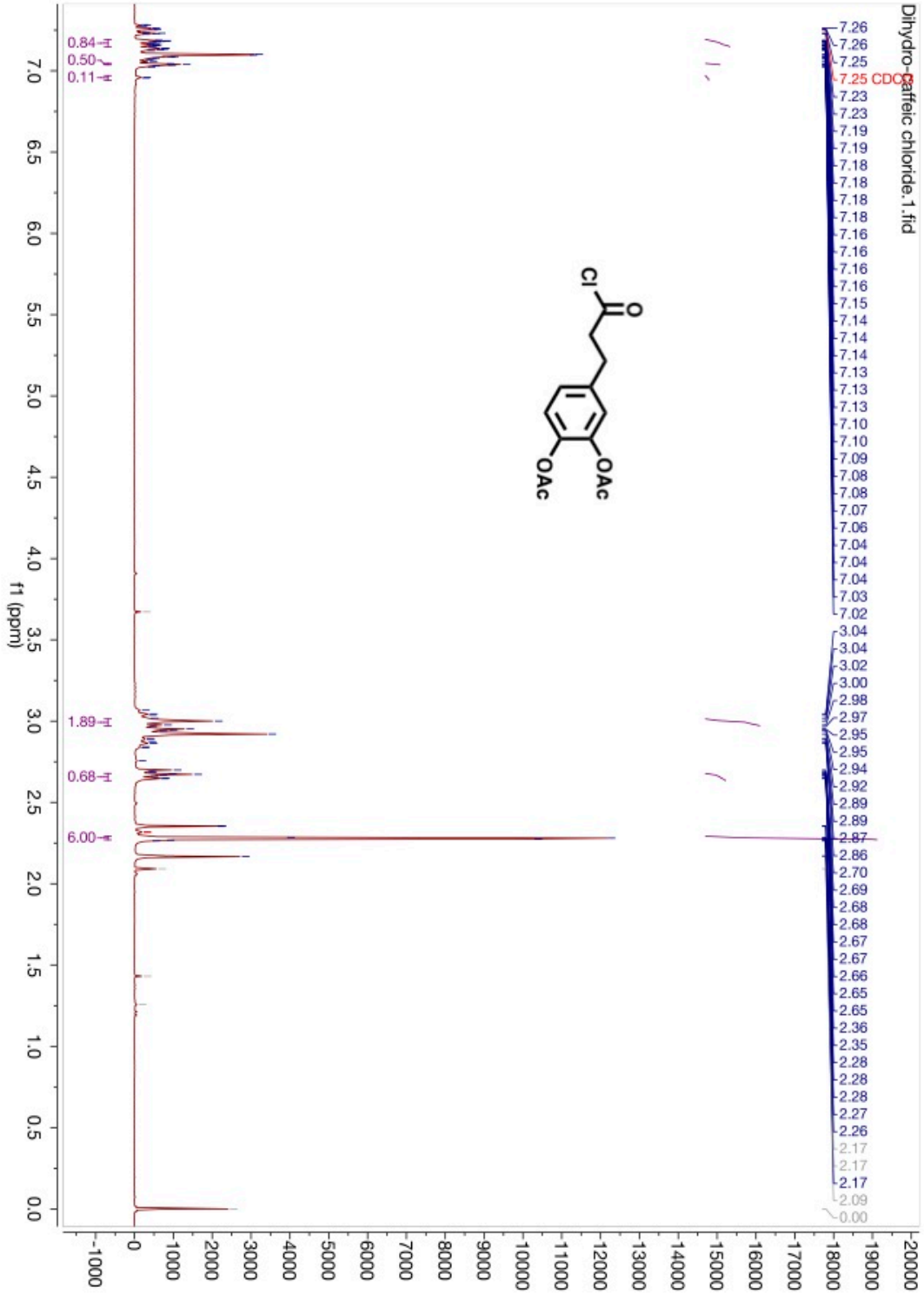
Cmpd 1, 0.3 min



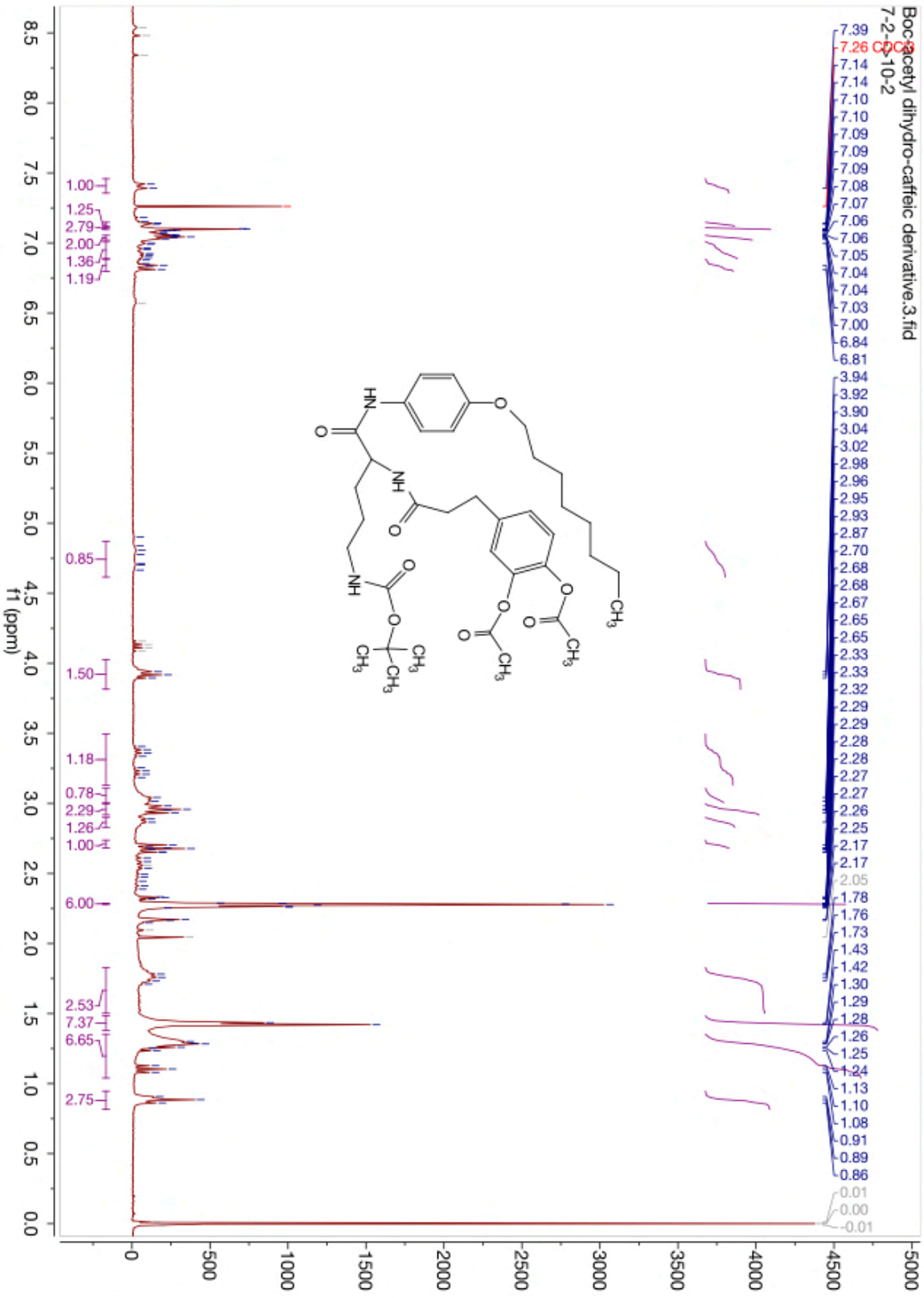
Mass List:

#	m/z	I
1	113.0	382292
2	192.0	178357
3	192.9	167644
4	193.0	814740
5	275.0	202614
6	315.2	252765
7	329.0	199071
8	334.2	369141
9	360.2	647501
10	362.2	1681711
11	363.2	375104
12	376.2	2797053
13	377.2	713057
14	384.9	226464
15	476.2	168414
16	494.2	253894
17	495.3	465923
18	496.2	12098787
19	497.2	3663420
20	498.2	662895
21	510.2	332389
22	511.2	182261
23	512.1	164299
24	610.2	2778797
25	611.1	918576
26	612.1	250861
27	632.1	785502
28	633.1	245481
29	723.3	153264
30	723.5	188497

3,4-diacetoxydihydro-caffeoyl chloride **19b**: ^{1}H NMR



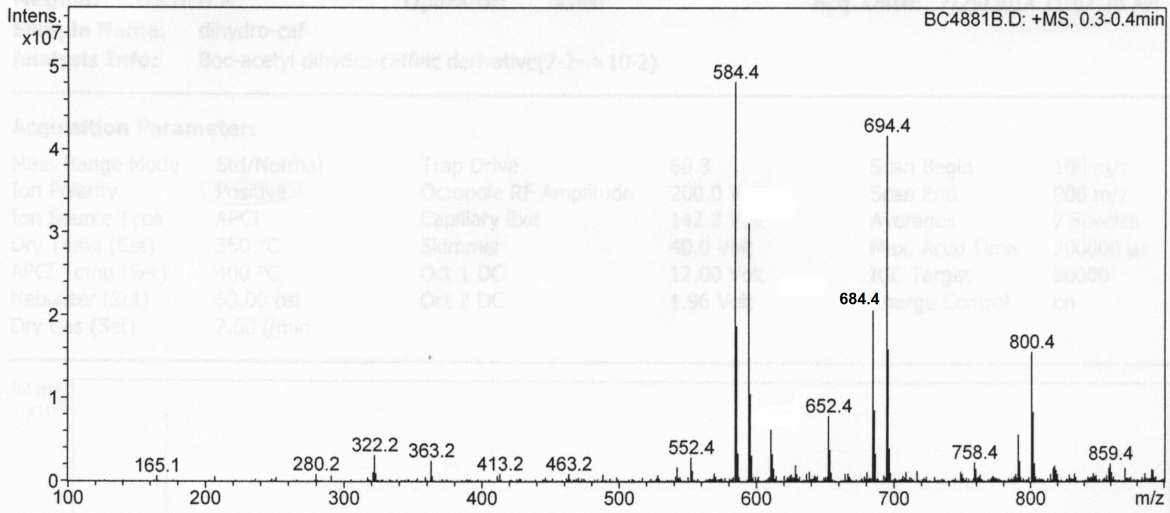
Dihydrocaffeic acid derivative **20a**: ^{1}H NMR



Dihydrocaffeic acid derivative **20a**: LC-MS (APCI-positive)

Compound Mass Spectrum List Report - MS

Cmpd 1, 0.3 min



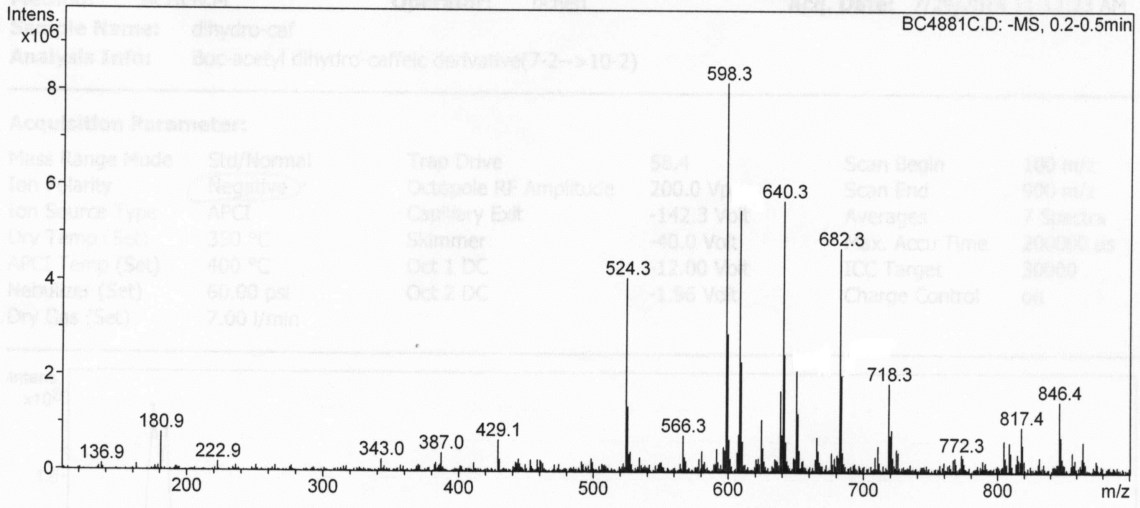
Mass List:

#	m/z	I
1	322.2	3119574
2	363.2	2417675
3	542.4	1706684
4	552.4	2851735
5	584.4	48089888
6	585.4	18666592
7	586.4	3316817
8	594.3	31041266
9	595.4	10520370
10	596.4	3077909
11	610.4	6195902
12	611.4	3237030
13	628.3	1919542
14	652.4	7846043
15	653.4	3747582
16	684.4	20583298
17	685.3	8522941
18	686.3	3275679
19	694.4	41626780
20	695.3	15889008
21	696.3	3965367
22	758.4	2258809
23	790.4	5580560
24	791.4	2400618
25	800.4	15581768
26	801.4	8319161
27	802.4	2168113
28	816.4	1621791
29	817.4	1874348
30	859.4	2138208

Dihydrocaffeic acid derivative **20a**: LC-MS (APCI-negative)

Compound Mass Spectrum List Report - MS

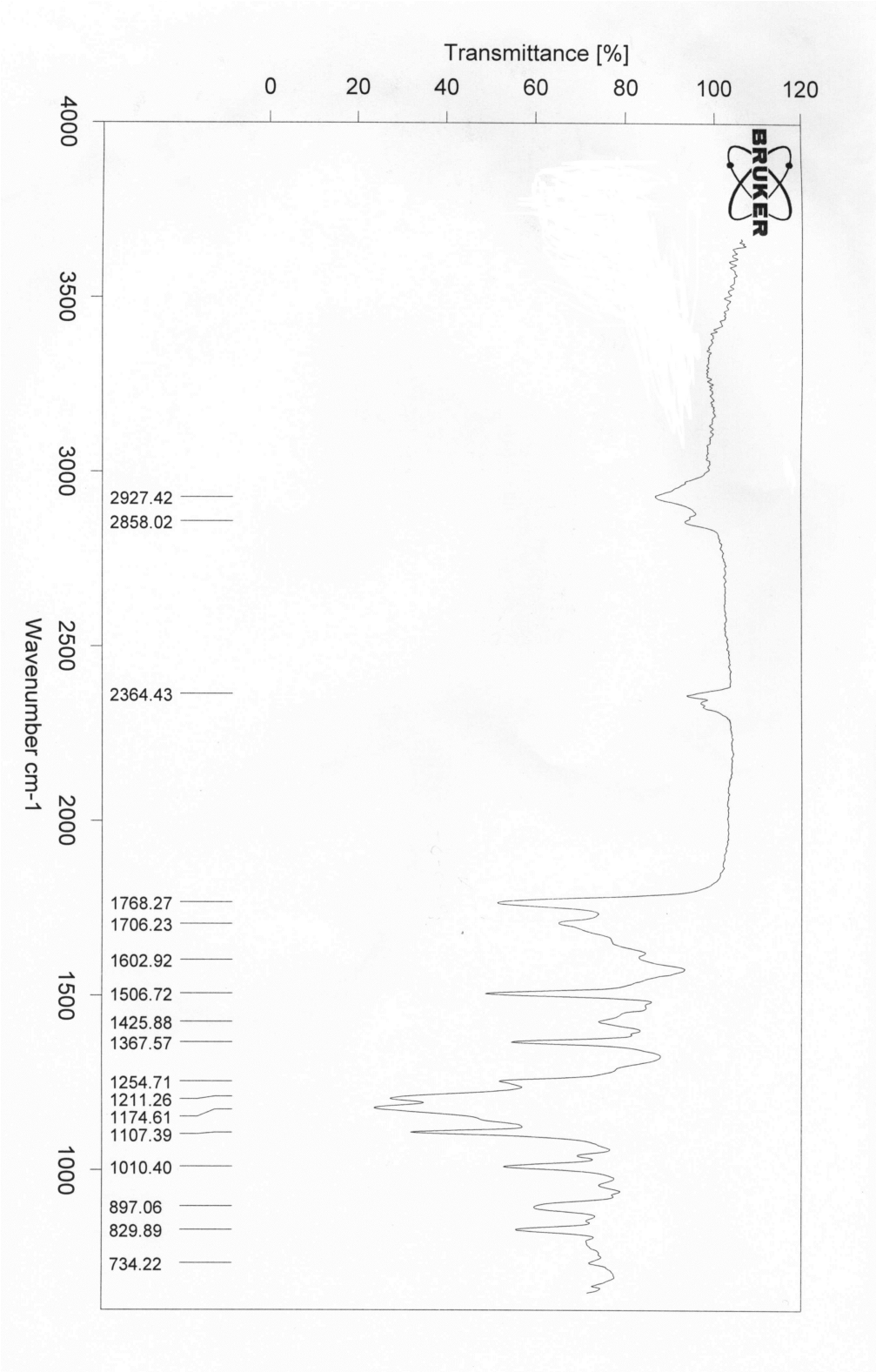
Cmpd 1, 0.3 min



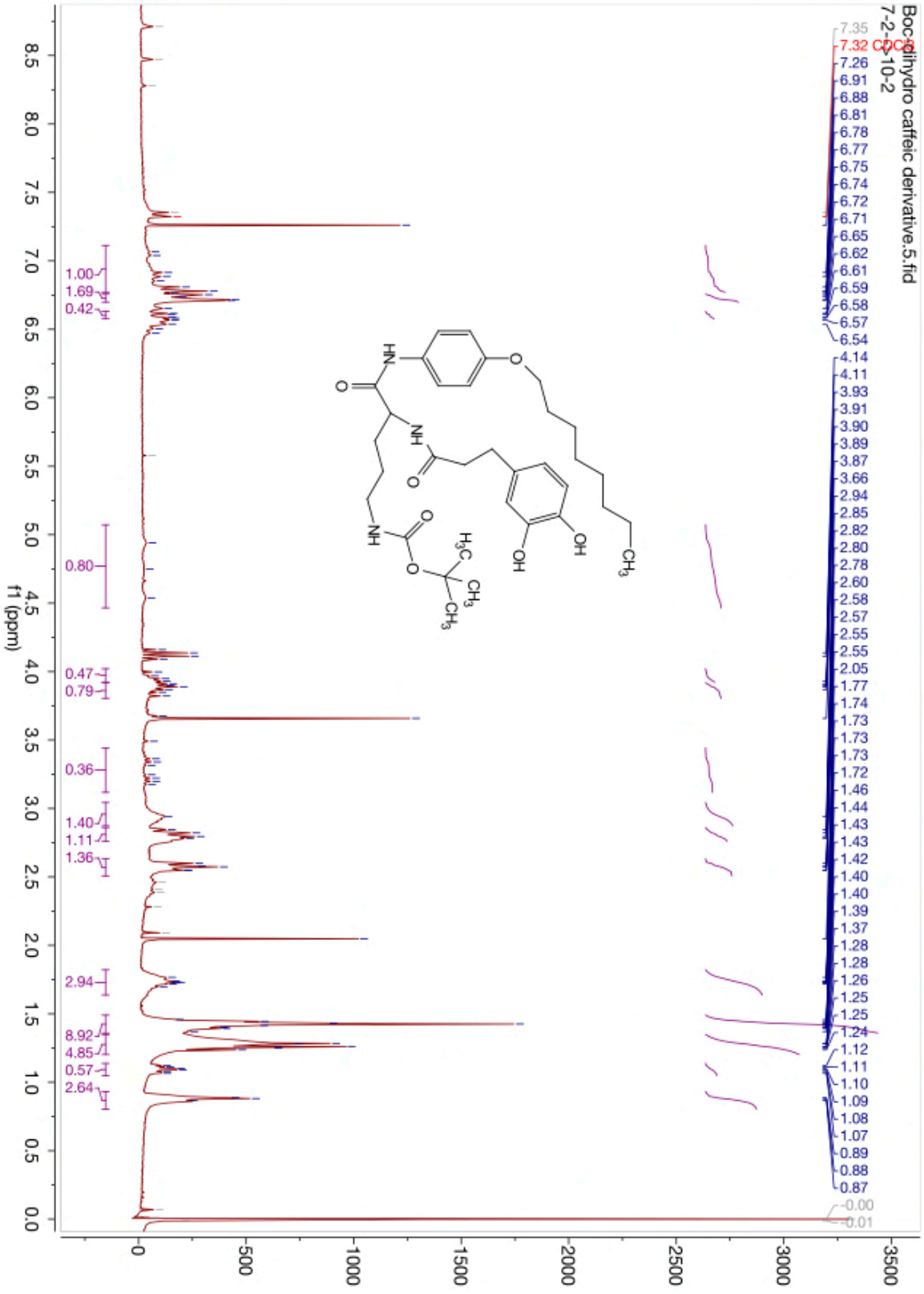
Mass List:

#	m/z	I
1	180.9	779126
2	429.1	649657
3	524.3	4073631
4	525.3	1368468
5	566.3	763904
6	598.3	8187980
7	599.3	2909301
8	600.3	679230
9	607.3	789569
10	608.3	5499682
11	609.3	2313110
12	610.3	642762
13	624.4	1099777
14	638.3	1708838
15	639.3	788626
16	640.3	5697731
17	641.3	2471050
18	650.3	2126800
19	651.3	1226662
20	665.3	732875
21	682.3	4705895
22	683.3	2036034
23	718.3	1862847
24	719.3	773649
25	720.3	885840
26	804.4	666154
27	817.4	948649
28	846.4	1488002
29	847.4	739220
30	864.3	642489

Dihydrocaffeic acid derivative **20a**: IR



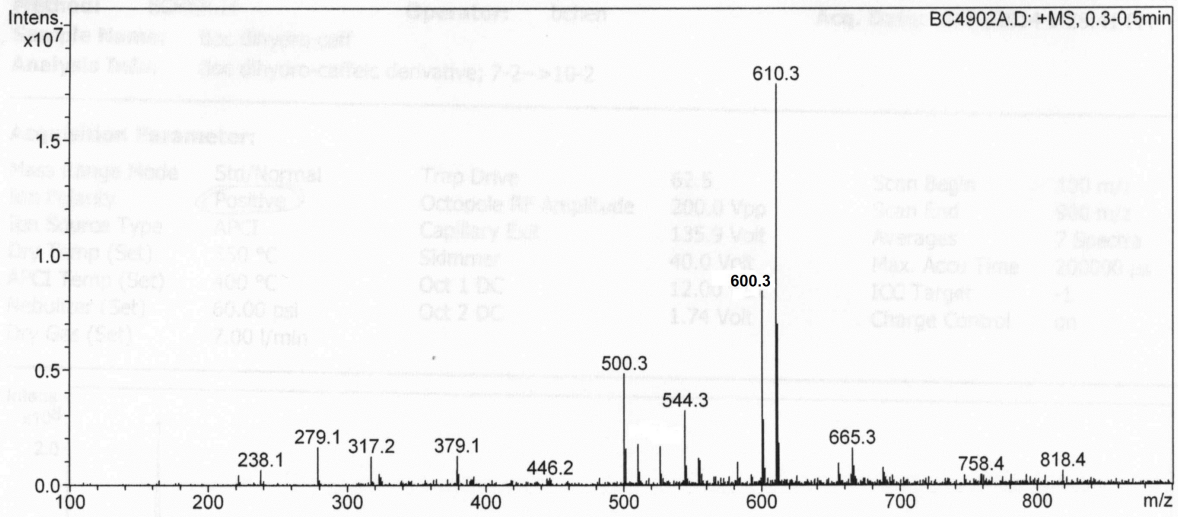
Dihydrocaffeic acid derivative **20b**: ^{1}H NMR



Dihydrocaffeic acid derivative **20b**: LC-MS (APCI-positive)

Compound Mass Spectrum List Report - MS

Cmpd 1, 0.3 min



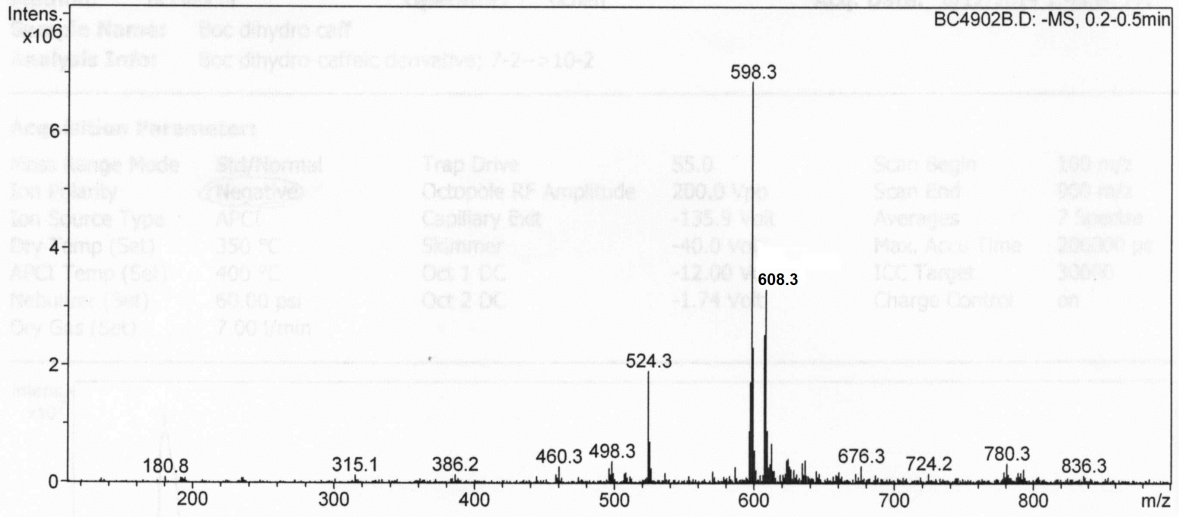
Mass List:

#	m/z	I
1	238.1	643752
2	279.1	1633169
3	317.2	1234999
4	323.0	473037
5	379.0	682736
6	379.1	1260097
7	380.2	476123
8	500.3	4840403
9	501.3	1568910
10	510.3	1748018
11	511.3	577233
12	526.3	1662072
13	527.3	478617
14	544.3	3216334
15	545.3	827842
16	554.2	1150479
17	555.3	1059304
18	582.4	982327
19	600.3	8430109
20	601.3	2822414
21	602.2	721609
22	610.3	17435040
23	611.3	6991838
24	612.3	1802712
25	655.3	928636
26	665.3	1591955
27	666.3	824088
28	687.3	751796
29	688.3	524491
30	818.4	595832

Dihydrocaffeic acid derivative **20b**: LC-MS (APCI-negative)

Compound Mass Spectrum List Report - MS

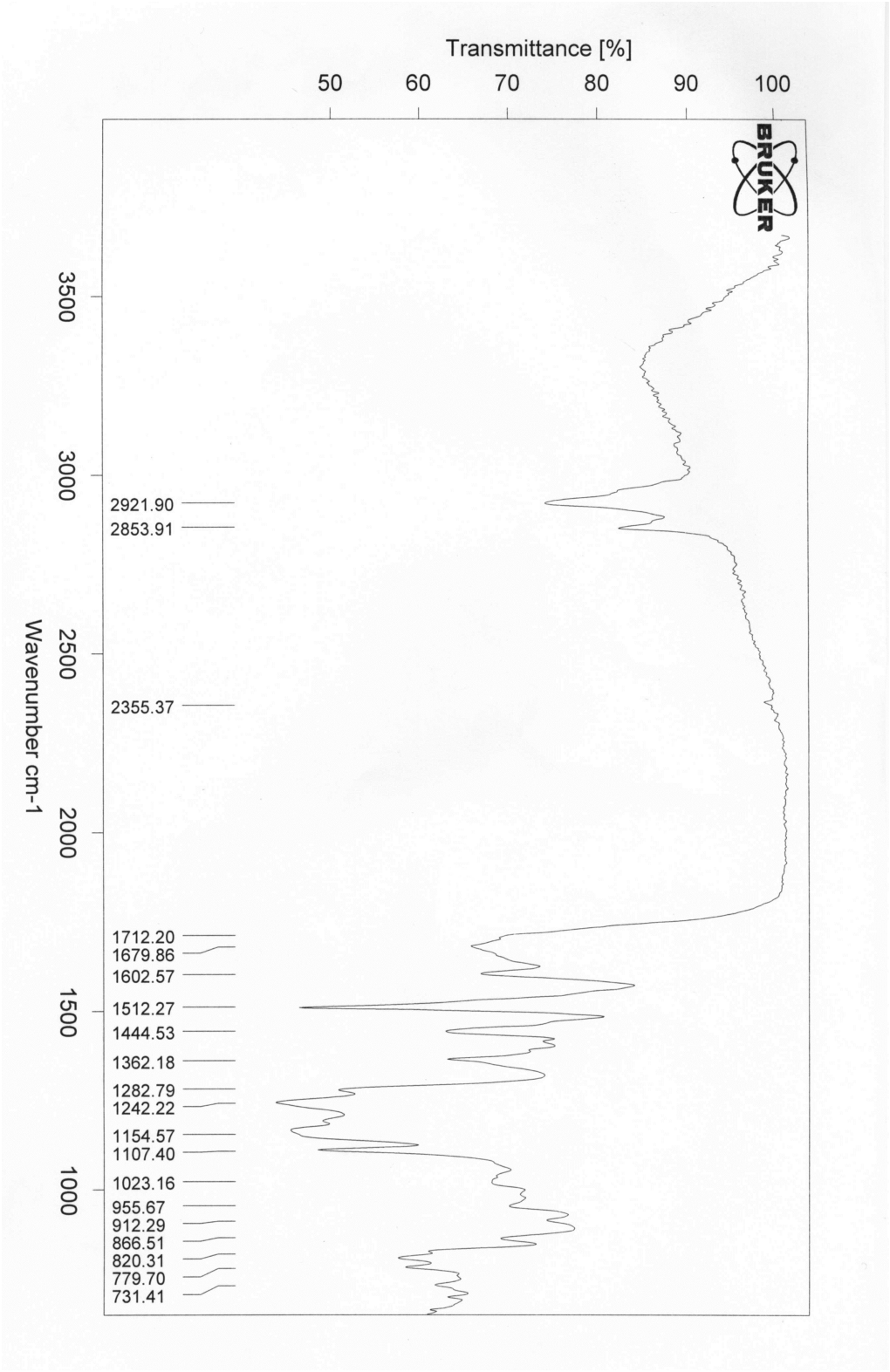
Cmpd 1, 0.3 min



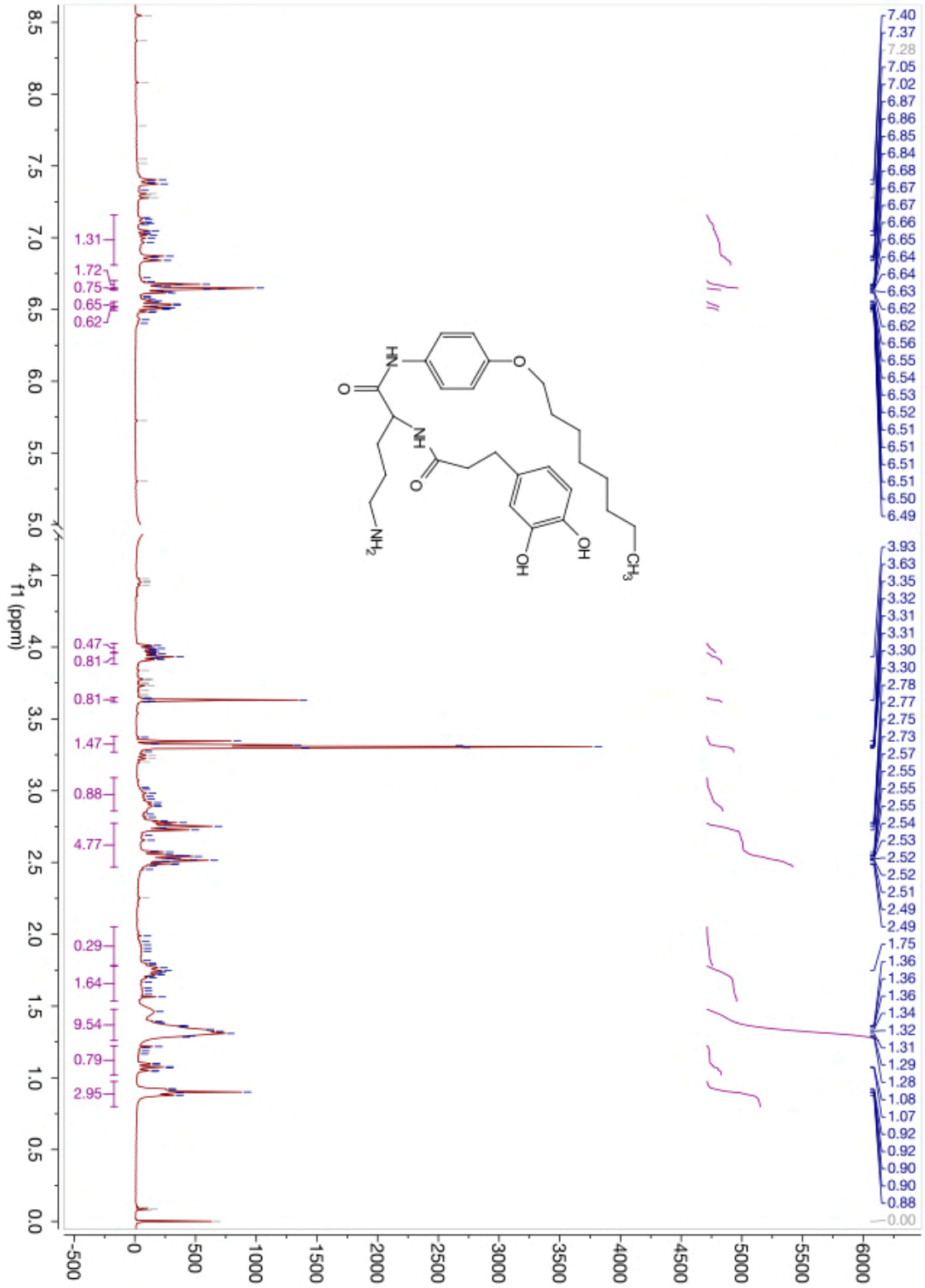
Mass List:

#	m/z	I
1	460.3	273857
2	496.3	250997
3	498.3	365602
4	524.3	1912697
5	525.3	700514
6	526.3	247652
7	586.3	266829
8	596.3	881783
9	597.4	1724521
10	598.3	6859878
11	599.3	2315779
12	600.3	549377
13	601.3	218801
14	607.3	2535299
15	608.3	3299905
16	609.3	886525
17	610.2	270043
18	611.3	320332
19	612.3	671214
20	623.2	384425
21	624.3	416135
22	625.3	281940
23	626.3	232998
24	628.2	223637
25	634.3	335599
26	636.2	387863
27	644.2	206883
28	676.3	288362
29	780.3	330796
30	792.3	234401

Dihydrocaffeic acid derivative **20b**: IR



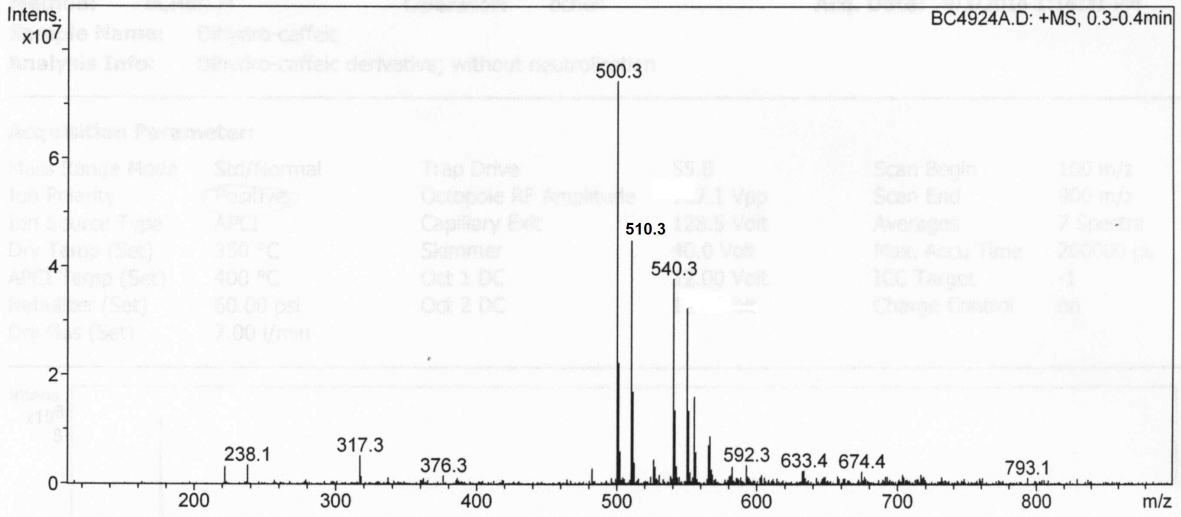
Dihydrocaffeic acid derivative **20c**: ^{1}H NMR



Dihydrocaffeic acid derivative **20c**: LC-MS (APCI-positive)

Compound Mass Spectrum List Report - MS

Cmpd 1, 0.3 min



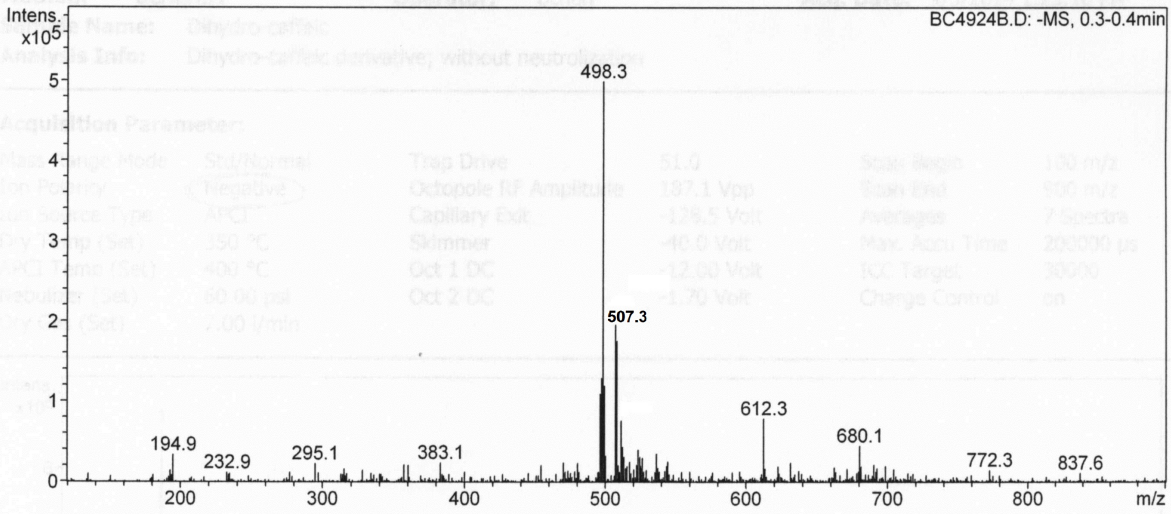
Mass List:

#	m/z	I
1	222.2	3124585
2	238.1	3413704
3	317.3	5199530
4	482.3	2775898
5	500.3	74212712
6	501.3	22206898
7	502.3	5985175
8	510.3	44757380
9	511.3	16917224
10	512.3	3885148
11	526.3	4501239
12	527.3	3148222
13	540.3	37724308
14	541.4	13539093
15	542.3	3297754
16	550.3	32367826
17	551.3	13391873
18	552.3	2120214
19	555.3	15970853
20	556.4	5805138
21	565.3	7137215
22	566.4	8701738
23	567.4	2620971
24	568.3	1724501
25	582.4	3122807
26	592.3	3489540
27	632.3	2359382
28	633.4	2447987
29	674.4	2253561
30	703.4	1705616

Dihydro-caffeic acid derivative **20c**: LC-MS (APCI-negative)

Compound Mass Spectrum List Report - MS

Cmpd 1, 0.4 min



Mass List:

#	m/z	I
1	194.9	33448
2	295.1	22471
3	357.3	20907
4	360.3	20217
5	383.1	23536
6	454.3	20156
7	470.2	23290
8	480.2	22890
9	496.3	109311
10	497.3	128489
11	498.3	498081
12	499.3	119110
13	500.2	32062
14	507.3	195213
15	508.3	174895
16	509.3	20214
17	511.2	75511
18	512.2	42864
19	513.2	30357
20	517.2	24279
21	522.2	20826
22	523.2	39298
23	524.3	30321
24	526.3	29248
25	536.3	34373
26	544.3	24808
27	612.3	78245
28	631.3	23392
29	680.1	44249
30	690.2	20392