Spring 5-17-2013

Synthesis and Antifungal Evaluation of Barbiturate Saponins And Progress Towards Cysteinyl Metal Peptides

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Synthesis and Antifungal Evaluation of Barbiturate Saponins

And Progress Towards Cysteinyl Metal Peptides

A Dissertation

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Chemistry

By

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May, 2013

Dedicated to my dear daughter Nyasa Madhav.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my research advisor, Professor Branko S. Jursic, for his patient guidance, enthusiastic encouragement, unwavering support and useful critiques throughout the years. His kindness and generosity made me feel very comfortable working with him. He has always been supportive and provided the freedom to pursue independent work. Without his encouragement and constant inspiration, it would have not been possible to achieve this stage in my life. My deep gratitude and sincere appreciation also goes to the members of my research advisory committee, Dr. Lee Roy Morgan, Dr. Mark L. Trudell and Dr. Ed Stevens, for all of their advice, help and guidance throughout this process.

Additionally, I would like to recognize all the former of Dr. Jursic's group -Dr Ravi Pingali, Dr. Sarada Sagiraju and Dr. Sunil Kumar Upadhyay, for being supportive, cooperative and having a friendly environment in the lab. I would also like to thank all current members of Dr. Jursic's group- Rajesh Komati and Rebecca Rhon for their support and help.

I would like to acknowledge the UNO Department of Chemistry, Department Chair Dr. Matthew Tarr for providing me a wonderful opportunity to use all the facilities at all the times to pursue my research. I am thankful to all the former and current faculty members Dr. Wiley, Dr. Rick, Dr. Cole and Dr. Gibb for all their support and advice. I can never forget how helpful Dr. Corrine was for solving NMR problems. I am very thankful to her for giving me NMR training.

Completion of this dissertation and subsequent PhD has been a long journey. During this period, I came across many people who helped me succeed in one or the other way. All their support is invaluable. Without these supporters, especially the select few I am about to mention, I may not have gotten to where I am today, at least not sanely. My special thanks go to Ms. Blanca

and her family for helping me during the toughest of the time. I cannot imagine writing this thesis without her help. I feel deep gratitude in knowing such a kind and generous person who offered selfless help to me and my daughter when we needed it the most. I extend my thanks to all my friends who filled my heart with joy and encouraged me throughout these days. I have been very fortunate to surround by such great people.

I would like to thank my father Mr. R. S. Singhal, mother Mrs. Veena Singhal and grandmother late Shrimati Darshan Devi, for their tremendous emotional support. My special thank to them for constantly motivating me and being much more than supportive through my entire journey. They are always there when I need them and have provided the best of everything. Most of all they gave me the wings of freedom and roots of responsibility so strong, that I never stopped believing in myself. My sister Shalini, brother Piyush have always filled my life with great joy, enthusiasm and stood by me for every moment in my life. Thanks to Jiju Vikas, neice Riya and nephew Aryan for filling our lives with tremendous joy and being there for us always. Great appreciation goes to my husband Maj. D B Madhav, for his love, encouragement and constant support. Acknowledgements are incomplete with the mention of our lovely daughter Nyasa for making our world so beautiful. She is a constant inspiration and encouragment for me to go on and never look back. Huge thanks to her for being there with me always. Waking up every day by my side with a bright smile and innocent eyes make each day very special and fill me with new energy to go on despite all the odds.

At the end, I would like to thank God for bestowing his blessings on me, giving me this opportunity and being so kind to me.

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ABBREVIATIONS

ZMC	Zinc Monocysteine
Ac	Acetyl
Bn	Benzyl
Bz	Benzoyl
DCE	Dichloroethane
DME	Ethylene glycol dimethyl ether
DMF	*N*,*N*-Dimethylforamide
DMSO	Dimethylsulfoxide
DCM	Dichloromethane
Ms	Mesylate
NBS	*N*-Bromosuccinimide
PE	Petroleum ether
ROS	Reactive Oxygen Species
THF	Tetrahydrofuran
TMS	Trimethylsilyl
TMSOTf	Trimethylsilyltriflate
Ts	Tosylate
PTSA	*p*-Toluenesulfonic acid

Abstract

Invasive fungal infections are a major threat to immune-compromised patients. There is a critical need to develop new antifungal agents because of increasing resistance to the common antifungal drugs.

In the first part of this dissertation, methods for preparation of novel barbiturate saponin as antifungals and their biological activities would be described. Barbiturates and steroidal saponins have shown remarkable antifungal activity in the biological assays. Therefore, attempts were directed to combine the barbiturate with the steroid to give novel antifungal agents. The need for extensive SAR studies and to better understand these compounds efforts were directed to synthesize novel saponin barbiturates.

Glycosylation of barbiturates was achieved under basic conditions to synthesize mono and disaccharide barbiturates. Saccharide molecules were directly introduced into the barbiturate without requiring protection and deprotection of saccharides. Efficient methods were developed for synthesis of 3β derivatized steroid derivatives containing ether, carbonate, ester and carbamate linker. Synthesized mono and disaccharide barbiturates were incorporated into the steroidal skeleton to give the novel antifungal agents. Several reaction conditions were explored to give the best yield under the most efficient reaction conditions. However, a better understanding and extensive SAR study needs to be done in order to develop more promising and potent antifungal compounds.

The second part of this dissertation describes the progress towards monocysteine metal complex synthesis and their biological activities. In this attempt, several protection deprotection strategies were explored and some novel protective groups were designed for peptide synthesis.

Keywords: L-cysteine, Zinc Monocysteine, Metal complexes, Amino acids, Peptides, L-tyrosine, A-007, Anticancer, Antioxidant, Radical Capture Compounds, Free Radicals, Trityl protection, Sulfhydryl group, Copper, Saponins, Steroids, Fatty-acids, Oligosaccharide, Stereoisomer, Aglycone, Cholestane, Cholesterol, Alkaloids, Cyclic-imides, Glycosylation, Barbiturates, Saccharides, Benzylic-Bromination, Antifungal agents, Azoles, Polyenes, Echinocandins, Sordarines, Protection, Deprotection, S to N Migration, Regioselective, Anomerization, Anomeric Effect, Catalytic, Phthalimides, Cysteinyl peptides, Succinimides, Tetrachlorophthalate, Pararosaniline, Copper sulphate, Benzyl, Isomerization.

CHAPTER-1: INTRODUCTION

The increasing burden of fungal infections, particularly in immune-compromised patients, has become a serious threat to human life. Fungal infections are generally categorized as superficial and systemic mycoses. Past two decades have witnessed a continuous increase in the incidence of fungal infections. Invasive fungal infections remain a leading cause of morbidity and mortality in patients suffering from haematological malignancies and that undergoing haematopoietic stem cell transplantation (HSCT)[1]. This significant increase, ironically, is believed to be due the advances in medical treatment, specifically the use of antibiotics, immunosuppressant drugs and steroids that suppress a patient's immune system thus making them more prone to fungal infections. As a result, invasive candidiasis has risen to become the fourth most common cause of hospital-acquired bloodstream infections in the U.S[2].

Several other factors that predispose a patient to invasive fungal infections include neutropenia caused due to chemotherapy, defective functioning T-lymohocytes caused as a result of organ transplant and HIV infection[2]. Prolonged usage and high doses of corticosteroids lead to impaired macrophage function. Various invasive and noninvasive medical procedures like vascular catheters, parentreal nutrition, hemodialysis and peritoneal dialysis cause barrier defect in compromised patients[3,4]. Medical procedures are both invasive and aggressive. This in turn disrupts the protective anatomical barriers and thus allows fungi to reach the normal sterile body site[12]. Major increase in invasive fungal infections have been observed not only in ever increasing community of organ transplant or AIDS patients, but also in patients who are hospitalized with severe illnesses and have to receive induction or consolidation chemotherapy and have to undergo bone marrow transplantation[5,6].

The search for new antifungal drugs is spurred by a dramatic increase in the incidence of fungal infections, growing primary and secondary resistance to popular drugs and the emergence of resistant strains to currently available antifungal therapy[7]. The past few years have seen the introduction of several new agents but the search for a new and better antifungal is ongoing. Antifungal research and development is challenging. Not only the fungal cell wall share similarity with the mammalian cells, but also a metabolic pathway similarity between the fungal and mammalian cells makes the target specific drug design a daunting task. This limits the availability of pathogen-specific targets[8]. Clinical development is hampered by challenges in timely and definitive diagnosis of the less common or more resistant fungi. Despite these issues, some novel products are progressing in development[9].

The systemic antifungals have been available since 1950s[10]. The development of the polyene antifungals represented a major advancement in medical mycology. The early development of antifungal agents such as nystatin and amphotericin B[11] was characterized by compounds with only limited efficacy because of their inherent toxicity and by the lack of substantial developments for decades. The 1980s saw the development of the triazoles[11], which revolutionized medical mycology, as these compounds were available both as intravenous and oral formulations, and were effective against fungal pathogens that were refractory to the polyenes (Fig. 1.1)[9].

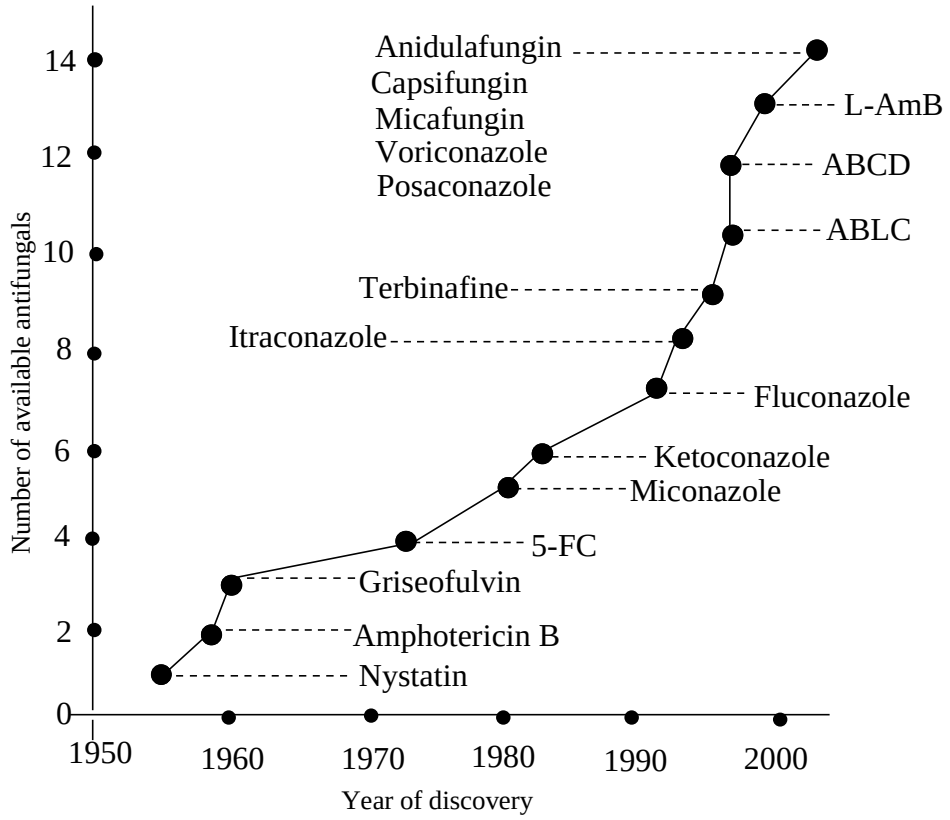

Figure 1.1 Timeline for systemic antifungal agent development [9]

The incidence and prevalence of invasive fungal infections saw a dramatic increase from 1980s. It was during this period that attention was diverted to develop new antifungals after a long period of slow development in antifungal discovery. During 1990s, triazoles were further modified and amphotericin B was reformulated with lipid compounds. Efforts were made to lessen the toxicity of these drugs and make them useful as systemic antifungals. The less toxic lipid based formulation of amphotericin B led to exploration of true efficacy of this drug[11].

The most common fungal pathogens continue to be the species of *Candida* and *Aspergillus*[13-15]. Parallel to the increase in fungal infections, the new millennium also saw the introduction of two new triazoles (voriconazole and posaconazole) and three echinocandins

(anidulafungin, caspofungin and micafungin) that have been licensed for the treatment and prevention of these infections[16]. The new class of antifungals 'echinocandins' are characterized by their inhibition of the synthesis of (1,3)-*β*-*d*-glucan (a key component of many fungal cell walls). These are the first class of antifungal agents that act against a specific component of the fungal organisms and not the mammalian cells [17,18]. Their safety profile is remarkable and has set the bar for new antifungals that are under development. The mechanism of action of antifungal agents for currently used drugs and those currently in development stage is illustrated in Fig.1.2.

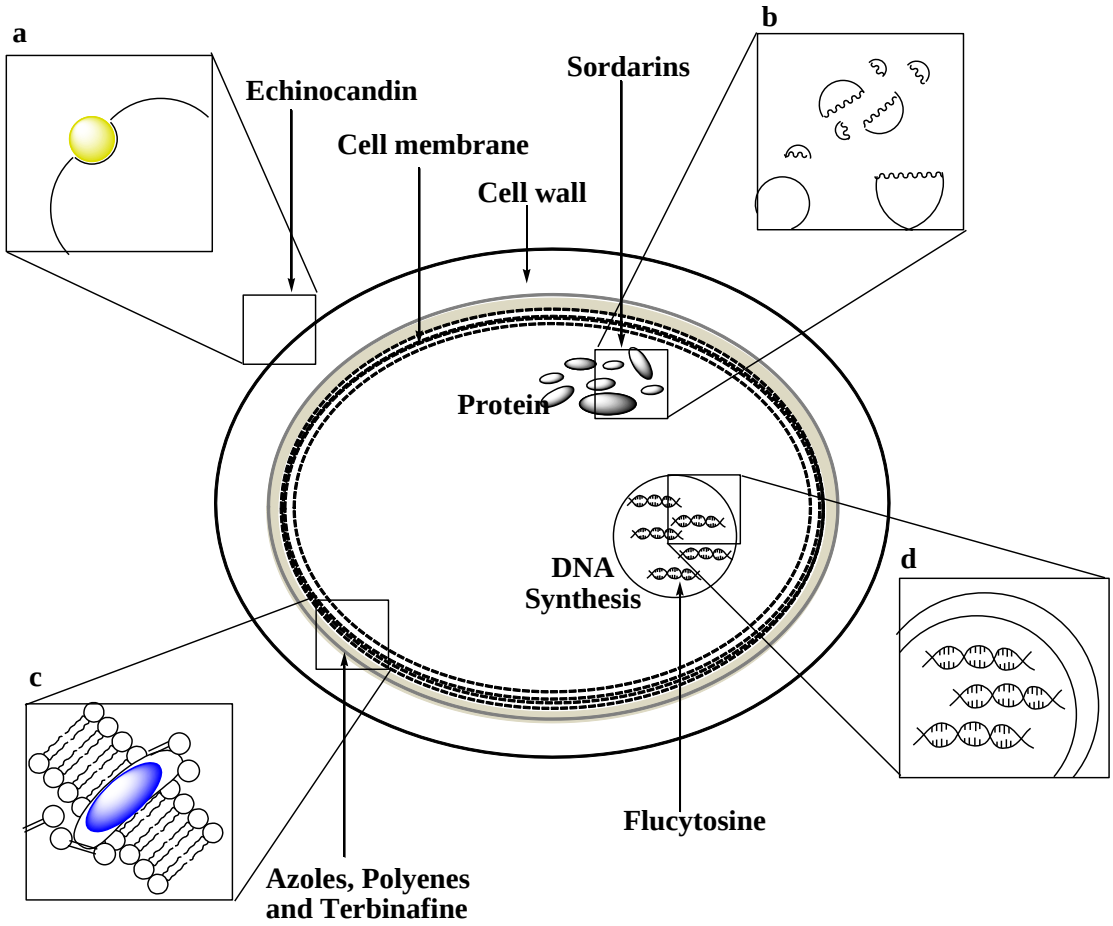

Figure 1.2 Mechanism of action of antifungal agents[9].

The antifungals acting on the cell wall are echinocandin that inhibit the cell wall formation. Sordarins act by interfering the assembling of proteins. Azoles, polyenes and terbinafine disrupt the fungal cell wall (Fig.1.2). Flucytosine interferes with the synthesis of DNA and antibodies and vaccines prevent the fungal infection by blocking them or destroying the fungal cells[9].

1.1 Systemic antifungal development

The discovery of the antifungal activities of griseofulvin, in 1940s was the first landmark in the development of active and safe antifungal agents. This was followed by azole, benzimidazole, by Wooley in 1944[19,20]. Elson's report on the fungistatic properties of propamidine followed in 1945[21]. This was followed by discovery of the first polyene macrolide antifungal, nystatin, by Hazen & Brown's in 1950[22]. Amphotericin B (AmB) was discovered in 1955 and introduced to treat several human cases of blastomycosis in 1957[23]. Later the introduction of oral griseofulvin and topical chlormidazole in 1958 and the subsequent introduction of IV AmB in 1960 ushered in the beginning of a new era of antifungal therapy[18b].After this initial introduction of AmB the advances in the search for new antifungal agents slowed down for almost three decades. The oral agent 5-fluorocytosine (flucytosine-5FC), developed in 1964 was initially effective in the treatment of infections caused by *Candida albicans* and *Cryptococcus neoformans* but soon developed resistance and limited its use as monotherapy. Nonetheless, 5FC is still used in combination with Amphotericin B[24].

Azole antifungals miconazole and clotrimazole were introduced as topical agents in 1969, and represented the only two additions to the antifungal agents in the 1960s. The 1980s saw the development of the triazoles, which revolutionized the antifungal agent discovery since these

compounds were available both as intravenous and oral formulations. In addition, these agents were effective against fungal pathogens that were refractory to the polyenes. The 1990s were perhaps the most prolific period in antifungal development. It is during this time that triazoles were further developed and amphotericin B was reformulated with lipid compounds. These lipid-based preparations were considerably less toxic and allowed the true efficacy of Amphotericin B to be explored[11].

The expansion in antifungal development continued into the new millennium with the advent of new class of antifungal called echinocandins. Since 2001, the echinocandin class has continued to expand with the introduction of micafungin and anidulafungin. The triazole class has also expanded with the addition of voriconazole and posaconazole, both of these have increased activity against fluconazole-resistant *Candida* spp. and filamentous moulds[15].

1.2. Pharmacological Targets for antifungal agents

Fungi are eukaryotes and relatively closely related to humans. Fungal DNA is organized into chromosomes within the cell nucleus and they have distinct cytoplasmic organelles. Fungi also have similar DNA replication and protein synthesis mechanism. This close resemblance of biosynthetic pathways to mammalian cells makes it extremely hard to have pathogen-specific targets. Cell membrane that serves important role in cell structure, division, and metabolism is made up of sterols. The only difference between the mammalian and the fungal cell is the sterol. This makes identification of new targets difficult. In the pathogenic fungi, the main sterol is ergosterol and this has been exploited as the target of antifungal drug action by several classes of antifungal agents including the polyenes, azoles, and allylamines. Mainly three targets i.e. plasma membrane sterols, nucleic acid synthesis and cell wall constituents (chitin, 1,3-β-glucan,

and mannoproteins) have been exploited extensively so far with varying degrees of success. Various fungal targets and mode of action is depicted in Fig.1.2.

1.3. Antifungal Agents Chemical Classification

Fungal infections can be either systemic or superficial. They are usually difficult to diagnose and treat. Since fungi are eukaryotic, antibacterial are ineffective in treating them. Fungi closely resemble the human cells therefore, the currently available antifungals are toxic and non-selective in their action. The currently available antifungals can be chemically divided into following classes namely polyene, azoles, pyrimidines, echinocandins, sordarins and saponins. These classes are discussed in detail below.

1.3.1. Polyenes Antifungals

These are also known as ergosterol disruptors. The polyene antifungals (Fig.1.3) act by complexing with ergosterol present in the fungal plasma membrane as shown in Fig.1.4. This binding leads to depolarization in the membrane, pore formation and increased membrane permeability. This results in the leakage of the cytoplasmic contents and ultimately death of the fungal cell. Thus, the polyenes are fungicidal[25]. The polyenes (AmB, nystatin) are large (26-28 carbon molecules) macrolide structures, with many hydroxyl groups, which confers the amphipathic nature to the compounds (Fig.1.3).

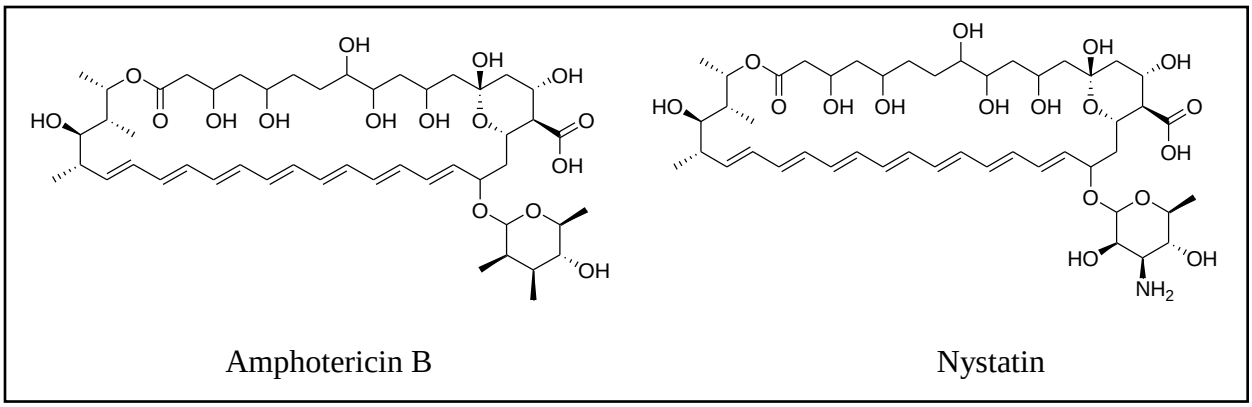

Figure 1.3 Polyene antifungals

Polyene Amphotericin B (Fig.1.3) was isolated from *S. nodosum* in 1956. It has broad spectrum of antifungal activity against *Blastomycesdermatitidis, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Paracoccidiodes brasiliensis, Sporotrichiumspecies and Torulopsis glabrata*[25,26].

Since, Amphotericin B is amphiphilic it can bind through both hydrophilic hydrogen bonds and hydrophobic, non-specific Van der Waals forces to ergosterol in fungal cell membrances[27]. Amphotericin B has a greater affinity to bind ergosterol and ergosterol-containing membranes than cholesterol or cholesterol-containing membranes[27a,28,29].

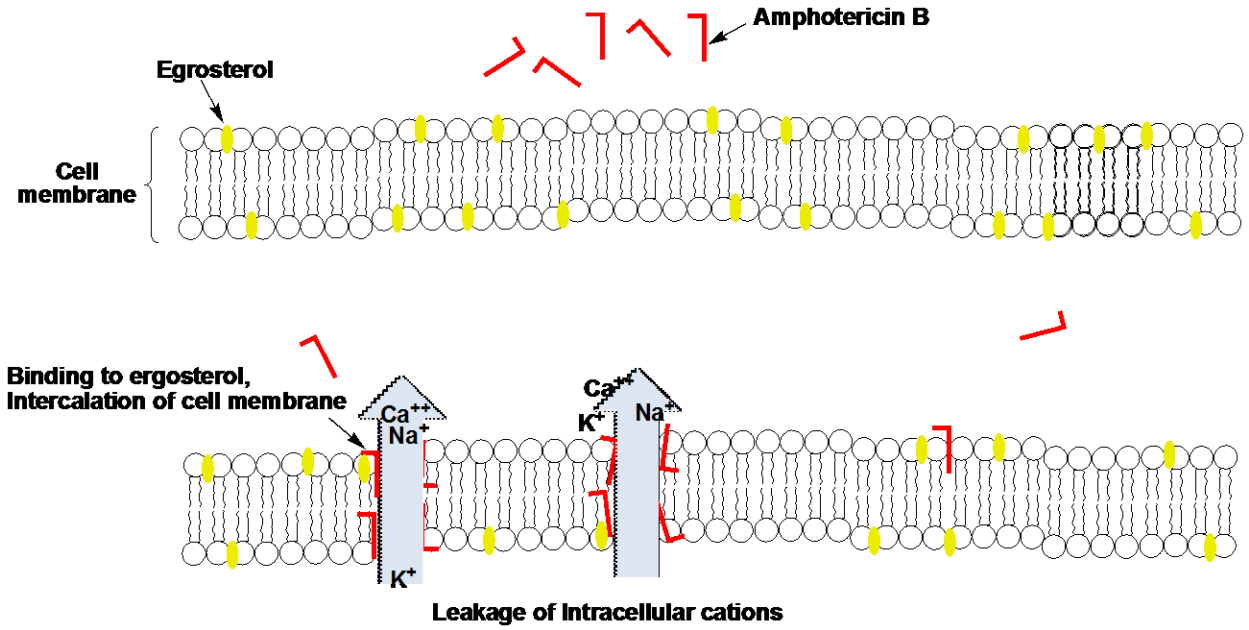

Figure 1.4 Disruption of fungal cell membrane by Amphotericin[30].

Amphotericin B binds with up to 10 times more avidity to ergosterol than to cholesterol in the mammalian cells[26]. This provides high specificity to amphotericin for fungal cells. Amphotericin shows a rapid lethal action against the fungal pathogens *in vitro*, but these antifungals agents are not lethal *in vivo*[31]. A direct antifungal effect of AmB stimulates release of cytokines and interleukin-1 from mammalian phagocytic cells and macrophage superoxide ion, all of which augments the antifungal activity[32-34].

However, amphotericin B has been shown to affect mammalian cells, and nephrotoxicity is a common side effect reported with the clinical usage[35]. Almost every patient develops some form of renal malfunction[2b]. Besides nephrotoxicity and infusion toxicity, amphotericin B also produces local thrombophlebitis, nausea and vomitting[36,37]. These toxicities can easily be minimized to varying degrees by pretreatment regimens. Second important polyene antifungal is Nystatin that was discovered in 1950 from the fermentation broth of *Streptomyces noursei*.

Because of unfavorable adverse effect profile of Nystatin, it is only used as a topical antifungal agent[38]. It is mostly non-absorbable after oral administration. It is effective in the treatment of oropharyngeal candidiasis[39].

1.3.2. Azoles

Azoles are fungistatic antifungal agents with a broad-spectrum activity. These synthetic compounds can be classified as either imidazoles or triazoles according to the number of nitrogen atoms in the five-membered azole ring, as indicated below (Fig.1.5).

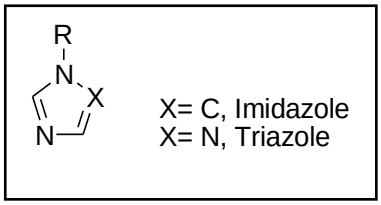

Figure 1.5 Azole nucleus

The imidazoles consist of ketoconazole, miconazole, and clotrimazole (Fig.1.6). The triazoles include itraconazole, fluconazole, voriconazole, posaconazole and ravuconazole. A triazole based pharmacophore has replaced the earlier imidazole pharmacophore in systemically active azoles because the triazole group enhances the specificity for fungal cytochrome p450 (erg11) targets and slows metabolism of the agents *in vivo*[40].

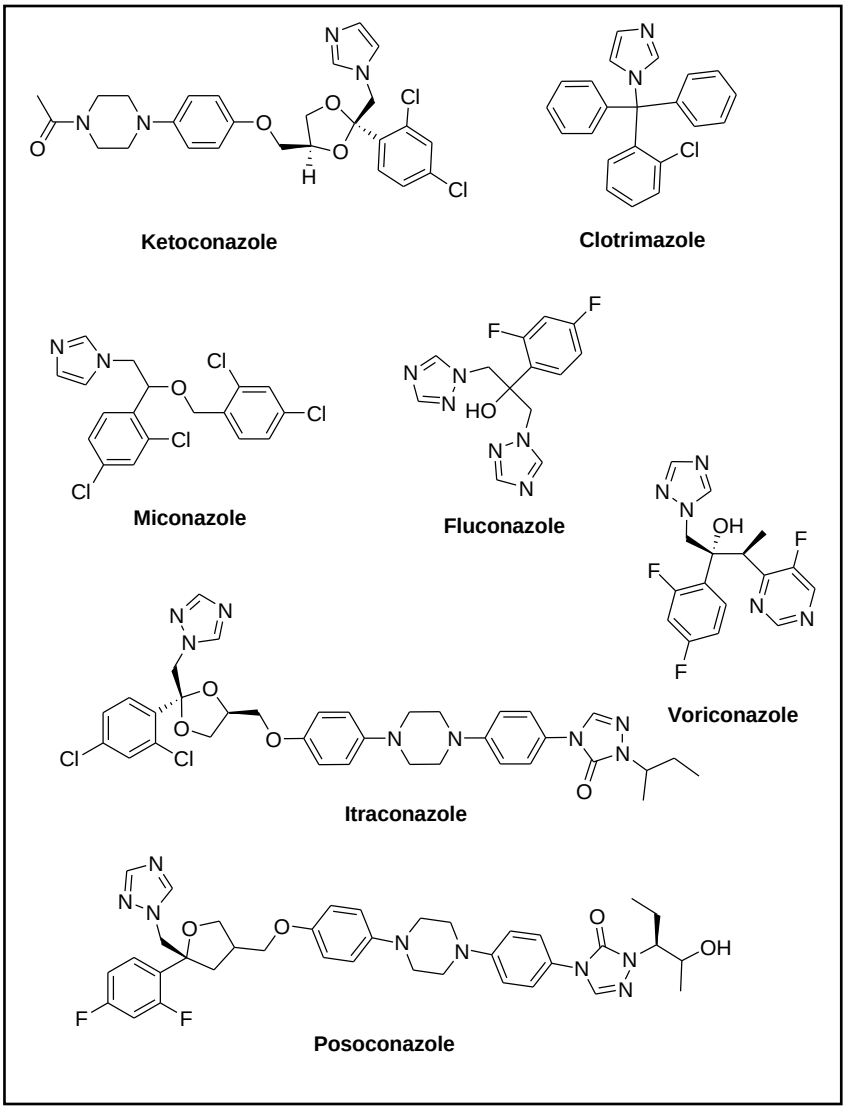

Figure 1.6 Chemical structure of imidazole and triazole class of azoles[5]

This class of antifungal agents has been most successful so far in terms of the number of different agents that have entered clinical use. However, because of toxicity and bioavailability problems associated with them, most of these antifungal imidazoles are formulated only for topical use and thus limited in their potential as systemic agents[41]. Agents licensed for clinical use in invasive fungal disease are fluconazole, itraconazole, posaconazole and voriconazole[9]. The antifungal activity of azole drugs results from the reduction of ergosterol synthesis by

inhibition of fungal cytochrome P450 3A-dependent C14-α-demethylase (Fig.1.7)[42,5]. Thus, azoles inhibit the conversion of lanosterol to ergosterol. This leads to depletion of ergosterol in fungal cell membrane[42-44].

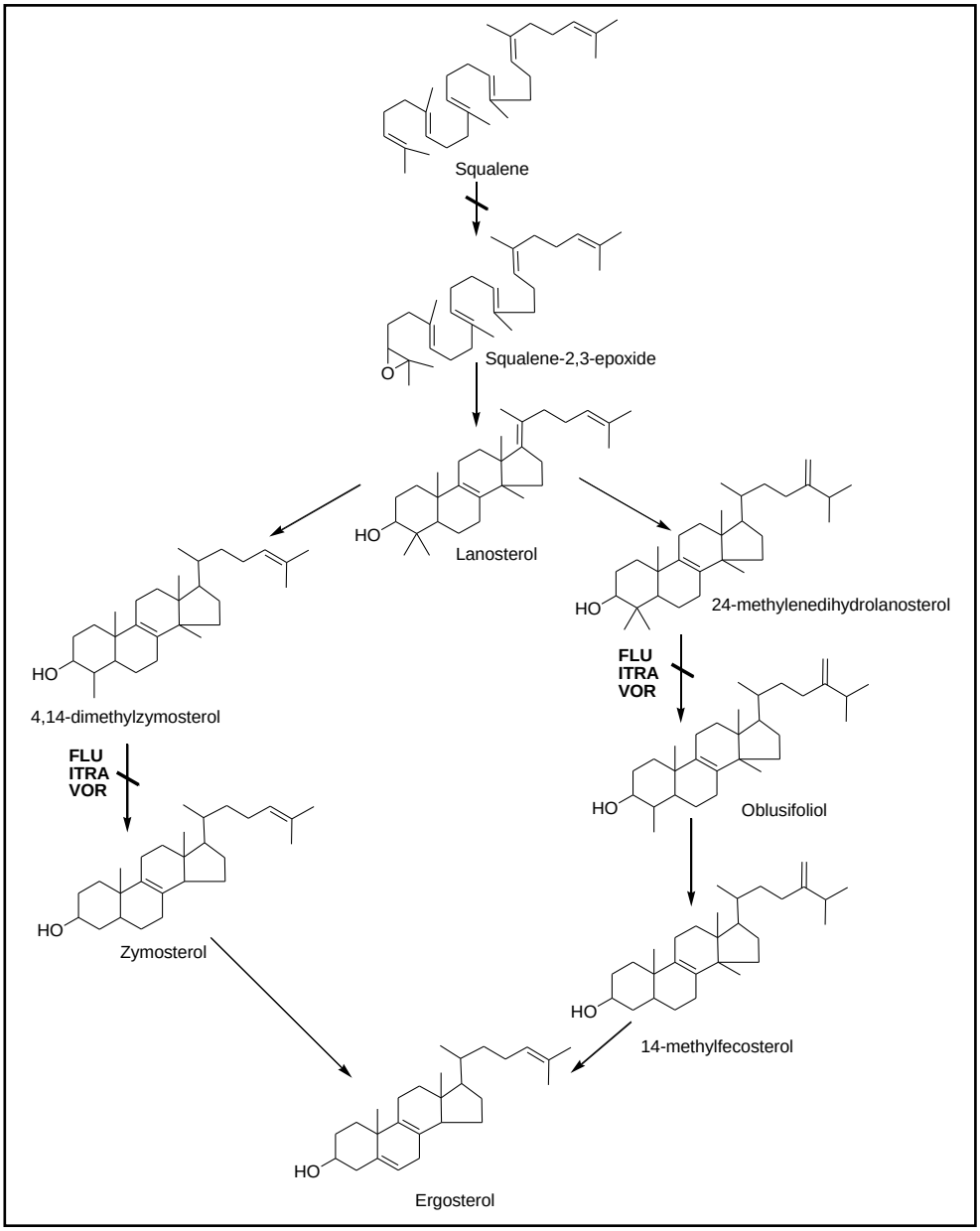

Figure 1.7 Pathway of sterol synthesis of ergosterol and blocks by azoles[60]. FLU, Flucaonazole; ITRA, Itraconazole; VOR, Voriconazole**.**

The selective action of azole drugs on fungal cells results from their greater affinity for fungal than for human cytochrome P450 enzymes. Imidazoles exhibit a lesser degree of selectivity than the triazoles, accounting for their higher incidence of drug interactions and side effects. The *in vitro* antifungal activity differs with each compound and the clinical efficacy may not coincide exactly with *in vitro* activity. The azoles are broad spectrum with primarily being active against *C. albicans, C. neoformans, C. immitis, H. capsulatum, B. dermatitidis, P. brasiliensis, C. glabrata, Aspergillus spp. And Fusarium spp*[2b,45a]. The azoles are relatively nontoxic with a relatively minor adverse reaction as gastrointestinal upset. Though all azoles have been reported to cause abnormalities in liver enzymes and, very rarely, clinical hepatitis[45].

Ketoconazole was introduced in 1981 as an oral azole antifungal for treatment of systemic fungal infection. It is a broad spectrum antifungal but causes hepatotoxicity and produce endocrine abnormalities by suppression of testosterone and ACTH-stimulated cortisol synthesis[2b,46a]. Ketoconazole has greater ability to inhibit mammalian cytochrome P450 enzymes. Therefore, it is less selective for fungal P450. As a result, systemic ketoconazole has fallen out of clinical use in the USA but is still used as a dermatologic cure.

Itraconazole is another triazole antifungal agent with broad spectrum of activity. It also has activity against *Aspergillus spp*. Itraconazole is available for oral and intravenous usage. Because of inconsistent bioavailability after oral administration, itraconazole is given with food. Also, low pH enhances drug absorption after oral administration[2b,41,46a]. Itraconazole interacts with hepatic microsomal enzymes just like other lipid-soluble azoles. When taken with rifamycins (rifampin, rifabutin, and rifapentine) bioavailability of itraconazole is reduced. However, it does not affect mammalian steroid synthesis, but its effect on the metabolism of other hepatically cleared medications is much less than those of ketoconazole. Although

itraconazole displays potent antifungal activity, its effectiveness can be limited by reduced bioavailability. Newer oral liquid and intravenous preparation utilizes cyclodextran as a carrier molecule to enhance solubility and bioavailability. Absorption of Itraconazole in cerebrospinal fluid, eye and saliva are minimal[2b,41]. Itraconazole has been replaced by voriconazole as the azole of choice for aspergillosis. It is used extensively in the treatment of dermatophytoses and onychomycosis[2].

A highly water-soluble azole is Fluconazole and display good cerebrospinal fluid penetration. Its oral bioavailability is high compared to ketoconazole and itraconazole. Since fluconazole has the least effect of all the azoles on hepatic microsomal enzymes, the drug interactions are also less common. Fluconazole has the widest therapeutic index of the azoles, allowing aggressive dosing for a variety of fungal infections. The drug is available in oral as well as intravenous formulations. It is the treatment of choice for secondary prophylaxis of cryptococcal meningitis. Intravenous fluconazole has been shown to be equivalent to amphotericin B in treatment of candidemia in ICU patients with normal white blood cell counts. Fluconazole is the agent most commonly used for the treatment of mucocutaneous candidiasis. Activity against the dimorphic fungi is limited to coccidioidal disease, and in particular for meningitis, where high doses of fluconazole often obviate the need for intrathecal amphotericin B. Fluconazole displays no activity against *aspergillus* or other filamentous fungi[2].

Voriconazole is water-soluble second generation triazole and is available in intravenous and oral formulations. Voriconazole is approved for first-line treatment of invasive aspergillosis, Oesophageal candidiasis[45b]. It is active against *Aspergillus spp*, *Fusarium spp*. and *Candida spp*. (including the fluconazole resistant or less susceptible spp. of *C. glabrata* and *C. krusei*)[26]. It also shows activity against the *Fusarium* and *Scedosporium* infections, which are hard to treat[46b].

Observed toxicities of Voriconazole therapy include skin rash and hepatic enzymes transaminase elevation[47], encephalopathy or hallucinations[48].

Posaconazole is the broadest spectrum member of the azole antifungal family, it is lipophilic second generation triazole antifungal with activity against most species of *candida* and *aspergillus*[50]. It was approved in 2006 by the FDA for prophylactic treatment of these[49]. It is the only azole with significant activity against the agents of zygomycosis and mucormycosis[51,52]. Posaconazole is rapidly distributed to the tissues, resulting in high tissue levels but relatively low blood levels. The toxicity associated with posaconazole therapy is gastrointestinal (14%), with hepatic transaminase elevation and hyperbilirubinemia occurring in 3%,[53] and headache[54,55], elevation of liver enzymes and skin rash[54].

1.3.3. Pyrimidines

Another important antifungal agent is flucytosine. It is a nucleoside analogue that was synthesized in 1957 as a cytosine analogue. It interferes with both DNA and RNA synthesis and function[5]. It was designed for treatment of leukemia, but did not display any cytotoxic activity and was ineffective[2b]. Later in 1963 its antifungal properties were discovered. It acts as an antifungal agent through conversion into 5-fluorouracil within the fungal cells and gains entry in the cell via cytosine permease. Fluorouracil becomes incorporated into RNA, causing premature chain termination, and it inhibits DNA synthesis through effects on thymidylate synthase[41,45,46,56]. This drug is selectively toxic to fungi because mammalian cells lack cytosine permease and do not convert large amounts of flucytosine to 5-fluorouracil. Flucytosine is primarily used in adjunct therapy with amphotericin B for *Cryptococcus neoform*[57-59], if used alone it develops resistance quickly. This is used for treatment of candida endophthamitis and

meningitis, cryptococcal meningitis and in trichosporonosis[41,46]. Most filamentous fungi lack these enzymes and hence useful spectrum of flucytosine is restricted to pathogenic yeasts[58].

1.3.4. Echinocandins

New millennium saw the introduction of a new class of antifungal agents called 'Echinocandins'. Presently there are three echinocandins that have been approved by the Food and Drug Administration (FDA). Caspofungin was approved first, in 2001, followed by micafungin in 2005 and anidulafungin in 2006[16].

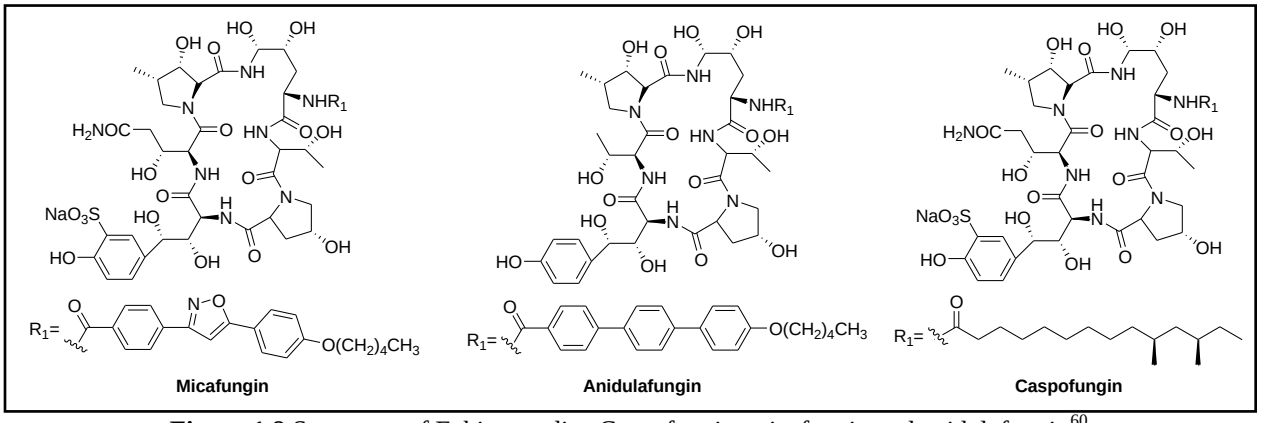

Figure 1.8 Structures of Echinocandins Caspofungin, micafungin and anidulafungin[60]

The echinocandins are large natural cyclic lipopeptide molecules that represent the fourth class of antifungal agents available for the treatment of systemic fungal infections after polyenes, azoles and pyrimidines. Echinocandins (Fig.1.8) are products of cyclopentamine, which is formed during the fermentation of some fungi such as *Zalerion arboricola* or *Aspergillus nidulans*[61,62]. Since the incidence caused by fluconazole-resistant non-*albicans* fungal species is on rise it is predicted that echinocandins are going to play a major role in the treatment of these

types of infections[63,64]. These agents are active against *candida* and *aspergillus*, but not against *C. neoformans* or the agents of zygomycosis and mucormycosis[64].

The fungal target of the echinocandins is the synthetic cell-wall enzyme complex *β*-(1,3)-*D*-glucan synthase[65]. Echinocandins inhibit the synthesis of *β*-(1,3)-*D* glucan, which is an essential component of the cell wall of several fungi, by noncompetitive inhibition of an enzyme UDP-glucose *β*-(1,3)-*D*-glucan- *β* (3)-*D*-glucosyltransferase (commonly referred to as 1,3-*β* -*D* glucan synthase)[66-67]. Fungi are eukaryotes like human beings but the cell-wall is different from the mammalian cells, and therefore represents a good target for antifungal drugs[72]. Fig.1.9 is a diagrammatic representation of the glucan synthase protein complex, and its regulatory network[17]. Mechanistic details of glucan synthesis and its inhibition by echinocnadins is still unclear, largely because a membrane-associated protein complex is involved. There is no doubt that the component to which echinocandins bind is *Fks1p*, but their non-competitive inhibitory effects on glucan synthesis do not necessarily imply that *Fks1p* itself is the catalytic subunit, nor is it clear whether the echinocandin-binding site is external or internal to the cell membrane.

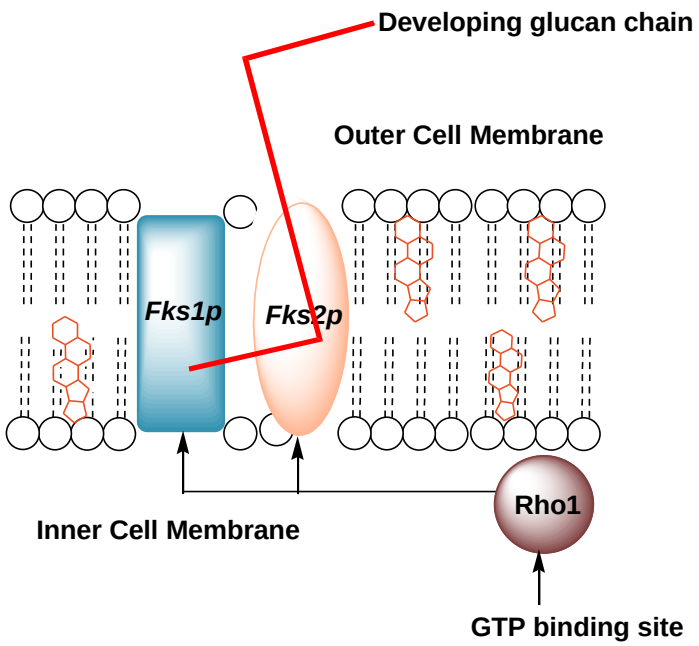

Figure 1.9 Transmembrane *Fks1p* and *Fks2p* protein complex involved in 1,3-*β* -*D* glucan synthesis fungal cell membrane[17]

Echinocandins specifically target the cell-cycle regulated *FKS1* gene transcription which is, linked to the cell-wall (Fig.1.9) remodeling and encodes for components of glucan synthase[70,71]. The inhibition of glucan synthase destabilizes the integrity of the fungal cell wall species of *Candida*, thus making the cell wall less rigid and unable to resist osmotic pressure, which ultimately leads to cell lysis[73-74].

All three echinocandins display good *in vitro* and *in vivo* fungicidal activity against most *Candida* spp., including strains of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*, and *C. krusei* that are amphotericin- or fluconazole-resistant[17]. Echinocandins are usually extremely well tolerated, with only minor gastrointestinal side effects and flushing. Because of the large molecular weight of the all three echinocandin, a poor oral absorption is observed. Therefore, all three compounds are available as intravenous formulations only. Although, the metabolism of

these compounds is not fully understood and is still under investigation, but almost the entire drug is degraded by non-oxidative pathways in the liver[75].

Caspofungin has potent *in vitro* inhibitory activity against *Aspergillus spp*. and moderate activity against some other moulds such as *H. capsulatum*, C. immitis and *B. dermatitidis*. Although it is also active against *P. carinii* and moderately against *dematiaceous fungi*[78], but it does not show any activity against *C. neoformans, Trichosporon spp., Fusarium spp., S. schenckii*, zygomycetes and hyalohyphomycetes[76,77]. Because of limited interactions of Caspofungin with cytochrome P-450 system as it is neither a substrate nor an inhibitor, it has only few side effects like headache, fever, nausea, rash, phlebitis at the site of infusion and reversible elevation of hepatic enzyme levels[78].

Micafungin was approved for the treatment and prophylaxis of *O. candidiasis* in patients undergoing stem cell transplantation[78]. Micafungin has less drug interaction compared to capsofungin[79,80]. Micafungin has been found to display superior activity than fluconazole when used as a prophylactic for stem cell transplant patients[80].

Anidulafungin was approved for use in the treatment of *O. candidiasis*, candidaemia, peritonitis and intra-abdominal abscesses due to *Candida spp*[78]. It is highly active *in vitro* against a wide range of *Candida spp*., including species that are resistant to azoles (*C. krusei*), amphotericin B (*C. lusitaniae*) or other echinocandins (*C. parapsilosis*)[82,84] and also against *Aspergillus spp*[85]. It is unique in echinocardin class of antifungals because of its ability to slowly undergo a process of biotransformation rather than metabolism in humans[78]. The most common adverse effects associated with it were found to be hypotension, vomiting, constipation, nausea, fever, hypokalaemia and elevated hepatic enzymes[78,83].

All echinocandins are fungicidal against most *Candida* species and retain activity against azole-resistant strains[67]. However, there are notable gaps in their fungal spectrum of activity. The toxic-effects profile of the three echinocandins is favourable, and certainly less of a problem compared to amphotericin B, whether in complex with lipid or not[75]. Caspofungin might have a narrower therapeutic window with respect to liver-function tests and concurrent use with cyclosporin than micafungin and possibly anidulafungin[75].

1.3.5. **Sordarins**

Sordarin (Fig.1.10), isolated from *Sordaria araneosa* in 1971[86], has been identified as selective inhibitors of fungal protein synthesis. These are semi-synthetic natural products that[87] have generated considerable interest as new antifungal agents. Several types of novel sordarin derivatives were developed preclinically in the 1990s by Glaxo–wellcome and merck[88]. These compounds primarily exert their antifungal effect by specifically inhibiting the protein synthesis elongation cycle without affecting protein synthesis machinery in mammalian systems[89].

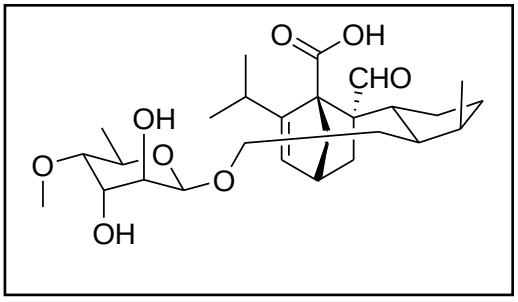

Figure 1.10 Chemical structure of Sordarin

The proposed mechanism of action of these compounds is the inhibition of a novel target for antifungal agents: elongation factor 2 in protein biosynthesis[90]. They exert their effect by blocking the function of fungal translation. Since these specific targets are, absent in mammalian

cells or electron transport chain their action is selective for the fungal cells[91]. Some sordarin derivatives have shown excellent *in vitro* activities against a wide range of pathogenic fungi, including *Candida spp., C. neoformans, P. carinii* and certain filamentous fungi and some emerging invasive fungal pathogens[92].

1.3.6. Saponins

Saponins have attracted a lot of attention in past few years. They are the emerging class of new antifungal agents as seen by scientist. Saponins are the secondary metabolites in the biosynthetic pathway of plant metabolism. Saponins have also been identified in some marine organisms and insects[95]. Their usefulness as antifungal agents recently has attracted a lot of attention from the researchers. Chemically, they generally occur as glycosides of steroids or polycyclic triterpenes[93]. These chemical compounds are amphiphilic in nature and are made up of two parts. The aglycone part is glycoside free termed as sapogenin. Because of their amphiphilic character, they are able to interact with cell membranes and are also able to decrease the surface tension of an aqueous solution. Because of this activity, they are named "saponin", which in turn is derived from the Latin word "sapo", meaning formation of a stable soap-like foam in aqueous solution[94]. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or ethylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid or steroid (Fig.1.11) in nature[96]. Based on the chemical nature of the aglycone (also known as sapogenin), saponins can be divided into steroidal, steroidal alkaloidal and triterpenoid saponins (Fig.1.11). The aglycone part of the saponins may contain one or more unsaturated C–C bonds. The oligosaccharide chain is normally attached at the C3 position (monodesmosidic), but many saponins have an additional

sugar moiety at the C26 or C28 position (bidesmosidic)[97]. The great complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone. Experiments demonstrating the physiological, immunological and pharmacological properties of saponins have provoked considerable clinical interest in these substances[97].

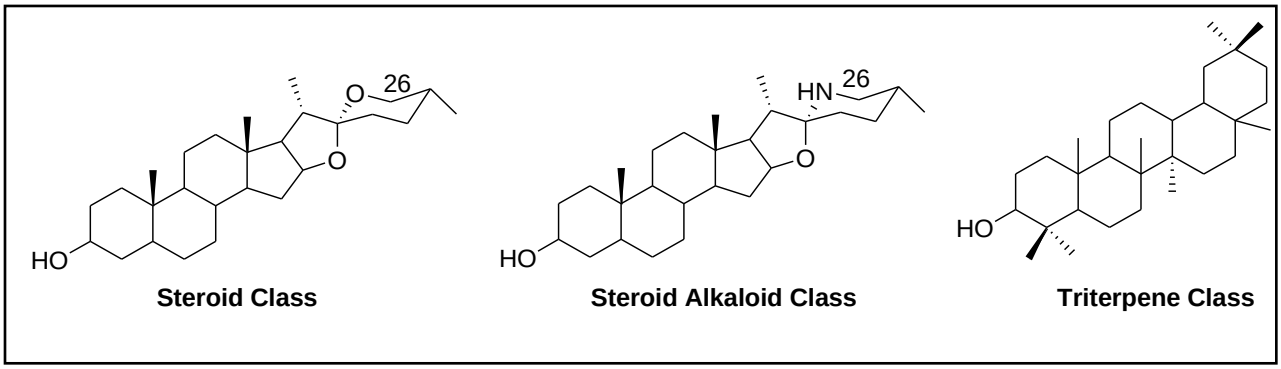

Figure 1.11 Classes of saponin.

Steroidal saponins derived from plant source are mainly the compounds containing 27 carbon atoms forming the core structures, i.e., spirostan (Fig.1.12) and furostan[98].

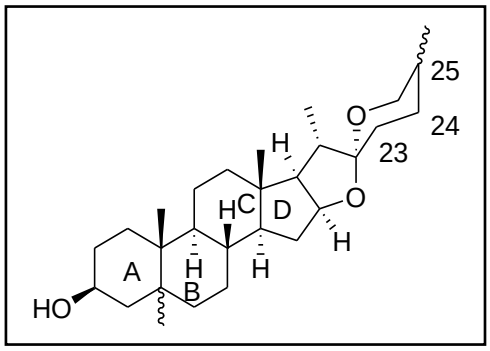

Figure 1.12 Core structure of spirostane

Saponins have been reported to have a numerous pharmacological activities[99]. Few of the important ones are permeabilizing of the cell membrane[99a], stimulation of luteinizing hormone

release leading to abortifacient properties[99c], immunomodulatory potential via cytokine interplay[99b], lowering of serum cholesterol levels[99c], cytostatic and cytotoxic effects on malignant tumor cells[99a], adjuvant properties for vaccines as immunostimulatory complexes[99a], and synergistic enhancement of the toxicity of immunotoxins[99b,c].

Both the aglycone and oligosaccharide moieties of saponins have been shown to be important for their biological activity[110-112]. The effectiveness of saponins has long been recognized in herbal medicine but they were not typically viewed as potential drug targets in the past due to their structural complexity. One exception to this rule is digoxin (Fig.1.13) which is a cardiac glycoside isolated from the leaves of the foxglove plant *Digitalis lanata* and has been marketed for over 200 years to treat congestive heart failure. Digoxin binds to Na^+/K^+ ATPase pumps in cardiac cell membranes and alters the membrane potential by changing the concentration of ions, which in turn increases the contraction strength of the cardiac muscles[111]. Digoxin is an unusual saponin and is available only by isolation from natural sources. The advances in the synthesis of glycoconjugates have not yet been successful in synthesis of this alkaloidal saponin.

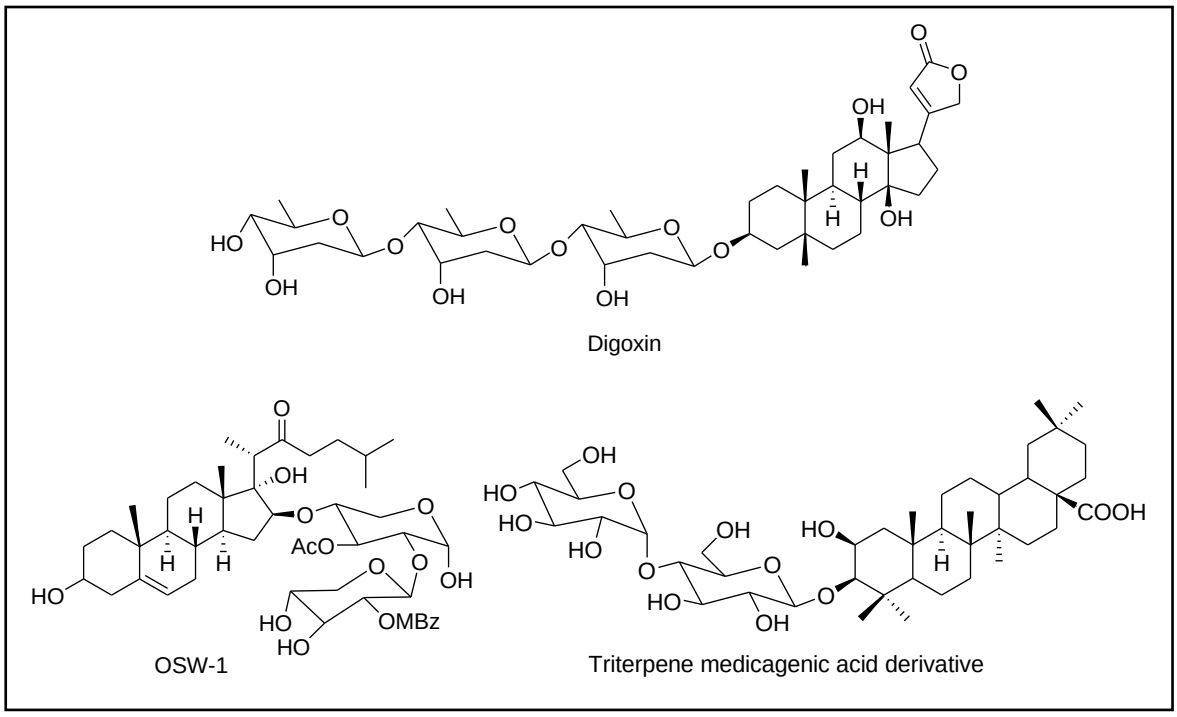

Figure 1.13 Antifungal saponins

A steroidal saponin isolated from bulbs of the lily plant *Ornithogalum saudersiae* OSW-1 (Fig. 1.13) has shown *in vitro* anti-tumor activity levels that are 10-100 times more effective than many well-known anticancer compounds including taxol and cisplatin[113]. Because of its unusual mechanism of action, it has the potential to be effective against cancers that are resistant to currently available chemotherapy. OSW-1 causes damage to the mitochondrial membranes, which in turn disrupts the calcium levels in the cytoplasm of the cell and ultimately leads to cell death by apoptosis. At the same time, OSW-1 has been documented to possess potent *in vitro* antifungal properties. For example, a derivative of the triterpene medicagenic acid (Figure 1.13), isolated from alfalfa root extracts was revealed to be effective against fungal skin infections in guinea pigs and did not show dermal toxicity in rhesus monkeys. It also showed promise toward the treatment of invasive fungal infections by successfully treating *Cryptococcus neoformans* infections in mice with a minimal inhibitory concentration of 4 μg/mL. This saponin was also

effective against amphotericin B-resistant strains of *Candida tropicalis*[113]. It was synthesized in three steps from medicagenic acid and acetobromomaltose in 49% overall yield.[112] Another important saponin CAY-1 has been identified to be effective in the treatment of invasive fungal infections (Fig.1.14). CAY-1 was isolated in 0.1 % yield from the fruit of the cayenne pepper plant, *Capsicum frutescens* to provide sufficient quantity for preliminary testing for antifungal activity and cytotoxicity toward mammalian cells[113]. The preliminary studies indicated CAY-1 to be an effective antifungal agent against sixteen fungal strains of *Candida* species, *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Neurospora crassa*. The most promising result was the activity of CAY-1 against *C. neoformans* (IC_{90} < 1μg/mL), a fungal species responsible for cryptococcal meningitis in HIV/AIDS patients, a fatal disease if left untreated.[113]

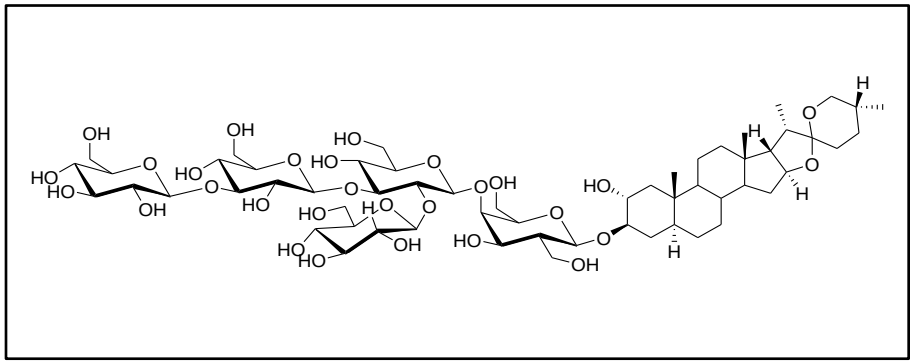

Figure 1.14 Structure of antifungal saponin CAY-1.

The predominant mechanism of antifungal activity of saponins is due to their ability to complex with sterols in fungal membranes which results in loss of membrane integrity (Fig.1.15). The precise mechanism is not fully understood and is still under investigation. Experimental analysis suggests the formation of transmembrane pores. Aggregation of the saponin-sterol complexes in the membrane may be mediated by interactions between the sugar residues of the saponin

molecules. The sugar chain attached to *C*-3 is usually critical for both the membrane-permeabilizing and antifungal properties of saponins, and removal of these sugar residues often results in loss of biological activity[102].

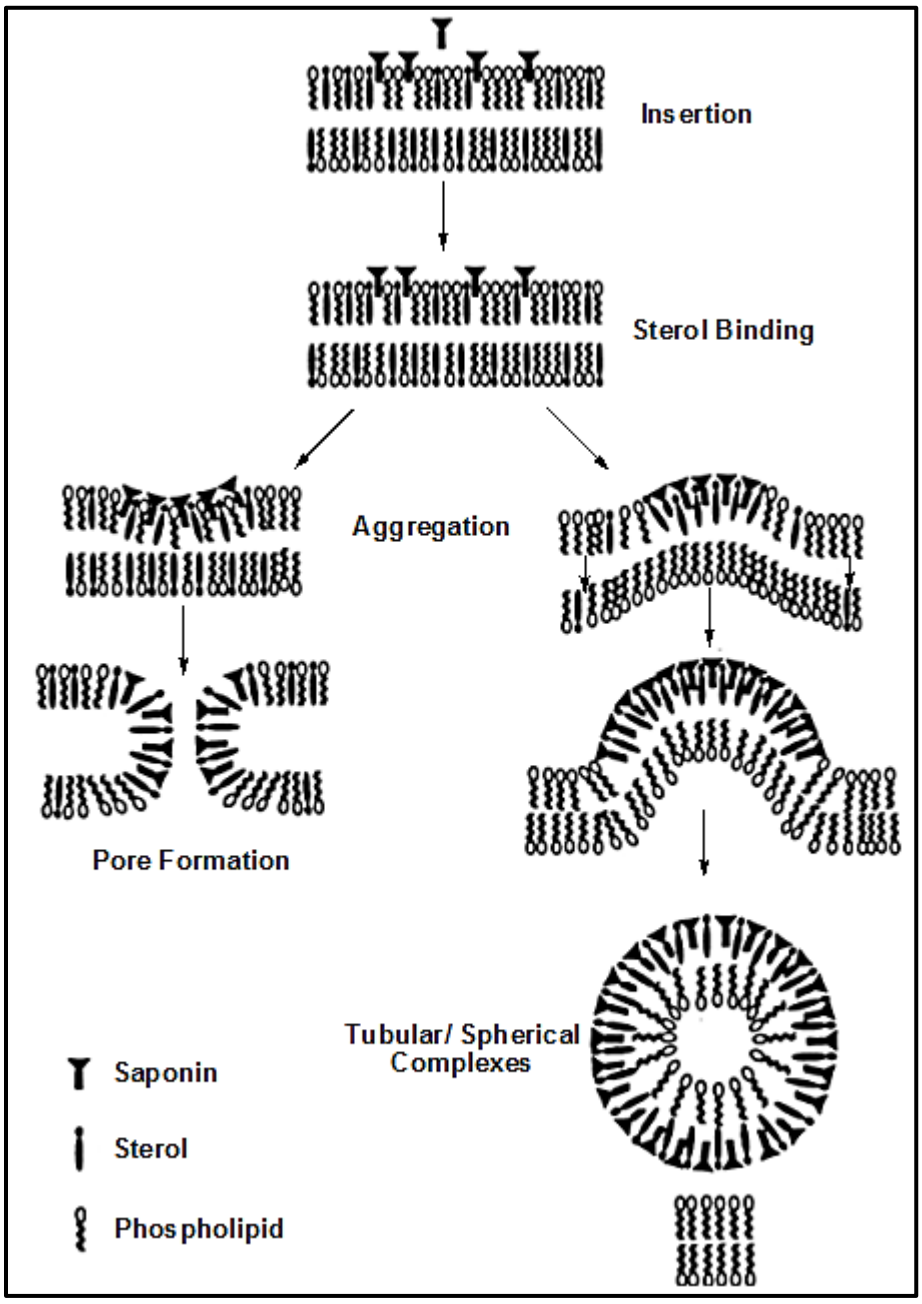

Figure.1.15 Proposed mechanism for membrane disruption by saponins[103].

1.4. Resistance to antifungal agents

Resistance to antifungals can be divided into two categories namely clinical resistance and *in vitro* resistance. A lack of clinical response to the antifungal agents used to treat the fungal infections is termed as clinical resistance. Low levels of the drug in the serum and/or tissue due to various reasons like immune-compromised state in AIDS patients and noncompliance with medication regimen can lead to clinical resistance. Even high doses of fungicidal agents are not able to eradicate the fungal infection from the host. The *in vitro* resistance has been divided into primary (innate or intrinsic resistance) and secondary (acquired) resistance. This form of resistance was rare in the past but is now most frequently reported in AIDS patients who suffer from recurrent azole-resistant oropharyngeal or esophageal candidiasis[114].

Azoles resistance

Resistance may be defined as a treatment failure in association with high or rising minimum inhibitory concentrations (MICs) for the same fungal strain while receiving a therapy. Many factors such as pharmacodynamic parameters, fungal virulence, host and differences in susceptibility testing methods further impair the analysis of a possible link between MIC and outcome[101]. Several mechanisms of azole-resistance have been identified, including enhanced efflux of the azole by up-regulation of multidrug efflux pumps, alterations in the cellular target of azoles (Erg11p), and modification of the ERG11 gene at the molecular level[101]. Resistant strains either exhibit a modification in the quality or quantity of target enzyme, reduced access to

the target, or some combination of these mechanisms. These changes bring about the increased efflux or alteration in drug metabolism or both. These mechanisms are summarized in Fig.1.16.

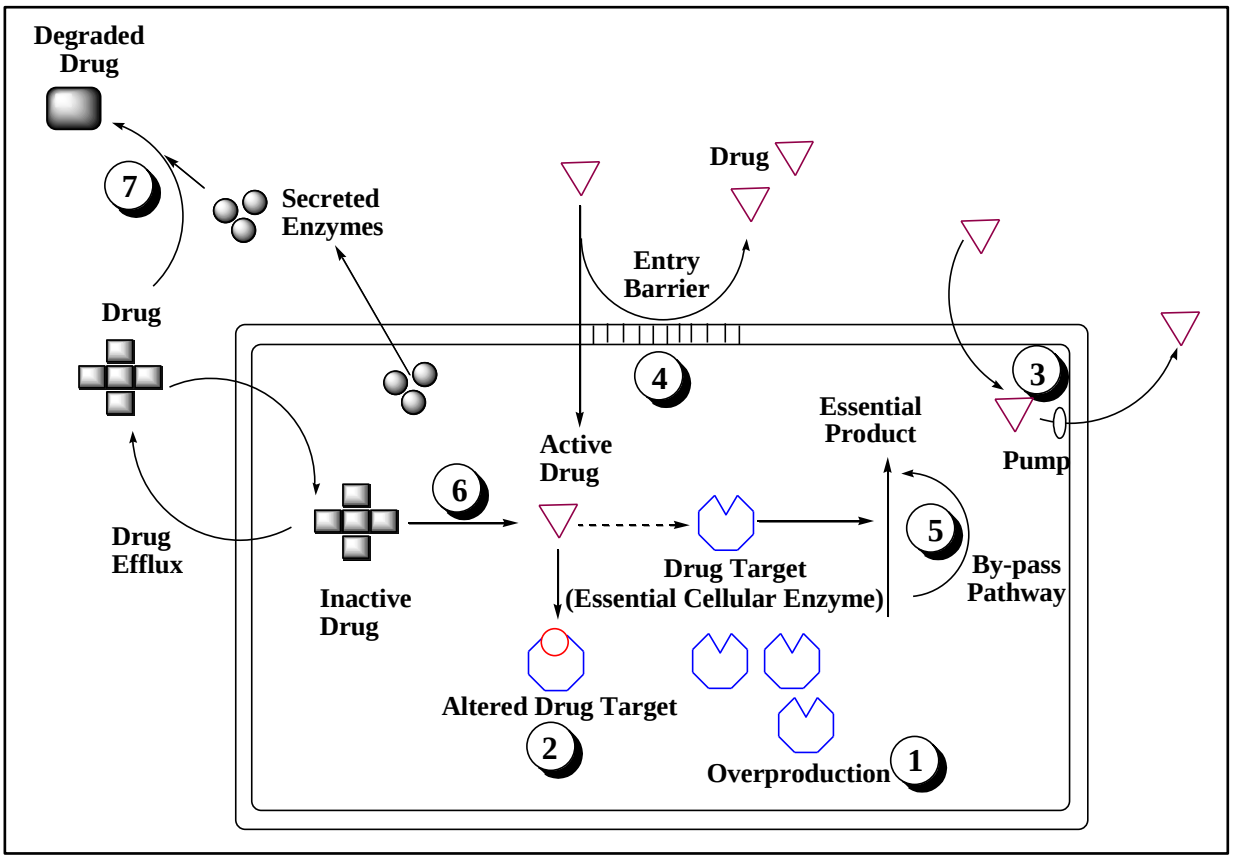

Figure 1.16 Mechanisms by which microbial cell might develop resistance. 1, The target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely. 2, The drug target is altered so that the drug cannot bind to the target. 3, The drug is pumped out by an efflux pump. 4, The entry of the drug is prevented at the cell membrane/cell wall level. 5, The cell has a bypass pathway that compensates for the loss-of-function inhibition due to the drug activity. 6, Some fungal "enzymes" that convert an inactive drug to its active form are inhibited. 7, The cell secretes some enzymes to the extracellular medium, which degrade the drug[102].

Resistance to the echinocandins has been attributed to mutations in the genes that code for 1,3-β-D-glucan synthase, specifically *Fks1* and, to a lesser extent, *Fks2*. Mutations in these genes result in alterations in the formation of the *Fks1* catalytic subunit of the glucan synthase complex which is the primary target of the echinocandins[73]. Another proposed mechanisms of resistance to the echinocandins include the presence of a drug efflux pump in the fungal cell wall

and overexpression of cell wall transport proteins. The incidence of resistance to echinocandins is rare[73].

1.5. Prevention and Control of Antifungal Resistance

There are definite guidelines and strategies for appropriate use of antimicrobials but none of them provide a strategic plan for combating antifungal resistance. Therefore, analogous comprehensive strategy to address the threat of antimicrobial resistance could be suggested for antifungals[106-108]. The strategy must aim to decrease the transmission of resistant organisms. This could be achieved by measures that include:

(i) Rational use of available antifungal agents.

(ii) Rapid and accurate diagnosis of fungal infections.

(iii) Appropriate dosing with special emphasis on avoiding treatment with low antifungal dosage.

(iv) Treatment with the appropriate antifungal.

(v) Therapy with combinations of existing agents.

(vi) Use of surveillance studies to determine the true frequency of antifungal resistance.

(vii) Treatment must aim to decrease the transmission of resistant organisms.

Advancement in rapid diagnosis of fungal infection is an urgent need. Diagnosis plays an important role in timely treatment and prevention of spread of infection. Unfortunately, progress in developing diagnostic methods specific to fungi has been slow.

1.6. Clinical need for New Antifungal Agents

Eight hundred million years of evolution have generated approximately 1.5 million fungal species that occupy many distinct ecological niches, yet only about 300 fungi cause

diseases in humans[103]. The identification of antifungals that act specifically against these pathogens is a particular challenge because of fungal diversity, individualized pathways for infection, and fungal use of multiple mechanisms that circumvent exogenous toxins. These highly regulated mechanisms include innate resistance to specific antifungal drugs, formation of biofilms, natural selection of spontaneous mutations that increase expression or decrease susceptibility of the drug target[103], stress-related tolerance that enhances short-term survival, modification of chromosomal ploidy, and overexpression of multidrug efflux pumps. However, the economic cost of fungal infection and its associated mortality, especially in debilitated and high investment patients, remain unacceptably high. The most prominent fungal pathogens affecting humans include *Aspergillus fumigatus, Candida albicans, C. glabrata, C. parasilosis, C. tropicalis, C. krusei, and Cryptococcus neoformans*. Although the skin, mucosal surfaces, and immune system usually provide robust defenses, weakened immune system dramatically increases susceptibility to debilitating and life-threatening opportunistic fungal infections. Fungal infections are normally treated with a modest repertoire of drugs derived from five antifungal classes that target DNA and RNA synthesis, ergosterol, the ergosterol biosynthetic pathway, or the biosynthesis of the cell-wall component 1,3-*β-D*-glucan. Unfortunately, the prophylactic use of fungistatic azoles such as fluconazole has been associated with an increased frequency of innate or acquired drug resistance in clinical isolates and the selection of non-albicans *Candida*, non-fumigatus *Aspergillus*, opportunistic yeast-like fungi, zygomycetes, and hyaline molds. Despite the fact that broader-spectrum third-generation azole drugs and the more expensive echinocandin class of antifungals prevent an increased proportion of life-threatening infections, *Candida* species still remains the fourth most common cause of hospital-acquired bloodstream infection and kill 40% of the patients, whereas disseminated *Aspergillus* infections kill up to

80% of affected patients[109]. Because of its economic and clinical impact, a focus is on multidrug therapy. Therefore, it is also important that physicians have access to a large number of antifungal medications to improve their chances of finding one that is both effective for treating the infection and will not interact with other medications the patient may be taking. The availability of molecular genetic tools has led to a rapid expansion in our understanding of the mechanisms by which microbial resistance emerges and spreads and promises to greatly improve efforts to develop novel and effective compounds for future use. With increased use and availability of different classes of antifungal agents, it is anticipated that we will see an increasing number and variety of fungal species resistant to these agents. Unfortunately, due to increased resistance and the emergence of new opportunistic infections, there is still a dire need for the development of new antifungal compounds.

1.7. Fungal Strains for Antifungal studies

Fungi are abundant worldwide and most of the fungi are inconspicuous. They are noticeable as mushrooms and molds. Although fungi play an essential role in decomposition of organic matter and have a fundamental role in nutrient cycling and exchange. They have also been used as source of food, fermentation of various food products, and production of antibiotics. Many fungal species produce bioactive compounds like alkaloids and polyketides that can be dangerous to humans. Fungi are ubiquitous and some species are responsible for causing serious diseases in humans, some of them can become fatal if left untreated for a long time. Mostly fungal infections are treatable in generally healthy individuals. However, the same infections become more difficult to treat in individual with weakened immune systems such as patients with HIV/AIDS, diabetes, undergoing organ transplant, chemotherapy or taking

antibiotics. In these cases, the fungal infections can get complicated and become life threatening. Generally there are three types of fungal infections namely, superficial, subcutaneous and systemic fungal infection. A fourth category is the opportunistic fungal infection mainly found in immune-compromised patients.

Opportunistic Infections	Causative Organism	Target Organs
Candidaisis, Thrush, Vulvovaginitis	*Candida albicans*	GI tract and vagina
Cryptococcal meningitis	C*ryptococcus neoformans*	Through inhalation, may cause mild lung infection. Mainly affects CNS
Aspergillosis	*Aspergillus* sp.	Lung, brain, sinuses and other organs
Mucormycosis	*Murcor* sp.	Sinuses, eyes, blood and brain
Pneumocystis carinii pneumonia	*Pneumocystis carinii*	Lungs (especially prevalent in HIV patients)

Organisms responsible for the opportunistic fungal infections may not be a threat to healthy individuals, but can cause life-threatening infections in the immune-compromised patients especially those suffering from HIV and other immune-deficiencies, cancer, diabetes, undergoing organ transplant, blood transfusion or receiving corticosteroid treatment. These organisms are prevalent throughout the U.S. and worldwide. Based on these the following fungal strains were chosen for our studies.

1. *Candida albicans*

Candida albicans is found on the skin and mucosal surfaces of all human beings. This remarkably successful and versatile human pathogen is harmless but can cause life-threatening

infections when the immune system is weakened[115]. This opportunist pathogen usually invades host tissues and eliminates the competing bacterial flora (e.g. from broad-spectrum antibiotic use). Invasive candidiasis is particularly common in intensive care units where mortality rates reach 45–49%[115b]. Prior colonization of mucosal surfaces with *C. albicans* can also lead to debilitating superficial infections in otherwise normal hosts. Approximately 75% of all women, usually have one episode of *Candida* vaginitis in their lifetime, with half having at least one recurrence[116].

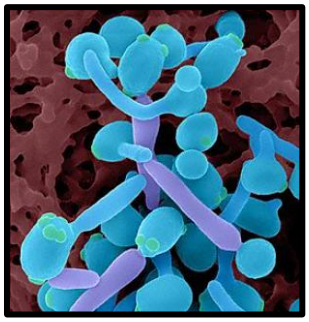

Figure 1.17 *Candida albicans*- yeast and new hyphae stages[115a]

C. albicans grows vegetatively as yeast or hyphae, and each form contributes to pathogenesis. *C. albicans* yeast cells colonize mucosal surfaces and facilitate dissemination of the organism through the blood stream[116]. Hyphae, by contrast, are important for host invasion and tissue destruction[115]. Factors affecting these diverse growth patterns during infection are poorly understood, but it is clear that innate immune mechanisms in mammalian epithelial cells normally prevent *C. albicans* from becoming a pathogen. Research has suggested the interactions between *C. albicans* and the mammalian innate immune system dictate the virulence potential of this specialized pathogen, yet relatively little is known about the molecular mechanisms underlying these interactions[116].

2. *Cryptococcus neoformans*

Cryptococcus neoformans is composed of two varieties namely *C. neoformans* variety *neoformans* and variety *grubii*. *Cryptococcus neoformans* grows as unicellular yeast and replicates by budding[117].

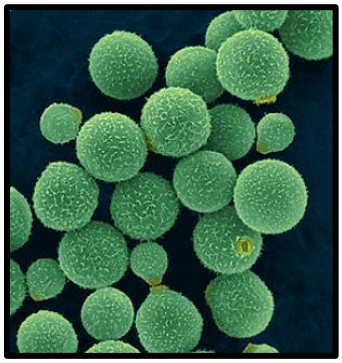

Figure 1.18 Encapsulated pathogenic yeast fungus *Cryptococcus neoformans*[117a]

During mating hyphae are made which eventually creates basidiospores at the end of the hyphae before producing spores. The cells are capable of producing a characteristic polysaccharide capsule[117b] under conditions like low glucose, serum, 5% carbon dioxide, and low iron to name a few. Also, on exposure to radiation such as gamma-radiation *C. neoformans* grows rapidly. Radiation appears to increase the electron-transfer capability of melanin in the fungus thereby increasing the total metabolic activity of fungal cells[118]. A fully grown *C. neoformans* has a prominent capsule which is mostly composed of polysaccharides. For microscopic identification, the India ink stain is used for easy visualization of the capsule in cerebral spinal fluid. As the particles of ink pigment do not enter the capsule that surrounds the spherical yeast cell, this results in a zone of clearance or "halo" around the cells and allows for quick and easy identification of *C. neoformans*. For identification in tissue, Mucicarmine stain provides specific staining of polysaccharide cell wall in *Cryptococcus neoformans.* Infection caused by *C.*

neoformans is termed cryptococcosis. It is responsible for infections predominantly for lungs, although fungal meningitis and encephalitis have been reported as a secondary infection especially for AIDS patients[118]. Those with a fully functional immune system rarely contract infections with this fungus. There *C. neoformans* is often referred to as an opportunistic fungus. It is a facultative intracellular pathogen[117].

3. *Candida glabrata*

Until recently *Candida glabrata* was considered a relatively nonpathogenic saprophyte of the normal flora of healthy individuals, rarely causing any serious infection in humans[114]. A widespread and increased use of immunosuppressive therapy together with broad-spectrum antimycotic drugs has increased the frequency of mucosal and systemic infections to a considerable amount[114]. *Candida glabrata* is haploid yeast of the genus *Candida*, previously known as *Torulopsis glabrata*. *Candida* species are most frequently isolated from the oral cavity and are detected in approximately 31 to 55% of healthy individuals[114]. *C. glabrata* has shown to be a highly opportunistic pathogen of the urogenital tract, and of the bloodstream (*Candidemia*). It is especially prevalent in HIV positive people, and the elderly.

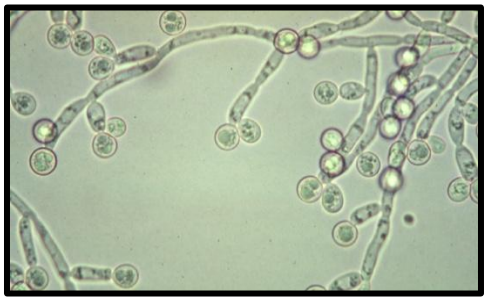

Figure 1.19 *Candida glabrata* [118b]

Depending on the site of infection, *C. glabrata* is often the second or third most common cause of candidiasis after *C. albicans. C. glabrata* infections can be mucosal or systemic and are common in immunocompromised persons. *C. glabrata* infections are difficult to treat and are often resistant to many azole antifungal agents, especially fluconazole. Consequently, *C. glabrata* infections have a high mortality rate in compromised, at-risk hospitalized patients. Increased colonizations have been observed with severity of illness and duration of hospitalization[114].

4. *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus that resides in soil, wherein it survives and grows on organic debris. It plays an essential role in recycling environmental carbon. Despite the fact that this fungal species is not the most prevalent fungus in the world, it is one of the most ubiquitous of those with airborne conidia[119]. Inhalation of conidia by immunocompetent individuals rarely has any adverse effect, since the conidia are eliminated relatively efficiently by innate immune mechanisms. Thus, until recent years, *A. fumigatus* was viewed as a weak pathogen responsible for allergic forms of the disease, such as aspergilloma, an overgrowth of the fungus on the surface of preexisting cavities in the lungs of patients treated successfully for tuberculosis, or farmer's lung, a clinical condition observed among individuals exposed repeatedly to conidia.

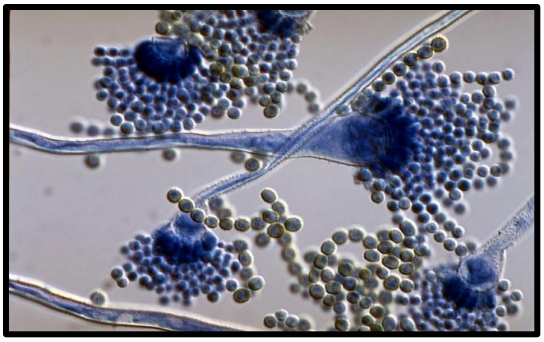

Figure 1.20 *Aspergillus fumigatus* [119a]

The increase in the number of immunosuppressed patients and the degree of severity of modern immunosuppressive therapies has changed the situation dramatically in recent years. Over the past 10 years, *A. fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immune-compromised patients in developed countries[119]. A fourfold increase in invasive aspergillosis (IA) has been observed in the last 12 years. *Aspergillus fumigatus* is an opportunistic filamentous fungus, which is the most frequent cause of invasive fungal infections in severely immune-compromised patients. *A fumigatus* is characterized by its angiotropism and propensity for angioinvasion[120]. Inhalation of asexual spores (conidia) by susceptible persons initiates invasive aspergillosis, which then germinates within the alveolar spaces, and penetrate the respiratory epithelium and vascular endothelium[120]. The release of proinflammatory cytokines and activation of the coagulation cascade is caused by the vascular invasion by *A fumigates*. This results in events like intravascular thrombosis and tissue ischemia that may cause sequestration of *Aspergillus*-infected tissue, limiting the delivery of immune effector cells and antifungal drugs to the site of infection[120]. Host with quantitative or qualitative defects in their polymorphonuclear leukocytes usually have extensive fungal proliferation and coagulative necrosis as prominent features of invasive aspergillosis. Even the

treatment with the advanced novel antifungal agents has not been able to reduce the mortality rate in immunosuppressed patients with invasive aspergillosis. Modulation of host angiogenesis by secreted *A fumigatus* secondary metabolites is a novel aspect of the pathogenesis of this important opportunistic fungus.

1.8. Aim of project

The aim of this dissertation for the research towards development of novel steroidal saponins is threefold. Firstly, literature clearly indicates saponins isolated from natural plant sources to be potential antifungal agents with minimal to no fungal resistance. Since the isolated yields of these saponins are exceptionally low, the final products are limited in their availability in sufficient quantity and purity for extensive biological studies. Therefore, development of practical synthesis of steroidal saponins would be able to provide sample in sufficient quantity and purity to explore the biological activity and understand underlying mechanism of action.

Secondly, a multi-drug therapy is usually a remedy to overcome drug resistance. Therefore, incorporation of barbiturate derivatives into the steroidal saponins would provide a new class of novel barbiturate saponins. These would be studied extensively for their biological activity, medicinal utility and their mechanism of action.

Thirdly, develop a practical synthetic strategy that could be employed at a commercial scale. One of the classic challenges of carbohydrate chemistry is to efficiently synthesize carbohydrate-based compounds and conjugates. In order to achieve the desired synthesis of novel barbiturate saponin derivatives develop methodology that is simple, efficient and cost-

effective by using unprotected saccharide. This would lead to a diverse array of saponin derivatives that could be investigated in future for extensive structure-activity relationship studies.

Finally perform antifungal assay for various steroidal saponin derivatives and sketch the SAR studies in order to identify the most potent antifungal agents of this class of compounds. For *in vitro* toxicity studies cell line assay would be performed followed by studies on animal models depending on toxicity profile.

Synthetic challenge

The structural complexity that makes carbohydrates important in so many biological processes sometimes renders their chemical synthesis difficult. Though it is possible to synthesize pure oligosaccharides in the laboratory, the regioselective protection of hydroxyl groups and the stereoselective assembly of glycosidic bonds present a number of challenges for synthetic chemists. The same regioselectivity challenges are also encountered when working with monosaccharides. The preparation of complex carbohydrates requires the strategic placement of protective groups that mask hydroxyl groups. Whilst the ability to remove one protecting group in the presence of another is a key feature, the steric and electronic nature of the protecting groups are also important as they greatly influence both the reactivity of the building blocks and the outcome of any glycosylation reactions. The properties of the protecting group next to the anomeric centre are very important. For example, a participating or non-participating functional group plays a significant role in the control of glycoside stereochemistry. Therefore, protecting groups on sites near the anomeric centre must be chosen carefully. The biggest challenges in carbohydrate synthesis are not only concerned with the stereoselective

glycosylation of two or more sugars but also the preparation of monosaccharide derivatives. In this case protecting group manipulation is also essential to afford a regioselectively protected saccharide so that other functional groups can be introduced at the desired position. Some protection strategies are the same for a number of monosaccharides for example glucose, mannose and galactose. This is generally the case for pathways to free primary hydroxyl groups. Standard methods for the selective protection of this position include tritylation or silylation followed by acetylation or benzylation of the remaining secondary hydroxyls. The primary protecting group can then be selectively removed. Since the reactivity differences between secondary hydroxyls are not that large, one step selective protection is challenging. Some methods developed for this purpose include Stannyl activation. By reacting carbohydrate hydroxyl groups with tin oxide reagents, stannylene ethers and acetals are formed. These stannylenes increase the nucleophilicity of the oxygens so that successive acylation or alkylation can be performed regioselectively. Therefore, we would like to develop synthetic strategy to use unprotected saccharides in our pursuit of developing novel barbiturate steroidal saponins.

1.9. References

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CHAPTER-2: SYNTHESIS OF NOVEL STEROIDAL BARBITURATE SAPONINS

The increasing burden of the opportunistic fungal infections and developing resistance to the currently available antifungals has created a need for new and more potent antifungal compounds. Advances in medical treatment, specifically the use of antibiotics, immunosupressant drugs and steroids suppress patient's immune system and make them more susceptible to fungal infections. At the same, the time invasive procedures also contribute to the rise in oppportunisitic fungal infections. Ironically even with this fungi seems to be the most neglected pathogens, as evident by the fact that amphotericin B, a polyene antibiotic discovered in 1956, is still used as a "gold standard" for antifungal therapy. The challenge is to develop effective strategies for the treatment of fungal infections considering the increase in opportunistic fungal infections in immunocompromised patients. A vast majority of clinically used antifungals suffer from various drawbacks in terms of toxicity, efficacy and cost, and developing resistance. With this there is a great need to develop novel antifungals belonging to a wide range of structural classes that have high selectivity and fewer side effects.

Recently synthetic derivatives of natural steroids have become a promising approach for discovery of new antifungal compounds. M. R. Banday *et al.*[9b] synthesized and investigated the antifungal activities of fatty acid analogues of cholesterol based on the fact that fatty acids and derivatives are reported as antimicrobial compounds. Of the 8 synthesized compounds only compounds **1** and **2** (Fig.2.1) showed good antifungal activity against all strains of fungi.

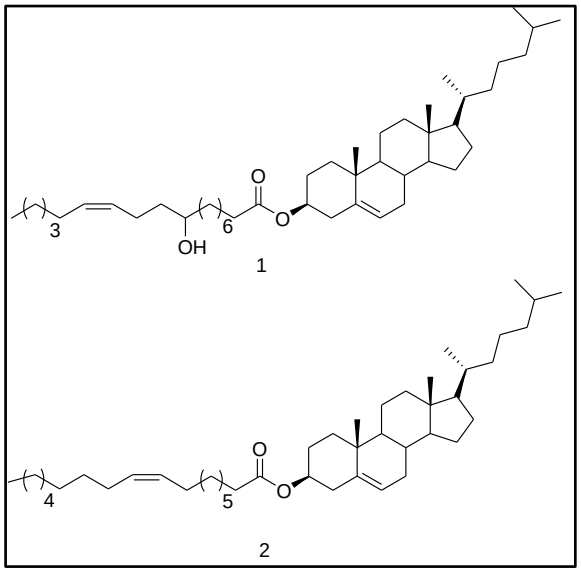
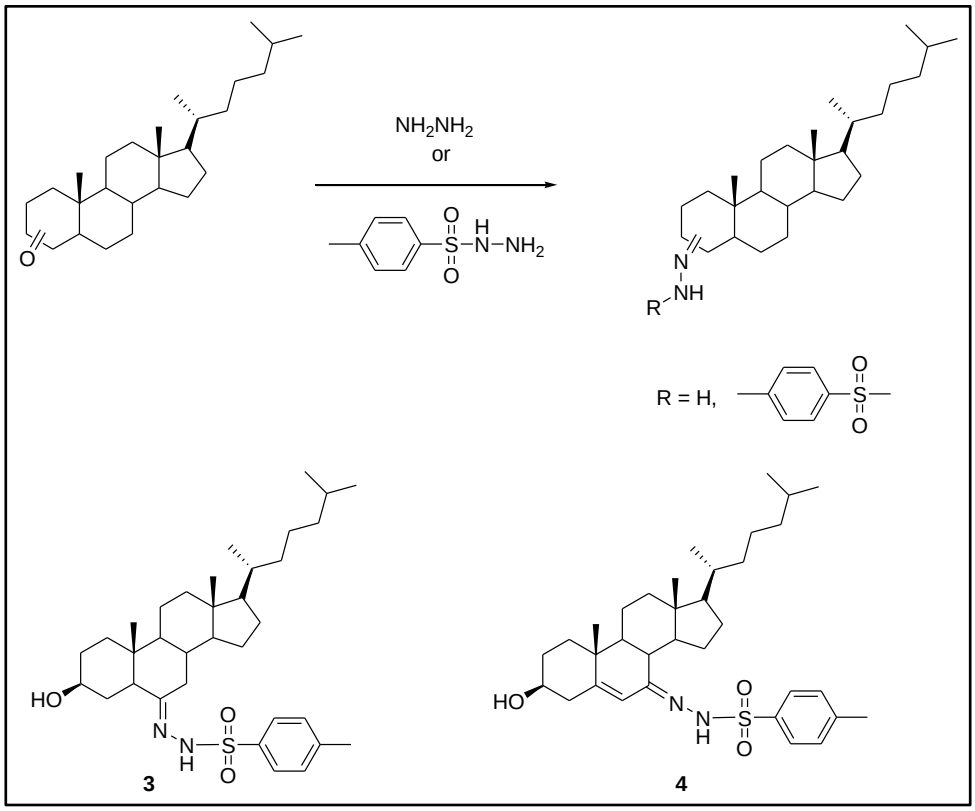


Figure 2.1 Active fatty acid analogues of cholesterol reported by M. R. Banday *et al.*[9b]

Additionally series of cholesterol-hydrazone derivatives were synthesized and evaluated for antifungal activity by C. Loncle *et al.* The studies indicated that the tosylhydrazone cholesterol derivatives **3** and **4** exhibited activities against *Candida albicans* at a concentration of 1.5μg/ml and against Amphotericin B and miconazole resistant strain *C. albicans* at a concentration of 25 and 12.5 μg/ml (Scheme 2.1)[6b].



Scheme 2.1 Synthesis of hydrazone derivatives of cholesterol by C. Loncle *et al.*[6b]

Several barbituric acid derivatives were synthesized and tested against *Candida albicans* and *Candida glabrata*. The nitrophenylhydrazone derivatives of barbituric acids were synthesized by refluxing the 5-acylbarbituric acid and the corresponding hydrazine in methanol, followed by the isolation and purification of the product[56]. A series of hydrazone and long chain barbituric acid derivatives were evaluated. It was observed that hydrazone derivatives of barbituric acid had a better antifungal activity compared to the long chain barbiturates. Compound **BA 22** was the most active of all the evaluated compounds with activity against *Candida albicans* and *Candida glabrata* at a concentration of 4 µg/mL and 2 µg/mL respectively. The antifungal activity of all the synthesized barbituric acid derivatives is summarized in table 2.1.

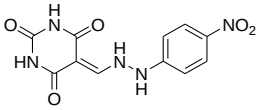
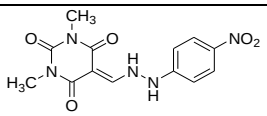
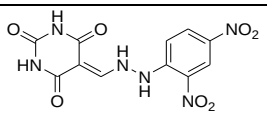
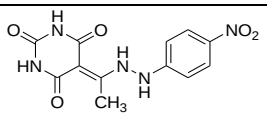
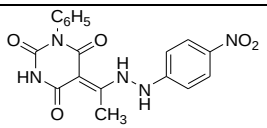
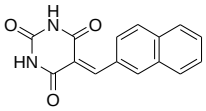
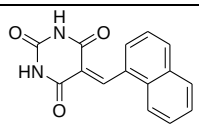
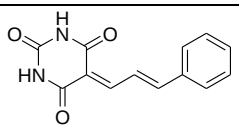
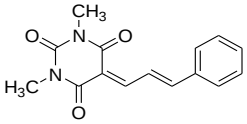
Compound	Structure	*C. albicans*	*C. glabrata*	*C. neoformans*	*A. fumigatus*
BA22		4 µg/mL	2 µg/mL	NA	NA
BA23		4 µg/mL	4 µg/mL	NA	NA
BA24		NC*	125 µg/mL	NA	NA
BA25		4 µg/mL	2 µg/mL	NA	NA
BA28		NC*	8 µg/mL	NA	NA
BA40		NC*	NC*	NA	NA
BA41		125 µg/mL	NC*	NA	NA
BA42		16 µg/mL	NC*	NA	NA
BA43		62 µg/mL	125 µg/mL	NA	NA

Table 2.1 Barbituric acid derivatives and their antifungal activities.

a. MIC values are reported only for compounds displaying (1) a prominent decrease in turbidity by visual comparison to the control wells containing no antifungal and (2) a >25% reduction in fungal growth compared to controls containing no antifungal, as measured spectroscopically by absorption at 530 nm. b. All values were determined after incubation at 30–35 °C for 48 h. c. Compounds that had (1) a slight reduction in turbidity to no change and (2) had less than a 10% reduction in growth compared to controls, as measured spectroscopically by absorption at 530 nm. NA (Not available)

The studies clearly indicated that the *N,N*-dimethyl barbituric acid and barbituric acid derivatives had the same range of activity against the fungal strains. Therefore, it was concluded that the presence or absence of methyl groups on the nitrogen of the barbituric acid derivatives has minimal to no effect on the antifungal activity. After having successfully tested barbiturate derivatives for antifungal activity, they were evaluated for toxicity profile. The *in vitro* toxicity studies were conducted on the liver cells, skin cells and the vero cells of animal models. It was observed that barbituric acid derivatives (table 2.1) were well tolerated even at concentrations as high as 200 µg/mL. With the promising antifungal activity of barbiturates, it was decided to develop a new class of novel barbiturate steroidal saponins and explore their antifungal SAR studies.

The need to synthesize novel antifungal compounds is necessary because of the increasing burden of the opportunistic fungal infections and developing resistance to the currently available antifungals. It is a well-known fact that multi-drug therapy could help overcome drug resistance. With this rationale, incorporation of barbituric acid derivatives coupled to saccharides into the steroidal skeleton would provide novel agents that would be active at two or more drug targets. This in turn could minimize the extent of drug resistance. To achieve the set target the synthesis would be carried out in three major steps.

Step 1: *C*-glycosylation of barbituric acid and its derivatives with unprotected monosaccharides and dissacharides under mild basic conditions in aqueous medium.

Step 2: Synthesis of a library of steroidal derivatives with various aliphatic and aromatic linker connected through carbamate, carbonate and ether linkage.

Step 3: Chemical ligation of *C*-glycosylated barbiturates from step 1 to steroid linker derivative (from step 2) into one molecule as shown (Fig. 2.2).

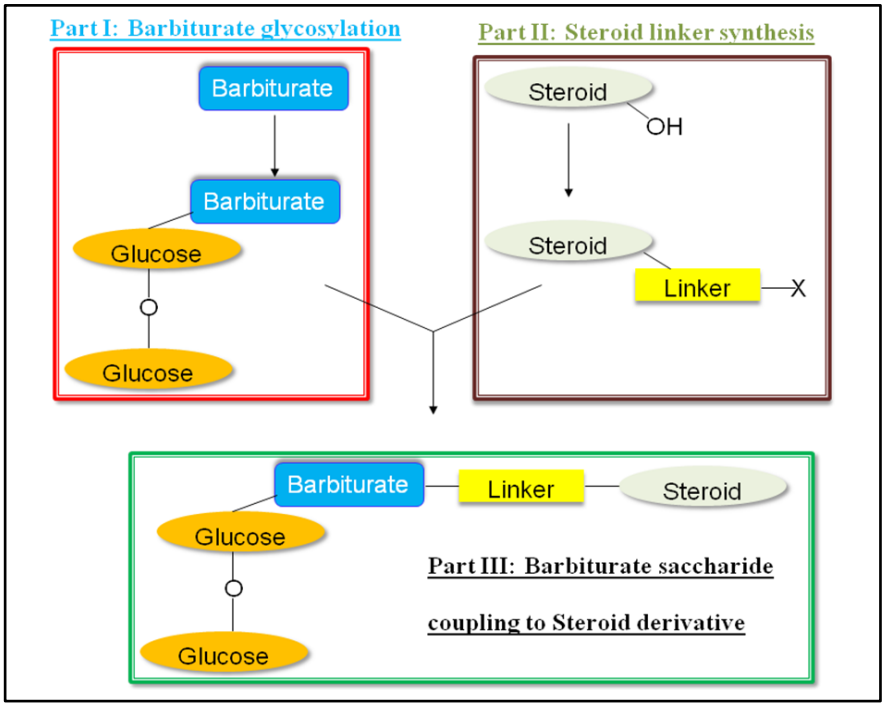


Figure 2.2 Steroidal barbiturate saponin proposed synthesis

In order to prepare the new class of novel barbiturate steroidal saponins *N*, *N*-dimethyl barbituric acid or barbituric acids could be coupled to various monosaccharides and dissacharides (Fig. 2.2) under glycosylation procedure without employing protection and deprotection techniques. This could be further coupled to the steroidal skeleton through various linkers like carbonate, cabamate, ether, or ester (both aliphatic and aromatic). A detailed reaction strategy with the problems encountered at various stages of synthetic procedure and solutions thereof are described in the subsequent sections.

2.1. Glycosylation methods

The usual chemical glycosylation process involves the coupling of glycosyl donor to a glycosyl acceptor to give a new covalent bond called glycosidic bond[1]. A glycosidic bond is a type of covalent bond that joins a carbohydrate molecule to another group, which may or may not be another carbohydrate. The saccharide moiety involved in the glycosylation has to be protected to carry out the synthesis of glycosidic bond. The resulting glycosidic bond can be a *O*-glycosidic bond (containing oxygen), *S*-glycosidic bond (thioglycosides, oxygen is replaced with a sulfur atom), glycosylamine (oxygen is replaced with a nitorgen atom) and *C*-glycosidic bond (oxygen replaced with carbon)[2]. For synthesizing the glycosides, the sugar is usually transformed into a fully protected glycosyl donor with an appropriate leaving group at its anomeric center. A suitable protected glycosyl acceptor containing one free hydroxyl group is then glycosylated with the former[3,4]. The Fischer–Helferich method (Fig. 2.3A) involving the direct replacement of the anomeric oxygen atom under acid-catalyzed reaction has been applied successfully for glycosylation of many substrates. However, the reversibility of the reaction limits its usefulness in the synthesis of complex oligosaccharides and glycoconjugates. In order to eliminate the reversible nature of this reaction, preactivation of the anomeric center through the introduction of a good leaving group is necessary.

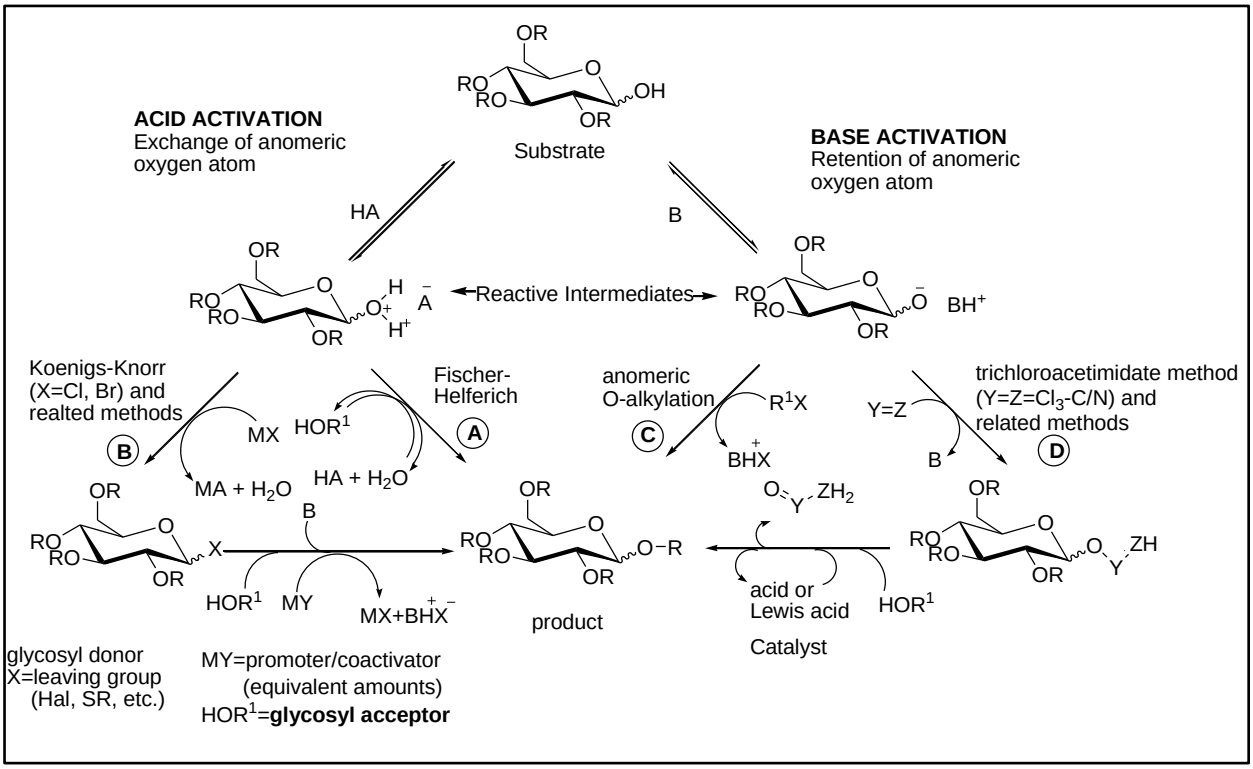


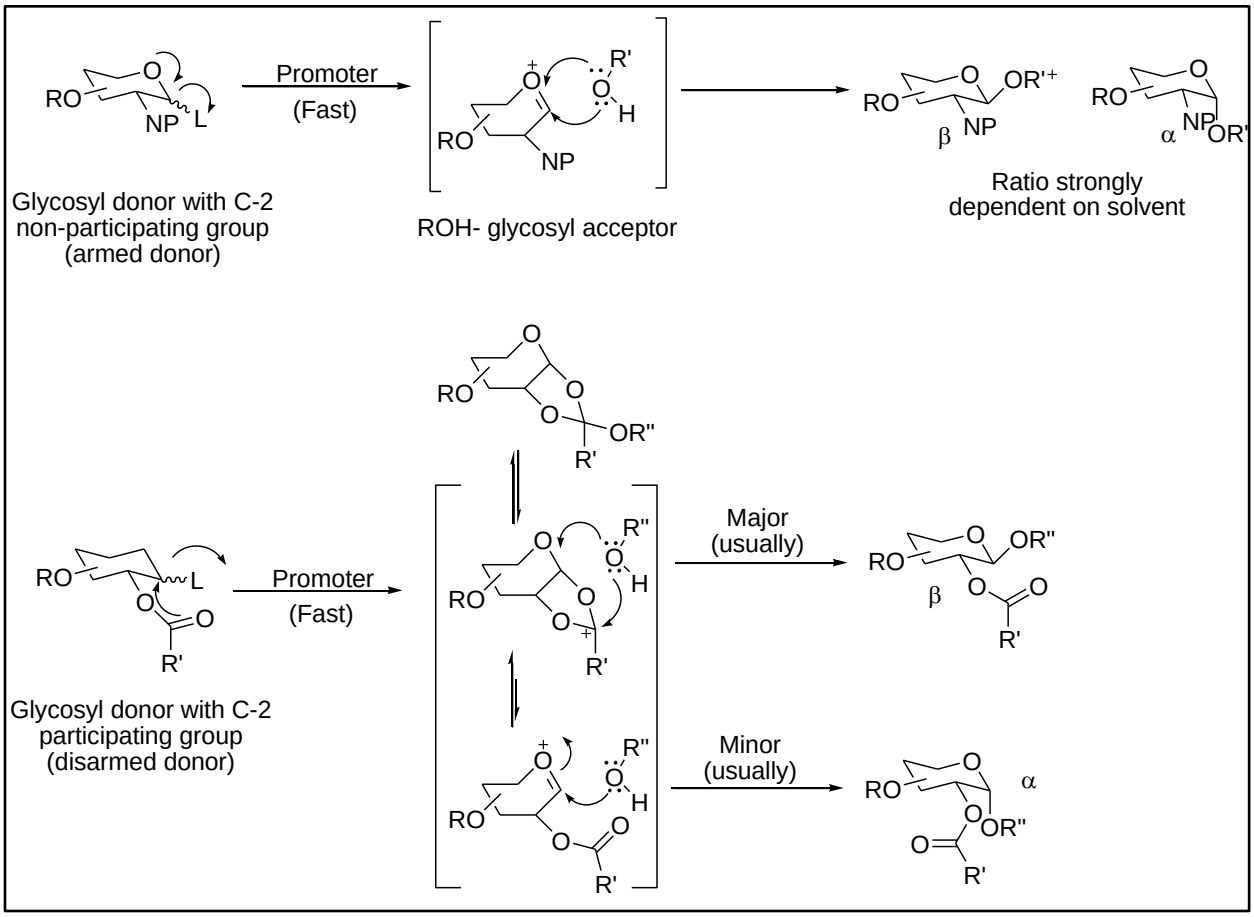
Figure 2.3 Generation of glycosidic and saccharide bonds[1]

Koenigs–Knorr method (Fig. 2.3B), introduced in 1901, is the best known for being irreversible. The method requires an exchange of the anomeric hydroxyl group with bromine or chlorine as the first step to generate a glycosyl-group donor. Followed by the second step in which the glycosyl group is transferred to the glycosyl acceptor in the presence of a heavy metal ion promoter. Because of some inherent disadvantages associated with this method, it becomes experimentally demanding and unsuitable for large-scale synthesis. For instance the reaction requires at least equimolar amounts of the heavy metal salt promoter, that is often incorrectly termed as "catalyst," is a limiting factor[5-7]. Therefore, alternative methods to overcome these shortcomings are of interest.

Other approaches involving the exchange of the anomeric oxygen atom for a fluorine, alkylthio, or arylthio as leaving group have been explored since these groups are not affected by manipulations of any involved protecting groups. In these methods, the anomeric carbon atom of

the sugar residue to be coupled serves as the electrophile and the alcohol as the nucleophile. An alternative method to the ones described above is a base-mediated deprotonation of the anomeric hydroxy group of a pyranose or furanose moiety to generate an anomeric oxide, which would undergo direct and irreversible anomeric *O*-alkylation to give the corresponding glycoside (Fig. 2.3C). Surprisingly, this simple "anomeric *O*-alkylation" method, as termed by Schmidt[2a,8, 9] had not been used for the synthesis of complex glycosides and glycoconjugates. The direct anomeric *O*-alkylation of various protected and even completely unprotected sugars in the presence of a base with triflates or Michael acceptors as alkylating agents has become a very convenient method for glycoside-bond formation[10-13].

The mechanism, of any well-developed glycosylation method for oligosaccharide synthesis follows the same general mechanism (Scheme 2.2). The leaving group of the glycosyl donor is freed from the molecule in the presence of a promoter[5,7,13,14]. The nature of the C-2 substituent of the glycosyl donor determines the structure of the oxocarbenium intermediate formed. Participating groups, such as aryl or alkyl esters and amides, can help stabilize the intermediate and tend to favor the formation of 1,2-*trans*-glycosides (Scheme 2.2). An important aspect of this reaction is the formation of an orthoester as a side reaction. This orthoester formation is reversible and can have a deleterious effect on the glycosylation depending on the nature of the glycosyl donor and acceptor and the reaction conditions employed. Glycosyl donors with non-participating groups at C-2, such as ethers, called armed donors, react faster than those with participating groups at C-2 (disarmed donors). This is because of the fact that the C-2 substituent of an armed donor is less electron withdrawing than that of a disarmed donor and therefore, the oxocarbenium intermediate forms faster (Scheme 2.2). In this case, the stereoselective outcome of the glycosylation is largely solvent dependent.



Scheme 2.2 Generalized mechanism for glycosylation reactions[4a]

A high anomeric stereoselectivity (Fig. 2.4) is often observed with pyranoses which can result from the enhanced nucleophilicity of equatorial oxygen atoms (owing to steric effects and the stereoelectronic kinetic anomeric effect due to repulsions of lone electron pairs, dipole effects, or both)[4a, 7, 8].

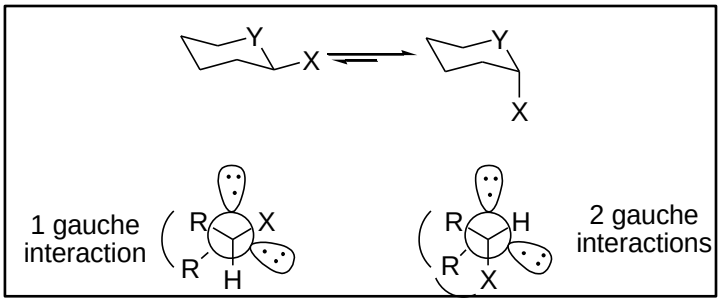


Figure 2.4 The anomeric effect[15]

The higher stability of products with an axial anomeric oxygen atom is observed. Chelation effects can also be used to promote anomeric stereoselectivity. There are three main requirements for an efficient glycosylation method in terms of small amounts of reagents must be used, the glycosyl donor must be generated in a simple process and the donor activated by a catalytic amount of a reagent and the glycosylation step must be stereoselective and high yielding. These demands are not met by any of the methods described above. However, the general strategy for glycoside bond formation is reasonable which involves the first step as the generation of the glycosyl donor involving the preactivation of the anomeric center (Fig. 2.4) via formation of a stable glycosyl donor, ideally through a catalytic reaction to attach a leaving group to the anomeric hydroxy group. The second step (activation of the glycosyl donor) consist of a sterically uniform high yielding glycosyl transfer to the glycosyl acceptor on the basis of activation of the glycosyl donor with a catalytic amount of a promoter and covalent binding of water released in this condensation reaction to the leaving group. In this way, the required amounts of reagents can be minimized (scheme 2.2).

C-GLYCOSIDES

C-glycosides (also known as *C*-nucleosides) are the compounds that contain a heterocyclic aglycone and a carbohydrate moiety that are connected by a C–C bond. Naturally occurring *C*-nucleoside pseudouridine **1** (first reported nucleoside)**,** 1-Methylpseudouridine **2**, 2'-*O*methylpseudouridine **3**[36] are shown in Fig. 2.5[16].

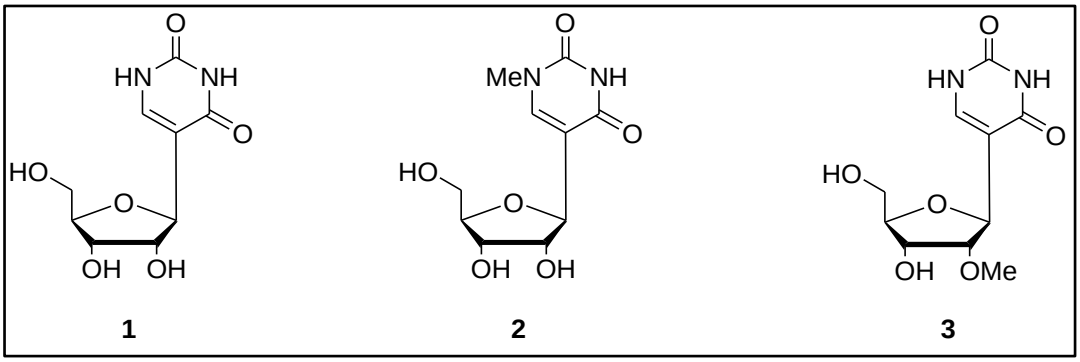
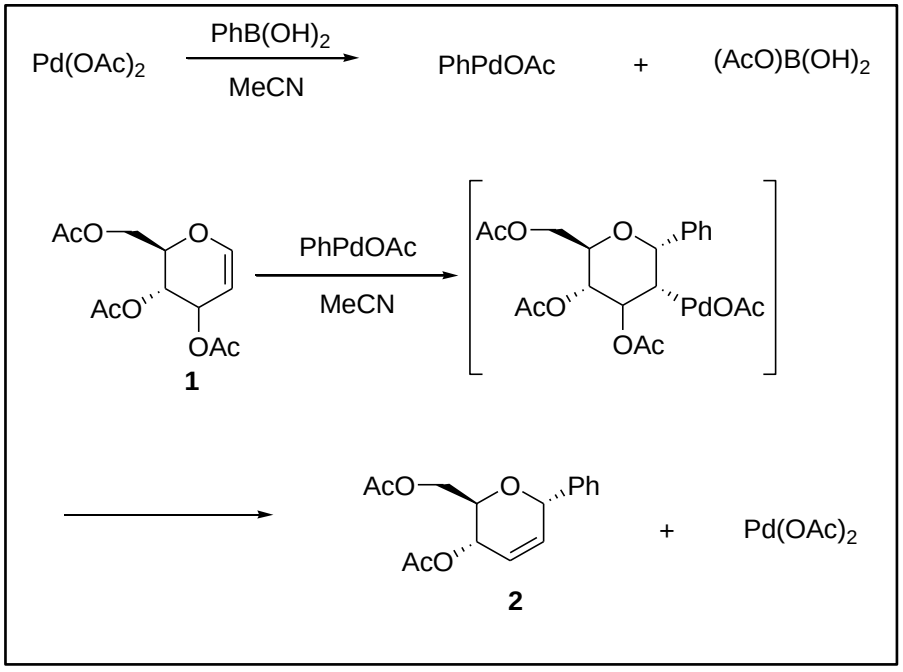


Figure 2.5 Naturally occurring *C*-nucleosides.

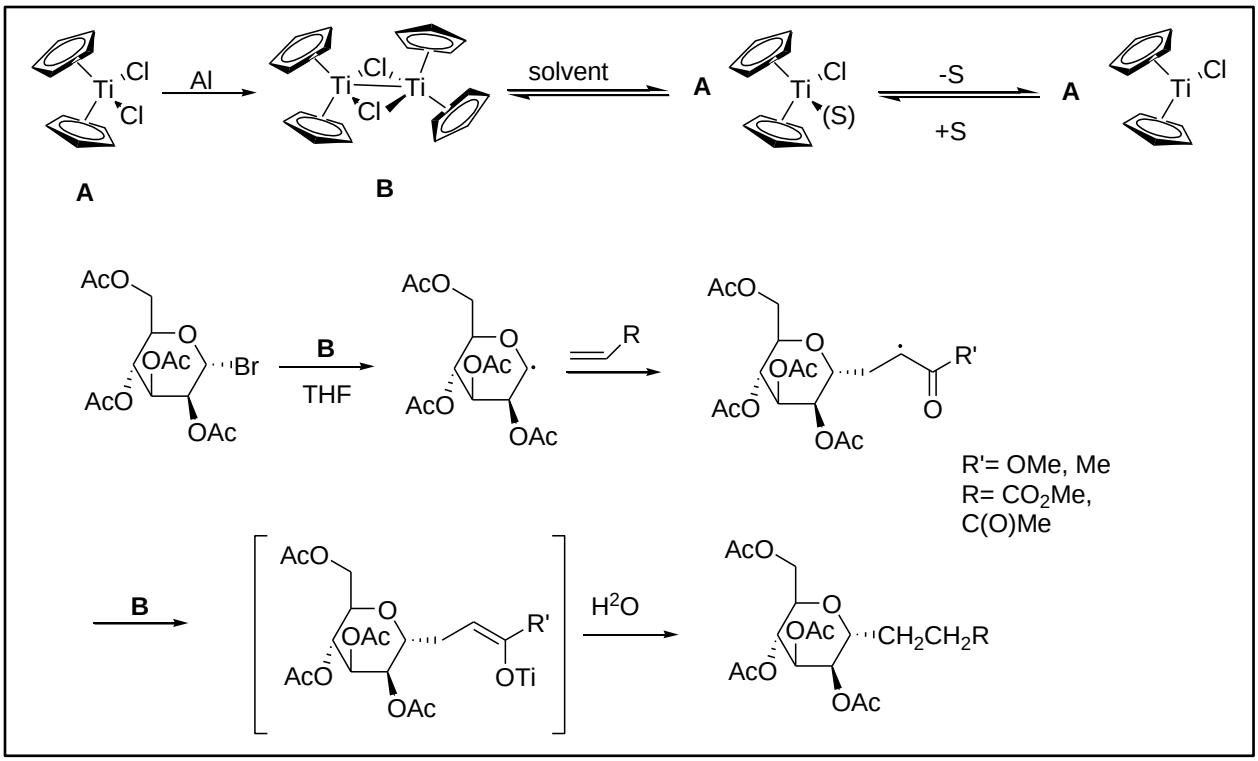
The naturally occurring *C*-nucleosides usually act as antibiotics, also exhibit anticancer and/or antiviral activity[17-19]. *C*-nucleosides have also been found to be the suitable candidates for use as building blocks of oligonucleotides for the construction of triplex DNA in gene therapy[20,21]. C–C bond of the *C*-nucleosides is resistant to hydrolytic and enzymatic cleavage[20]. Daves *et al.*[22-24] employed Heck-type coupling reactions to form C–C bonds between the anomeric carbon atom of sugar derivatives and heterocycles. Heck reaction conditions can be regioselective and stereoselective in the formation of a C–C bond[25,26]. The anomeric configuration of the *C*–nucleoside in the Heck reaction is controlled by presence of a suitable protecting group at the 3'-hydroxy function of the sugar moiety. This results in formation of glycosidic bond in the least sterically hindered face of the glycal ring during the attack by organopalladium reagents and results in the formation of the β-anomer[23].

Of the two major approaches for the synthesis of *C*-nucleosides in the literature[27,28], first approach uses a preformed aglycone that is coupled to a sugar derivative. In the other approach, a functional group is introduced at the anomeric position of the sugar derivative and is followed by construction of a heterocyclic base. Hainke *et al*. reported employing Friedel Crafts reaction, for the synthesis of *C*-glycosides. This involves the attack of nucleophilic species on an electrophilic ribose derivative[29] and lewis acid catalyzed processes[30]. Some synthetic routes involve the synthesis of a ring after the formation of a *C*-glycoside[31,32]. Ramnauth *et al.* reported steroselective *C*-glycosidation using aryl boronic acids *via* non-heck reaction conditions (Scheme 2.3)[33].



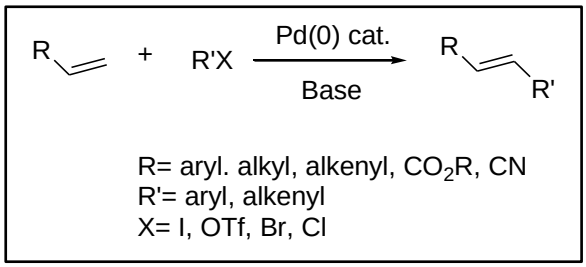
Scheme 2.3. Palladium(II) acetate mediated *C*-Glycosidation of tri-*O*-acetyl-D-glucal **1** [33]

Spencer *et al.* reported radical reactions for *C*-glycoside synthesis using Titanium (III) compounds and radical generation reaction conditions (Scheme 2.4)[34].



Scheme 2.4 Spencer *et al.* synthesis of simple *C*-glycosides using 1[34].

Heck coupling approach has also been employed for *C*-glycoside synthesis shown in Scheme 2.5[35].



Scheme 2.5 The Heck coupling reaction[35].

All the above described methods have some disadvantages associated with them either in terms of catalyst, percentage yield or non-applicability at commercial scale. There are usually three main requirements for an efficient glycosylation method[35]:

1. Use of small amounts of the reagents so that the glycosyl donor is generated in a simple process and activation of the donor by a catalytic amount of a reagent.

2. The glycosylation step must be stereoselective and high yielding.

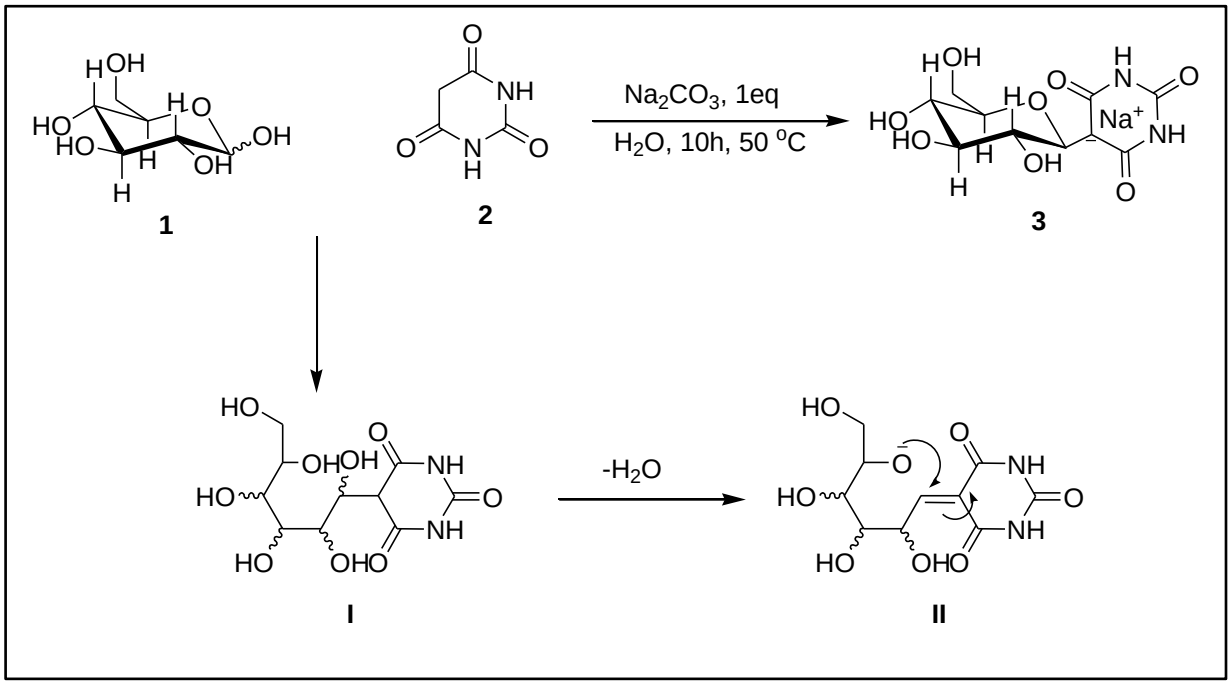
3. The method must be applicable on a large scale.

To achieve the above mentioned requirements use of unprotected sugar for glycosylation under basic conditions was explored. Use of unprotected saccharides offers various advantages like the total number of steps to get the final compound are drastically reduced, improved yields, no need for protection and deprotection steps, cost effective and easy applicability at large scale. This methodology is described in the following section.

2.1.2. Synthesis of Barbiturate Glycosides

One of the requirements of synthetic strategy for saponin barbiturates antifungal agents is its capability to be used at large commercial scale. To develop such a method it is proposed to avoid using the usual protection and deprotection of hydroxyl functionality. The usage of protection and deprotection methods where reaction conditions employ inert atmosphere is difficult to achieve at commercial scale. In order to develop an industry friendly methodology for *C*-glycosylation that is cost effective, efficient and reproducible at large scale it becomes imperative to utilize unprotected saccharide for barbiturate glycosylation. Only one report for converting the reducing monosaccharides into *C*-glucosidic derivatives exploiting the reactivity of the aldehydic form of the sugar was reported in 1986 by Gonzalez *et al*[36]. They developed a general methodology for the synthesis of pyrimidine *C*-nucleosides involving a one-step reaction

of aldohexose and aldopentose with barbituric acids in a water solution of sodium carbonate at 50°C (Scheme 2.6)[36].

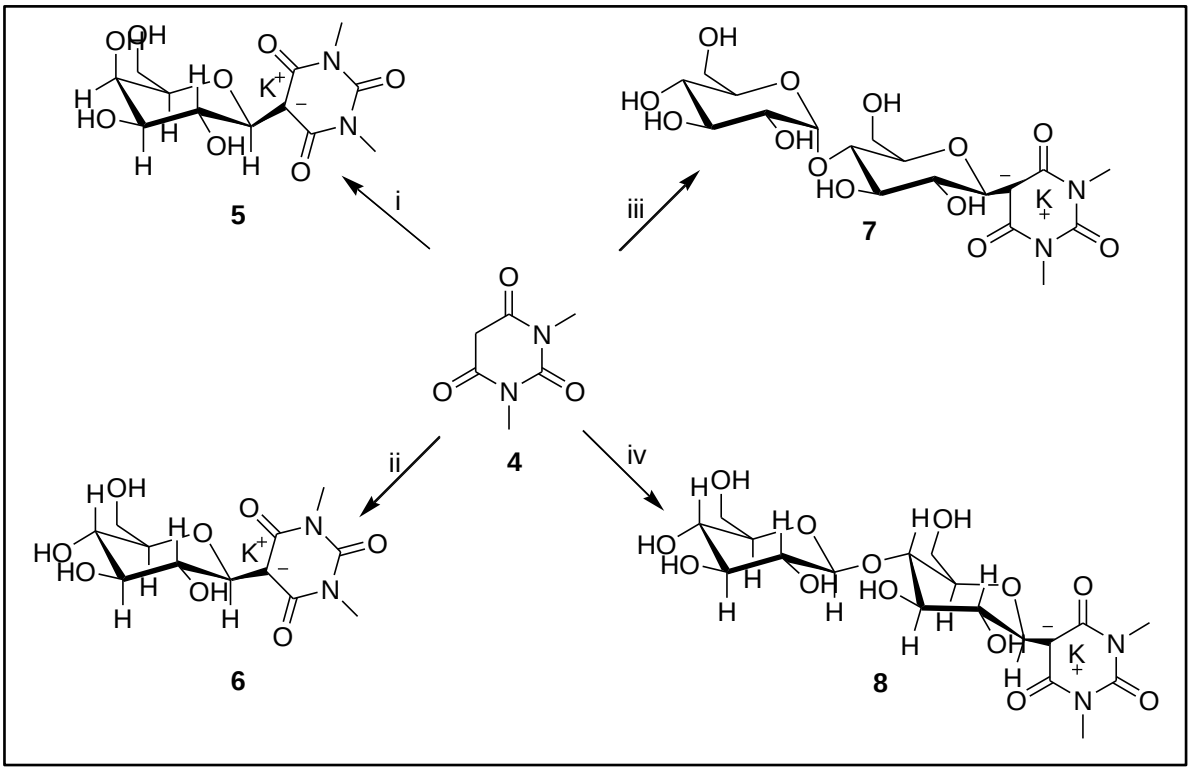


Scheme 2.6 *C*-glucosylbarbiturates synthesis under basic conditions and proposed reaction mechanism[36]

The *C*-glycosylation reaction of barbituric acid proceeds via a proposed mechanism shown in Scheme 2.6. It is proposed that the barbituric acid (pKa ~ 4) can act as a nucleophile even in absence of a strong base. The initially formed adduct **I** undergoes elimination to give an unsaturated intermediate **II**, which, can be attacked by another nucleophile. The most probable is the intermolecular reaction with the oxygen at carbon 6[36].

In our synthetic procedure, we achieved barbiturate glycosylation by employing glucose, galactose, lactose and cellobiose (table 2.2 and 2.3). The desired saccharide or disaccharide was added to *N*,*N*-dimethyl barbituric under mild basic condition in aqueous medium. The reaction mixture was carefully heated on a water bath at a temperature not exceeding 80 °C for 5 hours. Most of the water was evaporated to give slurry, which was carefully poured in stirring methanol

and continued to stir for an hour. During this period product precipitated as white to yellow solid material. This gave pure *C*-glycosylated barbiturate derivatives as a powdered material, which were filtered and washed with methanol. The reaction proceeds as per the Scheme 2.7 with a proposed mechanism similar to one shown in Scheme 2.6.



Scheme 2.7 *C*-glycosylation reaction of *N*,*N*-dimethylbarbituric acid **4** with (i) galactose, (ii) glucose, (iii) maltose and (iv) cellobiose, in K_2CO_3 (aq.) to form compounds **5, 6**, **7** and **8** respetively.

A variety of mono and disaccharide substrates could be used for glycosylation of barbituric acid and *N*,*N*-dimethyl barbituric acid to give a variety of *C*-glycosylated barbiturate salts as shown in table 2.2 and 2.3.

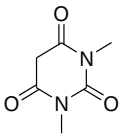
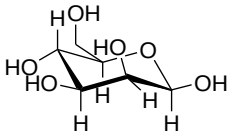
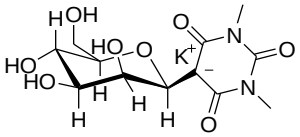
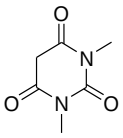
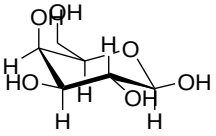
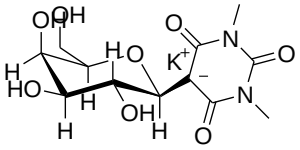
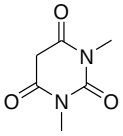
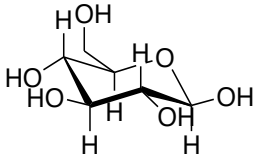
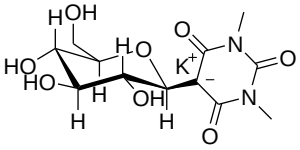
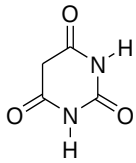
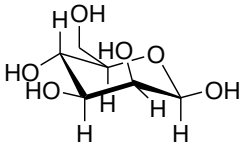
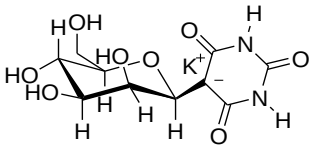
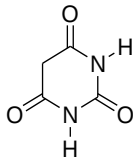
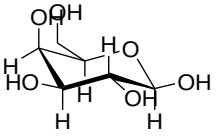
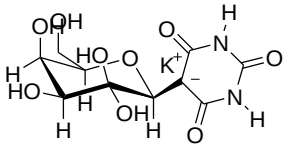
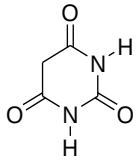
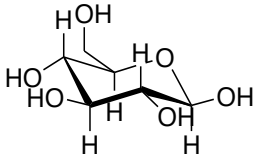
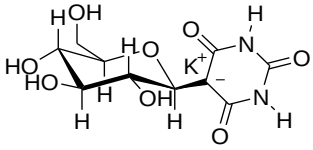
Entry	Babiturate	Monosaccharide	*C*-Glycosylated product
1.		 Mannose	
2.		 Galactose	
3.		 Glucose	
4.		 Mannose	
5.		 Galactose	 **5a**
6.		 Glucose	

Table 2.2 Glycosylated monosaccharide barbiturate derivates

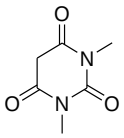
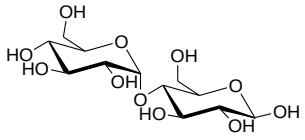
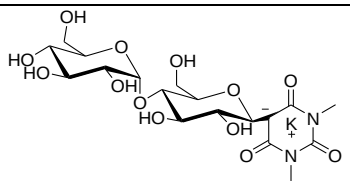
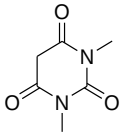
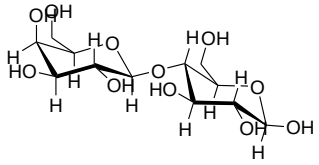
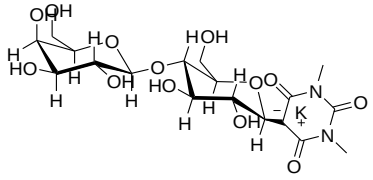
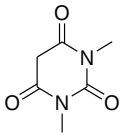
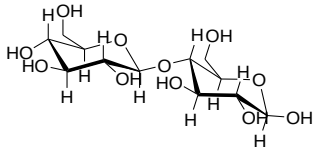
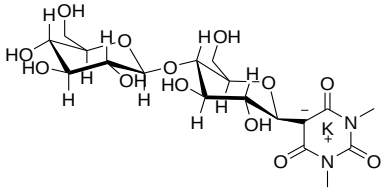
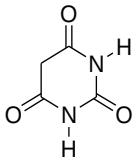
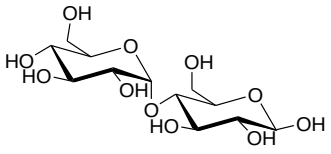
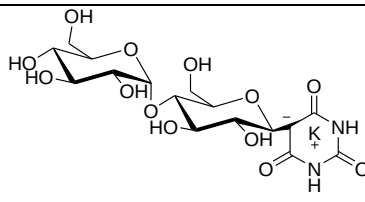
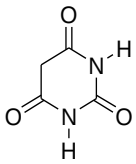
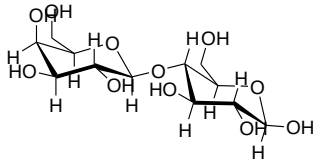
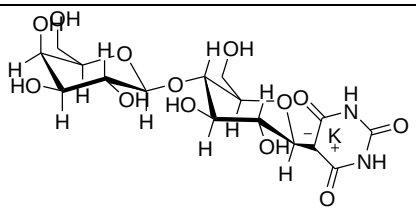
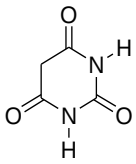
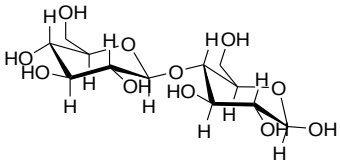
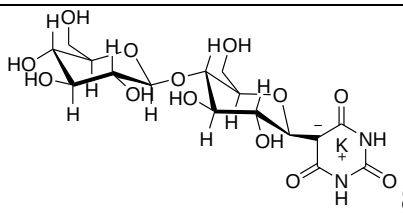
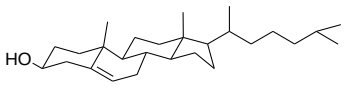
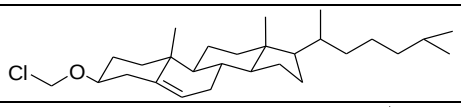
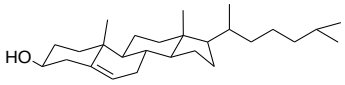
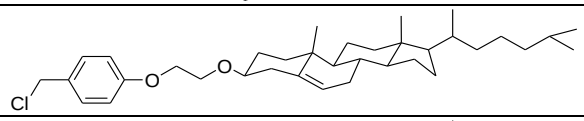
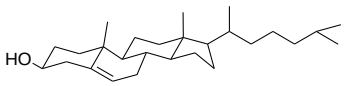
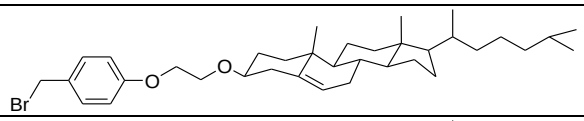
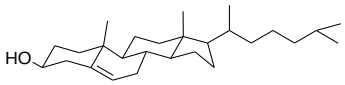
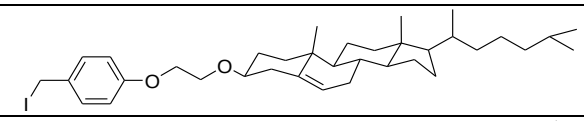
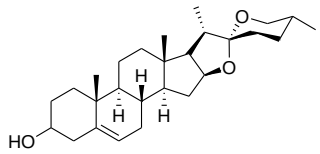
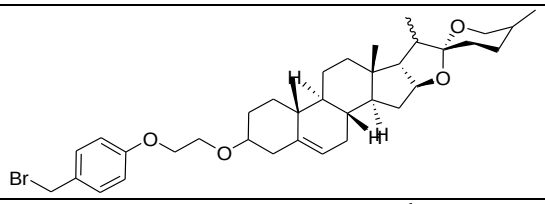
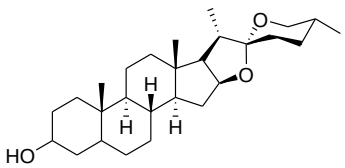
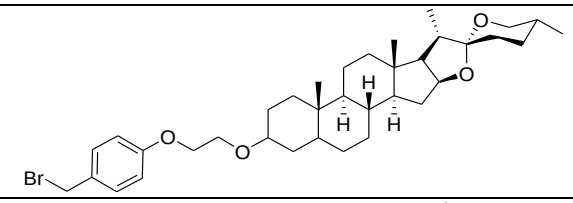
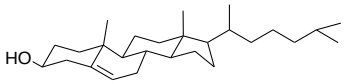
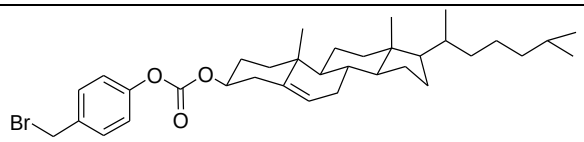
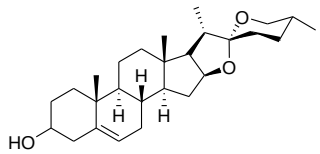
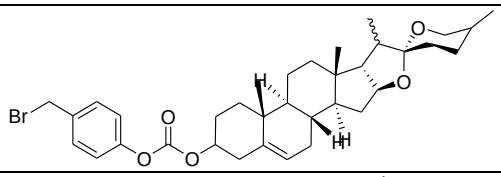
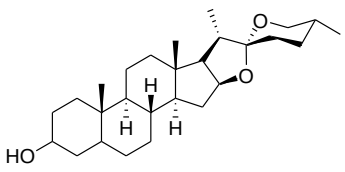
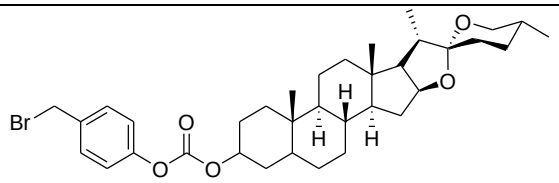
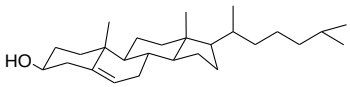
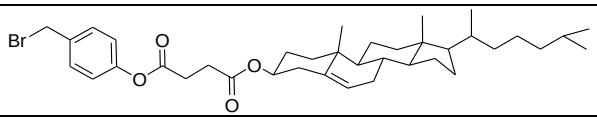
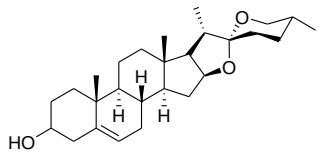
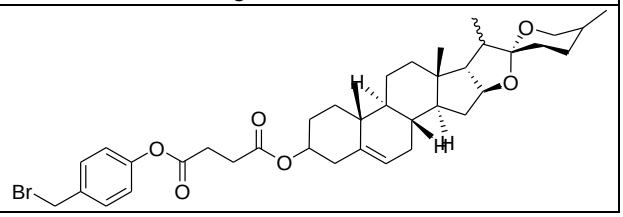
Entry	Barbiturate	Disaccharide	*C*-Glycosylated product
7.		 Maltose	
8.		 Lactose	
9.		 Cellobiose	
10.		 Maltose	 7a
11.		 Lactose	
12.		 Cellobiose	 8a

Table 2.3 Glycosylated disaccharide barbiturate derivates

The monosaccharide and disaccharide glycosylated barbiturate were synthesized in an isolated yield of 85-90% with sufficient purity. These were taken to the next step without further purification.

2.2. Synthesis of steroid linker derivatives

In order to synthesize the desired barbiturate saponin derivatives, the C-glycosylated barbiturate derivatives from step 1 need to be coupled to a variety of steroids through different linkers. The linker could be carbonate, cabamate or ether. It desired that a hydrolysable linker be used for the synthesis of antifungal compounds. This linker would be metabolized inside the human system to release two separate steroid and barbiturate molecules that can act on same or different drug targets to have the antifungal effects. A library of compounds with diverse steroidal skeleton would provide in depth understanding of the structure-activity relation of this novel class of compounds. Subsequently, help optimize the structure of compounds to provide most potent antifungal agent. To achieve this goal different steroid derivates with variable linkages were proposed as shown in Table 2.4.

Entry	Steroid	Linker	Derivative
13.		Ether	
14.		Ether	
15.		Ether	
16.		Ether	
17.		Ether	
18.		Ether	
19.		carbonate	
20.		carbonate	
21.		carbonate	
22.		Ester	
23.		Ester	

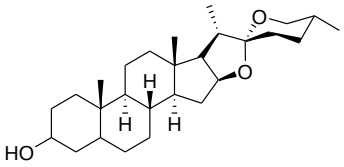
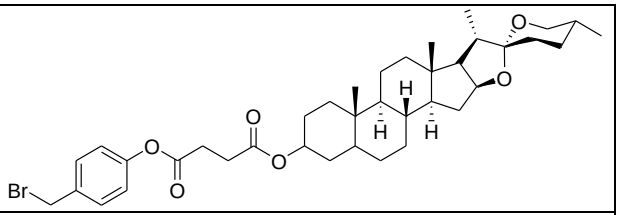
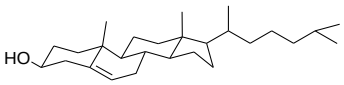
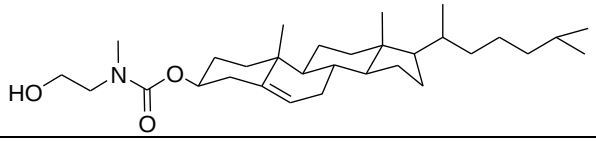
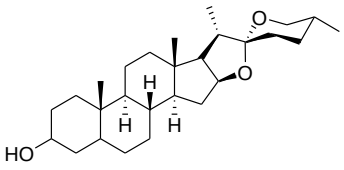
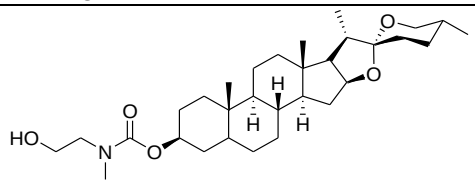
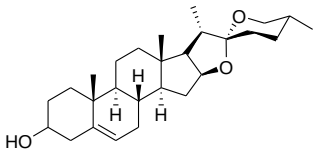
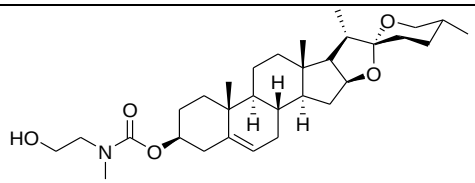
24.		Ester	
25.		Carbamate	
26.		Carbamate	
27.		Carbamate	

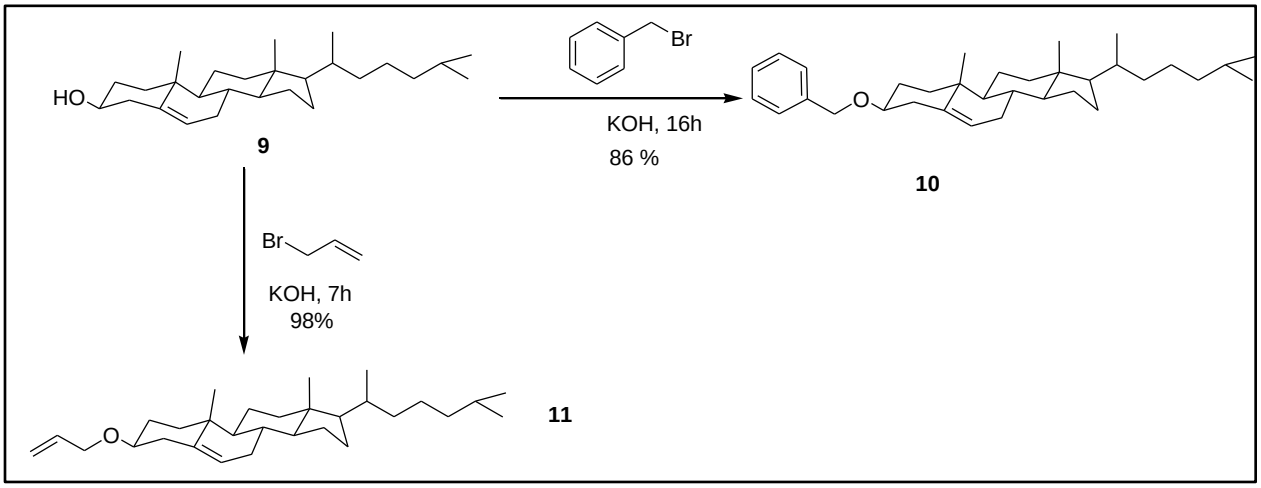
Table 2.4 Steroid linker derivatives

2.2.1. Ether linked steroid derivative synthesis

Literature is replete with the ether synthesis methods that can be applied to a variety of substrates. Ethers, in general can be synthesized by reacting an alkoxide nucleophile with a good alkyl electrophile. For instance, preparation of allyl ethers has been carried out from the corresponding alcohols using several reagents such as allyl bromide[38], allyl carbonate[39,40], allyl ethyl carbonate[41]. Similarly, benzyl ethers have been successfully prepared using benzyl bromide[42], benzyl iodide[43], phenyl diazomethane[44]. In addition, allyl bromide and benzyl bromide in presence of a base in a suitable solvent medium are frequently employed for the generation of allyl and benzyl ethers respectively.

Rao *et al.*[37] have reported Williamson synthesis in solvent-free environments[45]. The alcohol protection was carried out conveniently and efficiently with allyl and benzyl bromides,

using solid potassium hydroxide pellets without the use of any solvent (Scheme 2.8). As per them the reaction of cholesterol **9** under solvent free conditions with benzyl bromide and potassium hydroxide on stirring for more than 15 hours gives compound **10** in 86% yield.

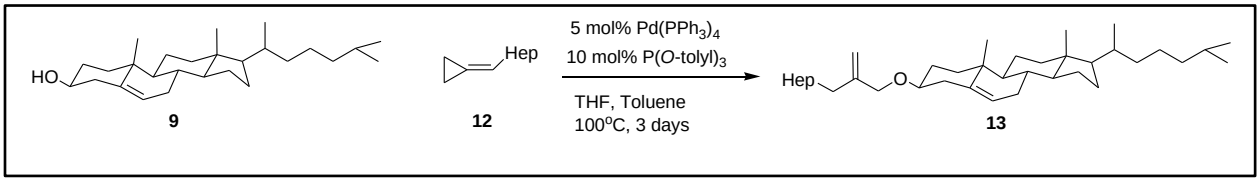


Scheme 2.8 Ether synthesis by Rao *et al.*[37]

Bogdal and coworkers have reported[47] solvent-free allyl and benzyl ether preparation using a combination of potassium carbonate and potassium hydroxide bases in the presence of tetrabutylammonium bromide under microwave irradiation. However, the reaction requires drastic conditions such as high temperature and specialized apparatus. Moreover, their study was limited to the preparation of ethers from alcohols.

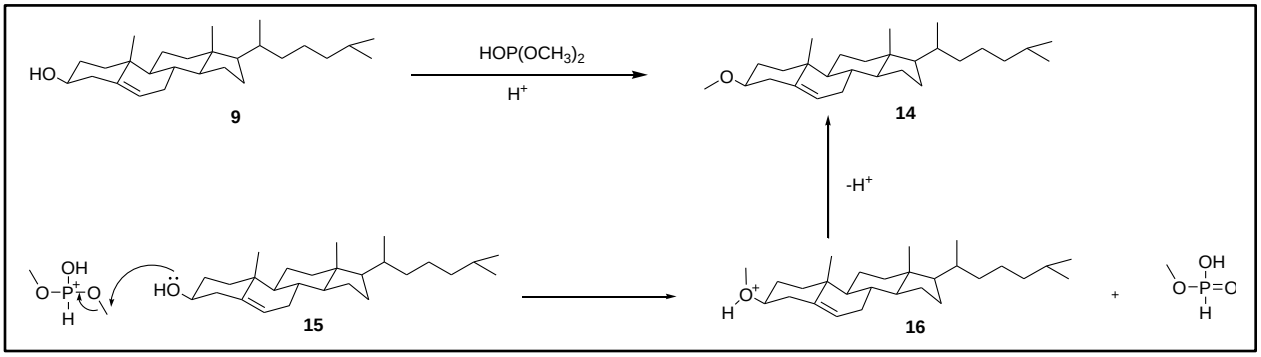
Camacho *et al.*[48] reported ether synthesis under Pd-catalyzed reaction conditions (Scheme 2.9). Addition of alcohols **9** to alkylidenecyclopropanes **12** proceeds presumably via activation of the alcohols **9**[49], that serves as a powerful tool in the synthesis of allylic ethers **13**. They successfully utilized Pd in addition of alcohol pronuclephiles to nonconjugated systems to give a regioselective product with wide range of substrates. A reperesentaive example from their

application to cholesterol **9** is shown in Scheme 2.9. This method clearly suffers from the drawback of long reaction times, high reaction temperatures, expensive catalyst and inefficiency at large scale.



Scheme 2.9 Cholesterol ether synthesis by Camacho *et al.*

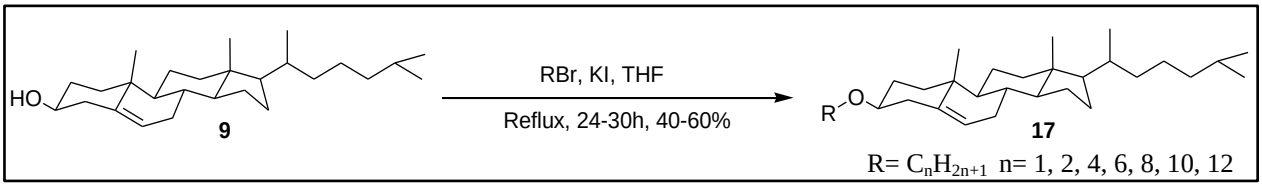
Kashman[50] *et al.* isolated sterol ether while performing phosphorous containing reactions with steroids. They reported that in the presence of catalytic amounts of acid, in a solution of a steroidal alcohol in dialkyl phosphite, corresponding ether formation was observed. According to Kashman[50] *et al.* procedure, if cholesterol is heated for several hours in HOP-$(OCH_3)_2$ containing p-TsOH, 3β-methoxycholest-5-ene is obtained as the product. The overall reaction and its mechanism are outlined in Scheme 2.10.



Scheme 2.10 3β-methoxycholest-5-ene synthesis by Kashman[50] *et al.*

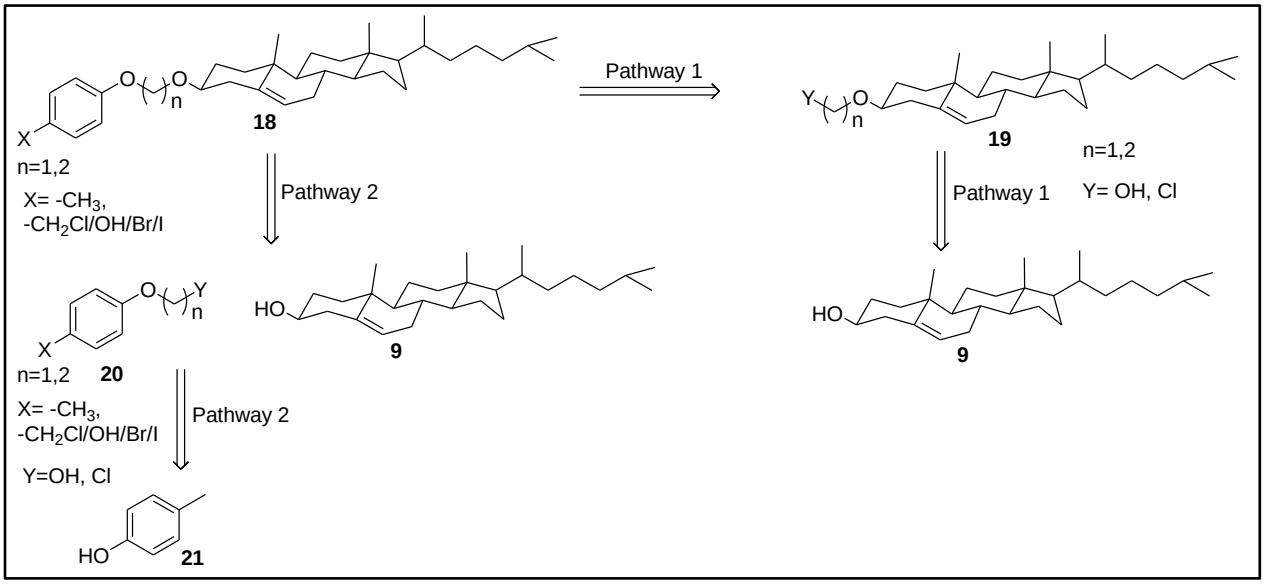
Another procedure reported by Wang *et al.*[51] (Scheme 2.11) successfully protects the β C-3 hydroxy by conversion to various alkoxy groups. The reaction involves use of desired

bromoalkyl, which forms the corresponding ether **17** under reflux conditions with the steroid **9** in presence of potassium iodide in yields of more than 90%.



Scheme 2.11 Cholesteryl ether synthesis by Wang *et al*[51].

Based on the literature review the steroidal linker derivatives could be synthesized in two ways. Either introduce the aliphatic linker into the steroidal skeleton followed by aromatization or *vice versa*. We proposed that the synthesis of compound **18** could be carried out either via pathway 1 or pathway 2. A reterosynthetic analysis to synthesize compound **18** is outlined in scheme 2.12.



Scheme 2.12: Retrosynthetic analysis for steroid linker synthesis

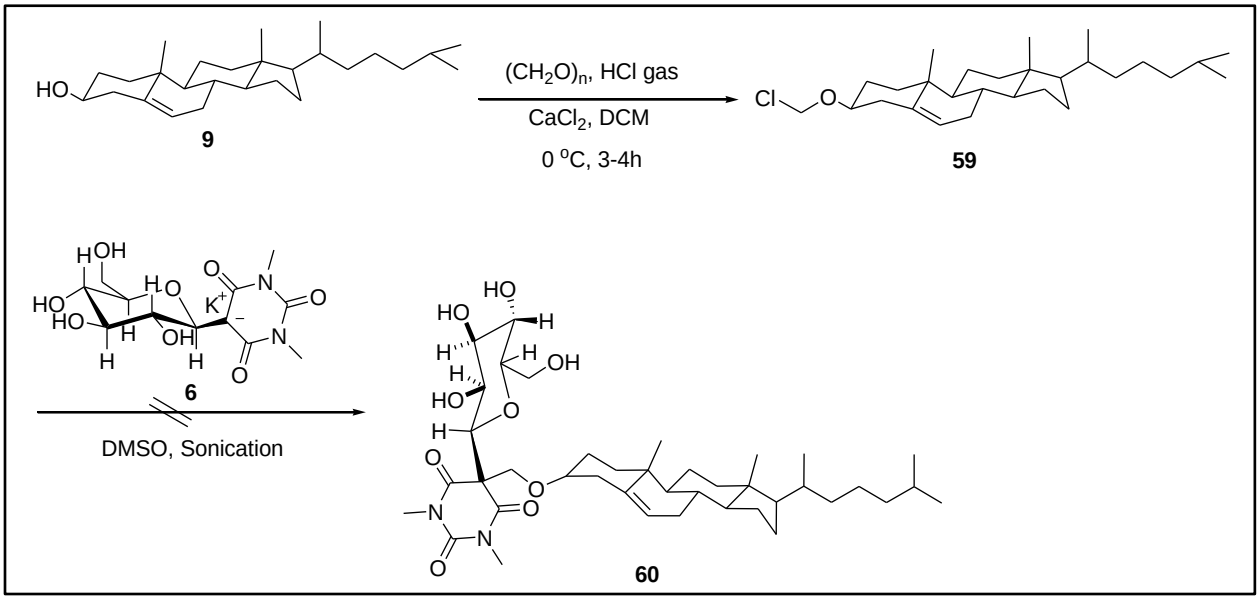
For the methodology development cholesterol was used as a model substrate. Pathway 1 involves reaction of cholesterol **9** with a suitable alkyl dihalide or dihydroxy substrate to give compound **19**, which on reaction with aromatic substrate provides compound **18**. Pathway 2

involves the attaching of aliphatic linker to the 4-hydroxytoluene **21**, which can be activated to give compound **20** having a leaving group. A further nucleophilic substitution reaction of compound **20** with cholesterol **9** would give the desired linker containing cholesterol derivative **18**. A detailed description of reaction conditions and their outcomes are discussed under results and discussions.

Results and Discussion

Cholesterol ether linked carbon spacer synthesis was attempted by introducing simple one carbon chain alkyl group by formylation method. A solution of Cholesterol **9** in dichloromethane was treated with parafomaldehyde by purging HCl gas through the solution at 0 °C (Scheme 2.13). The reaction was stirred at this temperature for 3-4 hours under dry conditions using calcium chloride pellets in the reaction mixture. It was observed that in the presence of any amount of water the reaction was reversible and goes back to the starting material. Calcium chloride pellets absorb all water formed in the reaction, thereby allowing reaction to progress in the forward direction. The reaction was continuously monitored at this stage. After successful completion of reaction product **59** was obtained as white powder in sufficient purity. Same procedure was used for cholestanol to give corresponding compound **59a**. With the success in selective conversion of 3β-hydroxyl group of cholesterol **9** to ether with retention of stereochemistry we were encouraged to explore the attachment of other ether linkers. Before progressing to the next step we tried coupling reaction of compound **59** with *C*-glycosylated barbiturate **6**. Because of limited solubility of sugar in organic solvent, DMSO was the solvent of choice for this reaction. Since heating at high temperatures has deleterious effect on sugars, the

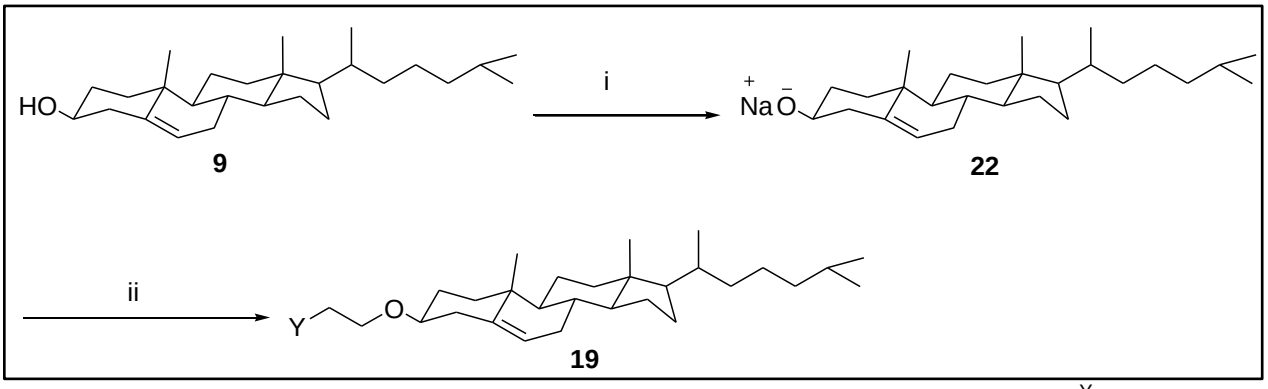
reaction has to be performed at moderate temperatures. Therefore, coupling of compound **59** to compound **6** was attempted with sonication in DMSO. Sonication was chosen as it has two fold advantages, firstly sonication enhances molecular interaction in the reaction medium of the reactants by providing maximum solubilty and secondly a moderate temperature of nearly 40 °C is appropriate for this reaction. Although the reaction did not yield the desired product even after several attempts, it helped in exploring reaction conditions for ether synthesis. Therefore, we proposed that a longer alkyl chain would be appropriate for reaction at the tertiary carboanion of **6.**



Scheme 2.13 Synthesis of 3β-Cholesteryl ether.

As per the reterosynthetic analysis (Scheme 2.12) first attempt for synthesis of long chain steroidal ether was made by following pathway 1 to develop methodology using cholesterol as the model substrate. A dry THF solution of the cholesterol **9** was treated with sodium hydride to

give the corresponding alkoxide **22**, which was reacted with various activated alkyl derivatives (Scheme 2.14).



Scheme 2.14 General scheme for synthesis of 3*β*-Cholesteryl ether,i) NaH, THF, reflux; ii) X Y where, X= Cl, Br, OTs; Y= OH, Cl, Br.

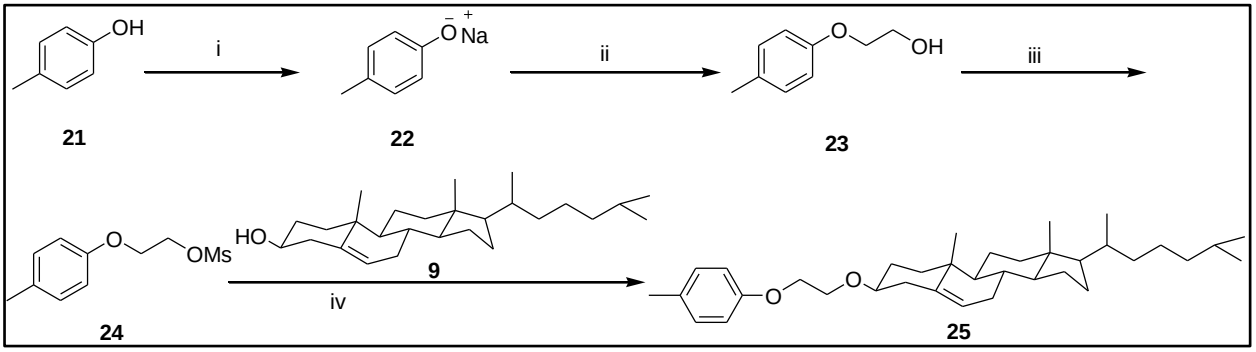
Entry	Reaction Conditions	X Y	Boiling Point (°C)
1.	Sodium hydride, THF, reflux	X=Y= Cl	84
2.	Sodium hydride, THF, reflux	X=Y= Br	133
3.	Sodium hydride, THF, reflux	X=Cl, Y=OH	131
4.	Sodium hydride, THF, reflux	X=OTs, Y= Cl	NA

Table 2.5 Reaction conditions and substrate for cholesteryl ether synthesis

The ether synthesis was attempted using a strong base like sodium hydride to abstract the hydroxyl proton to generate cholesteryl-3*β*-oxide **22** as intermediate under dry reflux conditions in THF (Scheme 2.14). One would assume that the intermediate would react with any suitable alkyl chain with an appropriate leaving group. Therfore, alkyl dihalide was chosen as a substrate. The rationale behind using dihalide as a substrate was that one halide group on the alkyl chain

would react with cholesteryl-3*β*-oxide **22**, and the second halide group would be available for reaction at the next step. When the reaction was attempted with dichloroethane (DCE) (Table 2.5) only the starting material was recovered. A more reactive substrate like dibromoethane (DBE) (table 2.5) with better leaving group (bromo) makes it more reative towards the in situ generated oxide ion. It also has a higher boiling point that would help the reagent to stay in the reaction medium longer under reflux conditions without escaping. Again only, the unreacted starting material was recovered. At this point, we decided to use alkyl substrate containing two different functional groups such as ethylene chlorohydrin that would make the alkyl substrate more stable on one terminal and reactive on the other. The presence of hydroxyl group on one terminal makes the substrate more stable at refluxing temperature by retaining it in the reaction medium via formation of hydrogen bond. When chlorohydrin was incorporated as the reagent once again only the unreacted starting substrate was recovered. It appeared that the chloro group either is not reactive enough or is being hydrolysed to hydroxyl group thereby rendering the substrate unreactive towards nucleophilic substitution by cholesteryl-3*β*-oxide **22** (Scheme 2.14). Therefore, the free hydroxyl of ethylene chlorohydrin was transformed to tosylate to provide the reagent. After attempting, the reaction under dry conditions using THF and sodium hydride as a base no desired compound was isolated except for the unreacted starting material. After several unsuccessful attempts we chose to follow pathway 2 (Scheme 2.12).

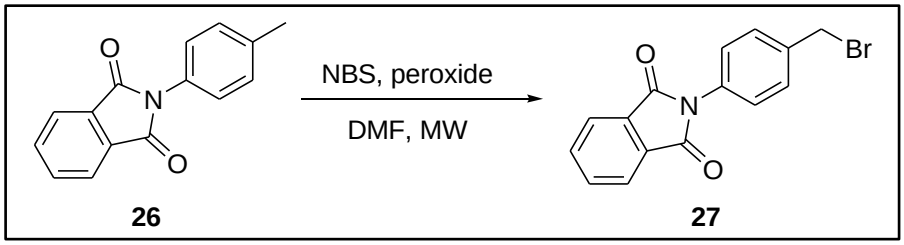
In our second attempt for synthesis of Cholesteryl-3*β*-ether derivative **25** (scheme 2.15) efforts were directed in synthesizing the activated aromatic linker **24** (Scheme 2.15).



Scheme 2.15 Cholesteryl-3β-ether derivative synthesis with *p*-cresol derivative; i) NaOH, H_2O; ii) Cl OH, MeOH- H_2O; iii) MsCl, py; iv) sodium hydride, THF, reflux.

Under basic conditions, the hydroxyl proton of *p*-cresol **21** is abstracted to give the corresponding sodium 4-methylphenolate **22**. A reaction with ethylene chlorohydrin gave compound **23.** The hydroxyl of compound **23** was converted to a mesylate leaving group by reaction with mesylate chloride under basic conditions to give compound **24.** Reaction of cholesterol **9** in presence of sodium hydride in refluxing THF with compound **24** afforded desired cholesteyl-3β-(2-(*p*-tolyloxy)ethoxy ether **25**. For reaction with *C*-glycosylate barbiturate, the methyl group in compound **25** has to be functionalized to a group that can act as a leaving group. This can be achieved by transformation to the corresponding bromo derivative **18** (Scheme 2.12).

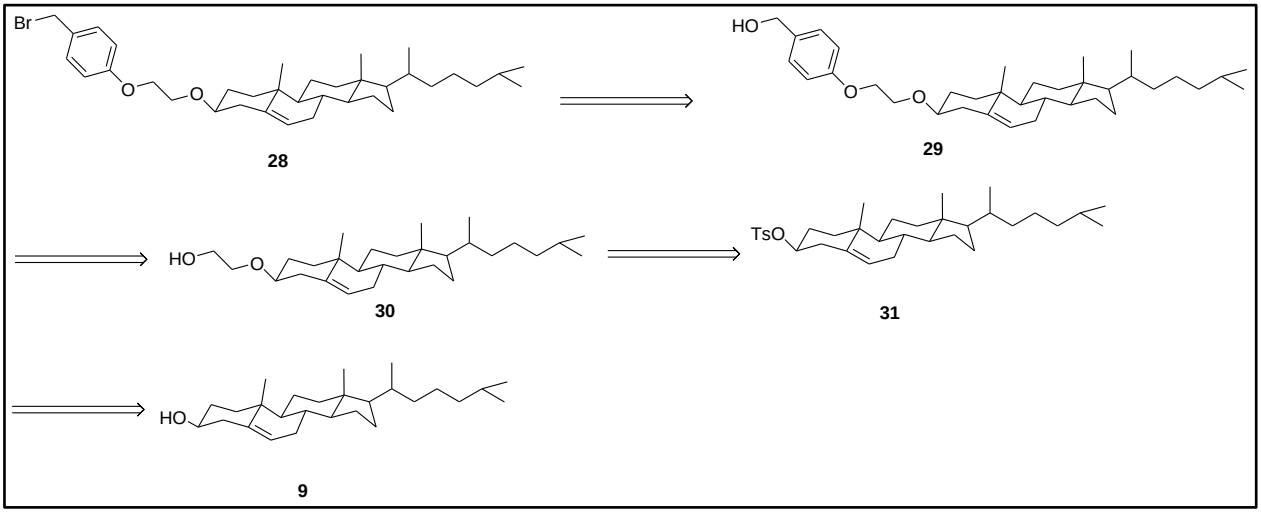
According to the literature procedure[52] bromination at the benzylic position can be achieved under microwave reaction conditions using NBS, benzoyl peroxide as shown in Scheme 2.16.



Scheme 2.16 Microwave bromination of 2-*p*-tolylisoindoline-1,3-dione[52]

The application of the same procedure to our substrate cholesteyl-3*β*-(2-(*p*-tolyloxy)ethoxy ether **25** resulted in a complex mixture of compounds that were difficult to isolate. Although the method is very successful for substrates like **26** but in case of cholesterol substrate **25** the mixture was a result of non selective mono and dibromination, under the microwave conditions, at several secondary, tertiary and allylic carbon centres in the steroid molecule. At this point, we had to reconsider our synthetic approach.

A third attempt for synthesis of ether linked carbon spacer cholesterol derivative was made. After careful analysis of the shortcomings of previous synthetic schemes and problems encountered, we decided to go a different route as represented by a reterosynthetic analysis in Scheme 2.17.

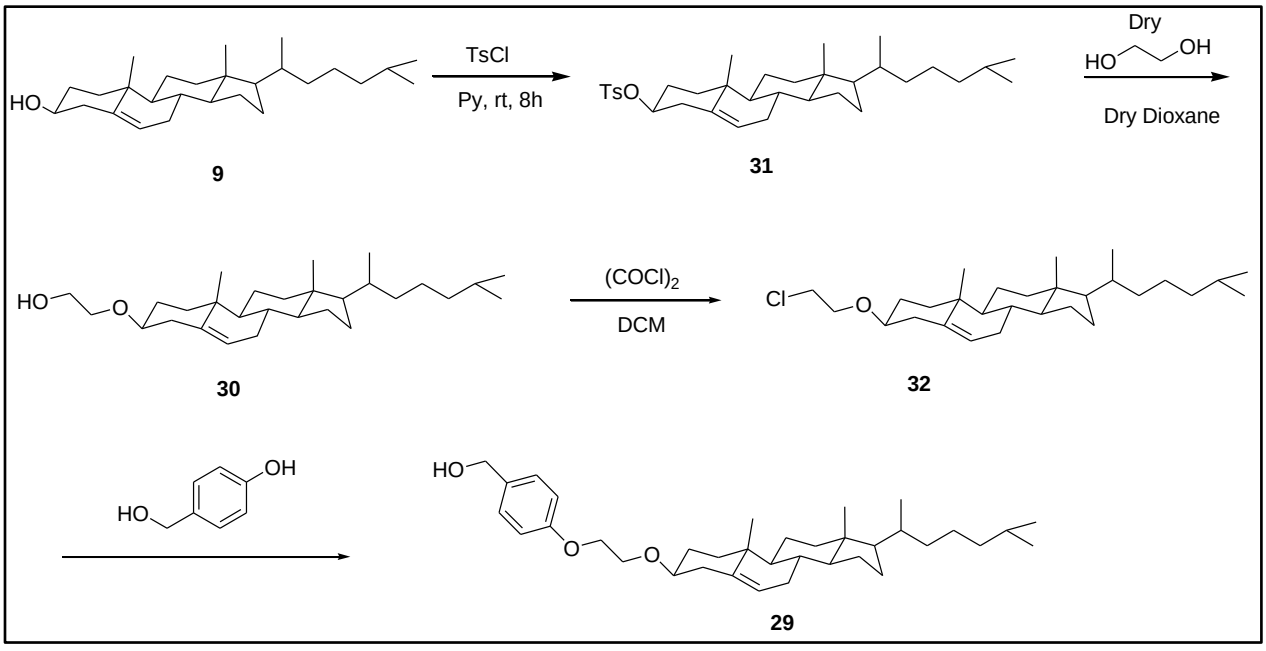


Scheme 2.17 Revised reterosynthesis analysis of cholesteryl-3*β*-ether linker

According to the reterosynthetic analysis (Scheme 2.17) cholesteryl-3*β*-(2-(4-(bromomethyl)phenoxy)ethoxy) ether **28** can be synthesized from its corresponding hydroxyl

derivative **29**. This in turn can be derived from cholesterol ether linker **30**. A cholesterol tosylate **31** on reaction with suitable alkyl derivative would afford compound **30** (Scheme 2.17).

The ether-linked carbon spacer cholesterol compound **29** and **30** (Scheme 2.18) were synthesized by modifying the methods of Bajaj[53] *et al*. To an ice-cooled solution of cholesterol **9** in pyridine, *p*-toluenesulfonyl chloride was added in fractions and stirred for 12h at room temperature. Cholest-5-en-3*β*-tosylate **31** was isolated in practical yield. Since the resulting tosylate was unstable on silica gel column, purification was carried out by recrystallization from hexane to get a free flowing white powder. It was taken to the next step without further purification. Cholest-5-en-3*β*-tosylate **31** and 7–10 equiv of appropriate diol in anhydrous dioxane were refluxed for 8 h under nitrogen atmosphere to afford Cholest-5-en-3*β*-oxyethan-2-ol **30** in 80–87% yield (Scheme 2.18).



Scheme 2.18 Synthesis of cholestery-3*β*-ether linker derivative *via* tosylate.

The compound **30** was converted to its corresponding chloro derivative **32** under dry conditions using oxalyl chloride at 0 °C. But a further attempt to attach the aromatic part by reaction with hydroxyl benzyl alcohol under reflux conditions resulted in hydrolysis of **32** to give back compound **30** instead of desired compound **29** (Scheme 2.18). Therefore, compound **30** was converted to a better leaving group like tosylate and bromo. This was subsequently reacted with hydroxyl aromatic substrate and the results are summarized in table 2.6. The reaction of compound **30** with 4-bromobenzylbromide under basic conditions using bases like sodium hydride, sodim hydroxide did not yield the desire compound **33**. Conversion of **30** to corresponding tosylate, bromo or chloro derivative was also not uselful for synthesizing compound **33**.

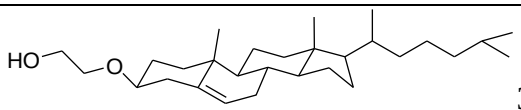
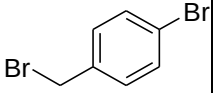
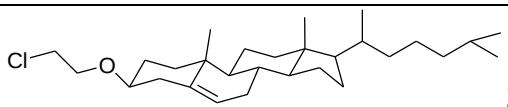
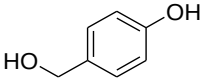
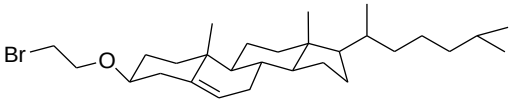
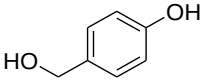
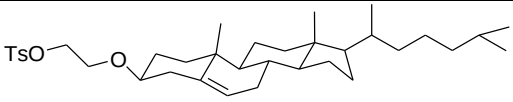
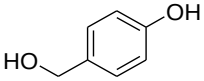
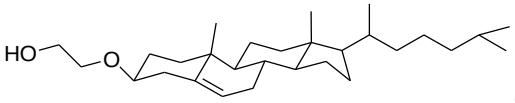
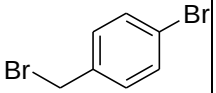
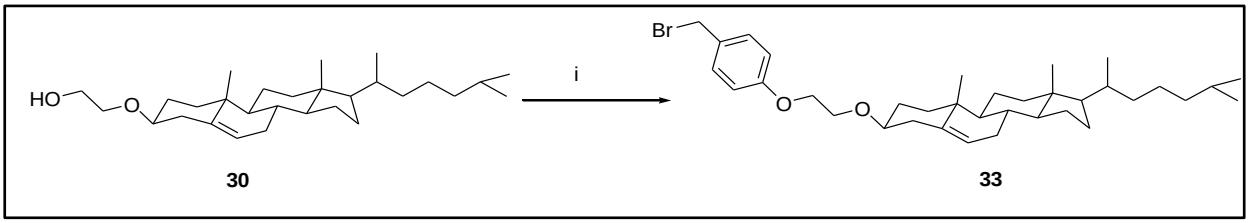
Entry	Steroid (number)	Benzyl compound	Reaction condition	Result
1.	 30		NaH, THF,	No product
2.	 32		NaOH, MeOH-H_2O,	No product
3.	 30A		NaOH, MeOH-H_2O,	No product
4.	 30B		KOH, Acetonitrile	No product
5.	 30		$Cu(acac)_2$, DMF, reflux	Product

Table 2.6 Cholesteryl-3*β*-aryl ether reaction conditions

After several attempts to synthesize compound **29**, we finally succeeded in our attempt. A reaction of compound **30** with 4-bromobenzyl bromide using $Cu(acac)_2$ under reflux conditions in DMF (Scheme 2.19) resulted in the formation of desired compound **33**.



Scheme 2.19 Synthesis of cholesterol-3β-(2-(4-(bromomethyl)phenoxy)ethoxy) ether linker; i) $Cu(acac)_2$, 4-bromobenzylbromide, DMF, reflux, 8h.

2.2.2. Synthesis of steroid linker containing carbonate, carbamate and ester.

The synthesis of barbiturate saponin requires bringing together two different substrates namely *C*-glycosylated barbiturate and steroid together in one molecule. The steroid linker plays the essential role of transporting the intact molecule to the site of action or inside the body where it can undergo hydrolysis to release two independently acting drug molecules. A good linker for this approach would be a carbonate, carbamate or ester. Using cholesterol as the model substrate the synthetic methodology was developed.

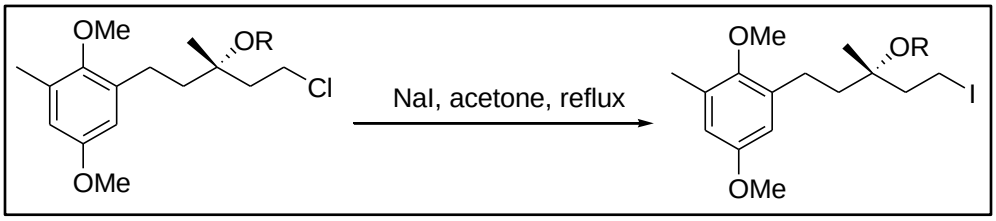
Cholesteryl chloroformate **34** was reacted with 4-hydroxybenzyl alcohol under basic reaction conditions to give Cholest-5-en-3β-yl-4-(hydroxymethyl)phenyl carbonate **35** (Scheme 2.22). Since hydroxyl by itself is not a good leaving group it has to be transformed to a better leaving group for reaction in the next step with *C*-glycosylated barbiturate **6**. We decided to transform the hydroxyl group to a chloro group by reacting Cholest-5-en-3β-yl-4-(hydroxymethyl)phenyl carbonate **35** with oxalyl chloride. This yielded Cholest-5-en-3β-yl-4-

(chloromethyl)phenyl carbonate **36** in good yield (Scheme 2.22). With the chloro derivative in hand, we attempted the coupling of steroid fragment **36** to the *C*-glycosylated barbiturate **6** (Scheme 2.23). The synthetic challenge was to find a reaction solvent for two different compounds **36** and **6** with different solubilities. Cholest-5-en-3*β*-yl-4-(chloromethyl)phenyl carbonate **36** is hydrophobic and does not dissolve in water, whereas *C*-glycosylated barbiturate **6** is hydrophilic and dissolves very well in water, DMSO or a mixture of water and DMSO at various concentrations. In addition, DMSO seemed to be a solvent of choice for this nucleophilic reaction as polar solvents favor these kinds of reactions. However, removal of DMSO and steroid solubility remains to be a challenge. After several trials with a variety of solvents like DMSO, THF, DCM, DCE, it was observed that a mixture of DMSO, water and DCM provides a suitable medium for this reaction. Cholest-5-en-3*β*-yl-4-(chloromethyl)phenyl carbonate **36** was dissolved in minimum amount of DCM and charged to a solution of *C*-glycosylated barbiturate **6** and allowed to sonicate (Scheme 2.23). The reaction was monitored continuously by ^{1}H NMR. After 3 days of continuous sonication, formation of product was evident by ^{1}H NMR. The observed rate of reaction was very slow which makes is impractical for a large-scale reaction. Therefore, it was decided to replace the chloro group with a functional group that can act as a better leaving group in the nucleophilic reaction.

Conversion of Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl carbonate **35** to corresponding tosylate **37** was achieved by reaction with *p*-toluene sulfonyl chloride under basic conditions (Scheme 2.22). Upon reacting compound **37** with *C*-glycosylated barbiturate **6** in a similar way for the chloro compound **36** (Scheme 2.23), it was observed that there was no conversion to product even after three days of continuous sonication instead some amount of tosylate hydrolysis was observed to give compound **35** (Table 2.7). At the end of fifth day some

product formation was observed in the ^{1}H NMR. Clearly, tosylate as a leaving group was not a solution for our problem. We needed a group more reactive than chloro and stable to hydrolysis. At this point it was decided to use the iodo derivative of Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl carbonate **35**.

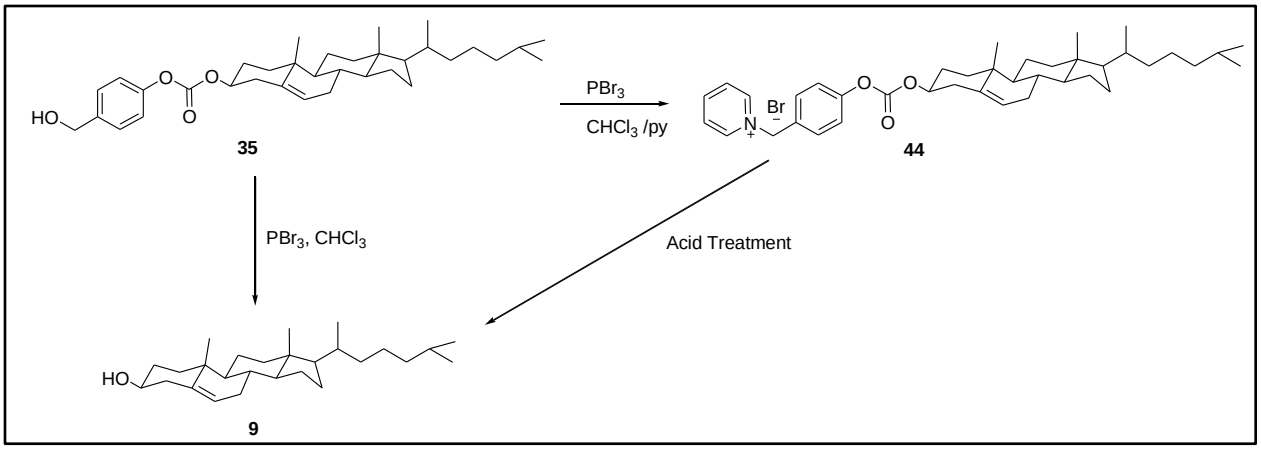
Several literature synthetic procedures indicate the usefulness of Finkelstein reaction for conversion of chloro derivatives to the corresponding iodo compounds. A representative example demonstrating application of this reaction has been reported by Maloney *et al.*[54] in their synthesis of *δ*-trans-Tocotrienoloic Acid (Scheme 2.20). Finkelstein reaction involves the treatment of a primary alkyl halide or pseudohalide with an alkali metal halide (e.g. KF, KI) that leads to replacement of the halogen via an S_N2 Reaction.



Scheme 2.20 An example of finkelstein reaction[54]

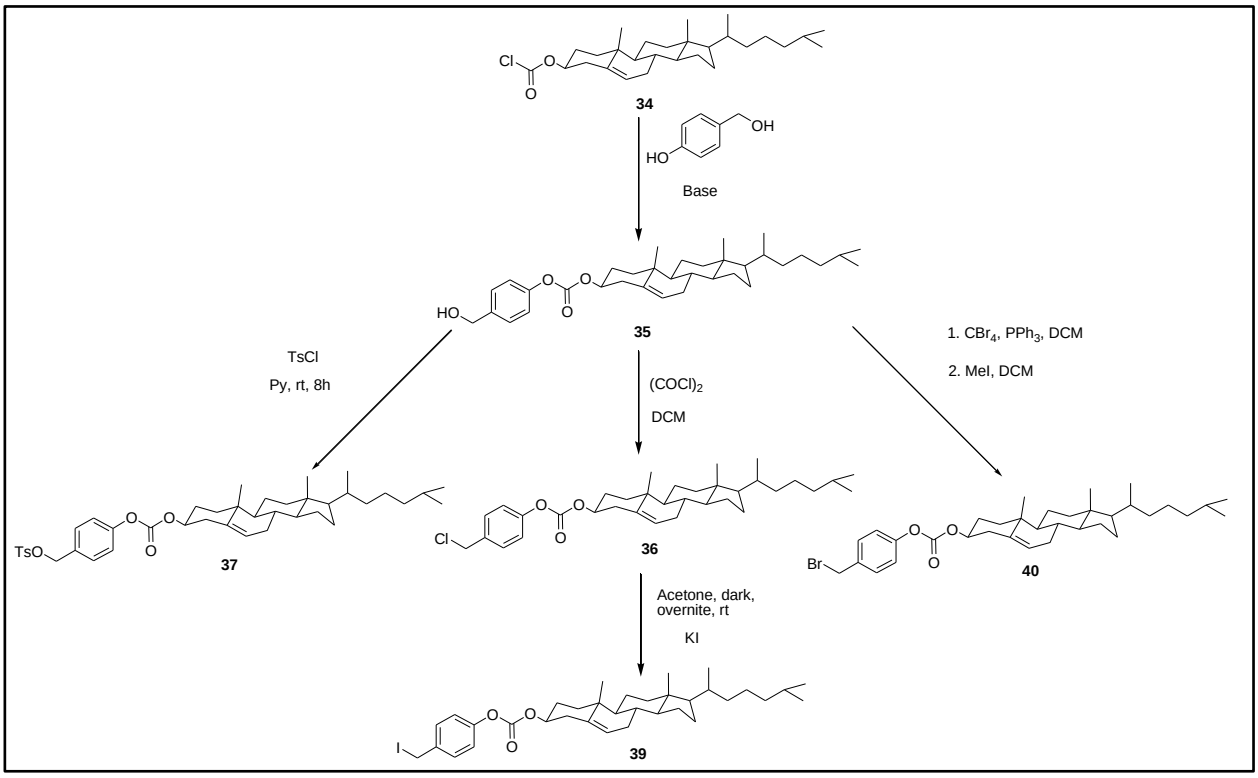
Encouraged by the reported synthetic application of Finkelstein reaction we tried it on our substrate Cholest-5-en-3*β*-yl-4-(chloromethyl)phenyl carbonate **36**. Conversion to Cholest-5-en-3*β*-yl-4-(iodomethyl)phenyl carbonate **39** was achieved via S_N2 reaction using potassium iodide in dark. Although the conversion to iodo derivative was successful but its application on a large scale was a challenge due to a very careful handling of iodo compound, reactions can only be carried out in dark and susceptible oxidations prompted us to look for better alternatives.

Another useful halide would be bromide. Since we had carbonate in our reaction substrate, we had to carefully select the reaction conditions to achieve formation of desired molecule. Use of PBr_3 alone led to the hydrolysis of Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl carbonate **35** and cholesterol **9** was recovered from the reaction (Scheme 2.21).



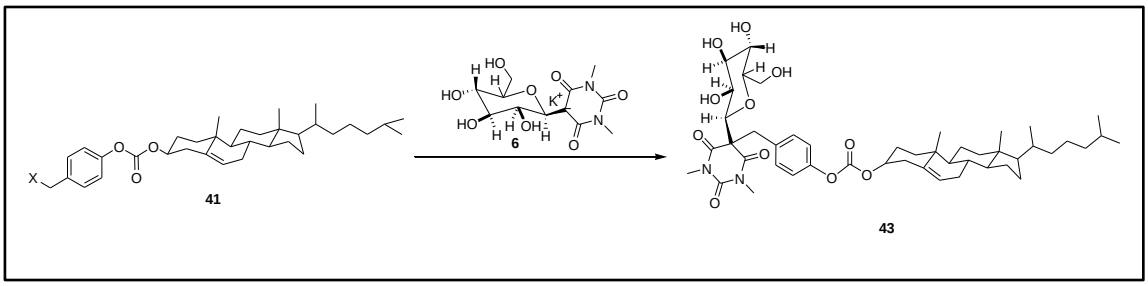
Scheme 2.21 Bromiation of Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl carbonate **35** using PBr_3

In order to avoid hydrolysis by *in situ* generation of hydrogen bromide in the phosphorus tribromide reaction an obvious choice was to do the reaction under basic conditions. When pyridine was employed in the reaction to maintain a favorable pH (above 7) it was observed that the bromination proceeded to form a pyridinium salt **44**. Any attempt to remove pyridine led to the hydrolysis of carbonate to give cholesterol **9**. The second attempt was made using carbon tetrabromide and triphenyl phosphine[55] (Scheme 2.22).



Scheme 2.22 Synthesis of Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl carbonate and its derivatization

The bromination[55] was efficiently carried out by using carbon tetrabromide in presence of triphenyl phosphine. Excess of triphenyl phosphine was removed by complexation with iodomethane and column chromatography. After successfully synthesizing Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl carbonate **40** it was employed in synthesis of saponin derivative **43** (Scheme 2.23).



Scheme 2.23 Reaction of derivatized cholest-5-en-3*β*-carbonate derivatives **41** with *C*-glycosylated barbiturate **42**

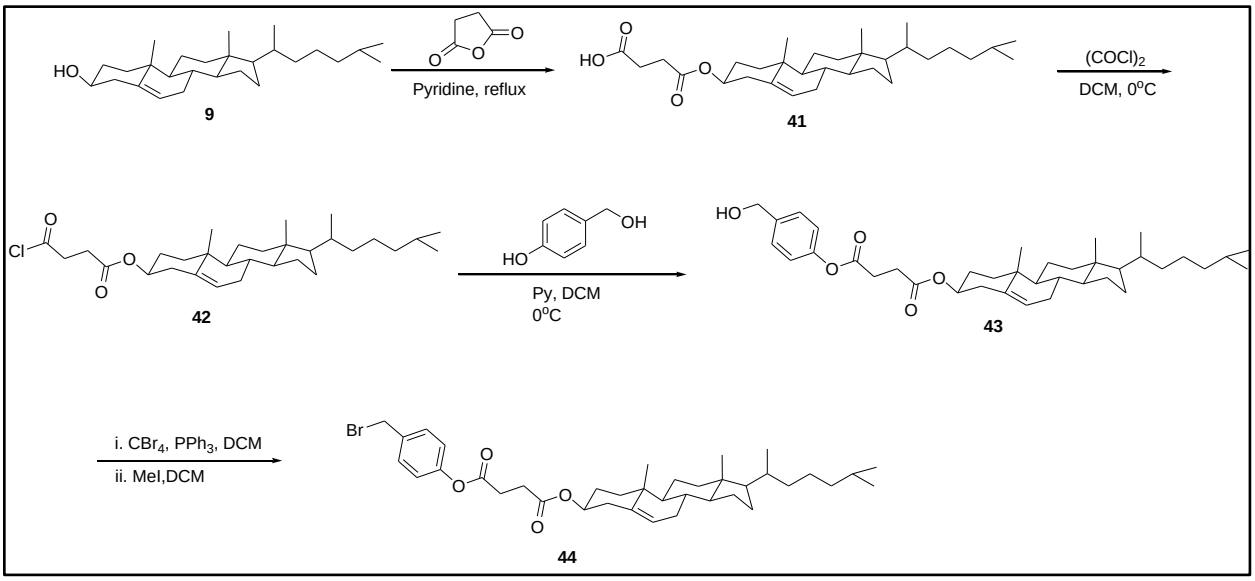
An outcome of various leaving groups in substrate **41** (Scheme 2.23) is summarized in table 2.7.

Entry	Compound 41 X=	Reaction Condition	Reaction time	Result (% conversion)
1.	-Cl	DMSO, sonication	3 days	Product (30%)
2.	-OTs	DMSO, sonication	3 days	Product + Hydrolysis (20%)
3.	-I	DMSO, sonication	NA	No Product, oxidation
4.	-Br	DMSO, sonication	15 min	Product (98%)

Table 2.7 Summary of leaving group reactivity in cholest-5-en-3β-carbonate derivatives

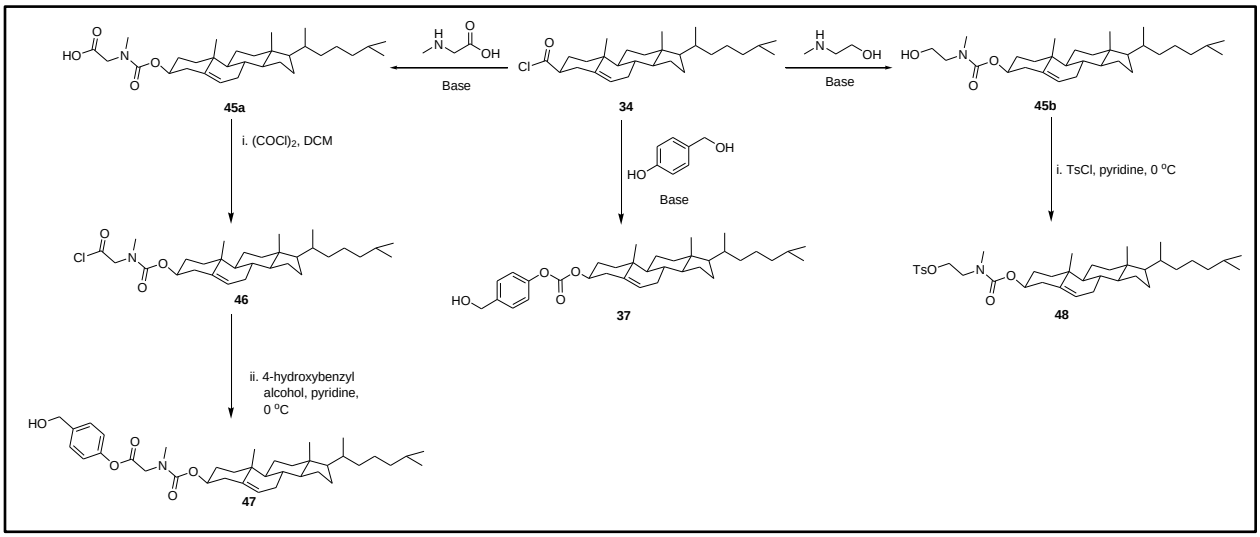
The ester-linked carbon spacer derivatives of cholesterol were synthesized using succinic anhydride. A solution of cholesterol **9** in pyridine was reacted with succinic anhydride for 8h at room temperature to afford Cholest-5-en-3β-yloxy-4-oxobutanoic acid **41** in quantitative yield (Scheme 2.24). The compound was purified by column chromatography using hexane-ethylacetate. In the next step, reaction with oxalyl chloride gave the corresponding acid chloride **42**. Since acid is unstable on the silica column it was taken to the next step without further purification. To an ice-cold solution of 4-hydroxybenzyl alcohol compound **42** was added. The reaction mixture was aalowed to stir at 0 °C for 6h to afford compound **43**, which was purified by column chromatography. Bromination was done at the next step where compound **43** was

treated with carbon tetrabromide and triphenylphosphine to give compound **44**. Removal of any excess triphenyl phosphine was achieved by complexation with iodomethane. Further purification of the compound was done by column chromatography using hexane-DCM to get the pure product **44**.



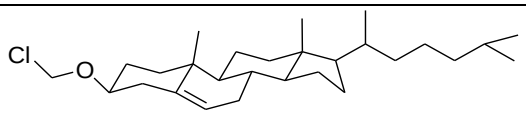
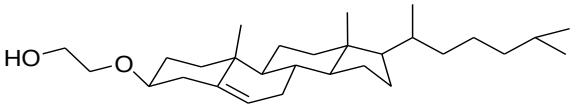
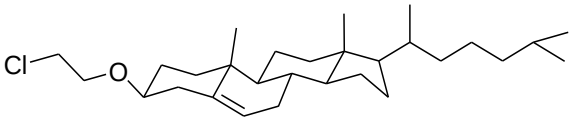
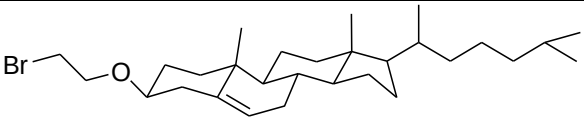
Scheme 2.24. Synthesis of Cholest-5-en-3β-yl-4-(bromomethyl)phenyl succinate

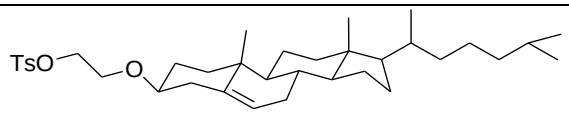
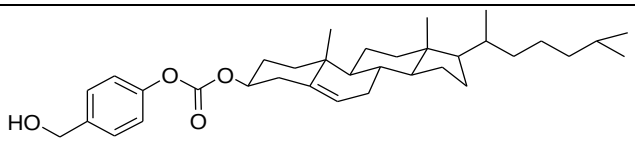
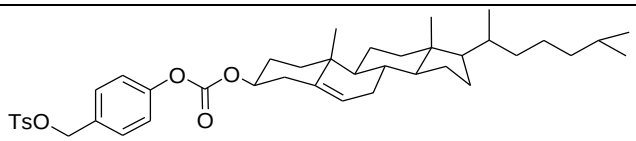
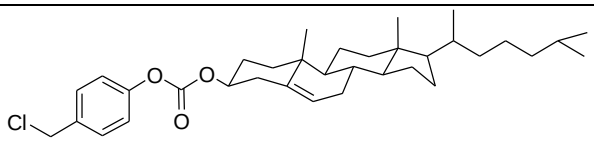
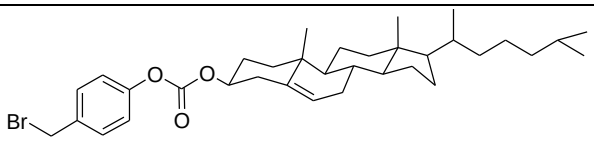
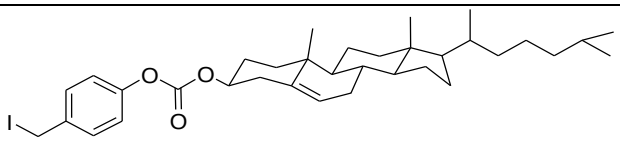
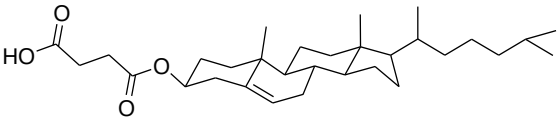
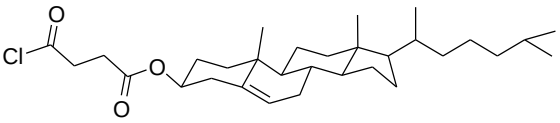
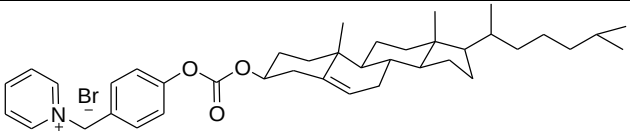
Similar to the synthesis of Cholest-5-en-3β-yl-4-(hydroxymethyl)phenyl carbonate **35** compound **45a** and **45b** can be synthesized. To a solution of cholesteryl chloroformate **34** was added *N*-methyl glycine (Sarcosine) and 2-(methylamino)ethanol to afford compound **45a** and **45b** respectively (Scheme 2.24).



Scheme 2.25 Synthesis of carbonate and carbamate linked cholesterol derivatives.

A variety of ether, ester, carbonate and carbamate linker were successfully synthesized as summarized in Table 2.8.

Entry	Linker	Spacer	Steroid derivative
1.	Ether	aliphatic	
2.	Ether	aliphatic	
3.	Ether	aliphatic	
4.	Ether	aliphatic	

5.	Ether	aliphatic	
6.	Carbonate	aromatic	
7.	Carbonate	aromatic	
8.	Carbonate	aromatic	
9.	Carbonate	aromatic	
10.	Carbonate	aromatic	
11.	Ester	aliphatic	
12.	Ester	aliphatic	
13.	Carbonate	aromatic	

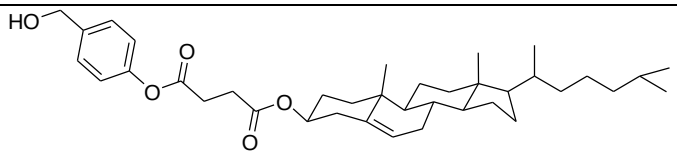
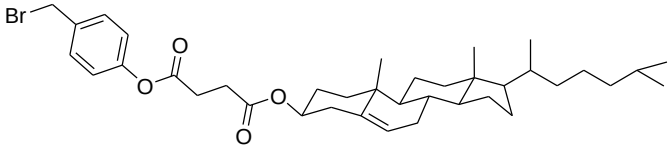
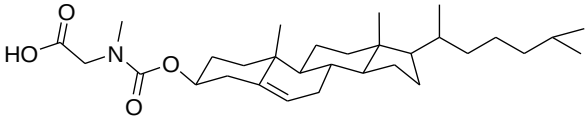
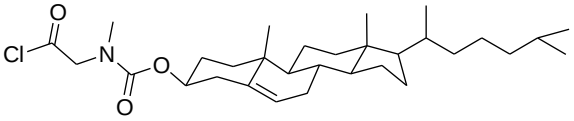
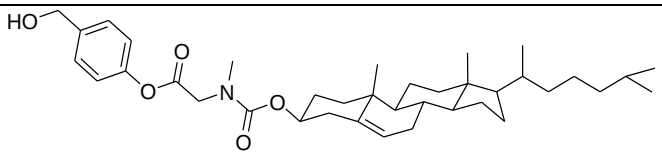
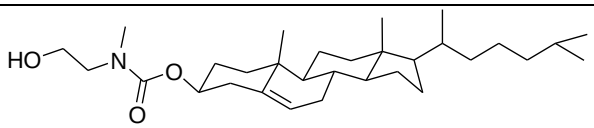
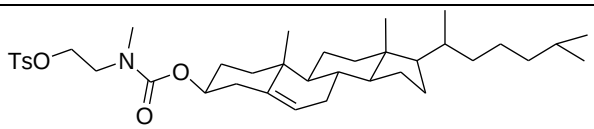
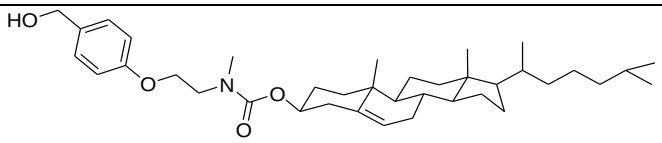
14.	Ester	aromatic	
15.	Ester	aromatic	
16.	Carbamate	aliphatic	
17.	Carbamate	aliphatic	
18.	Carbamate	aromatic	
19.	Carbamate	aliphatic	
20.	Carbamate	aliphatic	
21.	Carbamate	aromatic	

Table 2.8 Synthesized ester, ether, carbonate and carbamate linked carbon spacer cholesterol derivative

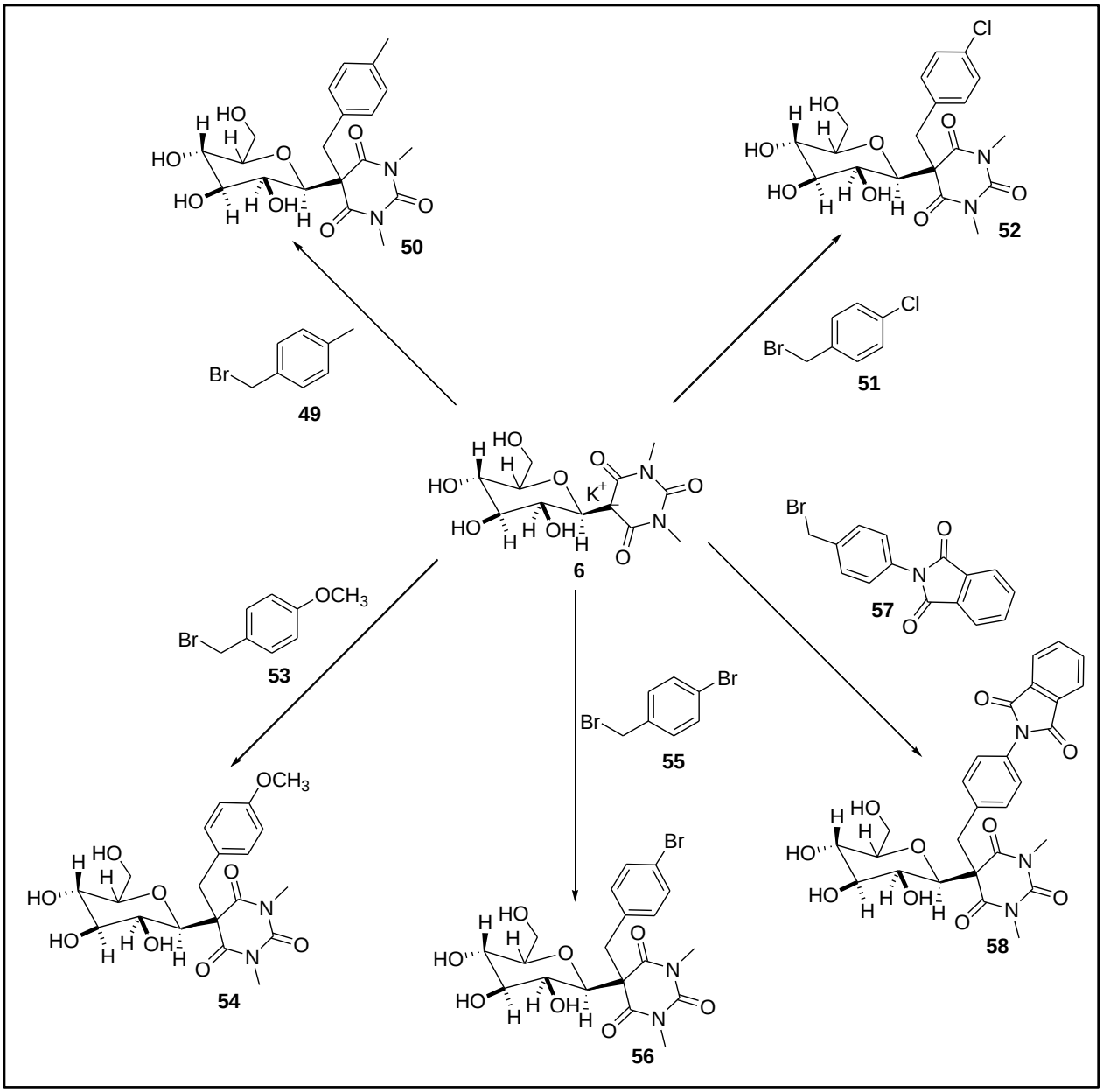
2.3. Synthesis of Novel Saponin Barbiturates as Antifungal Compounds

After successfully accomplishing the synthesis of two important fragments of the target molecule, the next step is to join them together into one molecule. One of the classic challenges in the field of carbohydrate chemistry is that carbohydrate-based compounds and conjugates are notoriously hard to synthesize. Not only the chemical synthesis, but the purification and isolation of these compounds containing fragments with diverse and completely opposite physical properties presents a serious challenge. At the same time, we desire to develop a synthetic procedure that can be successfully used at a commercial scale. Another challenge is to simplify the reaction strategy for its application at commercial scale.

Sugars are hydrophilic, so is the *C*-glycosylated barbiturate fragmement of our molecule. It dissolves in polar solvents like water, water-methanol and water-DMSO at various concentrations. On the other hand, the steroid linker derivatives are highly lipophilic and dissolve in common organic solvents like ethyl acetate, dichloromethane, chloroform, THF and to a lesser extent in DMSO. On coming in contact with water, they form micelle and do not dissolve in water at any temperature. At the same time heating at high temperatures is deleterious to the sugar containing fragment of the molecule i.e. *C*-glycosylated barbiturate salts. Therefore, the reaction has to be carried out at an ambient temperature so as not to decompose saccharide either in the substrate or in the formed product. The biggest challenge was to bring the hydrophilic *C*-glycosylated barbiturate derivatives and the hydrophobic steroid derivative in a common reaction medium. After several hit and trial runs, it was found that the reaction goes well in polar solvent like DMSO. Replacement with any other solvent did not give the desired product either because of the solubility issues or insufficient interaction between two substrates. Also, carrying out the reaction in DMSO is not a problem but its removal after the completion of

the reaction and monitoring reaction progress can become difficult. Because of susceptibility of sugar to degrade at high temperature the final compound cannot be heated to reamove the solvent at high temperature. High temperature also changes the stereochemistry of the molecule, which is not a desired outcome.

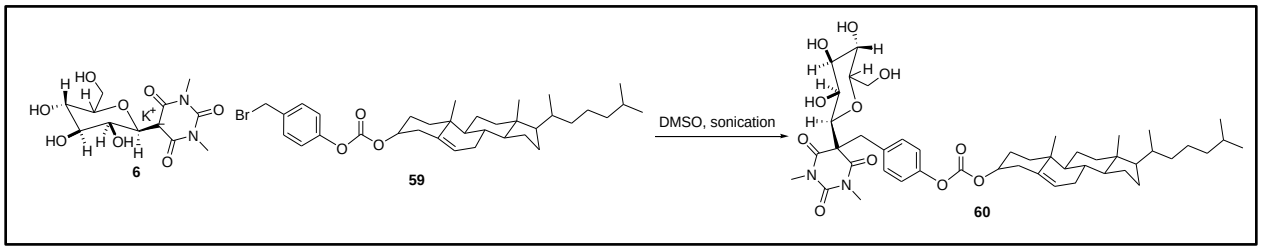
Therefore, to find out the best reaction conditions it was required to run some test reactions to optimize the reaction conditions. The model reactions were run using benzyl bromide derivatives to explore reaction conditions. After trying solubility in various solvents, it was soon found out that DMSO is an excellent solvent for carrying out this type of reaction. In this reaction, the *C*-glycosylated barbiturate derivatives acted as a nucleophile and the benzylic carbon with the leaving group undergoes substitution at benzylic position.



Scheme 2.26 Benzyl bromide derivative coupling to *C*-glycosylated-*N*,*N*-dimethyl barbiturate.

A variety of benzyl bromide substrates like 4-methylbenzyl bromide **49**, 4-chlorobenzyl bromide **51**, 4-methoxybenzylbromide **53**, 4-bromobenzyl bromide **55**, 2-(4-(bromomethyl)phenyl)isoindoline-1,3-dione **57** were reacted with *C*-glycosylate-*N*,*N*-

dimethylbarbituric acid **6** in DMSO under sonication reaction conditions to give the corresponding compounds as shown in Scheme 2.24. The resulting products were isolated using cosolvent precipitation. For instance, the isolation of compound **50** was achieved by using water and DCM. Water dissolved any unreacted sugar, potassium chloride. Addition of DCM precipitated the product and dissolved any unreacted 4-methylbenzylbromide **49**. Similar procedures were successfully employed for purification of all other compounds. When required a further purification was achieved by silica gel chromatography using DCM:Ethylacetate. In the same fashion *C*-glysolyated barbiturate **6** was used for coupling to steroid linker derivatives **59**. *C*-glycosylated-*N,N*-dimethyl barbiturate **6** was dissolved in DMSO and to it was added the corresponding solution of steroid linker derivative **59** dissolved in a minimum amount of DCM and soniacted for 1-2h to get the desired product **60.** It was isolated by cosolvent precipitation and a silica gel column chromatography using Hexane-EtOAc. The overall reaction for different substrates is outlined in Scheme 2.27.



Scheme 2.27 Representative example of conjugation of *C*-glycosylated barbiturate with the steroid linker segment

A series of steroidal saponin derivatives were synthesized using *N,N*-dimethyl barbituric acid and barbituric acid. A representative example is shown in Scheme 2.27 wherein compound **6** is reacted with compound **59** under sonication using DMSO as a solvent to give compound **60**. Several of these derivatives are exemplified in table 2.9 with detailed structure and linkages like, ether, carbonate, carbamate and ester.

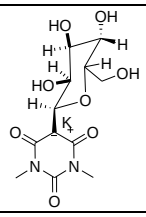
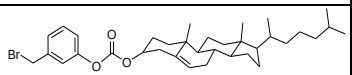
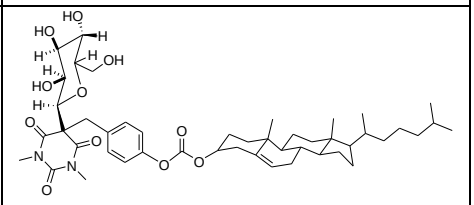
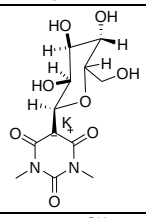
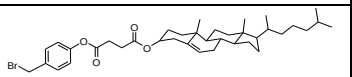
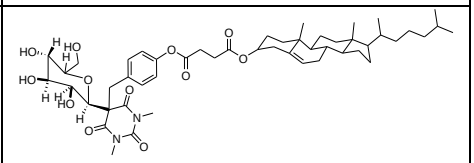
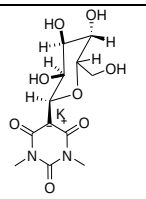
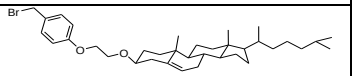
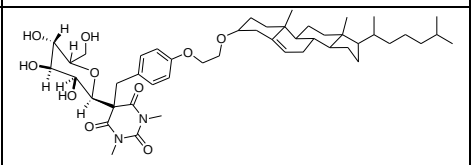
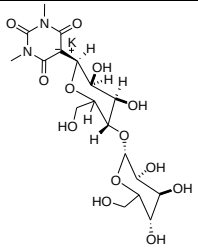
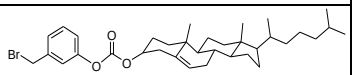
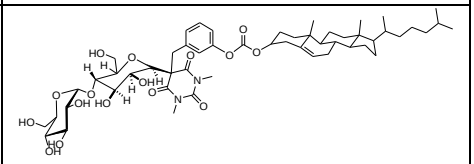
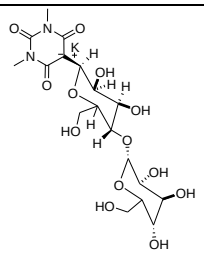
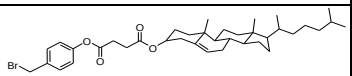
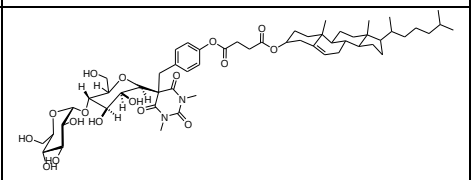
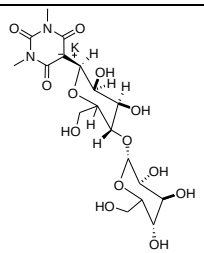
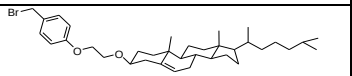
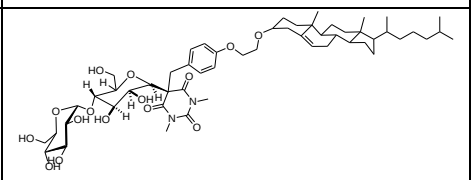
Compound	*C*-glycosylated barbiturate	Steroid derivative	Product
60			
61			
62			
63			
64			
65			

Table 2.9 Novel *N,N*-dimethyl barbiturate steroidal saponins derivatives

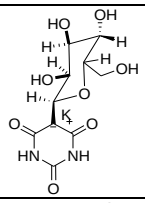
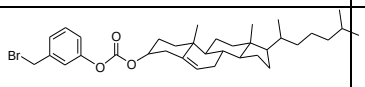
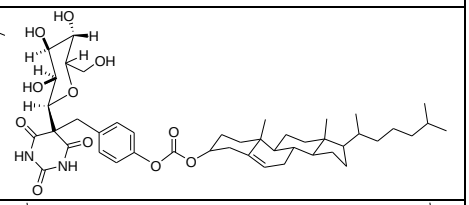
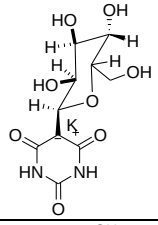
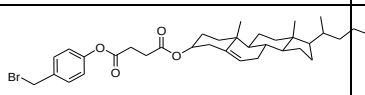
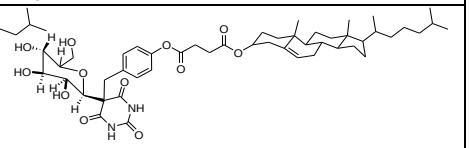
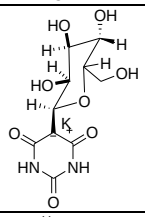
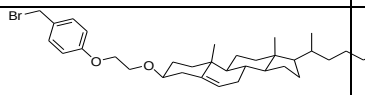
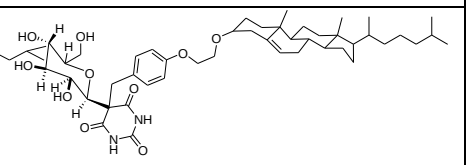
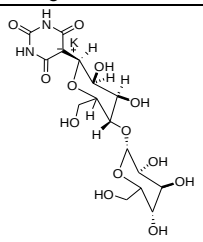
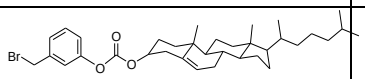
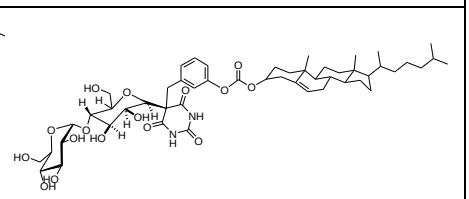
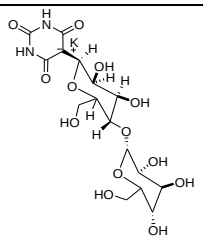
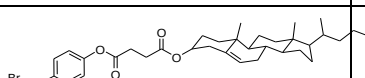
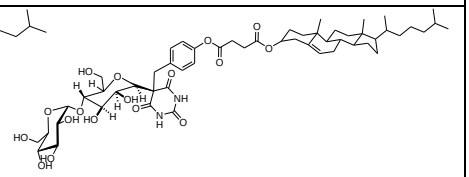
Compound	*C*-glycosylated barbiturate	Steroid derivative	Product
66			
67			
68			
69			
70			

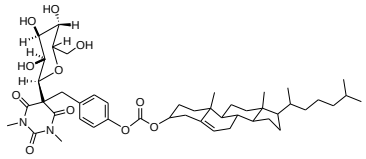
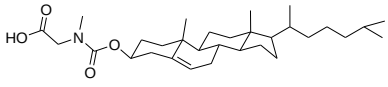
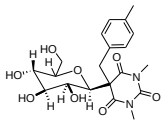
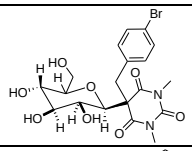
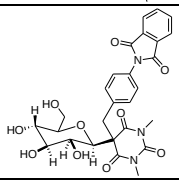
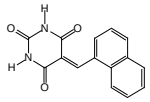
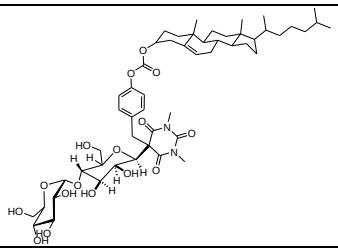
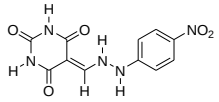
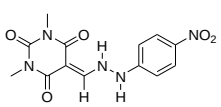
Table 2.9 Novel barbiturate steroidal saponins derivatives

2.4 Antifungal studies

The antifungal activity of the synthesized 3*β*-functionalized cholesterol, barbiturate and novel barbiturate saponins were evaluated *in vitro* using four species of fungi, including *Candida albicans, Cryptococcus neoformans, Candida glabrata,* and the filamentous fungus *Aspergillus fumigatus.* The results of these screenings are summarized in Table 2.10. Of the several barbituric acid derivatives tested for their antifungal activity 1,3-dimethyl-5-((2-(4-nitrophenyl)hydrazinyl)methylene) barbiturate **73** and its demethylated analogue **74** (Table 2.10) showed significant antifungal activity (>50% inhibition at the minimal concentration) against *Candida albicans, and Candida glabrata,* with *C. glabrata* being the most sensitive at the lowest concentration (2-4μg/mL). It was speculated that the presence or absence of methyl group on nitrogen of barbiturate has no significant effect on the antifungal activity since both compounds **73** and **74** showed same range of fungal activity. Barbiturate derivative **72** with basic naphthalene showed activity only against *Candida albicans*.

The intermediates of the steroidal segment compound **45a** tested against *Candida albicans* and *Candida glabrata* demonstrated comparable fungal activity to compounds **73** and **74**. Significant antifungal activity (>50% inhibition at the minimal concentration) against two fungal strains at concentration of 2-4μg/mL was observed. This encouraged synthesizing novel barbiturate steroidal saponin derivatives to search for more potent antifugal agents, and study the structure activity relationship of this class of compounds. Hydrolysis of the carbonate linker of **60** (yielding compound **50** and **9**) and **63** resulted in the total loss of antifungal activity against all tested species. The combination of the hydrolysis and insignificant binding led to the total loss of antifungal activity. Finally, it is speculated that the inversion of stereochemistry in compound **50**, **56** and **58** must have resulted in the total loss of antifungal activity.

Table 2.10. Antifungal activities of novel barbiturate saponins and analogs (MIC in μg/mL).

Compound	Structure	MIC_{50} (μg/mL)[a,f]			
		C. albicans [b]	*C. glabrata* [c]	*C. neoformans* [d]	*A. fumigatus* [e]
60		NC*	NC*	NC*	NC*
45a		2 μg/mL	2 μg/mL	NC*	NC*
50		NC*	NC*	NC*	NC*
56		NC*	NC*	NC*	NC*
58		NC*	NC*	NC*	NC*
72		125 μg/mL	NC*	NC*	NC*
63		NC*	NC*	NC*	NC*
73		4 μg/mL	2 μg/mL	NC*	NC*
74		4 μg/mL	4 μg/mL	NC*	NC*

[a]MIC values are reported only for compounds displaying 1) a prominent decrease in turbidity by visual comparison to the control wells containing no antifungal and 2) a >50% reduction in fungal growth compared to controls containing no antifungal, as measured spectroscopically by absorption at 530 nm. [b]ATCC no. 10231;[c]ATCC no. 48435;[d]ATCC no. 36556;[e]ATCC no. 16424 [f]All values were determined after incubation at 35°C for 48 h. *denotes compounds that had 1) a slight reduction in turbidity to no change and 2) had less than a 25% reduction in growth compared to controls, as measured spectroscopically by absorption at 530 nm.

2.5 Conclusions

In pursuit to synthesize novel barbiturate steroidal saponins, several new agents were synthesized. In absence of any reported literature, procedure for synthesis of this class of compounds synthetic methodology was developed using cholesterol as the model substrate. A variety of *C*-glycosylated *N,N*-dimethyl barbiturate and barbiturate salts were successfully synthesized employing mono and disaccharides in one pot synthesis under mildly basic conditions. Methodology was established for 3*β*-steroid linker chain for ether, ester, carbonate and carbamate.

Of the aliphatic and aromatic steroid linker, it was established that an aromatic linker is required for coupling to the *C*-glycosylated barbiturates. The reactivity of the halides towards the nucleophilic substitution in the steroidal substrates was studied. Out of the three halogenated derivatives, iodo was the most reactive making it nearly impossible to do reaction at the next step and mostly progressed with oxidation. Chloro derivatives reacted at a very slow and unacceptable rate. Best results were obtained with bromo derivatives as they offered better reactivity and yield. A good methodology has been successfully developed to functionalize 3*β* position of cholesterol using various linkers. The synthesized cholesterol derivatives were linked to the *C*-glycosylated barbiturates in an efficient manner with high yileds.

The antifungal activity of the synthesized 3*β*-functionalized cholesterol, barbiturate and novel barbiturate saponins were evaluated *in vitro.* The antifungal activity 1,3-dimethyl-5-((2-(4-nitrophenyl)hydrazinyl)methylene) barbiturate **73** and its demethylated analogue **74** were in the range of 2-4μg/mL and indicated that the presence or absence of methyl group on nitrogen of barbiturate may not have significant effect on the fungal activity of these agents. Compound **45a** had comparable antifungal activity to compounds **73** and **74**. Hydrolysis of the carbonate

linker of **60** and **63** rendered these agents with no significant antifungal activity against all tested species. It is speculated that stereochemistry and binding affinity of compound **50**, **56** and **58** interfered with their activity and resulted in the total loss of antifungal activity.

2. 6. Experimental

General Experimental

All reagents and solvents were purchased from Sigma-Aldrich and were analytical grade. Thin-layer chromatographic analysis (TLC) was performed using silica gel on glass plates and was detected under ultraviolet (UV) light and using PMA, Ninhydrin, $KMnO_4$ solution. Column chromatography was performed using silica gel porosity 60 Å, partial size 40-75 mm from Sorbent Technologies. The ^{1}H and ^{13}C NMR spectra were run on Varian 400 MHz Unity in $CDCl_3$, D_2O, CD_3OD or DMSO-d_6 as solvents and internal standards.

Synthesis of monosaccharide barbiturate derivatives, General procedure: A solution of dimethyl barbituric acid (15g, 0.1 mol) in 70 mL of water, saccharide (18g, 0.1 mol) in 70 mL and sodium carbonate were mixed together and heated at 80 °C for 5h. Reaction mixture was concentrated to one fifth of its original volume by evaporation, poured over methanol (1L) and stirred for 1h. White solid precipitate was isolated by filtration to give the pure product

Synthesis of potassium 1,3-dimethyl-2,4,6-trioxo-5-(β-D-glucopyranose)-hexahydropyrimidin-5-ide (6): A solution of *N,N*-dimethyl barbituric acid (15g, 0.1 mol) in 70 mL of water, glucose (18g, 0.1 mol) in 70 mL and sodium carbonate were mixed together and heated at 80 °C for 5h. Reaction mixture was concentrated to one fifth of its original volume by evaporation, poured over methanol (1L) and stirred for 1h. White solid precipitate was isolated by filtration to give the pure product in a yield of 90%. ^{1}H NMR (400 MHz, D_2O): δ 4.36 (d, J = 9.9, 1H), 4.21 (t, J = 9.4, 1H), 3.72 – 3.60 (m, 2H), 3.48 – 3.40 (m, 1H), 3.35 (t, J = 9.0, 1H),

3.29 (d, J = 9.5, 1H) and 3.03 (s, 6H) ppm. ^{13}C NMR (400 MHz, D_2O) δ 154.34, 95.58, 86.22, 79.76, 78.43, 76.21, 69.66, 69.48, 60.87 and 27.85 ppm.

Synthesis of sodium 1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-galactopyranose)-hexahydropyrimidin-5-ide (5): Galactose (10 g, 55.5 mmol) and 1,3-dimethylbarbituric acid 5 (10 g, 64.0 mmol) were dissolved in water (100 mL) and sodium carbonate (5.60 g) was added to it. The mixture was then heated at 80 °C for 5h. The solvent was reduced to one fifth of its volume by evaporation and the solution was precipitated by pouring into rapidly stirred methanol (500 mL). The white solid was collected by filtration. ^{1}H NMR (400 MHz, D_2O): δ 3.20 (s, 6H), 3.58 (dd, J 3.5, 9.7, 1H), 3.66 (br s, 3H), 3.93 (d, J= 3.5, 1H), 4.40 (d, J=9.7, 1H) and 4.50 (t, J= 9.7, 1H)ppm. ^{13}C NMR (D_2O): δ 28.1, 61.4, 67.8, 70.2, 75.5, 77.0, 79.1, 86.7, 154.7 and 164.7 ppm.

Synthesis of potassium 2,4,6-trioxo-5-(*β*-D-glucopyranose)-hexahydropyrimidin-5-ide (5a): A solution of barbituric acid (15g, 0.1 mol) in 70 mL of water, glucose (18g, 0.1 mol) in 70 mL and sodium carbonate were mixed together and heated at 80 °C for 5h. Reaction mixture was concentrated to one fifth of its original volume by evaporation, poured over methanol (1L) and stirred for 1h. White solid precipitate was isolated by filtration to give the pure product in a yield of 90%. ^{1}H NMR (400 MHz, D_2O): δ 4.36 (d, J = 9.9, 1H), 4.21 (t, J = 9.4, 1H), 3.72 – 3.60 (m, 2H), 3.48 – 3.40 (m, 1H), 3.35 (t, J = 9.0, 1H) and 3.29 (d, J = 9.5, 1H) ppm. ^{13}C NMR (400 MHz, D_2O) δ 154.34, 95.58, 86.22, 79.76, 78.43, 76.21, 69.66, 69.48 and 60.87 ppm.

Synthesis of disaccharide barbiturate derivatives, General procedure: A solution of dimethyl barbituric acid (15g, 0.1 mol) in 70 mL of water, saccahride (18g, 0.1 mol) in 70 mL and sodium carbonate (0.05 mol) were mixed together and heated at 80 °C for 5h. Reaction mixture was concentrated to one fifth of its original volume by evaporation, poured over methanol (1L) and stirred for 1h. White solid precipitate was isolated by filtration to give the pure product

Synthesis of sodium 1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5-ide (7): A solution of dimethyl barbituric acid (5g, 0.03 mol) in 20 mL of water, maltose (11.4g, 0.03 mol) in 20 mL and sodium carbonate (0.05 mol) were mixed together and heated at 80 °C for 5h. Reaction mixture was concentrated to one fifth of its original volume by evaporation, poured over methanol (1L) and stirred for 1h. White solid precipitate was isolated by filtration to give the pure product in a yield of 90%. ^{1}H NMR (400 MHz, D_2O): δ 5.39 (d, J = 1.8Hz, 1H), 4.5(d, J = 1.8Hz, 1H), 4.38 (t, J =10.8 Hz, 1H), 3.48- 3.85 (m, 9H), 3.38 (t, J =10.8 Hz, 1H) and 3.2 (s, 6H) ppm. ^{13}C NMR (D_2O): δ 154.44, 115.58, 99.92, 94.98, 86.20, 78.78, 78.29, 77.10, 76.00, 73.08, 72.70, 71.98, 69.46, 69.43, 60.75, 60.54, 48.96 and 27.73 ppm.

Synthesis of sodium 2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5-ide (7a): A solution of *N*,*N*-dimethyl barbituric acid (5g, 0.03 mol) in 20 mL of water, maltose (11.4g, 0.03 mol) in 20 mL and sodium carbonate were mixed together and heated at 80 °C for 5h. Reaction mixture was concentrated to one fifth of its original volume by evaporation, poured over methanol (1L) and stirred for 1h. White solid precipitate was isolated by filtration to give the pure product in a yield of 90%. ^{1}H NMR (400 MHz, D_2O): δ 5.39 (d, J = 1.8Hz, 1H), 4.5(d, J = 1.8Hz, 1H), 4.38

(t, *J* =10.8 Hz, 1H), 3.48- 3.85 (m, 9H) and 3.38 (t, *J* =10.8 Hz, 1H) ppm. ^{13}C NMR (D_2O): δ 154.44, 115.58, 99.92, 94.98, 86.20, 78.78, 78.29, 77.10, 76.00, 73.08, 72.70, 71.98, 69.46, 69.43, 60.75, 60.54 and 48.96 ppm.

Pottasium 1,3-dimethyl-5-(*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl)barbiturate (8)

Compound **8** (125 mg, 82%) was obtained as a white powdery solid ^{1}H NMR (400 MHz, D_2O): δ 3.16 (s, 6H), 3.28 (dd, *J*= 8.0, 8.7, 1H), 3.38 (t, *J* =9.3, 1H,), 3.46 (m, 2H), 3.52 (m, 1H), 3.57 (t, *J*= 9.5, 1H), 3.69 (dd, *J* =5.5, 12.1, 1H), 3.75 (dd, *J* =9.5, 9.8, 1H), 3.82 (br s, 2H), 3.87 (br d, *J* =12.1, 1H), 4.33 (dd, *J*= 9.5, 10.1,1H), 4.49 (d, *J*= 10.1, 1H) and 4.52 (d, *J* =8.0, 1H) ppm. ^{13}C NMR (D_2O): δ 165.5, 154.7, 102.9, 86.4, 78.9, 78.8, 77.0, 76.4, 76.2, 75.9, 73.6, 69.9, 69.7, 61.0, 60.5 and 28.0 ppm.

Pottasium 2,4,6-trioxo-5-(*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl) hexahydropyrimidin-5-ide (8a): Compound **8a** (100 mg, 80%) was obtained as a white powdery solid ^{1}H NMR (400 MHz, D_2O): δ 3.26 (dd, *J* =8.0, 8.7, 1H), 3.36 (t, *J*= 9.3, 1H,), 3.47 (m, 2H), 3.55 (m, 1H), 3.52 (t, *J*= 9.5, 1H), 3.71 (dd, *J*= 5.5, 12.1, 1H), 3.74 (dd, *J*= 9.5, 9.8, 1H), 3.85 (br s, 2H), 3.87 (br d, *J*= 12.1, 1H), 4.36 (dd, *J*= 9.5, 10.1,1H), 4.48 (d, *J* =10.1, 1H) and 4.55 (d, *J*= 8.0, 1H) ppm. ^{13}C NMR (D_2O): δ 165.3, 155.1, 103.2, 86.4, 78.9, 78.8, 77.0, 76.4, 76.2, 75.9, 73.6, 69.9, 69.7, 61.0 and 60.5 ppm.

Chloromethylation of steroid, General procedure: HCl gas was purged through a solution of desired steroid at 0 °C for 1-3h. The reaction mixture was kept dry by using calcium chloride pellets. Reaction was monitored by TLC. After the completion of reaction solvent was

evaporated and purified by column chromatography to give the desired steroid in almost 80-90% yield.

Synthesis of 1-(chloromethoxy)-3-methylbutane: To a solution of isoamyl alcohol (8.85 gm, 0.1 mmol) and paraformaldehyde (3 gm, 0.1 mmol) in 1, 2 dichloroethane (20 mL) was added Calcium chloride (10 gm). The reaction mixture was cooled to 0 °C and HCl gas purged through it for 2h. Solvent was removed under reduced pressure to give colorless liquid (13.20 mL. yield 95 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.51 (s, 2H), 3.89 – 3.63 (m, 2H), 1.70 (m, J = 15.6, 1H), 1.52 (m, J = 15.6, 2H), 0.91 (s, 3H) and 0.91 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): δ 66.7, 48.2, 23.2, 24.6 and 39.8 ppm.

Synthesis of Cholest-5-en-3*β*-oxymethyl chloride (59): A suspension of cholesterol (1 gm, 2.58 mmol) and paraformaldehyde (125 mg, 3.87 mmol) in dry DCM (5 mL) with calcium chloride (1 gm) was cooled to 0 °C. HCl gas (generated by carefully dropping sulfuric acid over sodium chloride) was purged through the solution for about 2h to give a clear solution. Reaction was continuously monitored by TLC. Solvent was evaporated under reduced pressure to give a white solid. Purified by column chromatography to give 1gm of white solid (88% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.45 (s, 2H), 5.36 (s, 1H), 3.42 (m, 1H) and 1.98-0.84 (m, 43H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): δ 141.8, 122.9, 80.3, 58.5, 56.9, 51.2, 46.1, 44.0, 39.7, 38.9, 37.5, 37.2, 36.3, 35.8, 31.7, 30.3, 29.98, 28.67, 28.12, 27.65, 27.32, 24.61, 23.22, 22.71, 20.71 and 19.45 ppm.

Synthesis of Cholestan-3*β*-oxymethyl chloride (59a): Synthesis was carried out in the same manner as for compound **59** to get 1gm of white solid (89% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.64 (s, 2H), 3.42 (m, 1H), 2.0-0.84 (m, 43H) and 0.63 (s, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): δ 79.3, 56.7, 56.5, 52.2, 46.1, 46.01, 41.0, 40.20, 39.7, 36.9, 36.3, 35.8, 34.5, 32.7, 32.0, 28.91, 28.38, 28.17, 27.65, 24.63, 24.22, 23.20, 20.71, 19.42, 16.22 and 16.14 ppm.

Synthesis of Cholest-5-en-3*β*-tosylate (31): A solution of cholesterol (10.0 g, 0.026 mol) in dry pyridine (10 mL) was cooled to 0 °C and tosyl chloride (7.4 g, 0.04 mol) was added in portions. The reaction mixture was then allowed to stir at 0 °C for 6 h. Diluted mixture with dichloromethane (50 mL) and was washed with 5% HCl (2 X 50 mL), water (50 mL), and brine (50 mL). Seperated the organic layer and dried over anhydrous Na_2SO_4. Dichloromethane was evaporated to give a white powder (13.2 gm, yield 93.2%). Mp: 132 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.80-7.77 (d, J = 7.8, 2H), 7.33-7.25 (d, J = 7.8, 2H), 5.27 (d, J = 4.5, 1H), 4.39 (m, 1H), 2.44 (s, 3H), 2.39 (m, 2H), 2.12-0.84 (m, 39H) and 0.64 (s, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): δ 144.61, 139.03, 134.93, 129.98, 127.82, 123.72, 82.51, 56.83, 56.31, 50.09, 42.47, 39.85, 39.72, 39.09, 37.08, 36.52, 36.39, 35.97, 32.04, 31.94, 28.82, 28.41, 28.19, 24.45, 24.03, 23.05, 22.80, 21.82, 21.19, 19.34, 18.93 and 12.04 ppm.

Synthesis of Cholest-5-en-3*β*-oxyethan-2-ol (30): Dry ethylene glycol (10 g, 0.16 mol) was added to a solution of Cholest-5-en-3β-tosylate (3.5 g, 6.5 mmol) in dry dioxane. The resulting mixture was refluxed for 4h under N_2. The reaction mixture cooled to room temperature and solvent removed under reduced pressure. The resulting residue was dissolved in DCM (50 mL) and washed with water (2 X50 mL), $NaHCO_3$ (2 X50 mL), brine (50 mL) and dried over sodium

sulfate. Solvent removed under reduced pressure. The compound was further purified by column chromatography over silica gel using a mixture of petroleum ether and ethyl acetate. Pure product was obtained as white solid (6.5 gm, yield 81.6%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.35 (s, 1H), 3.72 (t, *J* = 11.3, 2H), 3.64 – 3.54 (t, *J* = 11.3, 2H), 3.19 (m, 1H), 2.37 (d, *J* = 8.7, 1H), 2.22 (d, *J* = 11.1, 1H), 2.08 – 0.79 (m, 39H) and 0.68 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 140.8, 121.8, 70.5, 61.6, 58.3, 56.5, 50.8, 44.0, 39.6, 39.9, 37.7, 37.2, 36.1, 35.8, 31.9, 30.3, 29.9, 29.5, 28.1, 27.7, 27.3, 24.6, 23.2, 22.7, 20.7, 19.4 and 11.84 ppm.

Synthesis of Cholest-5-en-3*β*-oxyethyl-2-tosylate (30B): Solution of cholest-5-en-3*β* -oxyethan-2-ol 30 (4.3 g, 0.01 mol) in dry pyridine (15mL) was cooled to 0 °C, followed by addition of *p*-toluenesulfonyl chloride (3.5 g, 0.018 mol) in portions. The reaction mixture was allowed to stir at 0 °C for 6 h. To the reaction mixture dichloromethane (50mL) was added, and with 5% HCl (2 X 25 mL), water (2 X 25 mL), and brine (25mL). Dried the DCM layer over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The resulting product was purified by recrystallization using acetonitrile to give white powder (5.3 gm, yield= 91%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.80–7.83 (d, *J* = 8.1 Hz, 2H), 7.33–7.36 (d, *J* = 8.1 Hz, 2H), 5.33 (d, *J* = 4.5 Hz, 1H), 4.16–4.18 (t, *J* = 4.5Hz, 2H), 3.64–3.67 (t, *J* = 4.5Hz, 2H), 3.04–3.10 (m, 1H), 2.48 (s, 3H), 0.85–2.26 (m, 41H) and 0.68(s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 144.51, 138.87, 134.79, 129.86, 127.71, 123.84, 82.08, 60.78, 56.73, 56.17, 55.74, 49.94, 42.09, 39.66, 39.28, 38.71, 36.75, 36.41, 36.06, 35.55, 31.94, 31.56, 28.50, 28.06, 27.86, 24.09, 23.62, 22.93, 22.67, 21.72, 19.02, 18.51 and 11.86 ppm.

Synthesis of Cholest-5-en-3*β*-oxyethyl-2-chloride (32): A solution of Cholest-5-en-3β-oxyethan-2-ol (2.2gm, 5.1 mmol) in DCM (10 mL) was cooled to 0 °C, followed by addition of oxalyl chloride (0.97 gm, 7.65 mmol) and a few drops of DMF. The reaction was stirred at this temperature for 6h. Reaction was continuously monitored by TLC. Solvent evaporated under reduced pressure to get a white solid in almost quantitative yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.40 (d, J = 4.5 Hz, 1H), 5.35 (s, 2H), 4.49 (m, 2H), 3.79(m, 2H), 3.2 (m, 1H), 0.83–2.39 (m, 41H) and 0.65 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 140.1, 122.2, 80.6, 67.7, 65.8, 65.4, 64.7, 58.3, 56.5, 52.5, 50.8, 44.0, 39.9, 39.7, 37.8, 37.2, 36.1, 35.8, 31.9, 30.4, 29.6, 28.2, 27.7, 27.3, 24.7, 23.2, 22.7, 20.7, 19.1 and 11.6 ppm.

Synthesis of Cholest-5-en-3*β*-oxyethyl-2-oxy-benzyl alcohol (29): To a DMF (25 mL) solution of Cholest-5-en-3β-oxyethan-2-ol 30 (100 mg, 0.232 mmol) and 4-bromobenzyl bromide (57 mg, 0.232 mmol) was added $Cu(acac)_2$ (60 mg, 0.232 mmol) in portions. The reaction was allowed to reflux for 8 h. The crude reaction mixture was diluted with DCM (50 mL) and washed with water (2 X25 mL), brine (25 mL). The organic layer was dried over anhydrous sodium sulfate. Purification was achieved by silica column using hexane-DCM. A yellowish product weighing 41 mg (yield = 30%) was obtained. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.08 (d, J = 8.2 Hz, 2H), 6.9 (d, J = 8.2 Hz, 2H), 5.39 (d, J = 4.5 Hz, 1H), 4.80 (s, 2H), 4.29 (t, J = 4.5Hz, 2H), 3.82 (t, J = 4.5Hz, 2H), 2.89 (m, 1H) and 2.26–0.85 (m, 44H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 155.2, 141.2, 132.9, 128.42, 122.1, 115.65, 84.01, 69.89, 68.02, 58.32, 56.25, 50.80, 44.01, 40.01, 39.65, 37.76, 36.01, 36.0, 31.91, 30.13, 28.99, 28.12, 26.99, 27.35, 24.6, 23.22, 22.7, 20.17 and 19.5 ppm.

Synthesis of Cholest-5-en-3*β*-oxyethyl-2-bromide (33): To a solution of Cholest-5-en-3*β*-oxyethan-2-ol (2.2gm, 5.1 mmol) in DCM (10 mL) was added carbon tetrabromide (4.2 gm, 2.5 mmol) and triphenyl phosphine (1.1 gm, 2.5 mmol). The resulting solution was allowed to stir at room temperature for 4h. Reaction was monitored by TLC. After completion of reaction, the reaction mixture was charged with MeI and allowed to stir for 2h at room temperature. Washed reaction mixture with water (2 X 10 mL), Brine (10 mL). Organic layer dried over anhydrous sodium sulfate. Compound purified by column to give yellowish solid (2.1 gm, yield= 84%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.39 (d, 1H, J = 4.5 Hz), 3.8 (t, J = 10.5 Hz, 2H), 3.42 (t, J = 10.5 Hz, 3H), 3.2 (m, 1H), 0.83–2.38 (m, 41H) and 0.65 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 140.1, 122.2, 80.6, 67.7, 65.8, 65.4, 64.7, 58.3, 56.5, 52.5, 50.8, 44.0, 39.9, 39.7, 37.8, 37.2, 36.1, 35.8, 31.9, 30.4, 29.6, 28.2, 27.7, 27.3, 24.7, 23.2, 22.7, 20.7, 19.1 and 11.6 ppm.

Synthesis of Cholest-5-en-3*β*-yloxy-4-oxobutanoic acid (41): To a solution of Cholesterol (5 gm, 0.013 mol) in dry pyridine (15 mL), succinic anhydride (1.3 gm, 0.013 mol) was added and allowed to stir at room temperature for 8h. The reaction mixture was diluted with DCM (50 mL) and washed with water. The organic layer was washed with 5% HCl (2 X 50 mL) and brine (50 mL), dried over sodium sulfate. The solvent was evaporated under reduced pressure to give the crude mixture which was purified by column chromatography using hexane- DCM. Pure product was obtained as white solid (4.35 gm, yield=90%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.36 (d, J = 4.5 Hz, 1H), 4.6 (m, 1H), 2.6 (t, J = 10.2 Hz, 2H), 2.7 (t, J = 10.2 Hz, 2H), 2.32 (d, J = 4.5 Hz, 2H), 0.83–2.05 (m, 39H) and 0.65 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 176.98, 172.4, 141.02, 121.78, 74.22, 58.23, 56.65, 50.76, 44.09, 43.00, 40.01, 38.51, 37.5, 37.23, 36.22, 35.90, 32.01, 30.10, 29.88, 29.90, 28.45, 28.10, 27.77, 27.35, 24.69, 23.32, 22.70, 20.71 and 19.4 ppm.

Synthesis of Cholest-5-en-3*β*-yloxy-4-oxobutanoyl chloride (42): To an ice cold solution of Cholest-5-en-3β-yloxy-4-oxobutanoic acid 41 (2.5 gm, 4.2 mmol) in DCM (50 mL) was added oxalyl chloride (0.63gm, 5.04 mmol) with few drops of DMF and allowed to stir under nitrogen. Reaction was stirred at 0 °C for 4 hrs and solvent removed to give the product as white solid (2.6 gm) in quantitative yields which was taken to the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.41 (d, J = 4.5 Hz, 1H), 4.04 (m, 1H), 2.6 (t, J = 10.2 Hz, 2H), 2.4 (t, J = 10.2 Hz, 2H), 2.32 (d, J = 4.5 Hz, 2H) and 0.83–2.15 (m, 40H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 176.98, 172.4, 141.02, 121.78, 74.22, 58.23, 56.65, 50.76, 44.09, 43.00, 40.01, 38.51, 37.5, 37.23, 36.22, 35.90, 32.01, 30.10, 29.88, 29.90, 28.45, 28.10, 27.77, 27.35, 24.69, 23.32, 22.70, 20.71 and 19.4 ppm.

Synthesis of Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl succinate (43): To an ice cold solution of Cholest-5-en-3*β*-yloxy-4-oxobutanoyl chloride (2.5 gm, 4.2 mmol) in dry pyridine (10 mL) was added 4-hydroxybenzyl alcohol (0.52gm, 4.2 mmol) in portions and allowed to stir at 0 °C for 2h. Diluted the crude reaction mixture with DCM (50 mL) and washed with water. The organic layer was washed with 5% HCl (2 X 50 mL) and brine (50 mL), dried over sodium sulfate. The solvent was evaporated under reduced pressure to give the crude mixture which was purified by column chromatography using hexane- DCM. Pure product was obtained as white solid (4.35 gm, yield=90%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.22 (d, J = 9.1 Hz, 2H), 6.81 (d, J = 9.1 Hz, 2H), 5.38 (bs, 1H), 5.03(s, 2H), 4.51-4.58 (m, 1H), 2.87 (m, 2H), 2.61 (m, 2H), 2.28 (d, J = 9.1 Hz, 2H), 0.85–2.26 (m, 41H) and 0.68(s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 174.2, 173.1, 150.12, 141.2, 137.89, 127.7, 121.78, 74.2, 67.2, 58.00, 56.05, 50.09, 44.12, 39.89, 38.54,

37.42, 37.23, 35.89, 36.10, 31.89, 30.21, 29.89, 29.12, 28.45, 28.12, 27.83, 26.89, 24.6, 23.22, 22.71, 20.69 and 19.45 ppm.

Synthesis of Cholest-5-en-3*β*-yl-4-(bromomethyl)phenyl succinate (44): Bromination was carried out same way as for compound **33**. 100mg of compound was obtained as yellowish powder (yield =89%) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.39 (d, J = 10.5 Hz, 2H), 7.09 (d, J = 10.5 Hz, 2H), 5.38 (bs, 1H), 5.18 (s, 2H), 4.63-4.76 (m, 1H), 2.9 (t, J = 12.8 Hz, 2H), 2.79 (t, J = 12.8 Hz, 2H), 2.38 (, J = 10.5 Hz, 2H), 0.85–2.26 (m, 41H) and 0.68(s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 173.2, 172.89, 151.51, 141.2, 134.89, 129.7, 121.78, 121.42, 74.2, 58.03, 56.05, 50.09, 44.01, 39.89, 38.54, 37.42, 36.23, 35.89, 34.10, 31.89, 30.21, 29.89, 29.42, 29.15, 28.42, 28.12, 27.77, 27.33, 24.61, 23.22, 22.71, 20.69 and 19.45 ppm.

Synthesis of Cholest-5-en-3*β*-yl-4-(hydroxymethyl)phenyl carbonate (36): To a solution of cholesteryl chloroformate (1.5 gm, 3.33mmol) in THF was added 4-hydroxy-benzyl alcohol (414 mg, 3.33 mmol) and KOH (467mg, 8.35 mmol) in water (50 mL). The mixture was allowed to stir at room temperature for 30 minutes. THF was evaporated under reduced pressure and the pH was adjusted to approximately 6. The product was extracted in DCM. The organic layer was dried over anhydrous sodium sulfate. Solvent evaporated under reduced pressure to give a white solid crude product. Further purification was done by crystallization from methanol to give 1.2 gm (yield= 66%) of white solid powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.38-7.36 (d, J = 4.0, 2H), 7.18-7.15 (d, J = 4.0, 2H), 5.42 (s, 1H), 4.68 (s, 2H), 4.57 (dt, J = 11.0, 5.6, 1H), 2.47 (d, J = 8.7, 2H), 2.07 – 0.78 (m, 40H) and 0.68 (d, J = 4.0, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 151.48, 150.45, 139.36, 139.33, 129.89, 128.30, 123.45, 121.44, 79.13, 64.92, 56.88, 56.32,

50.18, 42.52, 39.90, 39.72, 38.14, 37.02, 36.75, 36.38, 36, 32.11, 32.03, 28.438, 28.22, 27.84, 24.48, 24.03, 23.03, 22.77, 21.25, 19.5, 19 and 12.1 ppm.

Synthesis of Cholest-5-en-3*β*-yl-(4-(chloromethyl) phenyl) carbonate (38): To a solution of Cholest-5-en-3*β*-yl-4-(hydroxymethyl) phenyl carbonate (100mg, 0.19 mmol) in DCM (10mL) was added oxalyl chloride (0.02 mL, 0.28 mmol) drop wise at 0 °C in presence of a few drops of DMF. Reaction was stirred at 0 °C for 4h. Solvent was removed under reduced pressure to get the pure product as a white powder (100 mg, yield=100%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.45-7.41 (d, J = 8.3, 2H), 7.25-7.21 (d, J = 8.3, 2H), 5.48 (s, 1H), 5.38 (s, 2H), 4.6 (m, 1H), 2.51 (d, J = 8.7, 2H), 2.17 – 0.88 (m, 40H) and 0.68 (d, J = 4.0, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 153.19, 149.89, 133.92, 131.04, 130.16, 122.57, 121.69, 115.61, 109.95, 79.25, 56.89, 56.33, 50.18, 42.52, 39.91, 39.72, 38.12, 37.02, 36.76, 36.39, 36.00, 32.79, 32.12, 32.04, 28.44, 28.23, 27.83, 24.49, 24.03, 23.04, 22.78, 21.25, 19.49, 18.92 and 12.09 ppm.

Synthesis of Cholest-5-en-3*β*-yl-4-(bromomethyl)phenyl carbonate (40): Bromination was done in the same way as for compound 33. The product was obtained as yellowish powder (118 mg, yield=82%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.41-7.39 (d, J = 5.3, 2H), 7.17-7.15 (d, J = 5.3, 2H), 5.43 (s, 1H), 4.68 (m, 1H), 4.48 (s, 2H), 2.51 (d, J = 8.7, 2H), 2.10 – 0.82 (m, 40H) and 0.68 (d, J = 4.0, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 153.18, 149.24, 139.32, 130.40, 130.14, 123.53, 121.69, 115.61, 109.95, 79.25, 56.89, 56.33, 50.18, 42.52, 39.91, 39.72, 38.12, 37.02, 36.76, 36.39, 36.00, 32.79, 32.12, 32.04, 28.44, 28.23, 27.83, 24.49, 24.03, 23.04, 22.78, 21.25, 19.49, 18.92 and 12.09 ppm.

Cholest-5-en-3*β*-yl-4-(tosylmethyl)phenyl carbonate (37): Procedure for synthesis is same as compound 31. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.78–7.8 (d, *J* = 8.1 Hz, 2H), 7.35–7.38 (d, *J* = 8.1 Hz, 2H), 7.18-7.15 (d, *J* = 4.0, 2H), 7.08-7.06 (d, *J* = 4.0, 2H), 5.39 (s, 1H), 4.49 (m, 1H), 2.50 (s, 3H), 2.47 (d, *J* = 8.7, 2H), 2.2.07 – 0.78 (m, 40H) and 0.68 (d, *J* = 4.0, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): *δ* 156.05, 151.48, 143.9, 139.36, 139.33, 138.92, 129.89, 129.65, 128.30, 122.45, 121.44, 79.13, 69.92, 57.88, 56.42, 50.18, 43.52, 39.90, 38.14, 37.02, 36.75, 36.38, 36, 32.11, 30.03, 28.23, 27.74, 24.48, 24.3, 23.03, 22.77 and 19.5 ppm.

Synthesis of Cholest-5-en-3*β*-yl-(4-(iodomethyl)phenyl) carbonate (39): To the acetone (10 mL) solution of Cholest-5-en-3*β*-yl-4-(chloromethyl) phenyl carbonate **38** (100mg, 0.154 mmol) potassium iodide (0.025 gm, 0.154 mmol) was added. The reaction mixture was allowed to stir in dark under nitrogen atmosphere for 5h. Reaction was continuously monitored by TLC and NMR. After the completion of reaction solvent was evaporated, diluted with DCM (25 mL), washed with water (2 X 10 mL), and dried over sodium sulfate. Solvent was removed to get a dark colored product weighing 80 mg. (yield= 69%). ^{1}H NMR ($CDCl_3$, 400 MHz) *δ* 7.41-7.39 (d, *J* = 5.3, 2H), 7.17-7.15 (d, *J* = 5.3, 2H), 5.43 (s, 1H), 4.71 (s, 2H), 4.64 (m, 1H), 2.51 (d, *J* = 8.7, 2H), 2.10 – 0.82 (m, 40H) and 0.68 (d, *J* = 4.0, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): *δ* 153.18, 149.24, 139.32, 130.40, 130.14, 123.53, 121.69, 115.61, 109.95, 79.25, 56.89, 56.33, 50.18, 42.52, 39.91, 39.72, 38.12, 37.02, 36.76, 36.39, 36.00, 32.79, 32.04, 28.44, 28.23, 27.83, 24.49, 24.03, 23.04, 22.78, 21.25, 19.49, 18.92, 12.09 and 7.06 ppm.

Synthesis of Cholest-5-en-3*β*-yloxycarbonyl(methylamino)acetic acid (44): To a solution of cholesteryl chloroformate (1gm, 2.30 mmol) in DCM (200 mL), 2-(methylamino)acetic acid

(0.89 gm, 0.01 mol) was added. The resulting reaction mixture was allowed to stir at room temperature for 1 h. Washed with 5% HCl (3 X 100 mL), water (3 X 100 mL). Organic layer was dried over anhydrous sodium sulfate and solvent removed under reduced pressure. A white solid was obtained which was purified by diluting with methanol and sonicating. Filtered and washed with methanol to give white solid (1.09 gm, yield= 95%) as pure product. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.35 (s, 1H), 4.50-4.48 (m, 1H), 4.03 (s, 1H), 3.97 (s, 1H), 2.96 (s, 3H), 2.36-2.22 (m, 3H), 1.97- 1.79 (m, 6H), 1.56-0.85 (m, 31H) and 0.66 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 174.32, 157.00, 156.17, 121.81, 115.57, 76.01, 75.80, 56.88, 56.35, 55.91, 50.66, 50.47, 50.19, 42.51, 39,95, 39.73, 38.71, 37.18, 36.76, 36.41, 36.03, 35.53, 32.13, 32.08, 28.46, 28.35, 28.23, 24.51, 24.08, 23.05, 22.79, 21.26, 19.58, 18.94 and 12.08 ppm.

Synthesis of Cholest-5-en-3*β*-yloxycarbonyl(methylamino)acetyl chloride (46): Compound was synthesized in the same way as compound 42. ^{1}H NMR ($CDCl_3$, 400 MHz) δ δ 5.35 (s, 1H), 4.60 (m, 1H), 4.50 (s, 2H), 3.27 (s, 3H), 2.36-2.22 (m, 3H), 1.97-0.85 (m, 38H) and 0.66 (s, 3H) ppm.

Synthesis of 4-(hydroxymethyl)phenyl-2-cholest-5en-3-yloxycarbonyl-*N*-methylaminoacetate (47): Compound was synthesized in the manner as compound 43. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.16 (d, J = 10.1 Hz, 2H), 6.98 (d, J = 10.1 Hz, 2H), 5.37 (m, 1H), 4.79 (s, 2H), 4.03 (s, 2H), 3.97 (s, 1H), 2.96 (s, 3H), 2.36-2.22 (m, 3H), 1.97-0.85 (m, 36H) and 0.66 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 169.32, 157.00, 151.17, 140.8, 139.0, 127.81, 121.8, 72.80, 69.00, 56.88, 58.35, 51.66, 50.66, 44.51, 39,95, 38.71, 37.18, 36.76, 35.41, 32.13, 30.08, 29.46, 28.35, 27.23, 27.13, 24.51, 23.05, 22.79, 21.26 and 19.58 ppm.

Synthesis of Cholest-5-en-3*β*-yl-2-hydroxyethyl(methyl)carbamate (45): To a solution of cholesteryl chloroformate (1.05gm, 2.32 mmol) in DCM (200 mL), 2-methylaminoethanol (1.30 gm, 0.01 mol) was added. The resulting reaction mixture was allowed to stir at room temperature for 1 h. Washed with 5% HCl (3 X 100 mL), water (3 X 100 mL). Organic layer was dried over anhydrous sodium sulfate and solvent removed under reduced pressure. A white solid was obtained which was purified by diluting with methanol and sonicating. Filtered and washed with methanol to give white solid (1.12 gm, yield= 95%) as pure product. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.35 (s, 1H), 4.48 (broad s, 1H), 3.73 (s, 1H), 3.41 (s, 1H), 2.94 (s, 3H), 2.34-2.28 (m, 3H), 1.99- 1.78 (m, 6H), 1.58-0.83 (m, 32H) and 0.66 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 400 MHz): δ 139.99, 122.75, 115.57, 56.88, 55.91, 52.10, 50.20, 42.51, 39,94, 39.73, 38.87, 37.22, 36.78, 36.39, 36.02, 32.12, 32.08, 28.46, 28.22, 24.50, 24.06, 23.05, 22.79, 21.26, 19.58, 18.93 and 12.07 ppm.

Synthesis of tosylate of Cholest-5-en-3*β*-yl-2-hydroxyethyl(methyl)carbamate (48): Synthesized same as compound **31**. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.78 (d, J = 7.6 Hz, 2H), 7.46 (d, J = 7.6 Hz, 2H), 5.35 (s, 1H), 4.46 (broad s, 1H), 3.72 (s, 1H), 3.39 (s, 1H), 2.95 (s, 3H), 2.43 (s, 3H), 2.34-2.28 (m, 3H), 1.98- 1.78 (m, 6H), 1.58-0.83 (m, 32H) and 0.66 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 400 MHz): δ 144.23, 138.69, 139.99, 130.12, 129.11, 122.75, 115.57, 56.88, 55.91, 52.10, 50.20, 42.51, 39,94, 39.73, 38.87, 37.22, 36.78, 36.39, 36.02, 32.12, 32.08, 29.32, 28.46, 28.22, 24.50, 24.33, 24.06, 23.05, 22.79, 21.26, 19.58, 18.93 and 12.07 ppm.

General Procedure for coupling of *C*-glycosylated barbiturates 6 with Benzyl bromide derivatives: Benzyl bromide derivative (0.1 mmol) was added to a clear solution of *C*-

glycosylated barbiturate **6** (0.1 mmol). The reaction mixture was sonicated to get a clear solution, else few drops of DCM were added to get a clear solution. The reaction mixture was allowed to sonicate for 15-30 mins depending on the substrate reactivity. After completion of reaction dilution with water and DCM precipitated the product. Filtered the mixture to get the pure product.

Synthesis of 1,3-dimethyl-5-(4-methylbenzyl)-5-(*β*-D-glucosyl)pyrimidine-2,4,6-trione (50): DMSO (5 mL) is added to compound 6 (100 mg, 0.280 mmol) and sonicated to get a clear solution, followed by addition of 1-(bromomethyl)-4-methylbenzene 49 (52 mg, 0.280 mmol). Continue to sonicate for another 15 minutes. Dilute with water (1 mL) and DCM (5mL). Collect the precipitate by decantation. Dissolve in EtOAc (10 mL) and wash with water (2 X 10 mL). Dried organic layer over anhydrous sodium sulfate and removed solvent under reduced pressure to get a yellowish solid (32 mg, yield=30%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.08 (d, J = 11.2 Hz, 2H), 6.98 (d, J = 11.2 Hz, 2H), 4.12 (m, 1H), 3.60-3.51 (m, 6H), 3.12 (s, 3H), 3.03 (s, 3H), 2.45 (s, 2H) and 2.05 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 179.02, 150.23, 128.96, 127.99, 135.45, 86.71, 84.01, 77.25, 71.25, 69.01, 62.12, 53.79, 36.78, 29.89 and 21.23 ppm.

1,3-dimethyl-5-(4-chlorobenzyl)-5-(*β*-D-glucosyl)pyrimidine-2,4,6-trione (52): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.32 (d, J = 9.6 Hz, 2H), 7.14 (d, J = 9.6 Hz, 2H), 4.12 (m, 1H), 3.55-3.48 (m, 6H), 3.12 (s, 3H) and 3.04 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 178.23, 150.67, 135.98, 129.05, 128.56, 86.07, 84.01, 77.25, 71.25, 69.01, 62.3, 53.79, 36.78 and 29.89 ppm.

1,3-dimethyl-5-(4-methoxybenzyl)-5-(*β*-D-glucosyl)pyrimidine-2,4,6-trione (54): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.08 (d, J = 11.2 Hz, 2H), 6.98 (d, J = 11.2 Hz, 2H), 4.12 (m, 1H), 3.78 (s, 3H), 3.60-3.51 (m, 6H), 3.12 (s, 3H), 3.03 (s, 3H) and 2.45 (s, 2H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 179.02, 150.23, 128.96, 127.99, 135.45, 86.71, 84.01, 77.25, 71.25, 69.01, 62.12, 53.79, 36.78, 35.8 and 29.89 ppm.

1,3-dimethyl-5-(4-bromobenzyl)-5-(*β*-D-glucosyl)pyrimidine-2,4,6-trione (56): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.62 (d, J = 9.6 Hz, 2H), 7.31 (d, J = 9.6 Hz, 2H), 4.12 (m, 1H), 3.62-3.58 (m, 6H), 3.10 (s, 3H) and 3.02 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 178.25, 151.06, 136.98, 132.0, 131.14, 120.56, 86.07, 84.01, 77.25, 71.25, 69.01, 62.23, 53.07, 36.78 and 29.77 ppm.

5-(4-(1,3-dioxoisoindolin-2-yl)benzyl)-1,3-dimethyl-5-((2*S*,3*R*,4*S*,5*S*)-3,4,5-trihydroxy-6-(hydroxymethyl)-tetrahydro-2H-pyran-2-yl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (58): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.98 (2H, m), 7.80 (2H, m), 7.55 (2H, d, J = 8Hz), 7.45 (2H, d, J = 8Hz), 4.12 (m, 1H), 3.62-3.58 (m, 6H), 3.10 (s, 3H) and 3.02 (s, 3H) ppm. ^{13}C-NMR ($CDCl_3$): δ 167.1, 140.0, 138.2, 135.1, 132.0, 130.2, 127.5, 124.3 86.07, 83.891, 77.51, 71.25, 66.01, 60.23, 52.07, 36.86 and 29.76 ppm.

General procedure for condensation of glycosylated barbituric acid and steroidal derivatives: To a solution of glycosylate barbiturate (0.2 mmol) in DMSO (5 mL) was added a solution of corresponding steroidal derivative (0.2 mmol) in DCM (2mL). The mixture was allowed to sonicate for 1-2h until precipitate is seen. After the completion of reaction diluted

with ethyl acetate (10 mL) washed with water (3 X10 mL). The organic layer was dried over sodium sulfate. Further purification was done by column chromatography using Ethyl acetate:Hexane as the eluent. The product was obtained in more than 50% yield and final sturcutre was confirmed by NMR.

1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-glucopyranose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-yl-4-methyl)phenyl carbonate (**60**): The product was obtained as yellowish powder (102 mg, yield=72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 (dd, J = 22.8, 8.5, 2H), 6.98 (dd, J = 22.8, 8.5, 1H), 4.55 (m, 1H), 4.08 (m, 2H), 3.84 (s, 1H), 3.53 (d, J = 33.8, 2H), 3.33 (s, 3H), 3.09 (s, 3H), 2.44 (d, J = 10.9, 2H), 2.05 (s, 2H), 2.01 – 0.80 (m, 40H) and 0.68 (s, 3H) ppm. ^{13}C NMR (400 MHz, D_2O) δ 172.01, 153.34, 150.7, 148.95, 140.82, 135.56, 128.76, 121.87, 121.45, 86.22, 79.76, 78.43, 76.21, 69.66, 69.48, 60.87, 58.23, 56.54, 52.7, 50.88, 44.09, 38.98, 37.23, 36.12, 36.78, 36.11, 35.78, 31.09, 30.06, 29.97, 29.79, 28.12, 27.35, 27.85, 24.67, 23.12 and 20.89 ppm.

1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-glucopyranose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-yl-4-methyl)phenyl succinate (**61**): The product was obtained as yellowish powder (72 mg, yield=67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.02 (dd, J = 22.8, 8.5, 2H), 6.89 (dd, J = 22.8, 8.5, 1H), 4.54 (m, 1H), 4.06 (m, 2H), 3.82 (s, 1H), 3.51 (d, J = 33.8, 2H), 3.31 (s, 3H), 3.06 (s, 3H), 3.21 (s, 2H), 2.76 (t, J = 12.8 Hz, 2H), 2.44 (d, J = 10.9, 2H), 2.05 (s, 2H), 2.01 – 0.80 (m, 40H) and 0.68 (s, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 172.3, 170.89, 151.7, 149.95, 141.82, 136.56, 128.76, 121.87, 121.45, 86.22, 79.76, 78.43, 76.21, 69.66, 69.48, 60.87, 58.23, 55.98,

51.97, 51.08, 44.09, 38.98, 37.23, 36.12, 36.78, 36.11, 35.78, 31.09, 30.06, 29.97, 29.79, 29.1, 29.6, 27.35, 27.85, 24.67, 23.12 and 20.89 ppm.

1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-glucopyranose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-Oxyethyl-2-oxy-)benzaote (**62**): The product was obtained as yellowish powder (45 mg, yield=42%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.08 (d, J = 8.2 Hz, 2H), 6.9 (d, J = 8.2 Hz, 2H), 4.6 (s, 2H), 4.36 (d, J = 9.9, 1H), 4.22 (t, J = 9.4, 1H),4.15 (t, J = 4.5Hz, 2H), 3.81 (t, J = 4.5Hz, 2H), 3.68 (m, 2H), 3.31 (t, J = 9.0, 1H), 3.19 (s, 3H), 3.01 (s, 3H), 2.89 (m, 1H) and 2.26–0.85 (m, 44H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 151.7, 141.82, 136.56, 128.76, 121.87, 121.45, 86.22, 79.76, 78.43, 76.21, 69.66, 69.48, 69.91, 67.23, 60.87, 58.23, 55.98, 51.97, 51.08, 44.09, 38.98, 37.23, 36.12, 36.78, 36.11, 35.78, 31.09, 30.06, 29.97, 29.79, 29.1, 29.6, 27.35, 27.85, 24.67, 23.12 and 20.89 ppm.

1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-yl-4-methyl)phenyl carbonate (**63**): Product was obtained as a pale yellow powder (32 mg, 57%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.22 (s, 2H), 7.07 (s, 2H), 5.43 (s, 1H), 5.12 (m, 1H), 4.5 (d, J = 6.8 Hz, 1H), 4.36 (t, J =12.8 Hz, 1H), 3.46- 3.80 (m, 9H), 3.36 (t, J =10.8 Hz, 1H), 3.12 (s, 6H), 3.12 (s, 2H,), 2.49 (d, J = 13.7 Hz, 2H), 2.10 – 0.82 (m, 40H) and 0.68 (d, J = 4.0, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 177.89, 154.44, 152.18, 148.89, 139.63, 131.04, 130.22, 124.30, 121.69, 116.58, 115.64, 108.95, 99.95, 95.98, 87.02, 79.28, 78.24, 78.19, 76.89, 75.98, 73.18, 72.59, 71.78, 69.41, 69.43, 61.15, 60.54, 56.83, 55.67, 50.18, 47.96, 42.52, 39.87,

38.98, 37.69, 37.12, 36.94, 36.56, 35.89, 32.69, 32.52, 32.14, 28.67, 28.23, 27.73, 26.53, 24.51, 24.03, 23.14, 22.78, 21.21, 19.79, 18.92 and 11.59 ppm.

1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5- (Cholest-5-en-3*β*-yl-4-(methyl)phenyl succinate (64): The product was obtained as pale yellowish powder (36 mg, Yield=45%) ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.08 (d, *J* = 12.8 Hz, 2H), 6.89 (d, *J* = 12.8 Hz, 2H), 5.39 (d, *J* = 12.8 Hz, 1H), 4.34 (d, *J* = 12.8Hz, 1H), 5.31 (bs, 1H), 4.61-4.71 (m, 1H), 4.28 (t, *J* =10.8 Hz, 1H), 3.58- 3.48 (m, 9H), 3.38 (t, *J* =10.8 Hz, 1H), 3.22 (s, 3H), 3.03 (s, 2H), 2.98 (s, 3H), 2.83 (t, *J* = 15.6 Hz, 2H), 2.76 (t, *J* = 15.6 Hz, 2H), 2.38 (d, *J* = 10.5 Hz, 2H), 0.85–2.26 (m, 41H) and 0.68(s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 178.2, 173.2, 172.89, δ150.44, 140.98, 135.02, 130.12, 122.08, 121.42, 115.48, 99.92, 95.01, 87.82, 78.86, 77.89, 77.10, 76.00, 74.42, 73.18, 72.09, 71.89, 69.45, 69.43, 60.75, 60.54, 58.13, 56.05, 51.09, 49.09, 44.10, 38.54, 38.25, 37.42, 36.23, 35.79, 34.12, 31.88, 31.01, 29.69, 29.12, 28.95, 28.12, 27.25, 27.77, 27.71, 27.33, 24.65, 23.12, 22.81, 21.69 and 19.25 ppm.

1,3-dimethyl-2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5- (Cholest-5-en-3*β*-oxyehtyl-2-oxo-benzyl ether (65): The product was obtained as pale yellowish powder (32 mg, Yield=38%) ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.02 (d, *J* = 18.4 Hz, 2H), 6.75 (d, *J* = 18.4 Hz, 2H), 5.31 (d, *J* = 9.2 Hz, 1H), 5.38 (d, *J* = 9.2 Hz, 1H), 4.69 (d, *J* = 9.2 Hz, 1H), 4.42 (t, *J* = 6.5 Hz, 2H), 4.36 (t, *J* =13.0 Hz, 1H), 3.82 (t, *J* = 6.5 Hz, 2H), 3. 78- 3.36 (m, 9H), 3.29 (t, *J* =13.0 Hz, 1H), 3.2 (s, 6H), 3.05 (s, 2H), 2.89 (m, 1H), 2.26–0.85 (m, 40H) and 0.63 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 178.2, 155.44, 154.2, 142.2, 131.9, 129.42, 121.90, 115.64, 115.85,

100.21, 95.81, 86.21, 84.15, 78.35, 78.25, 76.98, 76.12, 73.18, 72.71, 71.81, 69.89, 68.12, 61.28, 58.23, 56.39, 52.80, 44.01, 40.01, 38.65, 37.76, 36.84, 36.12, 36.01, 32.19, 28.21, 26.89, 27.25, 24.36, 23.22, 22.17, 20.17 and 19.6 ppm.

2,4,6-trioxo-5-(*β*-D-glucopyranose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-yl-4-methyl)phenyl carbonate (**66**): The product was obtained as yellowish powder (40 mg, yield=43%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.2 (s, 2H), 7.00 (dd, *J* = 20.8, 9.5, 2H), 6.88 (dd, *J* = 20.8, 9.5, 1H), 4.56 (m, 1H), 4.08 (m, 2H), 3.86 (s, 1H), 3.63 (d, *J* = 6.84, 2H), 2.54 (d, *J* = 13.2, 2H), 2.12 (s, 2H), 2.08 – 0.81 (m, 40H) and 0.68 (s, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 171.23, 153.87, 151.7, 149.05, 140.84, 135.65, 128.58, 121.96, 121.55, 82.11, 79.67, 77.83, 76.25, 69.56, 69.28, 61.07, 58.33, 56.52, 52.17, 50.48, 44.19, 38.98, 36.23, 36.11, 36.74, 36.31, 34.98, 31.13, 30.06, 28.02, 27.35, 27.85, 24.67, 23.12 and 19.86 ppm.

2,4,6-trioxo-5-(*β*-D-glucopyranose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-yl-4-methyl)phenyl succinate (**67**): The product was obtained as yellowish powder (61 mg, yield=37%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.02 (dd, *J* = 12.8, 8.5, 2H), 6.89 (dd, *J* = 12.8, 8.5, 1H), 4.57 (m, 1H), 4.04 (m, 2H), 3.80 (s, 1H), 3.49 (d, *J* = 12.8, 2H), 3.23 (s, 2H), 2.74 (t, *J* = 10.8 Hz, 2H), 2.45 (d, *J* = 12.8, 2H), 2.07 (s, 2H), 2.05 – 0.83 (m, 40H) and 0.66 (s, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 171.89, 170.26, 151.17, 149.75, 142.18, 136.46, 128.76, 122.08, 121.45, 86.12, 79.71, 78.45, 76.17, 69.56, 69.48, 61.19, 58.31, 56.12, 52.09, 51.08, 44.09, 38.98, 37.23, 36.12, 36.78, 36.15, 35.78, 31.09, 30.05, 29.97, 29.79, 29.1, 29.6, 27.35, 27.85, 24.67, 23.12 and 20.69 ppm.

2,4,6-trioxo-5-(*β*-D-glucopyranose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-Oxyethyl-2-oxy-)benzaote (**68**): The product was obtained as yellowish powder (25 mg, yield=40%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.02 (d, *J* = 12.2 Hz, 2H), 6.82 (d, *J* = 12.2 Hz, 2H), 4.56 (s, 2H), δ 4.26 (d, *J* = 8.9, 1H), 4.12 (t, *J* = 13.4, 1H), 4.15 (t, *J* = 14.5Hz, 2H), 3.85 (t, *J* = 14.5 Hz, 2H), 3.66 (m, 2H), 3.32 (t, *J* = 9.5, 1H), 2.89 (m, 1H) and 2.26–0.85 (m, 44H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 172.32, 151.7, 142.82, 135.56, 128.76, 122.87, 121.53, 85.22, 79.76, 78.43, 76.21, 69.66, 69.48, 69.92, 67.23, 60.87, 58.23, 55.98, 52.09, 51.08, 44.09, 38.98, 37.23, 36.12, 36.78, 36.11, 35.78, 31.09, 30.16, 29.1, 29.6, 27.35, 27.85, 24.65, 23.21 and 20.67 ppm.

2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5-(Cholest-5-en-3*β*-yl-4-methyl)phenyl carbonate (**69**): Product was obtained as a pale yellow powder (35 mg, 58%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.02 (s, 2H), 6.88 (s, 2H), 5.45 (s, 1H), 5.14 (m, 1H), 4.6 (d, *J* = 16.8 Hz, 1H), 4.29 (t, *J* =12.8 Hz, 1H), 3.45- 3.81 (9H), 3.36 (t, *J* =12.8 Hz, 1H), 3.14 (s, 2H), 2.52 (d, *J* = 16.7 Hz, 2H), 2.12 – 0.83 (m, 40H) and 0.68 (d, *J* = 4.0, 3H) ppm. ^{13}C NMR (DMSO-d_6, 400 MHz): δ 177.91, 154.45, 152.18, 147.89, 140.63, 132.04, 130.22, 125.31, 121.69, 116.48, 115.42, 108.35, 100.05, 95.48, 87.12, 79.32, 78.24, 78.19, 76.89, 75.98, 73.18, 72.59, 71.78, 69.41, 69.43, 61.15, 60.54, 56.32, 54.79, 50.18, 48.00, 42.54, 40.87, 38.98, 37.69, 37.12, 36.94, 36.56, 35.89, 32.69, 32.52, 32.14, 27.73, 26.53, 24.51, 24.03, 23.14, 22.78, 21.21, 20.79, 18.90 and 12.88 ppm.

2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5- (Cholest-5-en-3*β*-yl-4-(methyl)phenyl succinate (70): The product was obtained as pale yellowish powder (40 mg, Yield=56%) ^{1}H

NMR (DMSO-d_6, 400 MHz): δ 7.02 (d, J = 10.2 Hz, 2H), 6.84 (d, J = 10.2 Hz, 2H), 5.35 (d, J = 5.4 Hz, 1H), 4.36 (d, J = 10.28 Hz, 1H), 5.30 (bs, 1H), 4.59-4.69 (m, 1H), 4.28 (t, J =13.4 Hz, 1H), 3.55- 3.42 (m, 9H), 3.32 (t, J =13.8 Hz, 1H), 3.10 (s, 3H), 3.03 (s, 2H), 2.98 (s, 3H), 2.82 (t, J = 15.6 Hz, 2H), 2.76 (t, J = 15.6 Hz, 2H), 2.38 (d, J = 10.5 Hz, 2H), 2.26 –0.85 (m, 40H) and 0.65 (s, 3H) ppm. ^{13}C NMR (DMSO-d_6, 400 MHz): δ 177.2, 173.4, 172.68, 150.44, 141.08, 136.76, 130.13, 121.84, 121.21, 115.48, 99.82, 95.75, 87.82, 78.84, 77.89, 77.10, 76.00, 74.42, 73.18, 72.09, 71.89, 69.49, 69.40, 60.75, 60.54, 58.13, 56.05, 51.09, 49.09, 44.10, 38.54, 38.25, 37.42, 36.23, 35.79, 34.12, 31.88, 31.01, 29.69, 29.12, 28.95, 28.12, 27.25, 27.77, 27.71, 27.33, 24.65, 23.12, 22.81, 21.79 and 20.52 ppm.

2,4,6-trioxo-5-(*β*-D-maltose)-hexahydropyrimidin-5- (Cholest-5-en-3*β*-oxyehtyl-2-oxo-benzyl ether (71): The product was obtained as pale yellowish powder (22 mg, Yield=35%) ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.02 (d, J = 8.4 Hz, 2H), 6.75 (d, J = 8.4 Hz, 2H), 5.25 (d, J = 9.2 Hz, 1H), 5.36 (d, J = 8.2 Hz, 1H), 4.71 (d, J = 8.2 Hz, 1H), 4.41 (t, J = 13.2 Hz, 2H), 4.36 (t, J =13.0 Hz, 1H), 3.80 (t, J = 6.5 Hz, 2H), 3.78- 3.36 (9H), 3.25 (t, J =13.0 Hz, 1H), 3.05 (s, 2H), 2.89 (m, 1H), 2.26–0.85 (m, 40H) and 0.68 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 177.2, δ156.44, 153.2, 141.2, 132.1, 130.42, 122.50, 115.34, 115.85, 100.21, 95.81, 86.21, 84.15, 78.50, 77.25, 76.98, 76.02, 73.18, 72.71, 71.82, 68.12, 61.28, 58.25, 56.40, 51.80, 45.01, 41.01, 38.65, 37.76, 36.84, 36.12, 36.01, 32.19, 30.12, 28.89, 28.21, 26.89, 27.25, 24.36, 23.22, 22.17, 20.17 and 20.01 ppm.

Synthesis of **monobromoarene:** Benzene (10 ml) solution of methyl benzene derivative (5 mmol), *N*-bromosuccinamide (0.89 g; 5 mmol), and benzoylperoxide (0.12 g; 0.5 mmol) was

refluxed under microwave heating (magnetron power 600 W). After reaction was completed (See Table 1) solvent was evaporated; solid residue was dissolved in dichloromethane (100 ml) and washed with saturated water solution of sodium bicarbonate (3x15 ml) and water (3x15 ml). Dichloromethane was evaporated and the solid residue was purified by silica gel column chromatography with hexane-dichloromethane as an eluent.

4-(Bromomethyl)phenylphthalimide: Isolated yield (85%). ^{1}H-NMR ($CDCl_3$): δ 7.98 (2H, m), 7.80 (2H, m), 7.55 (2H, d, J = 8Hz), 7.45 (2H, d, J = 8Hz), and 4.55 (2H, s) ppm. ^{13}C-NMR ($CDCl_3$): δ 167.1, 140.0, 138.2, 135.1, 132.0, 130.2, 127.5, 124.3 and 33.0 ppm.

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Chapter-3: PROGRESS TOWARDS SYNTHESIS AND EVALUATION OF CYSTEINYL METAL PEPTIDES.

3.1 Introduction: Free radicals and oxidative Stress

Free radicals are defined as any atom/ molecule with an "unpaired electron" in the outer shell. These are highly reactive species formed in the body's cells during normal metabolic processes. Free radicals are involved in the body's host defense mechanism i.e. the immune system, cell signaling, inflammation, activation of gene transcription factors. This makes free radical an important component of everyday human life.

Various reactions that take place in the mitochondrial cells (Fig. 3.1.) as shown below[3]

1. $4H^{+} + O_2 \rightarrow 2H_2O$ (~95% of the time)

2. $O_2 \rightarrow {}^{*}O_2^{\bullet -} \rightarrow H_2O_2$ *Only 1-5% generate free radicals

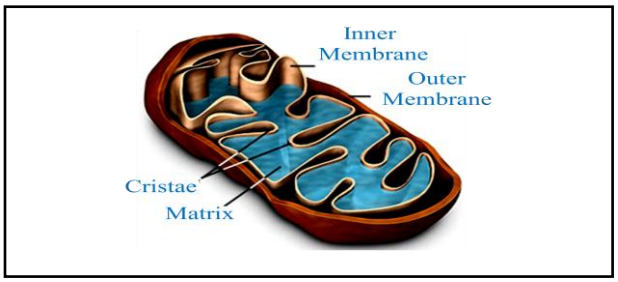


Figure 3.1 Structure of Mitochondrial Cell[3]

These reactions along with others generate Reactive Oxygen Species (ROS) like HO•, RO•, HOO•, ROO•, $\bullet O_2^-$, $^1O_2$, H_2O_2 and Reactive Nitrogen Species (RNS) likeNO•, NO_2•, $OONO^-$ [1].

These free radicals are responsible for the oxidative stress in the body. Oxidative stress is typically defined as an imbalance caused by the systemic manifestation of the reactive oxygen

species and inability of body's antioxidant defense systems to detoxify it or repair the damage. Various intrinsic and extrinsic factors contribute to the oxidative stress of body. Extrinsic factors like environmental pollutants, exposure to irradiation (like space travel) can lead to increases free radical production in the human body. Several processes taking place inside the human body (intrinsic factors) like regular wear and tear with aging, usual enzymatic and chemical reactions and interactions among ROS render the body with increased amount of oxidative stress.[4] The antioxidant defense system for the human body comes from the dietary intake of food rich in tocopherol, ascorbic acid or carotene. Several endogenously present antioxidant enzymes like superoxide dismutase, catalase, glutathione oxidase and melanin help fight the oxidative stress. Following (Fig.3.2) is an illustration to show the processes through which oxidative stress damages biological targets.

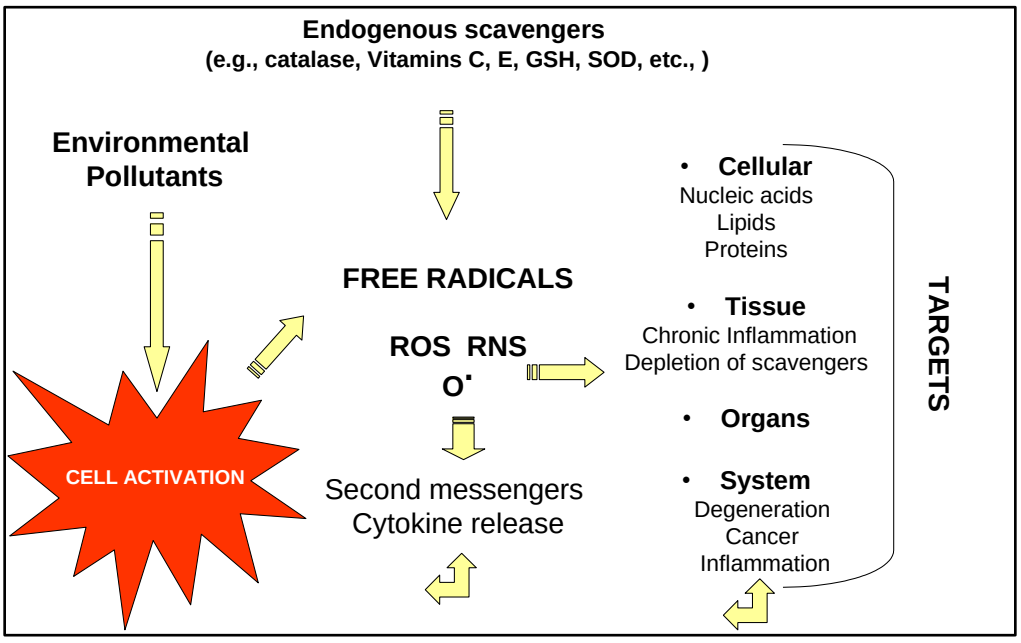


Figure 3.2 A simplistic representation of how exposure to environmental pollutants may induce an excess of free radical (ROS & RNS) and damage biological targets[4].

According to studies carried out by different scientific group it is thought that the oxidative stress in humans is involved in the development of many diseases or may exacerbate their symptoms by bringing out structural feature changes in DNA and proteins [5,6,7]. Dimerization or polymerization of lipids, cross linkage of proteins, fragmentation of DNA and formation of age pigment as some of the structural feature changes brought about by the free radicals. These in turn would lead to diseases like cancer[8], alzheimers[9], parkinsons, atherosclerosis, schizophrenia, bipolar disorder [10,11], heart failure, vasospasm, stroke, arthritis Sickle Cell Disease,[12] lichen planus,[13] vitiligo,[14]autism,[15] and chronic fatigue syndrome.[16]

3.2 Antioxidant

In order to protect the healthy cells and organ system of the human body against ROS, the humans have evolved a highly sophisticated and complex antioxidant defense system. A variety of endogenous and exogenous components, function interactively and synergistically to, neutralize the free radicals[17, 18]. These components include[18]

- Nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds such as glutathione and lipoicacid.
- Antioxidant enzymes, e.g., superoxide dismutase, glutathione peroxidase, and glutathione reductase, catalyze free radical quenching reactions.
- Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions are capable of catalyzing oxidative reactions.
- Skin pigment ‘melanin’ plays an important role in protecting against free radicals.

Numerous other antioxidant phytonutrients are present in a wide variety of plant foods. Several studies conducted have indicated amino acids like cysteine, homocysteine, tyrosine, histidine, tryptophan (Fig.3.3) as potential radical scavengers.

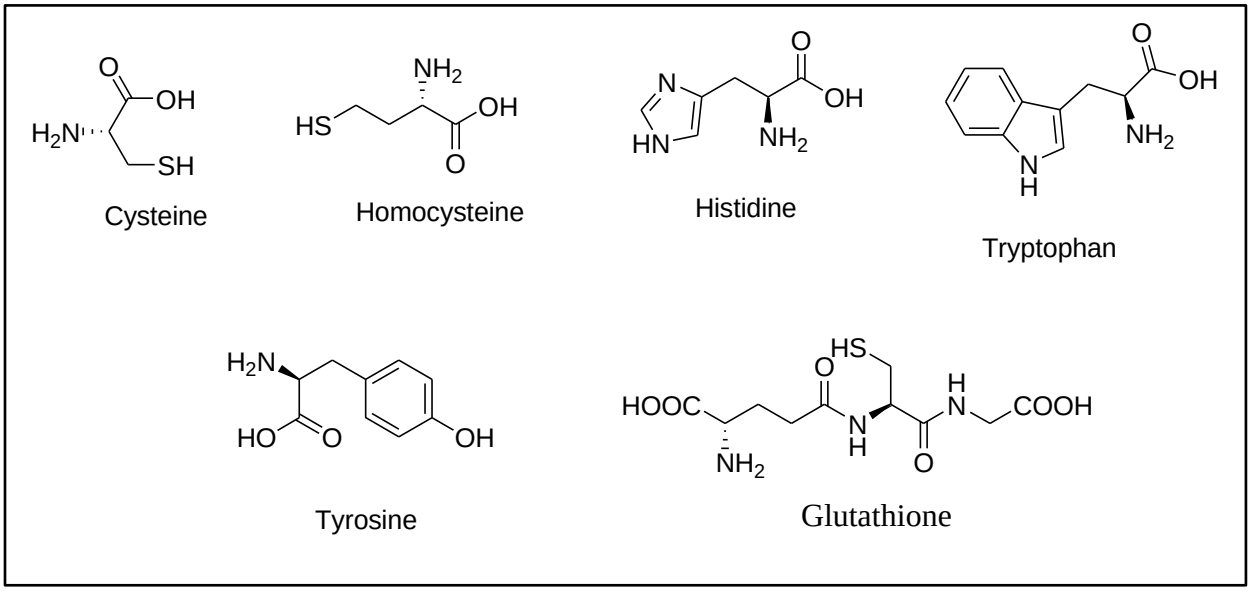


Figure 3.3 Amino acids and peptides as radical scavengers

The oxidative susceptibility of any given amino acid to the free radical attack is dictated in large part by its functional R-group on the side chain. The solvent accessibility and the chemical properties of vicinal residues are also important[18b].

All twenty biologically-derived amino acids are potentially oxidizable, the most reactive amino acids tend to be those containing either nucleophilic sulfur-containing side chains like cysteine and methionine or aromatic side chains as in tryptophan, tyrosine and phenylalanine from which hydrogen can be easily abstracted. Infact aminoacids like tyrosine and cysteine are intergarl part of melanin[21,22] biosynthesis which protects against harmful ultraviolet radiations. Melanin decreases effects of ionizing radiation in human and mouse somatic cells and act as

radioprotective agent. Therefore, it is thought that it imparts protection against certain types of skin cancers. Cysteine is the integral part of glutathione[23] and imparts radical scavenging properties to the molecule. Histidine's imidazole-contianing side chain is also oxidatively labile. The oxidation of these amino acids forms products that quench the free radicals by forming different product as shown in Table 3.1.

Amino acid	Oxidation product	Mechanism of formation
Cysteine	1. Cystine 2. Oxyacids	1. Hydrogen abstraction from SH group and subsequent radical dimerization 2. Hydrogen abstraction from SH group and subsequent reaction with oxygen and isomerization
Methionine	Methionine sulfoxide	Routes including both radical and non-radical reactions
Tryptophan	N-formylkynurenine, kynurenine, 5-hydroxytryptophan, 7-hydroxytryptophan,	Hydroxyl attack or one electron oxidation of ring
Tyrosine	3,4-dihyroxy-phenylalanine, di-tyrosine, 30chlorotyrosine, 3-nitrotyrosine, tyrosine hydroperoxide and subsequent cyclized materials	Hydroxyl attack or one electron oxidation of ring, radical-radical dimerization, HOCl, chlorination of tyrosine, formation of phenoxyl radical
Phenylalanine	O,m-tyrosine	Hydroxyl attack or one electron oxidation of ring,Reaction via nitrogen species, dimerization
Histidine	2-oxo-histidine	Hydroxyl attack or one electron oxidation

Table 3.1 Products generated on exposure of selected amino acids to radical species and postulated mechanism of formation[18b]

The antioxidant properties of peptides are more related to their composition, structure, and hydrophobicity[56]. Tyr, Trp, Met, Lys, Cys, and His are examples of amino acids that are responsible for antioxidant activity[69] of several peptides. Usually amino acids with aromatic residues can donate protons to electron deficient radicals and exert antioxidant effect. This inherent property is responsible for imparting the radical-scavenging properties of the amino acid residues[64]. His-containing peptides have antioxidant activity that comes form the hydrogen-donating, lipid peroxyl radical trapping and/or the metal ion-chelating ability of the imidazole group[54,64]. Cysteine has an SH group that is responsible for crucial antioxidant action due to its

direct interaction with radicals[63]. Not only presence of a proper amino acid but its positioning as well plays an important role in antioxidant activity of peptides[64]. Chen *et al.*[57] designed and studied antioxidant property of 28 synthetic peptides based on the structure of an antioxidative peptide (Leu-Leu-Pro-His-His) from digestion of a soybean protein, conglycinin[55]. Their studies revealed that the antioxidant activity of a peptide was more dependent on His-His segment in the Leu-Leu- Pro-His-His domain. Removal of His residue from the C-terminus dramatically reduced the antioxidant activity. The same study revealed that Pro-His-His sequence displayed the greatest antioxidative activity among all tested peptides. Saito *et al.*[65] have reported that any change in the arrangements of amino acid sequence in tripeptides resulted in different antioxidant activities. The specific structural features and peptide linkages have been claimed to influence antioxidant ability. For instance, when certain amino acids are incorporated in dipeptides they exert higher antioxidative properties[60]. Studies have also indicated that a peptide bond or its structural conformation can reduce the antioxidant activity of the constituent amino acids. Therefore, it was concluded that the peptide conformation could have both the synergistic and antagonistic effects for the antioxidant activity of free amino acids[58].

Several other factors can influence antioxidant activity of bioactive peptides like the operational conditions applied to isolate proteins, degree of hydrolysis, type of protease[62], peptide structure[65] and peptide concentration. Molecular weight (MW) of peptides can also influence antioxidant activity. It was found that antioxidant activity of peptides of MW 500–1500 Da was stronger than that of peptides above 1500 Da and peptides below 500 Da[59]. However, it has been postulated that the overall antioxidative activity must be ascribed to the integrative effects of these actions rather than to the individual actions of peptides[56]. The peptide

linkage between β-alanine, histidine and 1-methylhistidine was also involved in the activities of the dipeptides towards radical scavenging activity[70].

3.3 Aim of the project

Since reactive oxidative species (ROS) is implicated in the pathogenesis of many chronic diseases, such as cancer, cardiovascular and neurodegenerative diseases, finding novel antioxidants to combat ROS has attracted much attention[54]. To facilitate the discovery of antioxidants, some antioxidant pharmacophores have been identified, among which catechol has been given the most attention[54]. The great antioxidant potential of catechol is attributed to the fact that the semiquinone radical derived from H-atom donation of catechol can be stabilized by an intramolecular hydrogen bond and the electron-donating properties of the *ortho*-OH. Another amino acid with a great potential as a radical capture agent is cysteine. Since cysteine cannot be used in its free form because the sufhydryl group is higly susceptible to oxidation. It has to be trapped in a fashion where it is stable outside the human body and has a good shelf-life, but when it is inside the free sulfhydryl is easily available to capture free radicals in human body. To develop novel radical capture compounds based on the above mentioned facts a two-fold aim of this project is proposed. Firstly, develop an industrially viable, high yield procedure for Zinc monocysteine synthesis. Besides zinc, incorporate other metals like Ca, Mg, Cu and Cd into cysteine skeleton (Fig.3.4). Secondly, synthesize ‘Advanced Radical Capture Compounds’ by linking cysteine metal complex to other amino acids like tyrosine to get desired peptides. This approach will release two amino acids inside the body that have radical capture ability. Cysteine would be metabolized to sulfur-containing compound like taurine and glutathione that can fight the oxidative stress of body. The metal component would protect the free sulfhydryl and once

inside the body it can act as a supplement to induce metallothionein (MT) that has capability to scavenge free hydroxyl causing oxidative stress. In addition, studies have indicated an increased activity of antioxidant enzymes catalase and glutathione peroxidase by ZMC.

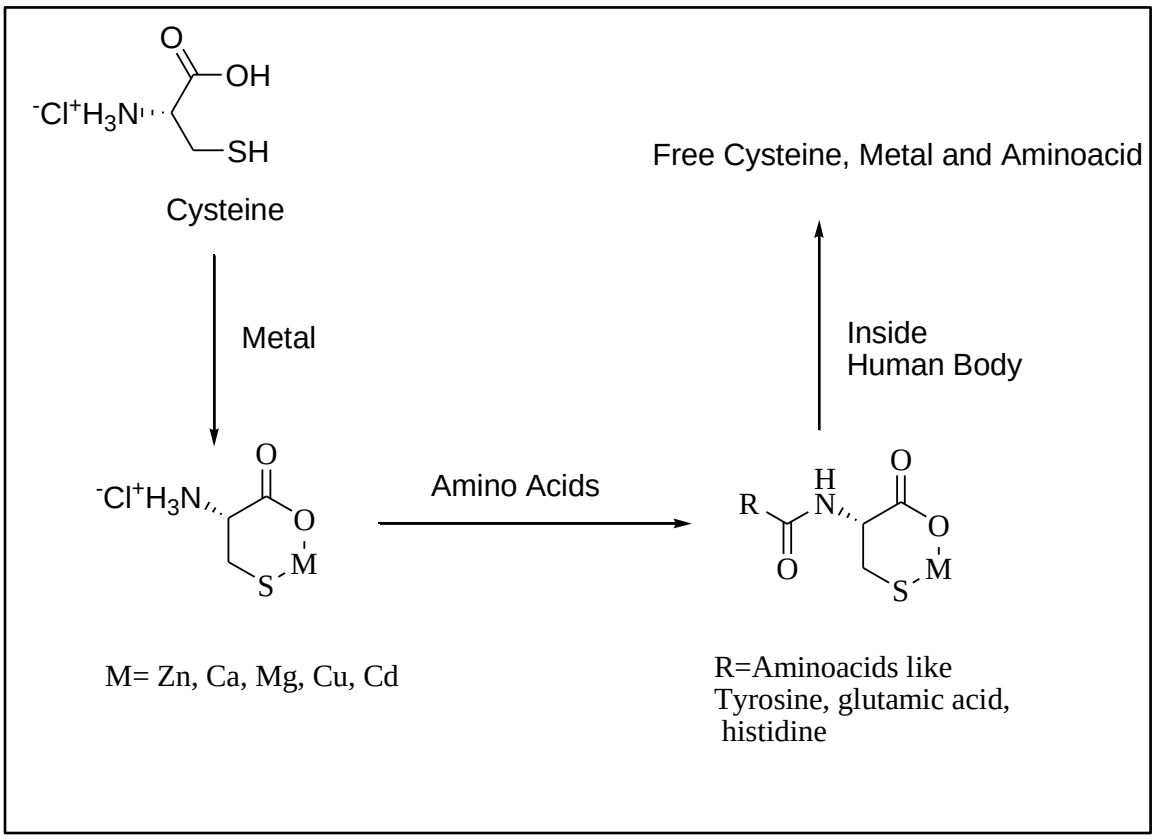


Figure 3.4 L-cysteine metal peptides

3.4. Cysteine metal complexes

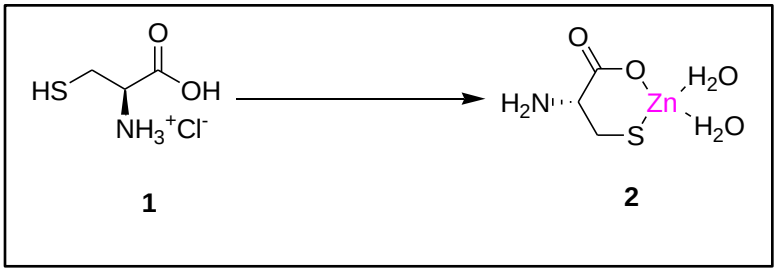
In the pursuit to synthesize, 'Advanced radical capture compounds' cysteine was chosen as one of the substrates. Cysteine is a non-essential amino acid that can be biosynthesized in the human body. The *β*-sulfhydyl group of cysteine often participates in several enzymatic reactions including antioxidant glutathione, where it acts as a precursor. Antioxidant ability of cysteine is

typically expressed in the tripeptide glutathione. The oral availability of glutathione is negligible and hence it is biosynthesized from L-amino acids cysteine, glycine and glutamic acid. Availability of cysteine through diet can sometimes be a limiting factor. Cysteine has also been implicated as an important source of sulfide in the human metabolism. In order to make cysteine available from outside source i.e diet it has to be masked inside other components. Since the β-sulfhydryl group is readily oxidized, it has to be protected in a way that is freely available inside the human system. To achieve this cysteine β-sulfhydryl group could be complexed with metals that do not readily oxidize the free sulfhydryl group. This will impart stability to cysteine and extend its shelf-life. One of these metals is zinc that was successfully used to trap free cysteine. Its synthesis and properties are discussed in the subsequent sections. At the same time, efforts were directed to synthesize other metal complexes of cysteine like calcium, magnesium, copper and cadmium besides zinc and are discussed later in this chapter.

3.4.1. Zinc Monocysteine Compound

Trace elements play a vital role in the human body to maintain a normal growth and development through a complex array of physiological functions. They are also responsible to balance the toxicity levels inside the human body. One such trace element is zinc, which occupies the second spot in being the most abundant trace element in the human body[1]. Zinc functions as a cofactor in more than 200 enzyme reactions and is known to be essential for the function of numerous transcription factors and nuclear regulatory elements.[2,3] In humans, severe zinc deficiency has been shown to result in stunted growth and impaired development.[4,5] Zinc can also act as an antioxidant by protecting the free sulfhydryl group from oxidation. Because some amino acids in various combinations with trace elements have been shown to exhibit

increased biologic effect over the individual substances, zinc-monocysteine conjugate was developed. Zinthionin (Oral ZMC) for Age Related Macular Degeneration (AMD) was developed by Dr. David Newsome[25]. The reported procedure for preparation of ZMC involves reaction of Cysteine HCl with zinc metal[25] (Scheme 3.1, Table 3.2, entry 1). The reported procedure suffers from several drawbacks including poor yields (20-30 %), leaves large solid residue as discard and finally use of lyophylization to dry final product[25] adds to the cost of preparation. All this makes the process non-viable at industrial scale. Therefore, we worked on improving the synthetic method for the preparation of ZMC. Our synthetic method was developed by using L-cysteine HCl and various zinc salts.



Scheme 3.1 Preparation of Zinc Monocysteine

The reaction was carried out using zinc salts like zinc acetate, zinc oxide, zinc chloride, zinc carbonate. The results are summarized in table 3.2 (entry 2 and 3).

S. No.	**Procedure**	**Yield**
1.	Zn^0/H2O, Reflux, 2h (reported procedure)	20-30%
2.	$Zn(OAc)_2$.$2H_2O$, $NaOAc.H_2O$, Heat 3h	98%
3.	$ZnCO_3$, AcOH, Na_2CO_3, Heat 3h	97%

Table 3.2 Reaction conditions for ZMC synthesis

The product was obtained in more than 95% yield and high purity. The isolated product was then analysed by NMR, Mass spectroscopy and elemental analysis. It was demonstrated that the zinc monocysteine compound was almost 100% pure and is accompanied by two molecules of water. A corresponding proton and carbon NMR was recorded in D_2O-HCl to confirm the structure (Fig 3.5). Structure was further confirmed by mass spectroscopy (Fig.3.6).

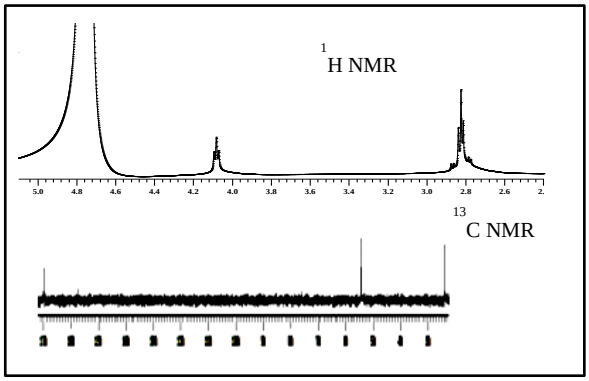


Figure 3.5 ^{1}H and ^{13}C NMR of ZMC

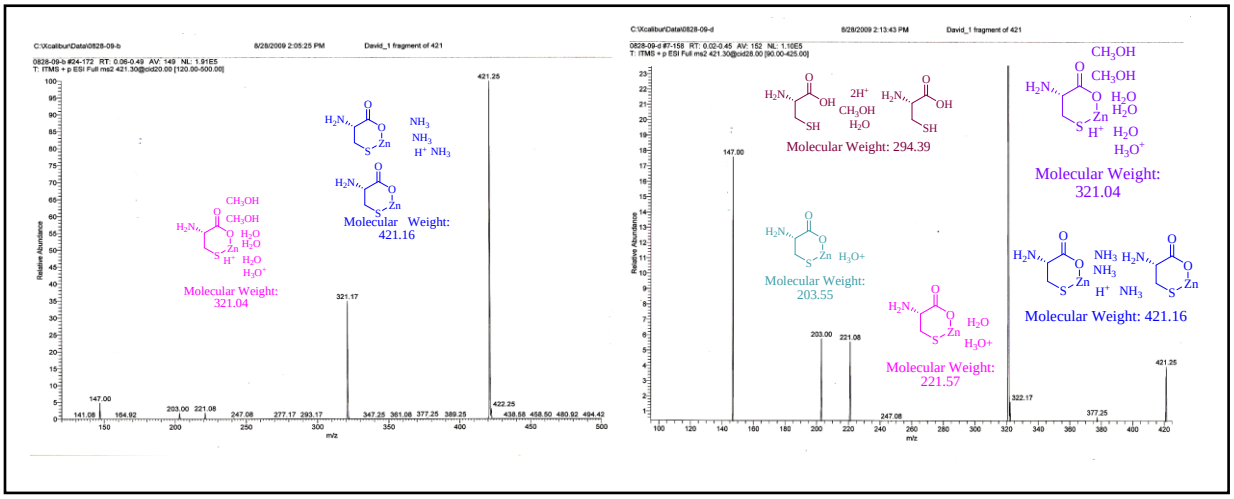
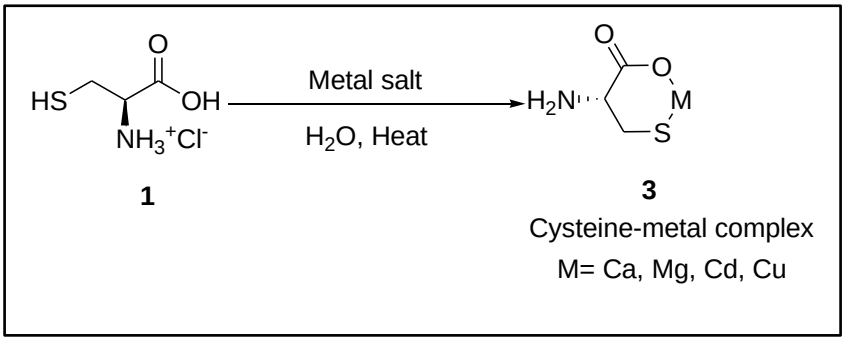


Figure 3.6 Mass Spectrum of ZMC

3.4.2 Synthesis of L-cysteine metal complexes

After successfully establishing the synthetic method for incorporation of zinc in L-cysteine hydrochloride other metals were incorporated into the Cysteine skeleton (Scheme 3.2).



Scheme 3.2 Cysteine metal complex synthesis

The reaction of L-cysteine HCl with other metal salts like calcium oxide, calcium hydroxide, calcium carbonate, magnesium carbonate, magnesium oxide, cadmium and copper salts were carried out in a similar way as for Zinc salts. The results are outlined in Table 3.3

Compound	Metal Salt	Cys-Metal Complex	Water Solubility
1.	CaO/ $CaCO_3$	Cys-Ca **(4)**	Soluble
2.	MgO/ $MgCO_3$	Cys-Mg **(5)**	Soluble
3.	$CdCl_2$	Cys-Cd **(6)**	Insoluble
4.	Cu salts	Oxidise cys	NA

Table 3.3 Cysteine metal complexes and their properties.

L-cysteine HCl was reacted with calcium carbonate and calcium oxide to give the corresponding L-cysteine monocalcium complex, which was water-soluble. The structure was confirmed by proton and carbon NMR and mass analysis (Fig.3.7). Similarly, L-cysteine monomagnesium

complex was synthesized and was also water soluble, whereas, the corresponding L-cysteine cadmium complex was water insoluble. All the synthesized L-Cysteine-metal complexes were characterized by mass spectroscopy and ^{1}H, ^{13}C NMR. A representative mass spectrum of L-cysteine calcium complex and its fragmentation pattern are presented in Fig.3.7.

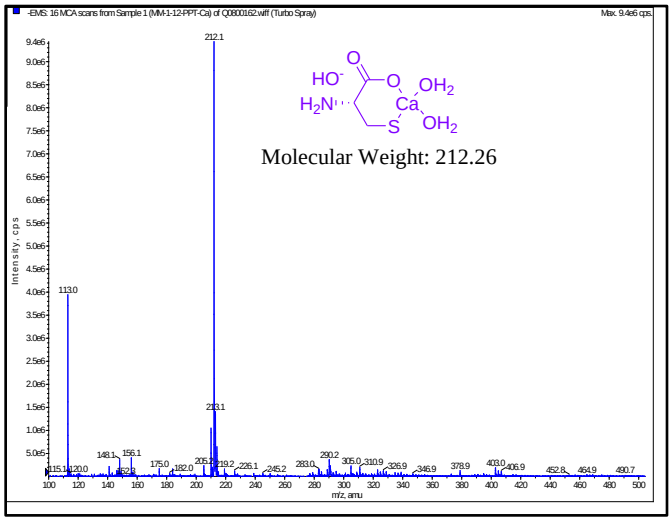


Figure 3.7 Mass spectrum of Cys-Ca complex

3.5. Cysteinyl Peptide Synthesis

Common elements in the solution phase peptide synthesis are the assembly of protected amino acids, their deprotection and finally purification to obtain the desired peptide molecules. This technique has been used successfully for the synthesis of small peptides containing a few amino acid residues (Fig.3.8). The main advantage of this technique is that the intermediate products can be isolated and purified after each step of synthesis, deprotected and recombined to obtain larger peptides of the desired sequence. This technique is highly flexible with respect to the chemistry of coupling and the combination of the peptidic blocks[26].

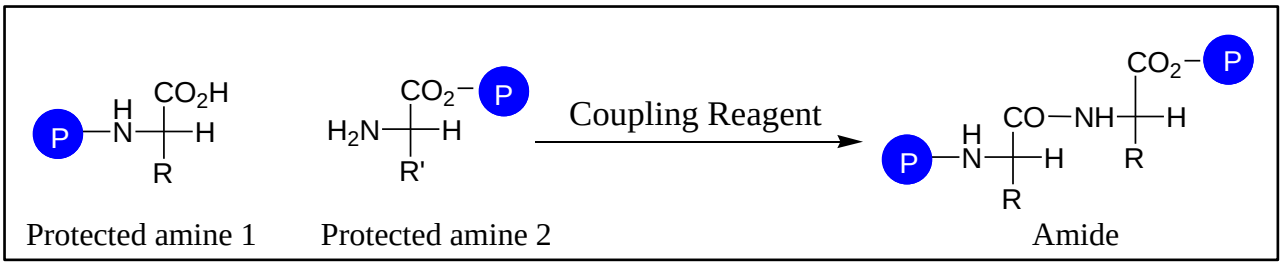
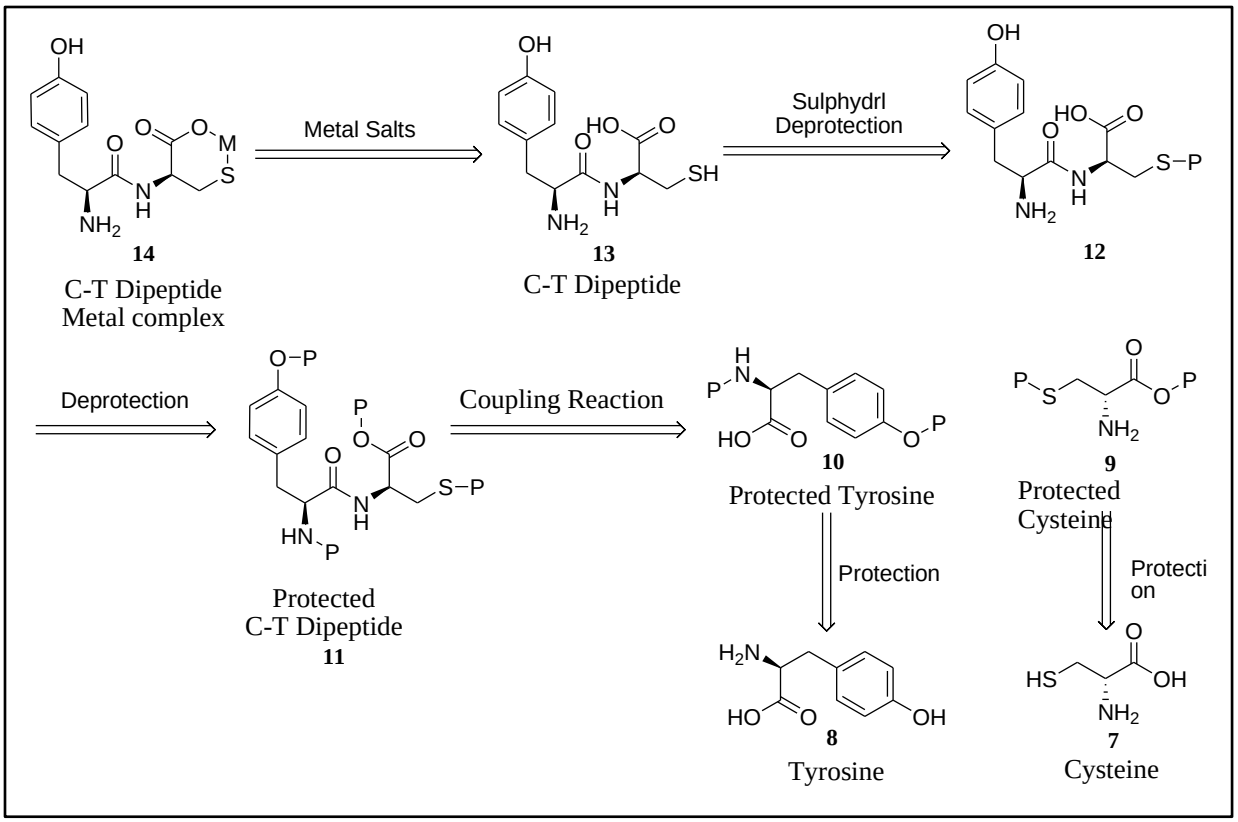


Figure 3.8. Solution phase peptide synthesis

New strategies for synthesis in solution have been developed, going from the design of functional groups for the side chains and condensation of fragments for the synthesis of large molecules[27] to the use of new coupling reagents[28]. The most serious problems for this chemical synthesis come from the poor solubility of some protected peptides in organic solvents. Researchers commonly experience the problem of limited solubility as to render peptide bond formation impossible[29]. This happens because of the fundamental tendency of the protected peptide chains to form intermolecular aggregates rather than interact with organic solvents[30]. The other serious problems include racemization of the activated *C*-terminal amino acid in peptides[31], and the lack and cost of high resolving methods for the purification of protected peptide fragments. At the same time, solution phase peptide synthesis gives better purity and high yields and it is a preferred method of peptide synthesis. In the last few years, more than 250 protecting groups have been proposed as suitable for peptide synthesis[32]; however, a relatively small number of those is actually used because of the stringent requirements that a protecting group should meet, particularly with respect to the requirement of the preservation of other functionalities.

In our case to synthesize cysteinyl peptide metal complex **14** employing L-tyrosine **8** the synthetic challenge was to protect and deprotect the side chain reactive groups in a cost effective and efficient manner that is industrially viable. Therefore, several methods for the protection and

deprotection of β-sulfhydryl group were tried to develop efficient and reproducible synthetic methodology. A retrosynthetic analysis of the desired C-T dipeptide **13** is outlined in Scheme 3.3. The desired metal complexed dipeptide **14** could be synthesized by reaction of the metal salts with the unprotected C-T peptide **13**. The dipeptide **13** in turn can be prepared from commercially available amino acids L-cysteine **7** and L-tyrosine **8**. These L-amino acids have side chain functional groups that could be suitably protected to give corresponding protected L-tyrosine **10** and protected L-cysteine **9**. Subsequently compound **9** and **10** could be coupled using peptide coupling reaction to yield dipeptide **11**. The desired coupling is through carboxy terminal of L-tyrosine bonding with the *N*-terminal of L-cysteine. Once the desired peptide bond has been synthesized the protective groups can be selectively removed to give compound **12** and the β sulfhydrl protection is removed right before capturing the free sulfhydryl group with a metal to yield dipeptide metal complex **14** (Scheme 3.3).



Scheme 3.3 Retrosynthetic analysis of cysteinyl peptide

3.5.1 Protecting groups for L-cysteine:

In order to accomplish cysteinyl peptide synthesis using L-Cysteine it is mandatory to have appropriate sulfhydryl protective groups. The nucleophilic thiol can otherwise be acylated, alkylated, or oxidized to disulfide by air during peptide synthesis. We wanted to develop a protective group that is convenient to synthesize from inexpensive starting material. It should have inherent properties of being recyclable, stablility to heat, light, and moisture and peptide synthesis reaction conditions. At the same time after the completion of peptide synthesis it should be easy to remove and purify. The protective group should be usable at large industrial scale processes. Due to the sensitivity of the cysteine molecule toward oxidation and elimination,

it usually is necessary to protect *β*-sulfhydryl function in addition to the amino group or the carboxyl group during peptide synthesis.

Even the protected Cysteine is susceptible to several side reactions. The commonly encountered side reactions of Cysteine during peptide synthesis are listed below.

1. Oxidation and alkylation: With thioether protection, cysteine can easily undergo oxidation and alkylation. Although this is less critical than in the case of Methionine, but it can occur.[38-40] The Cysteine residues can be protected against oxidation at deprotection stage by using 10% of water as scavenger.[41]

2. Elimination: Protected Cysteine on exposure to strong bases, such as sodium in liquid ammonia (Benzyl group removal), alkaline conditions, or hydrazynolysis, or exposed to strong acids such as HF can easily undergo elimination. The extent of elimination also depends strongly on the type of protecting group being used. The S*t*Bu is the worst case of elimination followed by Acm and Trt.[42, 43]. The Bn group used for protection can also produce elimination.

3. Reaction of Cysteine with carbocation: Carbocations generated at the deprotection step in acidic conditions in peptide synthesis can react with Cysteine. Formation of *S-tert*-butylated Cysteine has been observed after the removal of the Boc group or after deprotection in an Fmoc/*t*Bu strategy.[44]

4. Resin reattachment in the acidolytic cleavage resin-bound carbocations are generated that can react with both protected and unprotected Cysteine, thus causing reattachment of the peptide to the resin.[45]

5. Transfer of Acm (acetamidomethyl) group to Ser, Thr, Gln, and Tyr during Acm removal.[46-48]

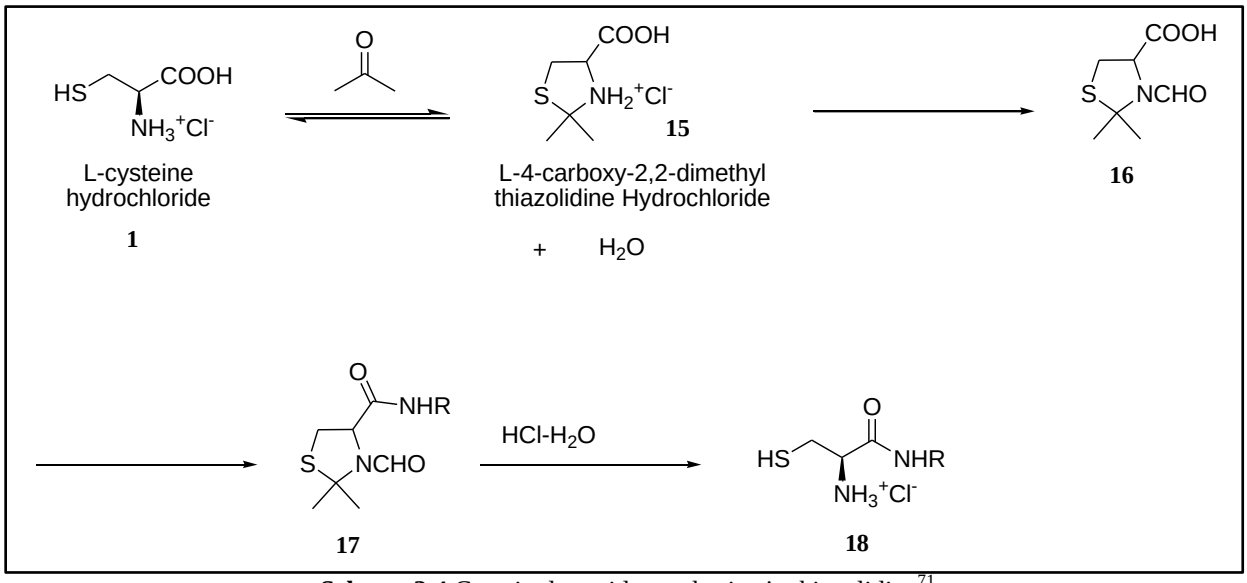
6. Thiazolidine formation: Thiazolidines of *N*-terminal Cysteine can result from Histidine-protecting groups that generate formaldehyde such as Bom (benzyloxymethyl) or Bum (*tert*-butyloxymethyl), when removed. Although this can be minimized using cysteine as scavenger.[49]

7. Racemization: Cysteine is highly prone to racemize during the anchoring to the solid support or during the couplings.[50] The extent of the racemization also depends on the *S*-protecting groups (S*t*Bu > Trt > Acm > MeBn > *t*Bu)[51,52] and coupling methods used (favored if preactivation in the presence of base is performed and in the coupling methods involving the use of base). Epimerization of the Cysteine linked to a hydroxyl resin can even take place during the synthesis as a result of the repetitive base treatments to remove the Fmoc group, with 2-chlorotrityl resin being the least prone to this process.[51-53]The most used protecting groups for the Fmoc/*t*Bu strategy are the Acm or Trt groups, when the desired product is the disulfide, and the Trt group, when the desired product is the free thiol. For the Boc/Bn strategy, the most used are Bn and Meb (*p*-methylbenzyl) to obtain the free thiol and Acm to obtain disulfides.

3.5.1.1. Thiazolidine protection

The synthesis of cysteinyl peptide is customarily carried out by blocking amino group usually by the N-carbobenzoxy procedure and transforming the *β*-sulfhydry1 group to a *S*-benzyl thioether[33] or oxidation to the cysteinyl derivative[34, 35]. After the condensation is complete, both the carbobenzoxy group and the S-benzyl group or the disulfide linkage can be cleaved readily by reductive means[36]. Of these several protective group methods for sulfhydryl protection of L-cysteine either they utilize harsh deprotection methods or lead to disulfide formation. In order to avoid these harsh deprotection conditions the procedure reported by Sheehan[71] *et al.* (scheme

3.4) was considered to provide an edge over these methods by not using the conventional reductive methods for deprotection after completion of peptide synthesis.



Scheme 3.4 Cysteinyl peptide synthesis *via* thiazolidine[71].

In our first attempt to prepare cysteinyl peptide we started by employing the procedure reported by Sheehan[71] *et al*. The procedure involved introduction of isopropylidine group that protects the sulfhydryl group from oxidation and tends to stabilize the Cysteine moiety against *β*-elimination. Since, the formation of the thiazolidine from cysteine and acetone involves a reversible equilibrium, the isopropylidine group can be removed readily in the presence of water. Using this approach, we were able to produce large quantities of L-4-carboxy-2, 2-dimethyl thiazolidine HCl **15** in excess of 98% yield, which was then used for further peptide synthesis as shown in Scheme 3.5.

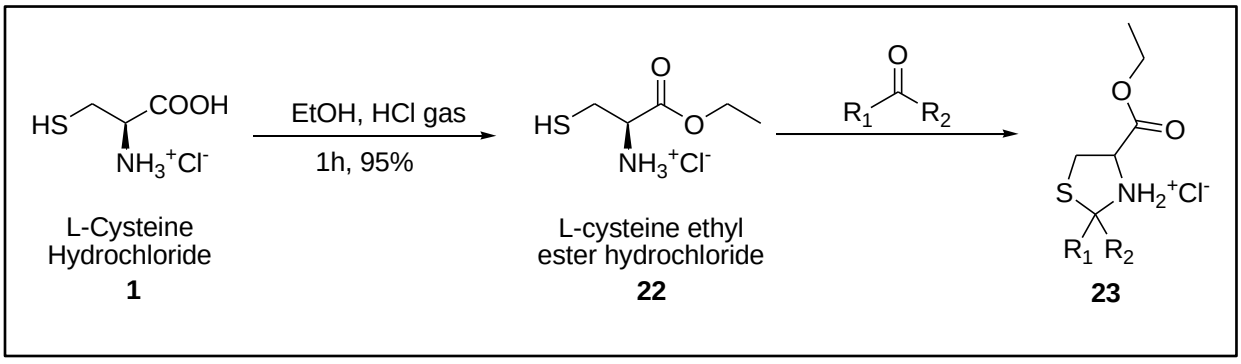
In order to improve handling of compounds a modification in physical properties is very helpful which can be achieved by functional group transformation. In case of L-cysteine HCl it can be achieved by transforming the free carboxyl group of the thiazolidine **15** to an ester. This

would make the handling of compounds better, change their physical properties, and improve stability. The peptide synthesis was then explored using the corresponding esters of L-4-carboxy-2, 2-dimethyl thiazolidine HCl (Table 3.4). Of the several esters synthesized using, methyl, ethyl, butyl, octyl alcohol. Only the butyl ester was found to be the best in terms of solubility, and purification.

S.No	**Alcohol**	**Product**	**Solubility in organic Solvents**
1.	Octanol	Cys octyl ester HCl (**19**)	Soluble
2.	Butanol	Cys butyl ester HCl (**20**)	Soluble in DCM
3.	Ethanol	Cys ethyl ester HCl (2**1**)	Insoluble in DCM, Soluble in Water

Table 3.4 L-cysteine ester and their solubility

The free NH group of the thiazolidine moiety **23** is readily available for peptide coupling in both the carboxy and the ester derivatives of thiazolidine **15** and **23** respectively (Scheme 3.5). It was observed that the thiazolidine protection reaction proceeds fairly easily with quantitive yields irrespective of the fact if the protection is done on L-cysteine hydrochloride **1** or its ester derivative **22** (Scheme 3.5).



L-Cysteine Hydrochloride **1**

L-cysteine ethyl ester hydrochloride **22**

23

Scheme 3.5 Synthesis of thiazolide ester derivatives

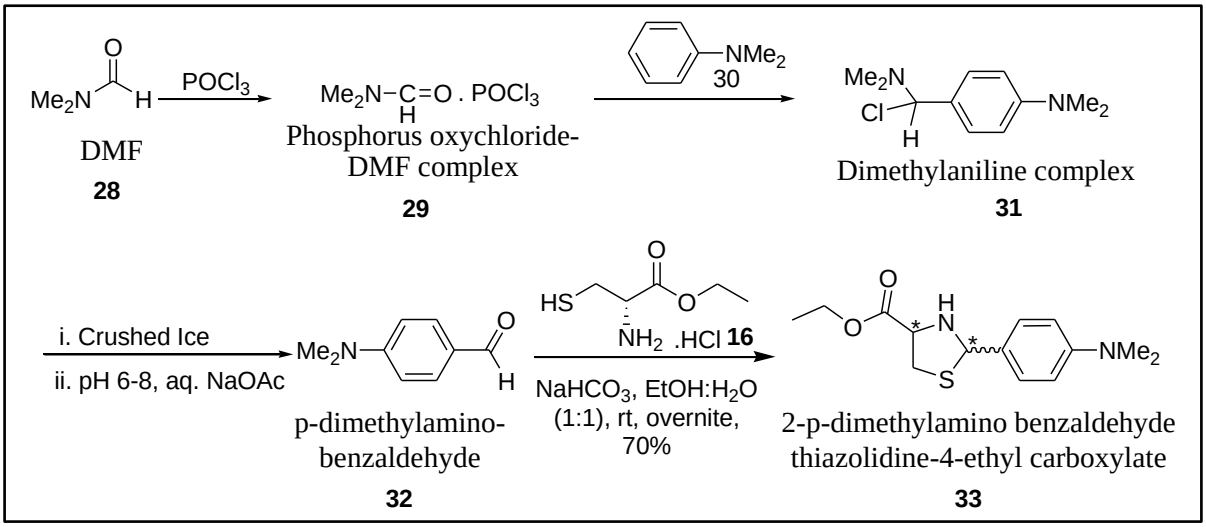
Even though thiazolidine protection reactions were carried out relatively easily but they suffer from several problems. Thiazolidine protection is stable as hydrochloride salt form, *in situ* generation of free NH is better for a reaction. The purification of the obtained peptide is difficult and often requires lyophilization. Since the isopropylidine group is not UV active monitoring of reaction was a tedious task. The group seems to be highly moisture, pH and heat sensitive (decomposition observed at 80 °C).

Therefore, in our pursuit to find a better sulfhydryl protective group several electron rich aromatic aldehydes or ketones were explored for thiazolidine formation using the procedure shown in scheme 3.6. As a model reaction benzaldehyde was used to give the corresponding thiazolidine derivative 25 (Table 3.5). Further, functionalized aromatic compounds were employed to synthesize aromatic thiazolidine derivatives. The thiazolidine synthesis was carried out under basic conditions using water and ethanol in a 1:1 mixture as solvent. The reaction was performed at room temperature and the product separated as a precipitate at the end of completion of reaction. Reaction with benzaldehyde gave the corresponding thiazolidine **25** with formation of daistereomers. Thiazolidine formation with 2,3,4-trimethoxy benzaldehyde proceeded with an equilibrium and yielded diastereomers. No product formation was observed using 2,4,5-trimethoxy benzaldehyde whereas in case of benzophenone pinacol was the only isolated product. The results are summarized in Table 3.5.

Entry	R_1 and R_2	Reaction Condition	Product (yield)
1.	R_1=R_2=-CH_3 (**24**)	Reflux, 6h	Equilibrium (98%)
2.	R_1= Phenyl (**25**) R_2=-H	EtOH-H_2O-$NaHCO_3$, 8h	Diastereomers (86%)
3.	R_1= 2,4,5-trimethoxy phenyl R_2=-H (**26**)	EtOH-H_2O-$NaHCO_3$, 8h	No product
4.	R_1= 2,3,4-trimethoxy phenyl R_2=-H (**27**)	EtOH-H_2O-$NaHCO_3$, 8h	Equilibrium, Diastereomers (65%)
5.	R_1=R_2=Phenyl (**28**)	EtOH-H_2O-$NaHCO_3$, 4h	Pinacol

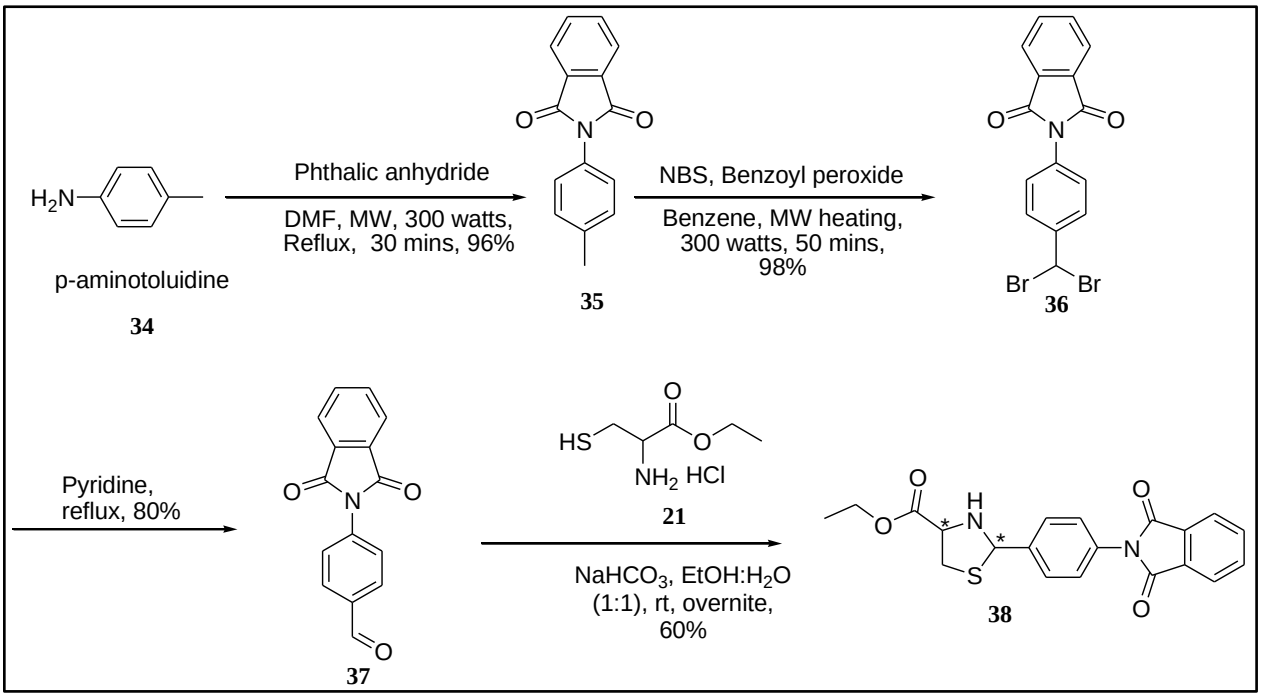
Table 3.5 Thiazolide ester derivatives

To have better understanding and establish a procedure for thiazolidine synthesis using L-cysteine hydrochloride another substrate used was *p-N,N* dimethyl amino benzaldehyde **32.** The synthesis of compound **32** was done as per standard method[71]. This was used for thiazolidine synthesis from L-cysteine HCl[72] under basic conditions in a mixture of Ethanol:water (1:1) as solvent for the reaction. The reaction proceeded well and the compound **33** was obtained as the desired product, which separated out a yellow colored precipitate (Scheme 3.6).



Scheme 3.6 Synthesis of aryl thiazolidine from *p*-dimethylaminobenzaldehyde

After successfully establishing procedure for aromatic thiazolidine synthesis several isoindole-1,3-dione derivatives were synthesized and employed as protection groups. These derivatives are discussed in detail in chapter 4. The isoindole-1,3-dione derivatives were synthesized under microwave reaction conditions. A representative example of this is outlined in Scheme 3.7. Microwave reaction were performed on 4-aminotoluidine **34** to give the corresponding compound **35**. This was dibrominated to give **36** and subsequently hydrolysed to give the corresponding aldehyde **37**. Compound **37** on reaction with ethyl 2-amino-3-mercaptopropanoate Hydrochloride **16** gave the corresponding thiazolidine derivative **38** (Scheme 3.7).

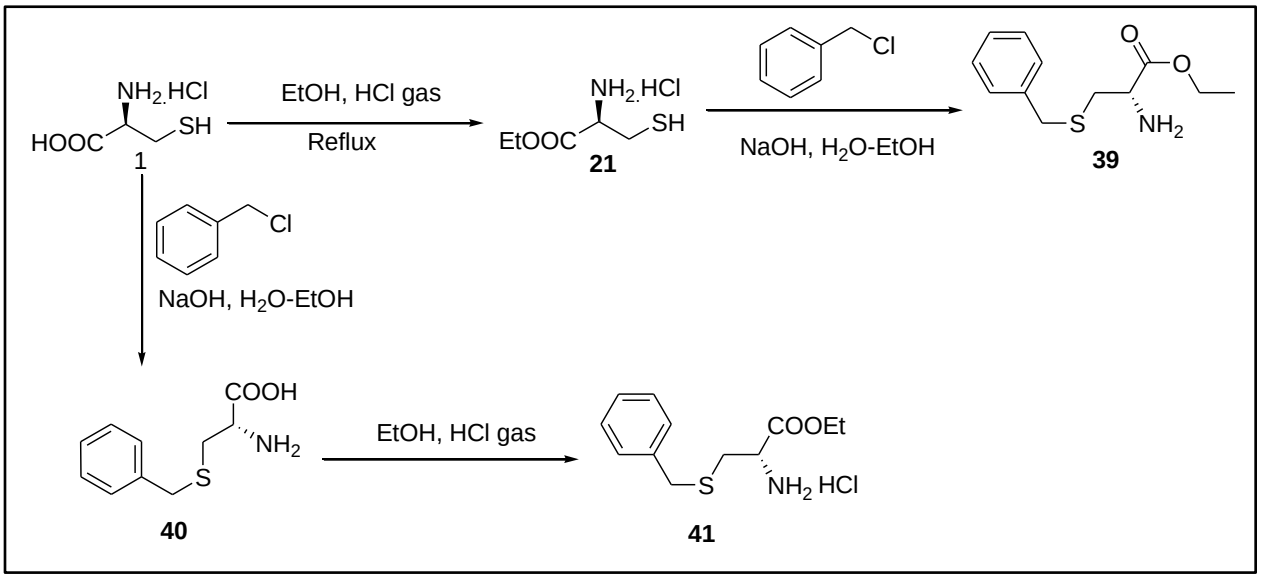


Scheme 3.7 Phthalate protected *p*-aminotoluidine derivatives as cysteine protection groups

Although various thiazolidine derivatives of L-cysteine hydrochloride were successfully synthesized, but a common problem encountered during this synthesis was racemization of the final product in most of the cases. Converting L-cysteine to isoproylidine derivative using acetone as protection for Cysteine racemization was highly evident and desired peptide synthesis could not be achieved as this group was too labile for any further reaction. When using aromatic aldehydes and ketones for preparation of thiazolidine derivatives formation of diastereomers was observed most of the times. It was not desirable for peptide synthesis as the purification of resulting diastereomers was a challenge. Therefore, a selective β-sulfhydryl protection of L-Cysteine hydrochloride was explored.

3.5.1.2. Selective *β*-Sulphydryl Protection of L-Cysteine hydrochloride

In order to selectively protect the *β*-sulphydryl group of L-cysteine HCl **1**, carboxy group was transformed to the corresponding ester **21** (Scheme 3.8). A model reaction was carried out using benzyl chloride under basic conditions to yield product **39** in quantitative yield. The *S*-benzylation reaction was successfully carried out with both L-cysteine hydrochloride **1** and its ester derivative **21**.



Scheme 3.8 *S*-benzyl cysteine derivatives synthesis

A peptide synthesis attempted on compound **41** was successful but the subsequent deprotection could not be achieved without oxidizing *β*-sulfhydryl functionality. Various deprotection methods including the hydrogenation were unsuccessful in generating a free sulfur group. An oxidized Cysteine (cystine) was recovered in these attempts. In order to synthesize the novel cysteinyl metal peptides we required a protective group that is easily removed at the completion of peptide synthesis without oxidizing the free sulfhydryl to disulfide derivatives. Therefore, analogues of benzylbromide were explored (Fig. 3.9).

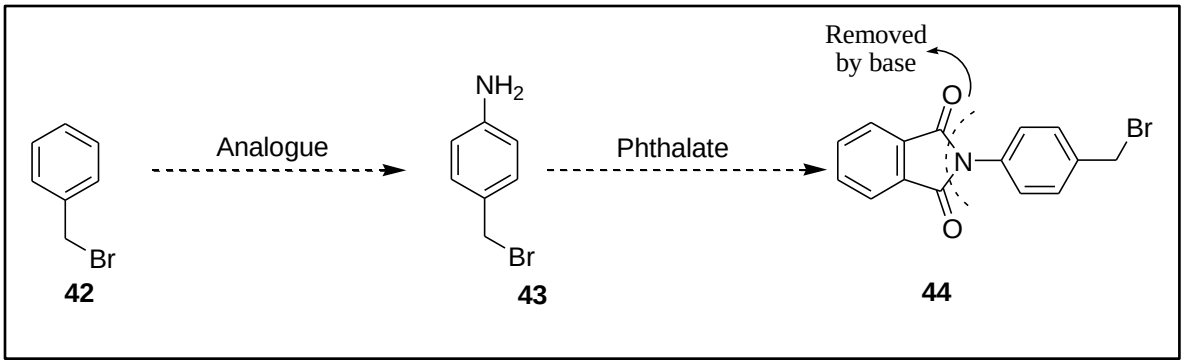
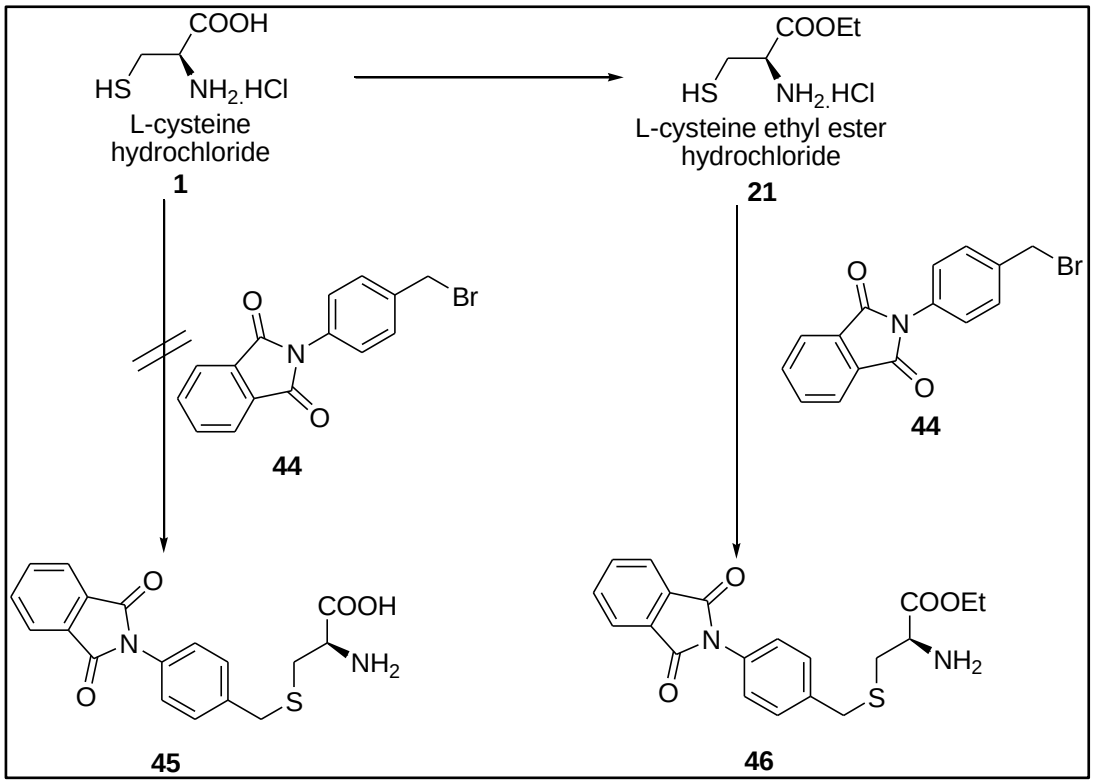


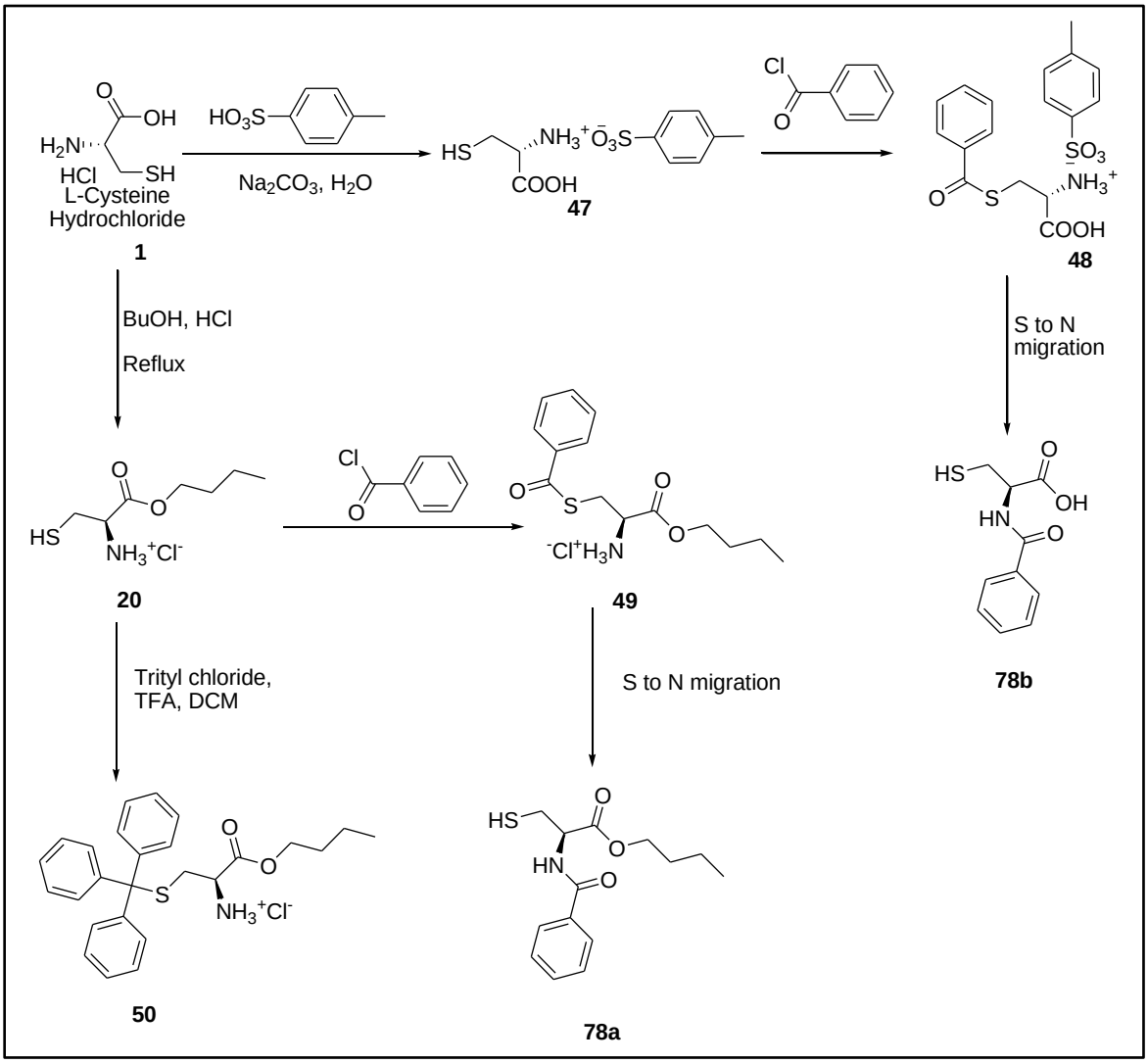
Figure 3.9 Proposed novel protecting group

The idea was to have a more labile group than benzyl such as phthalate protected amino benzyl bromide. After several trials, we successfully introduced compound **44** into **21** as a selective protection of *β*-sulfhydryl group (Scheme 3.9).



Scheme 3.9 Synthesis of phthalate based L-cysteine sulfur protection

Although we desire to use L-cysteine HCl for our reaction for selective *β*-sulfhydryl protection as a free carboxy group is required for metal complexation. But the free carboxy group changes the reaction pH along with limiting its solubility to polar solvent like water thereby making the selective sulfhydryl protection unachievable with 2-(4-(bromomethyl)phenyl)isoindoline-1,3-dione **44** under basic conditions. Conversion of the L-cysteine hydrochloride **1** to the corresponding ethyl ester **21** led to selective *β*-sulfhydryl protection under basic conditions. The problem was the insolubility of L-Cysteine hydrochloride in organic solvents. Therefore, to improve Cysteine solubility it was converted to tosylate and long chain esters. One such example of L-cystiene tosylate **47** for selective sulfhydryl is outlined in Scheme 3.10. Selective *β*-sulfhydryl protection of L-cysteine using trityl chloride (Scheme 3.10) and benzoyl chloride were successfully accomplished using the ester **20** and the tosylate **47** of L-cysteine hydrochloride **1**. These derivatives offered better solubility in organic solvents. The trityl group was found to be highly acid labile and unsuitable for peptide synthesis. For the desired peptide, synthesis at the *N*-terminal of compound **48** and **49** it was treated with a base. At this point, it was observed that the benzoyl group migrated from sulfur to the nitrogen to give compounds **78a** and **78b**. This is commonly known as Sulfur to Nitrogen migration. As Et_3N was added, new peaks appeared in NMR. Sulfur to Nitrogen benzoyl group migration was further explored for peptide synthesis and will be discussed later in this chapter.

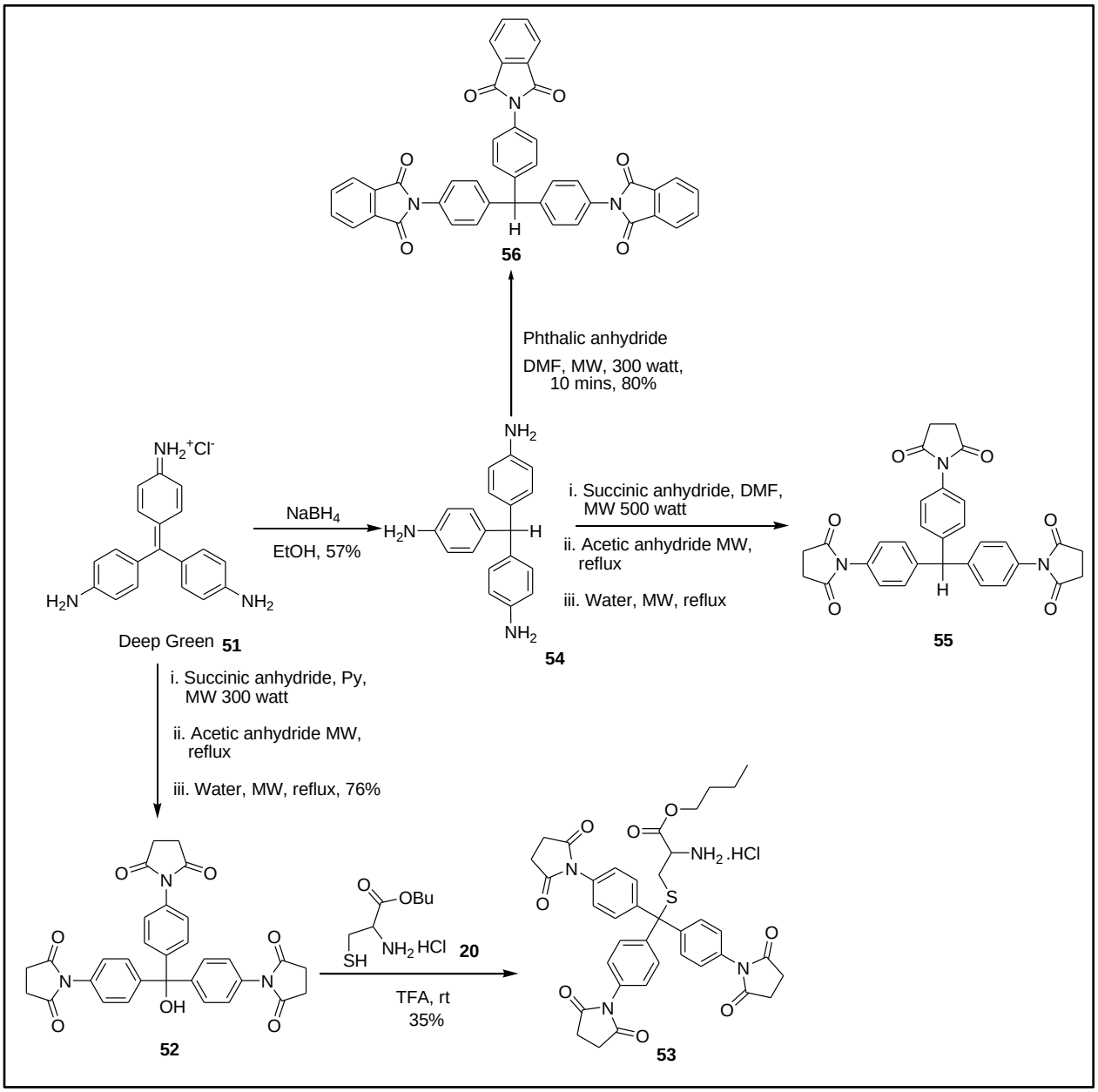


Scheme 3.10 Synthesis of sulfhydryl protection group

In our continued pursuit to find new protective groups pararosaniline was selected as a substrate to synthesize new protective groups. The rationale to choose pararosaniline is that it has three amino groups that can be protected with a suitable protective group and then introduced on L-Cysteine as selective *β*-sulfhydryl protection group. This would make the resulting L-cysteine derivative more stable for peptide synthesis compared to trityl group. For the deprotection the amino protection could be removed either under basic condition or by use of hydrazine hydrate. The free amine groups would stabilize the carbocation of the pararosaniline derivative thus

making it easier at the deprotection step. The amino protective group would provide stability under acidic conditions and make it more stable under low pH compared to trityl group. Therefore, it was chosen as an alternative to trityl to provide facile protective groups for our synthesis of target molecule.

Pararosaniline **51** has three free amino groups that were protected as phthalate (Scheme 3.11). Pararosanilne **51** was reduced using sodium borohydride to the corresponding derivative **54**. It was treated with phthalic anhydride under microwave conditions to yield compound **56** in ten minutes at microwave power of 300 watt. The resulting compound was not reactive enough to selectively protect the β-sulfhydryl group of L-cysteine hydrochloride (Scheme 3.11). Phthalate was then replaced with succinate and tetrachloro phthalate derivatives to get better results. Reaction of **54** with succinic anhydride under microwave power of 500 watt yielded compound **55** with better solubility. A further reaction with L-cysteine derivative did not yield the desired β-sulfhydryl protection because of lack of functionality at the tertiary carbon of **55**. Attempts to functionalize the tertiary carbon were unsuccessful. Therefore, pararosaniline hydrochloride **51** was directly reacted with succinic anhydride to yield compound **52**. A further reaction of **52** with 20 gave the desired compound **53**.



Scheme 3.11 Pararosaniline derivatives as selective β-sulfhydryl protective group

The obtained compound was further purified and characterized by NMR. A proton NMR spectrum of compound **53** is presented in Fig. 3.10.

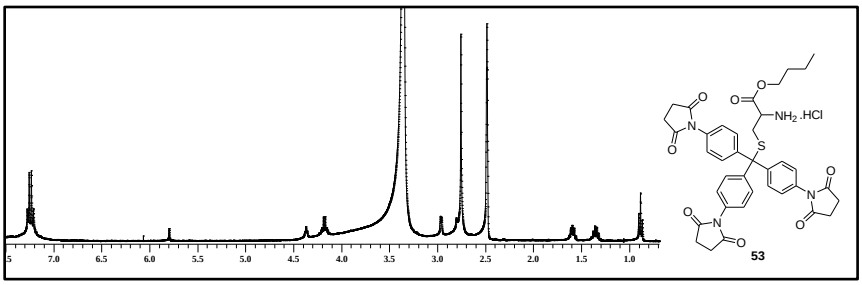
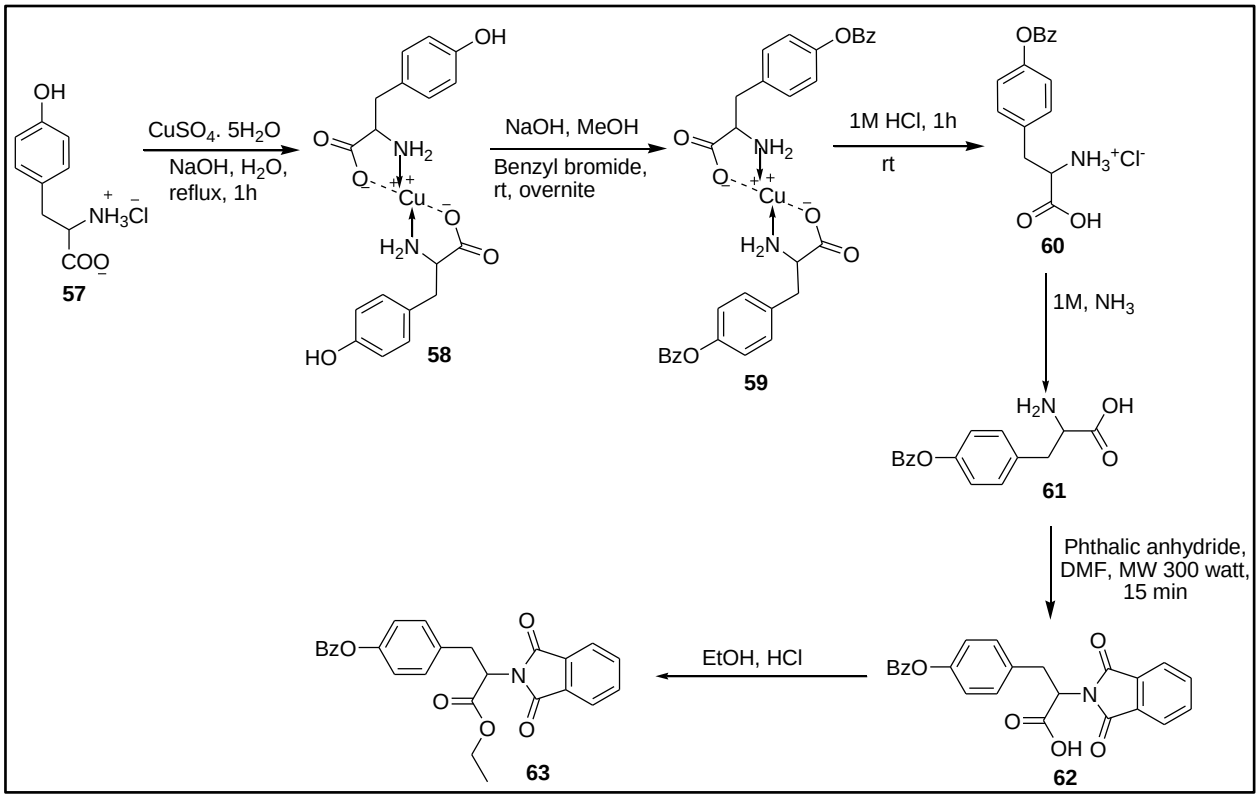


Figure 3.10 Protected cysteine compound **53** ^{1}H NMR

3.5.2. Protective groups for Tyrosine

The alkyl ether synthesis for L-tyrosine as reported by Weiss and co-workers[82] requires the protection of the amino group by formylation, and the over-all yield of the three-step method is only 30-40%. Earlier, Aberhalden and Guggenheim[83] reported the synthesis of the glycerine monoether, in unspecified yield, through the disodium salt of tyrosine. Solar[81] *et al.* reported complex method of alkylating L-tyrosine using DMSO under heating conditions with a yield of approximately 40%. Yet another method reported by Wunsh E *et al.*[85] and used by Capler *et al.*[84] is ether synthesis using copper complex formation. We were successfully able to apply the copper complex method to our synthesis that resulted in the overall yield of 70%-80%. Reacting L-tyrosine **57** with copper sulfate under basic conditions forms a copper complex **58** as outlined in Scheme 3.12. This mixture was then heated to 60 °C then cooled to room temperature, diluted with methanol and made more basic using sodium hydroxide. A further reaction with benzyl bromide to give **59** and a subsequent copper complex disintegration under acidic condition and neutralization with ammonium hydroxide gave the desired compound **60**. Free amino group in the compound **61** was protected as phthalate using microwave assisted phthalate synthesis. Benzyl protected L-tyrosine **61** was reacted with phthalic anhydride in a microwave at power of

300-400 watt for 15 mins to give a crude product **62**. Recrystallization was done using DCE to get a pure compound. A further esterification gave compound **63** (Scheme 3.12).



Scheme 3.12 Copper-mediated Tyrosine protection[84]

Benzyl ether protection using copper complex method served as a model reaction. We wanted to introduce novel protecting groups that are easier to remove at the end of synthesis. Therefore, amine group was protected as phthalate in L-tyrosine **57** and carboxy was converted to ester **64** outlined in Scheme 3.13. Microwave phthaloylation for both compounds **57** and **64** progressed well to give compounds **65** and **66** respectively. Compound **65** was further purified by recrystallization from dichloroethane and the NMR spectra are presented in Fig. 3.11.

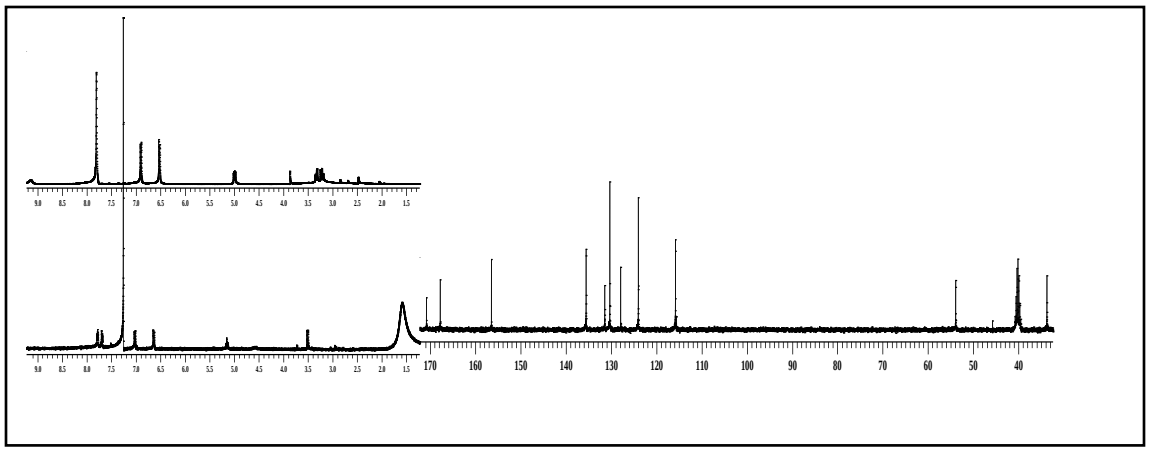
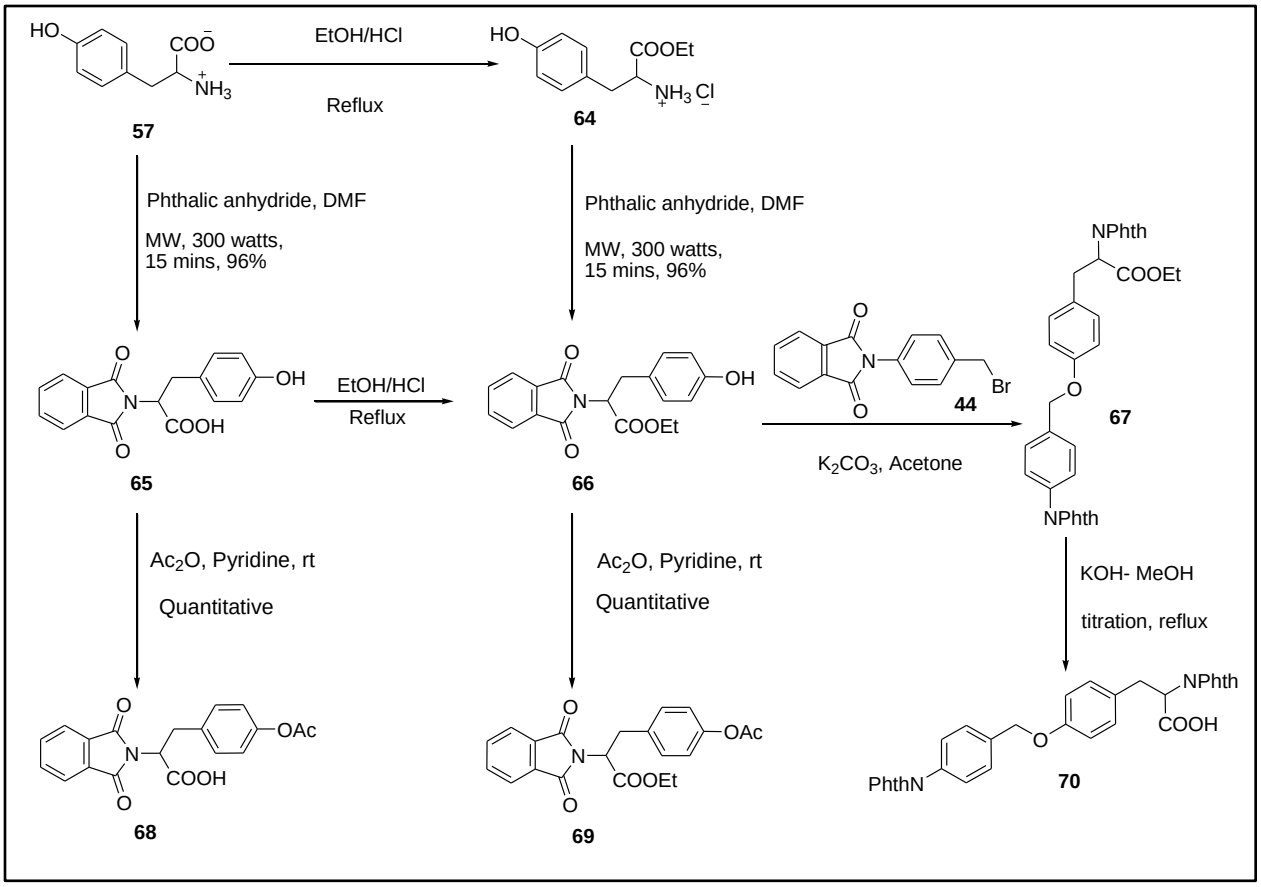


Figure 3.11 Protected tyrosine compound **62** ^{1}H and ^{13}C NMR

The hydroxyl group of **65** and **66** was selectively protected as acetate to give corresponding **68** and **69** (Scheme 3.13). Since the peptide bond formation was desired at the carboxy terminal of protected tyrosine, attempt to selectively hydrolyse ester led to removal of O-acetate in compound **69**. Therefore, compound **68** was prepared from **65** and used for peptide synthesis Scheme 3.13.



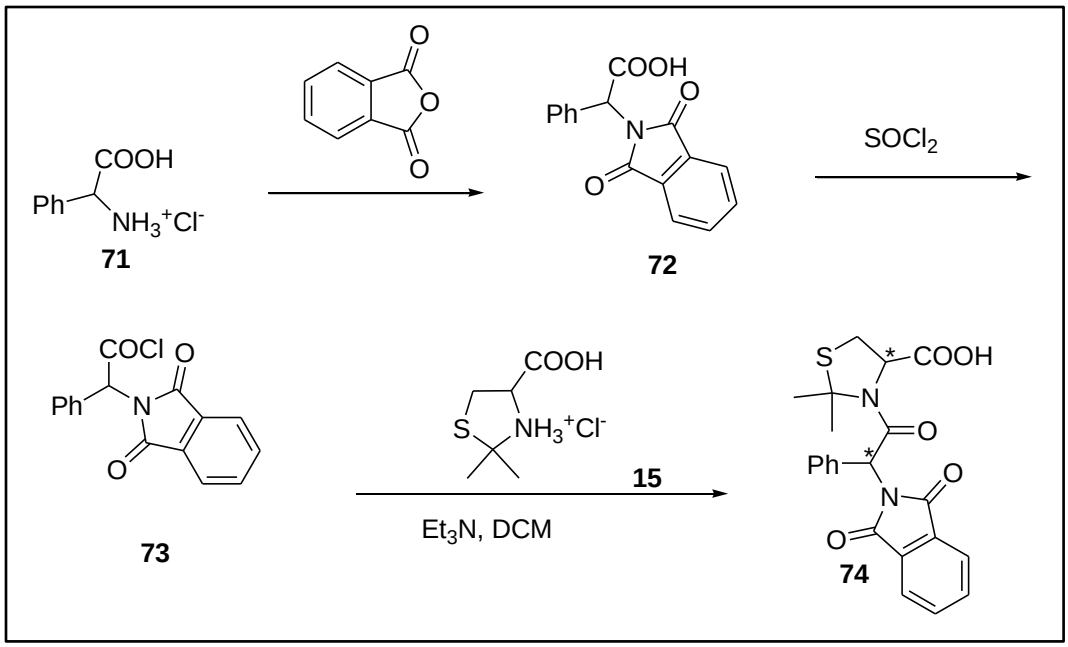
Scheme 3.13 α-carboxyl and amine protection in L-tyrosine

The successful results in protection of L-cysteine hydrochloride with our protective group, we were encouraged to introduce the same protecting group for L-tyrosine. This would provide the advantages at the deprotection step after peptide synthesis as all the protecting groups can be removed in one step under same reaction conditions yielding the desired peptide. Synthetic challenge for L-tyrosine protection was to specifically introduce the phthalate imide derivative **44** as a hydroxyl protective group. After several unsuccessful trials the best reaction conditions were found to be refluxing acetone using potassium carbonate as base to make the L-tyrosine ether compound **67** (Scheme 3.13) The compounds were further purified by crystallization and column chromatography as required and taken to peptide synthesis.

Selective ester hydrolysis of **67** was subsequently carried out under various basic conditions to find the optimum conditions. Under drastic basic conditions, phthalate hydrolysis was observed. After several experimental failures, the reaction was finally accomplished by using potassium hydroxide-Methanol titration under reflux conditions to give compound **70**. The reaction had to be performed carefully with titration method to avoid any unnecessary decomposition. The hydrolyzed pure compound **70** precipitated out of the reaction medium as the reaction progressed. L-Tyrosine was successful protected using efficient and industrially viable method to give the protected L-tyrosine compounds using the novel protective group **44**. The reactions were reproducible and no racemization was observed under these conditions.

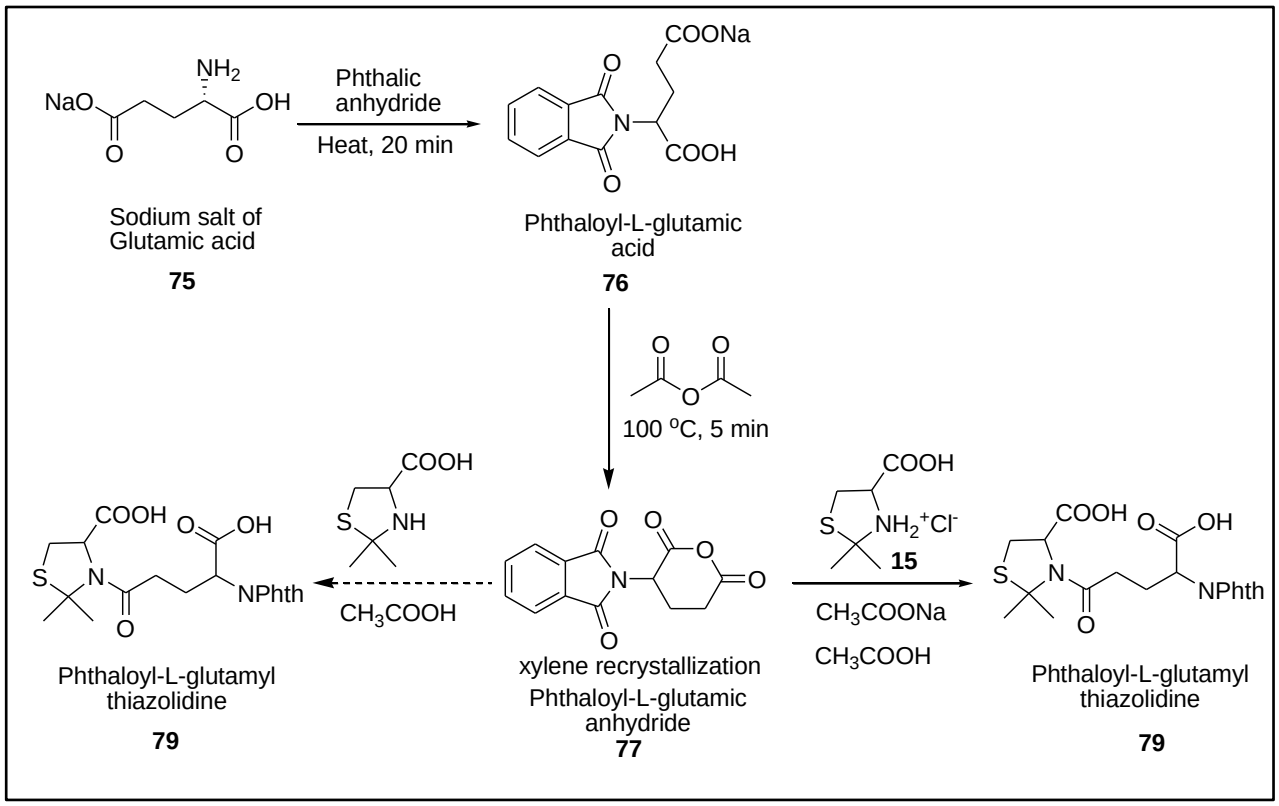
3.5.3. Peptide Synthesis

The sensitivity of the cysteine molecule towards oxidation and elimination makes it necessary to protect the β-sulfhydryl group during the peptide synthesis. We explored the peptide coupling by protecting the reactive β-sulfhydryl group of cysteine as a thiazolidine. This eliminated the need to protect the amino group separately and reduced the number of steps in peptide synthesis. The thiazolidine **15** was reacted with phthalate protected phenyl glycine **72** (scheme 3.14). Activation of the carboxy terminal of compound **72** was achieved by conversion to the corresponding acid chloride by using oxalyl chloride to yield compound **73** (Scheme 3.14). The reaction resulted in formation of the desired peptide **74** but with racemization. The product was obtained as mixture of diasteromers. Since an enantiomerically pure product was desired further purification remained a challenge and the diastereomer separation could not achieved at this stage.



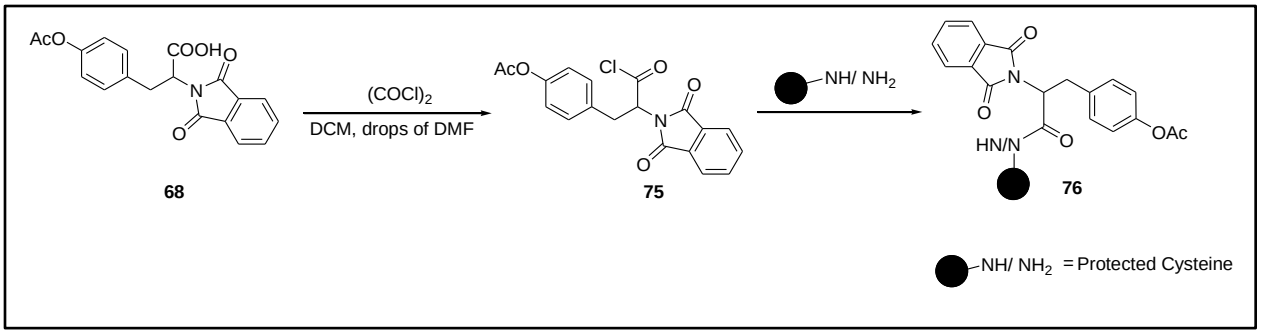
Scheme 3.14 Thiazolidine and cysteinyl phenyl glycine peptide synthesis.

Second peptide coupling reaction was attempted using glutamic acid **75** (Scheme 3.15). Phthaloyl-L-glutamic anhydride[71] **77** was synthesized from sodium salt of L-glutamic acid **75** without the intermediate isolation of phthaloyl-L-glutamic acid **76**. Recrystallization from xylene provided compound **77** in good yield and sufficient purity that it was taken to next step without further purification. Compound **77** on reaction with thiazolidine **15** provides compound **79**. A racemization was observed in this reaction leading to formation of diastereomers and making separation of enantiomerically pure compound difficult. When reaction was performed using free –NH derivative of **15** no product formation was seen, whereas *in situ* generation of free –NH using sodium acetate and acetic acid to maintain the pH balance favored the formation of product **79**.



Scheme 3.15 Coupling of thiazolidine to L-glutamic acid

A limited success with in peptide coupling of L-glutamic acid with thiazolidine in Scheme 3.15 prompted to explore alternate methods of peptide synthesis. The challenge was to synthesize peptide by coupling the *N*-terminal of cysteine with the carboxy terminal of tyrosine. To achieve the desired peptide bond formation the carboxy group of tyrosine was activated by converting to acid chloride by reaction with oxalyl chloride in DCM with few drops of DMF. The resulting acid chloride was reacted with thiazolidine ester of L-cysteine (Table 3.5 entry 1) under basic conditions (Scheme 3.16). Using DMAP or triethylamine as a base peptide bond was formed but with racemization, more so with triethylamine. In addition, with the progress of reaction thiazolidine decomposition was observed as a side reaction (Table 3.6).



Scheme 3.16 Cysteine-tyrosine peptide synthesis

A series of reaction were attempted for cysteinyl peptide synthesis. The results are summarized in Table 3.6. Attempts to react the activated acid chloride of protected L-tyrosine **75** with unprotected L-cysteine (Table 3.6 entry 2) under basic conditions did not yield the peptide formation. With trityl protected sulfhydryl cysteine (Table 3.6 entry 3) the peptide synthesis went well. The deprotection under acidic condition to remove the trityl protection reversed the reaction yielding tyrosine, cysteine and other decomposition products including cystine. The benzyl protected L-cysteine (Table 3.6 entry 4) was reacted with L-tyrosine to give the desired coupled product. Attempts to remove benzyl protection under palladium hydrogenation were unsuccessful. A possible explanation for no observed debenzylation would be poisoning of palladium catalyst by cysteine sulfur. Any other strong basic conditions employed for deprotection were deleterious to the coupled product. Instead, it was observed that the butyl ester of L-cysteine HCl when reacted with acid chloride of protected tyrosine compound **68** peptide coupling was successful (Scheme 3.16). We also explored the peptide synthesis using our protective group **52**. L-cysteine hydrochloride was selectively protected to give compound **53** which was coupled to protected L-tyrosine **68** to successfully give the desired dipeptide (Table 3.6 entry 5).

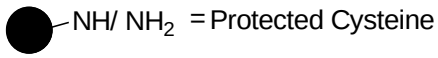
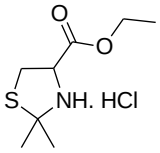
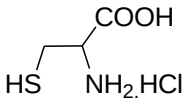
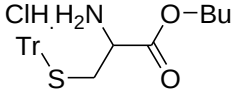
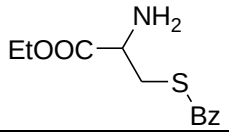
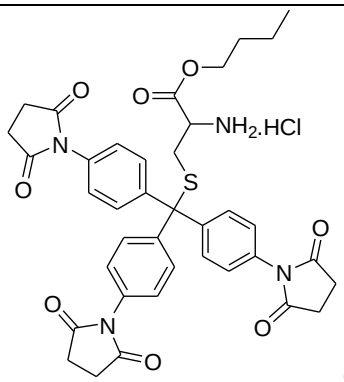
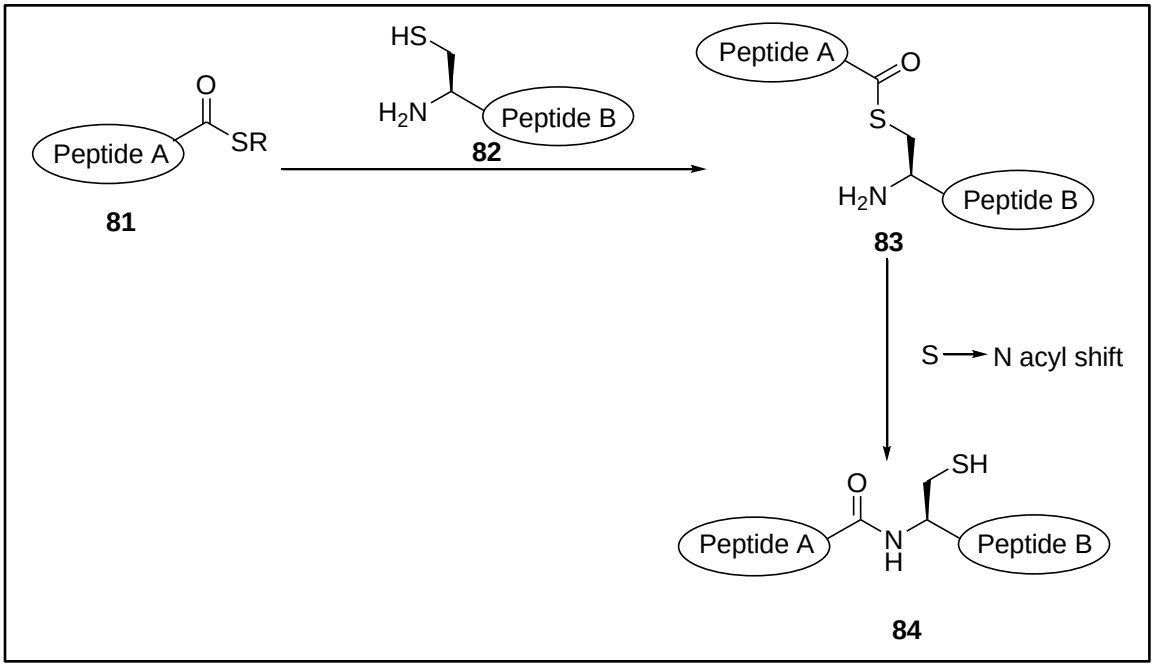
S.No.	Substrate 	Reaction Conditions	Peptide (product number)
1.	Thiazolidine ester 	K_2CO_3, DCM:THF	No Conversion
		DMAP, DCM:THF	Diastereomers (109)
		Et_3N, DCM	Product (110) (58%)
2.		NaOH, DCM:THF	No Conversion
		$NaHCO_3$, DCM:THF	No Conversion
3.		Et_3N, DCM	Product (111) (62%)
4.		Et_3N, DCM	Product (112) (62%)
5.	 (**53**)	Et_3N, DCM	Product (113) (71%)

Table 3.6 Cysteine-tyrosine peptide derivatives

Peptide synthesis involving selective functional group protection of amino acids, followed by activating carboxy functional group of one amino acid to add to the other is a promising way to synthesize peptide bonds. It suffers from several disadvantages in terms of removal of all protective groups at the end of peptide synthesis that lead to racemization and other issues. Protection deprotection of functional groups increases the total number of synthetic

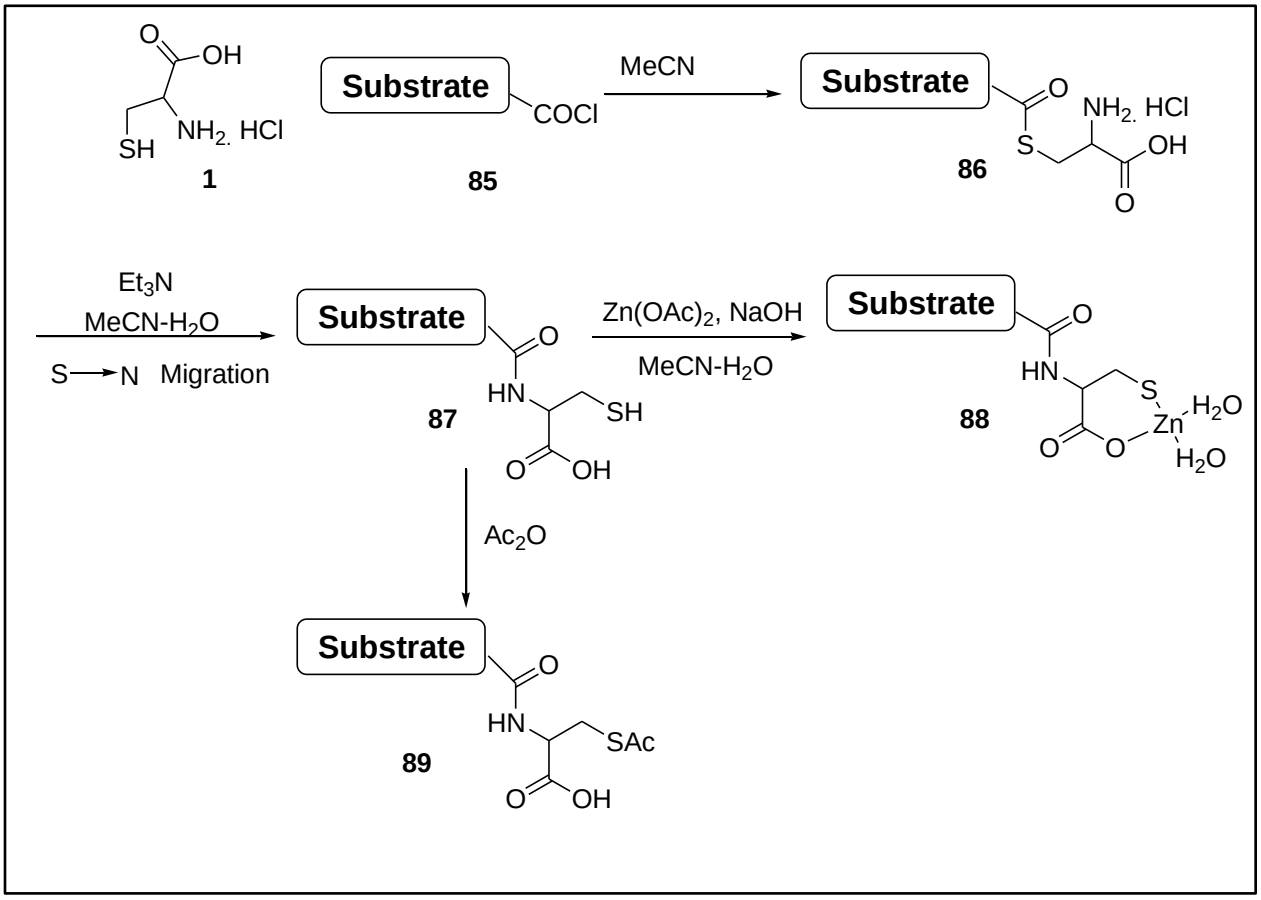
steps to arrive at the desired peptide molecule. There are other synthetic techniques mentioned in literature like Native Chemical Ligation (NCL)[74]. This strategy[75] is mostly used for synthesis of proteins, where a chemoselective reaction between two unprotected fragments i.e a C-terminal thioester and N-terminal cysteine occurs. As depicted in scheme 3.17 the thioester **81** undergoes trans-thioesterification with **82** to give **83**. This is a classical example of Sulfur to Nitrogen migration resulting in formation of peptide **84** having a free sulfhydryl group.



Scheme 3.17 *S*- and *N*- acylation of L-cysteine[76]

Based on the literature procedure of Katritzky *et al.*[76] we explored it for our peptide synthesis (Scheme 3.17). As a test reaction, *S*-benzoylation of L-cysteine HCl was performed (Table 6 entry 1). It was observed that under basic conditions the *S*-benzoyl group transfers to the amino functionality of L-cysteine giving compound **92** (Scheme 3.18). Since, we desired to trap the free sulfhydryl group as a metal complex, a further reaction with Zinc acetate under basic conditions

led to the formation of desired compound (Scheme 3.18). The overall reaction is summarized in Scheme 3.18.



Scheme 3.18 Cysteinyl metal peptides

Similarly, various substrates were explored and results are summarized in Table 3.6. The peptide synthesis of acid chloride of phthalate protected L-tyrosine (Table 3.6 entry 3) and phenyl glycine (Table 3.6 entry 2) with L-cysteine hydrochloride successfully yielded compounds **98** and **95** respectively with intermediate *S*-acyl formation. The reaction was carried out in

acetonitrile resulted in formation of *S*-acylated compound, which under basic conditions transforms to the desired peptide via *S* to *N* migration.

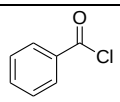
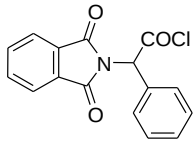
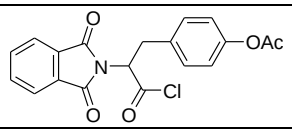
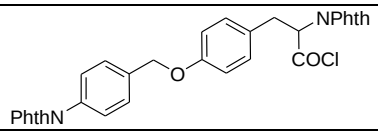
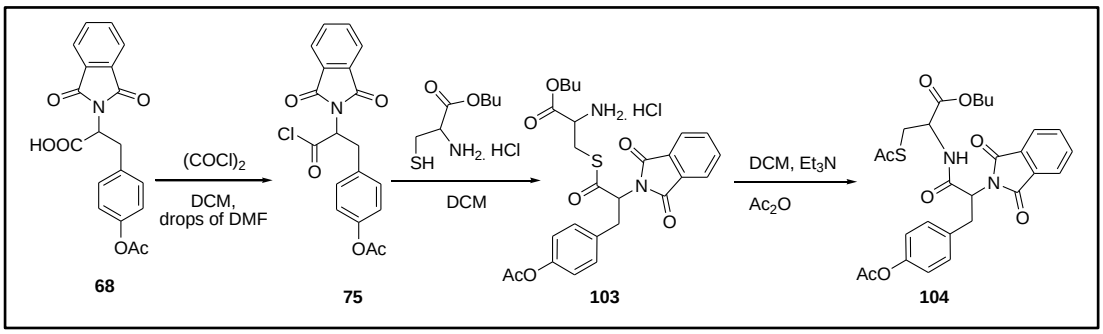
S.No	Substrate	*S*-Acylation percentage yield (product number)	*S* to *N* migration percentage yield (product number)	Zinc complex percentage yield (product number)
1.		65 (91)	65 (92)	72 (93)
2.		72 (94)	62 (95)	75 (96)
3.		71 (97)	65 (98)	70 (99)
4.		60 (100)	60 (101)	72 (102)

Table 3.7 Cysteinyl metal peptides derivatives

For better solubility of L-tyrosine derivatives and L-cysteine hydrochloride derivatives, we employed the butyl ester of L-cysteine hydrochloride (Scheme 3.19). Compound **68** was transformed to its corresponding acid chloride **75** via reaction with oxalyl chloride. This on subsequent reaction with L-cysteine butyl ester hydrochloride led to formation of compound **103**. Under basic conditions, *S* to *N* migration was achieved. Free sulfur was captured by a further reaction with acetic anhydride to give compound **104**.

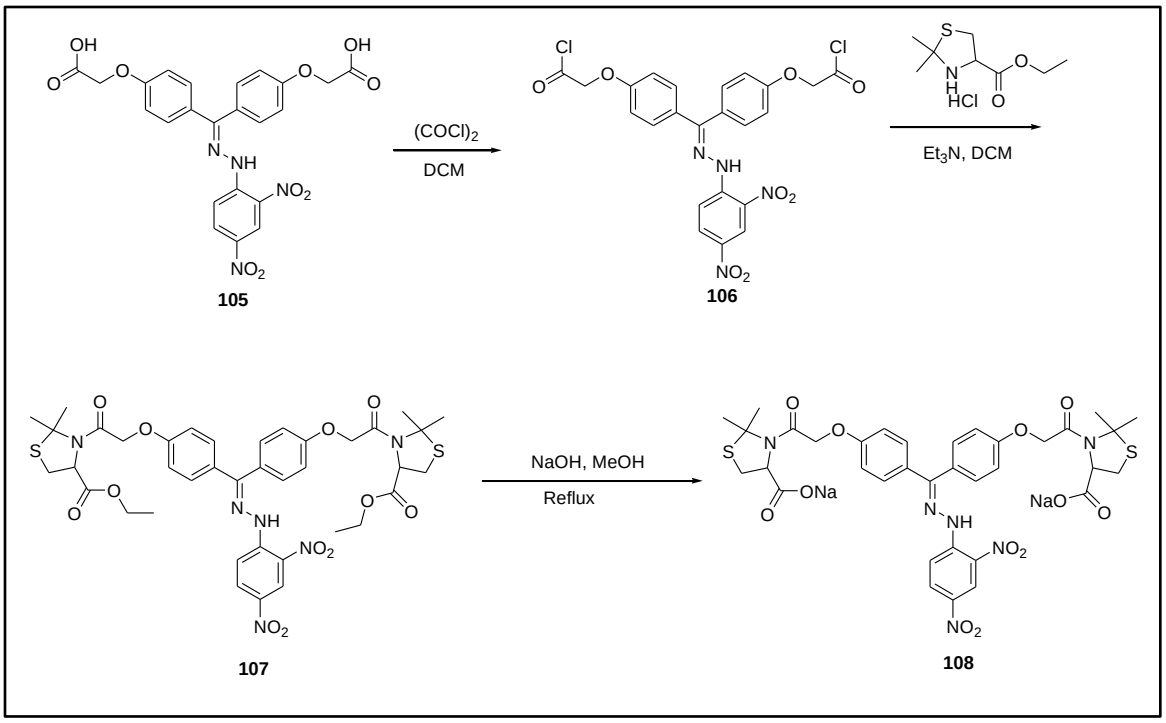


68 **75** **103** **104**

Scheme 3.19 *S* to *N* migration in butyl ester of L-cysteine

L-tyrosine derivative **68** was successfully incorporated to give L-cysteine based peptide with a free sulfhydryl group. The phthalate protected L-tyrosine was converted to acid chloride **75** and reacted with L-cysteine hydrochloride to give the corresponding thioester **103**. This under basic conditions undergoes *S* to *N* migration to give the desired product. At this point we wanted to confirm the presence of free thiol group therefore, a reaction with acetic anhydride was attempted which successfully gave *S*-acylated product **104**. The free sulfhydryl was then trapped as a metal complex by reaction with zinc acetate under basic conditions for peptide metal complex formation (Scheme 3.18).

Further exploration of phenols as radical capture agents was performed on anticancer agent A007. Studies indicated that it has potential to act as a radiation protection agent. To futher its ability as a radiation protection agent we successfully incorporated thiazolidine ester of cysteine in A007 structure (Scheme 3.20) to further explore its biological activities.



Scheme 3.20 A007-cysteine coupling reaction

Acid chloride **106** of compound **105** was obtained by reaction with oxalyl chloride. A further reaction with thiazolidine ethyl ester gave compound **107**. The ester hydrolysis was achieved by careful hydrolysis using sodium hydroxide and methanol under reflux conditions to give compound **108**. A further purification of compound **108** was achieved by fractional crystallization and careful stirring with silica gel.

3.6 Biological studies

The initial studies to understand the radiation exposure capability of compounds was conducted on phenolic compound namely A007. Mice were exposed to radiation for several days. This interferes with the white blood cell (WBC) count, which was monitored. Mice were given A007 and then exposed to radiation for several days and now their WBC count was monitored. The study revealed that the mice given A007 had their WBC count return close to

normal compared to those who were not given A007. The WBC count was monitored for days after radiation exposure and a graph representing the outcome is shown in Fig.3.12.

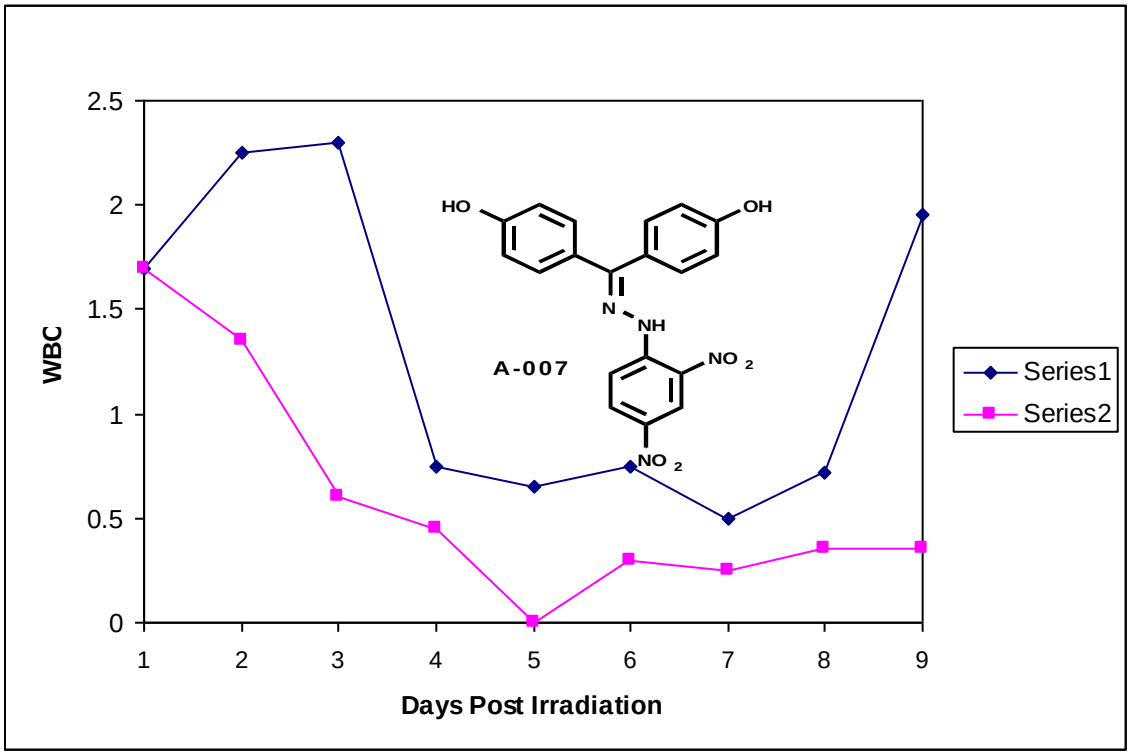


Figure 3.12 A-007 as radical scavenger (courtesy Dr. Lee Roy Morgan)

These results clearly indicate that A007 is acting as a radical capture agent. It was postulated that the free hydroxyl groups in A007 was able to scavange the free radicals thus returning the WBC count of mice to normal much faster than those not given A007. It is speculated that the inherent ability of A007 to capture free radicals is attributed to its aromatic nature and presence of hydroxyl functional groups. Once the free radicals are captured, they are stabilized over the aromatic system and avoid further reaction inside the animal models.

The biological studies of zinc monocysteine have shown to have promising anticancer activity. ZMC was used to treat the cancerous kidneys at 30 µM concentration. The kidneys were monitored continuously for cancerous growth. The images of the kidneys taken at day 7 of treatment with ZMC reveal the disappearance of cancerous cells (Fig. 3.13).

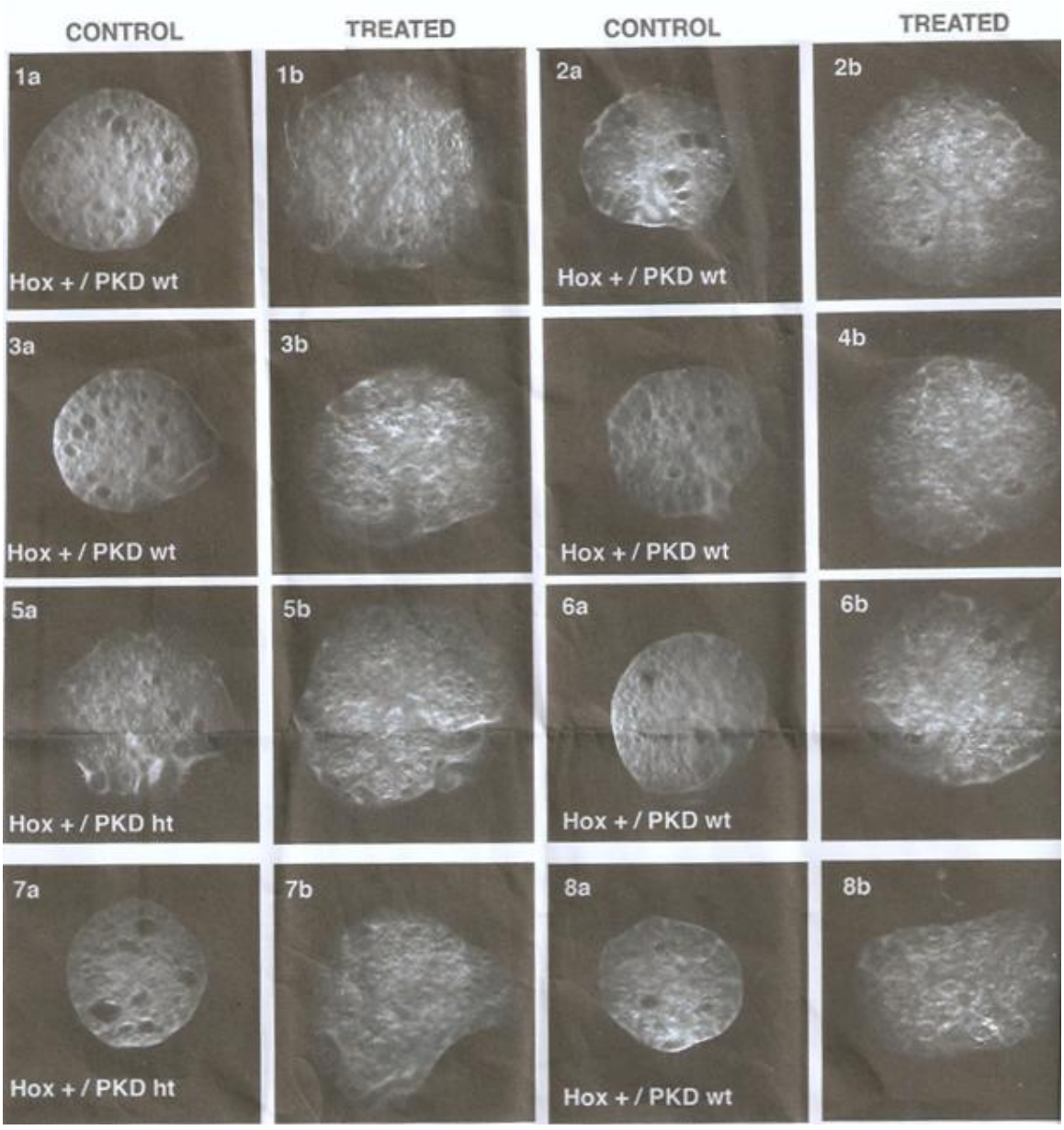


Figure 3.13 ZMC activity on cancerous kidneys: Kindneys were harvested at E13.5 and cultured for 24 hours in EOC media. At day 1 and 3 media was changed. 100µM 8-bromo-cyclic AMP was added to all cultures, and 30 µM Zincmonocysteine was added to contralateral (b) kidneys. Kidneys remained in culture until day 7 at which time images were taken. (courtsey Dr. David J Tate).

3.7 Conclusions

Reactive oxidative species (ROS) have been implicated in the pathogenesis of many chronic diseases, such as cancer, cardiovascular and neurodegenerative diseases therefore finding novel antioxidants to combat ROS has attracted a lot of attention in recent years. Amino acids like cysteine and tyrosine are the well-identified antioxidant pharmacophore among other hydroxyl rich compounds like catechol and A007. To explore the antioxidant activity A007 was studied for its effect in mice and was found to have radical capture ability. To this end A007 was successfully coupled to Cysteine derivatives to further study its biological activities.

Since cysteine cannot be used in its free form because the free β-sufhydryl group, it was complexed to several metals like zinc, calcium, cadmium and magnesium. These cysteine metal complexes are speculated to have better shelf life and stability. The commercial synthetic procedure for ZMC was improved to give better yields and improved methodology.

'Advanced Radical Capture Compounds' were synthesized by linking derivatized L-cysteine to L-tyrosine and other substrates and subsequently complexed to metal ions. In our effort to synthesize novel radical capture compounds containing L-cysteine and L-tyrosine we faced several problems of selective protection and deprotection of amino acids for peptide synthesis. To this end to meet the requirements of a suitable protective group in terms of stability, pH, moisture and heat sensitivity we successfully designed several protective groups and utilized them in our peptide synthesis. The *S* to *N* migration for cysteinyl peptide synthesis was explored with great success. Finally, the synthesized ZMC compound was tested for its biological activity. It was revealed to have anticancer activity in kidney cancer cells.

3.8. Experimental

General Experimental

All reagents and solvents were purchased from Sigma-Aldrich and were analytical grade. Thin-layer chromatographic analysis (TLC) was performed using silica gel on glass plates and was detected under ultraviolet (UV) light and using PMA, Ninhydrin, $KMnO_4$ solution. Column chromatography was performed using silica gel porosity 60 Å, partial size 40-75 mm from Sorbent Technologies. The ^{1}H and ^{13}C NMR spectra were run on Varian 400 MHz Unity in $CDCl_3$, D_2O, CD_3OD or DMSO-d_6 as solvents and internal standards.

Synthesis L-cysteine Zinc complex (2): Into hot water (2L) solution of zinc acetate (65.85g; 0.3 mol) and sodium acetate (54.4g; 0.4mol) water solution of cysteine hydrochloride monohydrate (52.7g; 0.3mol) was added with continuous magnetic stirring. A white precipitate formed immediately. White suspension was heated at ~90 °C for 3h with magnetic stirring. After cooling to room temperature solid was seperated by filtration, washed with water (3X200mL), acetone (3X100mL) and dried at 120 °C for 2h. Isolated yield of white powdery product was 54.8g (98%). Selected ^{1}H NMR (400 MHz, D_2O) δ 4.15 (t, J = 2.0, 1H) and 3.18-3.03 (m, 2H) ppm; ^{13}C NMR (D_2O): δ 171.66, 55.52 and 24.52 ppm. $C_3H_5NO_2SZn$ (MW184.53) m/z: 421.25, 321.00, 322.17, 221.08, 203.00 and 147.

L-cysteine Calcium complex (4): Preparation was carried out in the same way as L-cysteine Zinc compound. Selected ^{1}H NMR (400 MHz, D_2O) δ 3.52 – 3.41 (m, 1H) and 2.75 (ddd, J =

20.6, 13.7, 8.1, 2H) ppm. ^{13}C NMR (D_2O): δ 177.56, 65.42 and 30.12 ppm. $C_3H_5CaNO_2S$ (MW195.25) m/z: 212.1 and 113.0.

Synthesis of L-cysteine Magnesium complex (5): Preparation was carried out in the same way as L-cysteine Zinc compound. Selected ^{1}H NMR (400 MHz, D_2O) δ 3.52 – 3.41 (m, 1H) and 2.75 (ddd, J = 20.6, 13.7, 8.1, 2H) ppm. ^{13}C NMR (D_2O): δ 177.56, 65.42 and 30.12 ppm.

Synthesis of L-cysteine Cadmium complex (6): Preparation was carried out in the same way as L-cysteine Zinc compound. Selected ^{1}H NMR (400 MHz, D_2O) δ 3.52 – 3.41 (m, 1H) and 2.75 (ddd, J = 20.6, 13.7, 8.1, 2H) ppm. ^{13}C NMR (D_2O): δ 177.56, 65.42 and 30.12 ppm.

Preparation of L-4-carboxy-2,2-dimethyl thiazolidine hydrochloride (15): A suspension of L-cysteine hydrochloride monohydrate (5.0 g; 28.5 mmol) in 500 ml of anhydrous acetone was heated under reflux for six hours. The powdered cysteine hydrochloride dissolved and crystalline flakes started to separate slowly. The reaction mixture was concentrated under reduced pressure to slurry that was cooled overnight at 0-5 °C. Subsequent filtration gave 5.06 gm (90%) of white solid as pure product. Selected ^{1}H NMR (400 MHz, D_2O) δ 4.72 (t, J = 8.1, 1H), 3.41 (ddd, J = 52.6, 12.2, 8.1, 2H), 2.00 (s, 1H) and 1.61 (d, J = 5.1, 6H) ppm. ^{13}C NMR (D_2O): δ 172.1, 68.6, 66.8, 29.5, 26.8, and 26.8 ppm.

Preparation of (*R*)-ethyl 2-amino-3-mercaptopropanoate or L-cysteine ethyl ester hydrochloride (22): A suspension of L- cysteine hydrochloride monohydrate (1.0 g; 5.7 mmol)

in ethanolic HCl was refluxed for 5h to get a clear solution. The reaction was concentrated under reduced pressure to give pure product as a white powder in quantitative yield. Selected ^{1}H NMR (400 MHz, D_2O) δ 4.27 (t, J = 6.9, 1H), 4.19 (q, J = 8.0, 3H), 3.11 – 2.94 (m, 3H), 1.18 (t, J = 8.0, 4H).

Preparation of ethyl ester of L-4-carboxy-2,2-dimethyl thiazolidine hydrochloride (23): This compound was prepared in the same way as thiazolidine using the ethyl ester of L-cysteine HCl. ^{1}H Selected ^{1}H NMR (400 MHz, D_2O): δ 4.84 (t, 1H), 4.23-4.17 (q, J = 14.1 2H), 3.38-3.48 (ddd, J = 54.5, 12.1, 8.2, 2H), 1.68 (d, J = 7.9, 6H) and 1.18-1.15 (t, 3H) ppm. ^{13}C NMR (D_2O): δ 172.6, 66.2, 66.8, 61.3, 29.7, 26.7, 26.7 and 14.1 ppm.

Preparation of 2-Phenylthiazolidine-4-carboxylic Acid: L-cysteine hydrochloride monohydrate (3.0 g, 17.08 mmol) was dissolved in water (15 mL) and sodium bicarbonate (1.5 g, 17.08 mmol) was added followed by addition of benzaldehyde (2.17 g, 20.5 mmol) solution in ethanol (15 mL). The reaction mixture was stirred at room temperature for 2h, and the solid precipitated out was collected, washed with diethyl ether, and dried to afford 2-phenylthiazolidine-4-carboxylic acid with yields of 70%. Selected ^{1}H NMR (DMSO-*d*6): δ 7.25-7.55 (m, 5H), 5.67 (s, 0.6H), 5.50 (s, 0.4H), 4.22 (dd, J= 6.9, 4.5 Hz , 0.6H), 4.00 (dd, J =8.7, 7.2 Hz, 0.4H), 3.27-3.40 (m, 1H) and 3.04-3.16 (m, 1H) ppm.

Preparation of Ethyl 2-Phenylthiazolidine-4-carboxylate (25): L-cysteine ethyl hydrochloride (1.0 g, 5.385 mmol) was dissolved in water (15 mL) and sodium bicarbonate (0.45 g, 6.462 mmol) was added followed by addition of benzaldehyde (0.68 g, 6.462 mmol) solution in ethanol

(15 mL). The reaction mixture was stirred at room temperature for 1h. Ethanol was evaporated as much as possible, diluted with water and extracted in DCM. Product was obtained as oil in yields of 86%. Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 – 7.33 (m, 5H), 5.83 (s, 0.4H), 5.57 (s, 0.6H), 4.30 – 4.24 (q, *J* = 8.1, 2H), 4.02 and 3.50 (dd, *J* = 8.8, 1H), 3.45-3.50 (m, 1H), 3.10-3.15 (m, 1H) and 1.29 (q, *J* = 8.0, 3H) ppm.

Preparation of 2-(2,3,4-trimethoxyphenyl)thiazolidine-4-carboxylic Acid (27): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, *J* = 8.6, 1H), 6.60 (d, *J* = 8.7, 1H), 5.90 (s, 1H), 4.24 (m, 3H), 3.96 (s, 3H), 3.83 (s, 3H), 3.83 (s, 3H), 3.43 (dd, *J* = 10.1, 6.9, 2H) and 1.30 (t, *J* = 7.1, 1H) ppm.

Preparation of *p*-dimethylaminobenzaldehyde (32): Dimethylformamide (10g, 148.76 mmol) in a RBF was cooled in an ice bath. To it phosphorous oxychloride (6.32g, 41.32 mmol) was added carefully dropwise. Mixture was allowed to cool to room temperature after completion of addition. This was followed by addition of dimethylaniline (5g, 41.32 mmol), a yellow-green precipitate started to form. The reaction mixture was heated for 2hours. The mixture is then cooled to room temperature and subsequently poured on ice. Solution was neutralized with sodium acetate saturated aqueous solution to pH 6–8. *p*-dimethylaminobenzaldehyde began to precipitate. Stored the crude mixture in refrigerate overnite. Filtered the product, and washed several times with water on the filter. A very light-yellow product (12g, 82%) was obtained. Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.75 (s, 1H), 7.74 (d, 2H), 6.71 (d, *J* = 9.0, 2H), 3.09 (s, 3H) and 2.92 (s, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 191.02, 155.42, 130.18, 126.40, 114.82 and 40.22 ppm.

Synthesis of 2-*p*-dimethylamino benzaldehyde thiazolidine-4-ethyl carboxylate (33): Compound was synthesized in the same way as Ethyl-2-Phenylthiazolidine-4-carboxylate 18A. Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, J = 8.8, 2H), 6.7 (d, J = 8.8, 2H), 5.74 (s, 1H), 5.52 (s, 1H), 4.25-4.32 (m, 1H), 3.95-4.01 (m, 1H), 3.40-3.49 (m, 1H), 3.15-3.20 (m, 1H) and 2.97 (s, 6H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 171.5, 148.0, 129.62, 128.7, 114.25, 69.2, 62.65, 61.32, 40.21, 35.56 and 14.12 ppm.

Synthesis of phthalimides, general procedure: Phthalic anhydride (1.5 g; 0.01 mol), corresponding amine (0.01) and N,N-dimethylformamide (20 ml) were mixed together in a round bottom flask (100 ml). The reaction mixture stirred in the microwave reactor equipped with a condensor and magnetic stirrer. The reaction mixture was stirred under microwave irradiation with mild solvent refluxing (magnetron power between 300 to 400W). After completion of the reaction the resulting mixture was poured on crushed ice. This precipitated the compound as a white precipitate which was separated by filtration, washed thoroughly with water and dried under reduced pressure. The compounds were obtained in more than 95 percent purity. Further ^{1}H-NMR and ^{13}C-NMR spectra were recorded to establish structure of molecules.

4-methylphenylphthalimide (35): Isolated in quantitative yield. Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, 2H), 7.80 (d, 2H), 7.35(s, 4H) and 2.41(s, 3H) ppm. ^{13}C NMR(400 MHz, $CDCl_3$): δ 168.0, 138.2, 134.5, 132.0, 130.0, 129.5, 127.1 124.0, and 21.5 ppm.

Preparation of dibromoarene, general procedure: A mixture of methylbenzene derivative (3.3 mmol), *N*-bromsucinimide (10 mmol), and benzoylperoxide (0.31 mmol) in benzene (10 ml) was refluxed for three hours under microwave radiation (magnetron power 500W, magnetron power 200W with bead). After completion of the reaction the solvent was evaporated under reduced pressure and the solid residue was dissolved in dichloromethane (100 ml). Dichloromethane solution was washed with saturated sodium bicarbonate (3X15 ml), water (3X20 ml) and subsequently dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was further purified by filtration through short silica gel column with hexane-dichloromethane as solvent.

4,4'-(dibromomethyl)phenylphthalimide (36): The product was isolated as white powder (Yield=90%). Selected ^{1}H-NMR ($CDCl_3$): δ 7.98 (2H, m), 7.82 (2H, m), 7.72 (2H, d, J = 8.0 Hz), 7.51 (2H, d, J = 8.0 Hz), and 6.68 (1H, s) ppm. ^{13}C-NMR ($CDCl_3$): δ 167.1, 141.0, 135.3, 133.2, 131.0, 128.5, 127.1, 124.0 and 40.2 ppm. $C_{15}H_9Br_2NO_2$ (MW 395.05). MS (m/z) 396.90 (50%), 395.90 (10%), 394.90 (100%), 392.90 (50%).

Synthesis of 4-(1,3-dioxoisoindolin-2-yl)benzaldehyde (37): Refluxed 4,4'-(dibromomethyl)phenylphthalimide (28) in pyridine and water for 30 mins to get the corresponding ketone in quantitative yield. Selected ^{1}H-NMR ($CDCl_3$): δ 9.87 (1H, s), 7.85 (2H, m), 7.79 (2H, m), 7.72 (2H, d, J = 8.0 Hz), and 7.51 (2H, d, J = 8.0 Hz) ppm. ^{13}C-NMR ($CDCl_3$): δ 191.1, 170.0, 138.3, 133.2, 132.0, 129.5, 127.1 and 122.0 ppm.

Synthesis of ethyl 2-(4-(1,3-dioxoisoindolin-2-yl)phenyl)thiazolidine-4-carboxylate (38): Compound was synthesized in the same way as Ethyl-2-Phenylthiazolidine-4-carboxylate 18A. Compound was obtained as pale powder (Yield=82%). Selected ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.85 (2H, m), 7.79 (2H, m), 7.29 (2H, d, J = 8.0 Hz), 7.09 (2H, d, J = 8.0 Hz), 4.95 (m, 1H), 4.12 (m, 2H), 3.73 (m, 1H), 3.04 (m, 1H), 2.87 (m, 1H) and 1.30 (t, J=8.9, 3H) ppm. ^{13}C-NMR (400 MHz, $CDCl_3$): δ 171.1, 170.0, 136.3, 133.2, 132.0, 129.5, 128.67, 127.1, 69.23, 62.26, 61.3, and 61.5 ppm.

Synthesis of (S)-2-amino-3-(benzylthio)propanoic acid (40): To a rapidly stirring solution of L-cysteine (5 gm, 41 mmol) in 25 mL of 2N sodium hydroxide, benzyl bromide (4.91 mL, 7.06 gm, 41 mmol) in 48 mL ethanol was added. Reaction mixture was allowed to stir at rt for 30 minutes. After which it was neutralized to pH 6-7 by careful addition of 5% HCl solution and cooled to get precipitate. Filtered, washed with water, ethanol and ether to give 8.1 gm of compound 47 (yield= 93%). Selected ^{1}H-NMR (400 MHz, perdeuteriomethanol-trifluoroacetic acid): δ 7.3 (m, 5H), 6.8 (s, 2H), 4.06 (dd, J=7.2, 3.6 Hz, 1H), 3.75 (s, 2H), 3.05 (dd, J=14.4, 7.2 Hz, 1H) and 2.91 (dd, 1H, J=14.4, 3.6 Hz, 1H) ppm; ^{13}C-NMR (400 MHz, perdeuteriomethanol-trifluoroacetic acid): δ 174.79, 138.35, 128.69, 127.23, 57.75, 38.29 and 35.53 ppm.

***S*-Benzyl-L-cysteine ethyl ester hydrochloride (41):** Dry HCl gas (prepared by carefully pouring dropwise fuming sulfuric acid over sodium chloride) was purged through a solution of *S*-benzyl-L-cysteine HCl 47 (5.2 gm, 25 mmol) in ethanol 100 mL for 1 h, followed by refluxing 25h. Solvent evaporated to dryness and the residue was purified by recrystallization from

absolute ethanol-ether (1 : 10) to afford 6.2 gm (Yield= 91%) of white powder as pure product, mp 152–154 °C, Lit. 155 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.88 (s, 3H, NH_3^+), 7.34 (s, 5H), 4.20 (m, 3H), 3.86 (s, 2H), 3.0 (d, *J*=9.8 Hz, 2H) and 1.23 (t, *J*=10.6 Hz, 3H) ppm. ^{13}C-NMR (400 MHz, DMSO-d_6): δ 172.03, 136.27, 128.98, 127.21, 61.23, 53.99, 39.12 and 15.28 ppm.

Synthesis of monobromoarene, General procedure: A mixture of methyl benzene derivative (5 mmol), *N*-bromosuccinamide (5 mmol), and benzoylperoxide (0.5 mmol) in benzene (10 ml) was refluxed under microwave heating (magnetron power 500W, magnetron power 200W with bead). After reaction was completed solvent was evaporated to get a solid residue. This was dissolved in dichloromethane (100 ml) and washed with saturated sodium bicarbonate solution (3X15 ml) and water (3X15 ml). Solvent was evaporated and the resulting solid residue was purified by silica gel column chromatography with hexane-dichloromethane as an eluent.

4-(Bromomethyl)phenylphthalimide (44): Product was isolated as pale powder with isolated yield of 85%. ^{1}H-NMR ($CDCl_3$): δ 7.98 (2H, m), 7.80 (2H, m), 7.55 (2H, d, *J* = 8 Hz), 7.45 (2H, d, *J* = 8 Hz), and 4.55 (2H, s) ppm. ^{13}C-NMR ($CDCl_3$): δ 167.1, 140.0, 138.2, 135.1, 132.0, 130.2, 127.5, 124.3 and 33.0 ppm. $C_{15}H_{10}BrNO_2$ (MW 316.15). MS (m/z) 317.99 (10%), 314.99 (100%), and 316.99 (95%).

(*S*)-ethyl 2-amino-3-(4-(1,3-dioxoisoindolin-2-yl)benzylthio)propanoate (46): Same as compound 47. Isolated yield (69%). ^{1}H-NMR ($CDCl_3$): δ 7.88 (2H, m), 7.75 (2H, m), 7.44 (2H, d, J = 8Hz), 7.38 (2H, d, J = 8Hz), 4.25 (m, 2H), 3.76 (s, 2H), 3.45 (m, 1H), 3.17 (m, 1H), 2.97

(m, 1H) and 1.23 (t, *J*=10.6 Hz, 3H) ppm; ^{13}C-NMR ($CDCl_3$): δ 171.02, 167.12, 133.02, 131.29, 128.09, 121.45, 61.52, 54.22, 38.65, 35.89, and 14.19 ppm.

L-cysteine HCl tosylate salt (47): To the water (15 mL) solution of cysteine hydrochloride hydrate (7.88 g, 50 mmol), slowly added a solution of *p*-toluenesulfonic acid hydrate (11.41 g, 60 mmol) in 5 mL of H_2O. Allowed the reaction to stir at rt for 1h to recover white ppt as product. Filter ppt and dried under reduced pressure to give 12.3 gm, 90% yield of pure product 51. Selected ^{1}H NMR (D_2O, 400 MHz): δ 7.57 (d, *J* = 6.6 Hz, 2 H), 7.25 (d, *J* = 7.8 Hz, 2 H), 4.16 (dd, *J* = 5.4, 4.2 Hz, 1 H), 2.94-3.09 (m, 2 H) and 2.27 (s, 3 H) ppm.

***S*-benzoyl-L-cysteine-tosylate salt (48):** To a solution of **51** (0.1 gm, 0.25 mmol) in DCM, added benzoyl chloride (0.15, 0.25 mmol) and allowed to stir at room temperature for 20h. Solvent was evaporated under reduced pressure to get a ppt (0.15 mg, yield=89%) which was taken to the next step. Selected ^{1}H NMR (D_2O, 400 MHz): δ 7.88-7.59 (m, 5H), 7.34 (m, 2H), 4.72 (m, 1H), 3.86 (m, 1H), 3.75 (m, 1H) and 2.23 (s, 3H) ppm.

(*R*)-3-(benzoylthio)-1-butoxy-1-oxopropan-2-aminium chloride (49): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.89 (m, 2H), 7.76-7.66 (m, 3H), 4.54 (t, *J* = 4.3, 1H), 4.32-4.26 (m, 1H), 4.23-4.18 (m, 3H), 1.73 – 1.61 (m, 2H), 1.43-1.34 (m, 2H) and 0.93 (t, *J* = 7.4, 3H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz): δ 191.2, 172.36, 134.89, 134.59, 130.02, 128.90, 64.98, 52.24, 31.34, 31.22, 18.79 and 13.99 ppm.

(*R*)-1-butoxy-1-oxo-3-(tritylthio)propan-2-aminium chloride (50): L-Cysteine-O-butyl hydrochloride (0.5 gm, 2.15 mmol) and trityl chloride (0.601 gm, 2,15 mmol) were dissolved in 6 mL of trifluoroacetic acid obtaining a deep brown solution. Reaction mixture was allowed to stir at room temperature in an inert atmosphere. After this it was diluted with DCM (50 mL) and washed with water (2 X25 mL), dried over anhydrous sodium sulfate, solvent removed under reduced pressure. Product was obtained as sticky yellow substance (0.89 gm, yield=85%). Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42-7.20 (m, 15H), 4.53 (t, J = 4.3, 1H), 4.08 (t, J = 4.3, 2H), 3.63 (m, 1H), 3.36 (m, 1H), 1.73-1.61 (m, 2H), 1.43-1.34 (m, 2H), 0.93 (t, J = 7.4, 3H) ppm; ^{13}C NMR ($CDCl_3$, 400 MHz): δ 172.67, 143.99, 129.32, 128.20, 126.78, 67.34, 64.98, 55.24, 31.14, 23.22, 18.79 and 13.99 ppm.

4,4',4''-methanetriyltribenzenamine (54): Pararosaniline (100mg, 0.32mmol) was dissolved in 10 mL ethanol. Sodium borohydride (0.32 mmol) was added to it and allowed to stir at room temperature for 2h. The solvent was removed. Crude product was dissolved in ethyl acetate (50 mL) and washed with water, dried over sodium sulfate. Solvent removed under reduced pressure to get a light yellow product (yield= 57%). Selected ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.85 (d, J= 7.89, 6H), 6.54 (d, J= 7.89, 6H) and 5.58 (s, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 151.2, 135.67, 130.12, 115.87 and 54.67 ppm.

2,2',2''-(4,4',4''-methanetriyltris(4,1-phenylene))triisoindoline-1,3-dione (56): The synthesis was done in the same way as for compound 27. Product isolated as yellow powder in the yield of 80%. Selected ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.88 (s, 12H), 7.24 (d, J= 12.89, 6H), 7.02 (d,

J= 12.89, 6H) and 5.45 (s, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 169.02, 134.21, 130.23, 128.06, 127.54, 123.12 and 56.36 ppm.

Synthesis of 1,1',1''-(4,4',4''-(hydroxymethanetriyl)tris(4,1-phenylene))tripyrrolidine-2,5-dione (52): Procedure same as compound 27. Isolated as yellow powder (yield=76%). Selected ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.41 (m, 6H), 7.11 (m, 6H), and 2.90 (s, 6H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 180.02, 140.51, 132.23, 128.61, 123.54, 81.08 and 27.67 ppm.

Synthesis of butyl 2-amino-3-(tris(4-(2,5-dioxopyrrolidin-1-yl)phenyl)methylthio)propanoate (53): Product isolated as a powder a yellow precipitate with an isolated yield of 35%. Selected ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 – 7.09 (m, 12H), 4.36 (s, 2H), 4.27 – 4.07 (m, 1H), 2.96 (d, *J* = 10.1, 2H), 2.79 (d, *J* = 7.6, 1H), 2.75 – 2.67 (m, 2H), 1.48 (m, 1H), 1.33 (d, *J* = 7.1, 1H) and 1.02 – 0.76 (m, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 177.25, 172.15, 140.04, 132.78, 127.84, 121.23, 67.45, 65.71, 54.67, 32.12, 30.06, 27.65, 19.3 and 13.78 ppm

General Procedure for Benzylation of Amino Acids : A solution of an amino acid, (*S*-tyrosine) (50.0 mmol) in 2 M NaOH (25 mL, 50.0 mmol) and a solution of $CuSO_4.5H_2O$ (6.24 g, 25.0 mmol) in water (25 mL) were mixed together under stirring at room temperature. The blue precipitate of the Cu-complex separated immediately. After 1 h of reflux, the mixture was allowed to cool down to room temperature, and was dissolved in methanol (180 mL) and 2 M NaOH (25 mL, 50 mmol). Benzylbromide (6.25 mL, 52.5 mmol, 5 % excess) was added, and the mixture was stirred at room temperature overnight. The Cu-complex precipitate was collected,

washed with water and MeOH, stirred with 1 M HCl (100 mL) for 1 h to transform the Cu-complex into hydrochloride. The precipitate was filtered, washed with water (125 mL) and treated with 1 M NH3 (2X100 mL) to remove HCl, again washed with water (125 mL)and acetone (60 mL), and dried.

***O*-Benzyl-*S*-tyrosine (61):** Benzylation of L-tyrosine was accomplished using the general procedure for benzylation of amino acids as described above to give the product as white powder (5.2 gm, yield=73 %); m.p. 222 °C. Selected ^{1}H NMR (DMSO-d_6) δ 7.2–6.9 (m, 9H, Ph), 5.0 (s, 2H), 4.4 (m, 1H) and 3.4–3.1 (m, 2H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 175.07, 160.32, 137.01, 133.05, 131.33, 131.08, 130.65, 128.17, 119.32, 74.81, 58.00 and 36.99 ppm.

3-(4-(benzyloxy)phenyl)-2-(1,3-dioxoisoindolin-2-yl)propanoic acid (62): Procedure followed was the same as compound 27. Product was isolated as a white powder with a yield of 93%. Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 – 7.90 (m, 2H), 7.83 – 7.75 (m, 2H), 7.69 (t, J = 6.3, 1H), 7.51 (d, J = 8.4, 2H), 7.44 (d, J = 8.2, 2H), 7.09 (d, J = 8.4, 2H), 6.81 (d, J = 8.5, 2H), 5.11 (t, J = 10.4, 1H), 5.01 (s, 2H) and 3.63 – 3.39 (m, 1H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 175.12, 168.23, 156.98, 141.20, 133.23, 132.12, 129.18, 128.67, 127.67, 126.89, 126.12, 114.2, 70.18, 56.25 and 34.21 ppm.

Ethyl-3-(4-(benzyloxy)phenyl)-2-(1,3-dioxoisoindolin-2-yl)propanoate (63): Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 – 7.90 (m, 2H), 7.83 – 7.75 (m, 2H), 7.69 (t, J = 6.3, 1H), 7.51 (d, J = 8.4, 2H), 7.44 (d, J = 8.2, 2H), 7.09 (d, J = 8.4, 2H), 6.81 (d, J = 8.5, 2H), 5.11 (t, J = 10.4, 1H), 5.01 (s, 2H) and 4.25 (q, J = 9.3, 2H), 3.63 – 3.39 (m, 1H), and 1.26 (t, J = 7.1, 3H)

ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 175.12, 168.23, 156.98, 141.20, 133.23, 132.12, 129.18, 128.67, 127.67, 126.89, 126.12, 114.2, 70.18, 61.23, 56.25, 34.21 and 14.54 ppm.

L-tyrosine-ethyl ester (64): L-tyrosine (0.01 mol) was taken in ethanolic hydrochloride (20 mL) and refluxed for 5h. The solvent was removed under reduced pressure to obtain pure product as white crystalline compound (yield quantitative). Selected ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.12 (d, *J*= 5.85, 2H), 6.89 (d, *J*= 5.85, 2H), 4.23 (q, *J*= 12.85, 7.65, 2H), 3.82 (m, 1H), 2.97-3.02 (m, 2H) and 1.25 (t, *J*= 6.82, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 176.12, 153.87,132.16, 128.87, 115.67, 61.32, 53.21, 39.82 and 12.31 ppm.

2-(1,3-dioxoisoindolin-2-yl)-3-(4-hydroxyphenyl)propanoic acid (65): Synthesized using procedure for compound **24** and obtained in quantitative yield. Recrystallized from dichloroethane. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 5.5, 2H), 7.70 (d, *J* = 5.4, 2H), 7.11 (d, *J* = 8.3, 2H), 6.74 (d, *J* = 8.5, 2H), 3.99 – 3.70 (m, 2H) and 3.05 – 2.77 (m, 1H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 176.2, 167.89, 154.23, 131.75, 130.28, 128.32, 127.12, 115.98, 56.12 and 32.67 ppm.

Ethyl 2-(1,3-dioxoisoindolin-2-yl)-3-(4-hydroxyphenyl)propanoate (66): Isolated as white powder in quantitative yield. Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 5.5, 2H), 7.70 (d, *J* = 5.4, 2H), 7.11 (d, *J* = 8.3, 2H), 6.74 (d, *J* = 8.5, 2H), 4.23 (q, 2H), 3.99 – 3.70 (m, 2H), 3.05 – 2.77 (m, 1H) and 1.35 (t, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 176.2, 167.89, 154.23, 131.75, 130.28, 128.32, 127.12, 115.98, 62.24, 56.12, 42.67 and 32.67 ppm.

2-(1,3-dioxoisoindolin-2-yl)-3-(4-(methoxycarbonyloxy)phenyl)propanoic acid (68): Compound was isolated as pale powder (yield=95%). Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, J = 5.5, 2H), 7.70 (d, J = 5.4, 2H), 7.11 (d, J = 8.3, 2H), 6.74 (d, J = 8.5, 2H), 3.21(m, 1H), 2.99 (m, 1H) and 3.38 (s, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 176.2, 167.89, 154.23, 131.75, 130.28, 128.32, 127.12, 115.98, 58.23, 56.12 and 32.67 ppm.

Ethyl 2-(1,3-dioxoisoindolin-2-yl)-3-(4-(methoxycarbonyloxy)phenyl)propanoate (69): Isolated in quantitative yield. Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, J = 5.5, 2H), 7.70 (d, J = 5.4, 2H), 7.11 (d, J = 8.3, 2H), 6.74 (d, J = 8.5, 2H), 4.22 (q, 2H), 3.21(m, 1H), 2.99 (m, 1H), 3.38 (s, 3H) and 1.25(t, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 176.2, 167.89, 154.23, 131.75, 130.28, 128.32, 127.12, 115.98, 62.23, 58.23, 56.12, 32.67 and 14.23 ppm.

Ethyl 2-(1,3-dioxoisoindolin-2-yl)-3-(4-(4-(1,3-dioxoisoindolin-2-yl)benzyloxy)phenyl)propanoate (67): Yield (78%). Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 8.5, 2H), 7.72 (d, J = 8.5, 2H), 7.35 (d, J = 10.5, 2H), 7.19 (d, J = 10.5, 2H), 7.11 (d, J = 10.3, 2H), 6.74 (d, J = 10.5, 2H), 5.15 (s, 2H), 4.82(m, 1H), 4.22 (q, 2H), 3.23(m, 1H), 2.97 (m, 1H), and 1.32 (t, J = 15.5, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 168.2, 167.12, 156.85, 136.87, 132.00, 131.78, 131.16, 130.28, 128.72, 127.61, 127.06, 121.78, 114.25, 70.08, 62.23, 53.23, 32.67 and 14.23 ppm.

2-(1,3-dioxoisoindolin-2-yl)-3-(4-(4-(1,3-dioxoisoindolin-2-yl)benzyloxy)phenyl)propanoic acid (70): Yield (75%). Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 8.5, 2H), 7.72 (d, J = 8.5, 2H), 7.35 (d, J = 10.5, 2H), 7.19 (d, J = 10.5, 2H), 7.11 (d, J = 10.3, 2H), 6.74 (d, J =

10.5, 2H), 5.15 (s, 2H), 4.82(m, 1H), 3.23(m, 1H) and 2.97 (m, 1H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 168.2, 167.12, 156.85, 136.87, 132.00, 131.78, 131.16, 130.28, 128.72, 127.61, 127.06, 121.78, 114.25, 70.08, 53.23 and 32.67 ppm.

2-(1,3-dioxoisoindolin-2-yl)-2-phenylacetic acid (72): The compound was isolated as white powder in quantitative yield. Selected ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (m, 2H), 7.62 (m, 2H), 7.35 (d, J = 9.63, 2H), 7.21 (d, J = 9.65, 2H) and 5.47 (m, 1H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ 172.21, 170.02, 133.45, 129. 56, 128.67, 127.12 and 57.78 ppm

3-(2-(1,3-dioxoisoindolin-2-yl)-2-phenylacetyl)-2,2-dimethylthiazolidine-4-carboxylic acid (74): Yield (54%). Selected ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.78 (m, 5H), 7.35 (m, 2H), 7.21 (t, 1H), 7.08 (m, 2H), 5.45 (s, 1H), 4.75 (m, 1H), 3.05 (m, 1H), 2.91 (m, 1H) and 1.61 (s, 6H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 175.23, 169.12, 168.45, 131.89, 132.56, 127.89, 127.34, 65.42, 61.89, 53.23, 31.25 and 26.75 ppm.

Ethyl 3-(3-(4-acetoxyphenyl)-2-(1,3-dioxoisoindolin-2-yl)propanoyl)-2,2-dimethylthiazolidine-4-carboxylate (110): Yield 58%. Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (d, J= 9.23 Hz, 2H), 7.54 (d, J= 9.23 Hz, 2H), 7.09 (m, 2H), 7.01 (m, 2H), 5.02 (m, 1H), 4.71 (m, 1H), 4.24 (q, 2H), 3.58 (m, 1H), 3.32 (m, 1H), 3.12 (m, 1H), 2.89 (m, 1H), 2.29 (s, 3H), 1.61 (s, 6H) and 1.30 (t, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): δ 172.51, 169.00, 168.12, 167.12, 148.56, 137.18, 132.23, 131.90, 128.12, 127.56, 121.45, 62.32, 61.29, 61.35, 52.45, 35.67, 30.45, 27.91, 20.81 and 14.51 ppm.

Butyl 2-(3-(4-acetoxyphenyl)-2-(1,3-dioxoisoindolin-2-yl)propanamido)-3-(tritylthio)propanoate (111): Yield (62%). Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (d, *J*= 10.52, 2H), 7.54 (d, *J*= 10.52 Hz, 2H), 7.01-7.21 (m, 19H), 4.90 (m, 1H), 4.70 (m, 1H), 4.08 (t, *J*=8.25 Hz, 2H), 3.52 (m, 1H), 3.32 (m, 1H), 3.15 (m, 1H), 2.95 (m, 1H), 2.35 (t, *J*=15.25 Hz, 2H), 1.57 (m, 2H), 1.33 (m, 2H) and 0.98 (t, *J*=15.25 Hz, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): δ 172.12, 171.25, 169.02, 167.89, 148.25, 142.23, 136.78, 131.45, 128.98, 128.12, 127.89, 127.15, 126.31, 122.50, 67.84, 64.79, 54.23, 53.12, 34.68, 31.12, 27.89, 20.13, 18.78 and 13.23 ppm.

Ethyl 2-(3-(4-acetoxyphenyl)-2-(1,3-dioxoisoindolin-2-yl)propanamido)-3-(benzylthio)propanoate (112): Yield 62%. Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (d, *J*= 10.52, 2H), 7.54 (d, *J*= 10.52 Hz, 2H), 7.29-7.24 (m, 3H), 7.18-7.06 (m, 6H), 4.90 (m, 1H), 4.70 (m, 1H), 3.53 (m, 1H), 3.25 (m, 1H), 3.16 (m, 1H), 2.98 (m, 1H), 1.57 (m, 2H) and 1.33 (m, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): δ 172.10, 171.26, 169.02, 167.89, 148.25, 142.23, 136.78, 131.45, 128.98, 128.12, 127.89, 127.15, 126.31, 122.50, 67.84, 54.23, 53.12, 34.68, 31.12, 27.89, and 14.23 ppm.

Butyl 2-(3-(4-acetoxyphenyl)-2-(isoindolin-2-yl)propanamido)-3-(tris(4-(2,5-dioxopyrrolidin-1-yl)phenyl)methylthio)propanoate (113): Yield (71%): Selected ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.34 – 7.01 (m, 12H), 4.66 (m, 1H), 4.18 (t, *J*=7.29, 2H), 3.98 (m, 1H), 3.25 (m, 1H), 3.05 (m, 2H), 2.98 (m, 2H), 2.92 (d, *J* = 10.1, 2H), 2.32 (s, 3H), 1.57 (m, 2H), 1.35 (m, 2H) and 0.93 (t, *J* = 15.23, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 177.23, 171.89, 171.25, 168.98, 168.0, 148.25, 139.51, 135.81, 132.67, 132.02, 131.98, 128.1, 127.12, 122.24,

121.54, 68.19, 64.87, 54.35, 53.76, 34.81, 31.12, 27.89, 27.16, 21.02, 20.35, 18.50 and 12.87 ppm.

General Procedure for Preparation of *S*-Acylcysteines: L-cysteine hydrochloride 1 (2 mmol) solution in H_2O (3 mL) was added to a solution of the corresponding acid chloride of substrate (2 mmol) in CH_3CN (15 mL). The heterogeneous mixture was allowed to stir at room temperature for 18 h. Now the solid residue was filtered off, washed with water (3 X10 mL), dichloroethane (3 X10 mL), diethyl ether (3 X10 mL). The compound was completely dried under reduced pressure and vacuum to yield *S*-acylcysteine.

***S*-(Benzoyl)-L-cysteine hydrochloride (91):** Obtained as white precipitate in a yield of 65%; Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6): δ 8.64-8.66 (m, 2H), 8.45 (t, *J*=9.25 Hz, 1H), 8.20 (t, J=7.62 Hz, 2H), 5.56-5.57 (m, 1H), 4.73 (dd, *J* = 15.5, 4.2 Hz, 1H) and 4.47 (dd, *J* = 15.5, 3.8 Hz, 1H) ppm. ^{13}C NMR (400 MHz, TFA-DMSO-d_6): δ 201.01, 172.98, 138.7, 137.5, 132.00, 130.12, 57.65 and 30.78 ppm.

General Procedure for Preparation of *N*-Acylcysteines: L-cysteine hydrochloride 1 (2 mmol) and triethylamine (2 mmol) solution was prepared in CH_3CN (8 mL) and the corresponding acid chloride substrate was added to this mixture. After allowing the mixture to stir at room temperature from 2-5h solvent was removed under reduced pressure and diluted with ethyl acetate (10 mL). Ethyl acetate layer was washed with 2 N HCl and brine. Solvent was removed under reduced pressure and recrystallized (AcOEt:hexanes = 3:1) to get *N*-acylcysteines.

***N*-(Benzoyl)-L-cysteine (92)**: Obtained as a colorless oily compound (Yield=65%) ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.61 (br s, 1H), 7.78-7.85 (m, 2H), 7.41-7.58 (m, 3H), 7.20 (d, *J*=6.9 Hz, 1H), 5.06-5.14 (m, 1H), 3.10-3.28 (m, 2H) and 1.54 (t, *J* = 9.0 Hz, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 173.12, 168.45, 131.98, 129.02, 128.90, 127.71, 127.62, 127.52, 54.21 and 26.78 ppm.

General Procedure for Cysteinyl peptide Zinc complex: A solution of **C**ysteine coupled substrate (0.01 mol) and NaOH (0.02 mol) in water (100 mL) was mixed with zinc acetate (0.01 mol). The mixture sonicated for 8h. At this point product was isolated as white precipitate. It was washed with water (3 X100 mL) and acetone (3 X100 mL) and finally dried under vacuum to get white precipitate as product.

***N*-(Benzoyl)-L-cysteine zinc dihydrate (93)**: Yield (72%). Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 8.78 (br s, 1H), 7.85 (m, 2H), 7.70 (m, 2H), 7.50 (d, *J*= 16.9 Hz, 1H), 4.82 (m, 1H) and 3.02-3.21 (m, 2H) ppm. ^{13}C NMR (400 MHz, TFA- DMSO-d_6) δ 175.02, 169.15, 132.09, 130.02, 128.95, 127.72, 127.42, 127.12, 54.42 and 26.54 ppm.

1-carboxy-2-(2-(1,3-dioxoisoindolin-2-yl)-2-phenylacetylthio)ethanaminium chloride (94): Yield= 72%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.76 (m, 2H), 7.69 (m, 2H), 7.35 (m, 1H), 7.25 (m, 2H), 7.02 (m, 2H), 5.65 (s, 1H), 4.82 (m, 1H) and 3.02-3.21 (m, 2H) ppm. ^{13}C NMR (400 MHz, TFA- DMSO-d_6) δ 198.12, 175.02, 168.15, 135.09, 130.02, 129.3, 128.65, 127.72, 127.42, 65.72, 54.42 and 31.23 ppm.

2-(2-(1,3-dioxoisoindolin-2-yl)-2-phenylacetamido)-3-mercaptopropanoic acid (95): Yield= 72%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.76 (m, 2H), 7.70 (m, 2H), 7.31 (m, 1H), 7.25 (m, 2H), 7.04 (m, 2H), 5.82 (s, 1H), 4.82 (m, 1H) and 3.17-2.98 (m, 2H) ppm. ^{13}C NMR (400 MHz, TFA- DMSO-d_6) δ 175.02, 169.21, 168.24, 136.71, 132.35, 131.02, 129.78, 129.06, 127.72, 127.32, 57.72, 55.42 and 26.23 ppm.

2-(2-(1,3-dioxoisoindolin-2-yl)-2-phenylacetamido)-3-mercaptopropanate zinc dihydrate (96): Yield=75%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.78 (m, 2H), 7.72 (m, 2H), 7.51 (m, 1H), 7.28 (m, 2H), 7.04 (m, 2H), 5.81 (s, 1H), 4.81 (m, 1H) and 3.17-2.98 (m, 2H) ppm. ^{13}C NMR (400 MHz, TFA- DMSO-d_6) δ 175.02, 169.21, 168.24, 136.71, 132.35, 132.02, 129.76, 129.13, 127.72, 127.35, 60.72, 55.45 and 20.23 ppm.

2-(3-(4-acetoxyphenyl)-2-(1,3-dioxoisoindolin-2-yl)propanoylthio)-1-carboxyethanaminium chloride (97): Yield=71%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.78 (m, 2H), 7.72 (m, 2H), 7.12 (d, *J*=6.82 Hz, 2H), 7.02 (d, *J*=6.82 Hz, 2H), 4.87 (m, 1H), 4.56 (m, 1H), 3.85 (m, 1H), 3.67 (m, 1H), 3.32 (m, 1H), 3.08 (m, 1H) and 2.31 (s, 3H) ppm. ^{13}C NMR (400 MHz, TFA-DMSO-d_6) δ 202.12, 173.52, 170.12, 168.23, 149, 136.24, 132.32, 132, 128, 127.6, 121.45, 65, 54.42, 35.27, 30.09 and 21.37 ppm.

2-(3-(4-acetoxyphenyl)-2-(1,3-dioxoisoindolin-2-yl)propanamido)-3-mercaptopropanoic acid (98): Yield=65%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.78 (m, 2H), 7.75 (m, 2H), 7.10 (d, *J*=8.85 Hz, 2H), 7.00 (d, *J*=8.82 Hz, 2H), 4.89 (m, 1H), 4.75 (m, 1H), 3.58 (m, 1H), 3.45 (m, 1H), 3.17 (m, 1H), 3.01 (m, 1H) and 2.32 (s, 3H) ppm. ^{13}C NMR (400 MHz, TFA-

DMSO-d_6) δ 175.52, 171.12, 169.38, 168.23, 148.72, 136.24, 132.25, 132.12, 128.21, 127.16, 121.45, 57.42, 34.92, 27.09 and 20.31 ppm.

2-(3-(4-acetoxyphenyl)-2-(1,3-dioxoisoindolin-2-yl)propanamido)-3-mercaptopropanoate Zinc dihydrate (99): Yield=70%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.82 (m, 2H), 7.78 (m, 2H), 7.12 (d, *J*=10.12 Hz, 2H), 7.00 (d, *J*=12.65 Hz, 2H), 5.02 (m, 1H), 4.86 (m, 1H), 3.68 (m, 1H), 3.52 (m, 1H), 3.15 (m, 1H), 3.12 (m, 1H) and 2.45 (s, 3H) ppm. ^{13}C NMR (400 MHz, TFA- DMSO-d_6) δ 182.52, 172.12, 170.38, 169.23, 148.86, 136.42, 132.51, 132.27, 128.25, 127.16, 121.45, 58.42, 34.92, 27.58 and 21.31 ppm.

1-carboxy-2-(2-(1,3-dioxoisoindolin-2-yl)-3-(4-(1,3-dioxoisoindolin-2-yl)phenyl)propanoylthio)ethanaminium chloride (100): Yield=60%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.78 (m, 4H), 7.72 (m, 4H), 7.15 (m, 2H), 7.04 (m, 2H), 4.86 (m, 1H), 4.54 (m, 1H), 3.86 (m, 1H), 3.67 (m, 1H), 3.32 (m, 1H) and 3.08 (m, 1H) ppm. ^{13}C NMR (400 MHz, TFA- DMSO-d_6) δ 200.12, 174.54, 171.21, 169.45, 150.23, 137.44, 133.78, 131.21, 127.82, 127.54, 121.52, 66.75, 54.42, 36.17 and 31.09 ppm.

2-(2-(1,3-dioxoisoindolin-2-yl)-3-(4-(1,3-dioxoisoindolin-2-yl)phenyl)propanamido)-3-mercaptopropanoic acid (101): Yield=60%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.74 (m, 4H), 7.62 (m, 4H), 7.12 (m, 2H), 7.04 (m, 2H), 4.74 (m, 1H), 4.41 (m, 1H), 3.85 (m, 1H), 3.62 (m, 1H), 3.21 (m, 1H) and 3.04 (m, 1H) ppm. ^{13}C NMR (400 MHz, TFA- DMSO-d_6) δ 192.25, 175.54, 171.12, 169.35, 151.23, 136.54, 133.58, 130.32, 127.92, 127.52, 121.6, 65.75, 56.42, 34.17 and 29.09 ppm.

2-(2-(1,3-dioxoisoindolin-2-yl)-3-(4-(1,3-dioxoisoindolin-2-yl)phenyl)propanamido)-3-mercaptopropanoate Zinc dihydrate (102): Yield=72%. Selected ^{1}H NMR (400 MHz, TFA-DMSO-d_6) δ 7.78 (m, 4H), 7.67 (m, 4H), 7.21 (m, 2H), 7.02 (m, 2H), 4.98 (m, 1H), 4.45 (m, 1H), 3.9 (m, 1H), 3.67 (m, 1H), 3.3 (m, 1H) and 2.93 (m, 1H) ppm. ^{13}C NMR (400 MHz, TFA-DMSO-d_6) δ 195.5, 178.41, 172.2, 168.35, 152.23, 136.49, 132.58, 130.32, 128.12, 127.62, 122.6, 66.75, 55.42, 34.72 and 30 ppm.

A007-cysteine adduct (107): ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.14 (s, 1H), 8.81 (s, 1H), 8.42 (s, 1H), 8.21 (d, J = 9.6, 1H), 7.52 (s, 2H), 7.40 (s, 2H), 7.18 (d, J = 8.0, 2H), 6.95 (d, J = 11.6, 2H), 5.30 (s, 4H), 4.91 (m, 1H), 4.60 (t, J = 23.4, 4H), 4.13 (d, J = 30.0, 4H), 3.65 (s, 3H), 3.01 (d, J = 5.5, 1H), 2.83 (d, J = 22.7, 1H), 1.76 (s, 12H) and 1.29 – 1.07 (m, 3H) ppm.

3.9. References

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CHAPTER-4: SYNTHESIS OF NOVEL PROTECTIVE GROUPS FOR AMINO ACIDS

In the course of synthesis of cysteinyl peptides, several problems emerged while protecting the hydroxyl, sulfur and amino groups of the L-amino acids selected for peptide synthesis. Although there are several reported protective groups in literature. When applied in our peptide synthetic scheme they did not yield the desired results. In our pursuit to synthesize novel radical capture cysteinyl compounds (as described in chapter 3) we desired to trap the free *β*-sulfhydryl and *α*-carboxy functional group of L-cysteine hydrochloride by complexing with metals like zinc, calcium and magnesium. These on entering the body would liberate free sulfhydryl group that can act as a radical scavenger. Peptide synthesis by coupling the *N*-terminal of L-cysteine to carboxy terminal of the other amino acid like L-tyrosine presented problems in terms of racemization, yields and non-reproducibility of synthetic method when using the commonly used protective groups for our peptide synthesis. In the last few years, more than 250 protecting groups have been proposed as suitable for peptide synthesis[1]; however, a relatively small number of those are actually used because of the stringent requirements that a protecting group should meet, particularly with respect to the requirement of the preservation of other functionalities and stereochemistry.

4.1. Cyclic imides

Imide derivatives constitute an important class of organic compounds with numerous uses in biology[2], synthetic chemistry[3], nanomaterials[4] and polymer chemistry[5]. For instance, cyclic imides, particularly phthalimides, have been widely used as amino acid protective group[6] and have attracted considerable attention in peptide chemistry[7]. The relatively easy cleavage of

the phthaloyl group under mild condition makes it suitable as a protective group for the peptide synthesis[9]. Phthalimides are a subject of widespread interest[11] because of their interesting photophysical properties, which is still not completely understood[12], and their applications as fluoroprobes[11] and in synthetic chemistry. Phthalimide derivatives undergo various photochemical reactions that have attracted a lot of attention in recent years[10]. On the other hand, maleimides and succinimides are important constituents of peptide-conjugate haptens, antibody–antibody conjugates, immune conjugates, and enzyme inhibitors[8].

Phthalimide derivatives are reported to have local anesthetic properties comparable to Novesine by Settimo *et al*[13]. Among other biological activities are DNA cleaving agents[14] and tumericidal activity[15]. Pyromellitic dianhydride is an analogous compound, which undergoes facile polymerization reaction with diamines to give amide or imides containing polymers. Its relevance and application in nanomaterials has been studied by Holman *et al*[16]. Several phthalimides derivatives can self-assemble to form nano-tube like structures[17] and have interesting host to guest chemistry which is being investigated[18]. Another important amino acid phthaloyl derivative *N*-phthaloylglycine, a simple *N*-protected amino acid, has interesting supramolecular structural features on forming metal complexes[20]. Owing to the great importance of amino acids in physiological and pharmacological processes and biological interactions[19] and their importance in peptide synthesis, it is desired to find a simple, moderate, clean and convenient method for preparation of *N*-phthaloyl amino acid derivatives. The present work represents the synthesis of *N*-phthaloyl amino acids and their derivatives under mild conditions using microwave irradiation as a rapid, convenient method to give excellent yields and high purity of the products compared to conventional methods. The synthesized cyclic imide

derivatives were successfully used for protection of several amino acids and explored for peptide synthesis.

4.2. Cyclic imide Synthesis

Despite the wide applicability of cyclic imides, available routes for their synthesis are limited. Generally, most methods involve Lewis acid mediated condensation of an amine with maleic or phthalic anhydrides[21]. These methods are limited by low yields, by-product formation and harsh reaction conditions[22]. The development of simple and general synthetic routes for widely used organic compounds from readily available reagents is one of the major challenges in organic synthesis. Among the widely used method are the dehydrative condensation of an anhydride and an amine at high temperature[2] and the cyclization of the amic acid[23,24] in the presence of acidic reagents are the typical methods of choice[25]. The direct *N*-alkylation of maleimide with alcohols under Mitsunobu reaction conditions is an alternative method for the synthesis of imide derivatives in reasonably good yield[26]. However, each of these routes has its own synthetic problems when applied to a range of derivatives. For instance, synthesis of functionalized maleimide derivatives either by the direct condensation or through the intermediate amic acid cyclization method gives poor yields of the desired maleimide derivatives. Although the amic acid formation proceeds in quantitative yield, the subsequent cyclization results in only low yields of the desired maleimide derivatives together with extensive byproducts due to the incompatibility of the olefin functionality with harsh reaction conditions[27]. Similarly, in the Mitsunobu reaction only a narrow range of imide derivatives can be synthesized because alkanols are used as starting materials[28]. Recently, a solvent-free procedure using $TaCl_5$ +silica gel as catalyst has been described for the preparation of imides

under microwave irradiation[39]. Finally, Sandhu *et al.* [40] advocated the use of a more ecofriendly solvent-free system involving the reaction of equal amounts of anhydride and amines or amino acids in the absence of solvent in a domestic oven without any catalyst. In the case of the reaction of the anhydride with amino acids, a reaction between two solids was involved, for example between phthalic anhydride and glycine. Unfortunately, no temperature measurements were reported. Therefore, synthesis of functionalized imide derivatives is still a challenging endeavor. We describe herein an efficient and mild approach for the synthesis of imide derivatives of amino acids. These can be used as protection groups for peptide synthesis or exploration of other properties and activities. The development of environmentally friendly processes and economic reactions has attracted much attention of chemists[23]. Phthalimides exhibits a number of applications in biology and synthetic chemistry[29]. The easy cleavage of the phthaloyl group under mild condition makes it suitable as a protecting group for the amino group[9]. However, simple, efficient and environmentally benign processes are not available. Furthermore, in previously described procedures, amino acids with functionalized side chains, such as tryptophan and tyrosine, failed to give phthaloyl derivatives in good yields and in satisfactory purity. The use of phthaloyl moiety as a primary amine-protecting group is extensively documented in the literature[6b]. Several investigators have synthesized phthaloyl amino acids. The use of this protective group has not been practical in some sensitive substrates due to harsh conditions involved for protection and deprotection steps.

Billman and Harting[30] and Sheehan and Frank[31] obtained *N*-phthaloyl amino acids by heating mixtures of phthalic anhydride and appropriate amino acids at high temperatures. This resulted in racemization[32] as the experimental conditions were too drastic for *N*-phthaloylation of amino acids specially the ones containing additional functional groups[33] like tryptophan and

tyrosine. Sheehan and Frank[31] prepared *N*-phthaloyl amino acids by fusion methods in good yields for some cases but in others, the reaction conditions were too drastic. In order to control the temperature it is possible to perform reaction in the presence of boiling solvents like dioxane, pyridine or acetic acid, but then the reaction time is often too long. Balenovic *et al.*[33] used *O*-carboethoxythiobenzoic acid in their *N*-phthaloylation of amino acids. However, applications of this procedure are limited because the preparation of the reagent and the reaction procedure are laborious and the yields are very low. Phthaloyl amino acid esters, which on acid hydrolysis[31] can be converted into phthaloyl amino acids, are prepared more easily. However, the synthesis is carried out in two steps. First, a phthalimic acid derivative is obtained by reaction of phthalic anhydride and amino acid ester. The resulting compound was converted into *N*-phthaloyl amino acid esters with thionyl chloride[34] or a mixed anhydride[35]. In a modification of this method Bose, Greer and Price[36] obtained phthaloyl amino acid esters by heating an amino acid ester with phthalic anhydride in an inert solvent like benzene for several hours. The water formed in the dehydration of intermediate was collected in a separator. However, diketopiperazines was formed as by-products of this reaction. Nefkens *et al*.[37] reported N-phthaloylation by using *N*-(*O*-ethoxycarbonyl)phthalimide in aqueous sodium carbonate. It was claimed to be a simple preparation of phthaloyl amino acids and led to retention of chirality and high yields. However, their method failed in the case of tryptophan and diferent yields[38] were reported by other investigators in number of cases. Other investigations have been carried out in order to establish optimal reaction conditions, but the reported procedures do not seem to be satisfactory.

In order to obtain high yields without racemization it seems necessary to affect the synthesis of the phthaloylamino acids and their intermediate *N*-substituted phthalamic acids in solution and at moderate temperatures. The synthetic challenge is to selectively protect and

deprotect the side chain functional groups under mild conditions without racemization in an effective and efficient manner that is industrially viable. Therefore, we explored a rapid and one pot synthesis of phthaloyl derivatives of α-amino carboxamides under microwave irradiation. In this work, we report about an efficient and green process for the production of *N*-phthaloyl amino acids by fusing free amino acids or their esters with phthalic anhydride. We improved the fusion process, described earlier by Billman and Harting[30], by using microwave irradiation using a suitable solvent. The microwave reaction offered several advantages over the convention phthaloylation methods. Microwave reaction did not required any pressure to remove water instead exposing the reaction for 1-2 minutes can actually help get rid of water. The reaction did not have to be heated at high temperatures for long hours that lead to racemization. Microwave reaction usually provided the product in pure form and when required product was further purified by crystallization or a silica gel column using EtOAc-Hexane. The yield were almost quantitative and conversion happened in less than 30 minutes in most cases thus avoiding long heating exposures and any formation of undesired impurities. *N*-Phthaloylation of tryptophan was always difficult, because of the instability of the side chain. However, in our new procedure tryptophan was phthaloylated in good yields. L-Tyrosine and it ester derivatives were turned into the corresponding *N*-phthaloyl derivative in almost quantitative yield and high purity, which could not be obtained by Bose's procedure[36]. The fact that phthalic anhydride is contaminated with phthalic acid and could have adverse effect on amino acid phthaloylation was studied by Zeng *et al*[41]. We did not observe any adverse effect of presence phthalic acid. It is speculated that if any phthalic acid was present as contamination in the reaction mixture it would have been easily dehydrated to phthalic anhydride under microwave irradiation. Therefore, there was no interference observed by phthalic acid if it was present during the course of reaction.

Several parameters have been used to deal with protecting group during peptide-coupling reactions. A key issue is the use of appropriate N-protecting groups, such as the carbamate [tert-butyloxycarbonyl (Boc, **1**)[42], benzyloxycarbonyl (Cbz, Z, **2**)[43], 9-fluorenylmethy-loxcarbonyl (Fmoc, **3**)[44], or the recent type of base-sensitive amino protecting groups 1,1-dioxobenzo[b]thiophene-2-ylmethyloxycarbonyl (Bsmoc,**4**)[45], 2-(tert-butylsulfonyl)-2-propyloxycarbonyl (Bspoc, **5a**)[46], and 2- methylsulfonyl- 3-phenyl-1-prop-2-enyloxycarbonyl (Mspoc, **5b**)[47] (Fig.4.1).

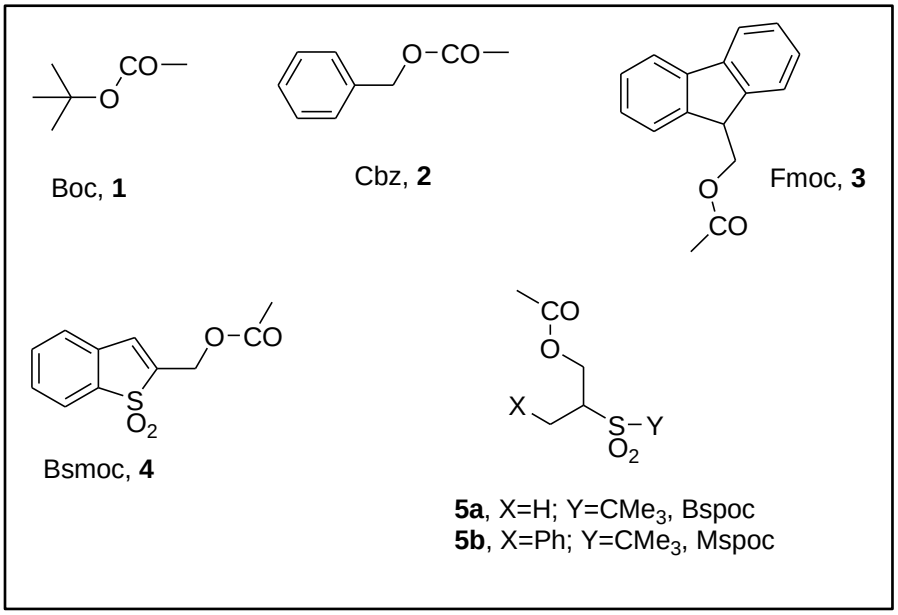
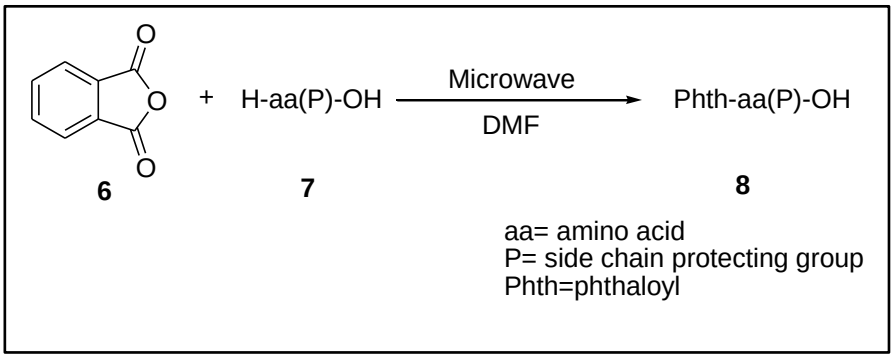


Figure 4.1 Structures of Protective Groups.

It is rarely mentioned about the *N*-Phthaloyl group in peptide synthesis, but it is very useful for synthesis[46] and other application, because it can be easily removed by aqueous HBr[47] or hydrazine in ethanol[48]. But this might not be a suitable deprotection condition for some longer peptide synthesis. Although the wide application of microwave-mediated synthesis of organic

compounds, only very little work appeared about *N*-phthaloylamino acids. Inspection in the literature cites two works where phthalic anhydride couples with amino acids under microwave-mediated conditions. In 1995 Bose *et al*.[49] reported that *N*-phthaloylamino acids could be synthesized within few minutes in good yields using microwave irradiation in DMF. Vidal *et al*.[50] reported examination of the latter technique for synthesis of phthalimides, among them phthaloylglycine which was obtained in 90 and 81% yields in xylenes and DMF, respectively. However, at the end of irradiation of these syntheses, the products are usually extracted with acetone. Here we re-examined this non-thermal methodology under solvent conditions for the preparation of *N*-phthaloyl- amino acids and their esters. We applied this procedure for the synthesis of target compounds, which proved to give excellent yields in short time reactions, while using conventional method takes 10–15h reflux in acetic acid or DMF. The synthetic pathway depicted in Scheme 4.1 outlines the chemistry of the present study. Full characterization of the synthesized compounds was based on the spectral data, particularly ^{1}H and ^{13}C NMR.



Scheme 4.1 Microwave assisted *N*-phthaloylation of amino acids.

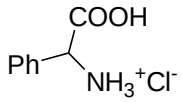
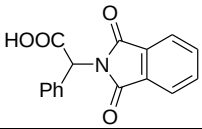
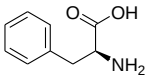
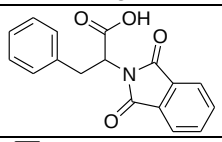
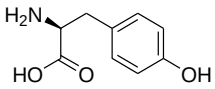
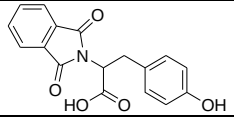
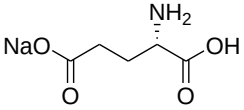
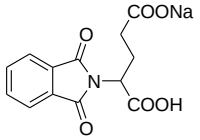
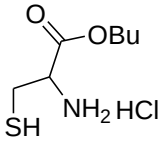
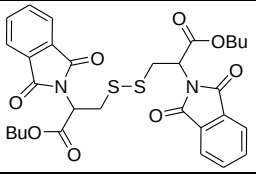
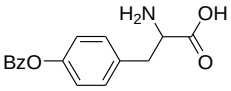
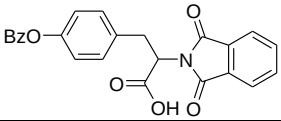
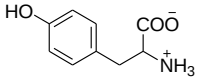
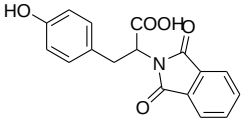
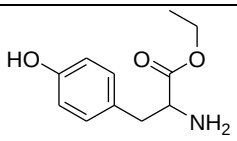
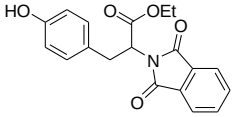
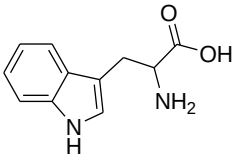
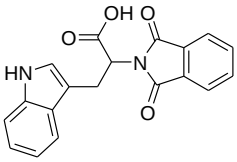
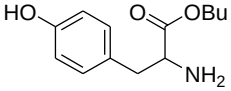
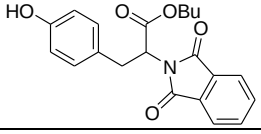
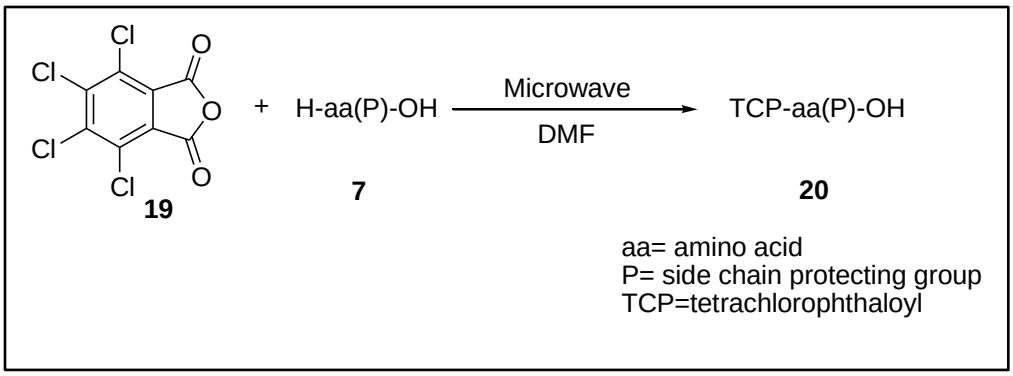
Product No.	Amino acid	*N*-phthaloylation	Microwave		Conventional	
			Time (mins)	Yield (%)	Time (hours)	Yield (%)
9.			20	95	15	51
10.			15	96	12	80
11.			15	100	15	86
12.			10	90	10	60
13.			15	95	10	75
14.			30	94	12	60
15.			15	96	14	86
16.			20	85	14	75
17.			20	75	-	-
18.			15	97	14	70

Table 4.1 *N*-Phthaloylated amino acids

DMF was found to be the most suitable solvent for the microwave *N*-phthaloylation reaction out of solvents like toluene, ethyl acetate and benzene for *N*-phthaloylation of amino acids. DMF was the superior solvent in comparison to others concerning both the reaction time and isolated yield. There are also reports of microwave-assisted synthesis without solvent[39]. However, the solvent free method can only be successful if one of the reactants absorbs microwave irradiation and has relatively low melting point. On the other hand, our procedure is applicable to broad range of amino acids regardless of their physical state or microwave radiation absorption ability because our identified solvent (DMF) is an excellent microwave reaction media, as it was demonstrated on the several amino acid reactions (Table 4.1). At the same time, it was observed that microwave-assisted reaction was far superior to the conventional *N*-phthaloylation reaction of amino acids.

The phthaloyl moiety as protection group is a very attractive approach. However, phthaloyl may not be practical in some sensitive substrates due to the harsh conditions required for its removal after achieving peptide synthesis. Addition of electron-withdrawing groups to the phthalimide aromatic ring greatly enhances deprotection under milder reaction conditions than those used for phthalimido group. Fraser-Reid and Schmidt[51] group have described use of tetrachlorophthalimido group while working on amino sugars. The application of tetrachlorophthalimido protective group is limited to sugar chemistry[52].



Scheme 4.2 Microwave assisted tetrachloro phthaloylation of amino acids.

Here we explored the use of tetrachlorophthalic anhydride for *N*-phthaloylation of amino acids (Scheme 4.2) and results are presented in Table 4.2. The reactions were carried out by mixing the tetrachlorophthalic anhydride with a desired amino acid in DMF and subjected to microwave irradiation. The reactions were usually completed in less than 15 minutes at a microwave power of 300 watt (Scheme 4.2). The microwave reaction offers simplicity and efficiency. The same reaction when performed under conventional heating took 20-22 hours of relux. When DMF was used as solvent, impurities were higher and decomposition observed because of long reaction time under reflux conditions. Therefore, much satisfactory results were obtained when reactions were carried out with dichloromethane[53]. Evidently, the reaction for tetrachlorophthaloylation under microwave irradiation proceeded well in terms of yield and reaction time (Table 4.2). The product was precipitated on addition of ice to the crude reaction mixture. When required a further purification was performed by crystallization using dichloroethane or a silica gel column using EtOAc-Hexane as eluent.

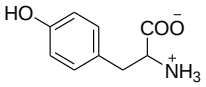
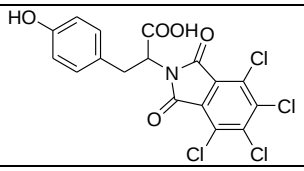
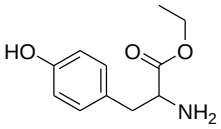
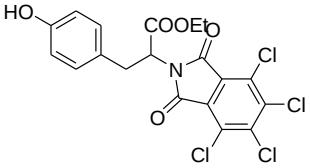
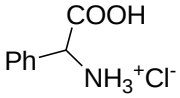
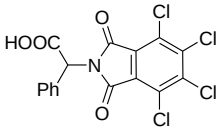
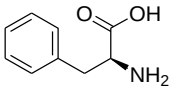
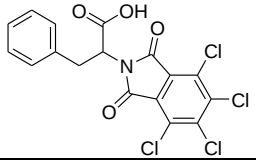
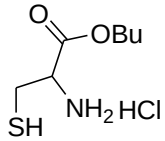
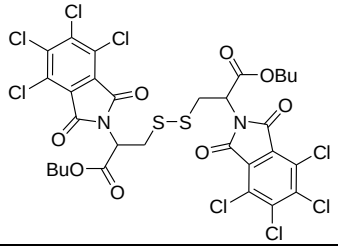
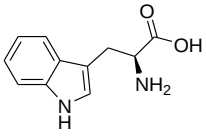
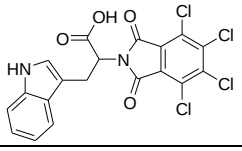
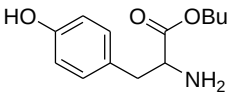
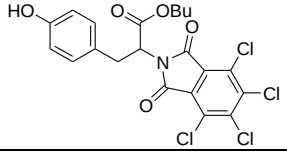
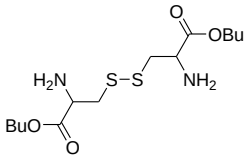
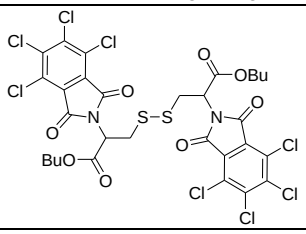
Product . No.	Amino acid	TCP-aa(P)-OH	Microwave		Conventional	
			Time (mins)	Yield (%)	Time (hours)	Yield (%)
21.			15	96	20	65
22.			10	98	22	70
23.			15	92	20	62
24.			15	95	20	90
25.			10	96	20	60
26.			15	94	20	62
27.			10	98	20	72
25.			5	95	20	60

Table 4.2 Tetrachloro phthaloylated derivatives of amino acids.

4.3. Cyclic imides derivatives as amino acid protecting groups

The synthetic challenge is to selectively protect and deprotect the side chain functional groups under mild conditions without racemization in an effective and efficient manner that is industrially viable. Therefore, several methods for the protection and deprotection of sulfhydryl group were attempted to improve the synthetic methodology. In order to accomplish cysteinyl peptide synthesis using L-Cysteine it is mandatory to have appropriate sulfhydryl protective groups. The nucleophilic thiol could be acylated, alkylated, or oxidized to disulfide by during peptide synthesis. In order to develop a protective group that is convenient to synthesize from inexpensive starting material with inherent properties of being recyclable, stable to heat, light, and moisture and peptide synthesis reaction conditions. At the same time after the completion of peptide synthesis, it should be easy to remove and purify to yield the desired peptide. Due to the sensitivity of the cysteine molecule toward oxidation and elimination, it is usually necessary to protect *β*-sulfhydryl function in addition to the amino or the carboxyl group during peptide synthesis. Even the protected Cysteine is susceptible to several side reactions. Elimination of the protected cysteine occurs on exposure to alkaline conditions, strong bases like sodium in liquid ammonia can remove the benzyl group, hydrazynolysis and strong acids like HF. The extent of elimination also depends strongly on the type of protecting group being used. Oxidation and alkylation of thioether protected cysteine[54-56]. This is less critical than in the case of Methionine but has been reported to occur. The cysteine residues can be protected against oxidation at deprotection stage by using ten percent water as scavenger[57].

Cysteine can easily react with the carbocations being generated at the deprotection step under acidic conditions. Formaiton of *S-tert*-butylated cysteine has been observed after the removal of the Boc group or after deprotection in Fmoc/*t*Bu protective strategy[58]. During

acidolytic cleavage resin-bound carbocations are generated that can react with the protected as well as unprotected cysteine which leads to reattachment of the peptide to the resin[59]. Another commonly observed phenomenon is the transfer of acetamidomethyl group to Ser, Thr, Gln, and Tyr during Acm removal[60]. Formation of thiazolidine of *N*-terminal cysteine can result from His-protecting groups that generate formaldehyde such as Bom (benzyloxymethyl) or Bum (*tert*-butyloxymethyl), at the deprotection stage. This has been minimized by using cysteine as scavenger[61]. A more common problem is cysteine is highly susceptible to racemize during the anchoring to the solid support or during the coupling reactions[62]. The extent of the racemization also depends on the *S*-protecting groups (S*t*Bu > Trt > Acm > MeBn > *t*Bu)[63,64] and coupling methods used. Racemization is favored if preactivation is achieved in the presence of base and if the coupling methods involve the use of a base. Epimerization of the cysteine linked to a hydroxyl resin can even take place during the synthesis as a result of the repetitive base treatments to remove the Fmoc group, with 2-chlorotrityl resin being the least prone to this process[65]. The most commonly used protecting groups for the Fmoc/*t*Bu strategy are the Acm or Trt groups, when the desired product is the disulfide, and the Trt group, when the desired product is the free thiol. For the Boc/Bn strategy, the most used are Bn and Meb (*p*-methylbenzyl) to obtain the free thiol and Acm to obtain disulfides. In the search for better protecting groups we were able to successfully synthesize protective groups that could be used for selective sulfhydryl protection. Large numbers of protective groups have been effectively and efficiently used for complex peptide synthesis[6b, 66]. In our case, the target molecules required additional functional properties that are not commonly associated with the traditional protection groups. For instance, one of the requirements is to alter the physical properties of the protected molecules in order to get intermediates that could be easily purified, characterized, and subjected to harsh reaction

conditions during our synthetic strategy. Secondly, the desired protecting groups should also be resistant to most reagents required for the preparation of our complex targeted molecules, allowing their convenient removal at the end of the synthetic procedure. Therefore, cyclic imides of *p*-aminobenzyl and pararoaniline were chosen as ideal candidates to be explored as protective groups for the purposes of target molecule synthesis (Fig.4.2).

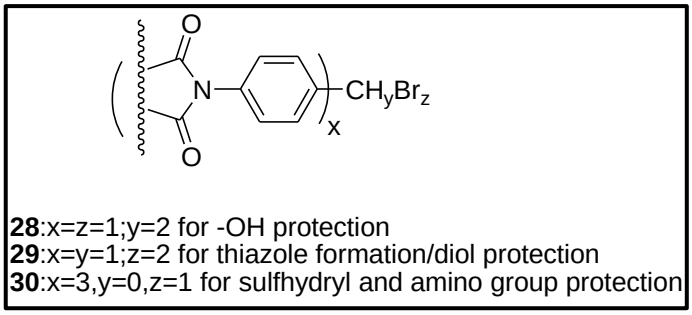
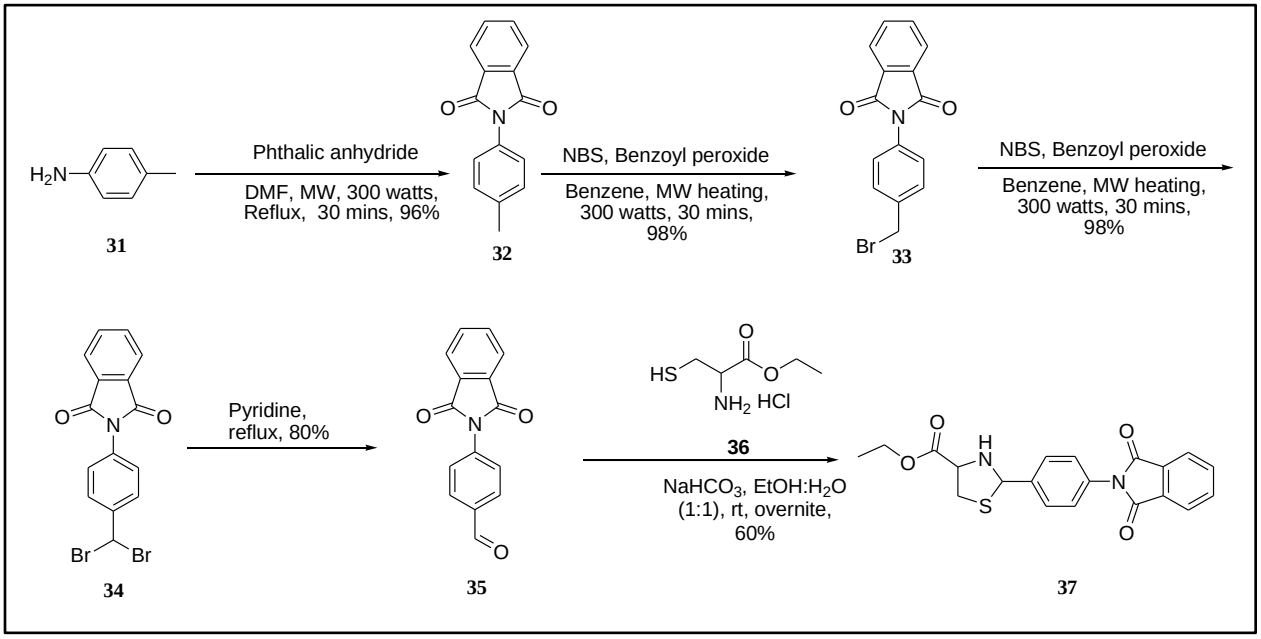


Figure 4.2 Novel sulhydryl, amino and alcohol protective groups.

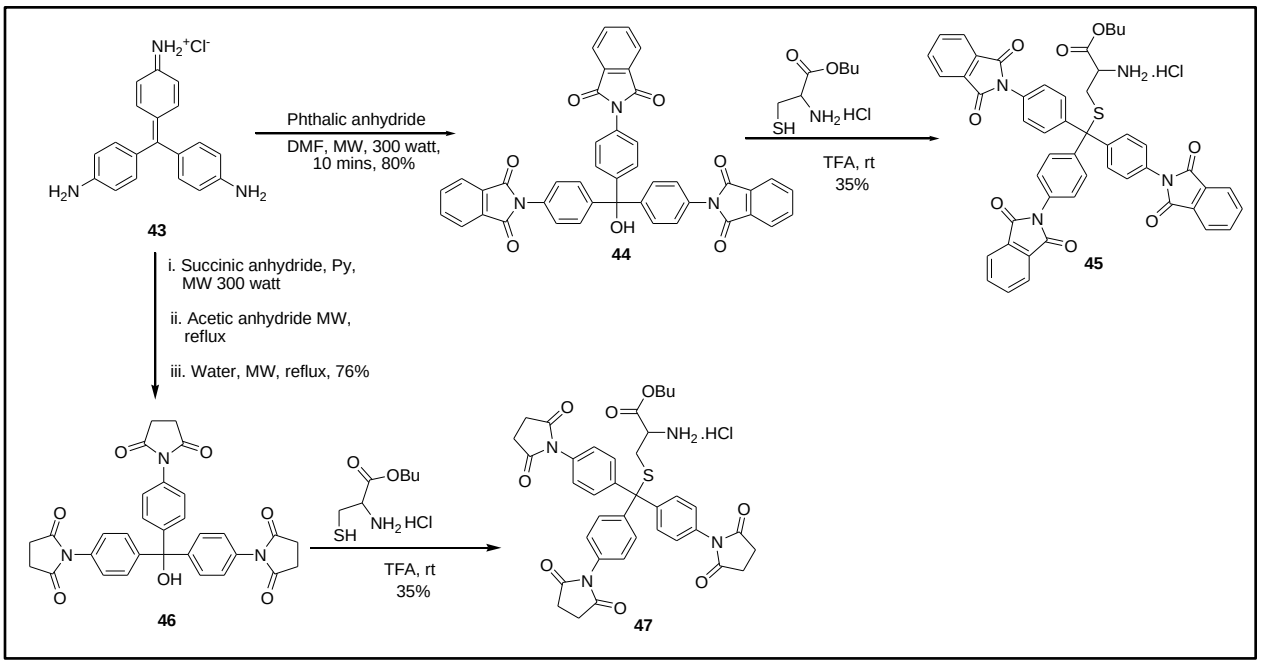
The rationale behind choosing cyclic imides is that these compounds crystallize well and are relatively stable in acidic, neutral, and basic reaction media thus making them easier to handle from milligram to grams scale[67]. Another important advantage offered by these protecting groups over other commonly used protective groups is their acid sensitivity can be easily altered by removal of the phthalate protection by treatment with hydrazine or a base. The resulting *p*-amino benzyl protection can be easily removed under moderate conditions without affecting the peptide bond. Similarly trityl is great protection group which was explored in selective *β*-sulfhydryl protection of L-cysteine hydrochloride, but it suffers from the disadvantage of being highly acid sensitive. Thus making it extremely difficult to use it in our target molecule that needs to be altered a various steps in synthetic strategy using acidic conditions. A better alternative to the trityl would be a cyclic imide of pararosaniline. It works on the same prinicipal as cyclic imides

of *p*-amino benzyl derivatives. For thiazolidine formation dibromo is hydrolysed to give the corresponding carbonyl to protect the sulfhydryl and amino groups of L-cysteine. For alcohol protection phenylmethyl bromide **28** is an ideal protective group, whereas for 1,2- and 1,3-diols the phenylmethylene dibromide **29** is the ideal protective group. Finally, for thiols and amines, the triphenylmethyl bromide **30** is the ideal protective group (Fig.4.2)[68-70]. Cyclic imides are compounds that crystallize well and are relatively stable in acidic, neutral, and dry basic reaction media[84]. When these groups are part of the protected aminoacids and derivatives either in the ether, thioether, or amine form, they are relatively stable in the mentioned reaction conditions. The major advantage of these benzyl-based protection groups over others that their acid sensitivity can be significantly increased by removal of imide protection using hydrazine or a base. The resulting *p*-amino benzyl ethers, thioethers, or amines are easily cleaved under moderate conditions.



Scheme 4.3 L-cysteine thiazolidine protection.

Pararosaniline **43** has three free amino groups that were protected as phthalate (Scheme 3.11). Pararosanilne **43** was reduced using sodium borohydride to the corresponding derivative. It was treated with phthalic anhydride under microwave conditions to yield in ten minutes at microwave power of 300 watt. The resulting compound was not reactive enough to selectively protect the β-sulfhydryl group of L-cysteine hydrochloride. Phthalate on reaction with compound **43** resulted in compound **44**, which selectively protected butyl ester of L-cysteine hydrochloride to give compound **45**. Reaction of **43** with succinic anhydride under microwave power of 500 watt yielded compound **46**, which on further reaction with butyl ester of L-cysteine, gave the desired β-sulfhydryl protected compound **47**.



Scheme 4.4 Pararosaniline derivatives as novel protective groups.

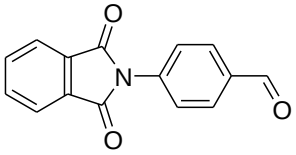
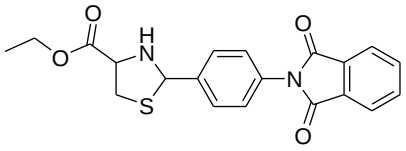
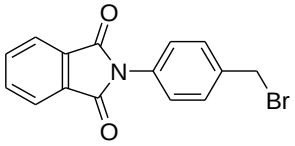
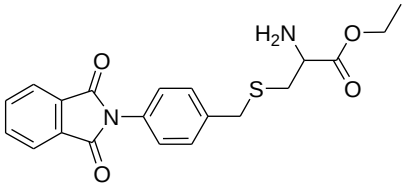
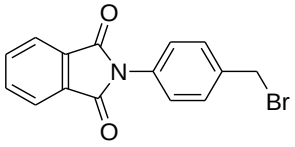
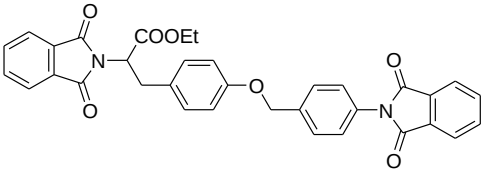
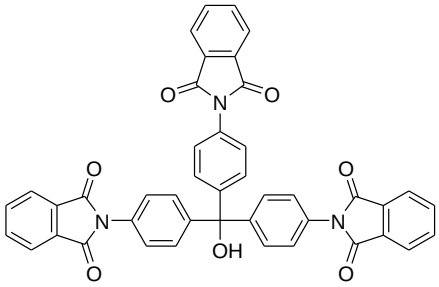
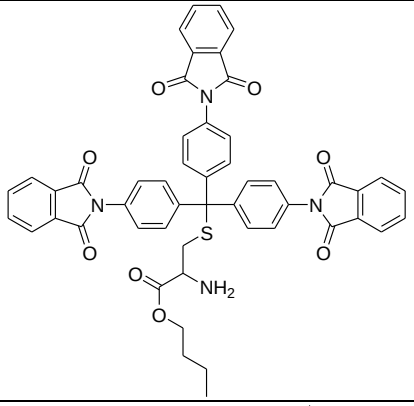
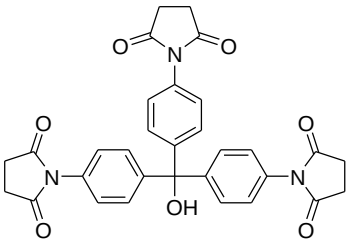
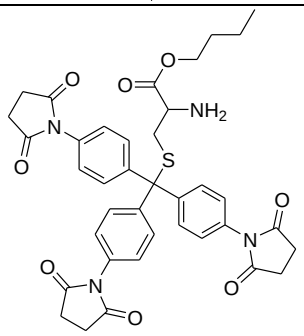
Product. No	Protective group	Cysteine protection
38.		
39.		
40.		
45.		
47.		

Table 4.3 Novel Protective groups for amino acids

In our continued pursuit to find new protective groups pararosaniline was selected as a substrate to synthesize new protective groups. The rationale to choose pararosaniline is that it has three amino groups that can be protected as phthalate and then introduced on L-Cysteine as selective β-sulfhydryl protection group. For the deprotection the phthalate group could be removed either under basic condition or by use of hydrazine hydrate. The free amine will stabilize the carbocation of the trityl derivative thus making it easier at the deprotection step. The phthalate group provides stability under acidic conditions and makes it more stable under low pH compared to trityl group. Therefore, it was chosen as an alternative to trityl to provide facile protective groups for our synthesis of target molecule. Since, pararoaniline cannot be used as such because of the presence of three free amino groups, it was protected. Therefore, phthalate was used as protective group but the resulting compound was found to have low solubility in the common organic solvents for our synthetic purpose. Phthalate was then replaced with succinate and tetrachloro phthalate derivatives to get better results. All these synthetic procedures were carried out using microwave assisted imide synthesis. This prompted us to explore new protection groups and new synthetic methodology to improve the reaction conditions and yields. One such improvement was developed for phthaloylation of α amino acids.

4.4. Conclusions

In conclusion, we have developed novel cyclic imides as protective groups for use in peptide synthesis. Synthesis for cyclic imides and their brominated derivatives were successfully achieved under microwave irradiation. The developed methods for protecting amino acids have potential to be used at large-scale industrial process for synthesis of cyclic imides derivatives very efficiently and in good yields under microwave conditions. N-phthaloylation of amino acids was achieved under microwave irradiation efficiently with improved yields compared to the reported procedures in the literature. The reactions were carried out under refluxing DMF for few minutes that avoided any racemization or denaturation of final products.

Tetrachlorophthaloylation was so far used for sugar chemistry or solid phase peptide synthesis. We explored the possibilities of synthesizing TCP derivatives under solvent conditions using microwave. Several amino acids including cysteine and tryptophan that have not been studied extensively in literature were explored in this dissertation. Introduction of electron withdrawing group on phthalate stabilized the molecule for use as protective groups in amino acids and facilitated the subsequent removal at the end of peptide synthesis.

Pararosaniline and *p*-amino benzyl compounds were explored for synthesis of protective groups in peptide synthesis. Efficient methods were developed that are reproducible at large scale with high yields. These methods were environment friendly and economic. All procedures are simple and materials can be obtained in large quantities. L-cysteine was selectively protected as thiazolidine and used for peptide synthesis. Selective hydroxyl, amino and β-sulfhydryl protection of amino acids including L-cysteine was successfully accomplished.

4.5. Experimental

General procedure for synthesis of N^{α}-phthaloyl-protected-L-aminoacids:

Method A: A suspension of phthalic anhydride (1.5 g; 0.01 mol) in *N*,*N*-dimethylformamide (20 ml) was taken in a round bottom flask (100 ml) and heated in microwave for 2 min to get a clear solution. The corresponding amine L-amino acid (0.01 mol) was then added to this clear solution. The reaction mixture was put inside the microwave equipped with a reflux condenser and a magnetic stirrer. It was allowed to stir under microwave irradiation with mild solvent refluxing (magnetron power between 200 and 300 W). Once the reaction is completed it was poured over ice to get the precipitate to give white powder as pure product in more than 95% yield. Further ^{1}H-NMR and ^{13}C-NMR spectra were recorded to establish the structure of compounds.

Method B: A suspension of phthalic anhydride (1.5 g; 0.01 mol) in *N*,*N*-dimethylformamide (20 ml) was taken in a round bottom flask (100 ml) and heated in microwave for 2 min to get a clear solution. The corresponding L-amino acid (0.01mol) was added to this clear solution. The reaction mixture was put inside the microwave equipped with a reflux condenser and a magnetic stirrer. It was allowed to stir under microwave irradiation with mild solvent refluxing (magnetron power between 200 and 300 W). Once the reaction was completed, it was cooled to room temperature diluted with ethyl acetate (100 mL) and washed with water (2 X50 mL), brine and dried over magnesium sulafte. Solvent was removed under reduced pressure. When required product was recrystallized from 1,2-dichloroethane. Product was obtained in more than 90%

yield. Further product formation was confirmed by ^{1}H-NMR and ^{13}C-NMR spectra to establish the structure of compounds.

2-(1,3-dioxoisoindolin-2-yl)-2-phenylacetic acid (9): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.99-7.85 (m, 4H), 7.55-7.28 (m, 5H) and 5.99 (s, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 168.37, 167.70, 135.27, 134.5, 131.88, 129.4, 127.9, 127.6, 123.1 and 56.9 ppm.

2-(1,3-dioxoisoindolin-2-yl)-3-(4-hydroxyphenyl)propanoic acid (15): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.14 (s, 1H), 7.81 (s, 4H), 6.91 (d, J = 8.3, 2H), 6.53 (d, J = 8.3, 2H), 5.01 (dd, J=12 Hz, J=4.8 Hz, 1H), 3.33(dd, J=13.8 Hz, J=5.4 Hz, 1H) and 3.21 (t, J=12 Hz, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 170.2, 167.1, 155.8, 134.9, 130.8, 129.6, 127.3, 123.4, 115.2, 53.2 and 33.1 ppm.

Butyl 2-amino-3-(tris(4-(1,3-dioxoisoindolin-2-yl)phenyl)methylthio)propanoate (45): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (m, 12H), 7.25 (m, 12H), 4.97 (m, 1H), 4.06 (m, 1H), 3.29 – 3.10 (m, 2H), 1.46 (m, 2H), 1.18 (m, 2H), 0.76 (t, J = 7.4, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 172.52, 167.09, 139.58, 132.34, 132.05, 128.45, 127.87, 123.01, 67.87, 65.05, 54.66, 31.21, 30.06, 18.87, 13.89 ppm.

Butyl 2-amino-3-(tris(4-(2,5-dioxopyrrolidin-1-yl)phenyl)methylthio)propanoate (47): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.24 (dd, J = 17.7, 8.5, 12H), 4.37 (t, J = 4.3, 2H), 4.28-4.06 (m, 1H), 2.97 (s, 1H), 2.80 (s, 1H), 2.76 (s, 12H), 1.71-1.51 (m, 2H), 1.35 (m, 2H) and 0.88 (t, J = 7.4, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 176.54, 171.45, 139.56, 132.78, 128.42, 122.09, 68.9, 65.2, 54.76, 31.12, 30.86, 27.67, 18.89 and 13.88 ppm.

2-(1,3-Dioxoisoindolin-2-yl)-3-phenylpropanoic acid (10): White powder, m.p. 156–157, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.94 (s, 1H) , 7.66 (m, 2H), 7.76 (m, 2H), 5.22 (t, *J*=8.8 Hz, 1H) and 3.58 (d, *J*=8.8 Hz, 2H), ppm ^{13}C NMR (400 MHz, DMSO-d_6) δ: 174.75, 167.53, 134.29, 131.51, 128.9, 128.7, 127.04, 123.66, 53.17 and 34.35 ppm.

Sodium 4-carboxy-4-(1,3-dioxoisoindolin-2-yl)butanoate (12)): ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.94 (s, 1H) , 7.68-7.87 (m, 4H), 4.82 (m, 1H) and 2.22-2.48 (m, 4H), ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ: 175.15, 170.23, 167.53, 134.29, 131.51, 124.28, 57.82, 32.25 and 22.58 ppm.

dibutyl 3,3'-disulfanediylbis(2-(1,3-dioxoisoindolin-2-yl)propanoate) (13): ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.90 (m, 8H), 4.97 (s, 2H), 4.07 (d, *J* = 27.0 Hz, 1H), 3.39 – 2.92 (m, 2H), 2.48 (s, 4H), 1.44 (s, 4H), 1.18 (s, 4H) and 0.76 (s, 6H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ: 167.53, 134.63, 132.05, 123.64, 115.62, 77.18, 66.25, 54.90, 30.45, 24.02, 19.15 and 13.63 ppm.

2-(1,3-dioxoisoindolin-2-yl)-3-(1H-indol-3-yl)propanoic acid (17): ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.74 (s, 1H), 7.80 (s, 4H), 7.26 (d, 1H, *J* = 8.2 Hz), 7.49 (d, 1H, *J* = 8.1 Hz), 7.00 (t, 1H, *J* = 8 Hz), 6.89 (t, 1H, *J* = 7.3 Hz), 5.13 (dd, 1H, *J*1 = 10 Hz, *J*2 = 6.7 Hz) and 3.55–3.62 (m, 2H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.4, 167.2, 136.1, 134.9, 130.9, 126.9, 123.4, 121.0, 118.5, 117.9, 111.5, 109.8, 52.7, and 24.1 ppm.

2-phenyl-2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)acetic acid (23): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.55-7.28 (m, 5H) and 5.99 (s, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 169.37, 168.70, 138.27, 135.5, 133.88, 129.4, 129.45, 127.9, 127.6, 125.1 and 56.9 ppm.

3-(4-hydroxyphenyl)-2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)propanoic acid (21): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.06 (d, *J*=8.5 Hz, 2H), 6.85 (d, *J*=8.5 Hz, 2H), 5.19 (dd, *J1*=6.1, *J2*=10.7 Hz, 1H), 3.60 (dd, *J1*=10.7, *J2*=14.4 Hz, 1H) and 3.50 (dd, *J1*=10.7, *J2*=6.1 Hz, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 172.05, 162.65, 154.24, 140.28, 131.23, 129.76, 129.19, 127.22, 124.48, 78.57, 53.83 and 33.56 ppm.

3-phenyl-2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)propanoic acid (23): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.40-7.29 (m, 5H), 5.42 (dd, *J1*=6.1, *J2*=11.7 Hz, 1H), 3.78 (dd, *J1*=5.2, *J2*=14.2 Hz, 1H) and 3.65 (dd, *J1*=11.7, *J2*=14.2 Hz, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 169.75, 163.65, 140.38, 138.03, 130.23, 129.76, 129.29, 128.45, 127.49, 54.89 and 34.56 ppm.

dibutyl 3,3'-disulfanediylbis(2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)propanoate) (25): ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.97 (s, 2H), 4.07 (d, *J* = 27.0 Hz, 1H), 3.39 – 2.92 (m, 2H), 2.48 (s, 4H), 1.44 (s, 4H), 1.18 (s, 4H) and 0.76 (s, 6H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ: 167.53, 134.63, 132.05, 123.64, 115.62, 77.18, 66.25, 54.90, 30.45, 24.02, 19.15 and 13.63 ppm.

Synthesis of phthalimides, general procedure: Phthalic anhydride (1.5 g; 0.01 mol), corresponding amine (0.01) and *N*,*N*-dimethylformamide (20 ml) were mixed together in a round bottom flask (100 ml). The reaction mixture stirred in the microwave reactor equipped with a

condenser and magnetic stirrer. The reaction mixture was stirred under microwave irradiation with mild solvent refluxing (magnetron power between 300 to 400W). After completion of the reaction, the resulting mixture was poured on crushed ice. This precipitated the compound as a white precipitate, which was separated by filtration, washed thoroughly with water and dried under reduced pressure. The compounds were obtained in more than 95 percent purity. Further ^{1}H-NMR and ^{13}C-NMR spectra were recorded to establish structure of molecules.

4-methylphenylphthalimide (32): Isolated in quantitative yield. Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, 2H), 7.80 (d, 2H), 7.35(s, 4H) and 2.41(s, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): δ 168.0, 138.2, 134.5, 132.0, 130.0, 129.5, 127.1 124.0, and 21.5 ppm.

Preparation of dibromoarene, general procedure: A mixture of methylbenzene derivative (3.3 mmol), *N*-bromsucinimide (10 mmol), and benzoylperoxide (0.31 mmol) in benzene (10 ml) was refluxed for three hours under microwave radiation (magnetron power 500W, magnetron power 200W with bead). After completion of the reaction, the solvent was evaporated under reduced pressure and the solid residue was dissolved in dichloromethane (100 ml). Dichloromethane solution was washed with saturated sodium bicarbonate (3X15 ml), water (3X20 ml) and subsequently dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was further purified by filtration through short silica gel column with hexane-dichloromethane as solvent.

4,4'-(dibromomethyl)phenylphthalimide (34): The product was isolated as white powder (Yield=90%). Selected ^{1}H-NMR ($CDCl_3$): δ 7.98 (2H, m), 7.82 (2H, m), 7.72 (2H, d, J = 8.0 Hz), 7.51 (2H, d, J = 8.0 Hz), and 6.68 (1H, s) ppm. ^{13}C-NMR ($CDCl_3$): δ 167.1, 141.0, 135.3, 133.2, 131.0, 128.5, 127.1, 124.0 and 40.2 ppm. $C_{15}H_9Br_2NO_2$ (MW 395.05). MS (m/z) 396.90 (50%), 395.90 (10%), 394.90 (100%), 392.90 (50%).

Synthesis of 4-(1,3-dioxoisoindolin-2-yl)benzaldehyde (35): Refluxed 4,4'-(dibromomethyl)phenylphthalimide (28) in pyridine and water for 30 mins to ge the corresponding ketone in quantitative yield. Selected ^{1}H-NMR ($CDCl_3$): δ 9.87 (1H, s), 7.85 (2H, m), 7.79 (2H, m), 7.72 (2H, d, J = 8.0 Hz), and 7.51 (2H, d, J = 8.0 Hz) ppm. ^{13}C-NMR ($CDCl_3$): δ 191.1, 170.0, 138.3, 133.2, 132.0, 129.5, 127.1 and 122.0 ppm.

butyl 3-(4-hydroxyphenyl)-2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)propanoate (27): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.06 (d, J=8.5 Hz, 2H), 6.85 (d, J=8.5 Hz, 2H), 5.19 (dd, $J1$=6.1, $J2$=10.7 Hz, 1H), 3.60 (dd, $J1$=10.7, $J2$=14.4 Hz, 1H), 4.10 (m, 2H), 3.50 (dd, $J1$=10.7, $J2$=6.1 Hz, 1H), 1.33 (m, 2H), 1.58 (m, 2H) and 0.95 (t, J=16.8, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 172.05, 162.65, 154.24, 140.28, 131.23, 129.76, 129.19, 127.22, 124.48, 78.57, 64.89, 53.83, 33.56, 31.26, 18.78 and 13.56 ppm.

Synthesis of monobromoarene, General procedure: A mixture of methyl benzene derivative (5 mmol), *N*-bromosuccinamide (5 mmol), and benzoylperoxide (0.5 mmol) in benzene (10 ml) was refluxed under microwave heating (magnetron power 500W, magnetron power 200W with bead). After reaction was completed, solvent evaporated to get a solid residue. The residue was

dissolved in dichloromethane (100 ml) and washed with saturated sodium bicarbonate solution (3X15 ml) and water (3X15 ml). Solvent was evaporated to dryness and the resulting solid residue was purified by silica gel column chromatography with hexane-dichloromethane as an eluent.

4-(Bromomethyl)phenylphthalimide (33): Product was isolated as pale powder with isolated yield of 85%. ^{1}H-NMR ($CDCl_3$): δ 7.98 (2H, m), 7.80 (2H, m), 7.55 (2H, d, J = 8 Hz), 7.45 (2H, d, J = 8 Hz), and 4.55 (2H, s) ppm. ^{13}C-NMR ($CDCl_3$): δ 167.1, 140.0, 138.2, 135.1, 132.0, 130.2, 127.5, 124.3 and 33.0 ppm. $C_{15}H_{10}BrNO_2$ (MW 316.15). MS (m/z) 317.99 (10%), 314.99 (100%), and 316.99 (95%).

Synthesis of butyl 2-amino-3-(tris(4-(2,5-dioxopyrrolidin-1-yl)phenyl)methylthio) propanoate (47): Product isolated as a powder a yellow precipitate with an isolated yield of 35%. Selected ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 – 7.09 (m, 12H), 4.36 (s, 2H), 4.27 – 4.07 (m, 1H), 2.96 (d, J = 10.1, 2H), 2.79 (d, J = 7.6, 1H), 2.75 – 2.67 (m, 2H), 1.48 (m, 1H), 1.33 (d, J = 7.1, 1H) and 1.02 – 0.76 (m, 1H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 177.25, 172.15, 140.04, 132.78, 127.84, 121.23, 67.45, 65.71, 54.67, 32.12, 30.06, 27.65, 19.3 and 13.78 ppm.

Synthesis of ethyl 2-(4-(1,3-dioxoisoindolin-2-yl)phenyl)thiazolidine-4-carboxylate (37): Compound was synthesized in the same way as Ethyl-2-Phenylthiazolidine-4-carboxylate (Compound 18A, Chapter 3). Compound was obtained as pale powder (Yield=82%). Selected ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.85 (2H, m), 7.79 (2H, m), 7.29 (2H, d, J = 8.0 Hz), 7.09 (2H, d, J = 8.0 Hz), 4.95 (m, 1H), 4.12 (m, 2H), 3.73 (m, 1H), 3.04 (m, 1H), 2.87 (m, 1H) and 1.30 (t,

J=8.9, 3H) ppm. ^{13}C-NMR (400 MHz, $CDCl_3$): δ 171.1, 170.0, 136.3, 133.2, 132.0, 129.5, 128.67, 127.1, 69.23, 62.26, 61.3, and 61.5 ppm.

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