DEVELOPMENT OF NOVEL ANTIFUNGAL AGENTS USING FUNGAL CELL WALL AS A TARGET

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CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Development of novel antifungal agents using fungal cell wall as a target"** submitted by Ms. P. M. Chaudhary was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Development of novel antifungal agents using fungal cell wall as a target"**, submitted for the degree of Doctor of Philosophy to the University of Pune, is the record of work carried out by me at Biochemical Sciences Division, National Chemical Laboratory, Pune - 411008, India, under the supervision of Dr. M. V. Deshpande (research guide) and Dr. B. G. Hazra (research co-guide). The work is original and has not formed the basis for the award of any other degree, diploma, associateship, fellowoship, titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

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ABBREVIATIONS

AIBN	Azaisobutyronitrile
BSA	Bovine serum albumin
CCDC	Chambridge crystallographic data center
CS	Chitin synthase
DCFH-DA	2',7'-dichlorfluorescein-diacetate
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DHR123	Dihydrorhodamine 123
DMF	N, N-Dimethylformamide
DMSO	Dimethyl sulfoxide
EDC	Ethylene dichloride
EDTA	Ethylenediamine tetra acetic acid
Fru-6-P	Fructose-6-phosphate
Glc-N-6-P synthase	Glucosamine-6-phosphate synthase
GlcNAc	N-Acetyl-D-glucosamine
h	Hour(s)
HCA	Hydrophobic cluster analysis
IR	Infra Red
MHz	Mega Hertz
µg	Microgram
MIC	Minimum Inhibitory Concentration
ml	Mililiter
mM	Milimolar
MS	Mass Spectrum
NBS	N-bromosuccinamide
NCCLS	National Committee for Clinical Laboratory Standards
NMR	Nuclear Magnetic Resonance
PI	Propidium iodide
p-TSA	*p*-Toluenesulfonic acid
p-TsCl	*p*-Toluenesulfonyl chloride
ROS	Reactive oxygen species

S_N2	Bimolecular nucleophilic substitution
TBAB	Tetrabutyl ammonium bromide
t-BuOH	*t*-Butanol
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
UDP	Uridine diphosphate
UDP-GlcNAc	Uridine diphosphate-*N*-acetyl-D-glucosamine
UPOC	Uracil polyoxin C
WGA	Wheat germ agglutinin
WGA-HRP	Wheat germ agglutinin-Horseradish peroxidase

GENERAL REMARKS

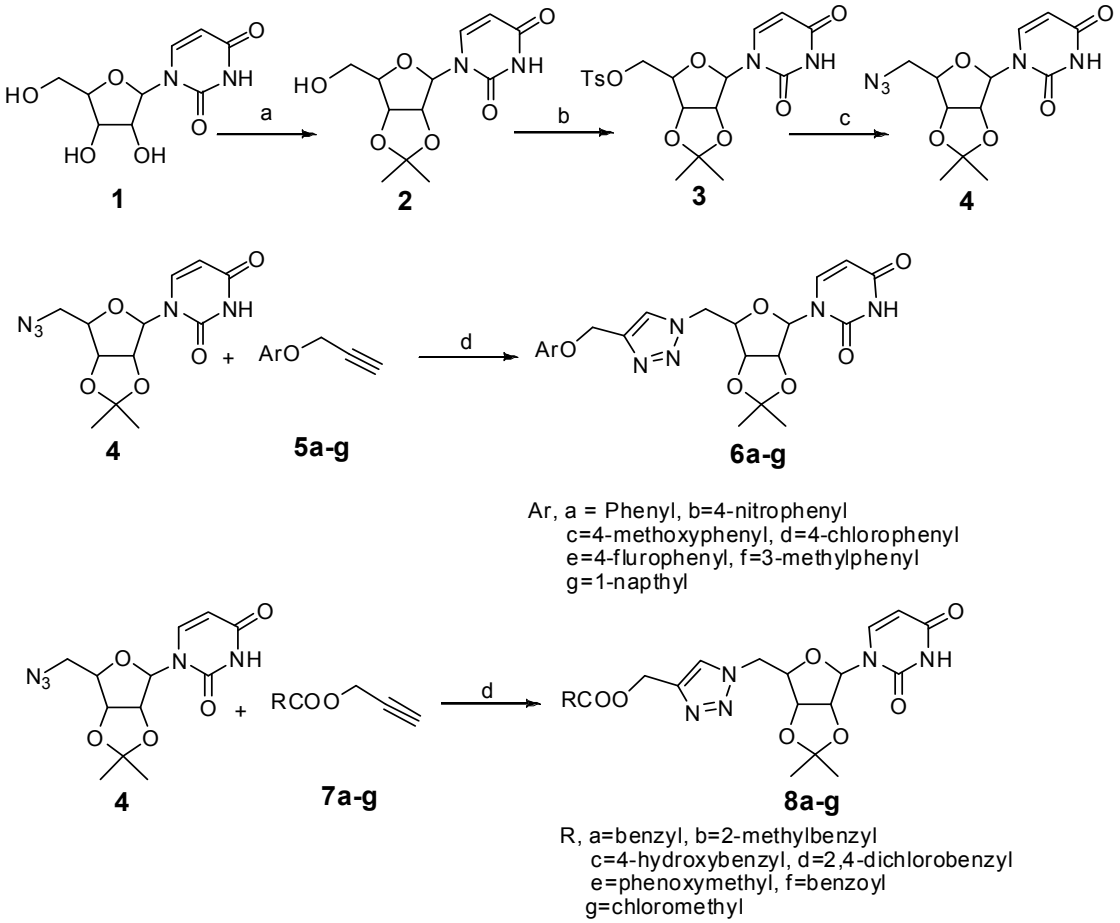
1. Independent references and compound numbering have been employed for abstract, as well as each chapter.
2. All the melting points were recorded on the Celsius scale using Buchi B 540 melting point apparatus and are uncorrected.
3. Petroleum ether used in the experiments was of 60 - 80 °C boiling range.
4. IR spectra were recorded as neat or Chloroform solution or Nujol mull, on a Shimadzu FT-IR spectrophotometer, using NaCl optics. IR bands are expressed in frequency (cm^{-1}).
5. Nuclear Magnetic Resonance spectra were recorded on Bruker AV 200 (200 MHz for ^{1}H and 50 MHz for ^{13}C NMR) or Bruker AV 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C NMR) spectrometers or Bruker DRX 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR). Chemical shifts (δ) are quoted in ppm and are referenced to tetramethylsilane (internal).
6. Mass spectra were recorded on Applied Biosystems API QSTAR Pulsar Mass Spectrometer (Electrospray ionization, direct infusion method, solvents used acetonitrile/methanol)
7. Elemental analysis was carried out on Thermo Finnigan Flash EA 1112 series Elemental Analyzer.
8. All reactions were monitored by thin-layer chromatography (TLC) using precoated silica plates (Merck F254, 0.25 mm thickness) and compounds were visualized by UV and Anisaldehyde reagent.
9. All evaporations were carried out under reduced pressure using Buchi rotary evaporator.
10. All solvents and reagents were purified and dried by following the procedures given in the book “Purification of Laboratory Chemicals” by Armarego and Perrin (3^{rd} edition).
11. Silica gel (60-120 and 230-400 mesh) used for column chromatography was purchased from Spectrochem Pvt. Ltd., India.

ABSTRACT

In recent years due to increase in the population of the immunocompromised patients, the risk of fungal diseases is increasing. Though a number of antifungal drugs are currently in use to treat these infections, different factors such as low potency, poor solubility, narrow clinical spectrum, emergence of resistance and toxicity affects their use. Hence, there is a widely recognized need for new antifungal agents. Polyoxins and nikkomycins are naturally occurring antifungal peptidyl nucleosides, which inhibit chitin synthase (CS) and subsequent cell wall formation. Even though these nucleosides exhibit activity against a variety of fungi, degradation of polyoxins by intracellular peptidases and low uptake of nikkomycin Z by *Candida albicans* cells, make them unsuccessful for clinical applications. The structural feature of these peptidyl nucleosides presents several sites for modification. The present study is aimed at synthetic modifications of nikkomycin and other CS inhibitor scaffolds for the development of new, effective and safer antifungal agents.

Introduction and review of literature (Chapter 1) comprises extensive literature survey of current status of antifungal agents, different important targets present in the fungi, importance and uniqueness of fungal cell wall, chitin and CS enzyme as unique target. Different methods for screening CS inhibitors are also included. Along with this synthetic modifications of polyoxins and nikkomycins reported in the literature are discussed.

Synthesis of 1,2,3-triazolyl uridine derivatives as a chitin synthase inhibitors (Chapter 2) In the present investigation modification of nikkomycin by substituting 1,2,3-triazoles at 5′ position of uridine **1** was undertaken. Synthesis of 1,2,3-triazole substituted uridine derivatives **6a-g** and **8a-g** was achieved using Cu(I)-catalysed Sharpless click chemistry approach. Uridine **1** was converted to 5′-azide-*O*-2′,3′-methylethylidene uridine **4** with 95% yield (Scheme 1). Propargyl ether of phenols **5a-g** were prepared by the reaction of phenols with propargyl bromide and K_2CO_3 in DMF. Propargyl esters **7a-g** were synthesized in excellent yield by heating (80 °C) respective acids in excess of propargyl alcohol. These **5a-g** and **7a-g** were then reacted with 5′-azidouridine **4** in the presence of copper sulfate and sodium ascorbate in *t*-Butanol/water (8:2) to yield exclusively 1,4-disubstituted-1,2,3-triazole derivatives of uridine **6a-g** and **8a-g** (Scheme 1). Structures of these final compounds



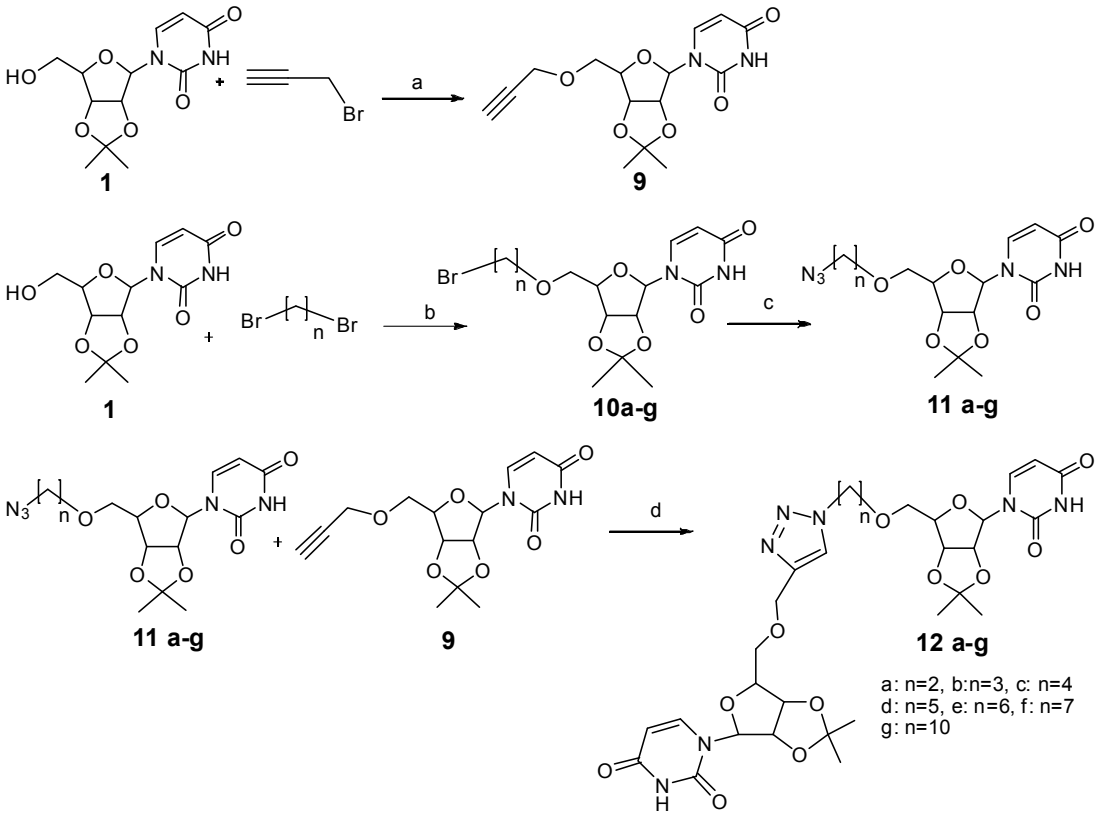
Scheme 1. Reagents and conditions: a) 2,2-Dimethoxy propane, *p*-TSA, acetone, 28 °C, 2 h, 71.5%; b) *p*-TsCl, pyridine, 0-28 °C, 10 h, 78%; c) NaN_3, DMF, 60 °C, 12 h, 70%; d) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 3-8 h.

were confirmed by IR, ^{1}H NMR, ^{13}C NMR, Mass Spectromtery, elemental analysis and X-ray crystallography.

Biological evaluation: Compounds **6a-g** were found to be active against *Candida albicans, Cryptococcus neoformans* and *Fusarium oxysporum* with MIC (minimum inhibitory concentration required for 90% inhibition of growth) values in the range of 8-128 μg/ml. The activity of compounds **6b-d** was higher against *C. neoformans* and *C. albicans* with MIC of 32 μg/ml. Compound **6a** was found to be most potent with MIC 8 μg/ml against *C. neoformans* as compared to nikkomyicn (MIC 32 μg/ml). Compounds **8a, 8b** showed MIC 32 μg/ml against human pathogens *C. neoformans* and *C. albicans,* respectively, which was comparable to nikkomycin. Compound **8g** showed MIC 64 μg/ml against *C. albicans* and potent inhibition of *F. oxysporum* with MIC value 16 μg/ml. Compounds **6b, 6c, 6g, 8a** and **8f** exhibited

>70% of yeast to hypha transition inhibition in *Benjaminiella poitrasii* at 4 μg/ml. Compound **6e, 6f, 8b, 8c, 8g** showed 60-70% of inhibition of transition. It was observed that compounds **6d, 6e, 6f** and **8f** at 4 μg/ml concentration inhibited 80-95% of *B. poitrasii* CS activity. Total seven compounds **6a, 6b, 6g, 8a, 8c, 8e** and **8g** exhibited 70-80% inhibition of CS activity. Remaining compounds except **8b** exhibited inhibition in the range of 60–70%. Based on the data of biological activities compounds **6a, 6b, 6d, 6f** and **8f** were selected as a lead compounds, and tested for their toxicity by haemolysis assay. For all compounds the HC_{50} (concentration required for 50% haemolysis) value was found to be >1024 μg/ml indicating the safety of these compounds for their use.

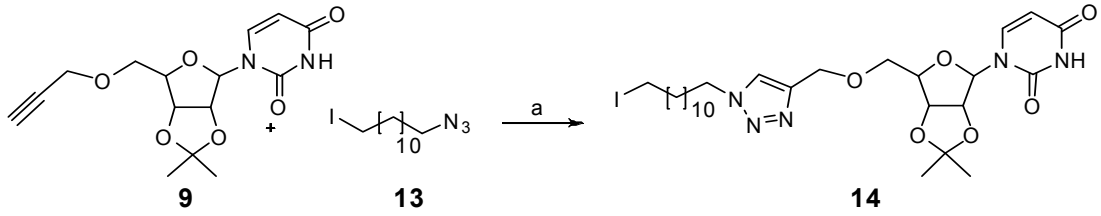
Synthesis of dimeric uridine derivatives as chitin synthase inhibitors (Chapter 3) Exact mechanistic details of chitin formation are still unresolved. In the extended structure of chitin polymer adjacent sugar residues have opposed orientations. It has been proposed that CS has two active sites in order to facilitate the simultaneous polymerization and formation of linear polymer with opposed



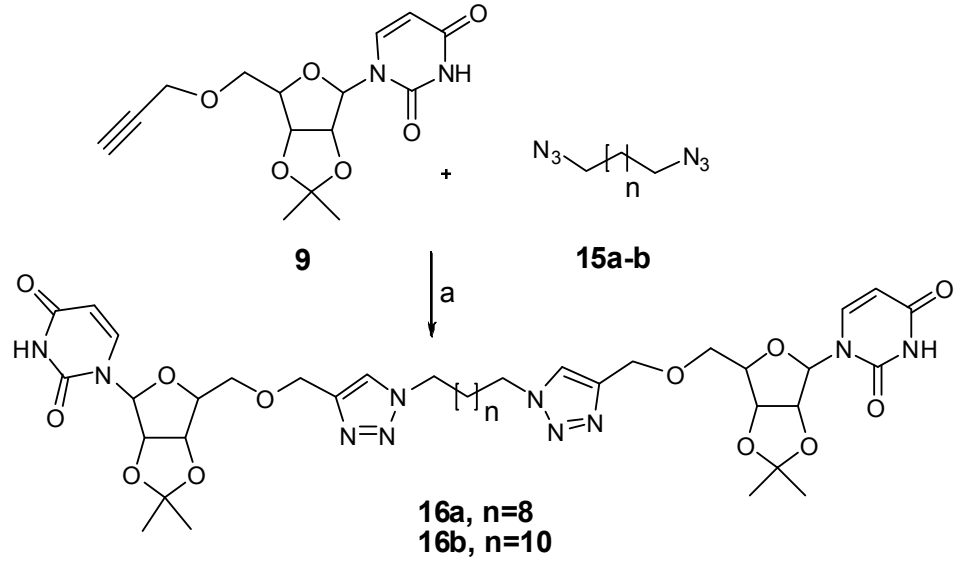
Scheme 2. Reagents and conditions: a) NaH, DMF, 0-28 °C, 6 h, 65%; b) NaH, DMF, 6 h, 0-28 °C; c) NaN_3, acetone, 65 °C, 6 h; d) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 3-6 h.

orientation of adjacent sugars. According to this hypothesis there should be two binding sites present for the UDP-GlcNAc binding. In such case appropriate dimeric nucleosides inhibitor would be more potential than corresponding monomeric inhibitor. With this rationale, few dimeric inhibitors were designed containing two uridine molecules connected together with varied length of aliphatic spacer in-between. For the synthesis of **12a-g** dimeric inhibitors click chemistry approach was used. Compound **9** was synthesized by propargylation of primary alcohol of uridine **1** using propargyl bromide (Scheme 2)**.** Synthesis of **10a-g** was achieved by o-alkylation of primary alcohol of uridine **1** with different dibromoalkanes in basic condition, further displacement of bromo with azide group led to **11a-g**. Uridine propargyl ether **9** was coupled with different azides **11a-g** in presence of copper sulphate and sodium ascorbate to afford final dimeric compounds **12a-g** (Scheme 2).

Momomeric inhibitor **14** was synthesized by coupling of uridine propargyl ether **9** with dodecaneiodo azide **13** in presence of copper sulphate and sodium ascorbate (Scheme 3). Along with this some symmetrical dimeric molecules were also



Scheme 3. Reagents and conditions: a) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 4 h, 70%.



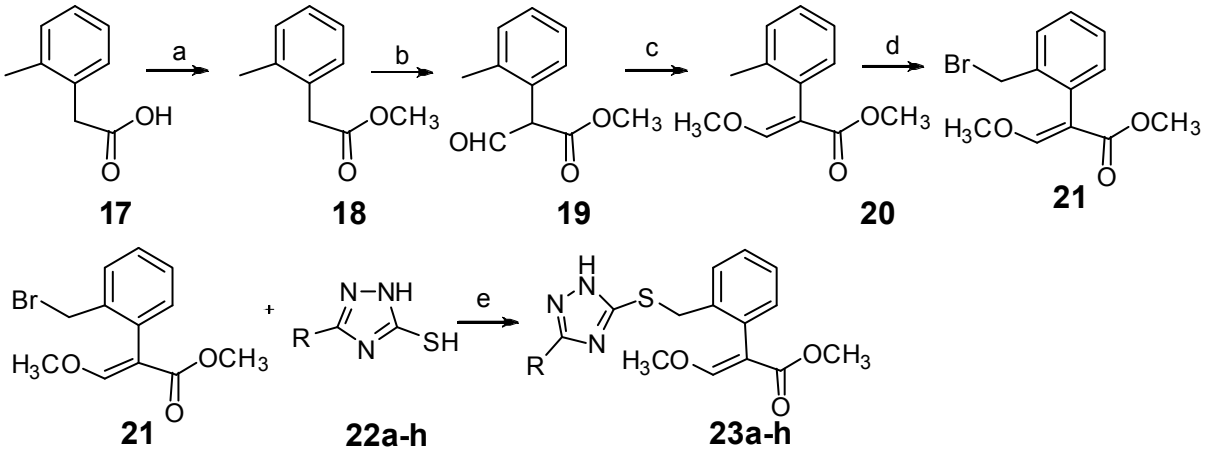
Scheme 4: Reagents and conditions: a) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 3-6 h.

synthesized. For this purpose diazides of higher alkanes such as decane and dodecane **15a-b** were synthesized and coupled with compound **9** to get **16 a** and **16b** (Scheme 4)**.**

Biological evaluation: All synthesized compounds **12a-g** were unable to inhibit the growth of tested organisms up to 500 μg/ml concentration. Only compound **14** showed MIC 32, 64 and 16 μg/ml against *C. glabrata*, *C. albicans* and *F. oxysporum* respectively. Compound **14** showed MIC of 4 μg/ml against all strains of *Cryptoccocus* sp. Dimeric compounds **12a-d** showed Y-H transition inhibition of *B. poitrasii* in the range of 45-58%, while compound **12e-g** and **16a-b** showed inhibition in the range of 60-70% respectively at 4 μg/ml concentration. Monomeric compound **14** inhibited the yeast to hypha transition to 68%. In case of dimeric compounds **12a-g**, the inhibition of CS from *B. poitrasii* at 250 μg/ml concentration was found to be in the range of 4-33%. The monomeric inhibitor **14** inhibited 57% CS activity at same concentration. The CS inhibition for even number of spacer length was found to increase with increase in the spacer length.

Mode of action of monomeric inhibitor using *Cryptococcus neoformans* as a model (Annexure I) Time kill assay of **14** against *C. neoformans* showed 98% killing of cells within 6 h. In propidium iodide (PI) staining, increased number of PI stained cells with increase in concentration of **14** indicated disruption of membrane integrity**.** Dihydrorhodamine 123 and 2′,7′-dichlorofluorescine diacetate staining showed increase in relative fluorescence with increased concentration of **14** indicating generation of intracellular reactive oxygen species. Hyperpolarization of mitochondrial membrane potential was evident in rhodamine123 staining of cells treated with compound **14.** Only 13% RBC haemolysis was observed for compound **14** at 128 μg/ml concentration. The results indicated that antifungal mode of action of **14** is by generation of intracellular reactive oxygen species, disruption of fungal cell membrane and loss of mitochondrial membrane potential.

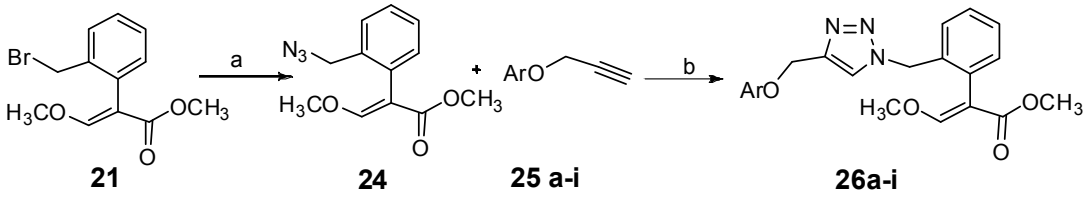
Synthesis of antifungal 1,2,3-triazole and 1,2,4-triazole thiol substituted strobilurin derivatives (Chapter 4) Strobilurins are antifungal compounds produced by fungus *Strobilurus tenacellus*. β-methoxyacrylate group is common structural element of all strobilurins. New strobilurin derivatives containing 1,2,4-triazole thiol and 1,2,3-triazole side chains were designed. Synthesis of 1,2,4-triazole thiol strobilurin derivatives **23a-h** was started with *o*-tolylacetic acid **17** which was converted to methyl ester **18.** Compound **18** on formylation gave **19,** which after



R, a= H, b= methyl, c= *t*-butyl, d =phenyl, e=*p*-chloro phenyl,
f= *p*-methoxyphenyl g=2-furyl , h =2-thiophene

Scheme 5. Reagents and conditions: a) MeOH, H_2SO_4, 28 °C, 12 h, 98%; b) Methyl formate, NaH, Toluene, 0-28 °C, 12 h, 98%; c) Dimethyl sulphate, EDC/water (1:1), TBAB, Na_2CO_3, 28 °C, 2 h, 98%; d) AIBN, NBS, CCl_4, 80°C, 3 h, 95%; e) K_2CO_3, DMF, 28 °C, 4 h.

reaction with dimethyl sulphate was converted to compound **20**. Bromination of **20** resulted in (*E*)-Methyl-2-(2-bromomethylphenyl)-3-methoxyacrylate **21.** In basic condition compound **21** was coupled with different 1,2,4-triazole thiols **21a-h** to yield final compounds **23a-h** (scheme 5). Compound **21** was converted to its respective azide **24** using sodium azide. This azide **24** was further reacted with different propargyl ether phenols **25a-i** in presence of copper sulphate and sodium ascorbate to



Ar, a= 4-chlorophenyl, b=2-chlorophenyll
c=2,6-dichlorophenyl, d= 3-methylphenyl
e=2,6-dimethyl phenyl, f=4-nitrophenyl
g=4-fluorophenyl, h=1-napthyl, i=2-napthyl

Scheme 6. Reagents and conditions: a) NaN_3, acetone, 60 °C, 6 h, 92.3%; b) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 3-6 h.

get final compounds **26a-i** (Scheme 6).

Biological evaluation: 1,2,4-triazole thiol compound **23a-h** specifically showed growth inhibition of *C. neoformans* and *Magnaporthe grisea.* **23a, 23c, 23f** showed MIC of 64 μg/ml against *C. neoformans* 3541. MIC of **23b and 23f** against

M. grisea was 16 and 64 μg/ml, respectively. Compound **23e** was found to be most potent with MIC 16 μg/ml against *C. neoformans* and *M. Grisea,* whereas its MIC against *C. neoformans* 3541 and *C. neoformans* 3542 was 64 μg/ml. From the 1,2,3-triazolyl strobilurin derivatives **26a-i,** only compound **26b** inhibited the growth of *C. albicans* 3557 with MIC 64 μg/ml. Y-H transition inhibition by compounds **23a-h** was in the range of 44-75%. In case of compounds **26a-i,** transition inhibition was observed from 21-55 %. Compounds **23a-f** at 64 μg/ml concentration showed CS inhibition in the range of 30-55%. For compounds **26a**, **26b**, **26d** and **26h** CS inhibition was in the range of 29-46%.

Compounds **30c, 30d, 30e** and **30f** showing good antifungal activity and CS inhibitory activity were tested further for their haemolytic activity. The HC_{50} values were >1024 μg/ml for these compounds.

Summary and conclusions Click chemistry approach was applied successfully for practical, reliable and efficient synthesis of forty one 1,4-disubstituted-1,2,3-triazolyl uridine derivatives, dimeric 1,2,3-triazolyl uridine derivatives, 1,2,4 triazole thiol and 1,2,3 triazole strobilurin derivatives. New potent antifungal agent **14** (Monomeric inhibitor) was synthesized and its mode of action was elucidated. Most of the synthesized compounds showed significant antifungal activity and have potential applications in agriculture and medicine.

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

Over the past two decades, fungal infections have increased significantly in frequency and becoming major reason of morbidity and mortality. As advances in medical care have improved the survival of patients with severe and life-threatening illness, the more aggressive nature of such care has led to a rapid increase in the number of immunosuppressed population which includes the patients with AIDS, autoimmune diseases, burns, radiotherapy, chemotherapy and transplantation [1]. These changes have been correlated with a substantial increase in the rate of invasive fungal infections (IFIs). Apart from the increased number of immunocompromised hosts, resistance development against antifungals have aggravated the situation [2].

1.1 Fungal infections

The Term mycosis refers to conditions in which fungi invade the resistance barriers of the human body and establish infections. These mycoses are of different types. Infections can be superficial that is situated at or close to the surface of the skin, or systemic, which means they can affect the body as a whole rather than individual parts or organs. Fungal infections are divided in the following groups

1. Superfical mycoses
2. Cutaneous mycoses
3. Subcutanneous mycoses
4. Systemic mycoses due to primary pathogens
5. Systemic mycoses due to opportunistic pathogens

The fungal pathogens are majorly divided in to two classes, primary true pathogens (*Histoplasma capsulatum, Blastomyces dermatitidis* and *Coccidioides immitis*) and opportunistic pathogens. Increase in the population of immunocompramized patients and increase in resistance against currently used drugs led to changing epidemiology and emergence of fungal pathogens which were previously considered clinically insignificant [3-5]. Most infections are caused by *Candida albicans* and *Aspergillus* sp. (*A. fumigatus* and *A. flavus*). However in last 2-3 decades, their percent share has declined due to emergence of pathogenic fungi which include - *Acremonium* sp., *Acremonium terreus,* non-albicans *Candida* sp. (*C. glabrata*, *C. parapsilosis*, *C. krusei*), *Cryptococcus neoformans, Fusarium* sp., *Microsporum* sp., *Paecilomyces* sp., *Penicillium marneffei, Rhizopus oryzae, Trichoderma* sp., *Trichophyton* sp., and *Trichosporon* sp. etc. [6-9].

1.2 Antifungal agents in current use

Different antifungal agents are currently in use for the treatment of fungal infections. In the early 1950s the available systemic antifungal drugs were nystatin and amphotericin B but toxicity limited their use. After two decades without any progress, the field of medical mycology was revolutionized by development of the triazoles in 1980s. Further development in triazoles and the reformulation of amphotericin B with lipid compounds was done in 1990s. These lipid-based preparations of amphotericin B were found to be less toxic with improved efficacy. Echinicandins, the new and safer antifungals targeting β-(1,3)-D-glucan synthase, were developed in late 1990s and the start of new millennium. The timeline for the development of antifungal agents is shown in Figure 1.1. These antifungal agents are broadly classified in to five classes as shown in Table 1.1.

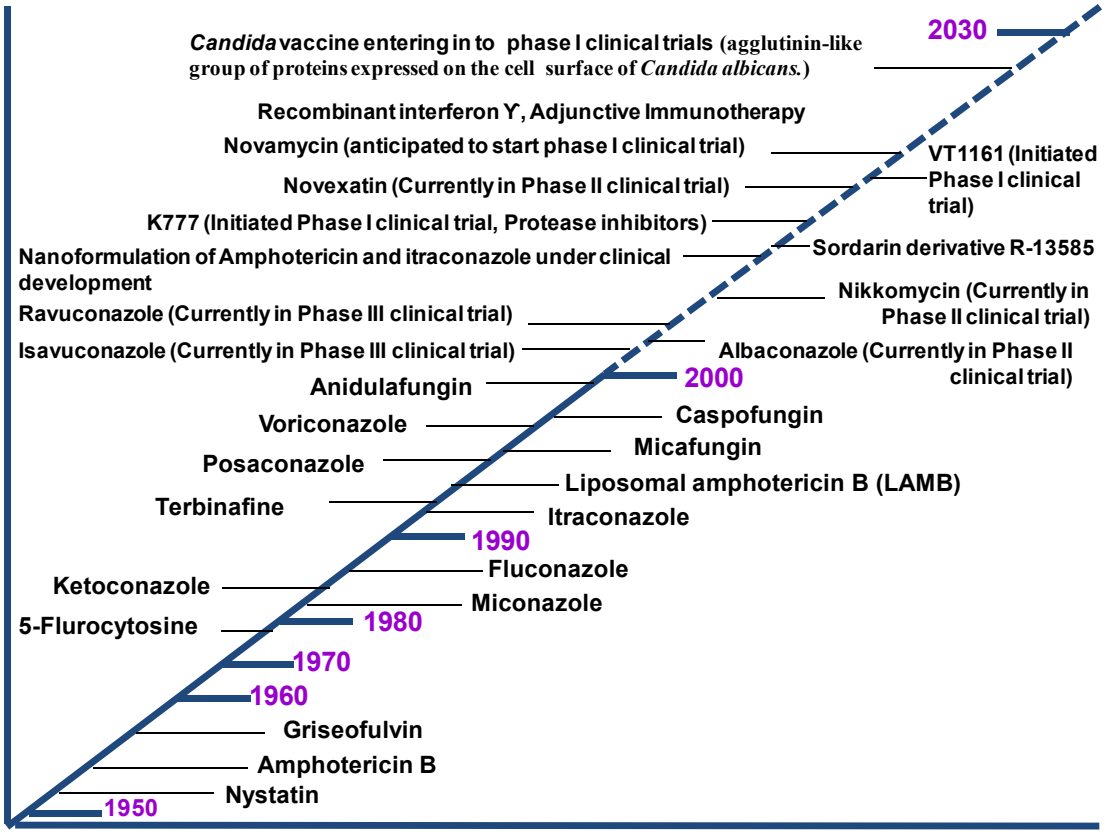


Figure 1.1 Timeline for the development of antifungal agents

Intensive use of ergosterol synthesis inhibitors for many years has resulted in resistance development. Secondly, due to the drawbacks of existing drugs such as acute and chronic side effects, less clinical efficiency, effect on non-targeted cells; the development of new antifungal agents is of major importance.

Table 1.1 Clinically used antifungal drugs

Class of antifungals	**Antibiotic**	**Target - Mode of action**
Polyenes	Amphotericin B, Nystatin	Ergosterol - Polyenes complex with ergosterol in the fungal cell membrane and compromise the integrity of the cell membrane.
Azoles	Imidazoles (e.g. ketoconazole, miconazole) Triazoles (e.g., fluconazole, itraconazole and voriconazole)	Ergosterol biosynthesis - Inhibition of lanosterol demethylase (cytochrome P450 dependent) enzyme leading to ergosterol depletion and accumulation of intermediates in the pathway. It disrupts the structure of the cell membrane, alters the activities of several membrane-bound enzymes.
Allylamines	Terbinafine	Ergosterol biosynthesis - Inhibit the enzyme squalene epoxidase in the ergosterol biosynthesis pathway
Fluoro-pyrimidines	5-fluorocytosine (5-FC)	Nucleic acid synthesis - Inhibit DNA and RNA synthesis
Echinocandins	Caspofungin	Glucan synthesis – Inhibit β-(1,3)-F-glucan synthesis resulting in disruption of the fungal cell wall

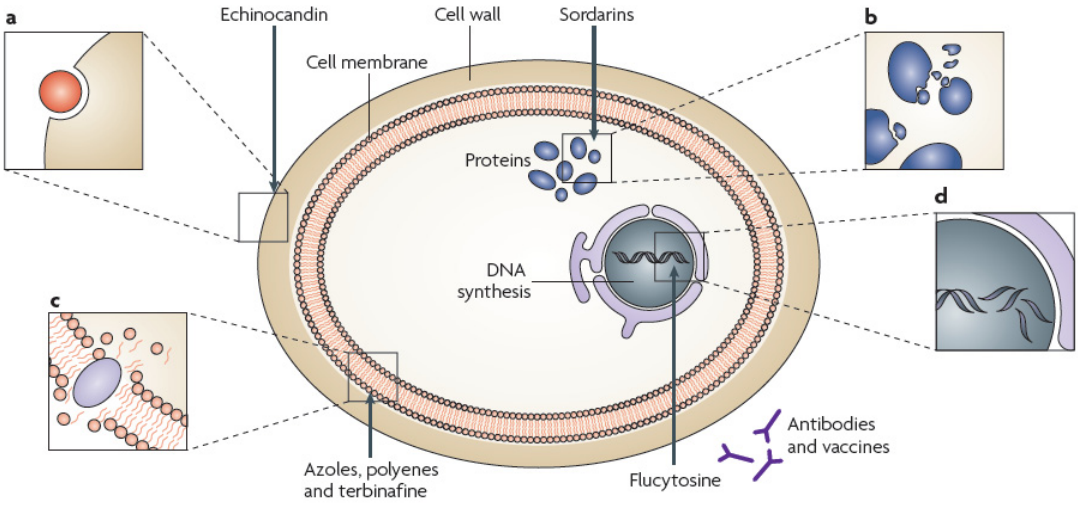


Figure 1.2 Mechanism of action of antifungal agents towards different targets (Source: Ostrosky-Zeichner et al. [12]).

For more details on current status of antifungal drug development and challenges in the treatment of fungal infections, please refer [10-12]. To achieve the goal of development of new potential drugs, one of the promising strategies is to identify novel, unexploited molecular targets almost exclusive to fungi. The main criteria for the selection of target includes

- The target should be important for either growth or virulence of the fungi
- Target should be universally present in all fungi
- It should be absent in the plants and animals.

As shown in Figure 1.2, protein synthesis, DNA synthesis, fungal cell membrane and fungal cell wall can be targeted for the development of antifungal agents. Out of these targets, fungal cell wall is unique target as it is present in all fungi, while absent in mammals.

1.3 Fungal cell wall as a antifungal target

Fungal cell wall is outermost rigid covering surrounding the metabolically active protoplast. The fungal cell wall is primarily composed of different biopolymers, which includes glycoproteins, mannoproteins and polycarbohydrates such as β- glucans, mannan and chitin (Figure 3.3). These polymers protect cell against mechanical stress as well as osmotic shock, decide morphology of actively

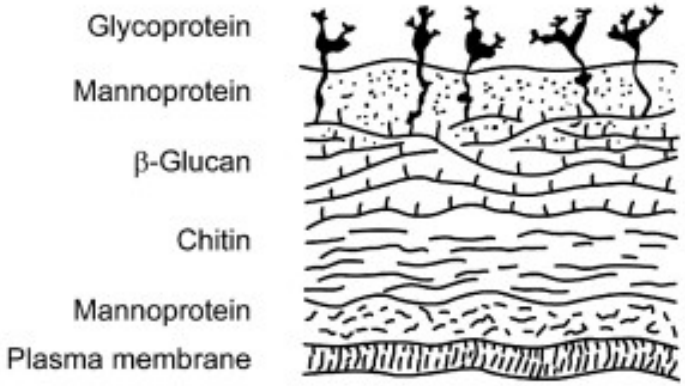


Figure 1.3 Ultra structure of fungal cell wall

growing cell. Therefore any kind of disruption to the cell wall would lead to the death of the fungi. Glucan, main structural component of fungal cell wall, consist of α-1,3-D-glucan, β-1,3-D-glucan and β-1,6-D-glucan [13]. Membrane bound enzyme β-(1,3)-D-glucan synthase catalyzes the biosynthesis of glucan from UDP-glucose.

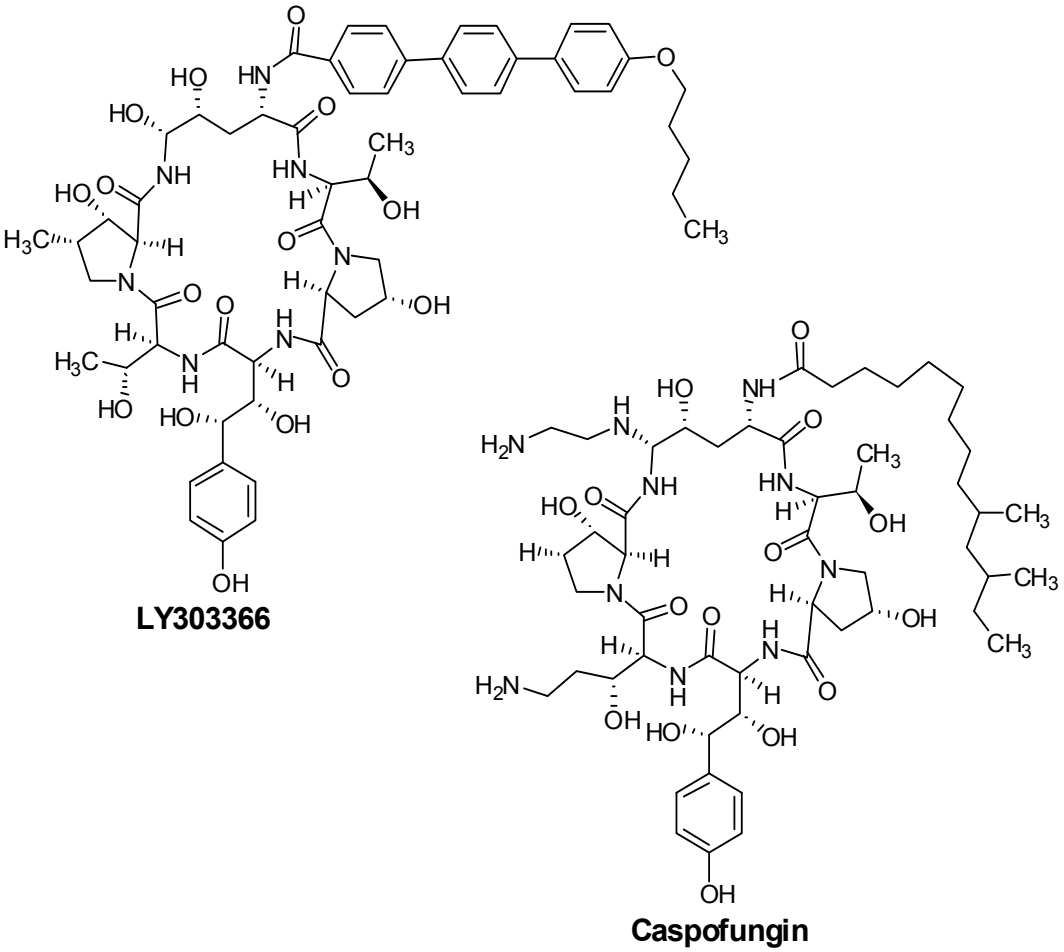


Figure 1.4 Glucan synthase inhibitors

Lipopeptide echinocandin class of compounds like LY303366 and caspofungin are known inhibitors of β-(1,3)-D-glucan synthase (Figure 1.4). These inhibitors act by non competitive mode of action [14].

Mannoproteins are homopolymers lacking crystalline skeleton and contains about 95% of polysaccharides. These polysaccharides are covalently bound to proteins through *N*-acetyl glucosamine residues (GlcNAc) by *N*-glycosylation of aspargine or *O*-glycosylation of threonine or serine. Compounds inhibiting this *N*- or *O*-glycosylation of protein may have antifungal potential. Pradimicin A and benanomicin A (Figure 1.5) are naturally occurring mannoprotein inhibitors [15, 16]. These mannoprotein inhibitors alter the cell membrane permeability by binding to cell wall manoproteins in presence of Ca^{2+} ions [17].

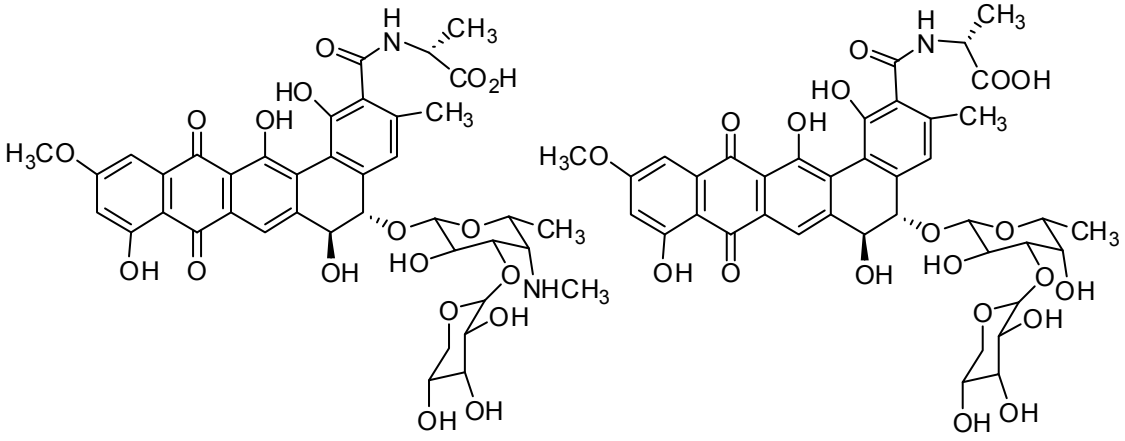


Figure 1.5 Mannoprotein inhibitors

From the different polycarbohydrates of cell wall, specifically chitin, a polymer of β-1,4-linked-*N*-acetylglucosamine, is present in broad range of fungi, while absent in plants and animals. Therefore, it is considered as a ideal target for antifungal agent development. Chitin biosynthesis, chitin synthase (CS) as target, different naturally occurring and chemically synthesized CS inhibitors are briefly discussed in following sections.

1.4 Chitin biosynthesis

In fungi, the amount of chitin in the cell wall varies from 2% in few yeasts to 61% in some filamentous fungi. Irrespective of the proportion, chitin appears to be essential for fungal growth and survival [18]. Glucosamine-6-phosphate synthase

(GlcN-6-P synthase), first enzyme of chitin synthesis pathway, brings together carbon and nitrogen metabolism pathways through conversion of fructose-6-phosphate to

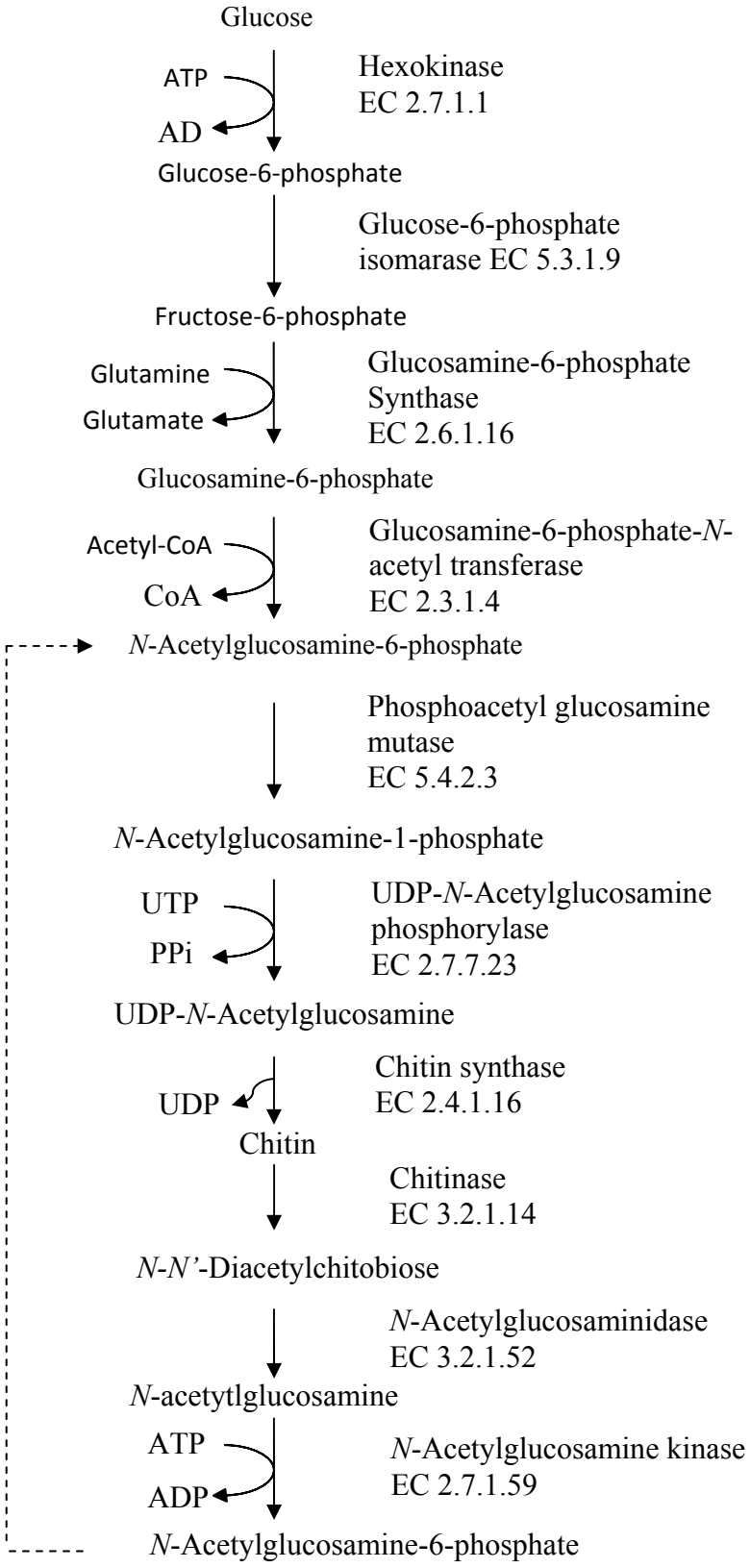


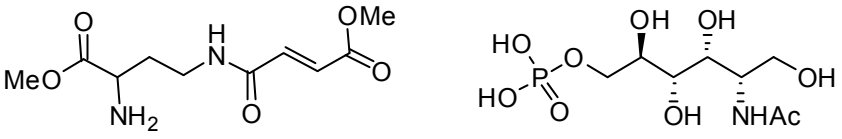
Figure 1.6 Chitin synthesis pathway

glucosamine-6-phosphate.

In subsequent steps, this glucosamine-6-phosphate is then converted to uridine diphosphate-*N*-acetyl glucosamine (UDP-GlcNAc). CS (EC 2.4.1.16) is crucial as it is involved in the synthesis of chitin from UDP-GlcNAc (Figure 1.6). In the cytoplasm, most of the zymogenic CS is accumulated in specialized structures called chitosomes. The chitosomes fuse to the plasma membrane, active CS is formed by proteases and eventually chitin is synthesized. The crystalline microfibrils have 20-400 chitin chains held together by hydrogen bonding [19, 20].

1.4.1 Glucosamine-6-phosphate synthase

GlcN-6-P synthase catalyses complex reaction involving ammonia transfer from L-glutamine to fructosamine-6-phosphate (Fru-6-P), followed by isomerisation of the formed Fru-6-P to glucosamine-6-phosphate. Several GlcN-6-P inhibitors have been reported from both natural and synthetic origin. Most of them were glutamine analogs acting as active site-directed alkylating agents. Tetaine was the first recognized selective inhibitor of GlcN-6-P synthase [21]. The most studied GlcN-6-P synthase inhibitor was N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP, Figure 1.7) a glutamine analog, acts as an active-site-directed inactivator, blocking the N-terminal, a glutamine binding domain of the enzyme [22, 23]. Another compound, 2-amino-2-deoxy-D-glucitol-6-phosphate (ADGP, Figure 1.7) is an analog of the putative transition state of the catalyzed sugar conversion reaction and is a strong inhibitor of the C-terminal domain of the GlcN-6-P synthase subunit.



FMDP **ADGP**

Figure 1.7 Glucosamine-6-phosphate synthase inhibitors

1.4.2 Chitin synthase

CS is present as isoezyme in fungi; the number varies from one in *Schizosaccharomyces pombe* [24], four in *C. albicans* [25], eight in *Benjiaminella poitrasii* [26] to ten in *Phycomyces blakesleeanus* [27]. CS, a glycosyltransferase type

enzyme, catalyzes a transglycosylation reaction in which sugar residues are transferred from UDP-GlcNAc to the growing chitin chain releasing uridine diphosphate (UDP, Figure 1.8). The different isoenzymes are involved in chitin synthesis at different sites or different time points in the life cycle of the fungus such as repair during cytokinesis, synthesis of primary septum, lateral cell wall integrity and hyphal tip growth soon and so forth. Roncero reviewed different CS in fungi, their functions, genetic complexity and particularly its regulation in *Saccharomyces cerevisiae* [28].

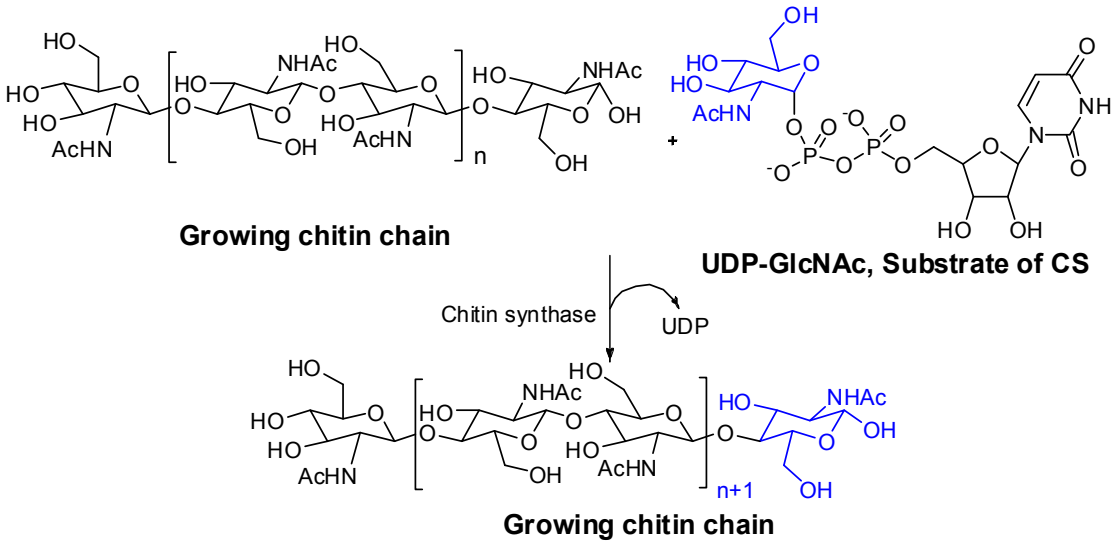


Figure 1.8 Mechanism of chitin synthesis

The universal motif of β-glycosytransferases - amino acid residue sequence glutamine-any amino acid-any amino acid-arginine-tryptophan (QXXRW) is present in CS. Along with it, CS active site has two conserved domains A and B, with two aspartic acid residues at domain A and one at domain B which are required for catalytic activity [29]. Based on the alternating orientation of the GlcNAc residues within the chitin chain; it was proposed that CS possesses two active sites. According to the hypothesis, CS is a processive glycosyl transferase (that adds a number of sugar residues) which forms two glycosidic bonds simultaneously by a specific mechanism resulting in the inversion of the anomeric configuration and release of two UDP molecules [29].

1.5 Chitin synthase inhibitors

Polyoxin and nikkomycin were the first reported CS inhibitors isolated from culture filtrates of *Streptomyces* sp., thereafter number of CS inhibitors were isolated

from natural sources like plants, microorganisms. Whereas, synthetic CS inhibitors have been designed and prepared mainly based on diversity-oriented synthesis of UDP-GlcNAc analogues.

1.5.1 Screening methods for chitin synthase inhibitors

The potential of antifungal agent is mainly adjudged by growth inhibition or spore germination inhibition assays. The assays are performed by disc diffusion method or broth microdilution technique as per Clinical and Laboratory Standards Institute (CLSI) guidelines.

Most of the pathogenic fungi (e.g. *C. immitis, B. dermatitidis, H. capsulatum, C. albicans*) change their morphology reversibly between yeast and filamentous hyphal form for survival and proliferation in the host, which in other words, is the change from saprophytic to pathogenic form. Therefore the compound which inhibits this reversible transition may have a potential as a antifungal drug. Usually in a dimorphic fungus, chitin contents increase during yeast to hypha transition [30]. Therefore, inhibition of transition can be used as a test for screening of potential CS inhibitors [31].

The filamentous fungi grow apically. Usually hypal tip is plastic, thin walled and a site for the deposition of nascent chitin. In the presence of specific CS inhibitor in osmotically stabilized medium, the hyphal tip bursting can be observed within few minutes under the microscope [32].

It has been reported earlier that initial chitin synthesis is essential for protoplast regeneration in *Benjaminiella poitrasii,* and specific CS inhibitors like nikkomycin have been shown to hamper the process of regeneration [33]. Similarly, Carrano *et al.* developed a method to detect CS inhibitors based on regeneration of *C. albicans* protoplast using fluorescent chitin precursor dansyl GlcNAc [34].

A yeast genome-wide drug induced haploinsufficiency screen using *S. cerevisiae* as a model organism has been developed by Giaever *et al.* [35]. Lowering the dosage of a single gene from two copies to one copy in diploid yeast results in a heterozygote. The resulting mutant strain becomes sensitive to any drug that acts on the product of this gene as compared to wild type strain. This haploinsufficient phenotype thereby identifies the gene product of the heterozygous locus as the likely drug target [36]. For instance, diploid wild type *S. cerevisiae* strain and its mutant

(haploid for the targeted gene *Chs2* – CS 2) are used for the screening of the CS inhibitors. As the mutant has only one copy of *Chs2,* the effect of CS inhibitors on growth will be more pronounced on it as compared to the wild type, whereas other agents will exert similar effect on both the strains. *S. cerevisiae* genes essential for growth and with no homologs in humans may be selected as specific antifungal target. However, around 30% of the genes essential for *C. albicans* yeast to filamentous transition, a switch central to its pathogenicity, lack homologs in *S. cerevisiae* and thus are better candidate antifungal targets [37]. Therefore, Roemer *et al.* employed a pathogen-focused approach for drug discovery using haploinsufficiency in *C. albicans* (termed as CaFT – *C. albicans* Fitness Test) and screened 45% of the *C. albicans* genome for the molecular targets of growth inhibitory compounds [38]. Using >2,800 heterozygous deletion strains and known inhibitors gene function elucidations were done. CaFT as screening test for drug discovery will confirm the mechanism of action of new compounds and their therapeutic potential as antifungal agents. Few of such molecular targets are mentioned in Table 1.2 and except *Chs2*; other targets are new with no known inhibitor.

Table 1.2 Few new* targets in yeasts and filamentous fungi for the development of antifungal agents

Name of the target	Biological role	Additional remarks
TRL1	Required for tRNA splicing and has phosphodiesterase, polynucleotide kinase, and ligase activities	Yeast is not viable due to deletion of targeted gene
ALR1	Plasma membrane Mg^{2+} transporter	Yeast is not viable due to deletion of targeted gene
RSC9	Component of the RSC chromatin remodeling complex; DNA-binding protein involved in the synthesis of rRNA	Yeast is not viable due to deletion of targeted gene
YPD1	Osmo-sensory signaling pathway via two component system	Yeast is not viable due to deletion of targeted gene.
CHS2	Chitin synthesis	In yeast, *CHS2* deletion results in loss of well-defined septa and growth is arrested.

*except *CHS2*

The pure form of the chitin is present only in diatoms. The extracellular β-chitin spines of the diatom, *Thalassiosira fluviatilis* are completely acetylated. The spines act as flotation device. Nikkomycin was reported to inhibit the β-spine formation in exponentially growing diatoms, which resulted in the sedimentation of treated cells. This sedimentation rate was found to be dose dependent [39].

For CS inhibitors screening, *in vitro* radiometric enzyme assay is routinely used in which CS activity in presence and absence of the inhibitor is estimated using radiolabelled substrate UDP-^{14}C-*N*-acetylglucosamine and by measuring the incorporation of ^{14}C in the formed chitin [40]. CS activity inhibition can also be estimated by non-radioactive spectrometric CS assay using horse radish peroxidase-WGA conjugate [41].

1.5.2 Naturally occurring chitin synthase inhibitors

1.5.2.1 Polyoxins

Isono *et al.* reported the protective and curative effects of the culture filtrates of three strains of *Streptomyces* against sheath blight disease of rice caused by *Rhizoctonia solani* [42]. Purification of the culture filtrates resulted in isolation of polyoxins A and B, which showed antifungal activities against other phytopathogenic fungi *Alternaria kikuchiana, Cochliobolus miyabeanus, Pyricularia oryzae* and *Pellicularia filamentosa* with minimum inhibitory concentration (MIC) in the range of 0.2-12.5 μg/ml [42, 43]. Subsequently, polyoxins A to L (Figure 1.9) were isolated and purified from the culture broths of *Streptomyces cacaoi* var. *asoensis* [44]. Polyoxins were designated as a new class of peptide nucleoside antibiotics based on the similarity of the core structure of all active polyoxins with CS substrate UDP-GlcNAc [44].

In vitro enzyme inhibition studies using *Saccharomyces carlsbergensis* CS preparation confirmed that the mode of action of the polyoxins was through competitive inhibition of the CS enzyme [45]. Further study on the relationship of structures of polyoxins to *P. oryzae* CS inhibition indicated that the oxygen atom at C-1′, amino group at C-2′, hydroxyl groups at C-3′ and C-4′, aliphatic carbon chain and terminal carbamoyloxy group moiety of polyoxins helps to stabilize the polyoxin-enzyme complex [46]. pKi-pH Plots for polyoxin C and other polyoxins indicated that the ionized amino group at C-2′ position plays an important role in the binding of

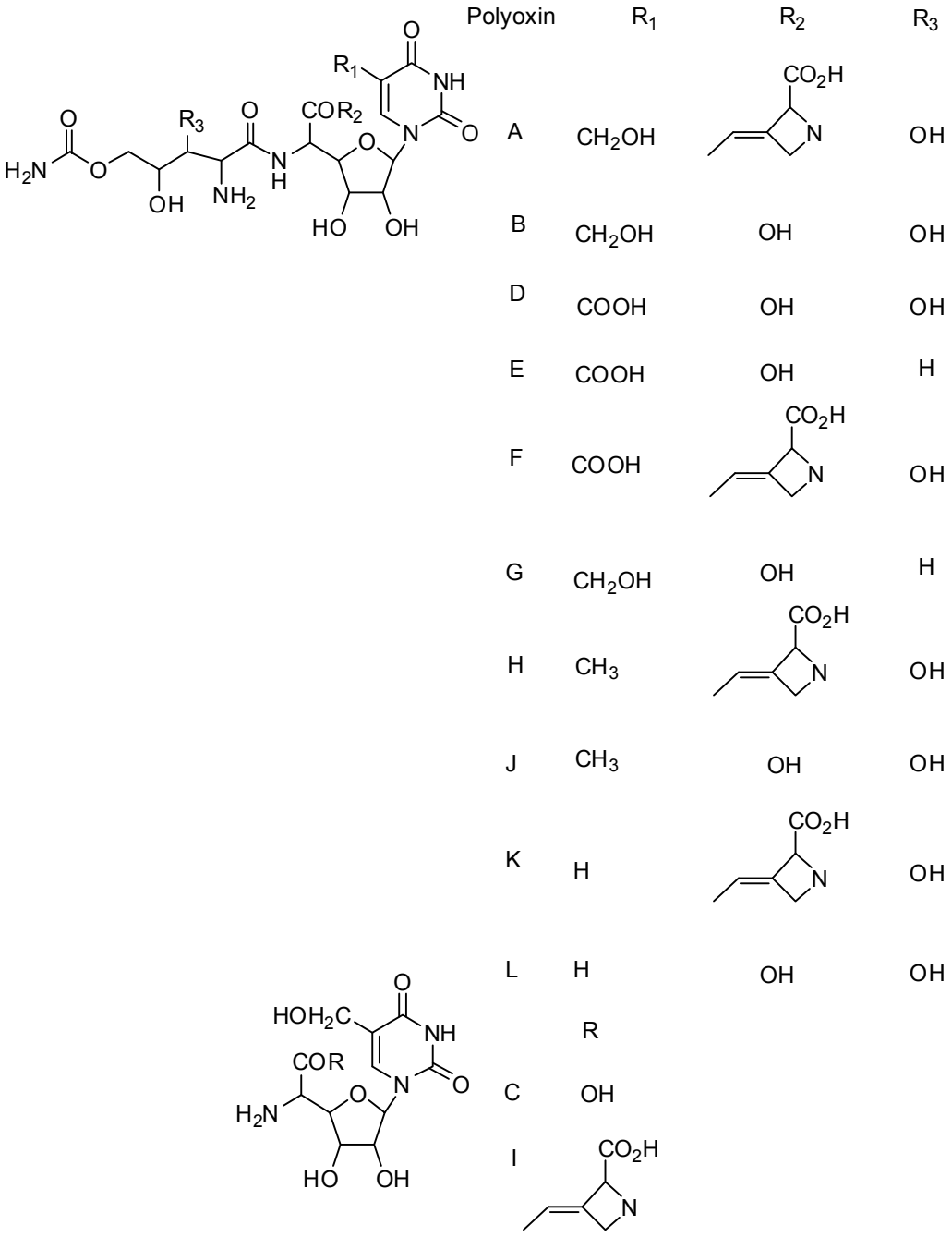


Figure 1.9 Structures of polyoxins

polyoxins to CS. Whereas, the carbonyl oxygen atoms at C-1′ and of the carbamoyloxy group might participated in the hydrogen bond formation with the enzyme [47].

Few studies demonstrated the *in vivo* effects of polyoxin D on cell wall of different fungi [48-50]. Though polyoxins were effective *in vitro* against CS, they were ineffective *in vivo* against yeast cells. The reasons were inability of polyoxins to cross the cellular membrane and accumulate intracellularly to required inhibitory

concentrations and presence of other peptides in the growth medium which inhibited polyoxin transport and action [50]. Though naturally occurring polyoxin D was completely resistant to degradation by cell extracts, many other polyoxins were rapidly degraded by intracellular peptidases to products that were not inhibitors of CS [51].

1.5.2.2 Nikkomycins

From the fermentation broth of *S. tendae* Tü 901, Dahn *et al.* isolated an antibiotic nikkomycin, which inhibited the growth of several fungi by affecting chitin biosynthesis [52]. Its structure was identified as a nucleoside-peptide antibiotic consisting of uracil, an amino hexuronic acid and an amino acid containing a pyridine ring. Bayer AG first evaluated nikkomycin as fungicide (U.S. Patent Nos. 4,315,922 and then patented a method for treating fungal infections with nikkomycin derivatives (U.S. Patent 5019560). Further from the fermentation studies with wild type and mutant strains of *S. tendae* Tü 901, 21 biologically active and 6 inactive nikkomycins were isolated and purified [53, 54]. Nikkomycins X, Z, pseudo-Z, I, J, N, M, D, E, B_Z, B_X, C_Z, C_X are produced by wild type *S. tendae* Tü 901. Mutant strains *S. tendae* Tü 901/395, Tü 901/172 and Tü 901/C37 produce the remaining nikkomycins O_Z, O_X, W_Z, W_X, K_Z, K_X, Q_Z, Q_X, R_Z, R_X, ZT, Z_H, J_T, J_H (Figure 1.10).

Based on *in vitro Coprinus cinereus* CS inhibition, *Ki* (inhibition constant) for the nikkomycins were found in the range of 0.81-4.5 μM, with the order B_X>X>Z_H>Z_T>B_Z>O_X>Z>W_X. The nikkomycins B_X and X exhibited better antifungal activity against different fungi including *C. albicans, Yarrowia lipolytica, Paecilomyces variotii, Mucor hiemalis* than nikkomycin Z [55]. Nikkomycin Z showed poor antifungal activity against *S. cerevisiae* as it inhibited only CS 3 and was ineffective against CS 2 [56]. Whereas, in *C. albicans,* nikkomycin Z inhibited vegetative growth by inhibiting all the three CS and IC_{50} value for CS 1, CS 2, CS 3 were 7.4, 0.4 and 6.4 μg/ml, respectively.

The problems associated with the polyoxins i.e. transport inside the cell and degradation by intracellular peptidases were overcome in nikkomycins. Nikkomycin Z, X and Bx were resistant to enzymatic hydrolysis but were unstable at neutral and alkaline pH, which can be attributed to presence of hydroxypyridyl residue. Replacement of hydroxypyridyl residue with hydroxyphenyl ring as in nikkomycins

W, Wz, Bx and B_Z resulted in stability at neutral and alkaline pH. The β-methyl group of the N-terminal amino acid of dipeptide nikkomycins might be protecting the molecule against peptidase activity in crude cell-extracts of different fungi. All the investigated nikkomycins except Cz and O_X* were transported via the peptide transport system in *Y. lipolytica* with more affinity for dipeptide nikkomycins than tripeptide [55].

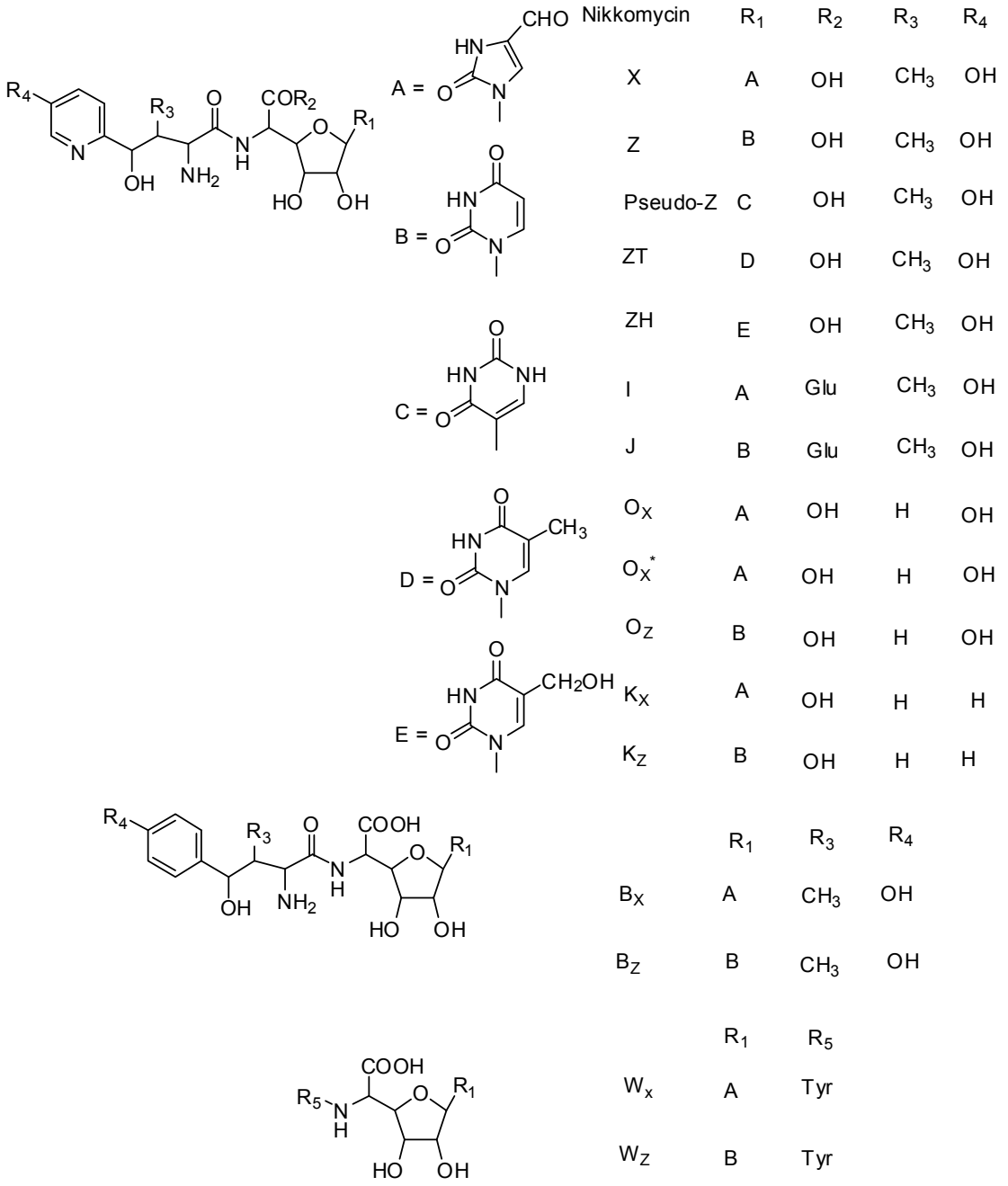
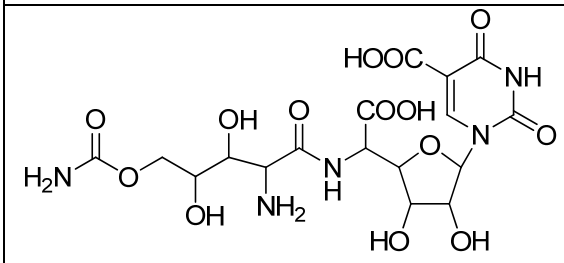


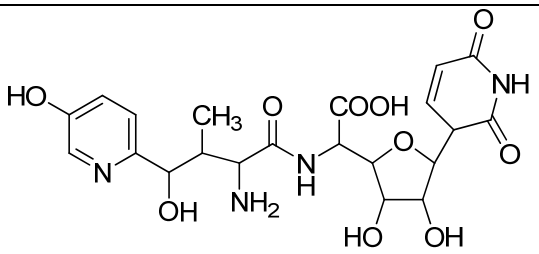
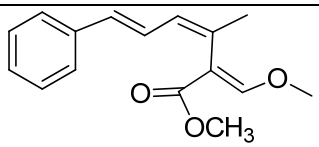
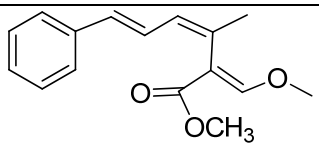
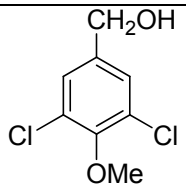
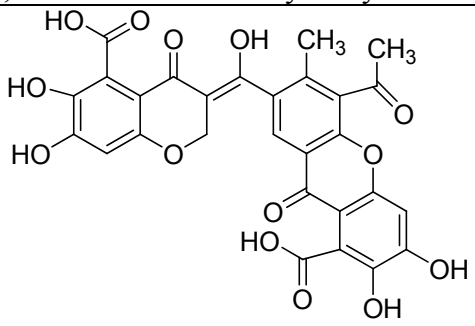
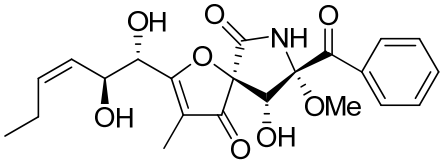
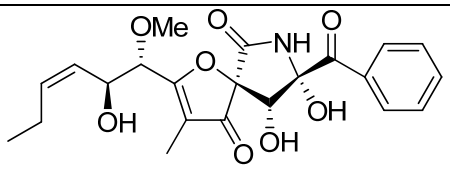
Figure 1.10 Structures of nikkomycins

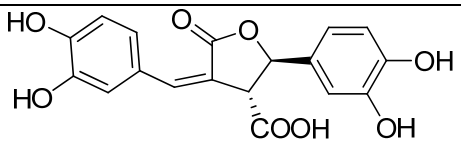
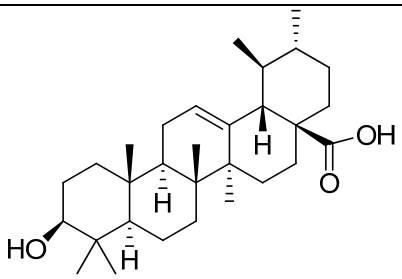
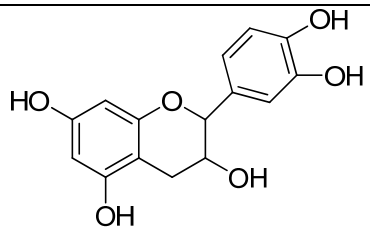
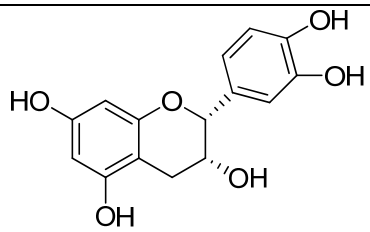
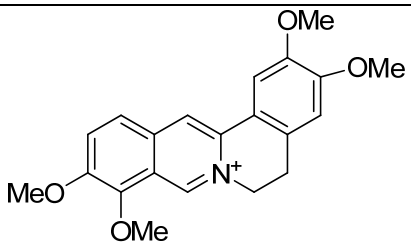
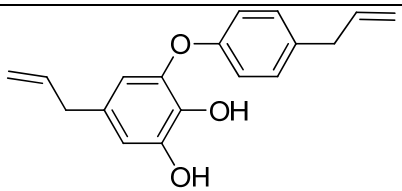
1.5.2.3 Other naturally occurring chitin synthase inhibitors

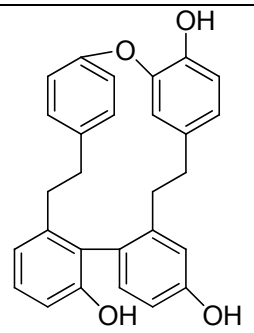
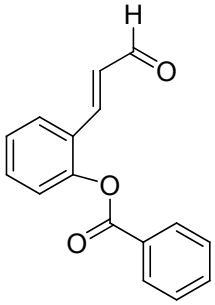
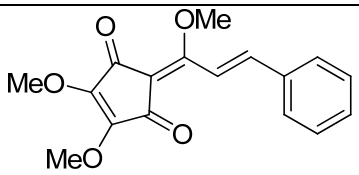
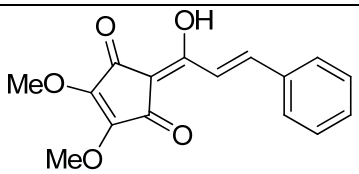
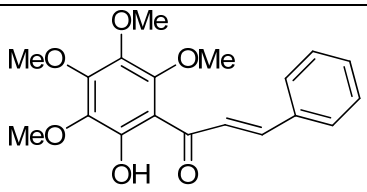
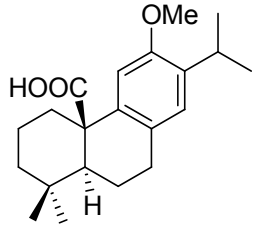
CS inhibitors isolated from natural sources, their structure, source and CS inhibitory activity are given in Table 1.3. Modification of few naturally occurring CS inhibitors were carried out to enhance their antifungal potential. Pfefferle *et al.* reported the inhibition of *Coprinus cinereus* CS activity using naturally occurring strobilurin A and B (24 and 30 % 0.25 mM) and further synthesized derivatives of strobilurin A and B containing different chlorine substituent to check the effect of chlorine substituent on CS inhibition [57]. It was found that strobilurin derivative with dichloro substituent showed maximum inhibition (50 % at 2.5 mM) of CS activity as compared to other derivatives. The phenolic OH groups in Phellinsin A determined the CS inhibitory activity. The antifungal activity with respect to the CS inhibition increased with increase in number of phenolic OH groups with maximum activity for compound having three phenolic OH groups [58]. Chlorinated compounds related to 3,5-Dichloro-4-methoxybenzyl alcohol (DCMB) isolated from *Stropharia* sp. were compared with DCMB for CS inhibition. Hexachlorophene and pentachlorophenol showed better CS inhibitory activity than DCMB [57]. 2-Benzoyloxycinnamaldehyde (2′-BCA) was isolated from *Pleuropterus ciliinervis* and its hydroxy, methoxy and halogenated derivatives were synthesized. Chloro and bromo derivatives of 2′-BCA exhibited 1.9 and 2.7 fold stronger CS 2 inhibitory activity than 2′-BCA, respectively [59].

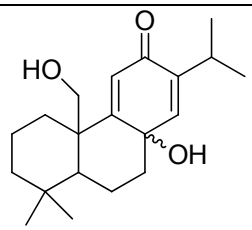
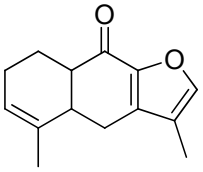
Table 1.3 Natural scaffolds for the possible development of chitin synthase inhibitors.

Structure and name of natural scaffold	**Source**	**Chitin synthase inhibition**
Bacterial Source		
 Polyoxin D	*Streptomyces cacaoi* var. *asoensis*	IC_{50} for *C. albicans* CS 0.45 µg/ml [44, 60]

 Nikkomycin Z	*S. tendae* Tü 901	IC_{50} for *C. albicans* CS 1, CS 2 and CS 3 - 7.4, 0.4 and 6.4 µg/ml respectively [52, 61].
Fungal source		
 Strobilurin A	*Strobilurus tenacellus*	IC_{50} for *C.cinereus* CS >64.5 µg/ml [57].
 Strobilurin B	*Strobilurus tenacellus*	IC_{50} for *C.cinereus* CS >64.5 µg/ml [57].
 3,5-Dichloro-4-methoxybenzyl alcohol	Submerged cultures of *Stropharia* sp.	IC_{50} for *C.cinereus* CS 51.70 µg/ml [57].
 Xanthofulvin	*Eupenicillium sp.*	IC_{50} for *S. cerevisae* CS 2 - 1.27 µg/ml [62].
 Pseurotin A	*Aspergillus fumigatus*	IC_{50} for *C. cinereus* CS 31.4 µg/ml [63].
 8-*O*-demethylpseurotin A	*Aspergillus fumigatus*	IC_{50} for *C. cinereus* CS 82.78 µg/ml [63].

 Phellinsin A	Culture broth of *Phellinus* sp. PL3	IC_{50} for *S. cerevisae* CS 1 and CS 2 - 76 and 28 µg/ml respectively [58, 64].
Plant Source		
 Ursolic acid	*Crataegus pinnatifida* Bunge leaves	IC_{50} for *S. cerevisiae* CS 2 0.84 µg/ml [65].
 Catechin	Stem bark of *Taxus cuspidata*	IC_{50} for CS 15 µg/ml [66].
 Epicatechin	Stem bark of *Taxus cuspidata*	IC_{50} for CS 29 µg/ml [66].
 Palmatine	*Phellodendron amurense*	IC_{50} for *C. albicans* CS 137.28 µg/ml [67].
 Obovatol	*Magnolia obovata*	IC_{50} for *S. cerevisiae* CS 2 - 10.86 µg/ml [68].

 Plagiochin E (PLE)	Liverwort *Marchantia polymorpha* L	IC_{50} for *C. albicans* CS2 and 3 - 16 µg/ml [69, 70].
 2-benzoyloxycinnamaldehyde (2-BCA)	Aerial parts of *Pleuropterus ciliinervis*	IC_{50} for CS 2 of *S. cerevisiae,* 70.8 µg/ml which was 2.5 times better than nikkomycin Z (175.6 µg/ml) [59].
 Methyllinderone	Stem bark of *L. erythrocarpa* Makino (Lauraceae)	IC_{50} for CS2 *S. cerevisiae* 23.3 µg/ml [71]
 Linderone	Stem bark of *L. erythrocarpa* Makino (Lauraceae)	IC_{50} for CS2 *S. cerevisiae* 21.4µg/ml [71].
 Kanakugiol	Stem bark of *L. erythrocarpa* Makino (Lauraceae)	IC_{50} for *S. cerevisiae* CS 2-23.8 µg/ml [71].
 *O*-methyl pisiferic acid	Leaves of *Chamaecyparis pisifera*	IC_{50} for CS 2 of *S. cerevisiae* 1.91 µg/ml [72].

 8,20-dihydroxy-9(11),13-abietadien-12-one	Leaves of *Chamaecyparis pisifera*	IC_{50} for *S. cerevisiae* CS 2 - 71.99 µg/ml [72].
 3,4,8a-trimethyl-4a,7,8,8a-tetrahydro-4a-naphto[2,3-b] furan-9-one	Whole plant of *Chloranthus japonicus* SIEB	IC_{50} for *S. cerevisiae* CS 2 - 39.6 µg/ml [73].

1.5.3 Synthetic modifications of polyoxins and nikkomycins

Numerous reports are available on modification of polyoxins and nikkomycins for the improvement of their properties. Being multifunctional molecules these nucleotide peptides offers different sites for the modification. General structure of the polyoxin and nikkomycin with different possible sites for modifications is given in Figure 1.11.

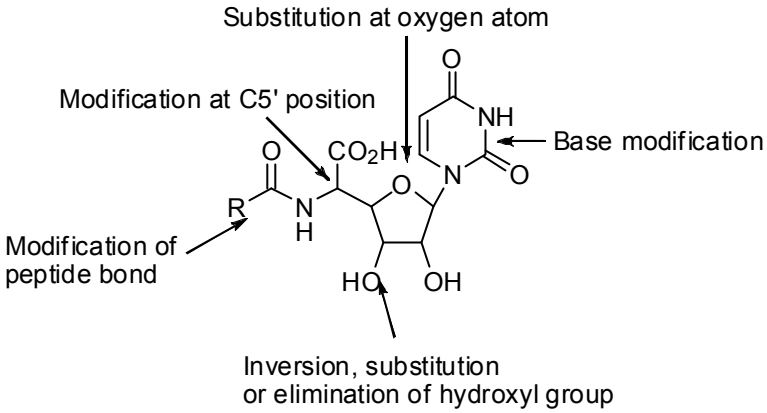


Figure 1.11 Different sites for the modification of nikkomycins and polyoxins

1.5.3.1 Peptide derivatives of polyoxins

A series of amino acyl derivatives of polyoxin C and L were synthesized and evaluated for CS inhibitory activity by Isono *et al.* [74-76]. Initially, they prepared 5′-decarboxylated analogs of polyoxins by synthesizing ω-substituted aminoacyl derivatives of 5′-amino-5′-deoxyuridine. No antimicrobial activity was observed for

all the 7 derivatives when tested against 15 different human and phytophatogenic fungi indicating the importance of 5′-carboxyl group for activity. 5′-carboxyl group was thought to be essential as it would place a negative charge in a position analogous to that of the phosphate groups in UDP-GlcNAc [74]. Therefore, *N*-aminoacyl derivatives of polyoxin C with amino acids such as glycine, alanine, ornithine and norvaline as the side chains were synthesized. Structure activity relationship based on the *in vivo* inhibition of phytopathogenic *A. kikuchiana*, *P. oryzae* and *C. miyabeanus* showed that: 1) the α-L-amino and 5′-carboxyl group were essential for the activity. 2) The alkyl chain length and the nature of ω-substituent affected the activity; polar groups such as ureido or carbamoyl increased the activity, whereas ionic groups such as amino or carboxyl groups decreased activity. However, these types of synthetic modifications did not show enhanced CS inhibition than the natural polyoxins [75, 76].

Polyoxins were ineffective against *C. albicans* due to inability of the antibiotic to penetrate the cell. Shenbagamurti *et al.* structurally altered the polyoxin molecule by introducing hydrophobic amino acids in the side chain so as to enhance the penetration into *C. albicans* [60]. The most active derivatives (**1-3**, Figure 1.12) were comparable in activity to ployoxin D (IC_{50} 0.45 μg/ml). Khare *et al.* reported the

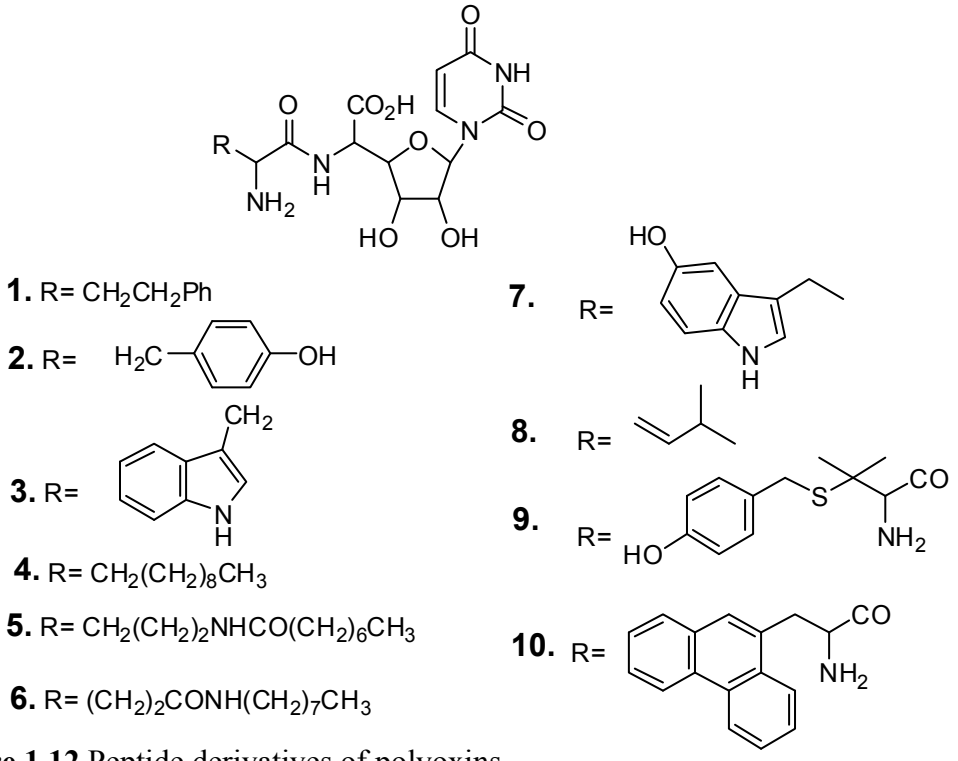


Figure 1.12 Peptide derivatives of polyoxins

synthesis and testing of polyoxins containing α-amino fatty acids (**4,** Figure 1.12) [77]. Compound **4** inhibited growth of *C. albicans* in culture at 40-80 μg/ml, whereas all other analogues were found to be less potent than polyoxin D (MIC 320 μg/ml). Polyoxins found to be unstable in the internal cell environment, to overcome this problem dipeptide and tripeptidyl analogues of polyoxins were synthesized [78]. However these analogues were rapidly metabolized to inactive uracil polyoxin C (UPOC) form releasing corresponding amino acids inside the cells. In order to improve metabolic stability, *Nϵ*-Oct-Lys-UPOC and *Nϵ*-Oct-Gln-UPOC (**5** and **6**, Figure 1.12**)** were synthesized and found to be more resistant to dipeptidase than above di- and tripeptidyl derivatives. But their antifungal efficacy against *C. albicans* (> 525 μg/ml) was still less than that of polyoxin D (MIC 26 μg/ml) [51]. Emmer *et al.* synthesized two L-alanine-UPOC analogs in order to obtain resistant analogs to peptidase hydrolysis. However, these modified molecules were ineffective in inhibiting CS activity [79].

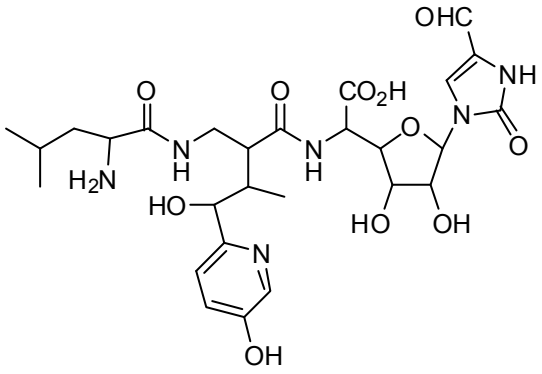
1.5.3.2 Peptide derivatives of nikkomycins

Cooper and coworkers reported a synthetic study of nikkomycin Z containing different amino acyl groups [80]. The activities of these derivatives were found to be much lower than that of natural analogs against *Candida* strains (**7** and **8**, Figure 1.12). Obi *et al.* synthesized nikkomycin Z analogues with different groups at the terminal amino acid moiety, e.g., *S*-aryl-L-Cys, *S*-aryl-L-methyl groups and phenanthrene (**9** and **10**, Figure 1.12) [81]. Among them, compound having phenanthrene group **10** was found to be a better *C. albicans* CS inhibitor with IC_{50} ~0.31 μg/ml (IC_{50} for nikkomycin was 0.393 μg/ml).

1.5.3.3 Peptidyl analogues of nikkomycin and polyoxin as a prodrug

Total nine tripeptidyl and dipeptidyl analogues of polyoxin and nikkomycin were synthesiszed as prodrugs [82]. Inside the cell, these analogues were expected to release active moiety upon hydrolysis.

Out of different analogues synthesized, only **11** (Figure 1.13) was found to release active component on hydrolysis inside the cells and act as prodrug. None of the compounds proved better anticandidal agents than nikkomycin.

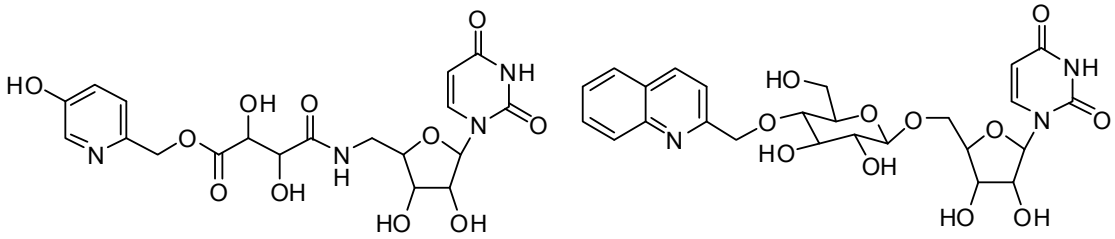


11

Figure 1.13 Peptidyl analogue of polyoxin as a prodrug**.**

1.5.3.4 Heteryl nucleoside derivatives of nikkomycin Z

Different heteryl nucleoside derivatives were synthesized by Beher *et al.* which conformationally mimic the transition state in presumed glycosyl transfer reaction [83]. In these derivatives the methyl quinoline and 2-methyl-5-hydroxypyridine served as the glycopyranosyl surrogate and malonic acid, tartaric acid and carbohydrate based non-hydrolysable linkers were chosen as a surrogate for the phosphate portion of the nucleoside (**12-13,** Figure 1.14). These linkers mimic the six membered ring pyrophosphate-M^{2+} complex formed in the active site of the CS enzyme. However all the compound showed weak *S. cerevisiae* CS inhibitory activity as compared to nikkomycin Z.

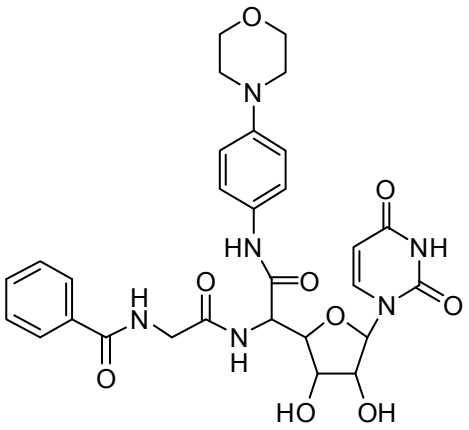


12 **13**

Figure 1.14 Heteryl nucleoside derivatives of nikkomycin Z

1.5.3.5 C5′ modification of polyoxin and nikkomycin

Using combinatorial concept and Ugi four component coupling reaction, Suda *et al.* synthesized active antifungal analogs of polyoxins [84]. All the Ugi products obtained were a mixture of diastereomers at C5′ position. Out of all these compounds



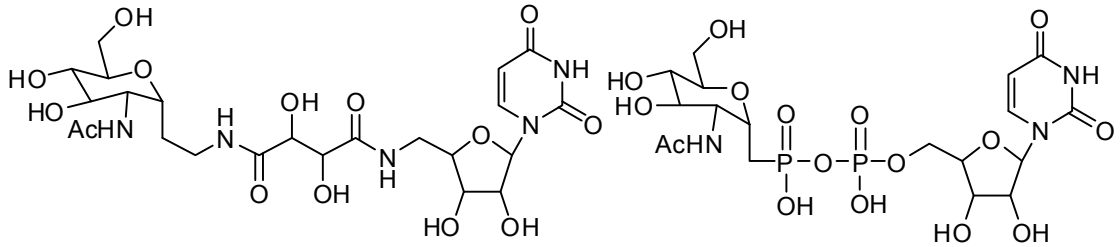
14

Figure 1.15 C5′ modified nikkomycin derivative

diasteriomer **14** (Figure 1.15) was found to be most active against *C. albicans* CS 1 with IC_{50} value 3.69 μg/ml (for nikkomycin IC_{50} value 4.69 μg/ml).

1.5.3.6 C-glycosyl nucleoside derivatives

A novel *C*-glycosyl nucleoside with tartarate as a linker residue between uridine moiety and GlcNAc was synthesized [85]. The IC_{50} value for the CS inhibition by synthesized compound **16** (Figure 1.16) was $12.06x10^3$ μg/ml, which appeared to be a very poor inhibitor of CS 1. Chang *et al.* reported synthesis of the C1-phosphonate analog of UDP-N-GlcNAc, as C-glycosyl phosphonates are well known isostere of corresponding phosphate [86]. Compound **17** (Figure 1.16) was weak inhibitor of CS (Ki > 10 mM) with competitive mode of action.



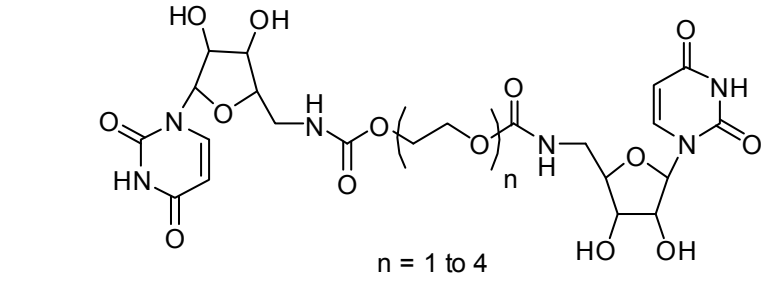
16 **17**

Figure 1.16 C-glycosyl nucleoside derivatives of UDP-*N*-acetylglucosamine

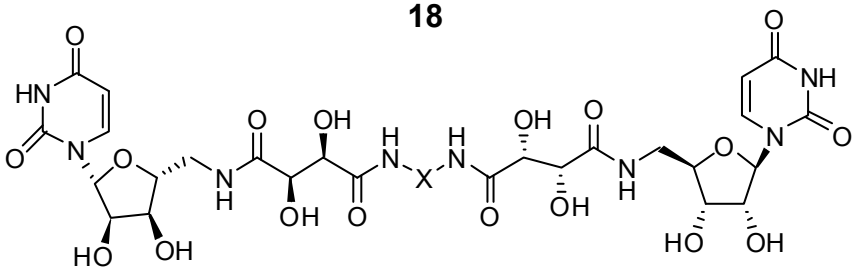
1.5.3.7 Dimeric inhibitors

Yeager *et al.* tested the hypothesis of presence of two active sites in CS by synthesizing dimeric nucleoside inhibiotors with the view that if two adjacent active

sites are present in close proximity, then dimeric inhibitors should show bivalent inhibition [87]. Dimeric inhibitors were synthesized by connecting 5′-deoxy-5′-aminouridine fragments by carbamate linkers of different lengths (**18,** Figure 1.17). It was found that efficacy of the dimeric inhibitors was dependent on the length of the spacer used, with shorter spacer (~14 Å) showing better efficacy. In the continuation of this work, uridine analogues joined by tartarate amide spacer (**19,** Figure 1.17) were synthesized and evaluated for their CS inhibition [88]. Use of tartarate amide spacer added incentives to the inhibitory activity with shortest dimer (~12Å) showing significant inhibition of activity. The results indicated the possibility of presence of two active sites in CS.



18



19

Figure 1.17 Dimeric nucleoside inhibitors

1.5.4 Biosynthetic approach for hybrid nucleoside peptide CS inhibitors

Recently, Li *et al.* used the combinatorial biosynthesis approach for the generation of new derivatives of polyoxin and nikkomycin [89]. Since the sturcutre of these two peptide nucleosides are similar, gene responsible for the biosynthesis of dipeptide of polyoxin from *S. cacaoi* was introduced in the *Streptomyces ansochromogenes* mutant, which produced nucleoside moiety of nikkonycin X. With this production of two hybrid antibiotics was achieved, out of which one was identified as polyoxin N **20** and another named as polynik A **21** (Figure 1.18). These

two hybrid antibiotics have better stability at neutral and alkaline conditions as compared to naturally occurring nucleosides. These antibiotics showed better antifungal activity against *C. albicans* and 5 different phytopathogenic fungi including *A. kikuchiana* and *Botrytis cinerea* as compared to polyoxin B.

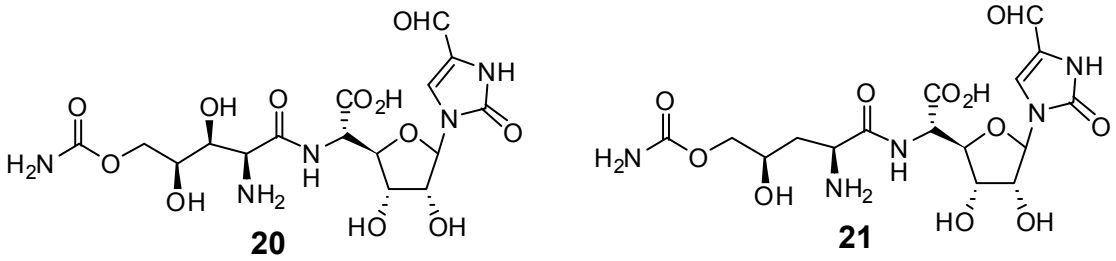


Figure 1.18 Hybrid nucleoside peptide inhibitors

1.5.5 Synergistic interaction of nikkomycin with other drugs

Nikkomycin Z exhibited additive and synergistic interactions when used in combination with either fluconazole or itraconazole against *C. albicans, C. parapsilosis, C. neoformans* and *C. immitis* [90]. Significant synergism was observed between nikkomycin and itraconazole against *A. fumigatus* and *A. flavus*. Powerful synergy for inhibition, killing, or both between nikkomycin Z and a glucan synthase inhibitor LY 303366 (semisynthetic analogue of echinocandian B) was observed against *A. fumigatus* and few other pathogens [91]. Nikkomycin Z (2 to 32 µg/ml) in combination with echinocandin FK463 (0.03 to 0.5 µg/ml) caused significant synergistic hyphal damage in filamentous fungi over a wide range of concentrations [92]. It also showed synergism with voriconazole and caspofungin against *C. albicans* [93]. The therapeutic efficacy of nikkomycin Z in *Candida vaginitis* murine model was enhanced when used in combination with zeamatin, a protein produced in corn which protects plants against fungal pathogens [94]. Synergism of nikkomycin with other drugs indicates that joining of two drug molecules may lead to a hybrid drug with dual mode of action and better efficacy.

1.5.6 Chemically synthesized chitin synthase inhibitors

Many chemically synthesized compounds structurally not related to the UDP-GlcNAc, but possessing antifungal and CS inhibitory activities have been reported. Ke *et al.* designed and synthesized two series of 1,3,4-oxadiazoline derivatives C1-10

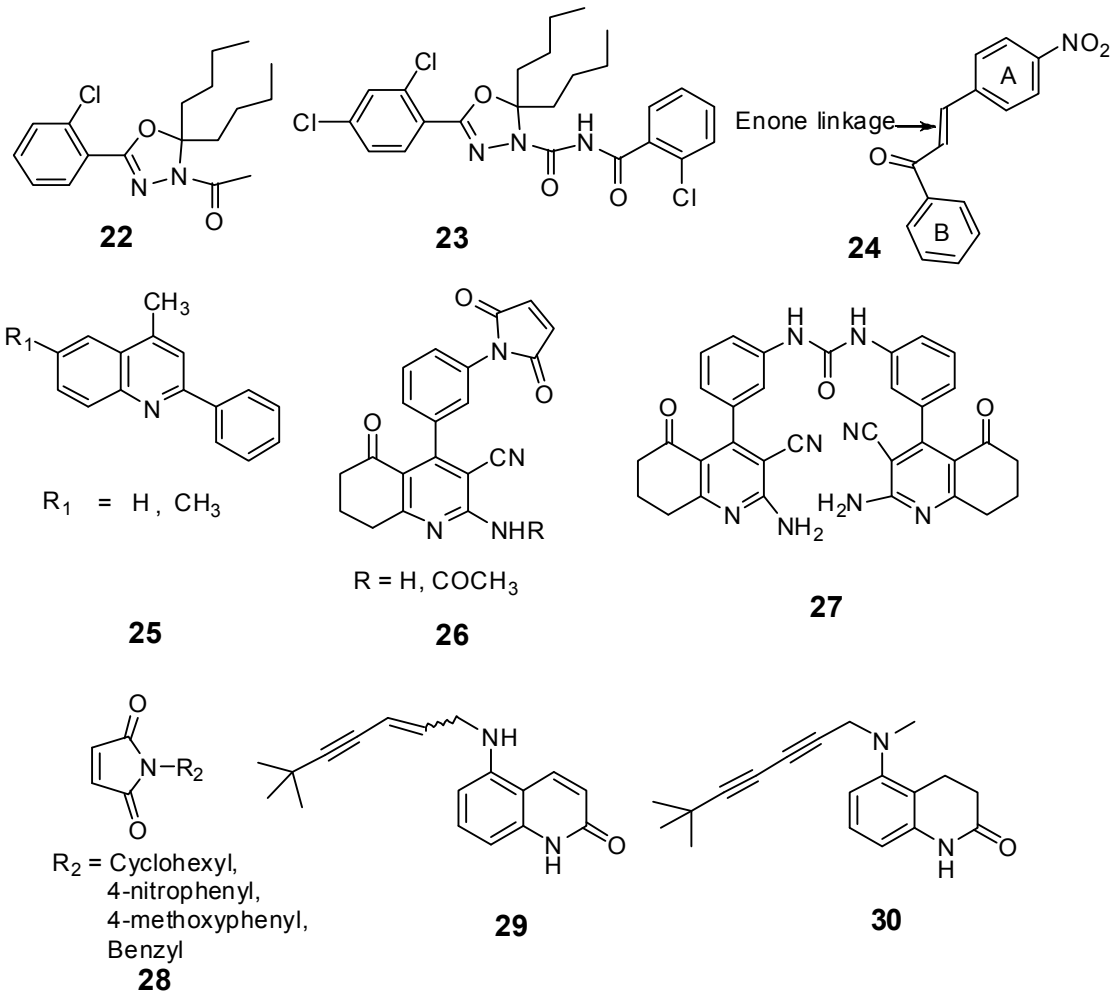


Figure 1.19 Chemically synthesized chitin synthase inhibitors

and C11-20 with the rationale that an oxadiazole ring improves pharmacokinetic and *in vivo* efficacy of drugs due to its higher hydrolytic stability [95]. All the compounds were found to inhibit CS activity *in vitro*. Structure-activity relationship study was done by introducing different electron donating (methyl, methoxy, ethyl etc.) and withdrawing groups (such as halogen atoms) into the aromatic ring. Compound C1 **(22,** Figure 1.19) containing *o*-chloro-substituent was most potent with 91% *S. cerevisiae* CS inhibition at 250 µM concentration, which may be due to increased lipophilicity by incorporation of halogen atoms into the aromatic ring. Introduction of acylurea moiety into 1,3,4-oxadiazolines heterocyclic structure resulted in lowered activities. Compounds C1 and C15 **(23,** Figure 1.19) exhibited CS inhibition with IC_{50} values of 2.66 and 2.85 µg/ml respectively.

Fourty one novel chalcone derivatives were synthesized by Claisen-Schmidt condensation and tested for antifungal activity against different human pathogens [96]. All these chalcones were inactive against *Candida* and *Cryptococcus* sp., but

found to be active against dermatophytes such as *Microsporum* sp., *Trichophyton* sp. and *Epidermophyton floccosum*. For structure activity relationship study, modification in A, B ring and enone linkage of chalcones was done. Introduction of electron donating groups decreased the antifungal activity, while electron withdrawing group specifically NO_2 at para position (**24,** Figure 1.19) increased the antifungal activity against *M. floccosum* with MIC value of 0.25 μg/ml. Replacement of benzene ring A by naphthalene resulted in loss of antifungal activity, while enone linkage was found to be important for antifungal activity. The IC_{50} values for CS inhibition was <220 μg/ml.

Urbina *et al.* synthesized a series of new 4-aryl- or 4-alkyl-N-arylamine-1-butenes and transformed some of them into 2-substituted 4-methyl-tetra-hydroquinolines and quinolines [97]. Unsubstituted homoallylamines were found to inhibit dermatophytes such as *Microsporum* sp., *Tricophyton* sp. and *Epidermophyton floccosum* with MIC <50 μg/ml. The most sensitive species was *E. floccosum*. When tested for *S. cerevisiae* CS 1 inhibition, it was observed that all homoallylamines were able to inhibit CS acitivity with IC_{50} >200 μg/ml. The derivative quinolines (**25**, Figure 1.19) were more effective with MIC values ranging from 0.75-25 μg/ml against above dermatophytes. The IC_{50} of the quinolines for *S. cerevisiae* CS were found to be 20-40 μg/ml.

Keeping in view the biological activity of the cyanopyridine derivatives Gholap *et al.* reported the synthesis of novel 2-amino-5-oxo-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile and its derivatives [98]. Out of all the synthesized compounds **26** and **27** (Figure 1.19) at 4 μg/ml concentration showed 91-95% *B. poitrasii* CS inhibition, while other compounds showed CS inhibition in the range of 60-90%. All the compounds exhibited antifungal activity against *C. albicans* and *Mucor* sp.

We have reported the synthesis of different N-substituted maleimides derivatives **28** [99]. Salewaska *et al.* reported that the same maleimide derivatives **28** at 50 μg/ml concentrations showed 20-25% reduction in chitin content and 14-21% inhibition of CS activity in *C. albicans* [100].

From random screening of Roche compound libraries a CS inhibitor RO-41-0986 (**29**, Figure 1.20) was obtained. The synthesis and structure activity relationship study of RO-41-0986 through modification of C-5 amino group, enediene side chain,

pyridine-2-one moiety and side chain substituent has been reported. Derivative RO-09-3143 (**30,** Figure 1.19) showed maximum and specific inhibition of *Candida* CS 1 with IC_{50} value of 0.000041 μg/ml and *in vitro* antifungal activity against *C. albicans* CY1002 with IC_{50} value 0.07 μg/ml [101]. In the presence of RO-09-3143, the *C. albicans* cells failed to form septa and displayed an aberrant morphology, confirming the involvement of the *C. albicans* CS 1 in septum formation [102].

1.6 Definition of the problem

Current status of fungal infections, emerging fungal pathogens, clinically used antifungal drugs and their limitations presented in the literature review prompted to search for novel antifungal agents with fungus-specific mode of action. CS, an enzyme catalyzing chitin formation, has long been recognised as a selective target for antifungal agent development. The literature review also signified that the research on CS inhibitors has progressed steadily and many advances have been made. However, only efforts related to nikkomycin Z led to the development of a potential candidate for the clinical treatment. Although nikkomycins were isolated in 1976, only recently nikkomycin Z is being developed as a specific drug for the treatment of coccidiomycosis, also known as Valley fever. The delay in the development of nikkomycin as a drug may be due to its efficacy essentially confined to highly chitinous fungi such as *C. immitis* and *B. dermatitidis* [103]. Secondly, one of the most dreadful fungal pathogen *C. albicans* is less susceptible and other *Candida* sp. are resistant to nikkomycin Z [104]. Attempts to make polyoxins/ nikkomycins resistant to dipeptidase and to enhance their uptake by the cell structural modification were unsuccessful as it either resulted in lower or complete loss of CS inhibitory activity. Therefore, development of CS inhibitors needs to be pursued further mainly due to presence of CS exclusively in fungi and availability of different sites for modification in nikkomycin. Directed efforts for CS inhibitors development may lead to a group of safe and effective antifungal drugs.

Based on this background, it was decided to undertake further research on CS inhibitors. Thus, present investigation is aimed at development of novel antifungal agents targeting fungal cell wall, specifically CS.

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Chapter 2

SYNTHESIS OF 1,2,3-TRIAZOLYL DERIVATIVES OF URIDINE AS CHITIN SYNTHASE INHIBITORS

2.1 Introduction

Chitin is the important structural component of fungal cell wall which is present universally in all fungi and absent in plants and animals. CS is involved in polymerization of UDP-GlcNAc which leads to chitin synthesis. Being important enzyme in chitin biosynthesis pathway, targeting CS could be better strategy for the development of novel antifungal agents. Nikkomycins and polyoxins are naturally occurring peptidyl nucleosides which inhibits CS activity. However, the efficacy of polyoxins and nikkomycins is limited due to hydrolysis in the internal cell environment [1], further lower uptake by pathogens like *C. albicans* [2].

In the present investigation, we pursued synthetic modifications of these peptidyl nucleosides at C5′ position of uridine (Figure 2.1) to overcome above-mentioned drawbacks. As uridine is main structural component of nucleoside inhibitors responsible for binding at active site of CS, uridine moiety has been retained and the nucleoside inhibitors with 1,2,3-triazoles substituted at 5′ position of uridine with aryl ether and aryl ester linkages have been designed. For the synthesis of these molecules Cu (I) catalyzed azide alkyne coupling was used [3].

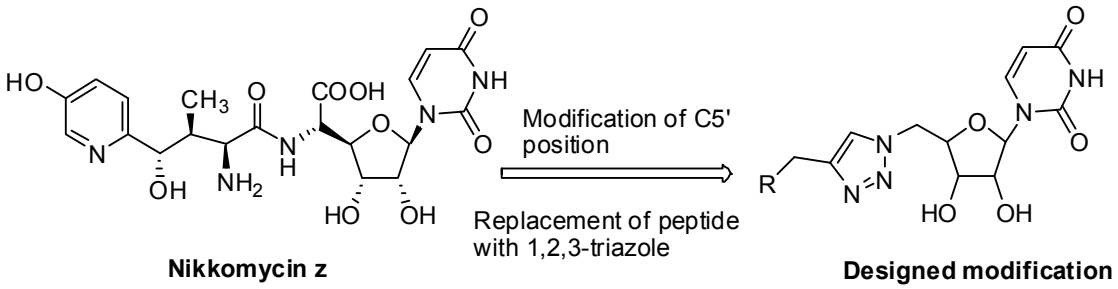


Figure 2.1 Designed modification of Nikkomycin

2.1.1 Importance of 1,2,3-triazoles

In medicinal chemistry triazoles appear to play vital role in increasing the potency as well as improving the pharmacokinetic properties of the drugs. 1, 4-Substituted 1,2,3-triazoles are bioisostere of Z-amide bond. The structural similarities include: a) three nitrogens of triazole mimicing the carbonyl group of amide, b) C-H bond of triazoles as hydrogen bond donor similar to N-H bond of amide and electrophilic, c) polarised two carbons of triazole are electronically similar to carbonyl carbon of amide (Figure 2.2).

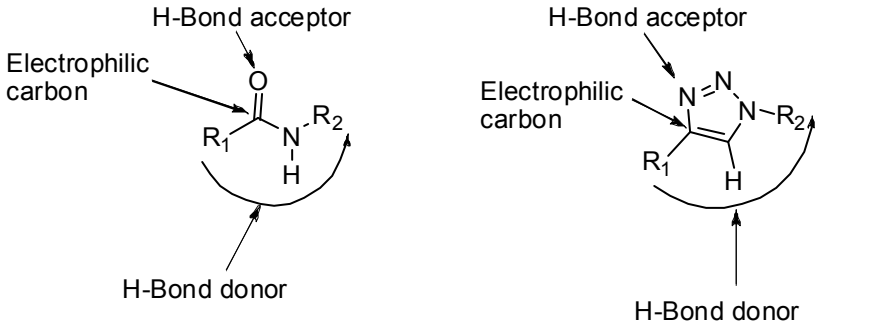


Figure 2.2 Structural comparison of amide and 1,2,3-triazole

The diopole moment of the triazole is high as compared to amide bond, which results in the increased hydrogen bonding capacity of triazoles and its enhanced peptide mimicry. Due to this 1,2,3-triazole unit may be considered as surrogate for a peptide bond [4, 5]. 1,2,3-triazole cores are known to strongly interact with biological targets due to hydrogen bonding to nitrogen atoms and large dipole moments [6]. Secondly, azoles are the largest class of antifungal agents in current use and under clinical investigations [7]. Though 1,23-triazoles are not present in the nature, molecules containing 1,2,3-triazoles shows diverse biological activities such as antifungal, antibacterial, antitubercular and anti-HIV etc. [8-11].

2.1.2 Importance of phenols and phenyl acetic acids

Phenols are present in a large number of biologically active compounds [12, 13]. Naturally occurring bioactive molecules like exophillic acid **1** inhibiting HIV-integrase and Methyl-4-[[(2E)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate **2,** which shows antitumor, antimicrobial and antioxidant activity (Figure 2.3) contains phenolic ether linkages [14]. Similarly, synthetic compound like C_3-symetric triantennary *N*-acetyl-(1-6)-β-D-glucosamine octadeacaoligosaccharide derivative **3** (Figure 2.3) with phenolic ether linkage has good antitumor activity [15].

Compounds containing phenyl acetic acid ester linkages such as Methyl-2-acetyl-3,5-dihydroxy phenyl acetate **4** (Figure 2.4) possesses antibacterial activity [16]. Some comb sugar polymers such as **5** (Figure 2.4) are useful in protein-carbohydrate interaction study [17].

For the synthesis of designed molecules, exploration of click chemistry is better strategy. Click chemistry is being rigorously used for the synthesis of different molecules containing 1,2,3-triazole moieties for number of different applications.

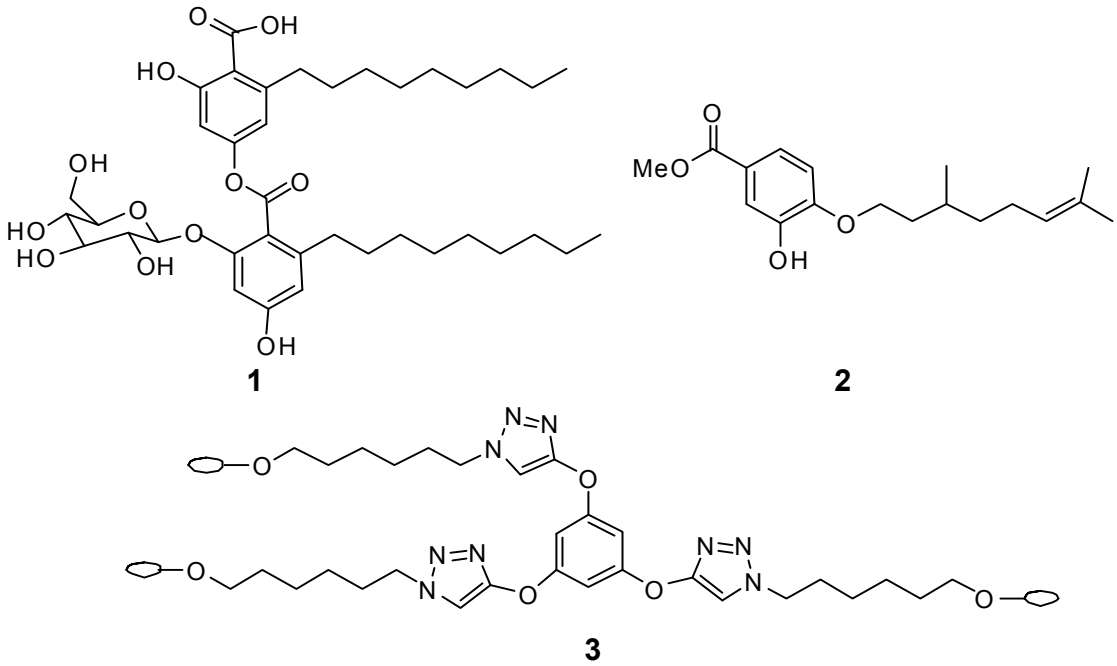


Figure 2.3 Phenolic ether linkages in bioactive molecules

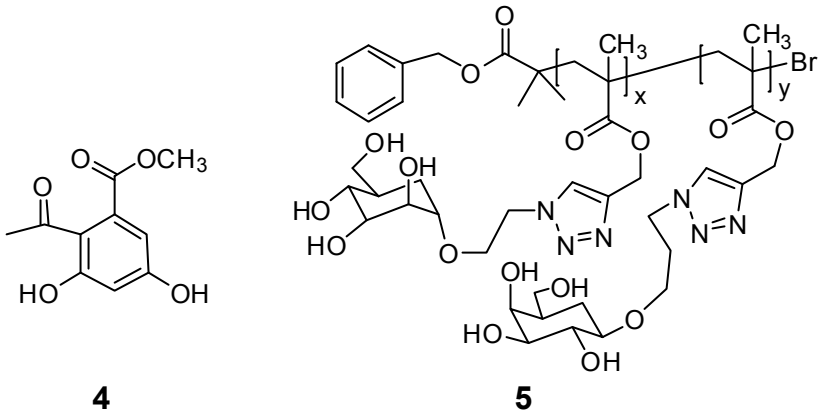


Figure 2.4 Phenyl ester linkages in bioactive molecules

2.1.3 Click Chemistry

The classical reaction of 1,3 dipolar cycloaddition of an azide and terminal or internal alkyne was first introduced by Huisgen [18, 19]. This traditional reaction requires high reaction temperature and pressure, longer reaction time. These reactions proceeds in concerted manner and always end up with formation of regioisomers, 1,4 and 1,5-substituted 1,2,3-triazoles. The ratio of regioisomers formed and the rate of reaction can be predicted by electronic and steric effects [20]. In 2001 Sharpless and Meldal came up with the azide alkyne cycloaddition reaction using Cu (I) catalyst, which improved the reaction rate dramatically up to 10^7 times (Figure 2.5) and termed

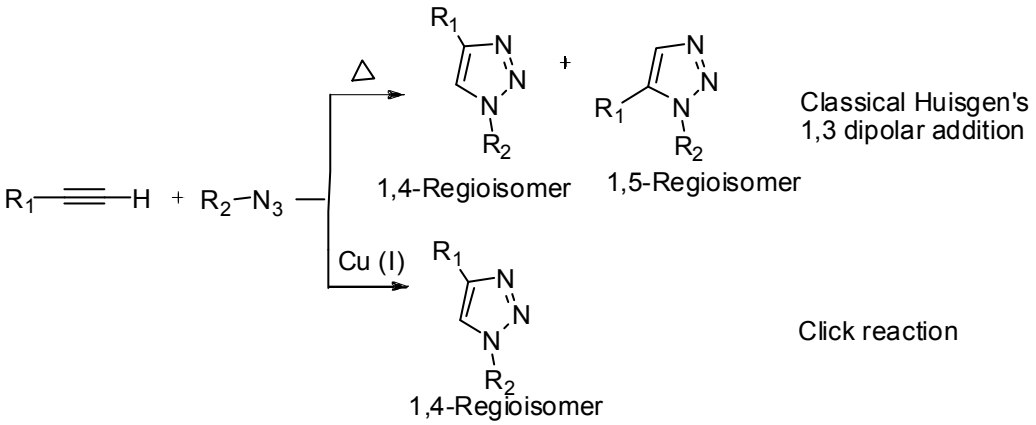


Figure 2.5 1,3 dipolar cycloaddition reaction between azide and alkyne

it as click reaction [3, 21, 22]. It is defined by the criteria - "*The reaction must be modular, wide in scope, give very high yields, generate only inoffensive by-products that can be removed by non-chromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions, readily available starting materials and reagents, the use of no solvents or a solvent that is benign (such as water) or easily removed, and simple product isolation*" [21].

In presence of Cu (I) catalyst the polarization of terminal alkyne takes place, which changes the reaction from concerted in to stepwise manner. The catalytic cycle for Cu (I) catalyzed azide alkyne coupling starts with formation of Cu (I) acetylide and after series of events, formation of 1,4-substituted 1,2,3-triazole takes place

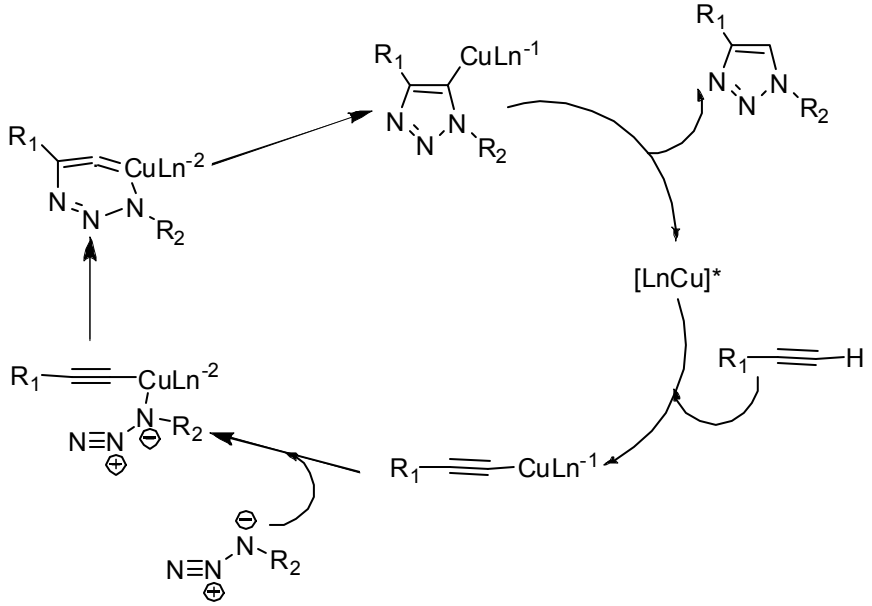


Figure 2.6 Mechanism of Cu (I) catalyzed azide and alkyne coupling reaction

(Figure 2.6) [3].

In medicinal chemistry click chemistry has been successfully employed for fragment based drug discovery, bioconjugation of lipids and carbohydrates,

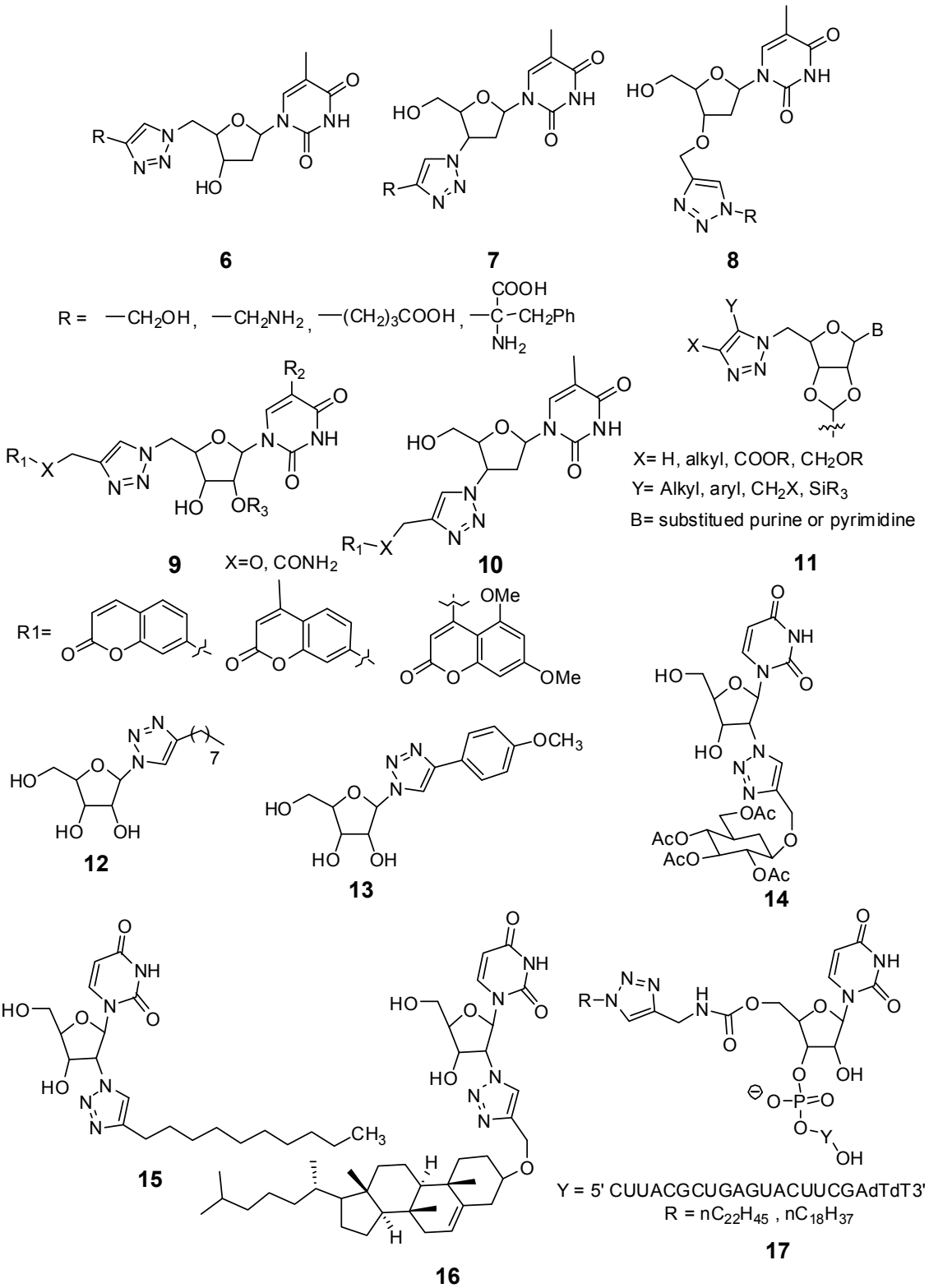


Figure 2.7 1,2,3-triazolyl nucleoside derivatives

bioconjugation of functional ligands to proteins, macrocyles synthesis, *in situ* click chemistry etc [23]. Click chemistry approach has been used for generation of 1,2,3-triazolyl nucleoside derivatives. Zhou *et al.* have reported the synthesis of 1,2,3-triazole functionalized thymidines as antiviral agents (Figure 2.7) [24]. The triazoles moiety was built at different sites of thymidine, but specifically the modification at 3′ and 5′ position was found to be effective leading to potent antiviral compounds **6, 7** and **8** (Figure 2.7).

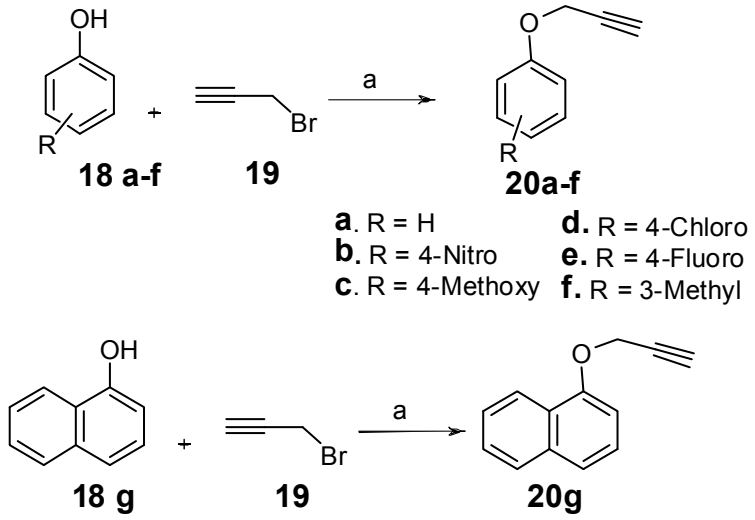
Synthesis of triazole linked coumarin nucleoside conjugates **9** and **10** (Figure 2.7) was reported by Kosiova *et al.* [25]. Coumarin derivatives were synthesized using Cu (I) catalyzed coupling of azidonucleosides and fluorescent coumarin propargyls. These materials have wide applications as fluorescent probes and signaling units. 1,2,3-Triazolyl uridines (**11**, Figure 2.7) were synthesized by Epple *et al.* [26]. Synthesis was carried out by coupling of 5′-azido uridine and carboxy-substituted alkyne components in presence of toluene. This procedure yielded high numbers of biologically active compounds in reasonable amount and purity. Akri *et al.* used click chemistry approach for microwave assisted synthesis of 4-substituted 1,2,3-triazolyl nucleosides **12** and **13** (Figure 2.7) [27]. These nucleosides showed cytostatic activity against CEM tumor cell lines. Kaczmarek *et al.* reported the 2′ functionalization of uridine by coupling 2′-azido-2′deoxyuridine with different alkynes [28]. These alkynes included lipids, fluorescent marker, penta acetylglucose, lysine and biotin (**14-16,** Figure 2.7).

Click conjugation strategy was used efficiently by Yamada *et al.* for the site-specific, high-throughput synthesis of oligonucleotide conjugates [29]. The reaction involved microwave-assisted coupling of azido-functionalized ligands to a fully protected solid-support bound alkyne-oligonucleotide prior to deprotection. The siRNA conjugates **17** (Figure 2.7) synthesized using this approach effectively silenced expression of a luciferase gene in a stably transformed HeLa cell line.

2.2 Results and discussion

2.2.1 Synthesis of propargyl ether of phenols

Propargyl ether of phenols **20a-f** and **20g** were prepared by the reaction of phenols **18a-f** and **18g** with propargyl bromide **19** and K_2CO_3 in DMF (Scheme 1). In ^{1}H NMR spectrum of the final compounds **20a-f** and **20g** the acetylenic proton was

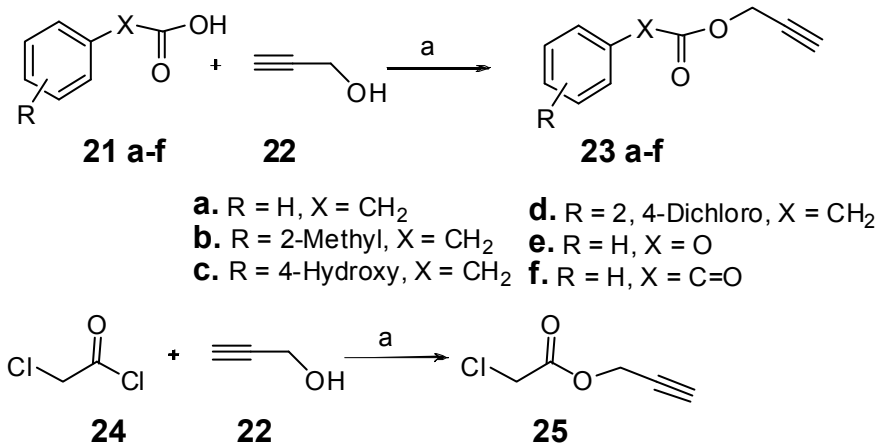


Scheme 1. Reagents and conditions: (a) K_2CO_3, DMF, 28 °C

identified as a triplet at δ 2.06 ppm and doublet at δ 4.78 ppm. The presence of acetylenic group was also evident from IR spectrum wherein the absorption was observed at 3307 cm^{-1}.

2.2.2 Synthesis of propargyl ester of phenyl acetic acids and chloroacetyl chloride

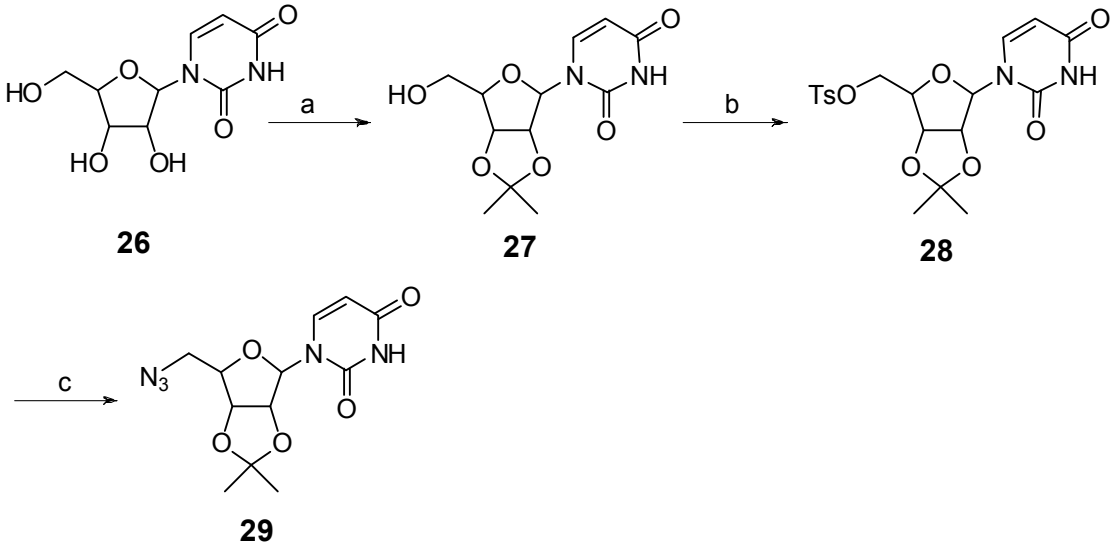
Propargyl esters **23a-f** were synthesized in excellent yield by heating acids **21a-f** and **24** in excess of propargyl alcohol **22** using catalytic amount of *p*-toluenesulphonic acid (*p*-TSA) at 80 °C (Scheme 2). Propargyl ester of chloroacetyl chloride **25** was synthesized by reacting chloroacetyl chloride **24** with propargyl alcohol **22**. Propargyl esters **23a-f** and **25** were characterized by ^{1}H NMR wherein the acetylenic protons appeared as a triplet at δ 2.45 and doublet at δ 4.76. In IR spectrum the stretching due to acetylene was observed at 3307 cm^{-1}.



Scheme 2. Reagents and conditions: (a) *p*-TSA, 80 °C

2.2.3 Synthesis of 5′-azido-2′,3′-*O*-methylethylidene uridine

5′-azido-2′,3′-*O*-methylethylidene uridine **29** was obtained from uridine **26** using reported procedure with 70% yield [30]. In first step uridine was reacted with 2,2′-dimethoxy propane to get 2′,3′-*O*-methylethylidiene uridine **27**. Further compound **27** was converted to its tosylate **28** by treating with *p*-tosyl chloride in pyridine. Formation of **28** was confirmed by ^{1}H NMR, where the resonance was seen at δ 7.76, 7.33 as doublet for aromatic protons at δ 2.44 as singlet for methyl protons of tolyl group and singlets at δ 1.54 and 1.32 for methyl protons of ketal. The tosylate **28** was transformed in to 5-deoxyazido-2′,3′-*O*-methylethylidene uridine **29** by S_N2 displacement of tosyl group in presence of NaN_3 (Scheme 3). The 5′-deoxyazido-2′,3′-*O*-methylethylidene uridine **29** showed characteristic stretching peak for azide group in IR spectrum at frequency 2104 cm^{-1}. In ^{1}H NMR spectrum resonance was seen at δ 7.36 as doublet for uracil proton and singlets at δ 1.57 and 1.36 for methyl protons of ketal.

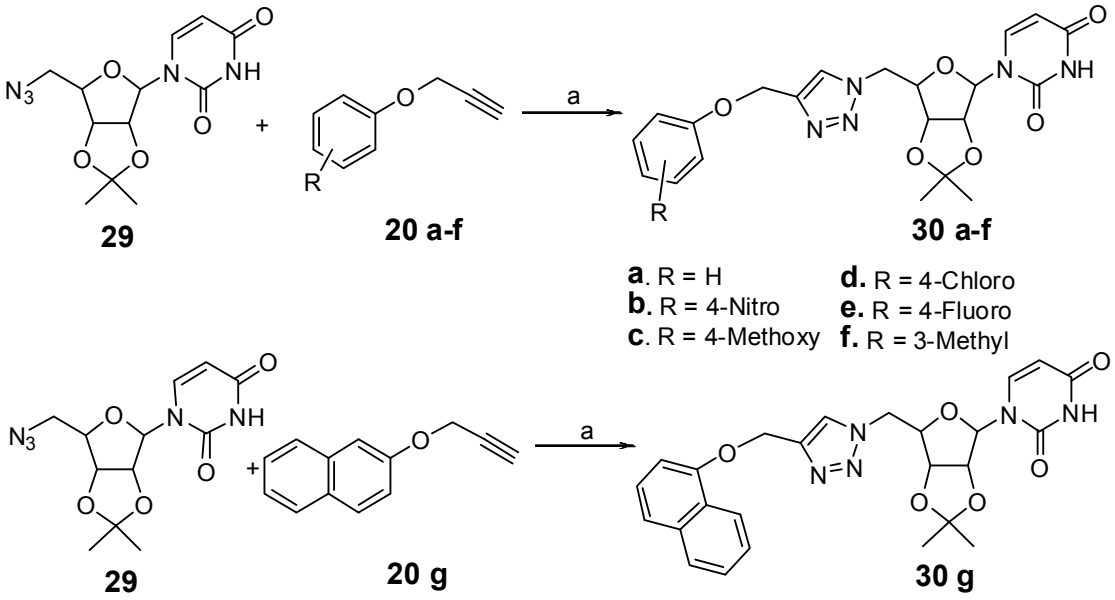


Scheme 3. Reagents and conditions: (a) Dimethoxy propane/acetone, *p*-TSA, 28 °C, 2 h, 71.5%; (b) *p*-TsCl, Pyridine, 0-28 °C, 10 h, 78%; (c) NaN_3, DMF, 60 °C, 12 h, 70%.

2.2.4 Synthesis of aryl ether linked 1,4-substituted-1,2,3-triazolyl uridine compounds

1,3 Dipolar cycloaddition of 5′-deoxyazido-2′,3′-dimethylethylidene uridine **29** with different propargyl ether of phenols **20a-f** and **20g** was carried out using click reaction. One equivalent of propargyl ether of phenols **20a-f** and **20g** and one

equivalent of 5′-deoxyazido-2′,3′-dimethylethylidene uridine **29** were dissolved in *t*-butanol/water (8:2) and resulting solution was treated with 5 mol% $CuSO_4.5H_2O$ and 10 mol% sodium ascorbate. The reaction mixture was stirred at room temperature (3-8 h) for completion of cycloaddition leading to final compounds **30a-f** and **30g** (Scheme 4, Table 2.1).

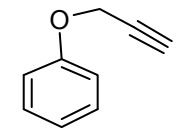
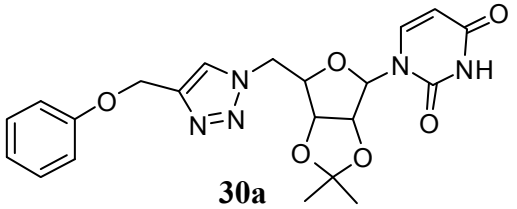
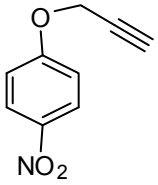
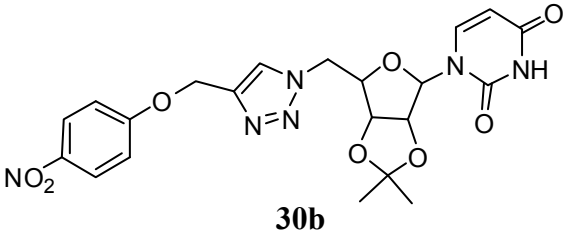
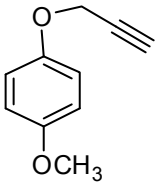
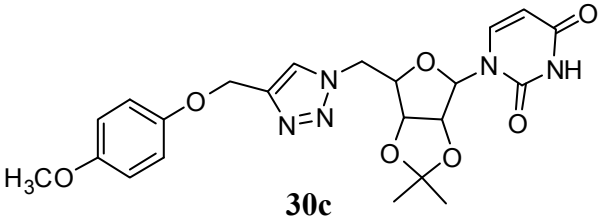
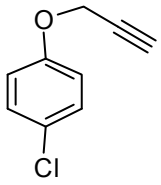
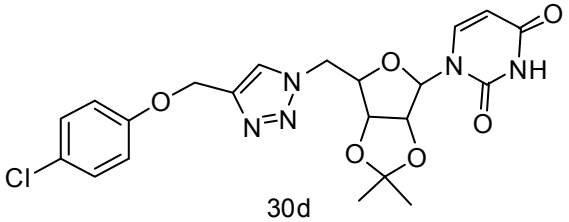
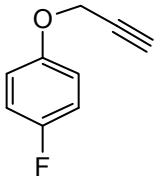
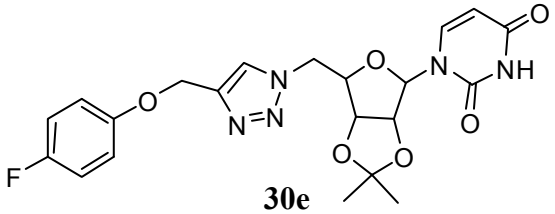
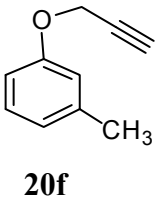
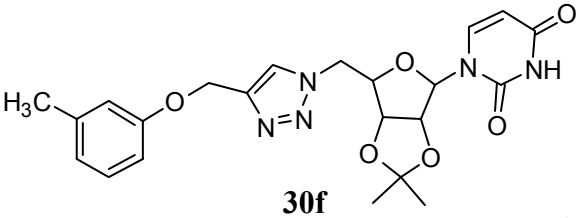
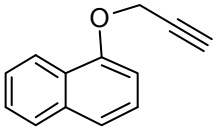
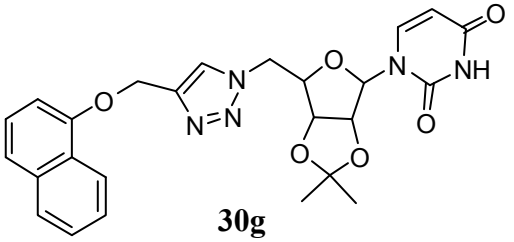


Scheme 4. Reagents and conditions: (a) $CuSO_4.5H_2O$, Sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C

All 1,2,3-triazolyl uridine derivatives **30a-f** and **30g** were characterized by IR, ^1H and ^{13}C NMR and Mass Spectrometry etc. For the representative compound, 5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-[(4-methoxyphenoxy)methyl]-1,2,3-triazol-1-yl}uridine (**30c**), the IR spectrum showed characteristic stretching frequencies at 1714, 1693 cm^{-1} etc. The ^1H NMR spectrum of **30c** showed resonance at δ 7.6 as a singlet which is specific for 1,4 substituted triazole proton. Along with this two singlets at δ 1.32 and 1.52 for methyl protons of ketal were observed. In ^{13}C NMR spectrum resonance at 174.9, 163.8 ppm for carbonyl carbon, 20.7 and 25.2 ppm for carbons of ketal was observed.

Structure of 5′-{4-[(4-chlorophenoxy)methyl]-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-methylethylidene) uridine **30d** was also confirmed by X-ray crystallographic study (Figure 2.8). The compound **30d** was crystallized by slow evaporation from ethyl acetate solution. The single crystal was subjected to X-ray analysis and the structure of 1,2,3-triazole **30d** was confirmed beyond any ambiguity.

Table 2.1 Synthesis of aryl ether linked 1,2,3-triazole derivatives of uridine **30a-f** and **30g.**

Sr. No	Proargyl ether of phenols	Product	% Yield
1	 **20a**	 **30a**	56
2	 **20b**	 **30b**	69
3	 **20c**	 **30c**	71
4	 **20d**	 30d	65
5	 **20e**	 **30e**	70
6	 **20f**	 **30f**	73
7	 **20g**	 **30g**	83

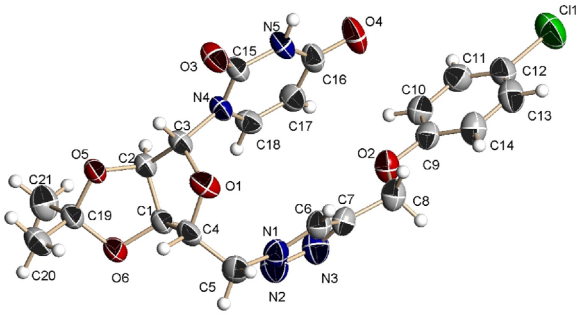
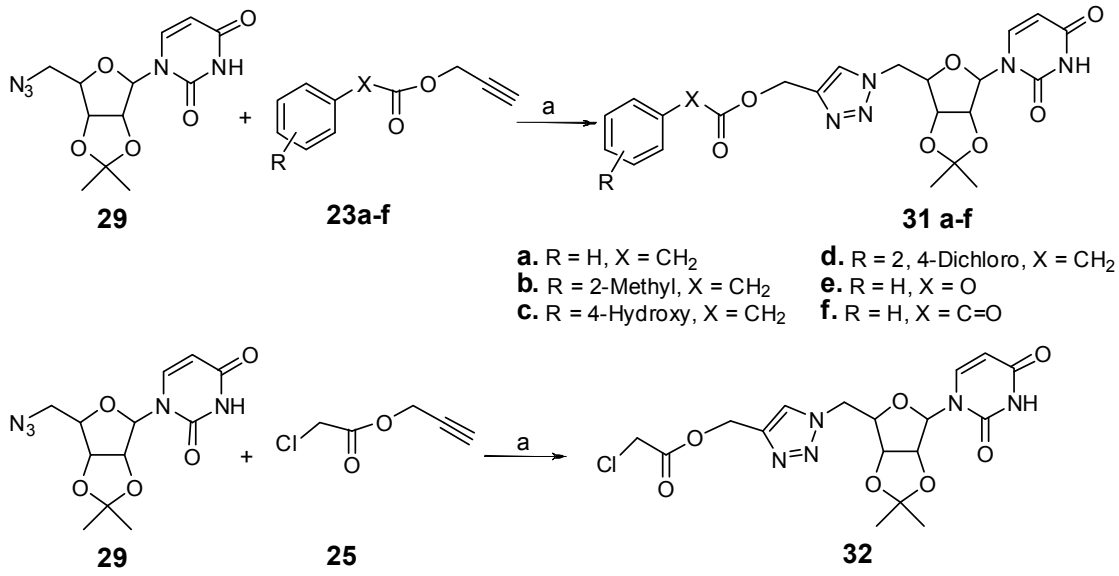


Figure 2.8 ORTEP diagram of compound **30d**

2.2.5 Synthesis of aryl ester linked 1,4-substituted-1,2,3-triazolyl uridine compounds:

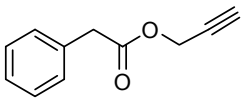
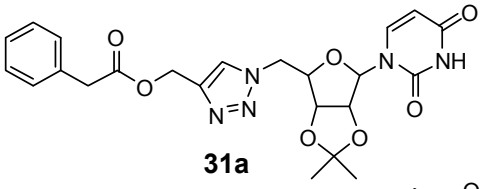
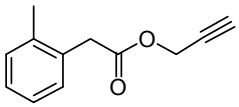
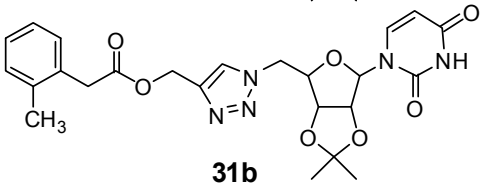
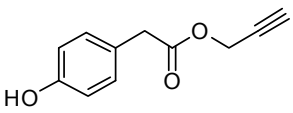
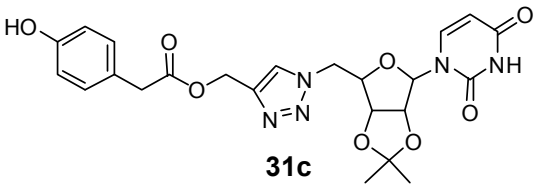
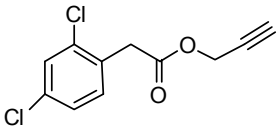
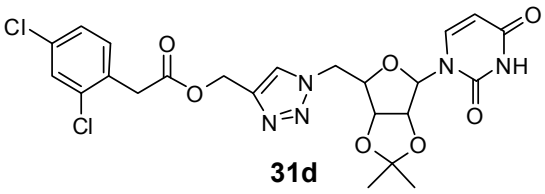
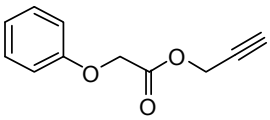
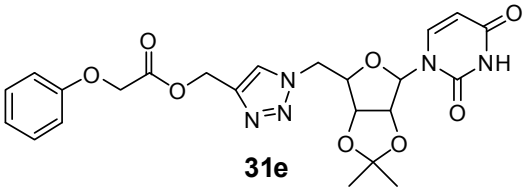
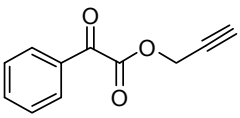
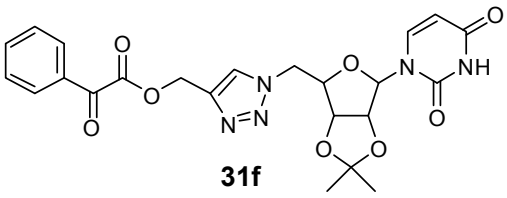
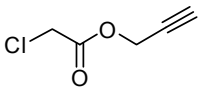
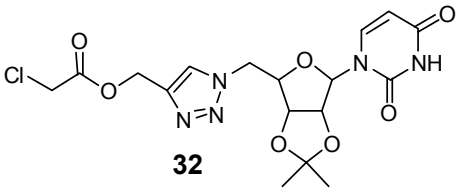
For the synthesis of aryl ester linked 1,2,3-triazole uridine derivatives **31a-f**, 5′-deoxyazido-2′,3′-*O*-methylethylidene uridine **29** was reacted with propargyl ester of acids **23a-f** using click reaction conditions (Scheme 5, Table 2.2).



Scheme 5. Reagents and conditions: (a) $CuSO_4.5H_2O$, Sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C

The structure of all aryl ester linked 1,2,3-triazolyl uridine compounds **31 a-f** were characterized using ^{1}H NMR, ^{13}C NMR and Mass Spectrometry etc. For instance, in case of 5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-[(2-methylphenyl) acetoxymethyl]-1,2,3-triazol-1-yl}uridine **31b**, the IR spectrum showed stretching frequencies at 1713, 1697, 1672 cm^{-1} etc.

Table 2.2 Synthesis of aryl ester linked 1,2,3-triazole derivatives of uridine **31a-f** and **32.**

Sr. No.	RCOO	Product	% Yield
1	 **23a**	 **31a**	60
2	 **23b**	 **31b**	74
3	 **23c**	 **31c**	50
4	 **23d**	 **31d**	57
5	 **23e**	 **31e**	55
6	 **23f**	 **31f**	53
7	 **25**	 **32**	60

In ^{1}H NMR spectrum resonance was seen at δ 7.56 as a singlet for triazole proton. Along with this three singlets were seen at δ 1.33, 1.53 and 2.23 for methyl protons of ketal and methyl protons of aromatic ring substituent. ^{13}C NMR spectrum

showed resonance at 171.4, 163.7 ppm for carbonyl carbon and at 19.5 and 25.6 ppm for carbons of ketal.

Similarly chloroacetyl chloride propargyl ester **25** was also reacted with 5′-deoxyazido-2′,3′-*O*-methylethylidene uridine **29** using click reaction conditions to get the final compound 5′-{4-(2-chloroacetoxymethyl)-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-(methylethylidene)uridine **32** (Scheme 5, Table 2.2). This compound **32** was synthesized so as to check the importance of aryl moiety in targeted enzyme inhibition.

2.2.6 Antifungal susceptibility testing of the synthesized compounds

All synthesized compounds **30a-g**, **31a-f** and **32** were evaluated for their antifungal activity using standard broth microdilution technique as per Clinical and

Table 2.3 Antifungal susceptibility testing by micro broth dilution method.

Compound no.	**Growth Inhibitory concentration in µg/ml**		
	F. oxysporum	***C. neoformans***	***C. albicans***
	MIC	**MIC**	**MIC**
30a	>128	8	>128
30b	64	32	>128
30c	128	64	32
30d	32	64	32
30e	>128	128	>128
30f	64	64	128
30g	64	64	>128
31a	32	32	32
31b	>128	64	32
31c	64	>128	>128
31d	>128	64	>128
31e	32	>128	>128
31f	128	>128	>128
32	16	>128	64
Nikkomycin	8	32	64
Fluconazole	8	32	32

Laboratory Standards Institute (CLSI, formerly NCCLS- National Committee for Clinical Laboratory Standards) guidelines [31, 32]. Antifungal activity was tested against human pathogens *C. albicans* and *C. neoformans* and plant pathogen *F. oxysporum*. The results of antifungal activity are given in Table 2.3 as MIC (Minimum Inhibitory Concentration for 90% growth inhibition) of the synthesized compounds.

All synthesized 1,2,3-triazolyl linked uridine compounds **30 a-g, 31a-f** and **32** were found to be active against all tested fungal pathogens with MIC values in the range of 8-128 μg/ml. Compound **31a** inhibited growth of all tested fungi with MIC 32 μg/ml, indicating the equal sensitivity of yeast and filamentous fungi for **31a**. Compound **31d** showed MIC 32 μg/ml against *F. oxysporum* and *C. albicans*, while MIC 64 μg/ml against *C. neoformans*.

MIC of nikkomycin against *C. albicans* cells was found to be 64 μg/ml. Yadan *et al.* reported that low *in vivo* efficacy of nikkomycin in *C. albicans* was due its lower uptake by the cells [2]. Compounds **30c**, **30d**, **31a** and **31b** were found to be potent inhibitor of *C. albicans* with MIC 32 μg/ml compared to nikkomycin.

This increase in the potency may be due to increase in the hydrophobicity of the compounds as compared to nikkomycin. Compound **30a** was most potent inhibitor of *C. neoformans* among all synthesized compounds with MIC 8 μg/ml, which is low as compared to nikkomycin (MIC 32 μg/ml). Compounds **30b** and **31a** were found to have comparable MIC 32 μg/ml against *C. neoformans* as that of nikkomycin and fluconazole. Compound **32** inhibited growth of *F. oxysporum* with MIC 16 μg/ml.

The MIC values of all aryl ether linked 1,2,3-triazolyluridine derivatives **30a-g** were found to be lower than aryl ester linked 1,2,3-triazole uridine derivatives **31a-f** and **32**. This may be attributed to increase in the hydrophilicity of **31a-f** and **32** due to increase in the heteroatoms.

Although polyoxins and nikkomycins show competitive *in vitro* CS inhibition, the ionized amino acid side chain significantly impedes their cellular transport [33]. In order to improve transport and intracellular stability, several analogues with diverse amino acid side chains were synthesized [34, 35], however, antifungal efficacy of the most active derivative against *C. albicans* was still less than that of polyoxin D. In case of aryl ester or aryl ether linked 1,2,3-triazolyl uridine compounds synthesized in

present investigation, the 5′ substitution of 1,2,3 triazole replacing peptide may have resulted in better permeability and concomitant improvement in antifungal activity.

2.2.7 Chitin synthase inhibition

Chitin synthase activity of *B. poitrasii* cells was estimated with (4 µg/ml) and without compounds using a non-radioactive chitin synthase assay, according to Lucero *et al.* [36]. The assay involved binding of synthesized chitin to WGA-coated surface followed by detection of polymer with horseradish peroxidase-WGA conjugate. Activity of horseradish peroxidase was determined by measuring the absorbance at 430 nm. It was observed that compounds **30d, 30e, 30f** and **31f** inhibited 80-95% of chitin synthase activity. Compounds **30a, 30b, 30g, 31a, 31c, 31e** and **32** exhibited 70-80% inhibition of CS activity. All other compounds except **31b** exhibited inhibition in the range of 60–70% (Table 2.4).

Obi *et al.* reported that in case of synthesized nikkomycin analogues anti-chitin synthase activity was found to be enhanced by introducing β-dimethyl group as β-methyl group of nikkomycin [37]. Furthermore, introduction of hydroxyl group in terminal aryl moiety resulted in significantly enhanced CS inhibitory activity. In present investigation introduction of methyl group at meta position of benzene ring of compound **30f** showed higher CS inhibitory activity. In contrast to the report by Obi *et al.,* in case of compound **31c** introduction of hydroxyl group in benzene ring did not show significant increase in CS inhibitory activity [37]. Whereas, in compound **31b** decrease in CS inhibition was observed, this could be attributed to steric hindrance exerted by methyl group at ortho position on benzene ring.

2.2.8 Yeast to hypha transition inhibition

Most of the pathogenic fungi are dimorphic and compounds which retard this dimorphic transition may have a potential as an antifungal drug. The effect of synthesized compounds on dimorphism i.e. yeast to hypha (Y-H) transition was checked using a nonpathogenic dimorphic fungus *B. poitrasii* as a model [38, 39]. *B. poitrasii,* a saprophytic zygomycetous fungus, shows temperature dependent transition similar to pathogenic fungi *H. capsulatum*, *P.brasiliensis. The* Y-H transition is rapid and occurs within 6 h. The yeast form of *B. poitrasii* is favoured by

glucose, high temperature (37 °C) and acidic pH whereas hyphal form is favoured by absence of glucose, low temperature (28 °C) and basic pH [38].

Yeast inoculum was grown in YPG (Glucose 1%) medium at 37 °C for 24 h and the transition to the hypha form was studied in YP medium at 28 °C. The yeast cells were inoculated in YP broth (with 4 μg/ml inhibitor and without inhibitor) and incubated at 28 °C for 6 h. The percentage of cells forming germ tubes was assessed as described earlier [40]. Nikkomycin Z (CS inhibitor) was used as a positive control in the Y-H transition experiment. It was observed that compounds **30b, 30c, 30g, 31a** and **31f** exhibited >70% of inhibition, whereas compounds **30e, 30f, 31b, 31c, 32** showed >60% of inhibition at 4 μg/ml. Remaining compounds showed moderate activity (Table 2.4).

Park *et al.* have reported HWY-289, a novel semi-synthetic protoberberine derivative, which showed inhibition of *C. albicans* CS1 and CS2 as well as Y-H transition [41]. The Y-H transition inhibition by these compounds may be partly due to the CS activity inhibition. *CS2* and *CS3* genes have been shown to exhibit transient peak of expression 1-2 h after induction of the hyphal growth phase [42]. Based on the transcription levels, Sudoh *et al.* have suggested an important role for CS 3 in *Candida* morphogenesis [43].

2.2.9 Comparative analysis of synthesized compounds based on biological activities

For the screening of antifungal compounds, the inhibition of spore germination or yeast to hypha differentiation assays are more sensitive than the growth inhibition assay [38]. Moreover, number of pathogens exhibit differentiation for the survival and proliferation in the host [38]. The comparative evaluation of compounds using *in vitro* inhibition of chitin synthase activity, inhibition of yeast to hypha transition (measured as % germ tube formation) and inhibition of yeast growth can be used to identify target specificity, hydrophobicity and toxicity, in general.

Results of inhibition of growth, germ tube formation and chitin synthase activity at 4 μg/ml concentration against *B. poitrasii* were compared (Table 2.4). With nikkomycin, inhibition of chitin synthase activity and % germ tube formation were >95% while that of growth was 45%. Based on *in vitro* inhibition of chitin synthase activity all the compounds were divided in three groups - Group I, >80%; Group II,

60-80%; Group III, <60% CS inhibition. From group I, however, **30e** did not show growth inhibition comparable to other two parameters which could be attributed to less uptake by the cells. While compounds **30c** and **31a** showed higher inhibition of germ tube formation as compared to chitin synthase activity. These compounds may be acting on some additional targets. Even for compound **31b** CS activity inhibition was very less as compared to growth or Y-H transition inhibition, indicating a different target (Table 2.4). Based on the comparison, compounds **30a, 30b, 30d, 30f,** and **31a** were identified as potent antifungal agents.

Table 2.4 Comparative analysis of all compounds based on biological activities in *Benjaminiella poitrasii.*

Group	Compound	% Inhibition		
		CS activity	% Germ tube formation	Growth
I	**Nikkomycin**	96.56	>95	45.45
	30d	95.08	58.13	34.65
	30e	84.06	64.70	9.43
	30f	82.14	69.23	34.43
	31f	80.04	72.00	51.52
II	**30a**	77.15	45.09	21.51
	30b	77.78	78.20	17.45
	30c	68.48	71.42	29.30
	30g	76.86	72.72	32.44
	31a	71.00	75.00	31.88
	31c	73.39	61.53	28.21
	31d	67.00	57.14	38.64
	31e	74.00	52.50	56.25
	32	75.71	60.00	30.21
III	**31b**	36.38	67.21	27.28

Concentration of compounds 4 μg/ml. Inhibition of chitin synthase activity- Group I, >80%; Group II, 60-80%; Group III, <60%.

2.2.10 Haemolysis testing

Major concern of employing these newly synthesized compounds for human application is their potential toxicity to mammalian cells. Cellular cytotoxicity of the

identified lead compounds **30a, 30b, 30d, 30f,** and **31a** was checked by haemolysis assay as described previously [44]. The results were given as HC_{50} (concentration causing 50% haemolysis). Concentrations of the inhibitors tested were in the range of 4-1000 μg/ml. For all inhibitors HC_{50} was found to be >1024 μg/ml which is very high as compared to their MIC values (8-32 μg/ml). Similarly nikkomycin also showed HC_{50} value >1024 μg/ml. These results suggested safety of these inhibitors for their use in human health care. However, further *in vitro* testing against different cell lines and *in vivo* animal studies are needed to ascertain the safety profile of these compounds.

2.3 Conclusions

The current endeavor enabled a practical, reliable and efficient synthesis of several novel 1,4-disubstituted-1,2,3-tiazolyluridine derivatives by 'click chemistry' approach, most of which showed significant antifungal activity. Compound **30a** showed better antifungal activity as compared to nikkomycin (MIC 32 μg/ml) against *C. neoformans* with MIC of 8 μg/ml. Compounds **30c, 30d, 31a** and **31b** demonstrated potent antifungal activity in comparison to nikkomycin (MIC 64 μg/ml) with MIC value of 32 μg/ml against *C. albicans.* Compounds **30b, 30c, 30g, 31a** and **31f** have profound suppressive effect on yeast-hypha transition, exhibiting >70% inhibition at concentration 4 μg/ml. Compounds **30d, 30e, 30f** and **31f** exhibited >80% inhibition of CS activity comparable to that of nikkomycin at a concentration 4 μg/ml. Comparing the results of all the three assays, compounds **30a, 30b, 30d, 30f** and **31a** were identified as lead molecules with potential application in medicine and agriculture for the control of fungal pathogens.

2.4 Experimental section

2.4.1 General procedure for the Synthesis of propargyl ether of phenols 20a-g

To the solution of specific phenol (1 mmol) in DMF, potassium carbonate (K_2CO_3, 1.2 mmol) was added. Propargyl bromide (1.2 mmol) was added drop wise to this mixture and the reaction mixture was heated at 60 °C for 4 h. Completion of reaction was monitored by TLC. Reaction mixture was cooled and diluted with 250 ml of ethyl acetate. Ethyl acetate layer was washed sequentially with 4 x 25 ml of

water, 25 ml of brine solution. The pooled organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo to yield final products **20a-g**.

(prop-2-ynyloxy)benzene (20a): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.20 (m, 2H), 6.89 (m, 3H), 4.76 (d, 2H), 2.58 (t, 1H).

1-nitro-4-(prop-2-ynyloxy)benzene (20b): ^{1}H NMR ($CDCl_3$, 200 MHz):δ = 8.16(d, 2H, *J*=9Hz), 7.06 (d, 1H, *J*=9 Hz), 4.56 (d, 2H, *J*=4 Hz), 2.48 (t, 1H, *J*=2 Hz).

1-methoxy-4-(prop-2-ynyloxy)benzene (20c): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.05 (d, 1H, *J*=8 Hz), 6.98 (d, 2H, *J*=8 Hz), 4.68 (d, 2H, *J*=4 Hz), 3.74 (s, 3H), 2.59 (t, 1H, *J*=2 Hz).

1-chloro-4-(prop-2-ynyloxy)benzene (20d): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.18 (d, 2H, *J*=8 Hz), 6.83 (d, 1H, *J*=8 Hz), 4.59 (d, 2H, *J*=4 Hz), 2.45 (t, 1H, *J*=2 Hz).

1-fluoro-4-(prop-2-ynyloxy)benzene (20e): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.06 (d, 1H, *J*=8 Hz), 6.93 (d, 2H, *J*=8 Hz), 4.60 (d, 2H, *J*=4 Hz), 2.49 (t, 1H, *J*=2 Hz).

1-methyl-3-(prop-2-ynyloxy)benzene (20f): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.03 (d, 1H, *J*=8 Hz), 6.77 (m, 3H), 4.76 (d, 2H, *J*=4 Hz), 2.58 (t, 1H, *J*=2 Hz), 2.31 (s, 3H).

1-(prop-2-ynyloxy)naphthalene (20g): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 8.19(m, 1H), 7.76 (m, 1H), 7.46 (m, 3H), 7.35 (d, 1H, *J*=6 Hz), 6.93 (dd, 1H), 4.73 (d, 2H, *J*=4 Hz), 2.59 (t, 1H, *J*=2 Hz).

2.4.2 General procedure for the synthesis of propargyl ester of acids 23a-f and 25

To the solution of specific acid (1 mmol) in excess of propargyl alcohol small pinch of pTSA was added. Reaction mixture was heated at 80 °C for 12 h. On completion of the reaction (monitored by TLC) the propargyl alcohol was removed under vacuum. The residue was dissolved in 150 ml of ethyl acetate and washed sequentially with 2 x 25 ml of dilute $NaHCO_3$, 25 ml of water, 25 ml of brine

solution. It was then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield products **21 a-f** and **25.**

Prop-2-ynyl-2-phenylacetate (21a): ^{1}H NMR ($CDCl_3$, 200 MHz):δ = 7.20 (m, 5H), 4.68 (d, 2H, *J*=4 Hz), 3.57 (s, 2H), 2.49 (t, 1H, *J*=2 Hz).

Prop-2-ynyl-2-o-tolylacetate (21b): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.13 (m, 4H), 4.69 (d, 2H, *J*=4 Hz), 3.63 (s, 2H), 2.48 (t, 1H, *J*=2 Hz).

Prop-2-ynyl-2-(4-hydroxyphenyl)acetate (21c): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.11 (d, 1H, *J*=8 Hz), 6.73 (d, 2H, *J*=6 Hz), 4.68 (d, 2H, *J*=4 Hz), 3.63 (s, 2H), 2.47 (t, 1H, *J*=2 Hz).

Prop-2-ynyl-2-(2,4-dichlorophenyl)acetate (21d): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.43 (s, 1H), 7.26 (d, 2H), 4.75 (d, 2H, *J*=4 Hz), 3.72 (s, 2H), 2.54 (t, 2H, *J*=2 Hz).

Prop-2-ynyl-2-phenoxyacetate (21e): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.20 (t, 2H), 6.69 (m, 3H), 4.68 (d, 2H, *J*=4 Hz), 4.57 (s, 2H), 2.56 (t, 1H, *J*=2 Hz)

Prop-2-ynyl-2-oxo-2-phenylacetate (21f): ^{1}H NMR [$CDCl_3$, 200MHz]: δ = 7.96(m, 2H), 7.64 (m, 1H, *J*=8 Hz), 7.47 (m, 2H, *J*=8 Hz), 4.58 (d, 2H, *J*=4 Hz), 2.54 (s, 1H, *J*=2 Hz).

Prop-2-ynyl-2-chloroacetate (25): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 4.68 (d, 2H, *J*=4 Hz), 4.07 (s, 2H), 2.54 (t, 1H, *J*=2 Hz).

2.4.3 Synthesis of 2′,3′-*O*-methylethylidene uridine (27)

To a solution of uridine **26** (1 gm, 4.0 mmol) in acetone, *p*-toluenesulfonic acid (0.001gm, 0.04mmol) was added. To this solution 2,2-dimethoxy propane (0.499 gm, 4.8mmol) was added dropwise over the period of 30 min. The reaction mixture was allowed to stir for 2 h. After completion of the reaction monitored by TLC, the acetone was removed. The residue was dissolved in 150 ml ethyl acetate which was

sequentially washed with 2 x 25 ml water, 2 x 25ml $NaHCO_3$, 25 ml water and 25 ml brine. Organic layer was dried on anhydrous Na_2SO_4 and evaporated in vacuo to get 0.82 gm of 2′,3′-methylethylidine uridine **27** (71.5%).

^{1}H NMR (MeOH D_4, 200 MHz): δ = 7.32 (d, 1H, *J*=8 Hz), 5.72 (d, 1H, *J*=8Hz), 5.60 (d, 1H), 4.87 (m, 2H), 4.30 (m, 3H), 1.54 (s, 3H), 1.32 (s, 3H).

2.4.4 Synthesis of 5′-*O*-tosyl -2′,3′-*O*-methylethylidine uridine (28)

To a solution of 2′,3′-O-methylethylidiene uridine **27** (1 gm, 3.5 mmol) in 10 ml of dry pyridine at 0 °C was added *p*-toluenesulfonyl chloride (0.793 gm, 4.2 mmol) under argon atmosphere. The reaction mixture was allowed to stir at 28 °C for 10 h. After completion of the reaction monitored by TLC, pyridine was removed in vacuo by forming pyridine-toluene azeotrope. The residue was dissolved in 150 ml of dichloromethane and washed sequentially with 2×25 ml of dilute HCl, 25 ml of water, 25 ml of brine solution. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield crude product. The crude tosylate was purified by silica gel column chromatography using ethyl acetate-petroleum ether mixture as eluent to get 1.2 gm (78%) final product **28**.

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.76 (d, 2H, *J*=8 Hz), 7.33 (d, 2H, *J*=8 Hz), 7.22 (d, 1H, *J*=8 Hz), 5.70 (d, 1H, *J*=8Hz), 5.64 (d, 1H), 4.83 (m, 2H), 4.29 (m, 3H), 2.44 (s, 3H), 1.54 (s, 3H), 1.32 (s, 3H).

2.4.5 Synthesis of 5′-azido-2′,3′-*O*-methylethylidene uridine (29)

To a solution of 5′-O-tosyl-2′,3′-methylethylidiene uridine **28** (1 gm, 2.2 mmol) in 15 ml of dry DMF sodium azide (0.286 gm, 4.4 mmol) was added. The reaction mixture was stirred at 60 °C for 12 hours under argon atmosphere. After completion of the reaction (monitored by TLC), it was cooled and diluted with 250 ml of ethyl acetate. Ethylcaetate solution was washed sequentially with 4×25 ml of water, 25 ml of brine solution and the aqueous extracts were extracted with another 25 ml of ethyl acetate. The pooled organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo to yield crude product. Purification of crude product by silica gel column chromatography using ethyl acetate-petroleum ether mixture as eluent afforded 0.49 gm (70%) 5′-deoxyazido-2′, 3′-methylethylidene uridine **29**.

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.36 (d, 1H, *J*=8 Hz), 5.75 (d, 1H, *J*=8Hz), 5.05 (m, 1H), 4.85 (m, 1H), 4.22 (m, 2H), 3.62 (m, 2H), 1.57 (s, 3H), 1.36 (s, 3H).

2.4.6 General procedure for the synthesis of 1,2,3-triazolyl uridine derivatives 30a-g

To a solution of uridine 5′-deoxyazido-2′,3′-*O*-methylethylidene uridine **29** (1 mmol) and specified propargyl ether phenols (1 mmol) in mixture of *t*-butanol and water (8:2), copper sulfate pentahydrate (24 mg, 5 mol%) and sodium ascorbate (40 mg, 10 mol%) were added. The reaction mixture was stirred at 28 °C for 3 to 8 h, after completion of reaction (monitored by TLC), reaction mixture was diluted with 200 ml of ethyl acetate. The organic layer was subsequently washed with 2×25 ml of water, 25 ml of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude products were purified by silica gel column chromatography using ethyl acetate and petroleum ether to obtain final compounds **30a-g**.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-(phenoxymethyl)-1,2,3-triazol-1-yl}uridine (30a): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.54 (s, 1H), 7.59 (s, 1H), 7.20 (m, 2H), 6.98 (d, 1H, *J*=8 Hz), 6.89 (m, 3H), 5.65 (d, 1H, *J*=8Hz), 5.40 (s, 1H), 5.12 (s, 2H), 4.97 (dd, 1H, *J*=1,6 Hz), 4.87 (m, 1H), 4.65 (d, 2H), 4.43 (m, 1H),1 .46 (s, 3H), 1.26 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 163.9, 158.2, 150.3, 144.1, 143.5, 129.5, 124.1, 121.3, 114.7, 102.1, 96.2, 86.3, 84.2, 81.8, 61.8, 51.9, 27.0, 25.2 ppm. MS *m/z*: (MH^+) 442.13. IR ($CHCl_3$) ν: 1711, 1693 cm^{-1}. Anal Calcd for $C_{21}H_{23}N_5O_6$: C, 57.14**;** H, 5.25; N, 15.56. Found: C, 56.89**;** H, 5.27; N, 15.28.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-[(4-nitrophenoxy)methyl]-1,2,3-triazol-1-yl}uridine (30b): ^{1}H NMR ($CDCl_3$, 200 MHz):δ = 9.87 (s, 1H), 8.16(d, 2H, *J*=9Hz), 7.7 (s, 1H), 7.08 (d, 1H, *J*=8Hz), 7.06 (d, 1H, *J*=9 Hz), 5.72 (d, 1H, *J*=8 Hz), 5.46 (s, 1H), 5.27(s, 2H), 5.10 (dd, 1H, *J*=1,8 Hz), 4.94 (m, 1H), 4.73 (m, 2H), 4.50 (m, 1H), 1.51(s, 3H), 1.32 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 164.1, 163.1, 150.1, 143.6, 141.9, 125.9, 124.3, 114.8, 102.8, 96.9, 86.5,84.3, 81.9, 62.9, 52.0, 27.0, 25.2 ppm. MS *m/z*:**:** (MH^+) 487.07. IR ($CHCl_3$) ν: 1693 cm^{-1}. Anal Calcd for $C_{21}H_{22}N_6O_8$: C, 51.85**;** H, 4.55; N, 17.27. Found: C, 51.00**;** H, 4.18; N, 17.31.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-[(4-methoxyphenoxy)methyl]-1,2,3-triazol-1-yl}uridine (30c): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.82(s, 1H), 7.63 (s, 1H), 7.05 (d, 1H, *J*=8 Hz), 6.84 (q, 4H, *J*=8 Hz), 5.17 (d, 1H, *J*=8Hz), 5.50 (s, 1H), 5.12(s, 2H), 5.03 (dd, 1H, *J*=1,6 Hz), 4.98 (m, 1H), 4.67 (bs, 2H), 4.48 (m, 1H), 3.74 (s, 3H), 1.52(s, 3H), 1.32 (s, 3H). ^{13}C ($CDCl_3$, 100MHz): 174.9, 163.8, 154.0, 152.3, 150.3, 144.3, 143.4, 124.1, 115.8, 114.7, 102.8, 96.1, 86.2, 84.2, 81.8, 62.6, 55.6, 51.8, 27.0, 25.2, 20.7 ppm. MS *m/z*: (MH^+) 472.10; IR ($CHCl_3$) ν: 1714, 1693 cm^{-1}. Anal Calcd for $C_{22}H_{25}N_5O_7$: C, 56.05**;** H, 5.35; N, 14.85. Found**:** C, 56.00**;** H, 5.25; N, 14.55.

5′-{4-[(4-chlorophenoxy)methyl]-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-methylethylidene) uridine (30d): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.59 (s, 1H), 7.59 (s, 1H), 7.18 (m, 2H), 7.01 (d, 1H, *J*=8 Hz), 6.83 (m, 2H), 5.67 (d, 1H, *J*=8 Hz), 5.43 (s, 1H), 5.09 (s, 2H), 5.00 (dd, 1H, *J*=1,6 Hz), 4.89 (m, 1H), 4.66 (m, 2H), 4.43 (m, 1H), 1.47 (s, 3H), 1.27 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 163.8, 156.8, 150.3, 143.7, 143.5, 129.4, 126.1, 124.2, 116.1, 114.8, 102.9, 96.4, 86.4, 84.3, 81.4, 62.1, 51.9, 27.0, 25.2 ppm. MS *m/z*: (MH^+) 476.05 IR ($CHCl_3$) ν: 1713, 1693 cm^{-1}. Anal Calcd for $C_{21}H_{22}ClN_5O_6$: C, 53.00**;** H, 4.65; N, 14.62. Found: C, 52.50**;** H, 4.25; N, 14.55.

5′-{4-[(4-flurophenoxy)methyl]1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-(methylethylidene) uridine (30e): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.74 (s, 1H), 7.64 (s, 1H), 7.06 (d, 1H, *J*=8 Hz), 6.93 (m, 4H), 5.72 (d, 1H, *J*=8 Hz), 5.49 (s, 1H), 5.13 (s, 2H), 5.05 (dd, 1H, *J*=2,8 Hz), 4.94 (m, 1H), 4.71 (m, 2H), 4.49 (m, 1H), 1.52 (s, 3H), 1.32 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 163.8, 159.8, 155.6, 154.3, 150.3, 143.9, 143.5, 129.3, 128.5, 124.1, 116.1, 114.8, 102.9, 96.3, 86.3, 62.5, 51.9, 27.0, 25.2 ppm. MS *m/z*: (MH^+) 460.08; IR ($CHCl_3$) ν: 1710, 1693 cm^{-1}. Anal Calcd for $C_{21}H_{22}FN_5O_6$: C, 54.90**;** H, 4.83; N, 15.24. Found: C, 54.40**;** H, 4.53; N, 15.34.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-[(3-methylphenoxy)methyl]-1,2,3-triazol-1-yl}uridine (30f): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.87 (s, 1H), 7.63(s, 1H), 7.03 (d, 1H, *J*=8 Hz), 6.77 (m, 3H), 5.71 (d, 1H, *J*=8 Hz), 5.51 (s, 1H), 5.17 (s, 2H), 5.02 (dd, 1H, *J*=1,8 Hz), 4.93 (m, 1H), 4.71 (m, 2H), 4.49 (m, 1H), 2.31 (s, 3H),

1.53(s, 3H), 1.33 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 163.8, 158.6, 150.4, 144.2, 143.4, 139.6, 129.2, 124.1, 122.1, 115.6, 114.8, 111.5, 102.9, 96.1, 86.3, 84.2, 61.8, 51.9, 27.0, 25.2, 21.5 ppm. MS *m/z*: (MH^+) 456.14; IR ($CHCl_3$) ν: 1718, 1710, 1693 cm^{-1}. Anal Calcd for $C_{22}H_{25}N_5O_6$: C, 58.02**;** H, 5.53; N, 15.38. Found: C, 57.60**;** H, 5.25; N, 15.00.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-(napthoxymethyl)-1,2,3-triazol-1-yl}uridine (30g): ^1H NMR ($CDCl_3$, 200 MHz): δ = 9.85(s, 1H), 8.19(m, 1H), 7.76 (m, 1H), 7.7 (s, 1H), 7.46 (m, 3H), 7.35 (d, 1H, *J*=6 Hz), 7.04 (d, 1H, *J*=8 Hz), 6.93 (dd, 1H), 5.66 (d, 1H, *J*=8 Hz), 5.47 (s, 1H), 5.36 (s, 2H), 5.05 (dd, 1H, *J*=1,8 Hz), 4.94 (m, 1H), 4.72 (m, 2H), 4.51 (m, 1H), 1.52(s, 3H), 1.32 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 163.7, 153.9, 150.3, 144.2, 143.4, 134.5, 127.5, 126.4, 125.8, 125.2, 124.0, 121.9, 120.8, 114.8, 105.4, 102.8, 96.4, 86.4, 84.3, 84.3, 81.9, 62.3, 51.9, 27.0, 25.2 ppm. MS *m/z*: (MH^+) 492.90. IR ($CHCl_3$) ν: 1714, 1693, 1668 cm^{-1}. Anal Calcd for $C_{25}H_{25}N_5O_6$: C, 61.10**;** H, 5.13; N, 14.25. Found: C, 61.00**;** H, 5.25; N, 14.00.

2.4.7 General procedure for the synthesis of 1,2,3-triazolyl uridine derivatives 31a-f and 32

Starting with 5′-deoxyazido-2′,3′-*O*-methylethylidene uridine **29** and specific propargyl ester of acids, compounds **31a-f** and **32** were synthesized by following the procedure mentioned in the section **2.4.6**

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-(phenylacetoxymethyl)-1,2,3-triazol-1-yl}uridine (31a): ^1H NMR ($CDCl_3$, 200 MHz):δ = 9.50 (s, 1H), 7.51 (s, 1H), 7.20 (m, 5H), 6.98 (d, 1H, *J*=8 Hz), 5.67 (d, 1H, *J*=8Hz), 5.42 (s, 1H), 5.16 (s, 2H), 4.97 (dd, 1H, *J*=1,8 Hz), 4.85 (m, 1H), 4.62 (m, 2H), 4.42 (s, 1H), 3.57 (s, 2H), 1.47 (s, 3H), 1.27 (s, 3H). ^{13}C ($CDCl_3$, 150MHz): 171.2, 163.5, 150.1, 143.2, 142.3, 133.3, 129.0, 128.2, 126.8, 124.7, 114.6, 102.6, 95.9, 86.0, 83.9, 81.6, 57.6, 51.6, 40.6, 26.7, 24.9 ppm. MS *m/z*: (MH^+) 484.13; IR ($CHCl_3$) ν: 1712, 1693 cm^{-1}. Anal Calcd for $C_{23}H_{25}N_5O_7$: C, 57.14; H, 5.21; N, 14.48. Found: C, 56.50**;** H, 5.25; N, 14.28.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-[(2-methylphenyl)acetoxymethyl]-1,2,3-triazol-1-yl}uridine (31b): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.60 (s, 1H), 7.57 (s, 1H), 7.13 (m, 4H), 7.03 (d, 1H, *J*=8 Hz), 5.72 (d, 1H, *J*=8 Hz), 5.47 (s, 1H), 5.21 (s, 2H), 5.03 (dd, 1H, *J*=1,8 Hz), 4.91 (m, 1H), 4.68 (m, 2H), 4.45 (m, 1H), 3.63 (s, 2H), 2.23 (s, 3H), 1.53(s, 3H), 1.33 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 171.4, 163.7, 150.3, 143.4, 142.7, 136.8, 132.4, 130.3, 130.2, 127.5, 126.1, 125.0, 114.8, 102.9, 96.3, 86.4, 84.3, 81.9, 57.9, 51.9, 38.9, 27.0, 25.6, 19.5 ppm. MS *m/z*: (MH^+) 498.73; IR ($CHCl_3$) ν: 1713, 1697, 1672 cm^{-1}. Anal Calcd for $C_{24}H_{27}N_5O_7$: C, 57.94**;** H, 5.47; N, 14.08. Found: C, 57.40**;** H, 5.25; N, 14.28.

5′-{4-[(4-hydroxyphenyl)acetoxymethyl]-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-(methylethylidene)uridine (31c): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.88. (s, 1H), 7.41 (s, 1H), 7.05 (d, 1H, *J*=8 Hz), 7.01 (d, 1H, *J*=6 Hz), 6.73 (d, 2H, *J*=6 Hz), 5.07 (d, 1H, *J*=8 Hz), 5.47(s, 1H), 5.20 (s, 2H), 5.04 (dd, 1H, *J*=1,6 Hz), 4.89 (m, 1H), 4.64 (m, 2H), 4.45 (m, 1H), 3.63 (s, 2H), 1.53(s, 3H), 1.33 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 176.2, 168.2, 159.8, 154.2, 147.6, 146.63, 134.2, 129.0, 128.4, 119.3, 118.7, 106.5, 100.0, 90.0, 88.1, 85.7, 61.5, 55.8, 44.0, 30.7, 28.9 ppm. MS *m/z*: (MH^+) 501.03; IR ($CHCl_3$) ν: 3338, 1683, 1693 cm^{-1}. Anal Calcd for $C_{23}H_{25}N_5O_8$: C, 55.31**;** H, 5.05; N, 14.02. Found: C, 55.00**;** H, 4.99; N, 14.00.

5′-{4-[(2,4-dichlorophenyl)acetoxymethyl]-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-(methylethylidene)uridine (31d): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.63 (s, 1H), 7.63 (s, 1H), 7.35 (s, 1H), 7.02 (s, 2H), 7.07 (d, 1H, *J*=8 Hz), 5.72 (d, 1H, *J*=8 Hz), 5.49 (s, 1H), 5.24 (s, 2H), 5.05 (d, 1H, *J*=6 Hz), 4.93 (m, 1H), 4.70 (m, 2H), 4.47 (m, 1H), 3.72 (s, 2H), 1.53(s, 3H), 1.33 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 170.0, 163.6, 150.2, 143.4, 142.3, 135.0, 133.8, 132.2, 130.6, 129.1, 127.1, 125.0, 114.7, 102.8, 96.3, 86.3, 84.2, 81.8, 58.1, 51.8, 38.2, 26.9, 25.1 ppm. MS *m/z*: (MH^+) 553.00; IR ($CHCl_3$) ν: 1730, 1693, 1672 cm^{-1}. Anal Calcd for $C_{23}H_{23}Cl_2N_5O_7$**:** C, 50.01**;** H, 4.20; N, 12.68. Found: C, 49.79**;** H, 4.55; N, 12.09.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-(phenoxyacetoxymethyl)-1,2,3-triazol-1-yl}uridine (31e): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.64 (s, 1H), 7.57 (s, 1H), 7.20 (t,

2H), 6.69 (m, 2H), 6.82 (d, 1H, *J*=8 Hz), 5.66 (d, 1H, *J*=8 Hz), 5.36 (s, 1H), 5.23 (s, 2H), 4.98 (m, 1H), 4.79 (bs, 1H), 4.62 (s, 2H), 4.57 (s, 2H), 4.36 (s, 1H), 1.46 (s, 3H), 1.26 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 168.8, 163.3, 157.5, 150.0, 143.4, 142.0, 135.0, 130.0 129.5, 128.8, 125.4, 125.4, 121.8, 114.8, 114.6, 102.9, 96.4, 85.9, 84.0, 81.4, 65.0, 58.0, 51.6, 27.0, 25.2 ppm. MS *m/z*: (MH^+) 500.00; IR ($CHCl_3$) ν: 1731, 1697, 1672 cm^{-1}. Anal Calcd for $C_{23}H_{25}N_5O_8$: C, 55.31**;** H, 5.05; N, 14.02. Found: C, 55.00**;** H, 4.99; N, 14.00.

5′-deoxy-2′,3′-*O*-(methylethylidene)-5′-{4-(benzoylcarboxymethyl)-1,2,3-triazol-1-yl}uridine (31f): ^{1}H NMR [$CDCl_3$, 200MHz]: δ = 9.77 (s, 1H), 7.96(m, 2H), 7.75 (s, 1H), 7.64 (m, 1H, *J*=8 Hz), 7.47 (m, 2H, *J*=8 Hz), 7.09 (d, 1H, *J*=8 Hz), 5.71 (d, 1H, *J*=8 Hz), 5.49 (s, 2H), 5.45(s, 1H), 5.08 (dd, 1H, *J*=1,8 Hz), 4.92 (m, 1H), 4.73 (m, 2H), 4.46 (m, 1H), 1.51(s, 3H), 1.31 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 164.1, 150.3, 142.8, 130.1, 128.1, 114.6, 102.7, 94.8, 86.8, 84.3, 82.1, 52.3, 44.2, 27.0, 25.1 ppm. MS *m/z*: (MH^+) 498.00; IR ($CHCl_3$) ν: 1715, 1693 cm^{-1}. Anal Calcd for $C_{23}H_{23}N_5O_8$: C, 55.53**;** H, 4.66; N, 14.08. Found: C, 55.10**;** H, 4.79; N, 13.90.

5′-{4-(2-chloroacetoxymethyl)-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-(methylethylidene)uridine (32): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.48 (s, 1H), 7.68 (s, 1H), 7.10 (d, 1H, *J*=8 Hz), 5.75 (d, 1H, *J*=8 Hz), 5.46 (s, 1H), 5.31(s, 2H), 5.08 (dd, 1H, *J*=1,8 Hz), 4.92 (m, 1H), 4.71 (m, 2H), 4.48 (m, 1H), 4.07 (s, 2H), 1.52 (s, 3H), 1.33 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 167.3, 163.8, 150.3, 143.7, 141.8, 125.4, 114.8, 102.9, 96.6, 86.4, 84.3, 81.9, 58.9, 51.9, 40.8, 27.0, 25.2 ppm. MS *m/z*: (MH^+) 442.73; IR ($CHCl_3$) ν: 1718, 1693 cm^{-1}. Anal Calcd for $C_{17}H_{20}ClN_5O_7$: C, 46.21**;** H, 4.56; N, 15.85. Found: C, 46.23**;** H, 4.79; N, 15.80.

2.4.8 Crystallographic data for 5′-{4-[(4-chlorophenoxy)methyl]-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-*O*-(methylethylidene)uridine (30d)

The single-crystal diffraction data were collected on a Bruker AXS Smart Apex CCD diffractometer at 297(2) K. The X-ray generator was operated at 50 kV and 30 mA using graphite-monochromatized (Mo K_{α}= 0.71073Å) radiation. Data were collected with ω scan width of 0.3° and with four different settings of φ (0°, 90°,

180° and 270°) keeping the sample-to-detector distance fixed at 6.145 cm and the detector position (2θ) fixed at -28°. $C_{21}H_{22}Cl_1N_5O_6$: M = 475.89, Crystal dimensions 0.62 x 0.38 x 0.19 mm^3, T = 297(2) K, monoclinic, space group $P\ 2_1$, a = 11.4808(15), b = 5.6022(7), c = 17.836(2) Å, β = 107.144(2)°; V = 1096.2(2) $Å^3$; Z = 2; ρ_{calcd} = 1.442 gcm^{-3}, μ *(Mo-K_α)* = 0.224 mm^{-1}, *F(000)* = 496, $2\theta_{max}$ = 52.00°, 11494 reflections collected, 4274 unique, 4141 observed ($I > 2\sigma(I)$) reflections, 300 refined parameters, R value 0.0324, $wR2$ = 0.0835 (all data R = 0.0334, $wR2$ = 0.0844), S = 1.036, minimum and maximum transmission 0.8737 and 0.9587; maximum and minimum residual electron densities +0.194 and –0.185 e $Å^{-3}$.

All the data were corrected for Lorentzian, polarization and absorption effects using Bruker's SAINT and SADABS programs. SHELX-97 (G. M. Sheldrick, SHELX-97 program for crystal structure solution and refinement, University of Gottingen, Germany, 1997) was used for structure solution and full-matrix least-squares refinement on F^2. All non-hydrogen atoms were refined anisotropically while all hydrogen atoms were refined with appropriate geometric constraints.

Crystallographic data (excluding structure factors) for the structure **30d** in this paper have been deposited with Cambridge crystallographic data center as supplementary publication number CCDC 709791.

2.4.9 Organisms and growth conditions

Screening of all the synthesized compounds was carried out using fungal strains *Candida albicans* and *Cryptococcus neoformans* (human pathogens), *Benjaminiella poitrasii* (saprophyte) were maintained on YPG (yeast extract, 0.3%; peptone, 0.5%; and glucose, 1%) agar slants, and *Fusarium oxysporum* (plant pathogen) was maintained on PD (potato, 20%; dextrose, 2%) agar slants at 28 °C.

2.4.10 Inhibitor stock preparation

i. The synthesized compounds were dissolved in 100% DMSO and the final concentration of DMSO in the assay was 2.5%.

ii. The sterility check of the stocks for bacterial contamination was done in NA broth by incubating for 24 h.

iii. 2.5% DMSO was not inhibitory for the fungal organisms used.

2.4.11 Antifungal susceptibility testing

The purified final compounds were evaluated for antifungal susceptibility testing by microbroth dilution method according to the recommendations of the NCCLS [32]. The cultures used were human pathogens – *C. albicans, C. neoformans,* phyotopathogen *F. oxysporum*, and saprophyte *B. poitrasii*. Appropriate amount of compounds were dissolved in DMSO to get 100X final strength. The stock was then diluted 1:40 in YPG medium and 200 µl from this was added to the first row of a 96-well microtiter plate. The compound was serially diluted two fold in successive wells to get a range of 1-128 µg/ml. Fungal yeast cells (~$2x10^4$ cfu/mL, spores for phytopathogen), freshly grown in YPG broth in logarithmic phase, were suspended in the medium and inoculated (100 µl) in the wells of the plate. The microtiter plate was incubated for 24-48 h, and the absorbance was measured at 600 nm by using microtiter plate reader to assess cell growth. The MIC was defined as the lowest concentration required for 90% inhibition of growth.

2.4.12 Effect of compounds on Yeast (Y) - hypha (H) transition in *Benjaminiella poitrasii*

For the Y-H transition in *B. poitrasii*, the yeast cells grown in YPG were harvested, washed in 0.85% saline, and 1×10^5 cells/ml were inoculated in YP (3 ml) medium. For the screening of compounds, 4 µg/ml compounds were added in the YP medium from the stocks prepared in DMSO. The tubes were incubated on rotary shaker (180 rpm) at 28 °C. Transition was observed after 6 h. Yeast or hypha cells were counted as described by Khale *et al.* [40]. In Y-H transition studies single or budding cells were counted as one yeast morphological unit; cells with one or more germ tubes were counted as one hyphal morphological unit using haemocytometer.

2.4.13 Chitin synthase assay

Chitin synthase activity was estimated using non radioactive chitin synthase assay reported by Lucero *et al.* [36]. The assay was carried out in 96 well plates, which involved plate coating. The total CS enzyme activity in cell extract was purified according chitins *et al.* [45].

Plate coating: WGA stock (1mg/ml) solution was diluted to 50 µg/ml with distilled water and 100 µl was added to each well. After incubation of plates at room

temperature for 16 h, WGA solution was removed by shaking. The remnant of WGA was removed by washing plates at least three times by immersion in running distilled water. Wells were blocked by adding 300 µl BSA blocking buffer (20 mg/ml in 50 mM Tris pH-7.5) and further plates were incubated at room temperature for 3 h. Coated plates were stored at -20 °C.

Extraction of chitin synthase enzyme: To obtain the hyphal mass, *B. poitrasii* ($2x10^5$ CFU/ml) was inoculated in YP medium and incubated at 28 °C for 24 h (180 rpm). Hyphal cells were collected on Whatman filter paper No. 1 and washed with 0.85% saline, followed by washing with Tris buffer (50 mM, pH 7.5) containing 2.5 mM $MgCl_2$ and 5 mM EDTA. Extraction buffer used was Tris (50 mM, pH 7.5) containing 2.5 mM $MgCl_2$. Hyphal cells of *B. poitrasii* (hypha, 0.5g/ml of extraction buffer) were disrupted using Braun's homogenizer with four cycles of 15 s each. The samples were centrifuged at 12,000 g for 10 min at 4 °C to obtain cell extracts for the estimation of enzyme activities.

Procedure: Coated plates stored at -20 °C were thawed at room temperature and emptied by shaking. In each well, 32 µl of Tris (50mM, pH 7.5), 10 µl of inhibitor, 50 µl of reaction mixture (1mM UDP-GlcNAc, 40 mM GlcNAc, 2.5 mM $MgCl_2$ and 50 mM Tris pH 7.5) and 8 µl enzyme (~ 40 µg protein) were sequentially added. Plates were incubated at room temperature for 90 min. followed by addition of 20 µl of 50 mM EDTA. Plates were gently shaken for 30 sec. 100 µl of 1 µg/ml WGA-HRP in BSA was added in each well, and incubated for 15 min. at room temperature. Plates were emptied by vigorous shaking and washed twice under running distilled water. In each well 100 µl of peroxidase substrate reagent was added. Reaction was stopped by adding 100 µl 1 N H_2SO_4, which leads to the formation of stable yellow colour. The absorbance was measured spectrophotometrically at 430 nm.

2.4.14 Protein estimation

Protein was estimated using folin-ciocalteu reagent as reported by Lowry *et al.* [46]. Crystalline bovine serum albumin was used as a standard.

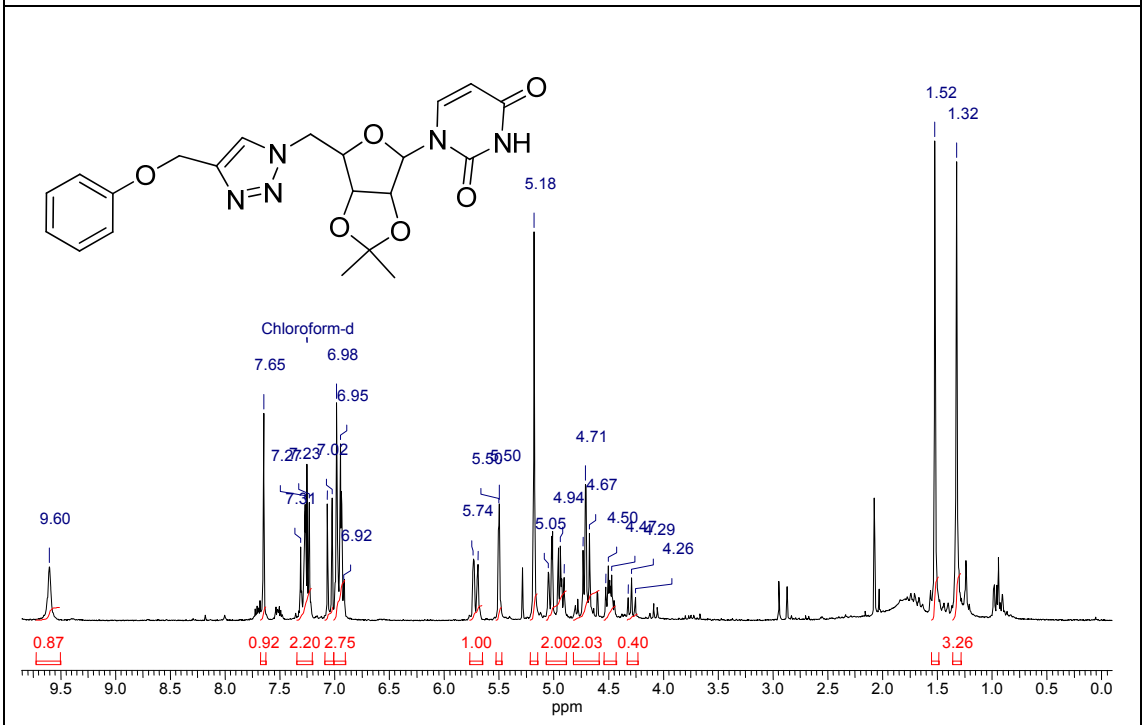
2.4.15 Haemolysis assay

The toxicity of synthesized compounds was checked by the red blood cell (RBC) lysis assay as described previously [44]. The concentrations tested were in the range of 4-

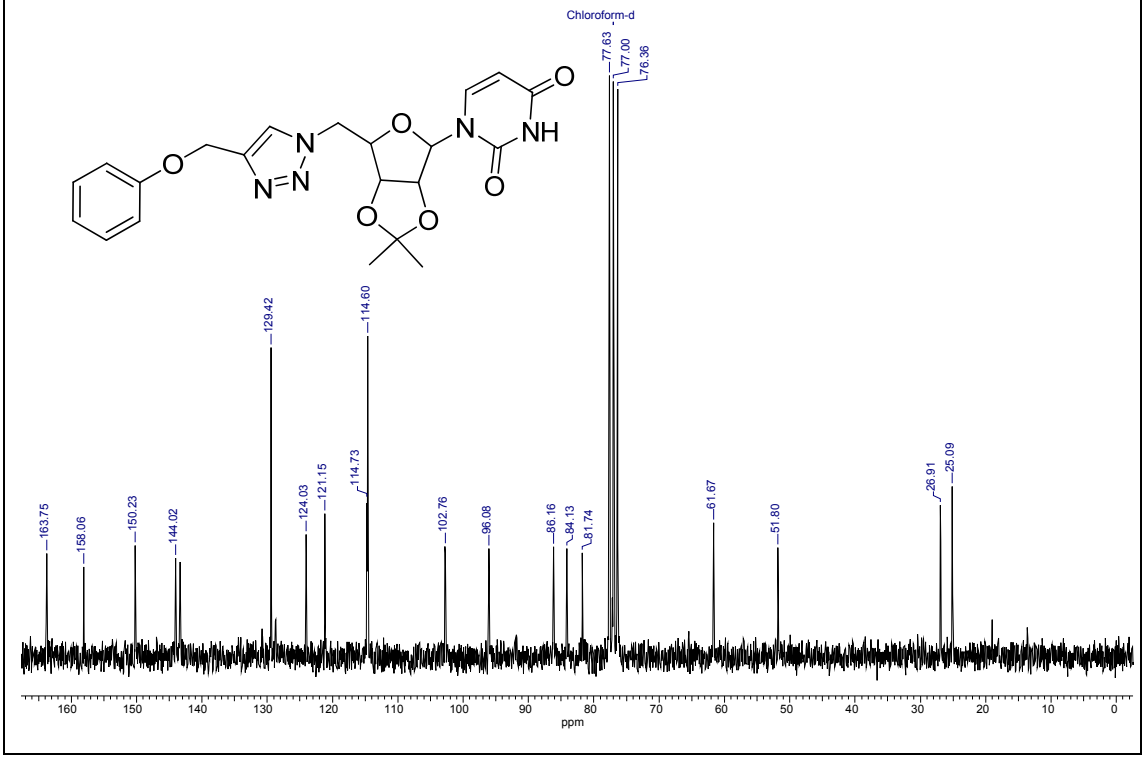
1024 µg/ml. The 2x desired concentration of the compound (in 750 µl of PBS) was mixed with 750 µl of 4% sheep RBC suspension in Eppendorf tubes and incubated at 37°C for 2 h. Triton X-100 (0.1% (v/v) in PBS was used as a positive control whereas 1% DMSO and PBS were used as negative controls. Tubes were centrifuged at 2,000 rpm for 10 min. and the absorbances of supernatant were read at 540 nm. Percent haemolysis was calculated as: [{(A-B)/(C-B)}x100], where A and B are the absorbance values of supernatant from the test sample and PBS (solvent control), respectively, and C is the absorbance value of supernatant from the sample after 100% lysis. The concentration (mean) causing 50% lysis (HC_{50}) was obtained from two independent experiments performed in triplicate.

2.5 Selected spectra

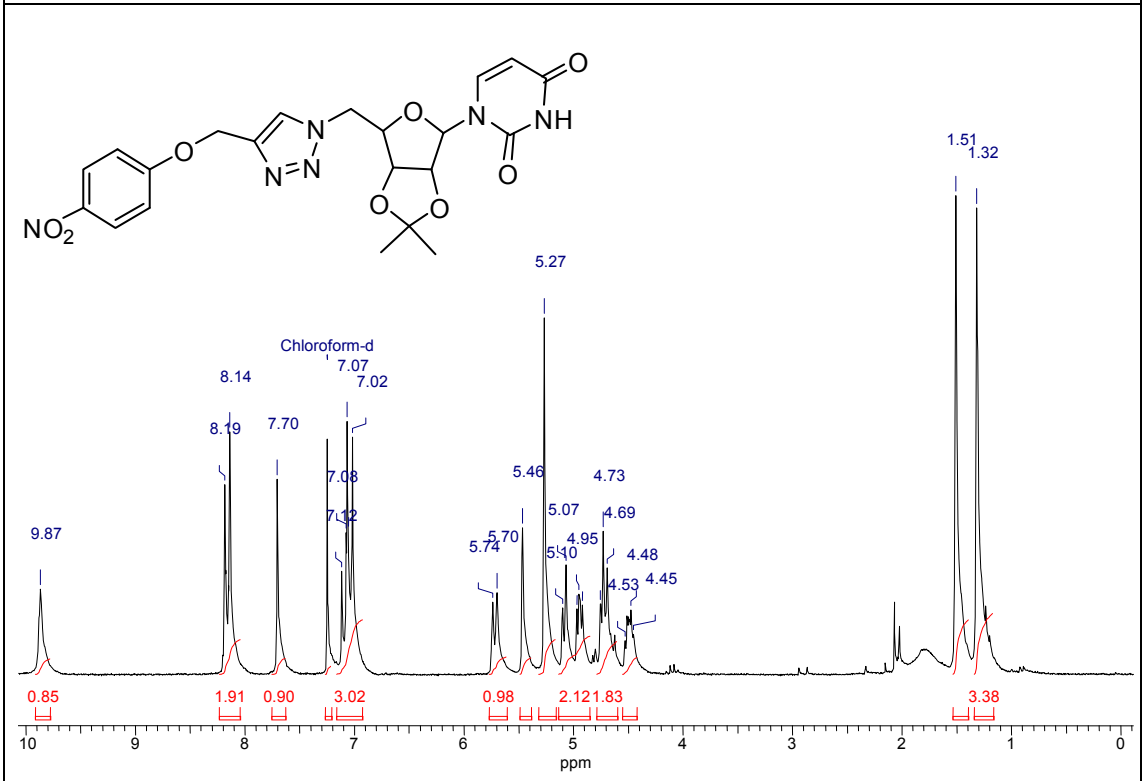
30a: ^{1}H NMR, 200 MHz, $CDCl_3$



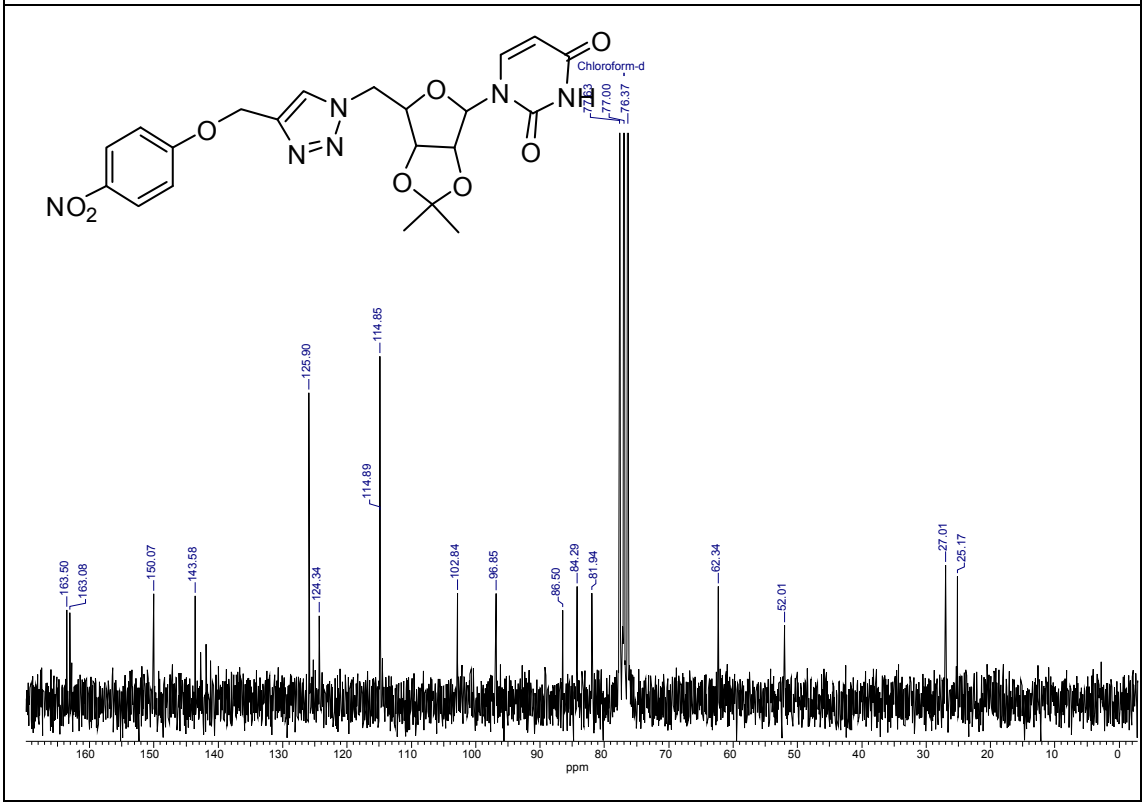
30a: ^{13}C NMR, 50 MHz, $CDCl_3$



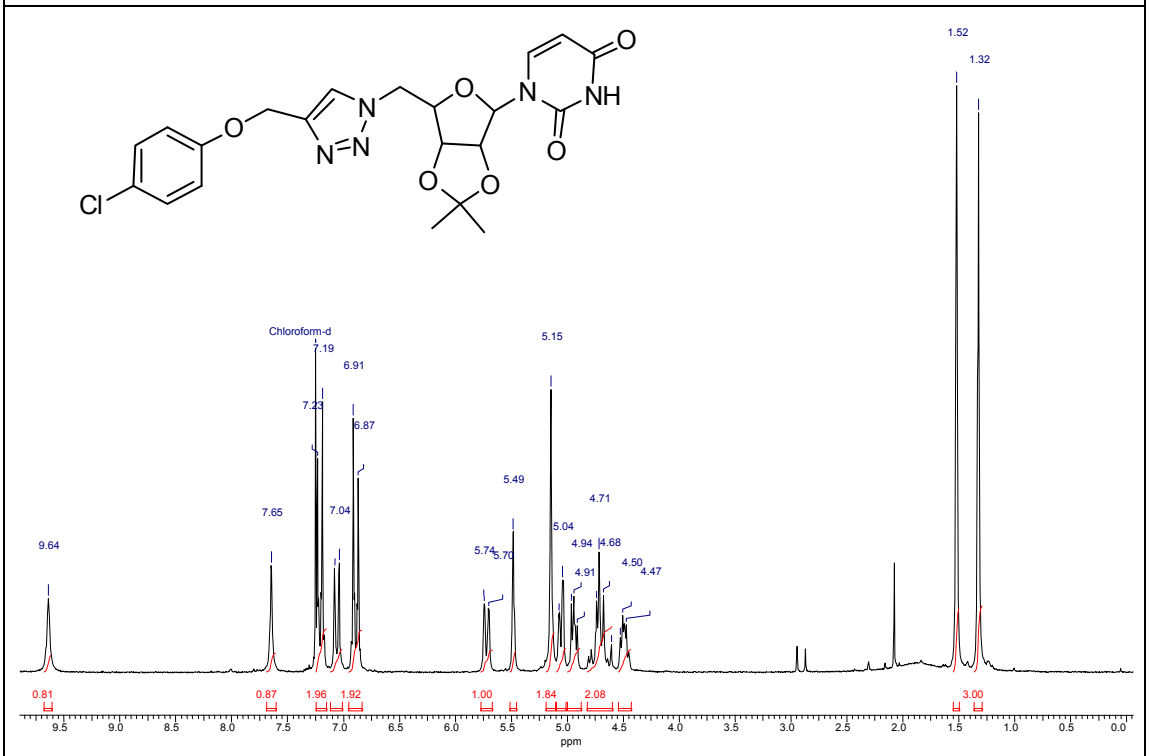
30b: ^{1}H NMR, 200 MHz, $CDCl_3$



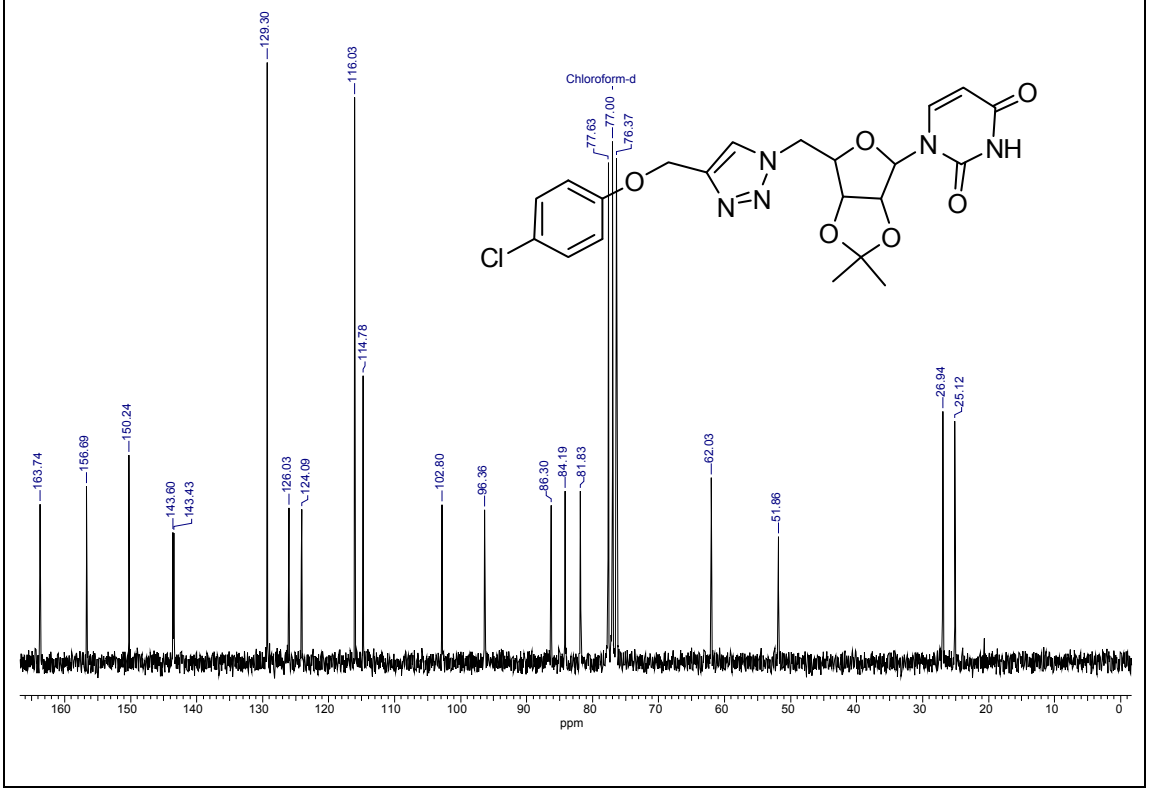
30b: ^{13}C NMR, 50 MHz, $CDCl_3$



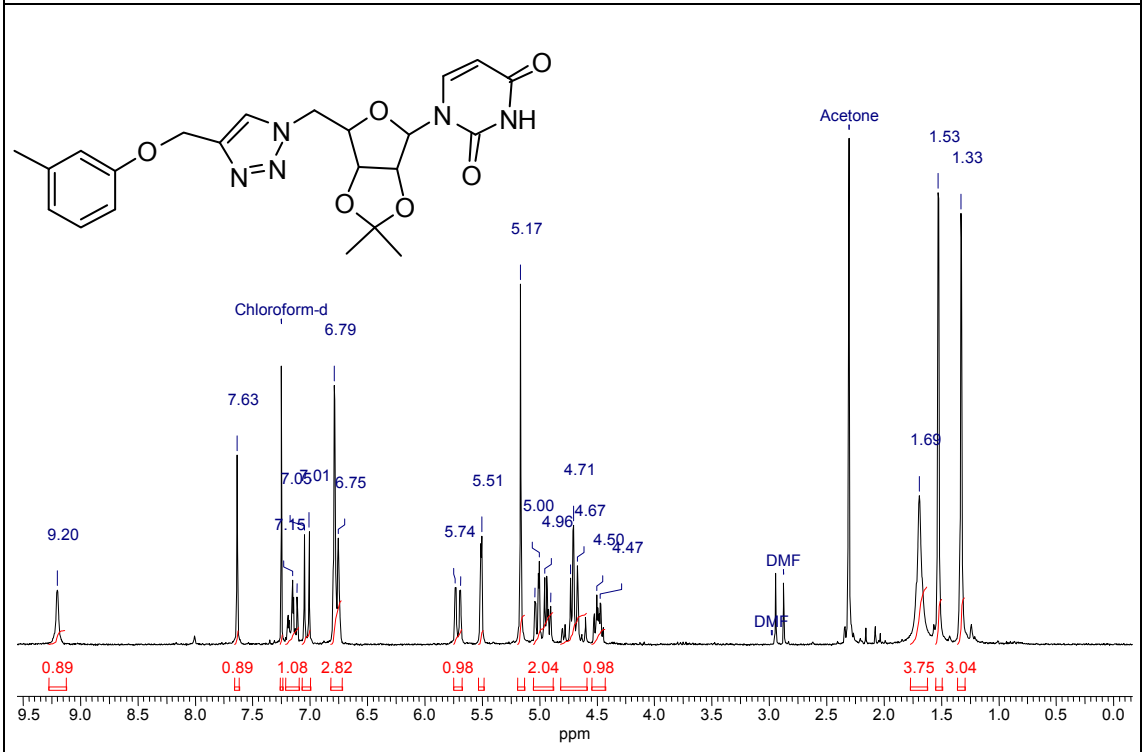
30d: ^{1}H NMR, 200 MHz, $CDCl_3$



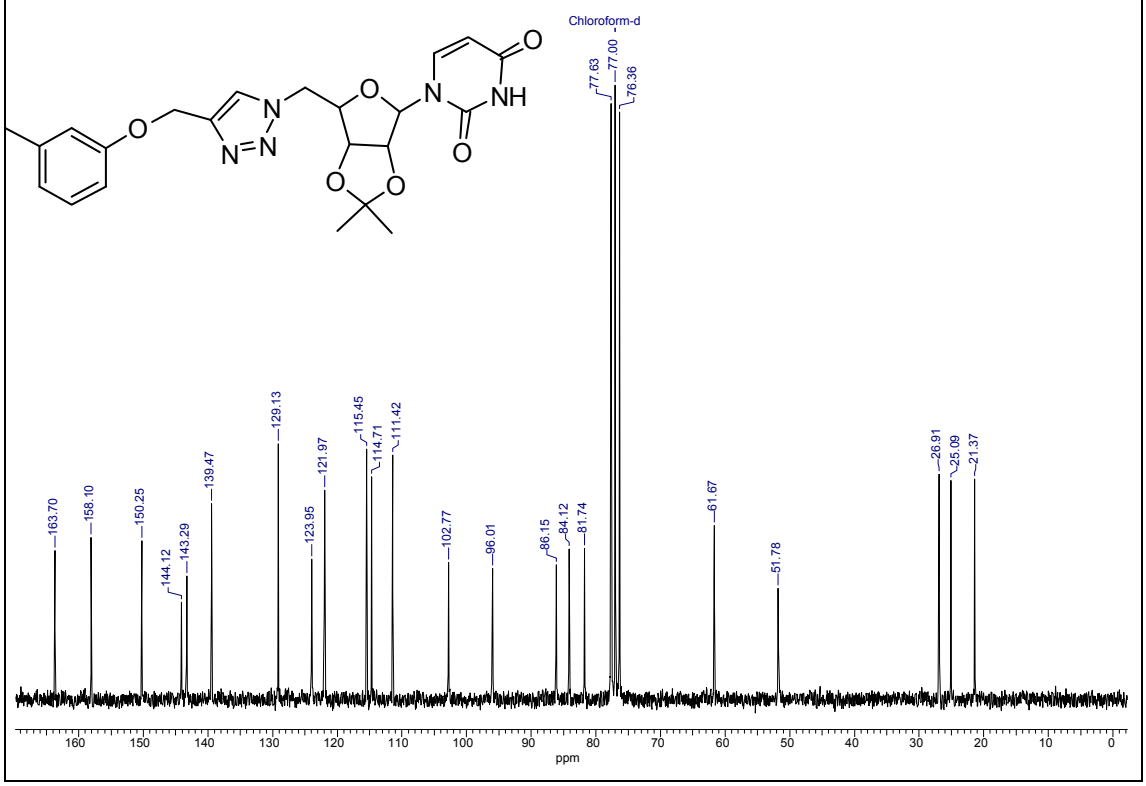
30d: ^{13}C NMR, 50 MHz, $CDCl_3$



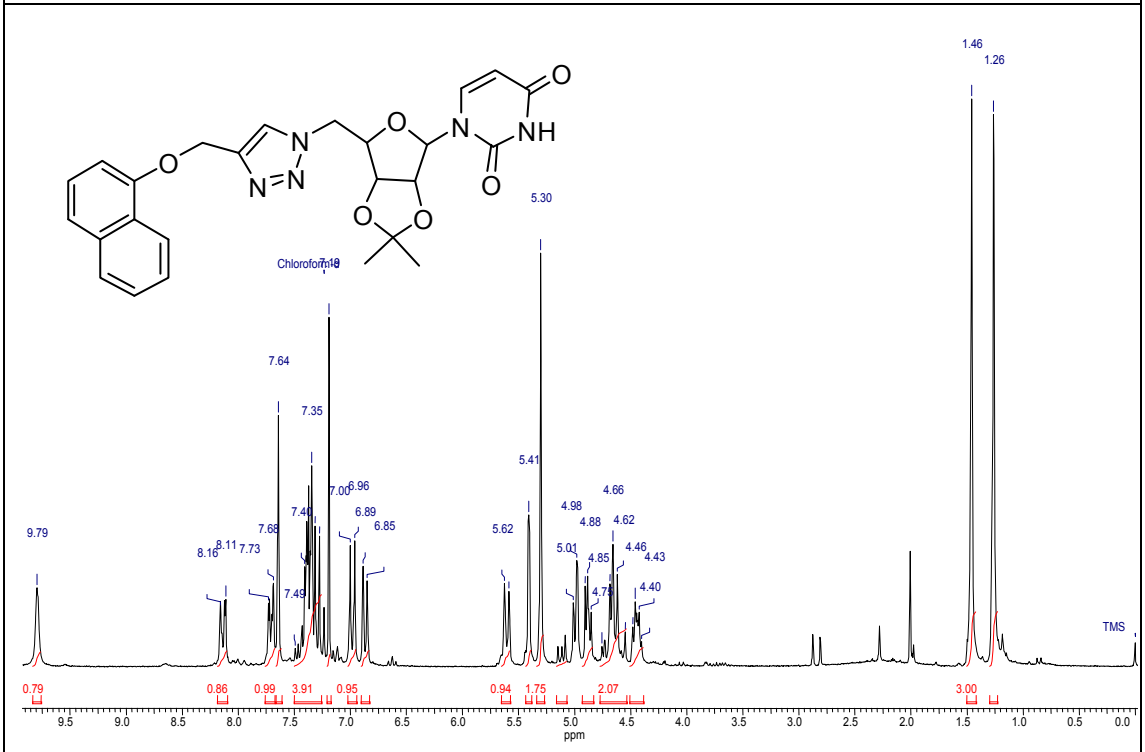
30f: ^{1}H NMR, 200 MHz, $CDCl_3$



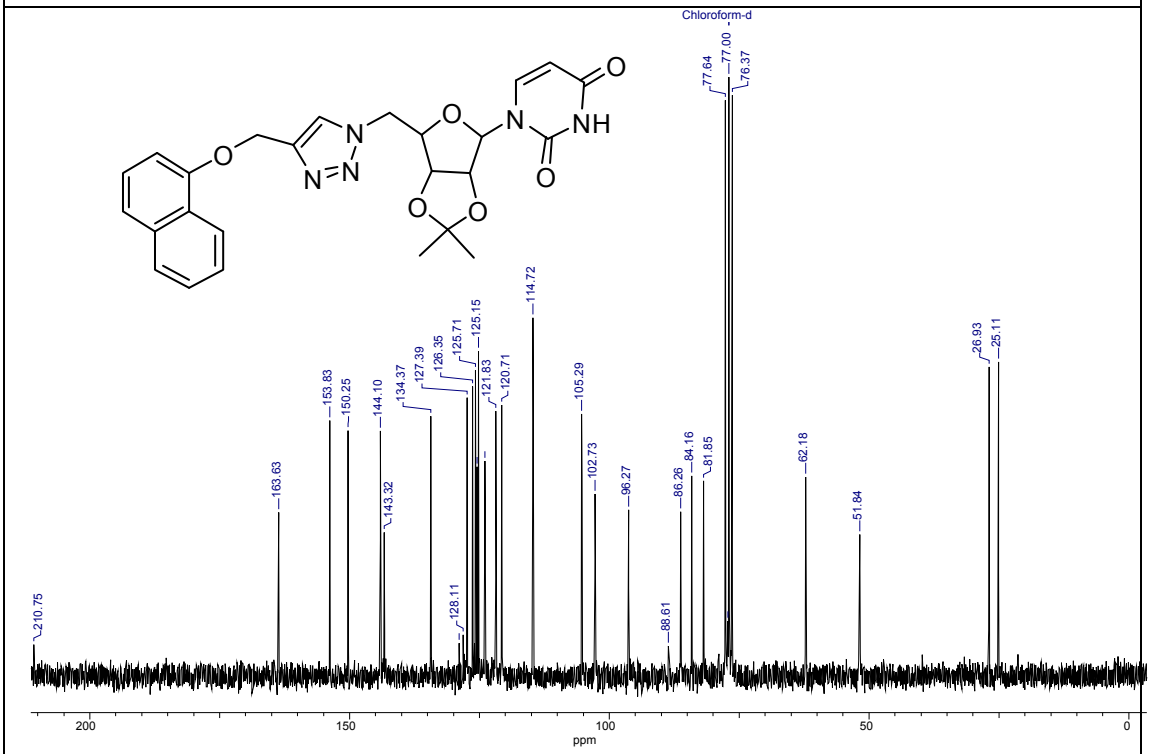
30f: ^{13}C NMR, 50 MHz, $CDCl_3$



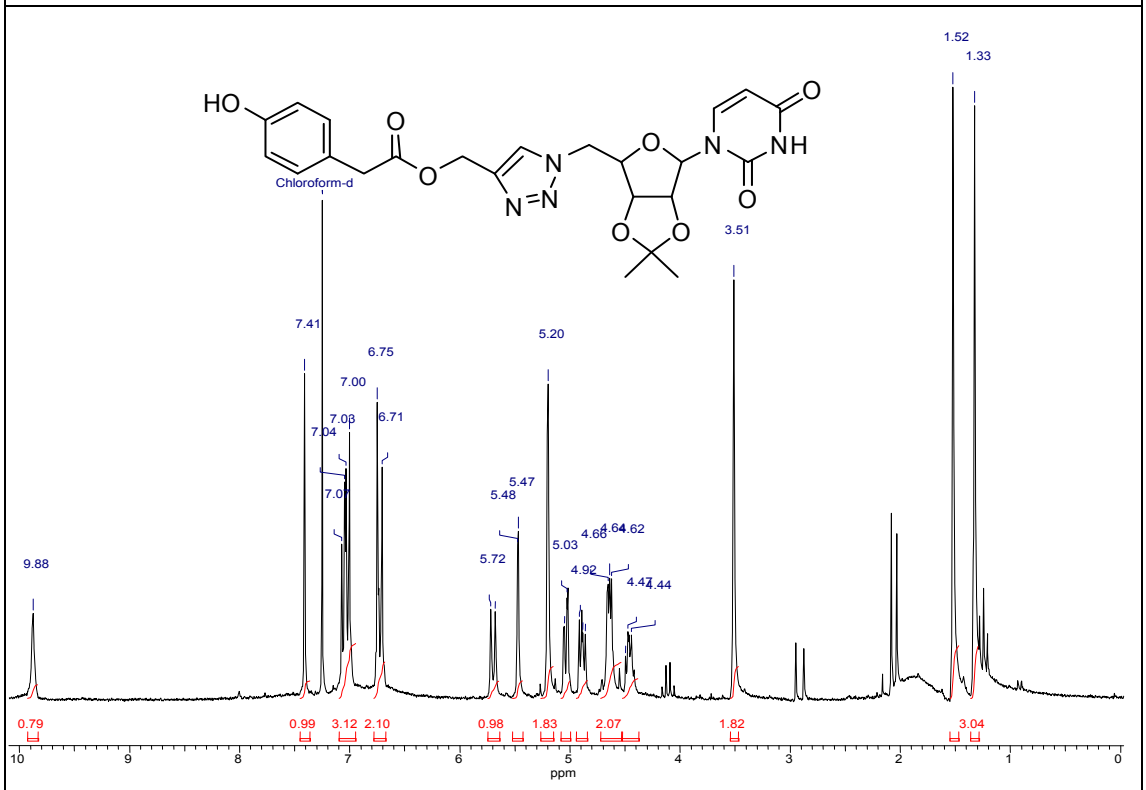
30g: ^{1}H NMR, 200 MHz, $CDCl_3$



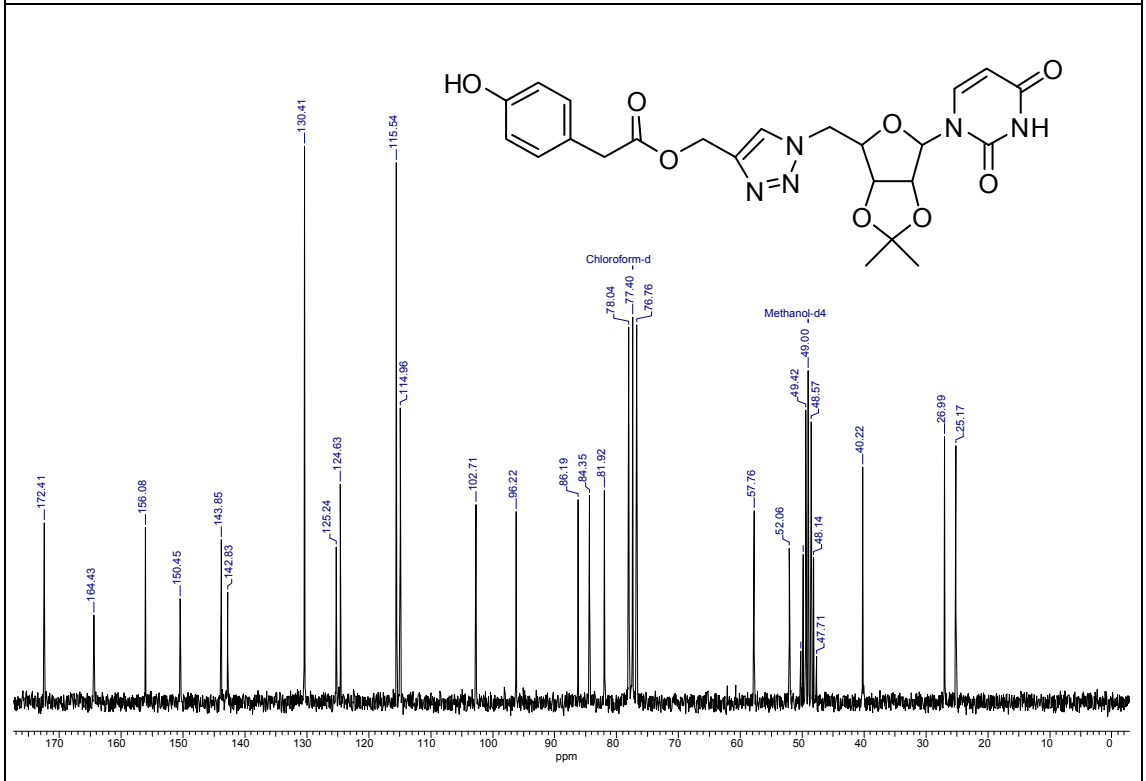
30g: ^{13}C NMR, 50 MHz, $CDCl_3$



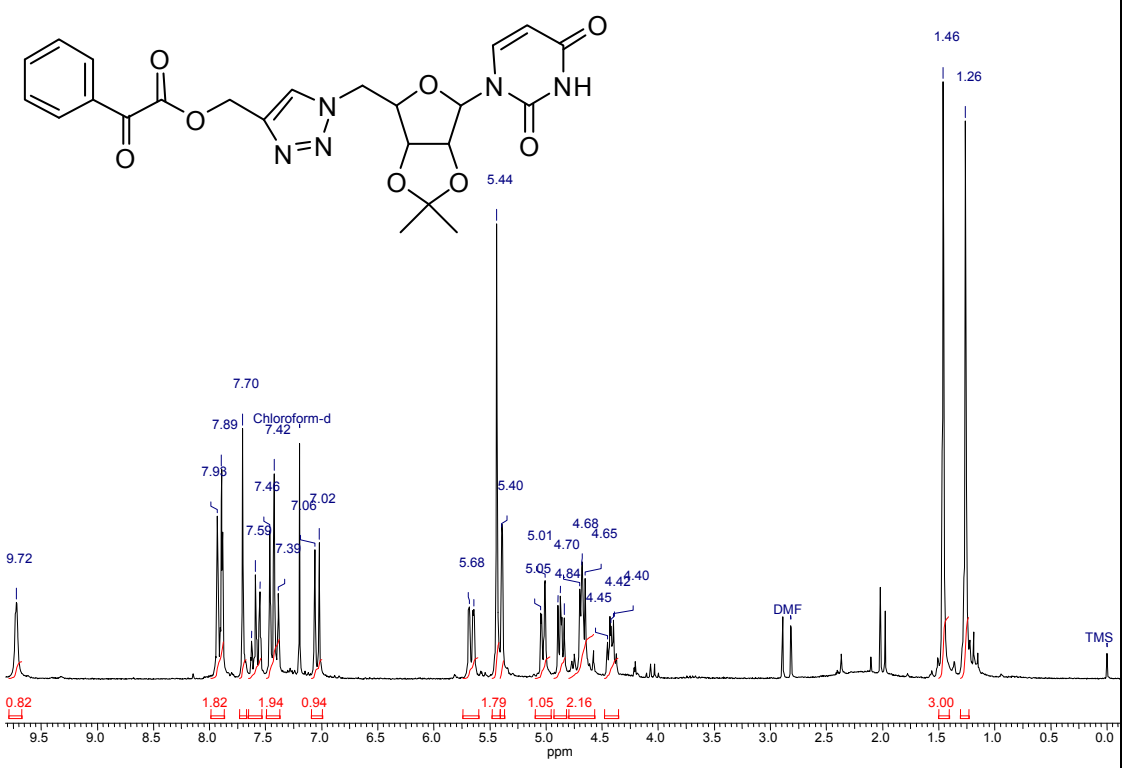
31c: ^{1}H NMR, 200 MHz, $CDCl_3$



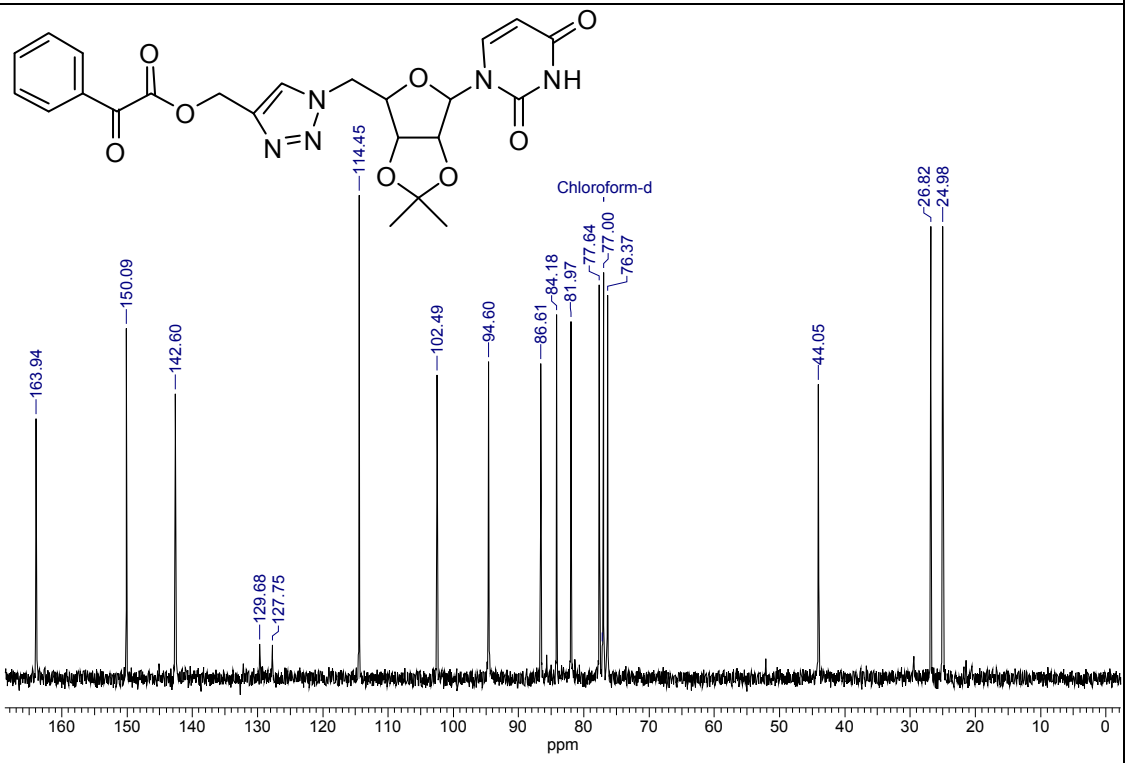
31c:^{13}C NMR, 50 MHz, $CDCl_3$



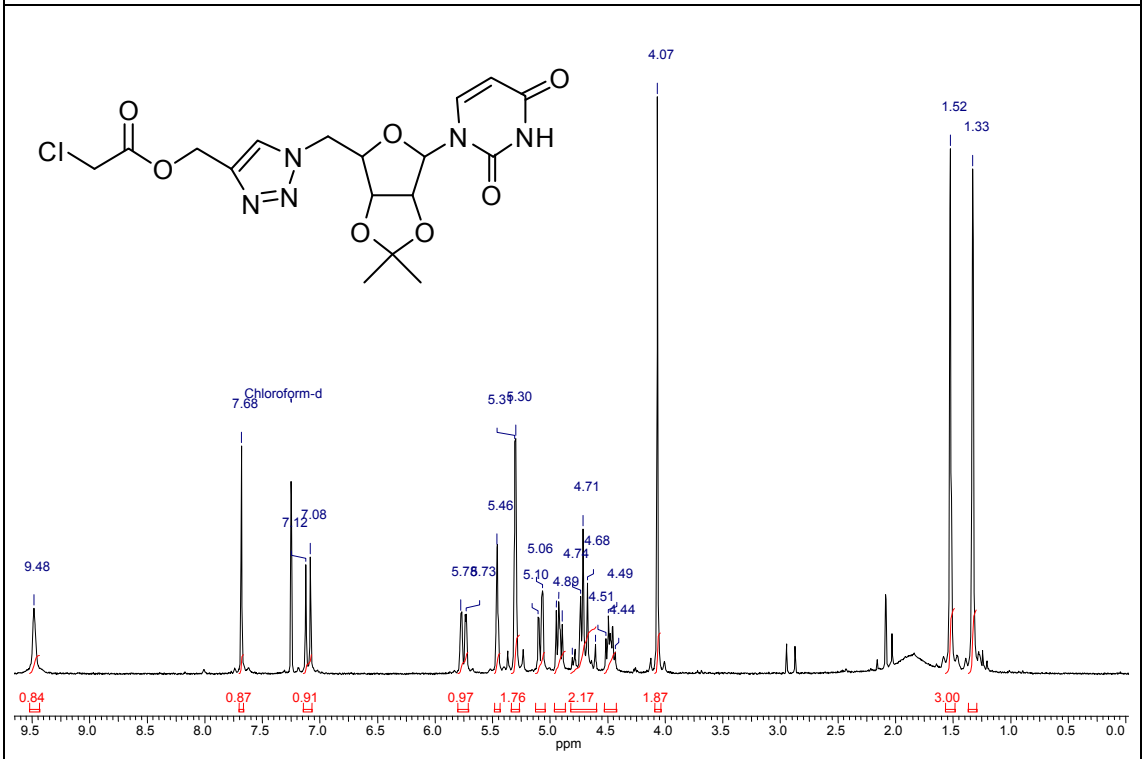
31f: ^{1}H NMR, 200 MHz, $CDCl_3$



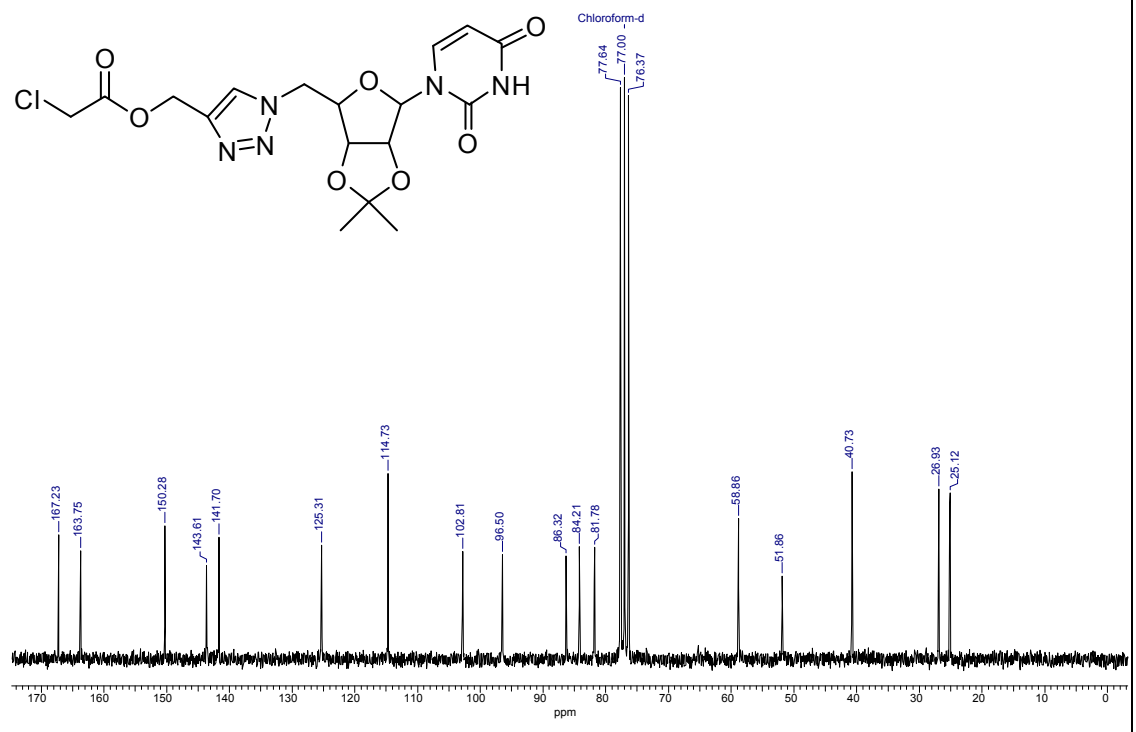
31f: ^{13}C NMR, 50 MHz, $CDCl_3$



32: ^{1}H NMR, 200 MHz, $CDCl_3$



32: ^{13}C NMR, 50 MHz, $CDCl_3$



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Chapter 3

SYNTHESIS OF DIMERIC URIDINE DERIVATIVES AS CHITIN SYNTHASE INHIBITORS

3.1 Chitin synthase: a glycosyltransferase with two active sites?

Glycosyltransferases (GTs) are the family of enzymes which are involved in the biosynthesis of polysaccharides and glycoconjugates. Majority of GTs transfer a sugar residue from an activated nucleotide sugar donor, to specific acceptor molecules forming glycosidic bonds. Transfer of the sugar residue occurs with either the retention or the inversion of the configuration at anomeric carbon [1]. GTs are classified in to two groups: processive enzymes that transfer multiple sugar residues to acceptor e.g. chitin synthase (CS), cellulose synthase and nonprocessive enzymes that catalyze the transfer of single sugar residue to the acceptor.

CS, a membrane bound glycosyltransferase type enzyme, catalyzes transglycosylation reaction in which sugar residues are transferred from UDP-GlcNAc to the growing chitin chain releasing uridine diphosphate. In the fungal cytoplasm, most of the CS in zymogenic form is accumulated in specialized structures called chitosomes. Synthesis of chitin takes place either intracellularly or at the interphase with the extracellular medium in the chitosomes. The chitosomes with the nascent polymer are translocated across the membrane and integrates with the cell surface. About 20-400 chains of the polysaccharide spontaneously assemble by hydrogen bonding to form crystalline microfibrils [2, 3]. Finally these microfibrils combine with other sugars, proteins, glucans and mannans to form fungal septa and cell wall.

Biosynthesis of polysaccharides has been studied widely in variety of organisms and the enzymes involved in it have been characterized. Genes coding for GTs have been identified in some of the cases, which helped in identification of other GTs along with understanding of their inter relationship. Study of sequence reveals the presence of certain conserved region for common function between various GTs. To detect three dimensional similarities in proteins showing very limited sequence relatedness hydrophobic cluster analysis (HCA) is a powerful sequence comparison method [4]. HCA revealed presence of two conserved domains which includes the N-terminal half (domain A) conserved in all the sequences studied and the C-terminal half (domain B) present in only half of the sequences. The chains of polysaccharides such as cellulose and chitin adopt a twofold screw axis [5]. The simultaneous or sequential addition of two monosaccharides by the GTs allows the twofold screw axis to be generated without the polymer chain or the protein having to rotate. This is of

particular significance in the case of cellulose or chitin, in which a number of polysaccharide chains are produced simultaneously and are subsequently assembled in a crystalline microfibril. The dual addition enables the production of heteropolysaccharides with alternating sugar residues such as in hyaluronic acid by specializing domain A for one sugar (e.g., *N*-acetyl D-glucosamine) transfer and domain B for a different sugar (e.g., D-glucuronic acid) transfer. This double transfer could take place at the two sites, simultaneously or sequentially, with the release of two UDP molecules. The empty sites created by the glycosidic bond formation would provide the necessary driving force for the chain to slide and grow two monomer units at a time. In the case of processive enzymes that have domain B functioning along with domain A, leading to the formation of two glycosidic linkages. The oligo- or polysaccharide chain now grows from its reducing end, as shown in Figure 3.1. The dual addition seems necessary for a processive reaction, because (i) GTs that only have domain A stop after a single addition, and (ii) a single addition leads to a totally different orientation of the reducing end with respect to the growing chain, whereas a second addition restores the orientation of this end [6].

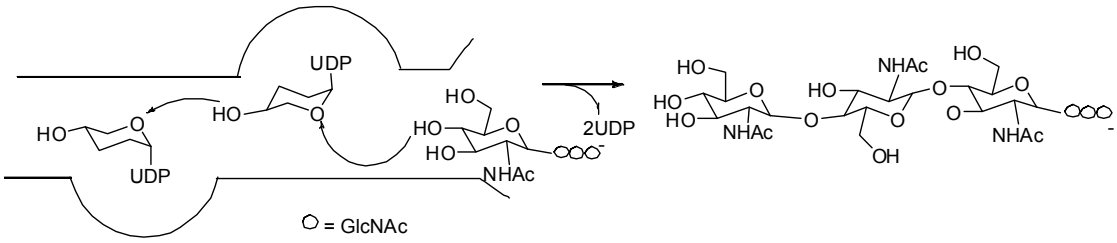


Figure 3.1 Two active site mechanism of chitin synthase

The universal motif of β-glycosytransferases - amino acid residue sequence glutamine-any amino acid-any amino acid-arginine-tryptophan (QXXRW) is present in CS. Along with it, CS active site has two conserved domains A and B, with two aspartic acid residues at domain A and one at domain B, which are required for catalytic activity of processive enzyme. Based on the alternating orientation of the GlcNAc residues within the chitin chain; it was proposed that CS possesses two active sites. According to the hypothesis, CS is a processive glycosyl transferase (that adds a number of sugar residues) which forms two glycosidic bonds simultaneously by a specific mechanism resulting in the inversion of the anomeric configuration and release of two UDP molecules. In such case dimeric CS inhibitor can work as shown

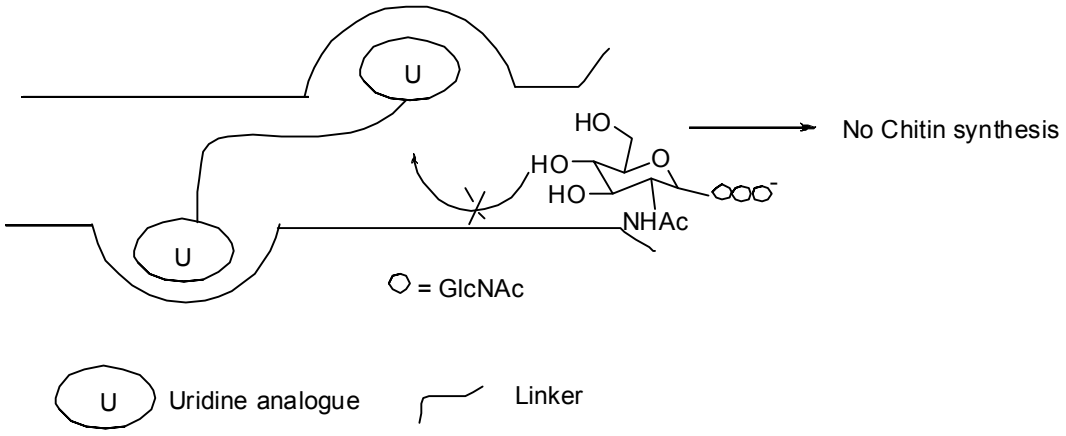


Figure 3.2 Mechanism of action of dimeric inhibitors

in figure 3.2. Dimeric uridine derivative may bind to the two active sites and block chitin synthesis.

3.1.1 Dimeric chitin synthase inhibitors

Yeager *et al.* tested the hypothesis of presence of two active sites in CS by synthesizing dimeric nucleoside inhibitors with the view that if two adjacent active sites are present in close proximity, then dimeric inhibitors should show bivalent inhibition [7]. Dimeric inhibitors **1** (Figure 3.3) were synthesized by joining two 5′-deoxy-5′-aminouridine fragments using carbamate linkers of different lengths. Methyl terminated monomer **2** (Figure 3.3) used as control for comparing the CS inhibitory activity.

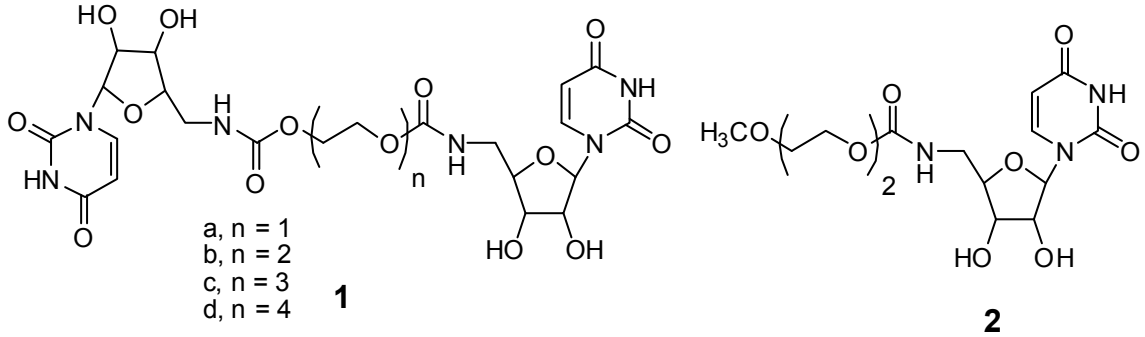
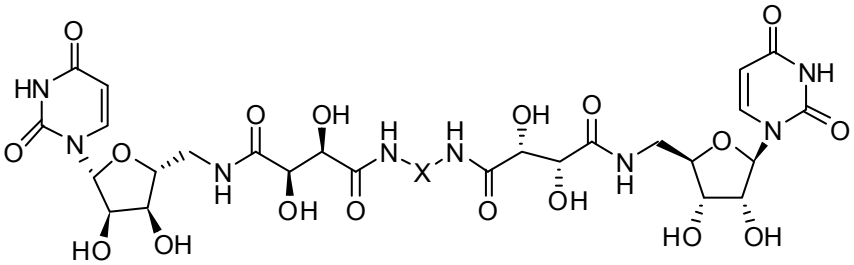


Figure 3.3 Dimeric chitin synthase inhibitors with carbamate spacer

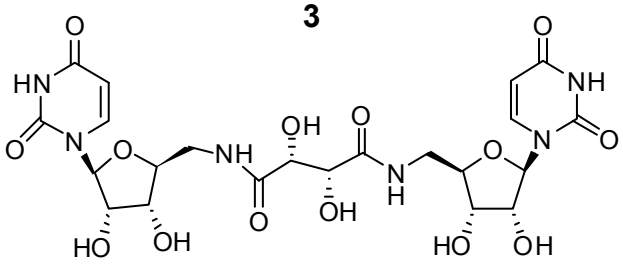
Shorter dimers with n=1 and 2 showed significant inhibition of CS at 1 mM concentration (IC_{50} 2.2 and 1.1 mM respectively) as compared to longer dimers and monomeric control (IC_{50} 11.8 mM). Doubling the concentration of monomeric inhibitor did not increase the inhibition. It was found that efficacy of the dimeric

inhibitors was dependent on the length of the spacer used, with shorter spacer (~14Å) showing better efficacy. In continuation of this work, dimeric uridine analogues with tartarate amide spacer (**3,** Figure 3.4) were synthesized and evaluated for their CS inhibition [8]. The monomeric inhibitor **5** (Figure 3.4) showed 8 % inhibition of CS activity which was lower as compared to dimeric inhibitor **4** (Figure 3.4). As the spacer length increased the CS inhibitory activity of compounds decreased. The shortest dimer **4** (Figure 3.4) with spacer length ~12Å showed potent inhibition of CS activity (35%) at 1 mM concentration. Use of tartarate amide spacer added incentives to the inhibitory activity and proved requirement of short and rigid spacer for better CS inhibitory activity. The results supported the possibility of presence of two active sites in CS.



X= $(CH_2)_2$, $(CH_2)_3$, $(CH_2)_4$, $(CH_2)_2O(CH_2)_2$

3



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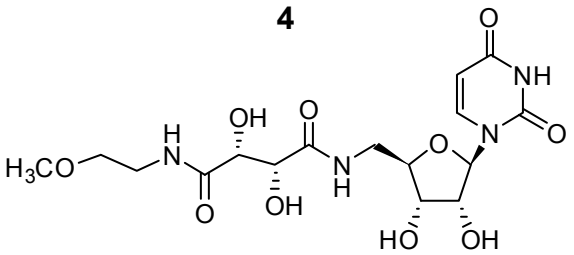


5

Figure 3.4 Dimeric chitin synthase inhibitors with tartarate spacer

Secondly, multivalent interactions involve the binding of different ligands simultaneously on one molecule. Number of such examples occurs in natural systems, wherein different carbohydrates and oligosaccharide ligands binds to protein receptors of cell surface [9]. Research efforts are directed to explore the concept of

multivalency for the discovery of potent new drugs [10, 11], with specifically many dimeric compounds being investigated as drug candidates. Due to dimerization, binding of one molecule brings another molecule closer which results increased potency of compound [12-21].

Based on this background, we tried to explore the possibility of presence of two active sites in CS through design, synthesis and biological evaluation of few dimeric CS inhibitors.

3.2 Click chemistry for the synthesis of dimeric inhibitors

Click chemistry conjugation approach has been used by many researchers for the synthesis of dimeric inhibitors containing 1,2,3-triazoles moiety as a linker. Suh *et al.* reported the synthesis of vitamin D dimers with 1,2,3-triazole linker using Cu (I) catalyzed 1,3 dipolar addition [22]. For the synthesis of these compounds, vitamin D C23 terminal acetylene was reacted with vitamin D side chain azide to form the dimer **6** (Figure 3.5). Although the dimers were synthesized successfully with regiospecificity, they were devoid of any antiproliferative activity.

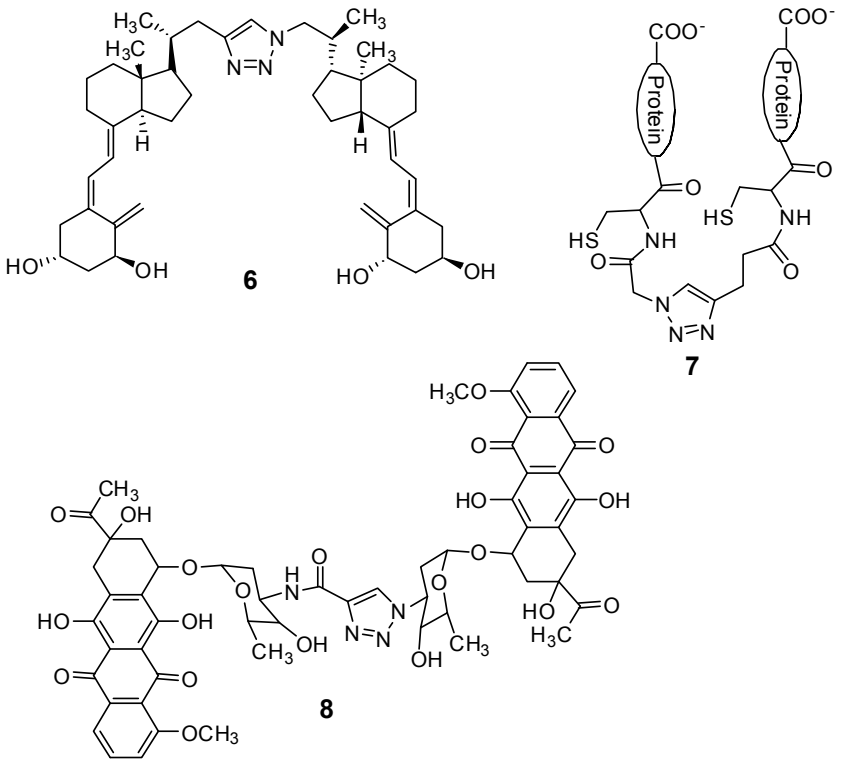


Figure 3.5 Dimeric inhibitors with 1,2,3-triazole

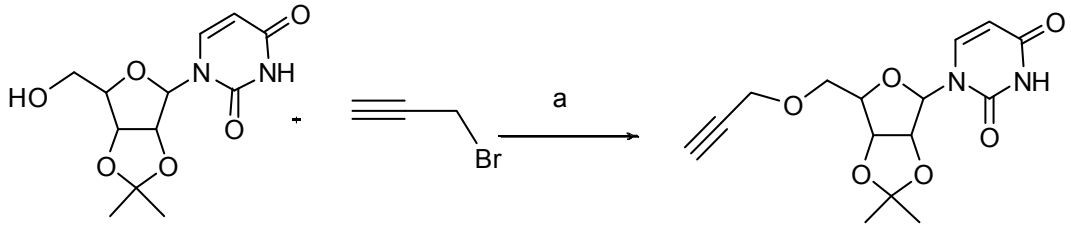
Xaio *et al.* used the Cu (I) catalyzed 1,3-dipolar cycloaddition approach for the synthesis of multimeric N-terminal linked protein G **7** (Figure 3.5) [23]. The azide and alkyne modification of N-terminal of protein G was carried out by native chemical ligation. Further, these azide and alkyne N-terminal protein G were coupled together in presence of Cu (I) catalyst.

Daunorubicin is a known potent anticancer drug. The mode of action of daunorubicin includes DNA intercalation, topoisomerase inhibition and production of free radicals for DNA damage. Since the bis-intercalator showed increased DNA binding affinity, Zhang *et al.* dimerized the daunorubicin using click reaction with $(EtO)_3PCuI$ as a catalyst [24]. Dimers with various spacer lengths were synthesized to check its effect on the cancer cells. Compound **8** (Figure 3.5) was most potent with IC_{50} value of 20 μM for the inhibition of leukemia cell line. The structure of the products suggested that the length and the flexibility of the linker are crucial factors for the resulting activity.

3.3 Results and discussion

3.3.1 Synthesis of *O*-propargyl ether of 2′,3′-*O*-methylethylidene uridine

The 2′,3′-*O*-methylethylidene uridine was prepared by following procedure as described in section 2.4.3. 2′,3′-*O*-methylethylidene uridine **9** was converted to its propargyl ether **11** by treating with sodium hydride and propargyl bromide **10** (Scheme 1). The IR spectrum of the propargyl ether **11** showed characteristic stretching frequencies at 3272.98 cm^{-1} for alkyne C-H and at 1712.96, 1666.38 cm^{-1}



9 **10** **11**

Scheme 1: Reagents and conditions: a) NaH, DMF, 0-28 °C, 6 h, 65%

for two carbonyl groups of uridine. The ^1H NMR showed resonances for alkyne C-H proton at δ 2.46 (t, J = 2 Hz), anomeric proton at δ 5.60 (d, J = 2.03 Hz) and in the ^{13}C spectrum resonances at 75.32 and 78.49 ppm were observed for two acetylinic carbons.

3.3.2 Synthesis of 5′-*O*-alkylazide-2′,3′-*O*-methylethylidene uridine compounds

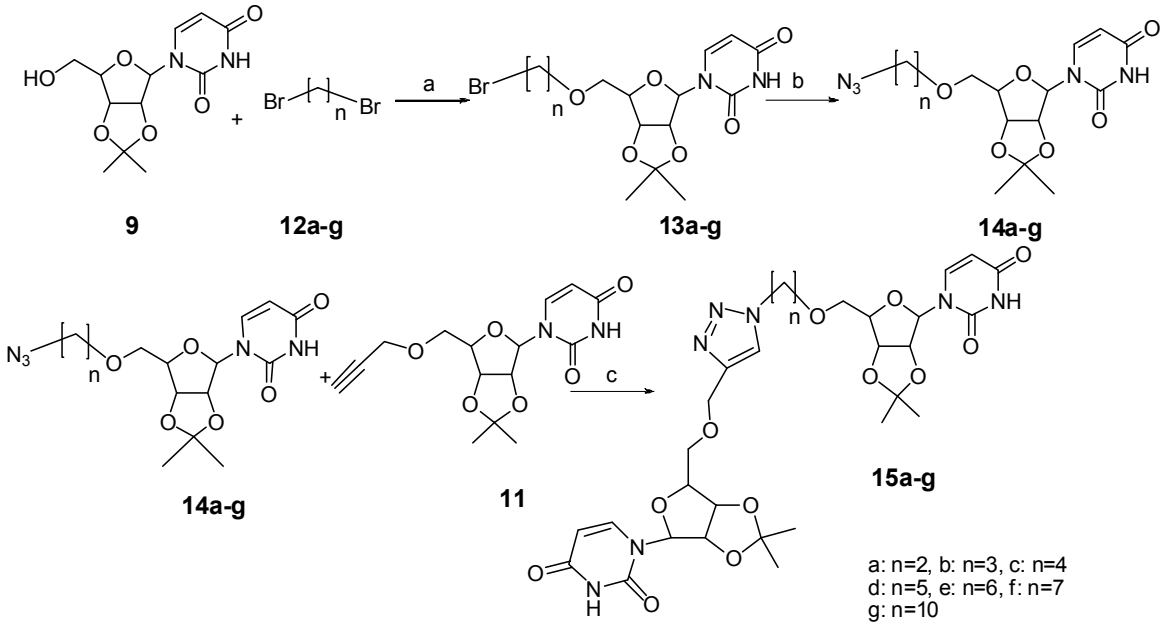
O-alkylation of 5′-hydroxy of 2′,3′-*O*-methylethylidene uridine **9** using different dibromoalkanes (**12a-g**) in basic condition led to the formation of O-alkyl substituted 2′,3′-*O*-methylethylidene uridine (**13a-g**). Formation of compounds **13a-g** was confirmed by ^{1}H NMR. For example, compound **13b** in ^{1}H NMR spectrum showed resonance at δ 3.79, 3.35 and 2.21 as a multiplets for methylene protons of spacers. Along with this two singlets at δ 1.55 and 1.33 were seen for methyl protons of ketal.

Compounds **13a-g** were further treated with NaN_3 in acetone, wherein the S_N^2 displacement of bromo group with azide gave compounds **14a-g.** Structure of compounds **14a-g** was confirmed by IR, ^{1}H NMR. In IR spectrum the strong stretching frequency was seen at 2118 cm^{-1} for the azido group. In case of compound **14b** ^{1}H NMR spectrum showed resonance at δ 3.84, 3.33 and 1.89 each for two protons as a multiplet. It also showed two singlets at δ 1.55 and 1.33 for ketal protons.

3.3.3 Synthesis of dimeric compounds

1,3-Dipolar cycloaddition of different azido uridine compounds **14a-g** with uridine propargyl ether **11** was carried out by click reaction. One equivalent of uridine propargyl ether **11** and one equivalent of compound **14a-g** were dissolved in mixture of *t*-butanol and water (8:2) and solution was treated with 5 mol% $CuSO_4.5H_2O$ and 10 mol% sodium ascorbate. The reaction mixture was allowed to stir at room temperature for 3-6 h for completion of cycloaddition and to get final compounds **15a-g** (Scheme 2).

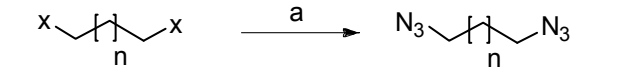
All dimeric uridine derived compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry. For instance, compound **15e** showed following observations in IR and NMR spectrum. The IR spectrum showed characteristic stretching frequencies at 1714, 1693 cm^{-1} etc. The ^{1}H NMR spectrum showed resonance at δ 7.59 as a singlet for triazole proton. Along with this two singlets at δ 1.32 and 1.52 for ketal protons and three multiplets at δ 4.28, 1.85, 0.94 for spacer protons were observed. In ^{13}C spectrum resonance was seen at 65.29, 62.09, 50.00, 37.73, 30.26, 18.88, 13.44 ppm for all methylenes of spacer and uridine. Resonance at 26.95 and 25.02 ppm was seen for methyl carbon of ketal.



Scheme 2: Reagents and conditions: a) NaH, DMF, 0-28 °C, 6 h; b) NaN_3, acetone, 65 °C, 6 h; c) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 3-6 h.

3.3.4 Synthesis of alkyl diazides

1,10-Dibromodecane **12g** was treated with NaN_3 in DMF to get 1,10-diazidodecane **16a** (Scheme 3). 1,12-diazidododecane **16b** was synthesized by reacting diiododododecane **12h** with NaN_3 in DMF. Formation of **16a** and **16b** was confirmed from IR and ^{1}H NMR spectrum. In IR spectrum the characteristic stretching frequency was observed at 2100 cm^{-1} for azide functionality. For compound **16a** in ^{1}H NMR spectrum the resonance was seen at δ 3.22 as triplet for four protons next to azide and at δ 1.56, 1.28 as a multiplet and singlet for four and twelve protons, respectively.



12g, x=Br, n=8
12h, x=I, n=10

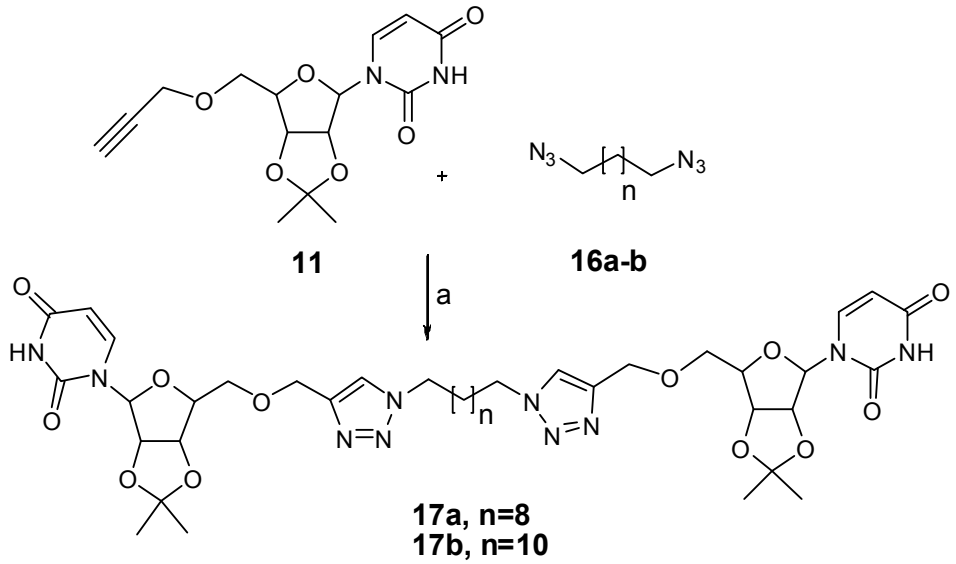
16a, n=8
16b, n=10

Scheme 3: Reagents and conditions: a) NaN_3, DMF, 28 °C, 10 h.

3.3.5 Synthesis of symmetrical dimers

1,3-Dipolar cycloaddition of uridine propargyl ether **11** with 1,10-diazidodecane **16a** and 1,12-diazidododecane **16b** were carried out by click reaction. Two equivalent of uridine propargyl ether **11** and one equivalent of compound **16a** or

16b were dissolved in mixture of *t*-butanol and water (8:2) and the solution was treated with 10 mol% $CuSO_4.5H_2O$ and 20 mol% sodium ascorbate. The reaction mixture was allowed to stir at room temperature for 3-6 h for completion of cycloaddition and to get final compounds **17a** and **17b** (Scheme 4). The dimers **17a** and **17b** were characterized by IR, ^{1}H NMR, ^{13}C NMR and Mass Spectrometry. In case of compound **17a** the IR spectrum showed characteristic stretching frequencies at 1716, 1690 cm^{-1} etc. The ^{1}H NMR spectrum showed resonance at δ 7.59 as a singlet for two protons of triazoles. Along with this two singlet were seen at δ 1.33 and 1.55 for methyl protons of ketal. The methylene protons of spacer showed three multiplets at δ 3.85, 1.83 and 1.24. In ^{13}C NMR spectrum spacer methylenes were seen at 62.26, 50.40, 35.75, 30.13, 29.08, 28.82, 27.23 ppm and ketal CH_3 at 26.36, 25.28 ppm.

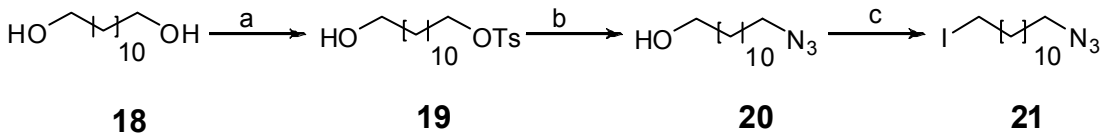


Scheme 4: Reagents and conditions: a) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 3-6 h.

3.3.6 Synthesis of 1-azido-12-iodododecane

1-Azido-12-iodododecane **21** was obtained from dodecane diol **18**. First selective monotosylation of dodecane diol **18** was carried out using tosylchloride in THF with one equivalent of NaH to get 12-hydroxydodecyl tosyl **19**. Small amount of ditosyl product was also formed, which was separated by column chromatography. In ^{1}H NMR spectrum the compound **19** showed resonance at δ 7.77, 7.33 as doublets and at δ 2.43 as singlet for tolyl protons. IR spectrum of **19** showed stretching at 1500 cm^{-1} which is characteristic of aromatic stretching. Further compound **19** was reacted

with NaN_3 to get 12-hydroxydodecyl azide **20.** The IR spectrum of 12-hydroxydodecyl azide **20** showed stretching at 2100 cm^{-1} characteristic to azide group. In ^1H NMR spectrum compound **20** showed resonance at δ 3.27 as doublet for four protons along with multiplet at 1.59 for four protons and singlet at δ 1.26 for remaining sixteen protons. Hydroxyl group of 12-hydroxydodecyl azide **20** was converted to iodo by treatment with iodine and PPh_3, leading to the formation of 1-azido-12-iodododecane **21** (Scheme 5)**.**



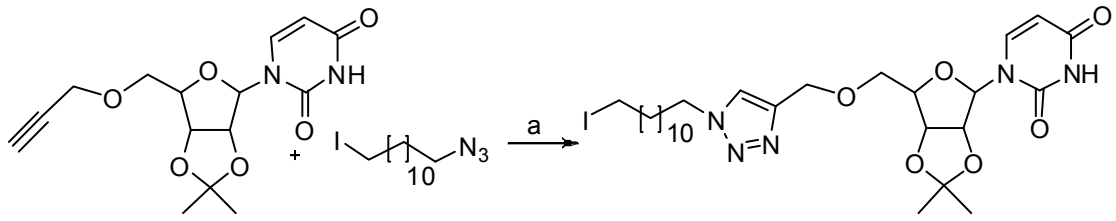
18 **19** **20** **21**

Scheme 5: Reagents and conditions: a) *p*-TsCl, NaH, THF, 0-28 °C, 6 h, 60%; b) NaN_3, dioxane/DMSO (7:1), 65 °C, 5 h, 94%; c) PPh_3, I_2, imidazole, DCM/H_2O (2:1), 28 °C, 1 h, 71%.

Formation of 1-azido-12-iodododecane **21** was confirmed by IR, ^1H NMR. In IR spectrum characteristic stretching frequency of azide at 2100 cm^{-1} was seen. ^1H NMR spectrum showed resonance at δ 3.24 as triplet for four methylene protons attached to iodo and multiplet at δ 1.56 for four methylene protons adjacent to azido group and a singlet was seen at δ 1.26 for remaining sixteen protons.

3.3.7 Synthesis of monomeric inhibitor

Monomeric inhibitor **22** was synthesized by reaction of one equivalent of uridine propargyl ether **11** with 1-azido-12-iodo dodecane **21** in presence of 5 mol% $CuSO_4.5H_2O$ and 10 mol% sodium ascorbate (Scheme 6). Structure of compound **22** was completely characterized from IR, ^1H NMR, ^{13}C NMR and Mass Spectrometry.



11 **21** **22**

Scheme 6: Reagents and conditions: a) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 70%.

Compound **22** showed characteristic stretching frequency in IR spectrum at 1732, 1697 cm^{-1}. In NMR spectrum resonance was seen at δ 7.57 as a singlet for triazole proton along with this resonance was seen at δ 3.89, 3.17, 1.78 and 1.24 as multiplet for spacer methylene protons. Methyl protons of ketal showed resonance at δ 1.56 and 1.34. In ^{13}C spectrum the resonance for spacer methylene carbons were seen at 61.28, 54.74, 52.68, 29.54, 29.45, 29.07 ppm.

All the synthesized dimers (**15a-g, 17a** and **17b**) and monomeric inhibitor **22** were checked for antifungal activity, CS inhibition and yeast to hypha transition inhibition.

3.3.8 Antifungal susceptibility testing

Antifungal activity of all synthesized compounds (**15a-g, 17a, 17b** and **22**) was checked against various human fungal pathogens including different strains of *C. albicans*, *C. neoformans* and *C. glabrata*. The dimeric inhibitors **15a-g, 17a** and **17b** did not inhibit the growth of any of the tested organism even at the highest concentration tested i.e. 512 μg/ml.

There are number of reports available on natural and synthetic dimeric antifungal agents. Abrunhosa *et al.* reported the growth inhibition of different *Aspergillus* sp. by novel chromene dimer [25]. Passiflin, a dimeric antifungal protein isolated from the seeds of passion fruit found to inhibit growth of *Rhizoctonia solani* with IC_{50} of 16 μM [26]. Lee *et al.* reported the dimeric sesquiterpene CHE-23C from stem and roots of *Chloranthus henryi* Hemsl, with potent inhibition of range of phytopathogenic fungi [27]. Ganendren *et al.* reported dimeric commercially available compounds alexidine dihydrochloride (compound A), 1,12 bis-(tributylphosphonium)-dodecane dibromide (compound P) and decamethonium dibromide (compound D) (structural analogues of phospholipid substrates) as specific inhibitors of fungal membrane bound phospolipase B [28].

However, in case of dimers synthesized during present study, no growth inhibition of any of the pathogen was observed. This loss of ability of dimers to inhibit whole cell growth of pathogenic fungi may be due to lower cell permeability. Dimerization may have increased overall polarity of the molecule as number of polar groups (such as hetero atoms) doubled, along with increase in steric bulk of the

molecule. These two factors may have affected the transport of the dimers inside the cell.

Monomeric inhibitor **22** inhibited the growth of *C. albicans* strain 1 with MIC 64 μg/ml, which is comparable to that of nikkomycin (MIC value 64 μg/ml) (Table 3.1). Monomeric inhibitor **22** showed potent growth inhibition of all *C. neoformans* strains (MIC 4-16 μg/ml) as compared to nikkomycin (MIC 32-512 μg/ml). Growth inhibition of *C. albicans* strains and *C. glabrata* by monomeric inhibitor **22** was also better than nikkomycin.

Table 3.1 Antifungal susceptibility testing of monomeric inhibitor **22** against human pathogens**.**

Sr. No.	Name of organism	MIC_{90} in μg/ml	
		22	**Nikkomycin**
1	*C. albicans* Strain 1	64	64
2	*C. albicans* 3471	16	16
3	*C. albicans* 3557	64	512
4	*Candida glabrata*	32	>512
5	*Cryptococcus neoformans*	4	32
6	*C. neoformans* 3541	4	64
7	*C. neoformans* 3542	4	512
8	*C. neoformans* 3378	16	>512

The monomeric inhibitor **22** was also tested against plant pathogens *Magnaporthe grisae, F. oxysporum* and *Dreschlera oryzae* and the observed MIC values were 32, 16 and 32 μg/ml respectively. Khare *et al.* reported that hydrophobic polyoxin L analogues with α-aminododecanoic acid side chain as a potent inhibitor of *C. albicans* [29]. This polyoxin analogue was also found to be most stable to internal cell hydrolysis. Similarly, monomeric inhibitor **22** with dodecane side chain showed potent inhibition of all tested pathogens. This potency may be due to increase in the lipophilicity of the inhibitor.

3.3.9 Chitin synthase inhibition

CS activity of *B. poitrasii* cells was estimated with (250 μg/ml) and without compounds using a non-radioactive spectrometric CS assay, according to Lucero *et*

al. as described in section 2.4.13 [31]. Monomeric inhibitor **22** showed maximum inhibition of CS activity (57.62 %). In case of dimeric inhibitors the spacer chain length was varied from two carbon chain to twelve carbon chain. For the dimeric inhibitors **15a, 15c, 15e** with 2, 4, and 6 methylene groups in the spacer chain, CS inhibition was 19-23%. Whereas, for dimers with odd number of spacer methylene groups CS inhibition was <10%. Maximum CS inhibition was observed for **15g** (29.46%) and **17b** (33.82%), wherein the spacer methylene chain length was 10 and 12 carbon respectively (Table 3.2). This suggested the chain length and flexibility contributes to CS inhibitory activity. The CS inhibitory activity was very low for the dimers (assay concentration 250 μg/ml) as compared to the aryl ether or aryl ester linked 1,2,3 triazole uridine derivatives (assay concentration 4 μg/ml). Potent antifungal activity but poor CS inhibition by monomeric inhibitor **22** indicated that CS inhibition is secondary mechanism and the compound may be acting by different mode of action. The mechanistic studies for monomeric inhibitor **22** are given in Annexure I.

Table 3.2 Chitin synthase inhibition by dimeric compounds

Inhibitor	Spacer methylene chain length	Chitin synthase inhibition at 250 μg/ml concentration
22	**Monomeric inhibitor**	57.62
15a	n = 2	19.76
15b	n = 3	4.50
15c	n = 4	22.46
15d	n = 5	6.43
15e	n = 6	22.94
15f	n = 7	9.48
15g	n = 10	29.46
17a	n = 10	16.79
17b	n = 12	33.82

Yeager *et al.* have reported the synthesis of uridine containing dimeric inhibitors with carbamate and tartarate spacer [7, 8]. It was found that in both the cases the dimeric inhibitors with shorter spacer **1b** (Figure 3.3) and **4** (Figure 3.4) showed maximum inhibition of CS at 1 mM concentration (45 and 35% respectively).

However, their respective monomeric controls **2** (Figure 3.3) and **5** (Figure 3.4) at 1 mM concentration showed only ~6 % inhibition of CS from *S. cerevisae.* Khare *et al.* reported that polyoxin L analogue with longer alkane (aminododecanoic acid) side chains are well transported through cell membrane and its increased hydrophobicity was well tolerated at the active site of the CS [29]. Due to this reason polymethylene chain was selected as spacer for the synthesis of dimers. The bivalent or improved inhibition of CS activity in case of dimers as against the monomeric inhibitor was not observed which may be due to use of nonpolar polymethylene chains as spacer.

The increased inhibitory efficacy of dimers reported by Yeager *et al.* might also be due to other mechanisms and do not prove the existence of two active sites unambiguously. The result of CS inhibition by dimers from present study does not support the hypothesis of presence of two active sites in CS and indicates towards the presence of alternative single-site mechanism. Hence, CS may be adding single monosaccharides in a sequential fashion to the non-reducing ends of the growing nascent polysaccharide chain. The answer to this controversy will only come through purification of enzyme to homogeneity and crystallization of at least the catalytic domain.

3.3.10 Yeast to hypha transition inhibition

B. poitrasii yeast inoculum was grown in YPG (Glucose 1%) medium at 37 °C for 24 h and the transition to the hypha form was studied in YP medium at 28 °C. The yeast cells were inoculated in YP broth (with and without compounds) at 28 °C for 6 h and the percentage of cells forming germ tubes was assessed as described by Khale *et al.* [32]. Nikkomycin Z was used as a positive control in the yeast-hypha transition experiment.

All the dimeric compounds **15a-g, 17a** and **17b** showed inhibition of *B. poitrasii* transition in the range of 45-73% at 4 μg/ml concentration. Compound **15a**, **15b** and **15d** showed transition inhibition in the range of 45-60%, while rest of the dimeric compounds showed inhibition in the range of 60-73% (Table 3.3).

In case of the dimers, even though they were unable to inhibit the growth of the fungi, they showed transition inhibition. Targeting the virulence factor is an emerging concept for the development of antifungal agents [33]. Morphological transition in medically important fungus *C. albicans* contributes to virulence and

affords the target for development of antifungal agent [34, 35]. Generally for the screening of the synthesized chemical compounds, NCCLS methodology MIC assays are used frequently [36, 37]. However this test is devised and optimized for testing drug candidates which are in current use and may not be useful for newer compounds. For instance, Brayman *et al.* reported a novel class of glucan synthase inhibitors with good *in vitro* activity but without defined MICs [38]. To prove the potential of such compounds they developed the method for the screening of these compounds using quantitative germ tube formation in *C. albicans*. They found that the glucan synthase inhibitor PNU143678E showed MIC 0.25 μg/ml for gem tube formation for *C. krusei,* while it did not show growth inhibition up to 200 μM (~85 μg/ml) tested by NCCLS method. In case of Amphotericin B complete inhibition of germ tube formation was seen at $1/10^{th}$ concentration corresponding to its MIC for *C. albicans* [39].

Table 3.3 Yeast to hypha transition inhibition by the dimeric compounds.

Inhibitor	Inhibitor methylene spacer length	% Y-H transition inhibition at 4 μg/ml concentration
22	Monomeric inhibitor	68.42
15a	2	45.66
15b	3	58.33
15c	4	73.33
15d	5	58.33
15e	6	69.23
15f	7	65.20
15g	10	61.53
17a	Symetrical 10	62.50
17b	Symetrical 12	60.00
Nikkomycin		>95

Hawser *et al.* reported that the known antifungal agents amphotericin B, mulundocandin and aculeacin showed MIC 0.06, 0.25 and 1 μg/ml respectively in *C. albicans,* while inhibition of morphological transition was seen at MIC 0.02, 0.05 and 0.012 μg/ml [40]. These results proved that morphological inhibition is sensitive compared to whole cell inhibition. Similarly dimeric inhibitors from present study did not inhibit growth of tested pathogenic fungi up to 512 μg/ml, however, they showed

inhibition of yeast to hypha in *B. poitrasii* at 4 μg/ml. Therefore, the dimers have potential for control of pathogenic fungi through inhibition of virulence factor (morphological transition) and needs to be further studied by synthetic modification (like use of polar spacer) and bioassays.

3.4 Conclusions

Based on the hypothesis of two active site mechanism of CS enzyme, total nine dimeric inhibitors **15a-g, 17a** and **17b** with aliphatic spacer chain and 1,2,3-triazoles as connecting moiety were designed and synthesized successfully by click chemistry. No inhibition of the growth of any of the tested organism was observed even at the highest dimer concentration tested i.e. 512 μg/ml. All the dimers showed poor CS inhibitory activity; however, they showed inhibition of *B. poitrasii* transition in the range of 45-73% at 4 μg/ml concentration. Monomeric inhibitor **22** was found to be potent antifungal agent as it inhibited the growth of all tested strains with MIC 4-64 μg/ml.

3.5 Experimental section

3.5.1 Synthesis of *O*-propargyl ether of 2′,3′-*O*-methylethylidene uridine (11)

NaH (0.101 gm, 4.22 mmol) was added to a solution of 2′,3′-*O*-methylethylidene uridine **9** (1 gm, 3.52 mmol) in dry DMF (15 ml) at 0 °C under argon atmosphere. The reaction mixture was allowed to stir for 1 h at 28 °C. After stirring the reaction mixture was again cooled to 0 °C and propargyl bromide (0.497 gm, 4.22 mmol) was added drop wise and stirred at 28 °C for 6 h. After completion of the reaction (monitored by TLC) the reaction mixture was quenched with ice and diluted with 300 ml of ethyl acetate. The ethyl acetate layer was washed sequentially with 3×35 ml of water, 35 ml of brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. Finally the crude propargyl ether was purified by silica gel column chromatography using ethyl acetate and petroleum ether as eluent to obtain 0.734 gm (65%) of *O*-propargyl ether of 2′,3′-*O*-methylethylidene uridine **11**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.41 (d, 1H, *J*=8 Hz), 5.79 (d, 1H, *J*=8 Hz), 5.60 (d, 1H, *J*=4 Hz), 4.98 (m, 2H), 4.66 (d, 2H), 4.28 (m, 1H), 3.85 (m, 2H), 2.17 (t, 1H), 1.56 (s, 3H), 1.340 (s, 3H).

3.5.2 General procedure for the synthesis of *O*-bromoalkyl 2′,3′-*O*-methylethylidene uridine 13a-g

NaH (1.2 mol) was added to a solution of 2′,3′-*O*-methylethylidene uridine **9** (1 mmol) in dry DMF (15 ml) at 0 °C under argon atmosphere. The reaction mixture was allowed to stir for 1 h at 28 °C. After stirring at room temperature, the reaction mixture was cooled to 0 °C and specific dibromoalkane (1.2 mmol) was added drop wise and reaction mixture further stirred at 28 °C for 6 h. After completion of the reaction, monitored by TLC, the reaction mixture was quenched with ice and diluted with 300 ml of ethyl acetate in a separating funnel. The ethyl acetate layer was sequentially washed with 3×35 ml of water, 35 ml of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Finally the crude product obtained was purified by silica gel column chromatography using ethyl acetate and petroleum ether as eluent to obtain final products **13a-g**.

5′-*O*-(2-bromoethyl)-2′,3′-*O*-methylethylidene uridine (13a): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.31 (d, 1H, *J*=8 Hz), 5.72 (d, 1H, *J*=8 Hz), 5.51 (d, 1H, *J*=4 Hz), 5.49 (m, 2H), 4.27 (m, 3H), 3.85 (t, 2H), 3.47 (t, 2H), 1.52 (s, 3H), 1.30 (s, 3H).

5′-*O*-(3-bromopropyl)-2′,3′-*O*-methylethylidene uridine (13b): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.48 (d, 1H, *J*=8 Hz), 5.68 (d, 1H, *J*=8 Hz), 5.63 (d, 1H, *J*=4 Hz), 4.78 (m, 2H), 4.27 (m, 1H), 3.97 (t, 2H), 3.79 (m, 2H), 3.35 (t, 2H), 2.21 (m, 2H), 1.55 (s, 3H), 1.33 (s, 3H).

5′-*O*-(4-bromobutyl)-2′,3′-*O*-methylethylidene uridine (13c): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.33 (d, 1H, *J*=8 Hz), 5.75 (d, 1H, *J*=8 Hz), 5.53 (d, 1H, *J*=4 Hz), 4.99 (m, 2H), 4.30 (m, 1H), 3.92 (m, 3H), 3.79 (m, 1H), 3.42 (t, 2H), 2.87 (m, 1H), 1.80 (m, 4H), 1.57 (s, 3H), 1.35 (s, 3H).

5′-*O*-(5-bromopentyl)-2′,3′-*O*-methylethylidene uridine (13d): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.39 (d, 1H, *J*=8 Hz), 5.72 (d, 1H, *J*=8 Hz), 5.55 (dd, 1H, *J*=4 Hz), 5.38 (d, 1H), 4.99 (m, 2H), 4.38 (m, 3H), 4.31 (m, 2H), 3.87 (m, 4H), 1.67 (m, 4H), 1.55 (s, 6H), 1.33 (s, 6H).

5′-*O*-(6-bromohexyl)-2′,3′-*O*-methylethylidene uridine (13e): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.38 (dd, 1H, *J*=8 Hz), 5.71 (dd, 1H, *J*=8 Hz), 5.58 (dd, 1H, *J*=4 Hz), 4.98 (m, 2H), 4.48 (m, 4H), 3.87 (m, 3H), 1.85 (m, 4H), 1.55 (s, 3H), 1.33 (s, 3H), 0.94 (m, 4H).

5′-*O*-(7-bromoheptyl)-2′,3′-*O*-methylethylidene uridine (13f): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.39 (dd, 1H, *J*=8 Hz), 5.75 (dd, 1H, *J*=8 Hz), 5.58 (dd, 1H, *J*=4 Hz), 5.22 (d, 2H), 4.31 (m, 4H), 3.89 (m, 3H), 1.84 (m, 4H), 1.58 (s, 3H), 1.36 (s, 3H), 1.34 (m, 6H).

5′-*O*-(10-bromodecyl)-2′,3′-*O*-methylethylidene uridine (13g): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.36 (dd, 1H, *J*=8 Hz), 5.70 (dd, 1H, *J*=8 Hz), 5.56 (dd, 1H, *J*=4 Hz), 5.00 (s, 2H), 4.67 (m, 3H), 3.80 (m, 4H), 2.49 (s, 4H), 1.84 (m, 2H), 1.56 (s, 3H), 1.35 (s, 3H), 1.24 (s, 10H).

3.5.3 General procedure for the synthesis of *O*-alkylazido-2′,3′-*O*-methylethylidene uridine 14a-g

To the solution of *O*-bromoalkyl-2′,3′-*O*-methylethylidene uridine (1 mmol) in acetone (15 ml) NaN_3 (1.5 mmol) was added. The reaction mixture was stirred at 65 °C for 6 h. After completion of the reaction (monitored by TLC) the reaction mixture was cooled and acetone was removed under reduced pressure. Then reaction mixture diluted with 250 ml of ethyl acetate. Organic layer was washed sequentially with 4×25 ml of water, 25 ml of brine. Organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to yield crude product. The purification of crude product was done by silica gel column chromatography using ethyl acetate-petroleum ether mixture as eluent to afford O-alkyl azide 2′,3′-*O*-methylethylidene uridine **14a-g**.

5′-*O*-(7-bromoheptyl)-2′,3′-*O*-methylethylidene uridine (13f): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.39 (dd, 1H, *J*=8 Hz), 5.75 (dd, 1H, *J*=8 Hz), 5.58 (dd, 1H, *J*=4 Hz), 5.22 (d, 2H), 4.31 (m, 4H), 3.89 (m, 3H), 1.84 (m, 4H), 1.58 (s, 3H), 1.36 (s, 3H), 1.34 (m, 6H).

5′-*O*-(10-bromodecyl)-2′,3′-*O*-methylethylidene uridine (13g): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.36 (dd, 1H, *J*=8 Hz), 5.70 (dd, 1H, *J*=8 Hz), 5.56 (dd, 1H, *J*=4 Hz), 5.00 (s, 2H), 4.67 (m, 3H), 3.80 (m, 4H), 2.49 (s, 4H), 1.84 (m, 2H), 1.56 (s, 3H), 1.35 (s, 3H), 1.24 (s, 10H).

5′-*O*-(2-azidoethyl)-2′,3′-*O*-methylethylidene uridine (14a): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.35 (d, 1H, *J*=8 Hz), 5.73 (d, 1H, *J*=8 Hz), 5.53 (d, 1H, *J*=4 Hz), 4.93 (m, 2H), 4.26 (m, 1H), 4.11 (t, 2H), 3.84 (m, 2H), 3.47 (t, 2H, *J*=6 Hz), 1.52 (s, 3H), 1.30 (s, 3H).

5′-*O*-(3-azidoproyl)-2′,3′-*O*-methylethylidene uridine (14b): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.58 (d, 1H, *J*=8 Hz), 5.73 (d, 1H, *J*=8 Hz), 5.68 (d, 1H, *J*=4 Hz), 4.94 (m, 2H), 4.00 (m, 3H), 3.84 (m, 2H), 3.33 (m, 2H), 1.89 (m, 2H), 1.55 (s, 3H), 1.33 (s, 3H).

5′-*O*-(4-azidobutyl)-2′,3′-methylethylidene uridine (14c): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.33 (d, 1H, *J*=8 Hz), 5.76 (d, 1H, *J*=8 Hz), 5.54 (d, 1H, *J*=4 Hz), 4.99 (m, 2H), 4.30 (m, 2H), 3.92 (m, 3H), 3.40 (t, 2H), 2.84 (m, 1H), 1.80 (m, 4H), 1.57 (s, 3H), 1.35 (s, 3H).

5′-*O*-(5-azidopentyl)-2′,3′-methylethylidene uridine (14d): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.36 (d, 1H, *J*=8 Hz), 5.71 (d, 1H, *J*=8 Hz), 5.55 (dd, 1H, *J*=4 Hz), 5.28 (d, 1H), 4.99 (m, 2H), 4.38 (m, 3H), 4.31 (m, 2H), 3.57 (m, 4H), 1.63 (m, 4H), 1.55 (s, 6H), 1.33 (s, 6H).

5′-*O*-(6-azidohexyl)-2′,3′-methylethylidene uridine (14e): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.34 (dd, 1H, *J*=8 Hz), 5.71 (dd, 1H, *J*=8 Hz), 5.56 (dd, 1H, *J*=4 Hz), 4.98 (m, 2H), 4.22 (m, 4H), 3.87 (m, 3H), 1.85 (m, 4H), 1.55 (s, 6H), 1.33 (s, 6H), 0.94 (m, 4H).

5′-*O*-(7-azidoheptyl)-2′,3′-methylethylidene uridine (14f): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.33 (dd, 1H, *J*=8 Hz), 5.75 (dd, 1H, *J*=8 Hz), 5.58 (dd, 1H, *J*=4 Hz), 5.21

(d, 2H), 4.34 (m, 4H), 3.89 (m, 3H), 1.84 (m, 4H), 1.56 (s, 3H), 1.33 (s, 3H), 1.34 (m, 6H).

5′-*O*-(10-azidodecyl)-2′,3′-methylethylidene uridine (14g): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.35 (dd, 1H, *J*=8 Hz), 5.68 (dd, 1H, *J*=8 Hz), 5.55 (dd, 1H, *J*=4 Hz), 5.13 (s, 2H), 4.67 (m, 3H), 3.73 (m, 4H), 2.49 (s, 4H), 1.81 (m, 2H), 1.56 (s, 3H), 1.35 (s, 3H), 1.24 (s, 10H).

3.5.4 General procedure for the synthesis of diazidoalkanes 16a and 16b

To the solution of specific dihaloalkane **12g** or **12h** (1 mmol) in DMF (10 ml), NaN_3 (1.2 mmol) was added. Reaction mixture was stirred at 28 °C for 10 h. On completion of reaction as checked by TLC, reaction mixture was diluted with 150 ml ethyl acetate. Further the ethyl acetate layer was washed sequentially with 4×25 ml of water, 25 ml of brine. The aqueous layer was again extracted with another 50 ml ethyl acetate. The organic layers were poooled together and dried over anhydrous Na_2SO_4 and concentrated under vacuo to get final diazides **16a** and **16b**.

1,10-Diazidodecane (16a): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 3.22 (t, 4H, *J* = 6, 8 Hz), 1.56 (m, 4H), 1.28 (s, 12H).

1,12-Diazidododeacane (16b): ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 3.24 (t, 4H), 1.56 (m, 4H), 1.26 (s, 16H).

3.5.5 Synthesis of 12-hydroxydodecyl tosyl (19)

To a solution of dodecanediol **18** (1gm, 4.9 mmol) in 30 ml of dry THF, NaH (0.129 gm, 5.38 mmol) was added at 0 °C under argon atmosphere. The reaction mixture was allowed to stir for 1 h at 28 °C. After stirring at 28 °C, the reaction mixture was again cooled to 0 °C and *p*-TsCl (1.02 gm, 5.38 mmol) was added in small portions under argon. Reaction mixture was stirred at 28 °C for 6 h. On completion of the reaction (monitored by TLC), reaction mixture was quenched with ice and THF was removed under vacuum. Reaction mixture was diluted with 300 ml of ethyl acetate. The ethyl acetate layer was washed subsequently with 3×35 ml of water, 35 ml of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced

pressure to get crude product. Finally the crude product was purified by silica gel column chromatography using ethyl acetate and petroleum ether as eluent to obtain 1.05 gm (60 %) of 12-hydroxydodecyl tosyl **19**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.77 (d, 2H, *J*=8 Hz), 7.33 (d, 2H, *J*=8 Hz), 3.99 (t, 2H, *J*=6 Hz), 3.62 (t, 2H, *J*=6 Hz), 2.43 (s, 3H), 1.51 (m, 6H), 1.20 (s, 12H).

3.5.6 Synthesis of 12-hydroxydodecyl azide (20)

To a solution of 12-hydroxydodecane tosyl **19** (1 gm, 2.8 mmol) in 16 ml of dioxane and DMSO mixture (14:2), NaN_3 (0.365 gm, 5.6 mmol) was added. The reaction mixture was stirred at 65 °C for 5 h. After completion of the reaction, (monitored by TLC) reaction mixture was cooled and dioxane was removed under reduced pressure. Then reaction mixture diluted with 250 ml of ethyl acetate and it was washed sequentially with 4×25 ml of water, 25 ml of brine. Organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to yield crude azido product. The crude azido product was purified by silica gel column chromatography using ethyl acetate-petroleum ether mixture as eluent to get 0.599 gm (94%) of 12-hydroxydodecyl azide **20**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 3.27 (t, 4H), 1.59 (m, 4H), 1.26 (s, 16H).

3.5.7 Synthesis of 1-azido-12-iodododecane (21)

To a solution of 12-hydroxy dodcyl azide **20** (1 gm, 4.4 mmol) in 20 ml mixture of DCM and water (2:1), iodine (1.67 gm, 6.6 mmol), PPh_3 (2.03 gm, 8.8 mmol) and imidazole (0.91 gm, 13.2 mmol) were added. The reaction mixture was stirred at 28 °C for 1 h. After completion of the reaction (monitored by TLC), DCM layer was separated and washed sequentially with 4×25 ml of water, 25 ml of brine. Organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to yield crude product. This crude product was purified by silica gel column chromatography using ethyl acetate-petroleum ether mixture as eluent to get 1.05 gm of final product **21** (71%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 3.24 (t, 4H), 1.56 (m, 4H), 1.26 (s, 16H).

3.5.8 General procedure for the synthesis of dimeric compounds 15a-g

To the stirred solution of propargyl ether of uridine **11** (1 mmol) and specific O-alkylazide uridine **14a-g** (1mmol) in 10 ml of *t*-butanol /water (8:2), $CuSO_4.5H_2O$

(24mg, 5 mole %) and sodium ascorbate (40 mg, 10 mol %) were added. Reaction mixture was stirred at 28 °C for 3 to 6 h. Completion of the reaction was monitored by TLC. After completion of reaction *t*-butanol was removed under reduced pressure. Then reaction mixture was extracted with 250 ml of ethyl acetate and washed sequentially with 4×25 ml of water, 25 ml of brine. Organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to yield crude product. The purification of crude product by silica gel column chromatography using ethyl acetate-petroleum ether mixture as eluent afforded final compounds **15a-g**.

Dimer 15a: Yield: 60%. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.61 (s, 1H), 7.35 (d, 2H, *J*=8 Hz), 5.72 (d, 2H, *J*=8 Hz), 5.51 (d, 2H, *J*=4 Hz), 5.45 (m, 4H), 4.27 (m, 6H), 3.85 (t, 2H), 3.47 (t, 2H), 1.52 (s, 3H), 1.30 (s, 3H); ^{13}C ($CDCl_3$+CH_3OH D4, 50 MHz): 162.70, 162.30, 152.35, 139.50, 130.33, 127.26, 123.60, 114.45, 100.48, 92.49, 86.54, 83.48, 80.61, 65.98, 60.20, 39.97, 36.98, 29.92, 28.63, 24.60 ppm. MS *m/z*: 712.27 $[M+Na]^+$; IR ($CHCl_3$) ν: 1718, 1693 cm^{-1}MS *m/z*: 698 $[M+Na]^+$; IR ($CHCl_3$) ν: 1720, 1692 cm^{-1}. Anal Calcd for $C_{29}H_{37}N_7O_{12}$: C, 51.55; H, 5.52; N, 14.51. Found: C, 52.00**;** H, 5.96; N, 14.82.

Dimer 15b: Yield: 55%. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.58 (s, 1H), 7.35 (d, 2H, *J*=8 Hz), 5.71 (d, 1H, *J*=8 Hz), 5.54 (s, 2H), 5.38 (d, 1H), 5.05 (m, 6H), 4.30 (m, 4H), 4.02 (m, 2H), 3.82 (m, 4H), 2.24 (m, 2H), 1.52 (s, 6H), 1.32 (s, 6H). ^{13}C ($CDCl_3$+CH_3OH D4, 50 MHz): 162.97, 162.49, 150.35, 139.99, 130.63, 128.26, 123.60, 113.48, 100.63, 93.40, 86.64, 84.48, 80.21, 65.16, 61.31, 37.97, 35.14, 29.92, 27.37, 26.33, 24.40 ppm. MS *m/z*: 712.27 $[M+Na]^+$; IR ($CHCl_3$) ν: 1718, 1693 cm^{-1}. Anal Calcd for $C_{30}H_{39}N_7O_{12}$: C, 52.25; H, 5.70; N, 14.22. Found: C, 52.32**;** H, 5.96; N, 14.02.

Dimer 15c: Yield: 58%. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.61 (s, 1H), 7.38 (d, 2H, *J*=8 Hz), 5.77 (d, 2H, *J*=8 Hz), 5.55 (dd, 2H, *J*=4 Hz), 5.38 (d, 1H), 5.11 (m, 4H), 4.91 (m, 2H), 4.38 (m, 6H), 3.87 (m, 4H), 1.67 (m, 4H), 1.56 (s, 6H), 1.37 (s, 6H); ^{13}C ($CDCl_3$, 50 MHz): 163.20, 162.62, 150.65, 138.30, 130.36, 127.96, 122.89, 113.48, 102.66, 93.40, 85.684, 82.84, 80.21, 66.61, 60.98, 37.33, 34.94, 27.32, 26.95, 26.33,

24.60, 18.65 ppm. MS *m/z*: 726.26 $[M+Na]^+$; IR ($CHCl_3$) ν: 1718, 1693 cm^{-1}. Anal Calcd for $C_{31}H_{41}N_7O_{12}$: C, 52.91; H, 5.87; N, 13.93. Found: C, 53.32**;** H, 5.86; N, 13.99.

Dimer 15d: Yield: 50%. ^1H NMR ($CDCl_3$, 200 MHz): δ = 7.61 (s, 1H), 7.38 (d, 2H, *J*=8 Hz), 5.77 (d, 2H, *J*=8 Hz), 5.55 (dd, 2H, *J*=4 Hz), 5.38 (d, 1H), 5.11 (m, 4H), 4.91 (m, 2H), 4.38 (m, 6H), 3.87 (m, 4H), 1.67 (m, 4H), 1.56 (s, 6H), 1.37 (s, 6H). ^{13}C ($CDCl_3$, 50 MHz): 162.63, 162.50, 152.65, 132.54, 130.06, 128.95, 127.23, 113.68, 100.29, 94.58, 94.02, 86.56, 83.65, 80.40, 65.65, 62.29, 50.23, 37.33, 31.06, 26.65, 25.14, 18.01, 14.98 ppm. MS *m/z*: (MH^+) 740.30 $[M+Na]^+$; IR ($CHCl_3$) ν: 1720, 1693 cm^{-1}. Anal Calcd for $C_{32}H_{43}N_7O_{12}$: C, 53.55; H, 6.04; N, 13.66. Found: C, 53.62**;** H, 6.36; N, 13.56.

Dimer 15e: Yield: 75%. ^1H NMR ($CDCl_3$, 200 MHz): δ = 7.59 (s, 1H), 7.38 (dd, 2H, *J*=8 Hz), 5.71 (dd, 2H, *J*=8 Hz), 5.58 (dd, 2H, *J*=4 Hz), 5.17 (m, 2H), 4.98 (m, 4H), 4.28 (m, 4H), 3.87 (m, 6H), 1.85 (m, 4H), 1.55 (s, 6H), 1.33 (s, 6H), 0.94 (m, 4H). ^{13}C ($CDCl_3$, 50 MHz): 167.49, 162.62, 150.65, 131.99, 130.70, 128.55, 128.43, 113.68, 101.29, 94.68, 94.34, 86.76, 84.44, 80.47, 65.29, 62.09, 50.00, 37.73, 30.26, 26.95, 25.02, 18.88, 13.44 ppm. MS *m/z*: 754.31 $[M+Na]^+$; IR ($CHCl_3$) ν: 1719, 1692 cm^{-1}. Anal Calcd for $C_{33}H_{45}N_7O_{12}$: C, 54.17; H, 6.20; N, 13.40. Found: C, 54.96**;** H, 6.28; N, 13.56.

Dimer 15f: Yield: 65%. ^1H NMR ($CDCl_3$, 200 MHz): δ = 7.60 (s, 1H), 7.39 (dd, 2H, *J*=8 Hz), 5.73 (dd, 2H, *J*=8 Hz), 5.59 (dd, 2H, *J*=4 Hz), 5.20 (d, 2H), 5.00 (m, 4H), 4.31 (m, 4H), 3.89 (m, 6H), 1.84 (m, 4H), 1.58 (s, 6H), 1.36 (s, 6H), 1.34 (m, 6H). ^{13}C ($CDCl_3$, 50 MHz): 162.67, 162.18, 150.79, 150.60, 142.44, 140.36, 123.56, 113.40, 101.50, 95.63, 95.11, 86.90, 84.32, 80.53, 62.35, 62.21, 50.20, 40.83, 35.99, 29.89, 28.30, 27.11, 26.35, 25.5 ppm. MS *m/z*: 768.33 $[M+Na]^+$; IR ($CHCl_3$) ν: 1718, 1693 cm^{-1}. Anal Calcd for $C_{34}H_{47}N_7O_{12}$: C, 54.76; H, 6.35; N, 13.15. Found: C, 54.94**;** H, 6.25; N, 13.62.

Dimer 15g: Yield: 55%. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.59 (s, 1H), 7.37 (dd, 2H, *J*=8 Hz), 5.72 (dd, 2H, *J*=8 Hz), 5.58 (dd, 2H, *J*=4 Hz), 5.19 (s, 2H), 4.99 (m, 4H), 4.27 (m, 4H), 3.85 (m, 6H), 2.49 (s, 4H), 1.84 (m, 2H), 1.56 (s, 6H), 1.35 (s, 6H), 1.24 (s, 10H). ^{13}C ($CDCl_3$, 50 MHz): 162.74, 162.34, 150.78, 150.73, 140.31, 139.97, 123.54, 113.92, 101.58, 95.30, 94.64, 86.77, 84.32, 80.46, 62.30, 50.31, 41.06, 35.56, 29.27, 29.05, 28.97, 28.68, 27.25, 27.08, 26.64, 26.25, 25.13 ppm. MS *m/z*: (MH^+) 810.38 $[M+Na]^+$; IR ($CHCl_3$) ν: 1718, 1696 cm^{-1}. Anal Calcd for $C_{37}H_{53}N_7O_{12}$: C, 56.41; H, 6.78; N, 12.44. Found: C, 56.62**;** H, 6.92; N, 12.65.

3.5.9 General procedure for the synthesis of symmetrical dimers 17a and 17b

For the synthesis of **17a** and **17b** two equivalents of propargyl ether of uridine **11** was used and the procedure followed was same as mentioned in 3.5.8.

Dimer 17a: Yield: 90%. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.59 (s, 2H), 7.50 (d, 2H, *J*=8 Hz), 5.68 (s, 2H), 5.64 (s, 2H), 5.17 (s, 4H), 4.94 (s, 4H), 4.27 (m, 6H), 3.85 (m, 4H), 1.83 (m, 4H), 1.55 (s, 6H), 1.33 (s, 6H), 1.24 (m, 12H). ^{13}C ($CDCl_3$, 50 MHz): 162.37, 150.77, 142.65, 140.33, 123.66, 113.92, 101.54, 94.58, 86.91, 84.69, 80.71, 62.26, 50.40, 35.75, 30.13, 29.08, 28.82, 27.23, 26.36, 25.28 ppm. MS *m/z*: 891.41 $[M+Na]^+$; IR ($CHCl_3$) ν: 1718, 1693 cm^{-1}. Anal Calcd for $C_{40}H_{56}N_{10}O_{12}$: C C, 55.29; H, 6.50; N, 16.12. Found: C, 54.99**;** H, 6.55; N, 16.13.

Dimer 17b: Yield: 64%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.60 (s, 2H), 7.45 (d, 2H, *J*=8 Hz), 5.72 (d, 2H, *J*=8 Hz), 5.66 (d, 2H), 5.19 (m, 4H), 4.99 (m, 4H), 4.29 (m, 6H), 3.88 (m, 4H), 1.86 (m, 4H), 1.58 (s, 6H), 1.35 (s, 6H), 1.26 (m, 16H). ^{13}C ($CDCl_3$, 200 MHz): 162.30, 150.77, 142.58, 140.58, 123.60, 114.08, 101.75, 95.50, 86.97, 84.37, 80.58, 62.41, 50.43, 35.77, 30.17, 29.28, 29.15, 28.29, 27.25, 26.40, 25.28 ppm. MS *m/z*: 919.44 $[M+Na]^+$; IR ($CHCl_3$) ν: 1798, 1693 cm^{-1}. Anal Calcd for $C_{42}H_{60}N_{10}O_{12}$: C, 56.24; H, 6.74; N, 15.62. Found: C, 56.23**;** H, 6.99; N, 15.80.

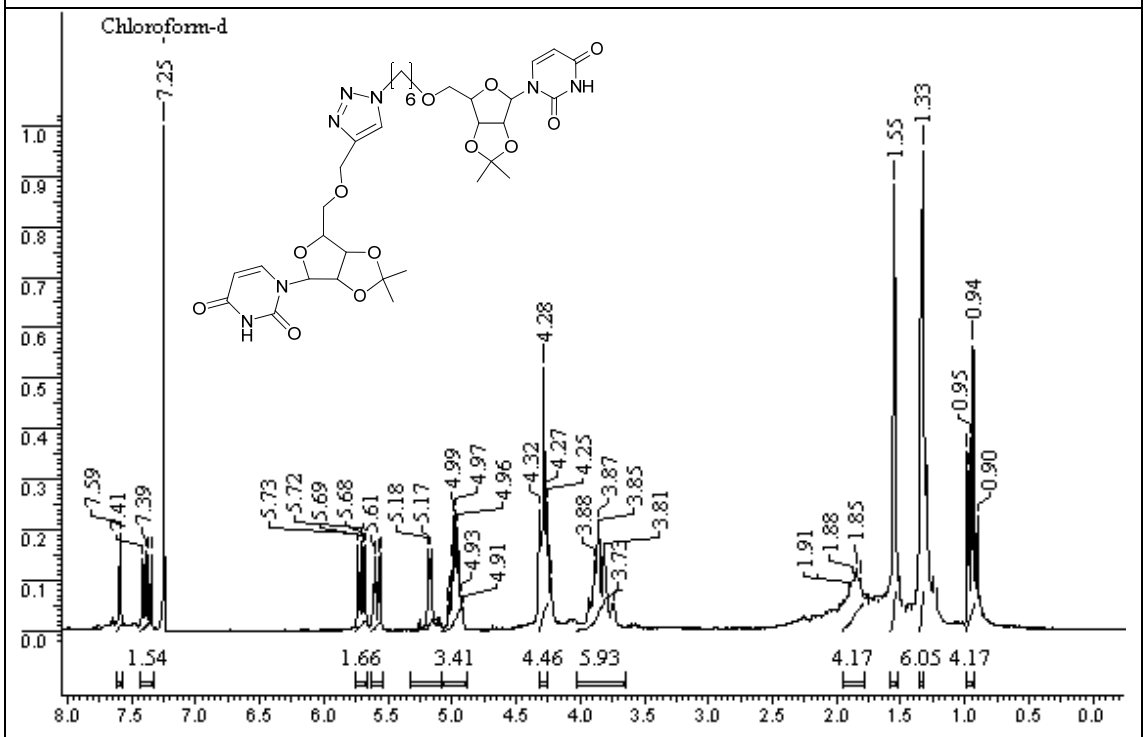
3.5.10 Synthesis of 5′-methoxy-[1-(12-iododododecyl)-1,2,3-triazole-4-yl]-2′,3′-*O*-(methylethylidene) uridine (22)

Procedure used for the synthesis of **22** was same as mentioned in section 3.5.8.

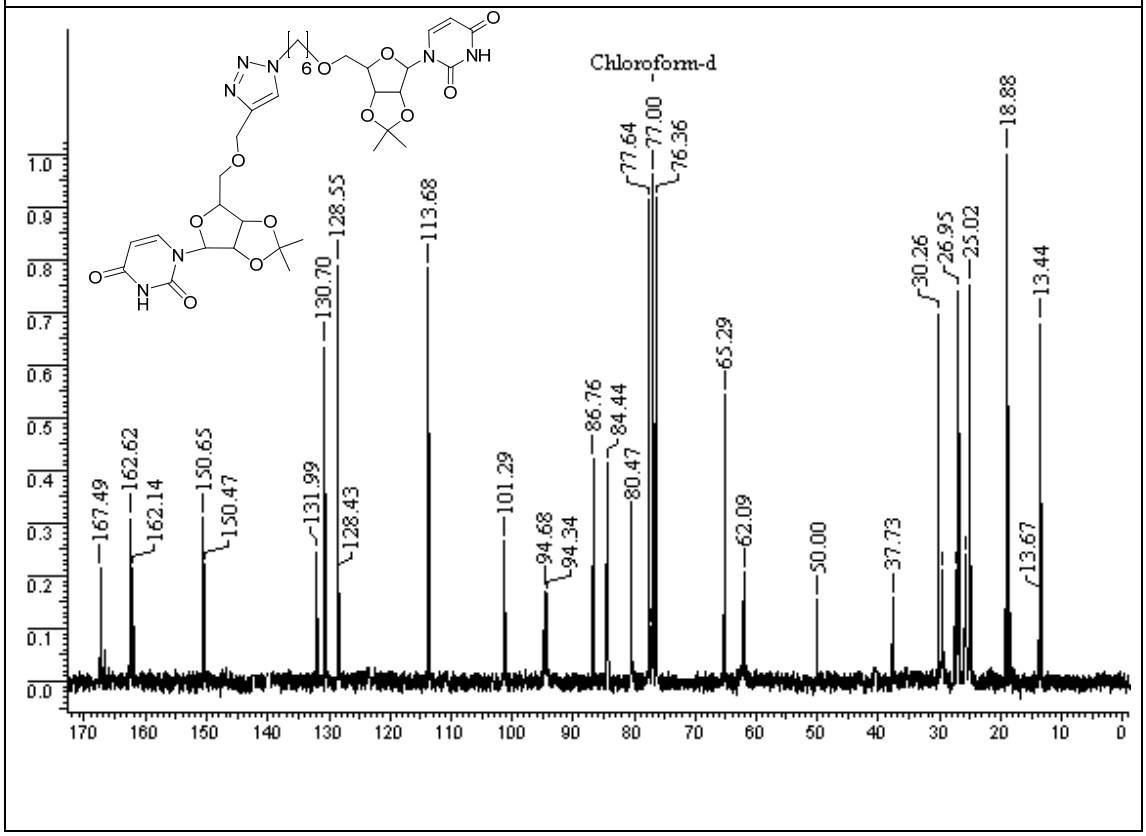
5′-methoxy-{1-(12-iododododecyl)-1,2,3-triazole-4-yl}-2′,3′-(methylethylidene) uridine (22): Yield: 70%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.57 (s, 1H), 7.38 (d, 1H, *J*=8 Hz), 5.71 (d, 1H, *J*=8 Hz), 5.59 (d, 1H), 5.19 (m, 2H), 4.99 (m, 2H), 4.27 (m, 3H), 3.89 (m, 2H), 3.17 (t, 2H), 1.78 (m, 6H), 1.56 (s, 3H), 1.34 (s, 3H), 1.24 (s, 14H). ^{13}C ($CDCl_3$, 50 MHz): 162.85, 151.19, 141.02, 138.80, 132.09, 101.63, 90.91, 85.66, 74.70, 70.14, 61.28, 54.74, 52.68, 29.54, 29.45, 29.07, 26.53, 26.41 ppm. MS *m/z*: 682.22 $[M+Na]^+$; IR ($CHCl_3$) ν: 1719, 1697 cm^{-1}. Anal Calcd for $C_{27}H_{42}IN_5O_6$: C, 49.17; H, 6.42; I, 19.24; N, 10.62. Found: C, 49.52**;** H, 6.25; I, 20.02 N, 10.95.

3.6 Selected spectra

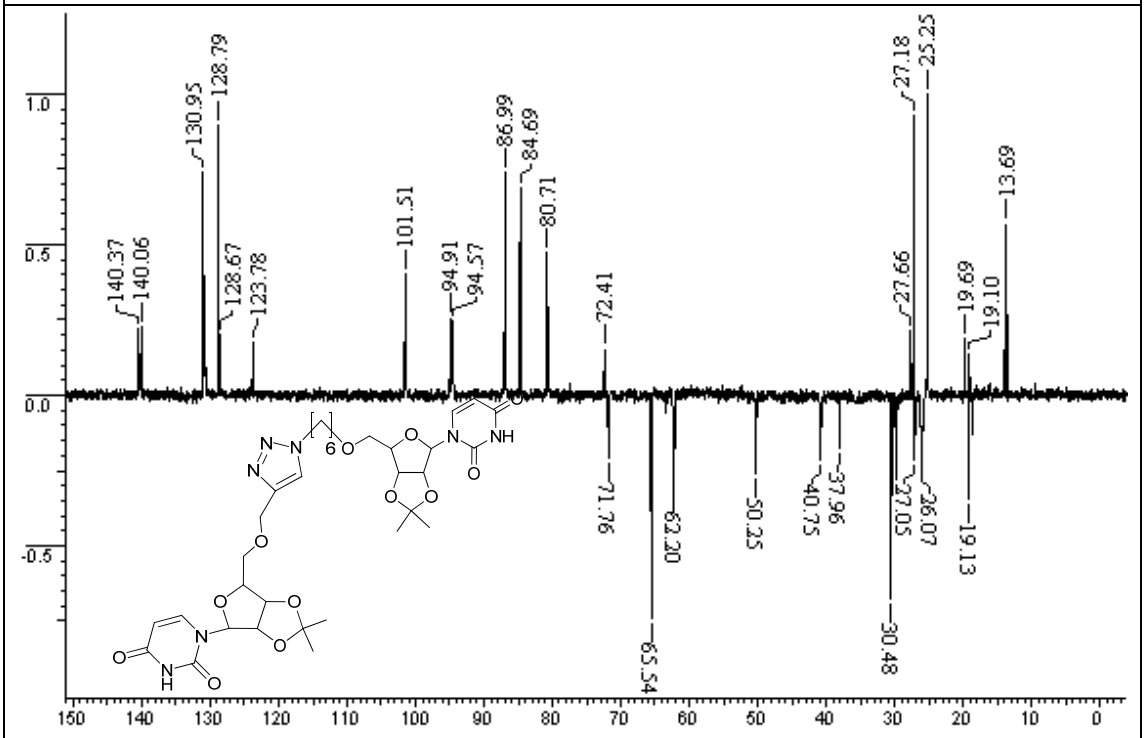
15e: ^{1}H NMR, 200 MHz, $CDCl_3$



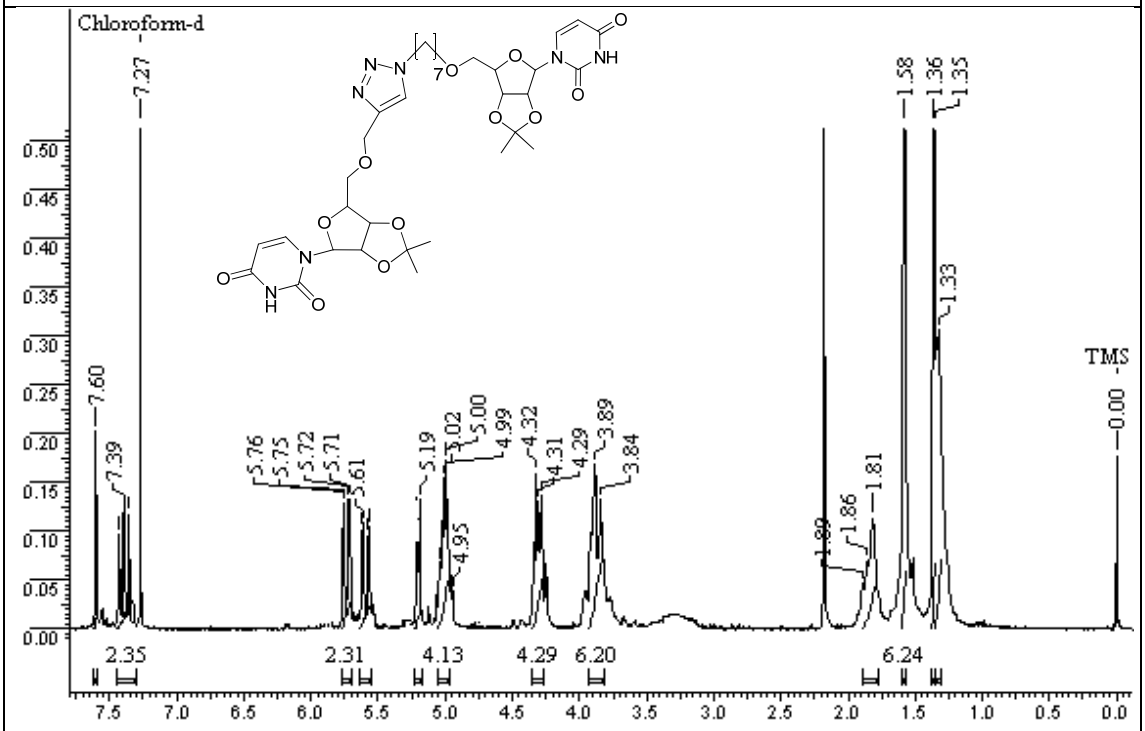
15e: ^{13}C NMR, 50 MHz, $CDCl_3$



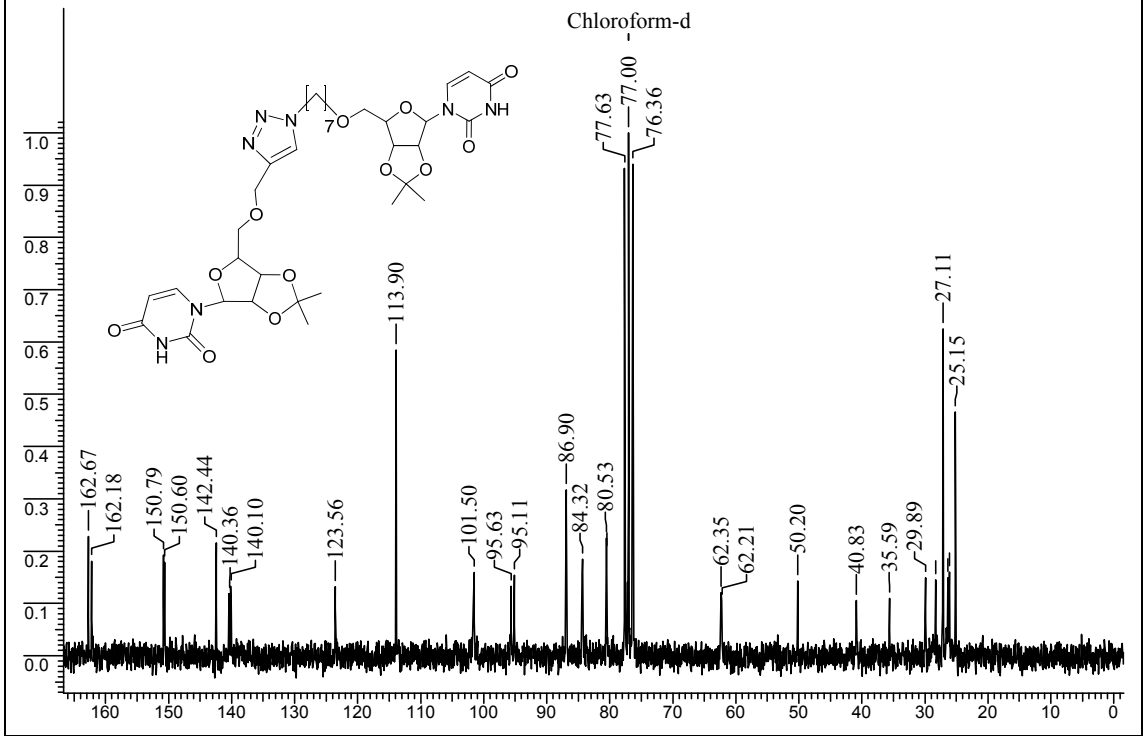
15e:DEPT, 50 MHz, $CDCl_3$



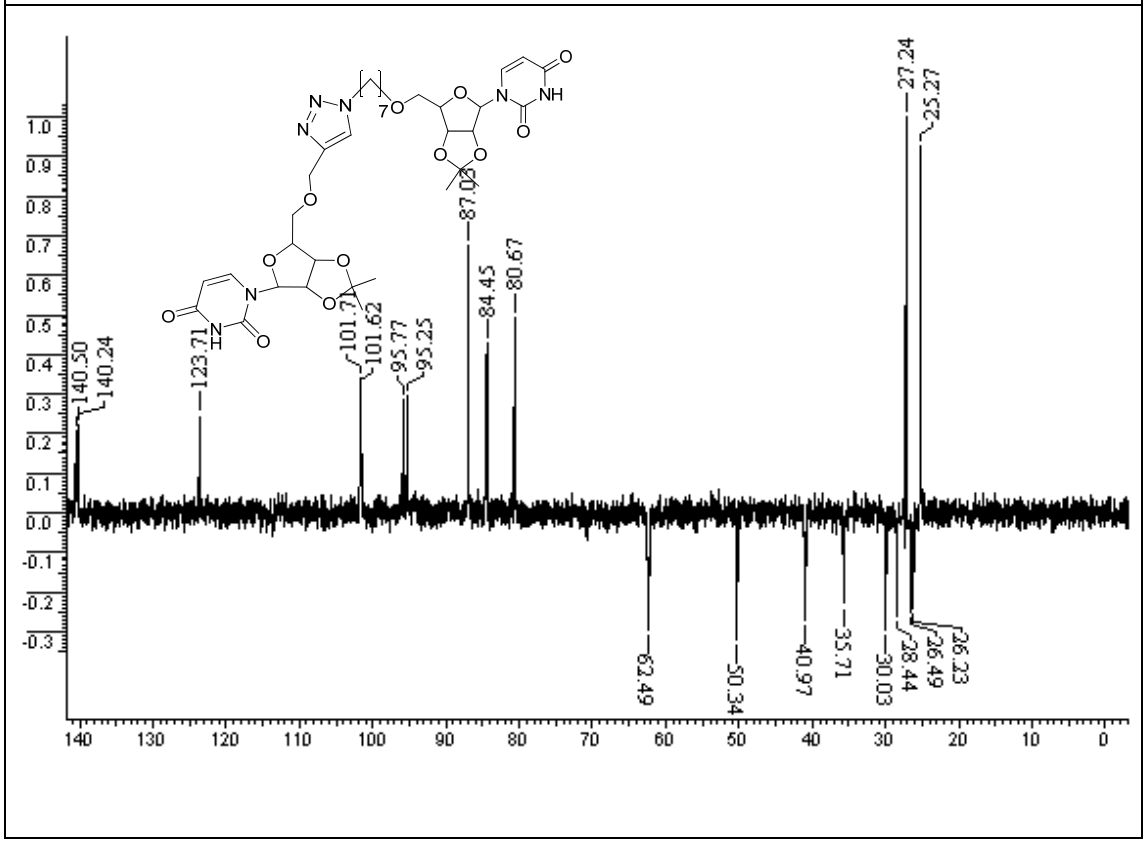
15f: ^1H NMR, 200 MHz, $CDCl_3$



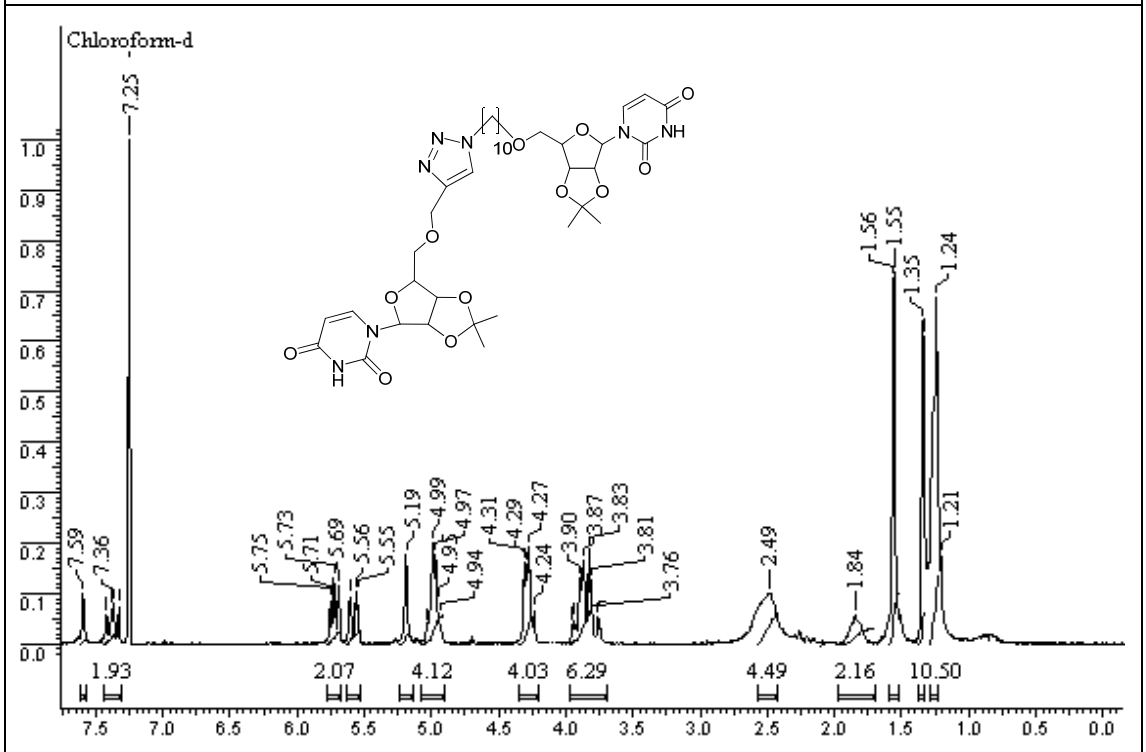
15f: ^{13}C NMR, 50 MHz, $CDCl_3$



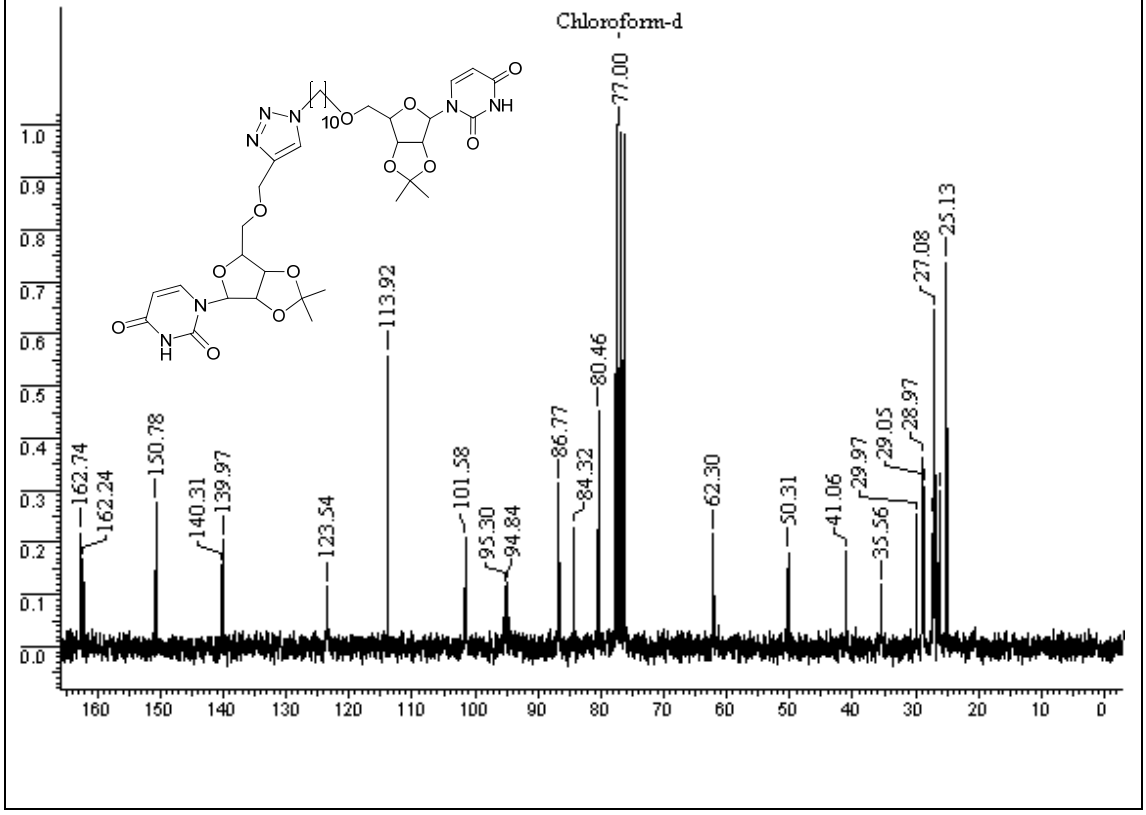
15f: DEPT, 50 MHz, $CDCl_3$



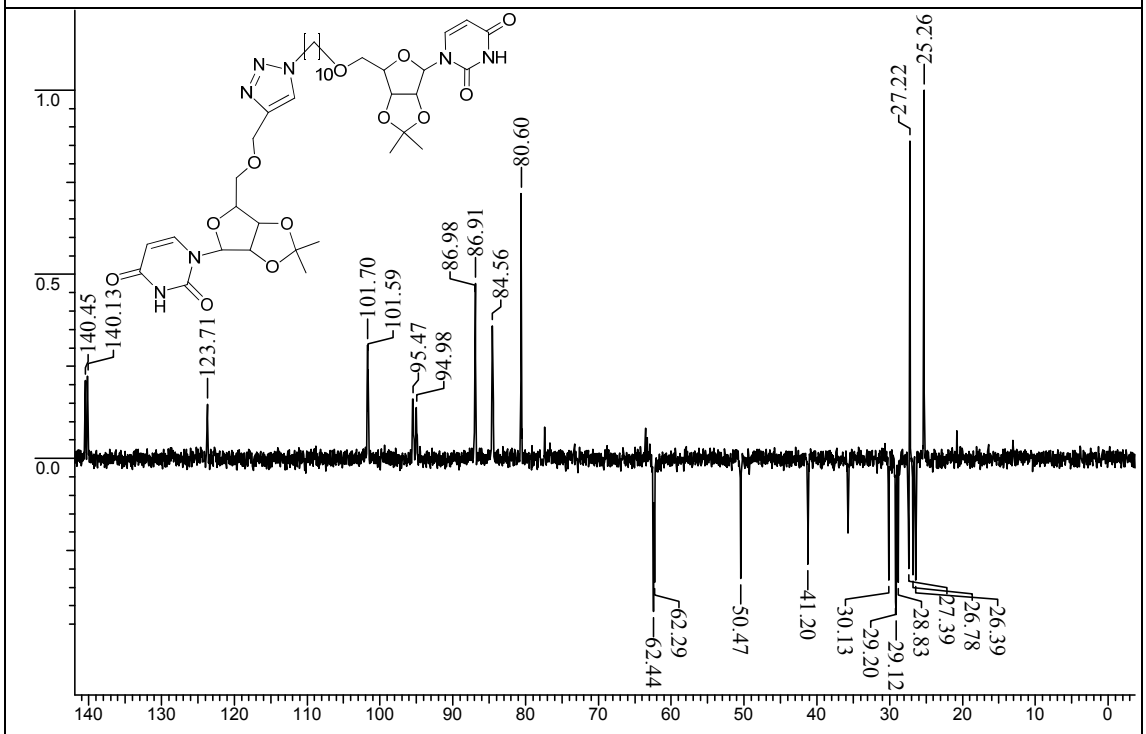
15g: ^{1}H NMR, 200 MHz, $CDCl_3$



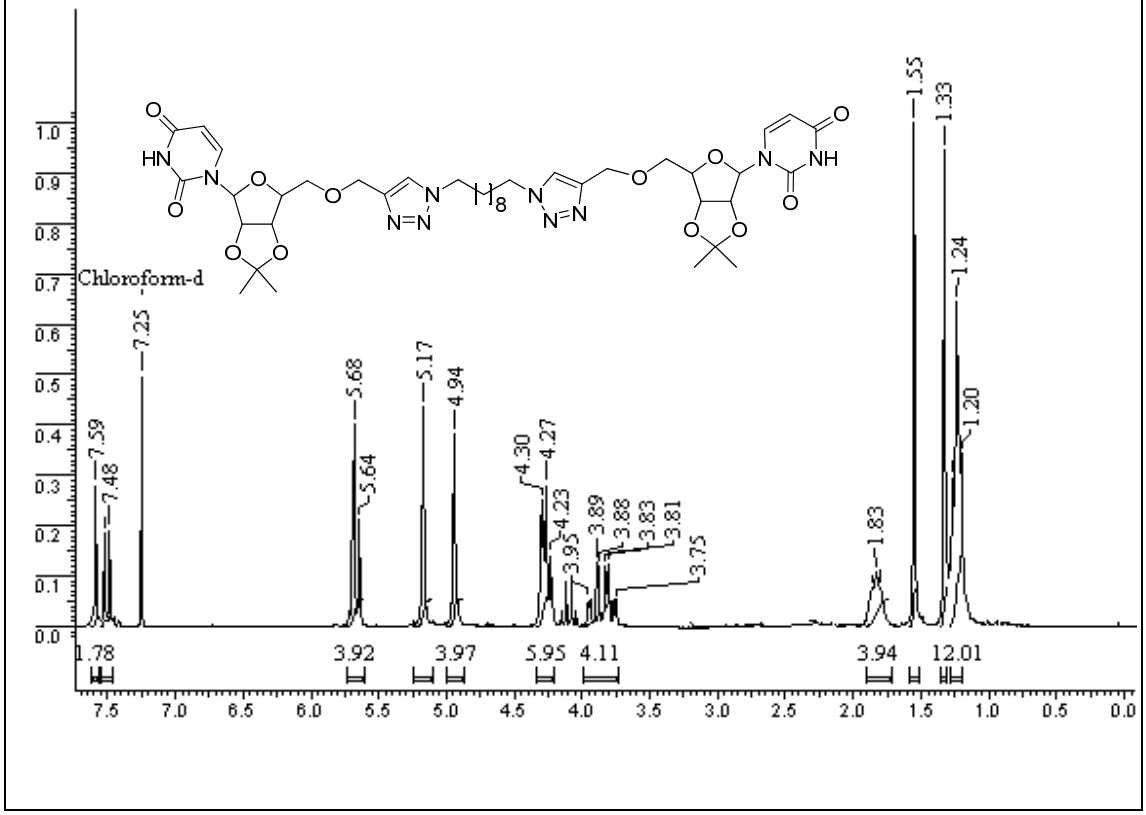
15g:^{13}C NMR, 50 MHz, $CDCl_3$



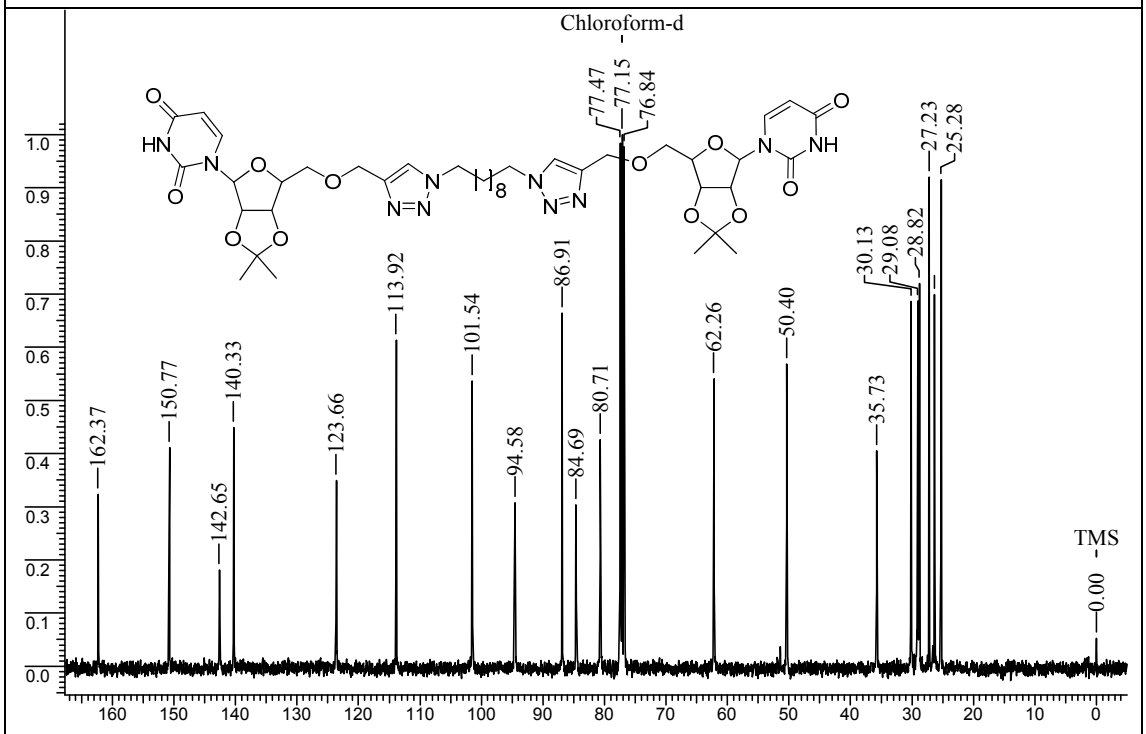
15g: DEPT, 50 MHz, $CDCl_3$



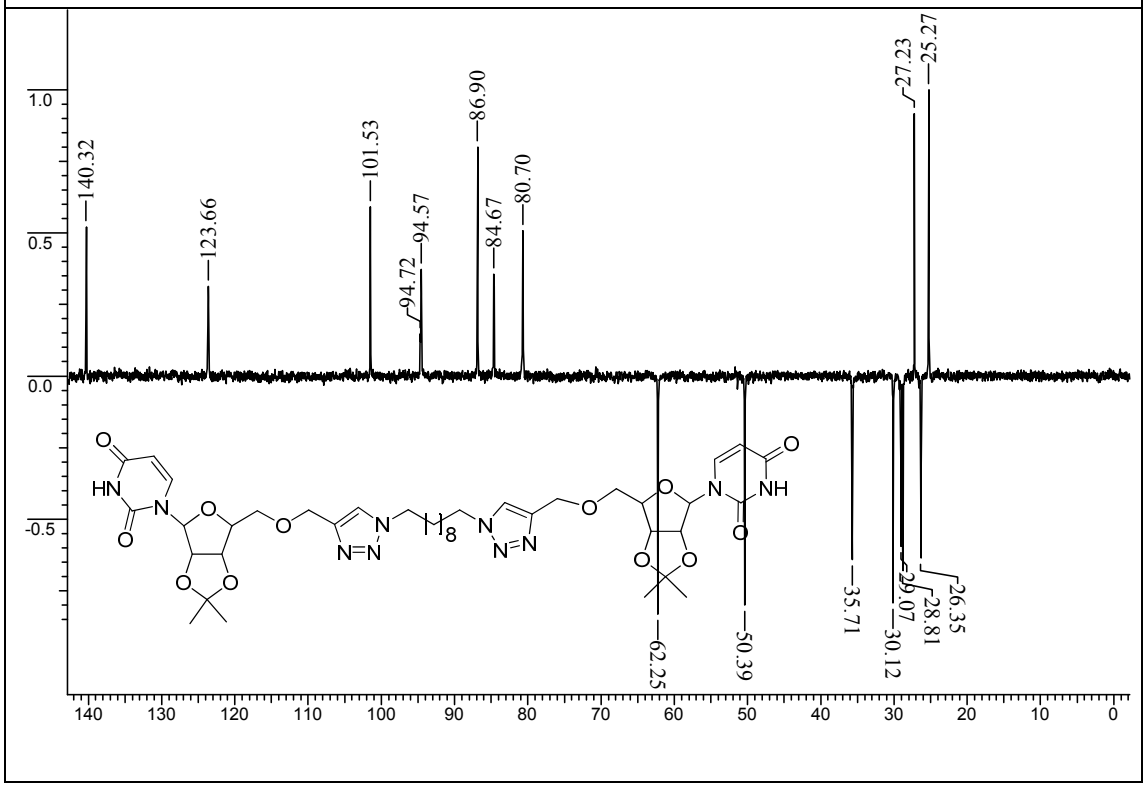
17a: ^{1}H NMR, 400 MHz, $CDCl_3$



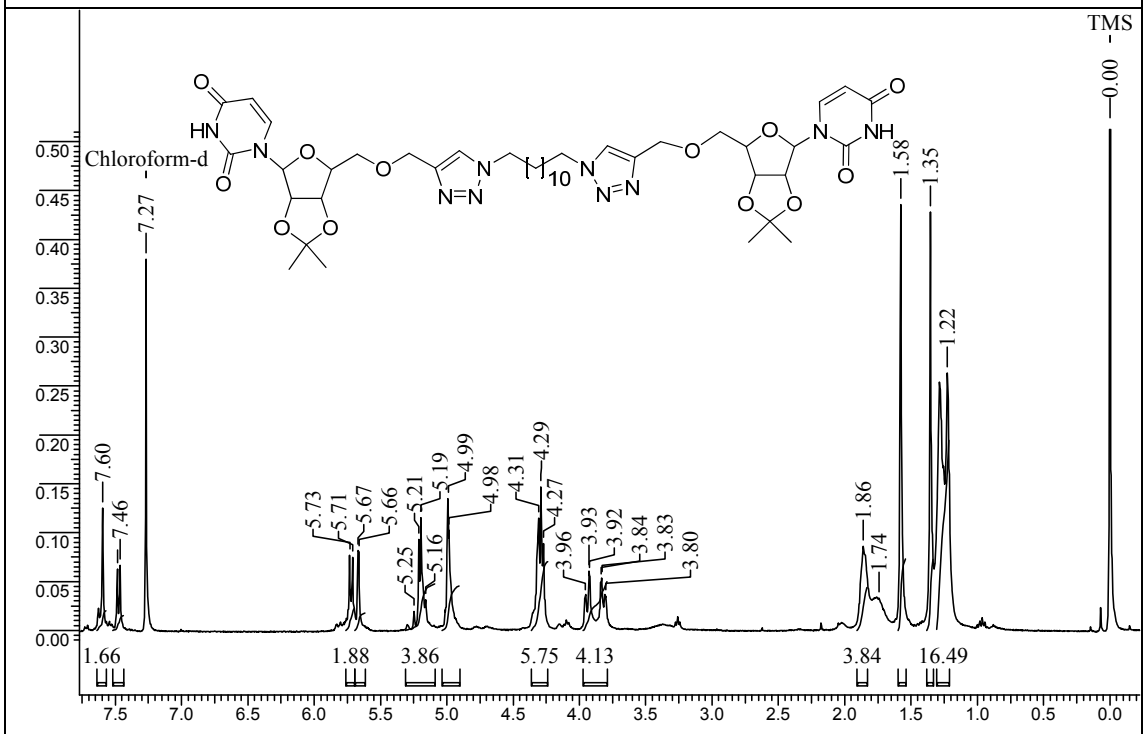
17a: ^{13}C NMR, 50 MHz, $CDCl_3$



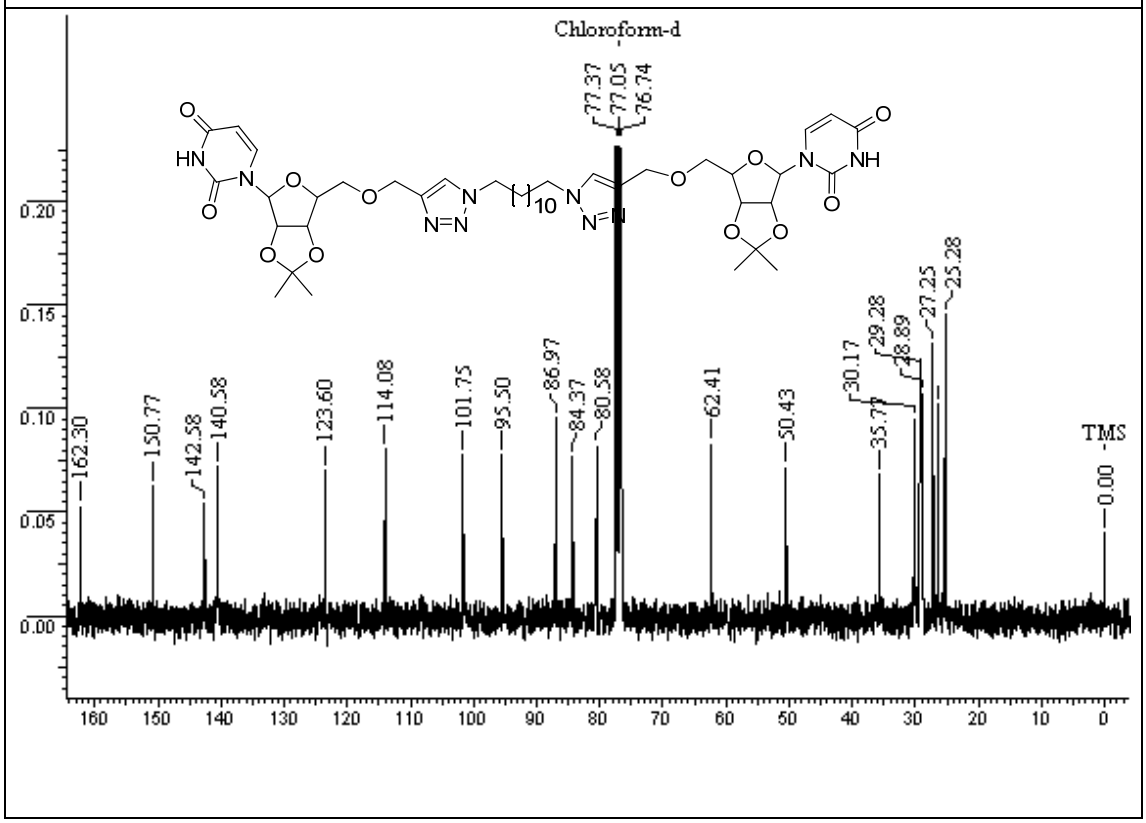
17a: DEPT, 50 MHz, $CDCl_3$



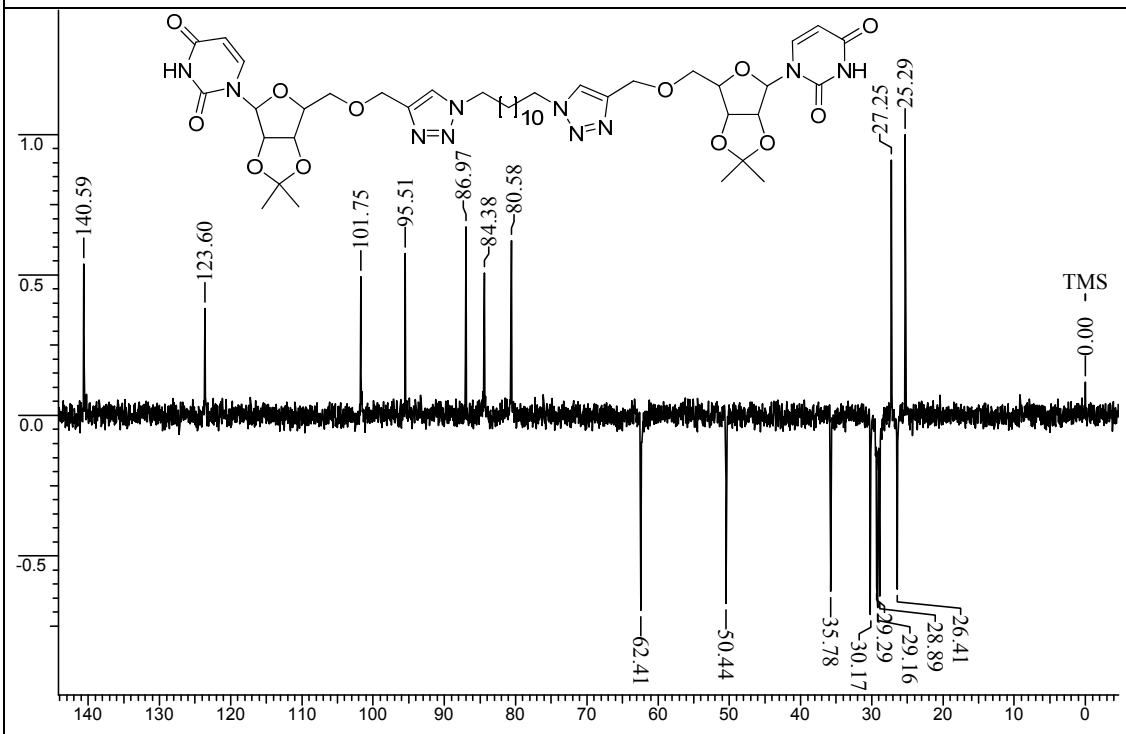
17b: ^{1}H NMR, 400 MHz, $CDCl_3$



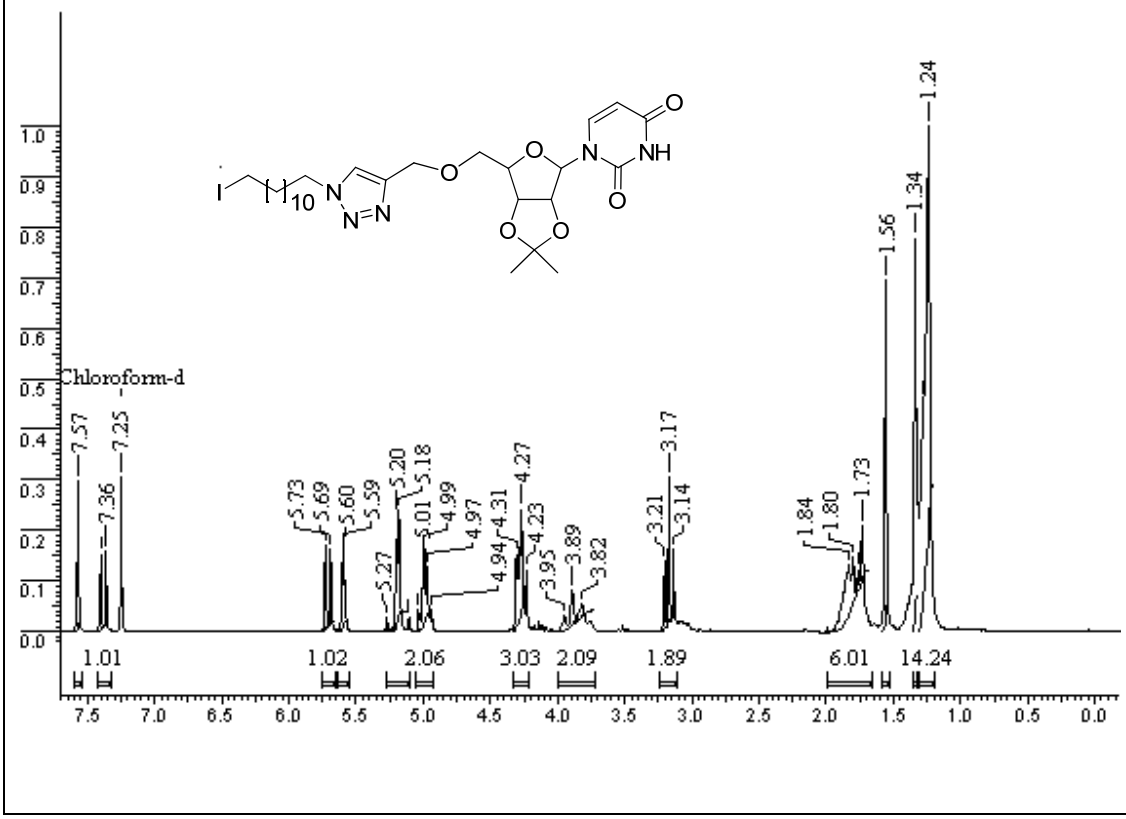
17b: ^{13}C NMR, 100 MHz, $CDCl_3$



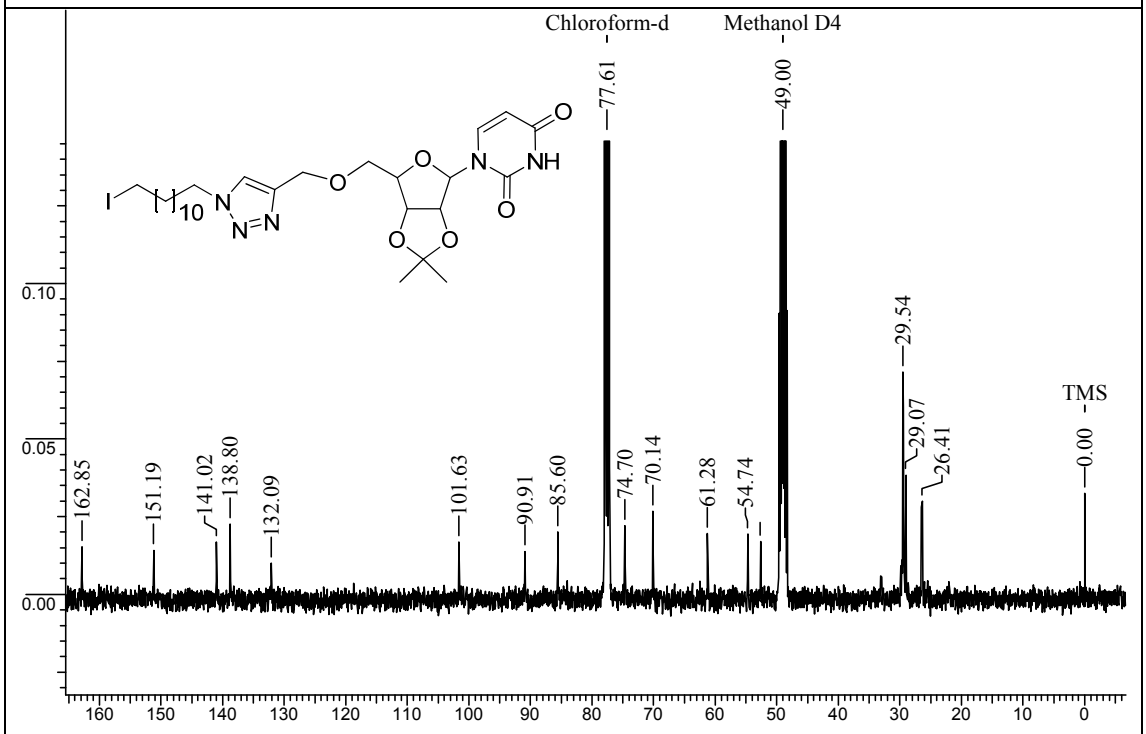
17b: DEPT, 100 MHz, $CDCl_3$



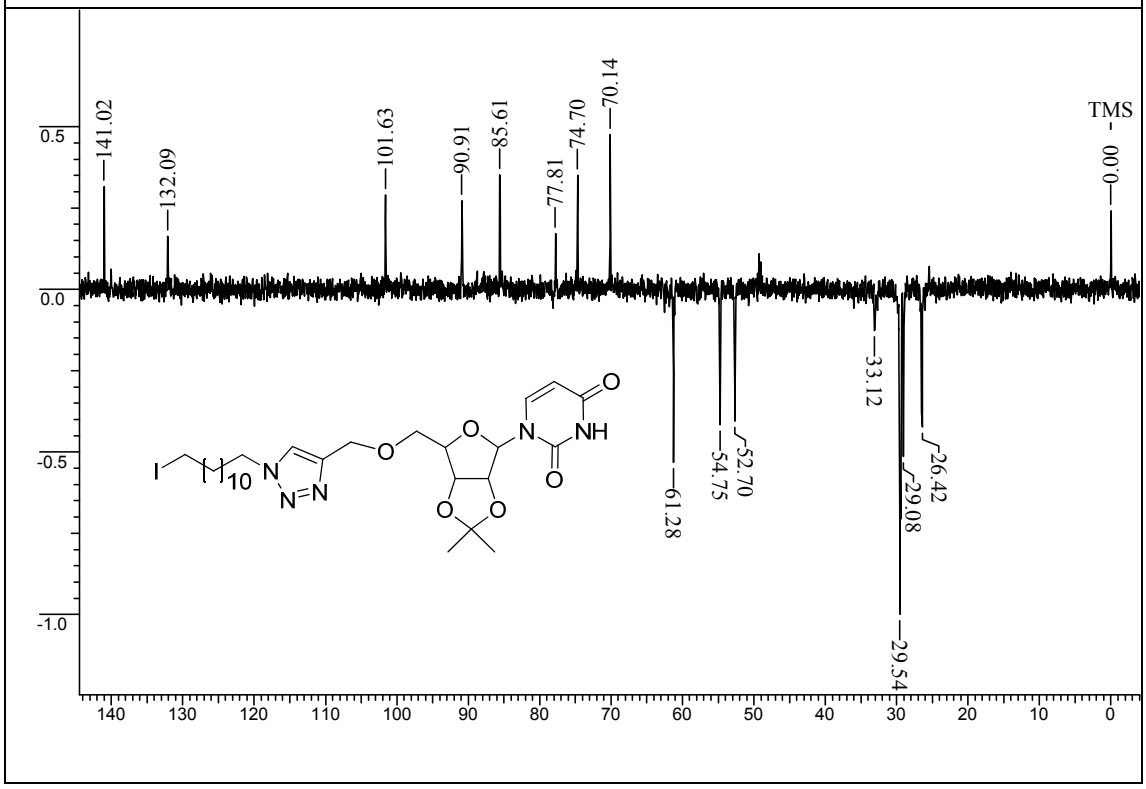
22: ^1H NMR, 200 MHz, $CDCl_3$



22: ^{13}C NMR, 50 MHz, MeOH D_4+$CDCl_3$



22: DEPT, 50 MHz, MeOH D_4+$CDCl_3$



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Chapter 4

SYNTHESIS OF ANTIFUNGAL 1,2,3-TRIAZOLE AND 1,2,4- TRIAZOLE THIOL SUBSTITUTED STROBILURIN DERIVATIVES

4.1 Introduction

Strobilurus tenacellus, a fungus that grows on decaying pine cones shows antifungal activity on solid agar and submerged liquid culture. In 1977, Anke *et al.* reported the isolation of two antibiotics named strobilurins A and B from the mycelium of *Strobilurus tenacellus* strain No. 21602 (Figure 4.1) [1]. After the discovery of strobilurin A and B, a number of different strobilurins such as strobilurin C, strobilurin E, strobilurin H, 9-methoxystrobilurins A and K, strobilurin D and hydroxystrobilurin A were isolated (Figure 4.1) [2-5]. The compounds were found to

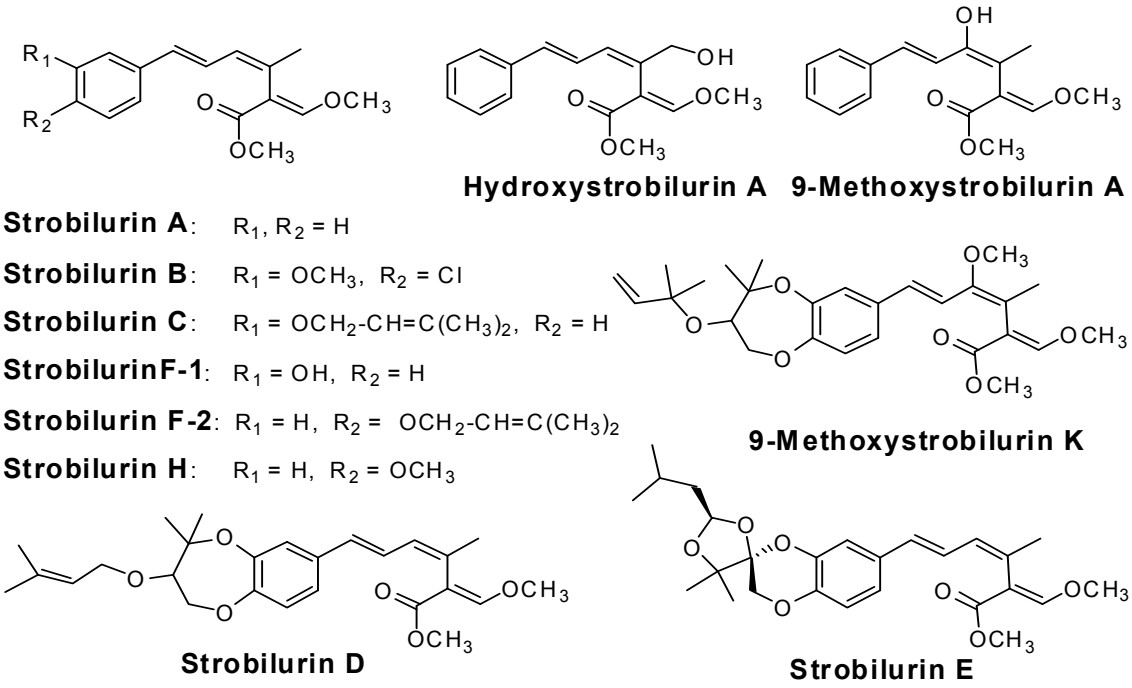


Figure 4.1 Naturally occuring strobilurin derivatives

inhibit many yeasts and filamentous fungi including phytopathogens *Botrytis cinerea*, *Rhizoctonia solani, Phytophthora infestans, Cladosporium cladosporioides, Curvularia lunata* etc. The strobilurins were further developed as major class of agricultural fungicides, with β-methoxyacrylic acid as main structural component [6]. Strobilurins shows the fungicidal mode of action, by binding to the Q_o site of cytochrome b which results in inhibition of mitochondrial respiration [7]. Cytochrome b is situated in the inner mitochondrial membrane of fungi and is part of cytochrome bc_1 complex. When strobilurins bind to cytochrome bc_1 complex it changes the conformation of the complex leading to disruption of electron transfer between

cytochrome b and cytochrome c_1, this stops the production of ATP and disturbs the energy cycle within the fungus.

Pfefferle *et al.* reported that culture filtrate of *Oudemansiella radicata* and *Psathyrella* sp. inhibited CS [8]. From these cultural filtrates oudemansins and strobilurins were already isolated by same group. They tested these isolated strobilurin A and strobilurin B for their CS inhibitory activity and it was found that strobilurin A and B (Figure 4.1) inhibited CS activity of *Coprinus cinereus* by 24 and 30 % of at 0.25 mM concentration. Three synthetic strobilurin derivatives **2-4** (Figure 4.2) containing chlorine atom along with a non chlorinated derivative **1** (Figure 4.2) were tested for CS inhibition in order to check the effect of chlorine substituent. Strobilurin derivative **4** with dichloro substituent showed maximum inhibition (49% at 0.25 mM) of CS activity as compared to other derivatives, whereas derivative **1** showed only 12% CS inhibition.

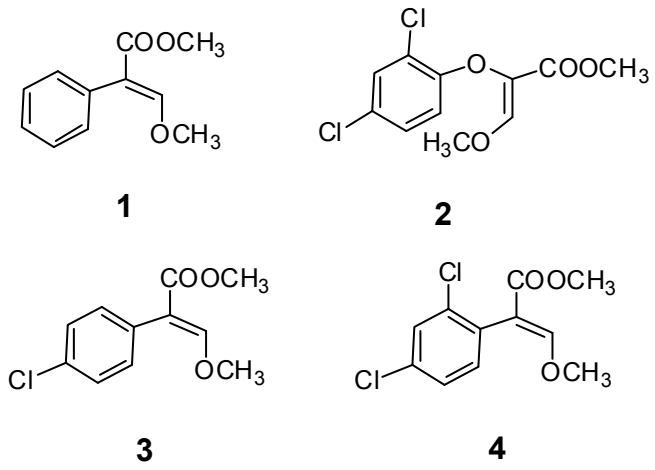


Figure 4.2 Chlorinated derivatives of strobilurin

Though strobilurin A was found to be effective against the fungi growing on agar, it failed to control the phytopathogens growing on plants under greenhouse conditions. The reasons for the failure of strobilurin A was its volatility and photochemical instability [9]. On the basis of the knowledge of their structure and physical properties, research programme for synthetic modifications of strobilurins was undertaken by Syngenta and BASF. In 1996, first synthetic strobilurin azoxystrobin was launched in the market by Syngenta [6, 10]. Currently different synthetic strobilurins are available in the market, out of which few are depicted in Figure 4.3. Even though these strobilurins were used successfully in the fields for the

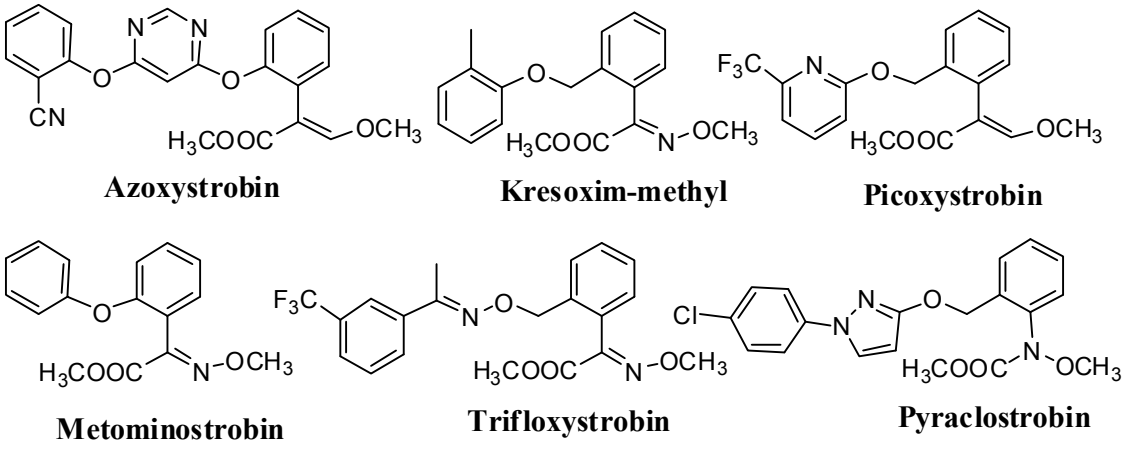
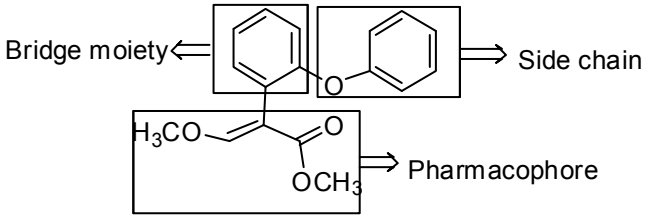


Figure 4.3 Commercially available strobilurin fungicides

control of fungal diseases, significant resistance has been developed by some important phytopathogenic fungi against them [11, 12]. Therefore, research efforts are directed towards the synthesis of new effective strobilurin analogues.



5

Figure 4.4 General structure of strobilurin

General structure of strobilurin fungicides **5** (Figure 4.4) consists of mainly three parts - side chain, aromatic bridge and pharmacophore (*E*)-methyl-β-methoxyacrylate group or (*E*)-methylmethoxyiminoacetate group, which are present in all active strobilurins.

Li *et al.* reported the synthesis of strobilurins containing phenyl substituted pyrazole ring with methyl and dimethyl modifications [13]. Most of the compounds showed better fungicidal activity against phytopathogens *Pyricularia oryzae, Botrytis cinerea, Pseudoperonospora cubensis* and *Erysiphe graminis*. Out of all synthesized compounds, derivative **6** (Figure 4.5) was found to be most effective even at lowest concentration tested (3.2 μg/ml). Strobilurins with different heterocyclic side chains (**7**, Figure 4.5) were synthesized by Huang *et al.* [14] using microwave assisted synthetic procedures. These compounds inhibited 50-91 % growth of *F. oxysporum, R. solani, B. cinerea, Gibberella zeae, Colletotrichum gossypii* at 50 μg/ml

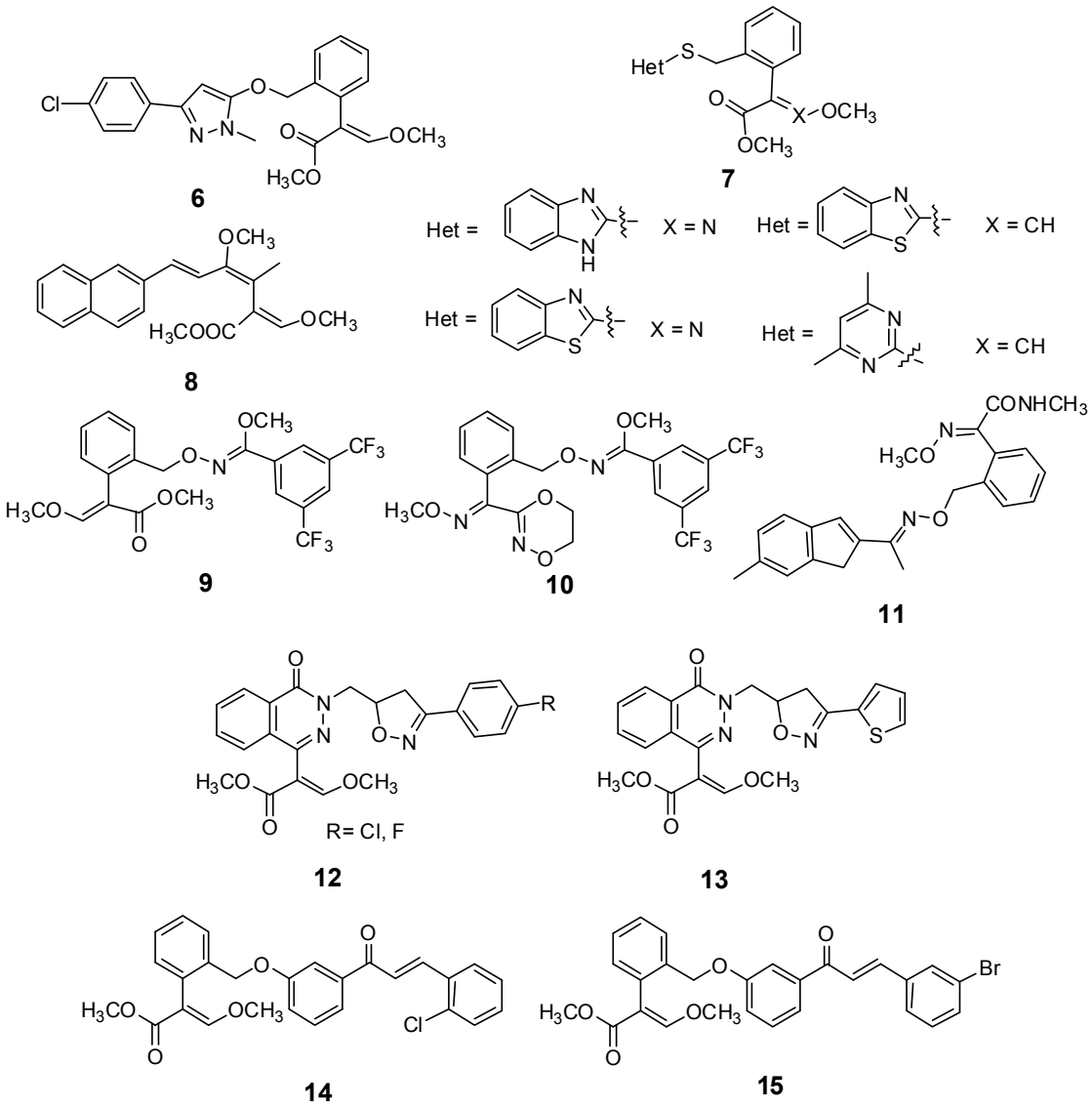


Figure 4.5 Synthetic strobilurin derivatives

concentration. Uchiro *et al.* reported the synthesis of nine derivatives of β-methoxyacrylate antibiotics with different aryl structures and compound **8** (Figure 4.5) was found to be potent inhibitor against broad range of fungi [15]. Liu *et al.* synthesized novel bis(trifluoromethyl)phenyl-based strobilurin analogues by the reaction of 1-bis(trifluoromethyl)phenyl based methanone oxime with corresponding strobilurin pharmacophores in the basic condition [16]. Total twenty two strobilurin derivatives were synthesized, out of which compounds **9** and **10** (Figure 4.5) showed >95% growth inhibition of *E. graminis* at 1.5 μg/ml concentration. Nineteen indene-substituted oxime ether strobilurins were reported by Tu *et al.* [17]. Compound **11** (Figure 4.5) completely inhibited the growth of *P. oryzae*, *P. infestans*, *B. cinerea* at 2500 μg/ml. New 3-isoxazoline substituted strobilurin derivatives were synthesized

from phthalic anhydride with the view to get an advantage of biological activity of isoxazoline pharmacophore [18]. Out of all synthesized compounds the derivatives **12** and **13** (Figure 4.5) showed good antifungal activity against *C. albicans* and *A. niger* with MIC in the range of 31-62 µg/ml. Chalacone based strobilurin derivatives were synthesized by Zhao *et al.* [19]. Compound **14** and **15** (Figure 4.5) exhibited IC_{50} 118.52 µg/ml and 113.64 µg/ml against *P. cubensis*.

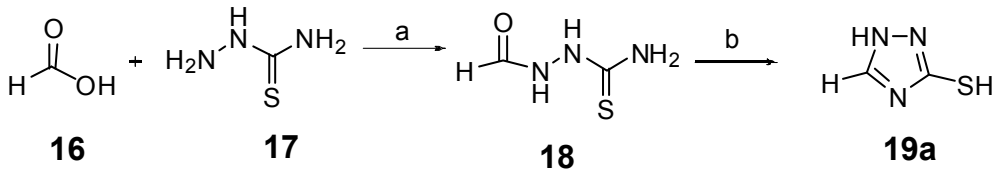
The detailed literature survey of different strobilurin derivatives showed that synthesis and biological evaluation of 1,2,3 and 1,2,4-triazole strobilurin derivatives have not been explored rigorously. Since some chlorinated strobilurins were able to inhibit CS activity, we have designed and synthesized 1,2,3-triazole and 1,2,4-triazole thiol strobilurin derivatives. 1,2,4-Triazoles are known antifungal agents. Number of marketed antifungal agents such as fluconazole, voriconazole, posaconazole etc. consists of 1,2,4-triazole as a main pharmancophore [20-22]. Though 1,2,3-triazoles are not present in the nature, it is a potential pharmacophore owing to its moderate dipole character and rigidity and can be readily incorporated into a design strategy [23, 24].

4.2 Results and discussion

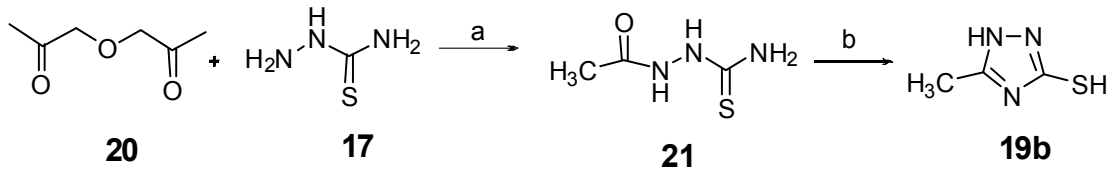
4.2.1 Synthesis of 5-substituted-1,2,4-triazole-3-thiol

Synthesis of 5-substituted-1,2,4-triazole-3-thiol was carried out according to reported procedure [25, 26]. Reaction of formic acid **16** with thiosemicarbazide **17** gave compound **18,** which on further cyclization in presence of alkali afforded 1-H-1,2,4-triazole-3-thiol **19a** (Scheme 1).

Reaction of acetic anhydride **20** with Thiosemicarbazide **17** gave acylthiosemicarbizide **21**, which on cylcization gave 5-methyl-1H-1,2,4-triazole-3-thiol **19b** (Scheme 2).

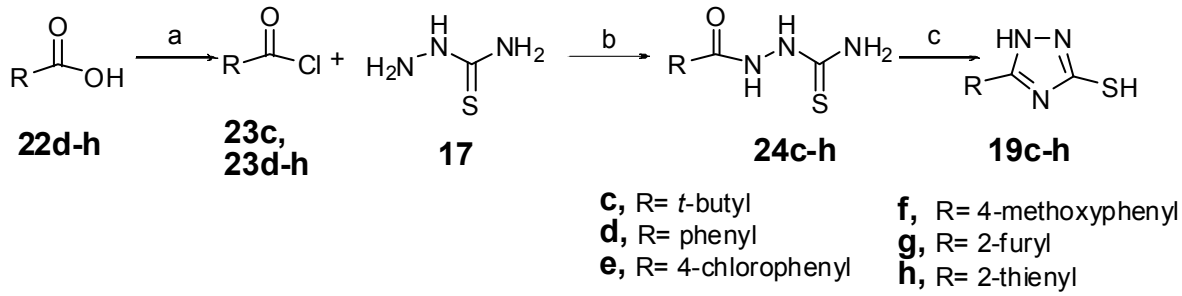


Scheme 1. Reagents and conditions: a) Water, 80 °C, 1 h; b) 2 N NaOH, 80 °C, 3 h.



Scheme 2. Reagents and conditions: a) Water, 80 °C, 1 h; b) 2 N NaOH, 80 °C, 3 h.

The acid chlorides of benzoic acid, *p*-chlorobenzoic acid, *p*-methoxy benzoic acid, furan-2-carboxylic acid and thiophene-2-carboxylic acid (**23d-h**) were obtained by reaction of respective acids (**22d-h**) with thionyl chloride in dichloromethane. On the other hand the pivaloyl chloride **23c** (purchased from SRL Pvt. Ltd., India) was used directly for the synthesis of **19c** (Scheme 3).



Scheme 3. Reagents and conditions: a) $SOCl_2$, DCM, 3 drops of pyridine, 50 °C, 3-6 h ; b) Pyridine, 28 °C, 10-12 h; c) 2 N NaOH, 80 °C, 3 h

Thiosemicarbazide **17** was acylated with acid chlorides (**23c-h**) to get *N*-acetyl thiosemicarbazide derivatives **24c-h.** These *N*-acetyl thiosemicarbazide derivatives **24c-h** on cyclization in presence of NaOH gave 5-substituted-1,2,4-triazole thiols **19c-h** (scheme 3). Compounds **19a-h** were characterized by ^{1}H NMR and ^{13}C NMR. ^{1}H NMR spectrum of compound 1-H-1,2,4-triazole-3-thiol **19a** showed resonance at δ 13.63 and 13.10 as singlet for NH and SH protons, along with this triazoles C-H proton showed singlet at δ 8.30. In ^{13}C NMR spectrum two triazoles carbons were seen at 166.98 and 141.52 ppm.

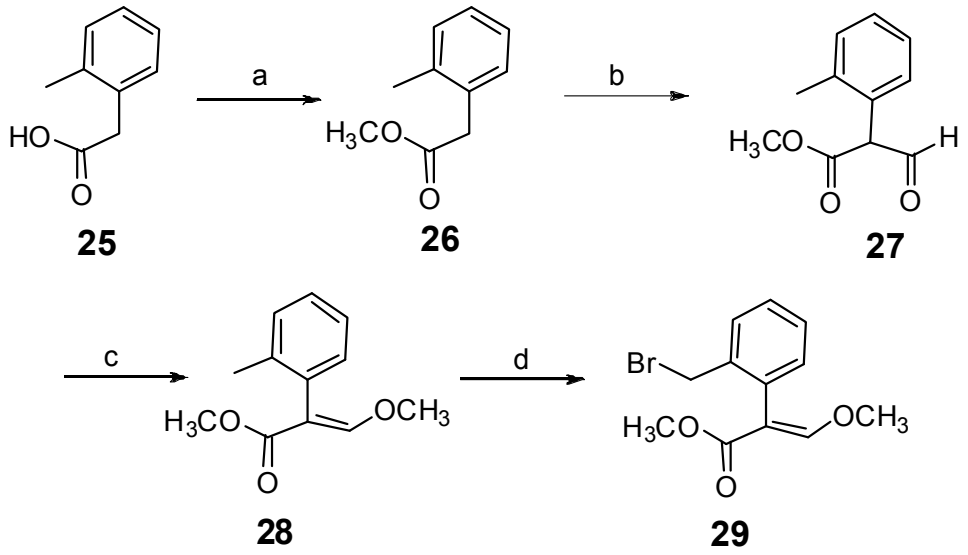
4.2.2 Synthesis of (E)-methyl-2-(2-(bromomethyl)phenyl)-3-methoxyacrylate

The synthesis of (E)-methyl-2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29** was carried out using reported procedure [27]. *o*-Tolylacetic acid **25** on stirring in dry methanol using catalytic amount of H_2SO_4 gave corresponding ester **26**. Formylation of methyl o-tolylacetate **26** was carried out using methyl formate in presence of NaH leading to formation of compound **27.** Compound **27** was

characterized by IR, ^{1}H NMR, ^{13}C NMR and Mass Spectrometry. The IR spectrum of this compound showed characteristic stretching frequencies at 1724, 1658, 2981 cm^{-1} etc. The ^{1}H NMR spectrum showed resonance at δ 11.83 for formyl proton, along with this resonance at δ 3.64, 2.21 as a singlet was seen for ester methyl and aromatic methyl protons.

Compound **27** was further reacted with dimethyl sulphate in presence of tetrabutyl ammonium bromide (TBAB) to get (E)-methyl-2-(methyl phenyl)-3-methoxyacrylate **28.** Using phase transfer catalyst TBAB the yield of (E)-methyl-2-(methyl phenyl)-3-methoxyacrylate **28** was improved (98%) and specifically E isomer was obtained. Compound **28** showed characteristic stretching frequencies at 1706, 1631 and 2947 cm^{-1} in IR spectrum. The ^{1}H NMR spectrum of (E)-methyl-2-(methyl phenyl)-3-methoxyacrylate **28** showed resonance at δ 7.56 as a singlet for vinylic proton and resonance at δ 3.80 and 3.69 as a singlet for methoxy protons.

Bromination of (E)-methyl-2-(methyl phenyl)-3-methoxyacrylate **28** was carried out using NBS in CCl_4 and AIBN as a initiator to give (E)-methyl-2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29** (scheme 4). Compound (E)-methyl 2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29** was characterized using IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy. Characteristic stretching frequency was observed at 1735, 1631, 765 cm^{-1} in IR spectrum of compound **29**. The ^{1}H NMR



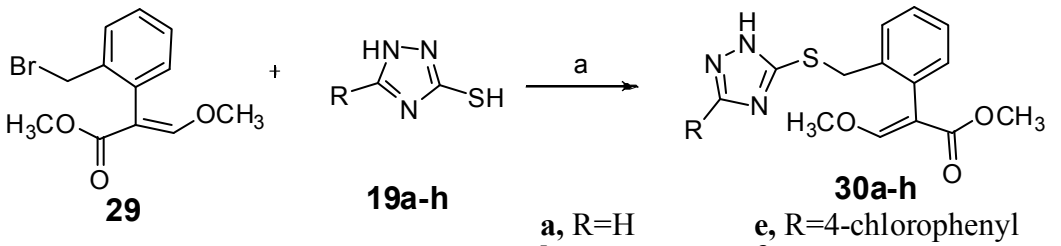
25 **26** **27** **28** **29**

Scheme 4. Reagents and conditions: a) MeOH, drop of H_2SO_4, 28 °C, 12 h, 98%; b) Methyl formate, NaH, Toluene, 0-28 °C,12 h, 98%; c) Dimethyl sulphate, EDC/H_2O (1:1), TBAB, Na_2CO_3, 28 °C, 2 h, 98%; d) AIBN, NBS, CCl_4, 80 °C, 3 h, 95%.

spectrum showed the resonance at δ 4.40 as a singlet for two protons of CH_2-Br, along with this two singlets were seen at δ 3.82 and 3.69 for methoxy protons. In ^{13}C NMR spectrum the resonance was seen at 101.00, 60.20, 52.30, 51.56 ppm.

4.2.3 Synthesis of 1,2,4-triazole thiol substituted strobilurin derivatives

(E)-methyl-2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29** was finally coupled with different 5-substituted-1,2,4-triazole-3-thiols **19a-h** in presence of K_2CO_3 and DMF at 28 °C yielding compounds **30a-h** (scheme 5, Table 4.1). Compounds **30a-h** were fully characterized using IR, ^1H NMR, ^{13}C NMR and Mass spectrometry.



a, R=H
b, R=Methyl
c, R=*t*-butyl
d, R=phenyl
e, R=4-chlorophenyl
f, R=4-methoxyphenyl
g, R=2-furyl
h, R= 2-thienyl

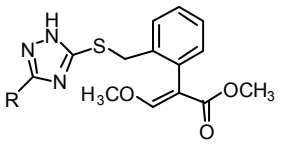
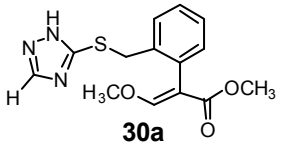
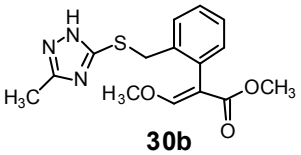
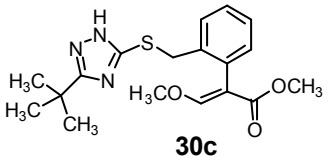
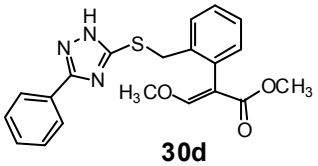
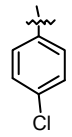
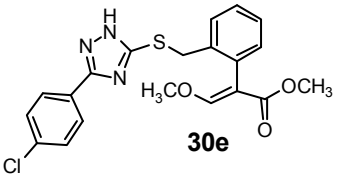
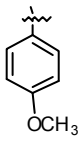
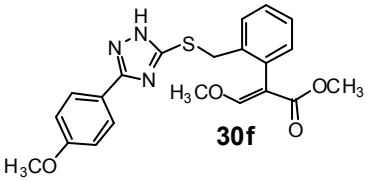
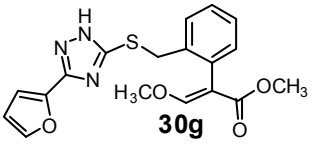
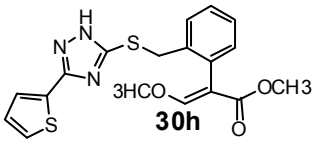
Scheme 5. Reagents and conditions: a) K_2CO_3, DMF, 28 °C, 4 h.

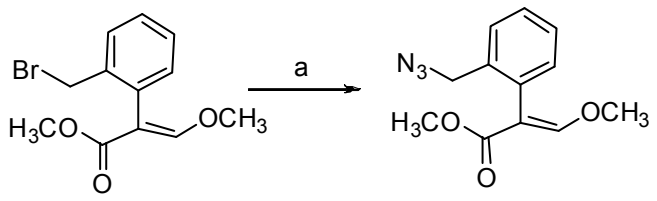
In case of compound (E)-methyl-3-methoxy-2-(2-((5-(4-methoxyphenyl)-1H-1,2,4-triazole-3-ylthio)methyl) phenyl)acrylate **30f**, IR spectrum showed characteristic stretching frequencies at 3419, 2948, 1710, 1660, 1500 and 1132 cm^{-1}. In ^1H NMR spectrum the resonance was seen at δ 7.9 and 6.99 as a doublets, which are characteristic four aromatic protons. Protons of S-CH_2 and two O-CH_3 groups showed resonance at δ 4.24, 3.88 and 3.79 respectively as a singlet. In ^{13}C NMR spectrum the carbon resonance appeared at 51.87, 55.23. 61.99 ppm for methoxy carbons, 36.62 ppm for S-CH_2, 160.45, 159.55 ppm for triazole carbons.

4.2.4 Synthesis of (E)-methyl-2-(2-(azidomethyl)phenyl)-3-methoxyacrylate

Further (E)-methyl 2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29** was transformed in to (E)-methyl 2-(2-(azidomethyl)phenyl)-3-methoxyacrylate **31** by S_N2 displacement of bromo group in presence of NaN_3 with 92% yield (scheme 6).

Table 4.1 1,2,4-triazolethiol substituted strobilurin derivatives

R		Yield %
 **19a**	 **30a**	70
 **19b**	 **30b**	70
 **19c**	 **30c**	75
 **19d**	 **30d**	75
 **19e**	 **30e**	82
 **19f**	 **30f**	71
 **19g**	 **30g**	73
 **19h**	 **30h**	52



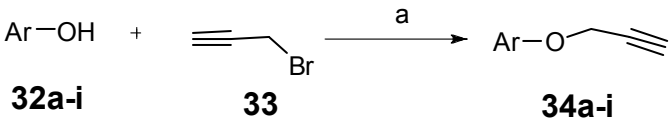
29 **31**

Scheme 6. Reagents and conditions: a) NaN_3, acetone, 60 °C, 6 h, 92%.

The azido compound **31** showed characteristic stretching peak for azide group in IR spectrum at frequency 2102 cm^{-1}. ^1H NMR spectrum showed resonance at δ 2.67 characteristic to protons of CH_2-N_3.

4.2.5 Synthesis of propargyl ether of phenols

Propargyl ether of phenols (**34a-i**, scheme 7) were obtained by reacting phenols **32a-i** with propargyl bromide in presence of K_2CO_3 in DMF.



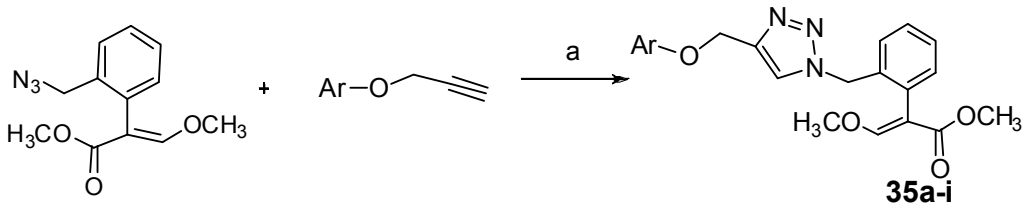
32a-i **33** **34a-i**

a, Ar= 4-Chlorophenyl
b, Ar= 3-Chlorophenyl
c, Ar= 2,6-Dichlorophenyl
d, Ar= 3-Methylphenyl
e, Ar= 2,6-Dimethylphenyl
f, Ar= 2-Nitrophenyl
g, Ar= 4-Fluorophenyl
h, Ar= 1-Napthyl
i, Ar= 2-Napthyl

Scheme 7. Reagents and conditions: a) K_2CO_3, DMF, 28 °C, 4 h.

4.2.6 Synthesis of 1,2,3-triazole derivatives of strobilurin

1,3-Dipolar cycloaddition of (E)-methyl-2-(2-(azidomethyl)phenyl)-3-methoxyacrylate **31** and different phenol propargyls **34a-i** was carried out by click reaction.

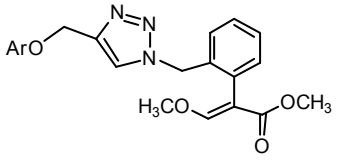
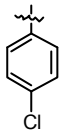
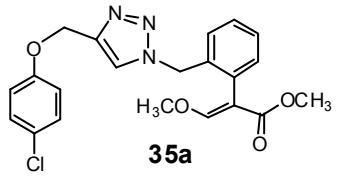
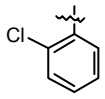
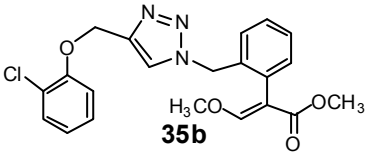
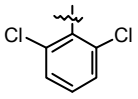
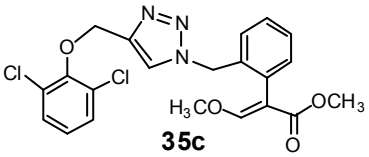
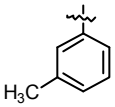
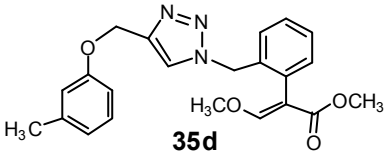
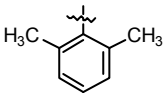
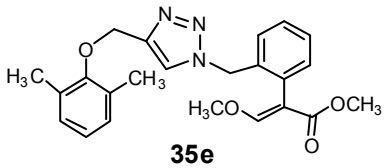
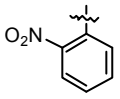
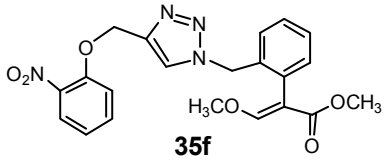
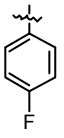
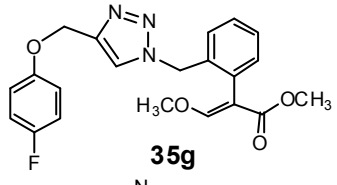
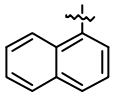
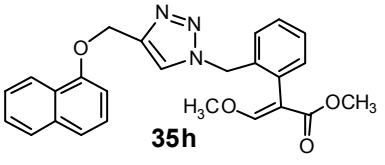
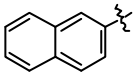
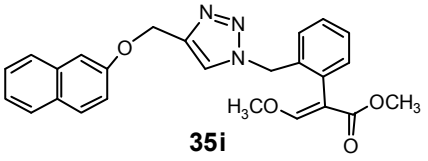


31 **34a-i** **35a-i**

a, Ar= 4-Chlorophenyl
b, Ar= 2-Chlorophenyl
c, Ar= 2,6-Dichlorophenyl
d, Ar= 3-Methylphenyl
e, Ar= 2,6-Dimethylphenyl
f, Ar= 2-Nitrophenyl
g, Ar= 4-Fluorophenyl
h, Ar= 1-Napthyl
i, Ar= 2-Napthyl

Scheme 8. Reagents and conditions: a) $CuSO_4$, sodium ascorbate, *t*-BuOH/H_2O (8:2), 28 °C, 3-6 h

Table 4.2 1,2,3-Triazole strobilurin derivatives .

Ar		Yield
 **34a**	 **35a**	68
 **34b**	 **35b**	60
 **34c**	 **35c**	56
 **34d**	 **35d**	70
 **34e**	 **35e**	55
 **34f**	 **35f**	50
 **34g**	 **35g**	65
 **34h**	 **35h**	70
 **34i**	 **35i**	74

One equivalent of propargyl ether of phenols **34a-i** and one equivalent of (E)-methyl 2-(2-(azidomethyl)phenyl)-3-methoxyacrylate **31** were dissolved in *t*-butanol/water (10 ml) and resulting solution was treated with 5 mol% $CuSO_4.5H_2O$ and 10 mol% sodium ascorbate. The reaction mixture was allowed to stir for 3-6 h for completion of cycloaddition to get compounds **35a-i** (Scheme 8, Table 4.2).

All 1,2,3-triazolyl strobilurin derivatives were characterized by IR, ^{1}H NMR, ^{13}C NMR and Mass Spectrometry. Representative compound (E)-methyl-2-(2-((4-((2,6-dimethylphenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)-3-methoxyacrylate (**35e**) showed following observations in IR, ^{1}H NMR and ^{13}C NMR spectrum. The IR spectrum showed characteristic stretching frequencies at 1708, 1631 cm^{-1} etc. The ^{1}H NMR spectrum showed resonance at δ 7.49 as a singlet for triazole proton. Singlet at δ 7.59 for vinylic proton, two singlets at δ 5.46, 4.90 for N-CH_2 and O-CH_2 protons, two singlets at δ 3.79, 3.69 for O-CH_3 protons were observed. In ^{13}C spectrum the resonance was seen at 65.23, 61.80, 51.78, 51.45, 15.99 ppm.

4.2.7 Antifungal susceptibility testing

All synthesized compounds **30a-h** and **35a-i** were evaluated for their antifungal activity against different strains of human pathogens *C. albicans, C. neoformans* and plant pathogen *F. oxysporum, Drechslera oryzae, Magnaporthe*

Table 4.3 Antifungal activity of 1,2,4-triazole thiol substituted strobilurin derivatives.

Inhibitor	**MIC_{90} value in µg/ml**				
	Cryptococcus neoformans	***C. neoformans* NCIM 3541**	***C. neoformans* NCIM 3542**	***Fusarium oxysporum***	***M. grisae***
30a	>512	64	>512	>512	512
30b	>512	256	>512	>512	128
30c	>512	64	>512	>512	>512
30d	256	64	128	64	>512
30e	16	64	64	>512	16
30f	>512	64	256	>512	32
30g	>512	256	>512	256	64
30h	>512	>512	>512	>512	128
Azoxystrobin	32	16	128	32	4

grisea. All synthesized strobilurin derivatives were unable to inhibit the growth of different strains of *C. albicans* and *D. Oryzae* up to 512 μg/ml. For other fungi, the 1,2,4-triazole thiol strobilurin derivatives **30a-h** showed growth inhibition in the range of 16-512 μg/ml. Compound **30e** inhibited the growth of *C. neoformans* with MIC of 16 μg/ml which was found to be better than that of standard azoxystrobin (MIC 32 μg/ml). Compounds **30a** and **30c-f** showed MIC 64 μg/ml against *C. neoformans* NCIM 3541, while MIC of compound **30b** and **30g** was 256 μg/ml. Compounds **30e, 30f** and **30g** showed MIC 16, 32 and 64 μg/ml respectively against plant pathogen *M. Grisea*. Compound **30e** was found to be potent inhibitor as it inhibited growth of *C. neoformans*, *C. neoformans* 3541, *C. neoformans* 3542 and *M. Grisea* with MIC in the range of 16 to 64 μg/ml. *C. neoformans* strain 3541 was found to be sensitive towards all 1,2,4-triazole thiol substituted strobilurin derivatives except **30h**.

Shridhara *et al.* reported the synthesis of different 3-isoxazoline methyl-3-methoxy-2-(4-oxo-3,4-dihydrophthalazin-1-yl)prop-2-enoate derivatives [18]. Out of all derivatives, compounds with 4-chloro phenyl and 4- fluoro phenyl substituent showed potent inhibition of *A. niger* and *C. albicans* with MIC 62.5 μg/ml. For pyrazole strobilurin derivatives, compound with *p*-chlorophenyl substituent inhibited 100% growth of *P. oryzae, B. cinerea* at 400 μg/ml*, P. cubensis* at 3.12 μg/ml and *E. graminis* at 1.56 μg/ml [13]. Zhao *et al.* also showed that in case of chalcone based derivatives, 4-chlorophenyl substituted derivative showed 100 % growth inhibition of *P. cubensis*, *Sphaerotheca fuliginea* at 200 μg/ml concentration [19]. Similarly, in present study compound **30e** with 4-chlorophenyl substituent showed potent antifungal activity against different strains of *C. neoformans*, and *M. Grisae.*

With 1,2,3-triazole strobilurin derivatives **35a-i**, no growth inhibition of any tested pathogenic fungi was observed even at 512 μg/ml concentration except for compound **35b**. Compound **35b** inhibited the growth of *C. albicans* 3557 with MIC 64 μg/ml, which was better than azoxystrobin (MIC >512 μg/ml). The results showed that 1,2,4-triazole thiol group played important role in the antifungal activity exhibited by **30a-h**.

4.2.8 Chitin synthase inhibition

All the synthesized 1,2,4-triazole thiol and 1,2,3-triazole strobilurin derivatives were tested for their *in vitro* CS inhibition at 64 μg/ml concentration using

CS preparation from *B. poitrasii*. 1,2,4-Triazole thiol substituted strobilurin derivatives **30a-h** inhibited CS activity in the range 10-51% (Table 4.4). Compound **30c** with *t*-butyl substituent showed maximum CS inhibition (50.87%). Whereas, aromatic 1,2,4-triazole thiol substituted derivatives **30d-f** showed 32-40 % of CS inhibition. On the other hand, the heterocylic substituted derivatives **30g** and **30h** showed very less CS inhibition (10-12%), suggesting that heterocyclic substituent is not favourable modification.

Pfefferle *et al.* reported that strobilurin A and B inhibited 24 and 30 % of CS activity of *Coprinus cinereus* at 0.25 mM concentration [8]. Synthetic chlorinated strobilurin derivatives **2-4** (Figure 4.2) were also tested for the CS inhibitory activity and it was found that compound **4** with dichloro substituent showed 49% inhibition of CS activity at 65 μg/ml whereas compound **2** with dichloro substituent but different methoxyacrylate unit showed only 24 % inhibition of CS at 69 μg/ml [8]. These results suggested the importance of β-methoxyacrylate unit present in compound **4** for CS inhibitory activity. We have also used the same β-methoxyacrylate unit as that of compound **4** for the synthesis of strobilurin derivatives. In our case compounds containing chlorine substituent **30e** showed 38 % inhibition of CS activity at 64 μg/ml.

Compounds **35a-i** inhibited the CS activity in the range of 5-46% at 64 μg/ml concentration (Table 4.4). Compound **35d** with methyl substituent at meta position in phenyl ring showed potent inhibition of CS activity (46.46%), which may be because of the similarity of meta methyl substituent in benzene ring to that of β-methyl group of nikkomycin responsible for higher CS inhibitory activity [28]. Compounds **35a** and **35b** showed 29 and 33% CS inhibition, suggesting that position of chloro substituent in phenyl ring did not affect CS inhibitory activity significantly. Compound **35c** with dichloro substituent showed loss of CS inhibitory activity (4% CS inhibition), which may be due to steric hindrance exerted by dichloro substituent. Substitution of strong electron withdrawing groups such as 2-nitro and 4-fluoro in **35f** (CS inhibition 11.19%) and **35g** (CS inhibition 19.50%) respectively led to lower CS inhibitory activities. *In vitro* CS inhibition and whole cell growth inhibition results of all synthesized strobilurin derivatives reveal the importance of chlorine substituent in enhancement of antifungal activity.

Table 4.4 Chitin synthase inhibition by 1,2,4-triazole thiol substituted and 1,2,3-triazole substituted strobilurin derivatives

Inhibitor 64 µg/ml	% chitin synthase inhibition	Inhibitor 64 µg/ml	% chitin synthase inhibition
30a	33.44	**35a**	29.81
30b	29.32	**35b**	33.10
30c	50.87	**35c**	4.03
30d	40.35	**35d**	46.46
30e	38.09	**35e**	24.17
30f	32.960	**35f**	11.19
30g	10.64	**35g**	19.50
30h	15.99	**35h**	29.49
		35i	28.76

In case of **30e,** 38% of CS inhibition was seen at 64 µg/ml concentration; on the other hand its MIC against tested fungal pathogens was in the range of 16 to 64 µg/ml. Similar trend was observed for other 1,2,4-triazole thiol substituted strobilurin derivatives. These results indicate that CS inhibition is secondary mechanism for antifungal action of **30a-h**, and they may be acting primarily through respiratory inhibition similar to other strobilurins.

4.2.9 Yeast to hypha transition inhibition

All 1,2,3-triazole and 1,2,4-triazole thiol substituted strobilurin derivatives were tested for their potential to inhibit Y-H transition inhibition of *B. poitrasii* at 4 µg/ml concentration. 1,2,4-Triazole thiol substituted strobilurin derivatives **30a-h** inhibited yeast to hypha transition in the range 41-75 % (Table 4.5). Compound **30c** with *t*-butyl substituent was found to be the most potent inhibitor of transition (75.5%). Compound **30e** with *p*-chlorophenyl substituent showed 66% inhibition of Y-H transition.

1,2,3-Triazole strobilurin derivatives **35a-i** showed 21-56% inhibition of Y-H transition in *B. poitrasii* at 4 µg/ml concentration (Table 4.5). Hawser *et al.* tested

Table 4.5 Yeast to hypha transition inhibition by 1,2,4-triazole thiol substituted and 1,2,3-triazole strobilurin derivatives.

Inhibitor 4 µg/ml	% Yeast to hypha transition inhibition	Inhibitor 4 µg/ml	% Yeast to hypha transition inhibition
30a	47.24	**35a**	30.00
30b	41.71	**35b**	24.90
30c	75.50	**35c**	21.36
30d	45.50	**35d**	40.05
30e	66.13	**35e**	55.89
30f	44.18	**35f**	34.93
30g	41.93	**35g**	32.60
30h	47.61	**35h**	43.69
		35i	44.79

eleven antifungal agents for their morphogenetic transformation inhibition and growth inhibition in *C. albicans* [29]. It was observed that antifungal agents amphotericin B, mulundocandin and aculeacin inhibited morphogenetic transformation at concentration lower than their MIC concentrations. This proves that inhibition of germ tube formation is more sensitive for antifungal agent action than inhibition of growth. Similarly for compounds **35a-i** even though there was no growth inhibition of all tested fungi up to 512 µg/ml, Y-H transition inhibition was seen at 4 µg/ml in the range of 21-56%. Compound **35d** and **35f** with methyl substituent showed 40 and 55 % of Y-H transition inhibition respectively. Napthyl substituted 1,2,3-triazole strobilurin derivatives **35h** and **35i** showed 44 % of transition inhibition. Compounds **35a-c**, **35f** and **35g** with electron withdrawing substituent showed lower transition inhibition (21-34%).

4.2.10 Haemolysis testing

After comparing results of growth and CS activity inhibition, compounds **30c, 30d, 30e** and **30f** were identified as lead molecules. These compounds were tested for RBC haemolysis as mentioned in chapter 2 section 2.4.15 [30]. It was found that all the tested compounds along with azoxystrobin did not show any haemolysis up to 1024 µg/ml (HC_{50} >1024 µg/ml). As MIC values for all these compounds were low

(16-512 μg/ml) as compared to HC_{50} concentration, these compounds may be considered as safe for use.

4.3 Conclusions

Total seventeen new strobilurin derivatives containing different 1,2,3-triazoles and 1,2,4-triazole thiols were synthesized with good yields. The methods used for the synthesis of the compounds were simpler. 1,2,4-triazole thiol substitute strobilurin derivatives were found to inhibit the growth of different human pathogenic and phytopathogenic fungi with MIC values in the range of 16-512 μg/ml. CS activity was inhibited in the range of 30-50 % by compounds **30a-f** at 64 μg/ml concentration. Compounds **35a**, **35d-i** showed CS inhibition in the range of 30-55%. Growth and CS activity inhibition results indicated that CS inhibition is secondary mechanism for antifungal action of **30a-h**, and they may be acting primarily through respiratory inhibition similar to other strobilurins.

4.4 Experimental section

4.4.1 1-H-1,2,4-triazole-3-thiol (19a)

To the solution of thiosemicarbazide **17** (1 gm, 10.9 mmol) in hot water (15 ml) formic acid (2.54 ml, 67 mmol) was added. The reaction mixture was heated at 80 °C for 1 h. After completion of the reaction (monitored by TLC) excess formic acid was concentrated under vacuum. The reaction mixture was cooled and filtered to get 9.5 gm of formylthiosemicarbazide **18**. Formylthiosemicarbazide **18** (0.58 gm, 4.32 mmol) 10% aq. NaOH (15 ml) was added and reaction mixture was heated at 80 °C for 3 h. After completion of reaction (monitored by TLC), reaction mixture was cooled and acidified with conc. HCl leading to formation of a precipitate. The precipitate was filtered and washed with water to get 9.5 gm of 1-H-1,2,4-triazole-3-thiol **19a** (95%). Mp: 213-216 °C; ^{1}H NMR (DMSO d_6, 400 MHz): δ = 13.63 (s, 1H), 13.10 (s, 1H), 8.30 (s, 1H); ^{13}C (DMSO d_6, 100MHz): 166.98, 141.51 ppm.

4.4.2 5-methyl-1H-1,2,4-triazole-3-thiol (19b)

To the solution of thiosemicarbazide **17** (1 gm, 11 mmol) in hot water (20 ml) acetic anhydride **20** (1.1 ml, 11 mmol) was added in small portions with cooling and stirring. This solution was stirred at 28 °C overnight. Water was completely removed

to get solid 1-acetylthiosemicarbazide **21**. 1-Acetylthiosemicarbazide **21** (1 gm, 10 mmol) was added to 10% aq. NaOH (15 ml) and heated at 80 °C for 3 h. On completion of reaction (checked by TLC), the reaction mixture was acidified using conc. HCl. The precipitate formed was separated by filtration to get 10 gm of 5-methyl-1,2,4-triazole-3-thiol **19b** (95%). Mp: 272-274 °C (lit. 282 °C); ^{1}H NMR (DMSO $d_{6,}$ 400 MHz): δ = 13.30 (s, 1H), 13.13 (s, 1H) 2.40 (s, 3 H); ^{13}C (DMSO d_{6}, 100MHz): 166.98, 140.98, 10.33 ppm.

4.4.3 General procedure for preparation of 5-substituted-1,2,4-triazole-3-thiol 19c-h

To the solution of specific acid **22d-h** (1 mmol) in CH_2Cl_2 (10 ml), $SOCl_2$ (2 mmol) was added over a period of 1 h at 0 °C along with 3 drops of pyridine. The reaction mixture was stirred overnight and then heated at 50 °C for 3-6 h. Completion of reaction was checked by TLC and excess of thionyl chloride was removed by distillation to get corresponding acid chlorides **23d-h**.

The specific acid chloride **23c-h** (1 mmol) was added drop wise to the solution of thiosemicarbazide **17** (1 mmol) in dry pyridine (25 ml) at 0 °C. The reaction mixture was stirred at 28 °C for 10-12 h. On completion of the reaction (monitored by TLC), excess of pyridine was removed under vacuum. Residue was poured on ice water and extracted with 150 ml ethyl acetate. Ethyl acetate layer was washed with 3×50 ml of water, 1×25 ml brine. Organic layer was dried over anhydrous Na_2SO_4 then concentrated to get respective *N*-acetyl thiosemicarbazide **24c-h.** Specific *N*-acetyl thiosemicarbazide was added to 10% aq. NaOH (15 ml) and heated at 80 °C for 3 h. After completion of reaction (checked by TLC), the reaction mixture was acidified using conc. HCl. The precipitate formed was separated by filtration to get final 5-substituted-1,2,4-triazole-3- thiol **19c-h.**

5-tert-butyl-1H-1,2,4-triazole-3-thiol (19c): Yield: 95%; ^{1}H NMR (DMSO $d_{6,}$ 400 MHz): δ = 13.10 (d, 1H) 1.24 (s, 3 H); ^{13}C (DMSO d_{6}, 100MHz): 166.98, 160.18, 29.00 ppm.

5-phenyl-1H-1,2,4-triazole-3-thiol (19d): Yield: 71%; Mp: 253-255 °C; ^{1}H NMR (DMSO $d_{6,}$ 400 MHz): δ = 13.10 (d, 1H), 7.46 (m, 5H);

5-(4-chlorophenyl)-1H-1,2,4-triazole-3-thiol (19e): Yield: 82%; Mp: 295-297 °C (Lit. 297 °C); ^{1}H NMR (DMSO d_6, 400 MHz): δ = 14.20 (s, 1H), 13.80 (s, 1H), 7.90 (d, 2 H), 7.43 (d, 2H); ^{13}C (DMSO d_6, 100MHz): 169.98, 149.18, 138.60, 127.89, 128.07, 122.10 ppm.

5-(4-methoxyphenyl)-1H-1,2,4-triazole-3-thiol (19f): Yield: 78%; ^{1}H NMR (DMSO d_6, 400 MHz): δ = 13.50 (s, 1H), 13.40 (s, 1H), 7.90 (d, 2 H), 6.99 (d, 2H), 4.10 (s, 3H); ^{13}C (DMSO d_6, 100MHz): 169.98, 160.02, 131.20, 125.89, 116.36, 113.63, 111.62, 58.60 ppm.

5-(furan-2-yl)-1H-1,2,4-triazole-3-thiol (19g): Yield: 52%; Mp: 220-223 °C; ^{1}H NMR (DMSO d_6, 400 MHz): δ = 13.92 (s, 1H), 13.70 (s, 1H), 7.91 (d, 1H, J=4 Hz), 7.14 (d, 1H, J=4Hz), 6.70 (dd, 1H, J=4Hz); ^{13}C (DMSO d_6, 100MHz): 169.98, 150.02, 134.20, 132.25, 129.33, 125.28 ppm.

5-(thiophen-2-yl)-1H-1,2,4-triazole-3-thiol (19h): Yield: 73%; Mp: 220-224 °C; ^{1}H NMR (DMSO d_6, 400 MHz): δ = 13.65 (s, 1H), 13.20 (s, 1H), 7.64 (d, 1H, J=4Hz), 7.35 (d, 1H, J=4Hz), 6.99 (dd, 1H, J=4Hz); ^{13}C (DMSO d_6, 100MHz): 163.98, 146.78, 135.11, 133.67, 129.63, 128.66 ppm.

4.4.4 Synthesis of methyl 2-tolylacetate (26)

o-Tolylacetic acid **25** (1 gm, 6.6 mmol) was dissolved in methanol. Catalytic amount of sulfuric acid was (0.2 ml) added to this solution. The mixture was stirred at room temperature for 12 h. Reaction completion was checked by TLC. Excess methanol was evaporated and 25 ml water was added. Product was extracted in 2×150 ml ethyl acetate. Ethyl acetate layer was washed sequentially with 2×25 ml $NaHCO_3$ solution, 25 ml water and 25 ml brine solution. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to yield 1.09 gm (98%) of methyl 2-tolylacetate **26** in the form of colorless oil. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.29 (m, 4H), 3.81 (s, 2H), 3.63 (s, 3H), 2.28 (s, 3H); ^{13}C ($CDCl_3$, 50MHz): 171.92, 162.98, 131.13, 130.12, 129.84, 128.02, 127.58, 125.71, 51.67, 38.79, 19.87 ppm. MS *m/z*: 187 $[M+Na]^+$; IR ($CHCl_3$) ν: 1724, 1369, 1174, 1024 cm^{-1}. Anal Calcd for $C_{11}H_{12}O_3$: C, 73.15**;** H, 7.37. Found: C, 73.10**;** H, 7.37.

4.4.5 Synthesis of methyl-3-oxo-2-o-tolylpropanoate (27)

To the solution of methyl 2-tolylacetate **26** (1 gm, 6.09 mmol) in toluene (5 ml), methyl formate (5.6 ml) was added. Sodium hydride (0.29 gm, 12 mmol) was added to this mixture in small portions over a period of 1 h at 0 °C under argon atmosphere. Reaction mixture was stirred at 28 °C for 12 h. After completion of reaction (monitored by doing TLC), excess methyl formate and toluene were evaporated under reduced pressure and reaction mixture was quenched with ice. Product was extracted with 150 ml of ethyl acetate. Organic layer was washed sequentially with 2×50 ml of water and 50 ml of brine solution and dried over the Na_2SO_4. Organic layer was then evaporated under reduced pressure to get brown colored oil which on further purification by column chromatography using ethyl acetate-petroleum ether afforded 1.05 gm (98%) of methyl-3-oxo-2-o-tolylpropanoate **27**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 11.93 (d, 1H), 7.29 (m, 4H), 3.81 (s, 3H), 3.73 (s, 1H), 2.28 (s, 3H); ^{13}C ($CDCl_3$, 50MHz): 171. 92, 162.98, 131.13, 130.12, 129.84, 128.02, 127.58, 125.71, 51.67, 38.79, 19.87 ppm. MS *m/z*: 215 $[M+Na]^+$; IR ($CHCl_3$) ν: 2981, 1724, 1658, 1369, 1174, 1024, 827 cm^{-1}. Anal Calcd for $C_{11}H_{12}O_3$: C, 68.78**;** H, 6.24. Found: C, 68.82**;** H, 6.00.

4.4.6 Synthesis of (E)-methyl-2-(methylphenyl)-3-methoxyacrylate (28)

The methyl 3-oxo-2-o-tolylpropanoate **27** (1 gm, 5.2 mmol) was taken in 10 ml ethylene dichloride/water (1:1). To this heterogeneous solution sodium carbonate (0.66 gm, 6.24 mmol) and catalytic amount of tetrabutylammonium bromide (TBAB) was added, followed by addition of dimethyl sulfate (0.787 gm, 6.25 mmol). This heterogenous mixture was stirred at room temperature for 2 h. Completion of reaction was checked by TLC. Organic layer was separated from water layer and sequentially washed with 2×25 ml of water, 25 ml brine and dried over solid Na_2SO_4. Further, organic layer was evaporated to get final product (E)-methyl 2-(methyl phenyl)-3-methoxyacrylate **28** (1.05 gm, 98%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.56 (s, 1H), 7.18 (m, 4H), 3.80 (s, 3H), 3.69 (s, 3H), 2.18 (s, 3H); ^{13}C ($CDCl_3$, 50MHz): 164.5, 158.3, 136.1, 134.6, 131.3, 127.8, 126.3, 125.26, 104.0, 60.2, 52.3, 19.2 ppm. MS *m/z*: 229.24 $[M+Na]^+$; IR ($CHCl_3$) ν: 2948, 1708, 1631, 1255, 1130 cm^{-1}. Anal Calcd for $C_{12}H_{14}O_3$: C, 69.88**;** H, 6.84. Found: C, 69.92**;** H, 6.79.

4.4.7 Synthesis of (E)-methyl-2-(2-(bromomethyl)phenyl)-3-methoxyacrylate (29)

To the solution of (E)-methyl-2-(methylphenyl)-3-methoxyacrylate **28** (1 gm, 4.8 mmol) in carbon tetrachloride (10 ml) azaisobutyronitrile (AIBN, 0.078 gm, 0.48 mmol) and N-bromosuccinamide (1.02 gm, 5.76 mmol) was added. The reaction mixture was refluxed for 3 h. After completion of reaction (checked by TLC), carbon tetrachloride was removed under reduced pressure and residue was extracted in ethyl acetate. Ethyl acetate layer was sequentially washed with 2 x 25 ml of water, 25 ml brine and dried over solid Na_2SO_4. Organic layer was then evaporated to get crude product, which on purification by column chromatography using ethyl acetate and petroleum ether as a eluent afforded 1.31 gm (95%) of (E)-methyl 2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29.** ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.63 (s, 1H), 7.44 (m, 2H), 7.33 (m, 2H), 4.40 (s, 2H) 3.82 (s, 3H), 3.69 (s, 3H); ^{13}C ($CDCl_3$, 50MHz): 164.5, 158.3, 136.1, 134.6, 131.3, 127.8, 126.3, 125.26, 101.0, 60.2, 52.3, 51.56 ppm. MS *m/z*: 307 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631, 1434, 765 cm^{-1}. Anal Calcd for $C_{12}H_{13}BrO_3$: C, 50.55**;** H, 4.60. Found: C, 50.70**;** H, 4.50.

4.4.8 General procedure for the preparation of 1,2,4-triazole thiol substituted strobilurin derivatives 30a-h

3-substituted-1,2,4-triazole thiols **19a-h** (1 mmol) was dissolved in DMF (10 ml) to which K_2CO_3 (1.2 mmol) was added, followed by the dropwise addition of solution of (E)-methyl 2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29** in DMF (5 ml). Reaction mixture was stirred at room temperature for a period of 4 h. After completion of reaction (checked by TLC), reaction mixture was quenched with ice and extracted with ethyl acetate. The ethyl acetate layer was washed subsequently with 3×35 ml of water, 35 ml of brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. Finally the crude product obtained was purified by silica gel column chromatography using ethyl acetate and petroleum ether as eluent to afford **30a-h.**

(E)-methyl-2-(2-((1H-1,2,4-triazol-3-ylthio)methyl)phenyl)-3-methoxyacrylate (30a): ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.95 (s, 1H), 7.61 (s, 1H), 7.15 (m, 4H), 4.13 (s, 2H), 3.85 (s, 3H), 3.77 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 160.30, 150.01, 135.58, 132.18, 131.52, 129.43, 128.44, 127.89, 110.27, 62.16, 51.08, 36.02 ppm. MS *m/z*:

328.08 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631cm^{-1}. Anal Calcd for $C_{14}H_{15}N_3O_3S$: C, 55.07; H, 4.95; N, 13.76, S, 10,50. Found: C, 55.20**;** H, 5.0, N, 13.89; S, 11.02.

(E)-methyl-3-methoxy-2-(2-((5-methyl-1H-1,2,4-triazol-3-ylthio)methyl)phenyl) acrylate (30b): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.62 (s, 1H), 7.21 (m, 4H), 4.18 (s, 2H), 3.86 (s, 3H), 3.76 (s, 3H), 2.43 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 168.58, 160.75, 155.62, 135.68, 132.43, 131.39, 129.70, 128.30, 127.72, 109.82, 62.18, 51.93, 35.30, 12.03 ppm. MS *m/z*: 342.38 $[M+Na]^+$; IR ($CHCl_3$) ν: 3419, 1710, 1660 cm^{-1}. Anal Calcd for $C_{15}H_{17}N_3O_3S$: C, 56.41; H, 5.37; N, 13.16. Found: C, 56.30**;** H, 5.50, N, 12.90.

(E)-methyl-3-methoxy-2-(2-((5-*t*-butyl-1H-1,2,4-triazol-3-ylthio)methyl)phenyl) acrylate (30c): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.60 (s, 1H), 7.16 (m, 4H), 4.12 (s, 2H), 3.85 (s, 3H), 3.76 (s, 3H), 1.34 (s, 9H). ^{13}C ($CDCl_3$, 400MHz): 160.20, 135.93, 132.24, 131.41, 129.65, 128.83, 128.26, 127.73, 110.38, 71.81, 62.09, 51.98, 35.89, 32.47, 29.14, 19.15 ppm. MS *m/z*: 384.15 $[M+Na]^+$; IR ($CHCl_3$) ν: 1719, 1632cm^{-1}. Anal Calcd for $C_{18}H_{23}N_3O_3S$: C, 59.81; H, 6.41; N, 11.63. Found: C, 59.99**;** H, 6.55, N, 11.70.

(E)-methyl-3-methoxy-2-(2-((5-phenyl-1H-1,2,4-triazole-3-ylthio)methyl)phenyl) acrylate (30d): ^1H NMR ($CDCl_3$, 400 MHz): δ = 8.07 (m, 2H), 7.61 (s, 1H), 7.40 (m, 4H), 7.15 (m, 3H), 4.21 (s, 2H), 3.83 (s, 3H), 3.75 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 169.74, 162.25, 137.54, 134.10, 132.65, 131.34, 130.73, 130.00, 129.62, 129.29, 128.94, 128.65, 128.44, 127.38, 111.01, 62.44, 52.07, 36.25 ppm. MS *m/z*: 404.11 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1633 cm^{-1}. Anal Calcd for $C_{20}H_{19}N_3O_3S$: C, 62.97; H, 5.02; N, 11.02. Found: C, 62.95**;** H, 4.98; N, 11.56.

(E)-methyl-3-methoxy-2-(2-((5-(4-clorophenyl)-1H-1,2,4-triazole-3-ylthio)methyl) phenyl)acrylate (30e): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.98 (d, 2H, J=12Hz), 7.63 (s, 1H), 7.38 (d, 2H, J=8 Hz), 7.15 (m, 4H), 4.13 (s, 2H), 3.87 (s, 3H), 3.80 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 168.86, 160.59, 135.80, 135.40, 132.18, 131.47, 129.53, 128.85, 128.42, 127.81, 127.67, 110.05, 62.11, 52.03, 35.89 ppm. MS *m/z*: 438.89 $[M+Na]^+$; IR

($CHCl_3$) ν: 1708, 1633 cm^{-1}. Anal Calcd for $C_{20}H_{18}ClN_3O_3S$: C, 57.76; H, 4.36; Cl, 8.52; N, 10.10. Found: C, 57.86**;** H, 4.20; Cl, 9.52; N, 10.40.

(E)-methyl-3-methoxy-2-(2-((5-(4-methoxyphenyl)-1H-1,2,4-triazole-3-ylthio) methyl)phenyl)acrylate (30f): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.95 (d, 2H, J=8 Hz), 7.61 (s, 1H), 7.18 (m, 4H), 6.93 (d, 2H, J=12 Hz), 4.16 (s, 2H), 3.85 (s, 3H), 3.84 (s, 3H), 3.77 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 168.69, 160.74, 160.44, 136.03, 132.17, 131.32, 129.60, 128.32, 127.83, 127.61, 114.01, 110.10, 62.03, 55.25, 51.92, 35.68 ppm. MS *m/z*: 434.47 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{21}H_{21}N_3O_4S$: C, 61.30; H, 5.14; N, 10.21. Found: C, 61.39**;** H, 5.50; N, 9.98.

(E)-methyl-3-methoxy-2-(2-((5-(furan-2-yl)-1H-1,2,4-triazol-3-ylthio)methyl) phenyl)acrylate (30g): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.64 (s, 1H), 7.51 (d, 1H, J = 4Hz), 7.21 (m, 4H), 6.99 (d, 1H, J=4Hz), 6.51 (dd, 1H, J=4Hz), 4.17 (s, 2H), 3.88 (s, 3H), 3.80 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 168.66, 160.48, 144.78, 143.42, 135.55, 132.20, 131.35, 129.51, 128.34, 127.72, 111.51, 109.96, 62.03, 51.91, 35.61 ppm. MS *m/z*: 394.09 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{18}H_{17}N_3O_4S$: C, 58.21; H, 4.61; N, 11.31. Found: C, 58.90**;** H, 4.85; N, 11.65.

(E)-methyl-3-methoxy-2-(2-((5-(thiophen-2-yl)-1H-1,2,4-triazol-3-ylthio)methyl) phenyl)acrylate (30h): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.64 (d, 1H, J=4Hz), 7.62 (s, 1H), 7.32 (d, 1H, J = 4Hz), 7.16 (m, 4H), 7.07 (dd, 1H, J=4Hz), 4.12 (s, 2H), 3.86 (s, 3H), 3.79 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 168.92, 160.45, 135.59, 132.15, 131.54, 129.51, 128.54, 127.95, 127.70, 126.68, 126.39, 110.22, 62.17, 51.11, 36.08 ppm. MS *m/z*: 410.07 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{18}H_{17}N_3O_3S_2$: C, 55.80; H, 4.42; N, 10.84. Found: C, 56.01**;** H, 4.10; N, 10.98.

4.4.9 General procedure for the synthesis of propargyl ether of phenols 34a-i

The procedure used for the synthesis of propargyl ethers of phenols was same as mentioned in section 2.4.1.

4.4.10 Synthesis of (E)-methyl-2-(2-(azidomethyl)phenyl)-3-methoxyacrylate (31)

To the solution of (E)-methyl 2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **29** (1gm, 3.05 mmol) in 15 ml of acetone, sodium azide (0.237 gm, 3.66 mmol) was added. The reaction mixture was stirred at 60 °C for 6 h. On completion of the reaction (monitored by TLC), acetone was removed under vacuum and extracted with 256 ml of ethyl acetate. Ethyl acetate layer was washed sequentially with 4×25 ml of water, 25 ml of brine solution. Organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to yield crude product. The purification of crude product by silica gel column chromatography using ethyl acetate-petroleum ether mixture as eluent gave 0.8 gm (92.3%) of (E)-methyl 2-(2-(azidomethyl)phenyl)-3-methoxyacrylate **31**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.60 (s, 1H), 7.40 (m, 4H), 3.90 (s, 2H), 3.72 (s, 3H), 3.69 (s, 3H); ^{13}C ($CDCl_3$, 50MHz): 164.5, 158.3, 136.1, 134.6, 131.3, 128.8, 126.3, 125.62, 111.0, 60.2, 52.3, 49.00 ppm. MS *m/z*: (MH^+) 283; IR ($CHCl_3$) ν: 2110, 1708, 1631, 1434, 765 cm^{-1}. Anal Calcd for $C_{12}H_{13}N_3O_3$: C, 58.29; H, 5.30; N, 16.99. Found: C, 58.70**;** H, 5.45; N,16.40.

4.4.11 General procedure for the preparation of 1,2,3-triazole derivatives of strobilurin 35a-i

To the stirred solution of (E)-methyl 2-(2-(azidomethyl)phenyl)-3-methoxyacrylate **31** (1 mmol) and specific propargyl ethers of phenols **34a-i** (1 mmol) in 10 ml of tertiary butanol/water (8:2), copper sulphate (24 mg, 5 mol %) and sodium ascorbate (40 mg, 10 mol %) were added. Reaction mixture was stirred at 28 °C for 3-6 h. On completion of the reaction (monitored by TLC), *t*-butanol was removed under reduced pressure and reaction mixture was partitioned between ethyl acetate and water. The ethyl acetate layer was washed subsequently with 3×35 ml of water, 35 ml of brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure to get crude product. Finally, the crude product was purified by silica gel column chromatography using ethyl acetate and petroleum ether as eluent to obtain pure 1,2,3-triazole derivatives of strobilurin **35a-i**.

(E)-methyl-2-(2-((4-((4-chlorophenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl) phenyl)-3-methoxyacrylate (35a): ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.57 (s, 1H), 7.44 (s, 1H), 7.35 (m, 2H), 7.20 (m, 4H), 7.09 (d, 1H, J=8Hz), 6.88 (d, 1H, J=8Hz),

5.43 (s, 2H), 5.11 (s, 2H), 3.76 (s, 3H), 3.67 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.10, 160.77, 156.82, 133.49, 131.75, 129.39, 129.04, 128.78, 128.68, 116.07, 109.38, 62.27, 62.11, 52.23, 51.80 ppm. MS *m/z*: 436.11 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{21}H_{20}ClN_3O_4$: C, 60.95; H, 4.87; N, 10.15. Found: C, 60.40**;** H, 5.01; N,10.94.

(E)-methyl-2-(2-((4-((2-chlorophenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl) phenyl)-3-methoxyacrylate (35b): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.57 (s, 1H), 7.50 (s, 1H), 7.35 (m, 3H), 7.20 (t, 3H, J=8Hz), 7.09 (d, 1H, J=8Hz), 6.90 (t, 1H, J=8Hz), 5.43 (s, 2H), 5.23 (s, 2H), 3.76 (s, 3H), 3.67 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.43, 160.85, 153.73, 143.68, 133.49, 132.46, 131.69, 130.28, 130.28, 128.97, 128.68, 128.60, 127.80, 123.16, 122.91, 121.97, 114.22, 109.28, 63.14, 62.11, 52.16, 51.76 ppm. MS *m/z*: 436.11 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{21}H_{20}ClN_3O_4$: C, 60.95; H, 4.87; N, 10.15. Found: C, 60.40**;** H, 5.01; N,10.10.

(E)-methyl-2-(2-((4-((2,6-dichlorophenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl) phenyl)-3-methoxyacrylate (35c): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.58 (s, 1H), 7.49 (s, 1H), 7.37 (m, 2H), 7.21 (m, 1H), 7.00 (m, 4H), 5.56 (s, 2H), 4.90 (s, 2H), 3.79 (s, 3H), 3.68 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.12, 160.44, 155.00, 144.54, 134.31, 132.04, 130.74, 130.69, 128.61, 128.50, 128.32, 123.85, 122.47, 109.03, 65.23, 61.80, 51.78, 51.45 ppm. MS *m/z*: 470.08 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{21}H_{19}Cl_2N_3O_4$: C, 56.26; H, 4.27; N, 9.37. Found: C, 56.62**;** H, 4.52; N, 9.32.

(E)-methyl-2-(2-((4-((3-methylphenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl) phenyl)-3-methoxyacrylate (35d): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.56 (s, 1H), 7.43 (s, 1H), 7.36 (m, 2H), 7.12 (m, 3H), 6.76 (t, 3H, J=8Hz), 5.43 (s, 2H), 5.13 (s, 2H), 3.75 (s, 3H), 3.67 (s, 3H), 2.31 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.23, 160.64, 158.08, 144.05, 139.35, 133.41, 132.32, 131.53, 129.03, 128.85, 128.50, 128.42, 122.71, 121.81, 115.33, 111.39, 109.12, 61.89, 61.73, 51.98, 51.55, 21.31 ppm. MS *m/z*: 416.17 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{22}H_{23}N_3O_4$: C, 67.16; H, 5.89; N, 10.68. Found: C, 67.96**;** H, 6.01; N, 10.94.

(E)-methyl-2-(2-((4-((2,6-dimethylphenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl) phenyl)-3-methoxyacrylate (35e): ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.59 (s, 1H), 7.49 (s, 1H), 7.37 (m, 2H), 7.21 (m, 2H), 6.96 (m, 3H), 5.46 (s, 2H), 4.90 (s, 2H), 3.79 (s, 3H), 3.68 (s, 3H), 2.22 (s, 6H). ^{13}C ($CDCl_3$, 400MHz): 167.12, 160.44, 155.00, 144.31, 133.31, 132.04, 131.34, 130.69, 128.61, 128.50, 128.32, 123.85, 122.47, 109.03, 65.23, 61.80, 51.78, 51.45, 15.99 ppm. MS *m/z*: 430.18 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{23}H_{25}N_3O_4$: C, 67.80; H, 6.18; N, 10.31. Found: C, 67.50**;** H, 6.99; N,10.64.

(E)-methyl-2-(2-((4-((2-nitrophenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl) phenyl)-3-methoxyacrylate (35f): ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.81 (dd, 1H, J=8Hz), 7.60 (s, 1H), 7.55 (s, 1H), 7.34 (m, 3H), 7.18 (d, 2H, J=8Hz), 7.05 (t, 1H, J=8Hz), 5.44 (s, 2H), 5.31 (s, 2H), 3.78 (s, 3H), 3.68 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.46, 160.88, 151.49, 140.08, 134.24, 133.46, 132.41, 131.69, 128.88, 128.71, 128.62, 125.58, 121.00, 115.42, 109.30, 63.64, 62.17, 52.20, 51.79 ppm. MS *m/z*: 447.14 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{21}H_{20}N_4O_6$: C, 59.43; H, 4.75; N, 13.20. Found: C, 59.94**;** H, 4.63; N, 13.62.

(E)-methyl-2-(2-((4-((4-fluorophenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl) phenyl)-3-methoxyacrylate (35g): ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.57 (s, 1H), 7.34 (m, 2H), 7.20 (d, 1H, J=4Hz), 7.16 (d, 1H, J=4Hz), 6.90 (m, 4H), 5.42 (s, 2H), 5.08 (s, 2H), 3.74 (s, 3H), 3.66 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.23, 160.66, 159.60, 154.86, 154.17, 154.12, 133.36, 132.31, 131.54, 128.80, 128.53, 128.43, 115.72, 109.09, 62.32, 61.91, 52.04, 51.56 ppm. MS *m/z*: 420.14 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{21}H_{20}FN_3O_4$: C, 63.47; H, 5.07; F, 4.78; N, 10.57. Found: C, 63.74**;** H, 5.60; F, 5.02; N,10.98.

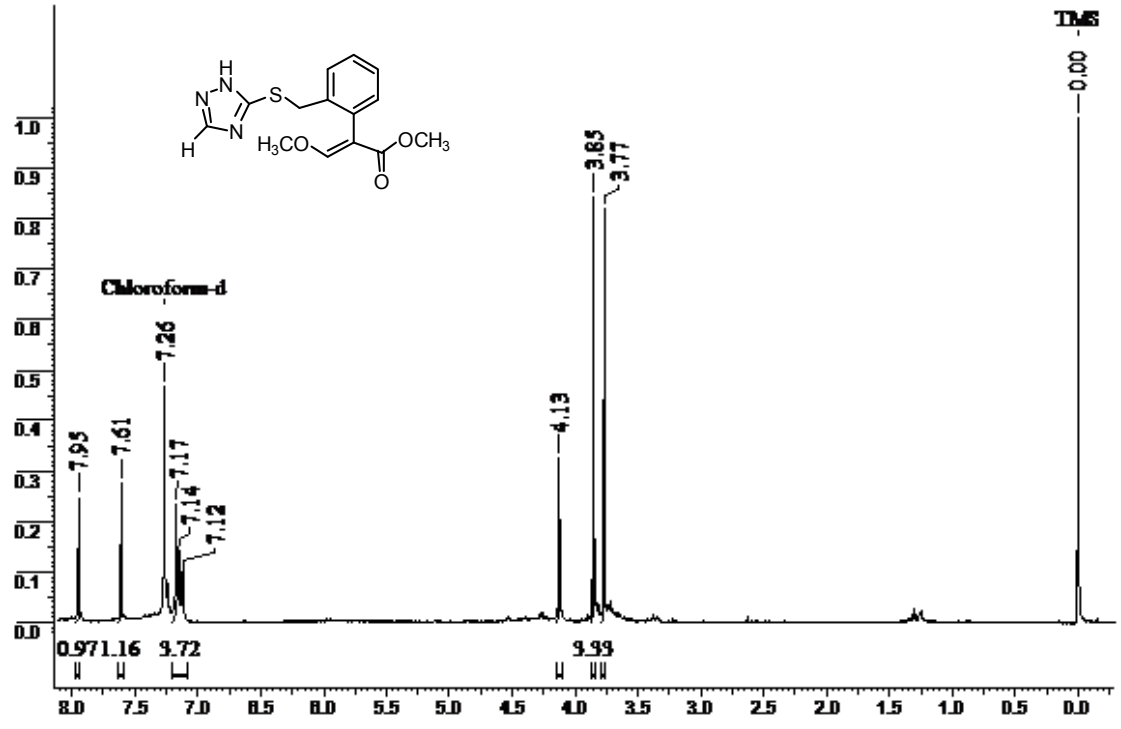
(E)-methyl-3-methoxy-2-(2-((4-((naphthalen-1-yloxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)acrylate (35h): ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.19 (d, 2H, J=8Hz), 7.78 (d, 2H, J=8Hz), 7.57 (s, 1H), 7.52 (s, 1H), 7.40 (m, 6H), 7.27 (t, 2H, J=8Hz), 6.95 (d, 1H, J=8Hz), 5.46 (s, 2H), 5.36 (s, 2H), 3.72 (s, 3H), 3.66 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.28, 160.64, 153.76, 134.29, 133.43, 132.25, 131.55, 128.76,

128.51, 128.47, 127.28, 126.25, 125.65, 125.37, 125.04, 122.81, 121.79, 120.59, 109.14, 105.19, 62.19, 61.89, 52.01, 51.59 ppm. MS *m/z*: 452.17 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{25}H_{23}N_3O_4$: C, 69.92; H, 5.40; N, 9.78. Found: C, 70.06**;** H, 5.94; N,10.06.

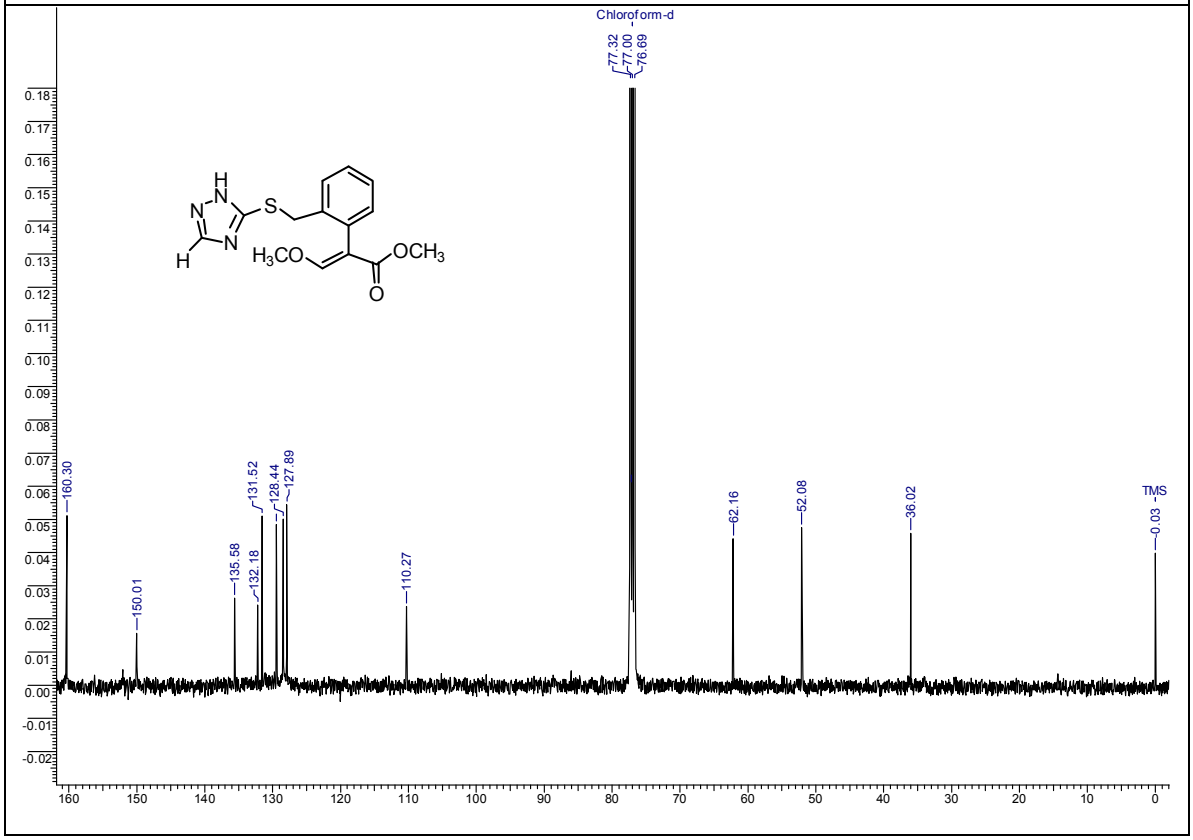
(E)-methyl-3-methoxy-2-(2-((4-((naphthalen-2-yloxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)acrylate (35i): ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.76 (m, 3H), 7.59 (s, 1H), 7.51 (s, 1H), 7.38 (m, 4H), 7.21 (m, 4H), 5.47 (s, 2H), 5.30 (s, 2H), 3.77 (s, 3H), 3.69 (s, 3H). ^{13}C ($CDCl_3$, 400MHz): 167.05, 160.41, 155.75, 134.00, 133.14, 132.10, 131.33, 129.11, 128.67, 128.34, 128.26, 127.21, 126.50, 126.06, 123.47, 122.61, 118.36, 108.94, 106.74, 61.68, 51.84, 51.39, 37.77 ppm. MS *m/z*: 452.17 $[M+Na]^+$; IR ($CHCl_3$) ν: 1708, 1631 cm^{-1}. Anal Calcd for $C_{25}H_{23}N_3O_4$: C, 69.92; H, 5.40; N, 9.78. Found: C, 70.06**;** H, 5.94; N,10.06.

4.5 Selected spectra

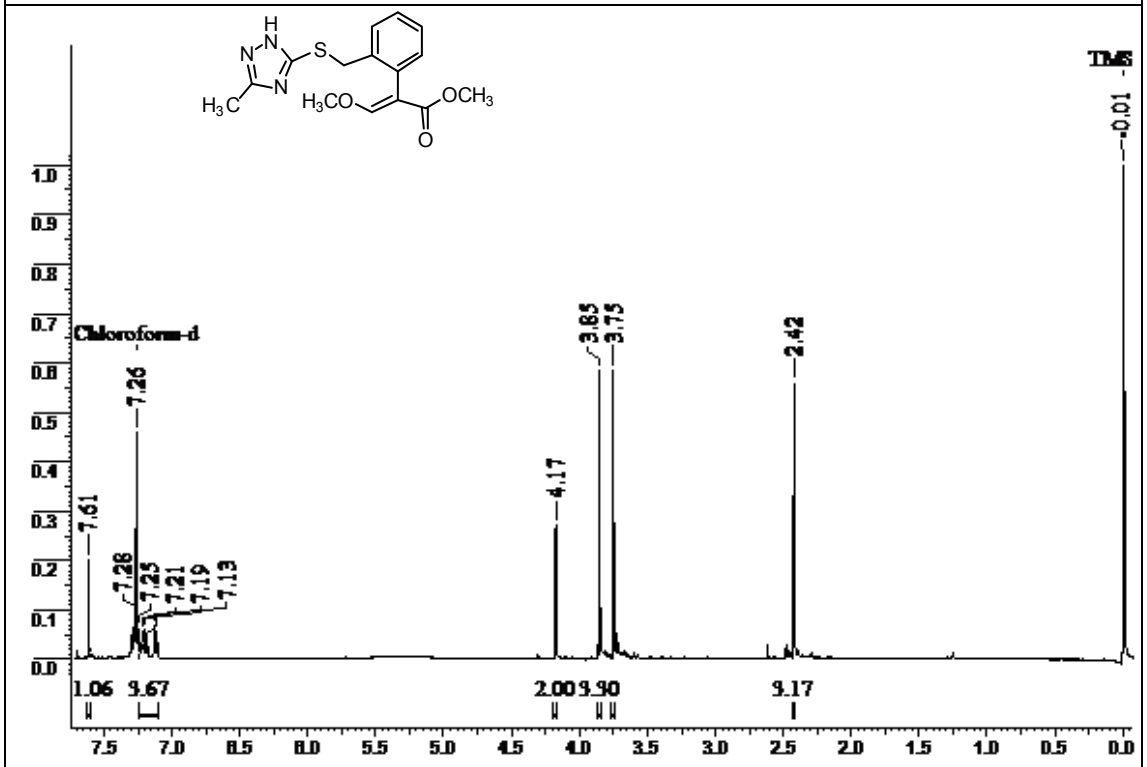
30a: ^{1}H NMR, 400 MHz, $CDCl_3$



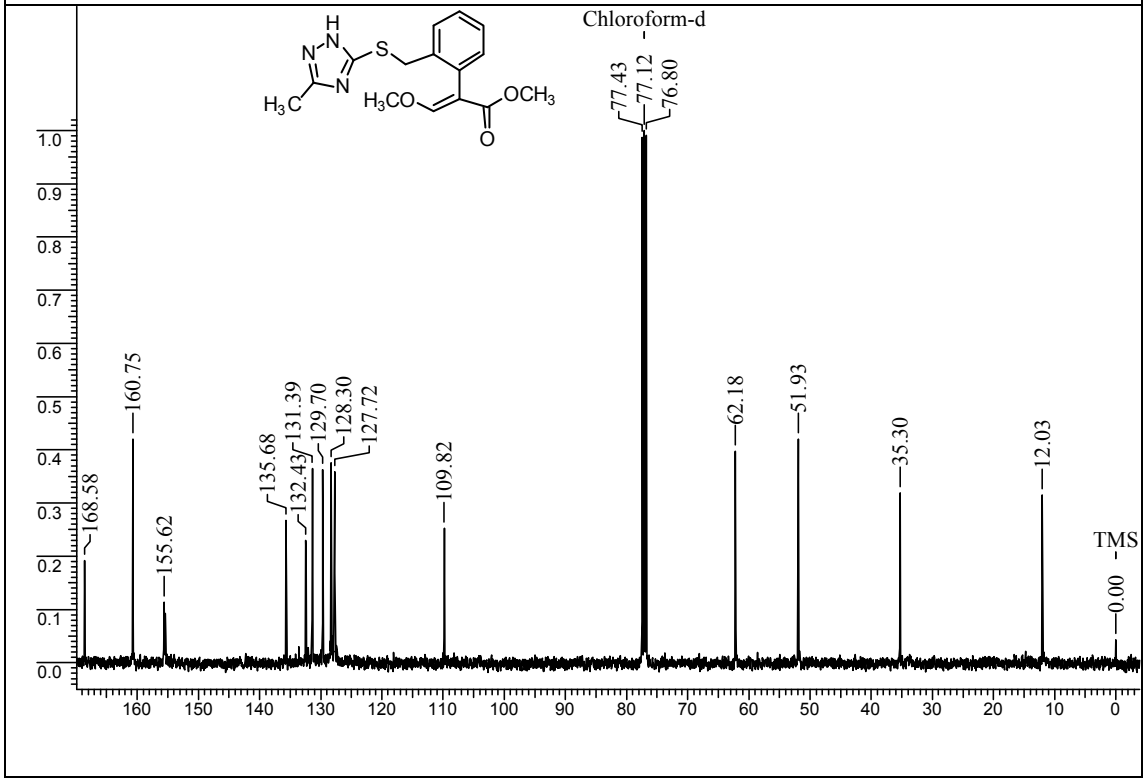
30a: ^{13}C NMR, 100 MHz, $CDCl_3$



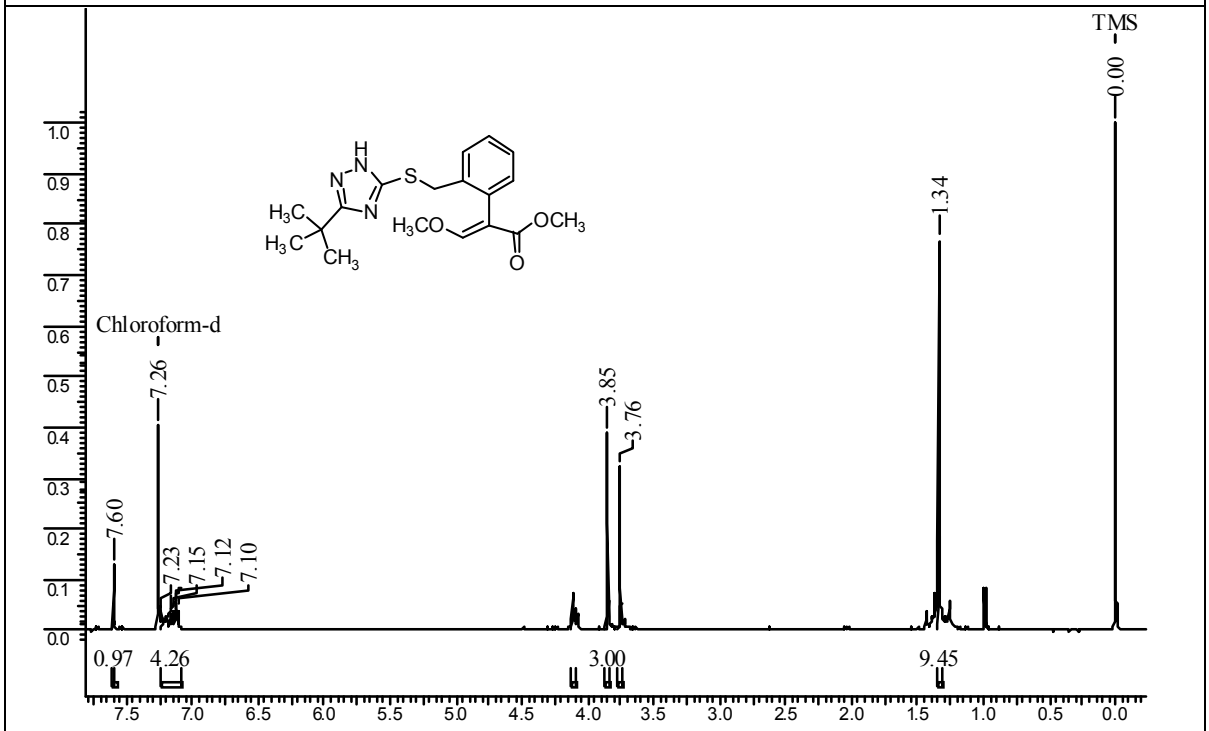
30b: ^{1}H NMR, 400 MHz, $CDCl_3$



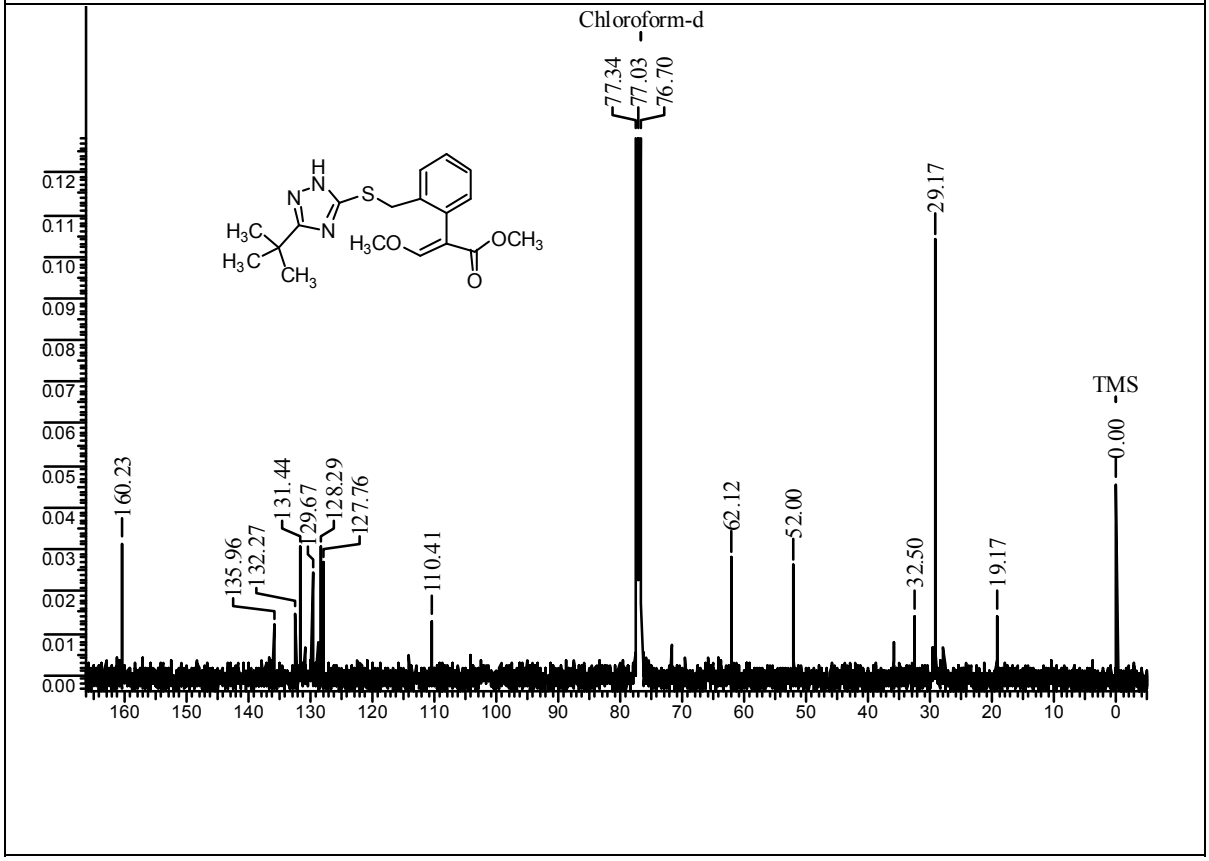
430b: ^{13}C NMR, 100 MHz, $CDCl_3$



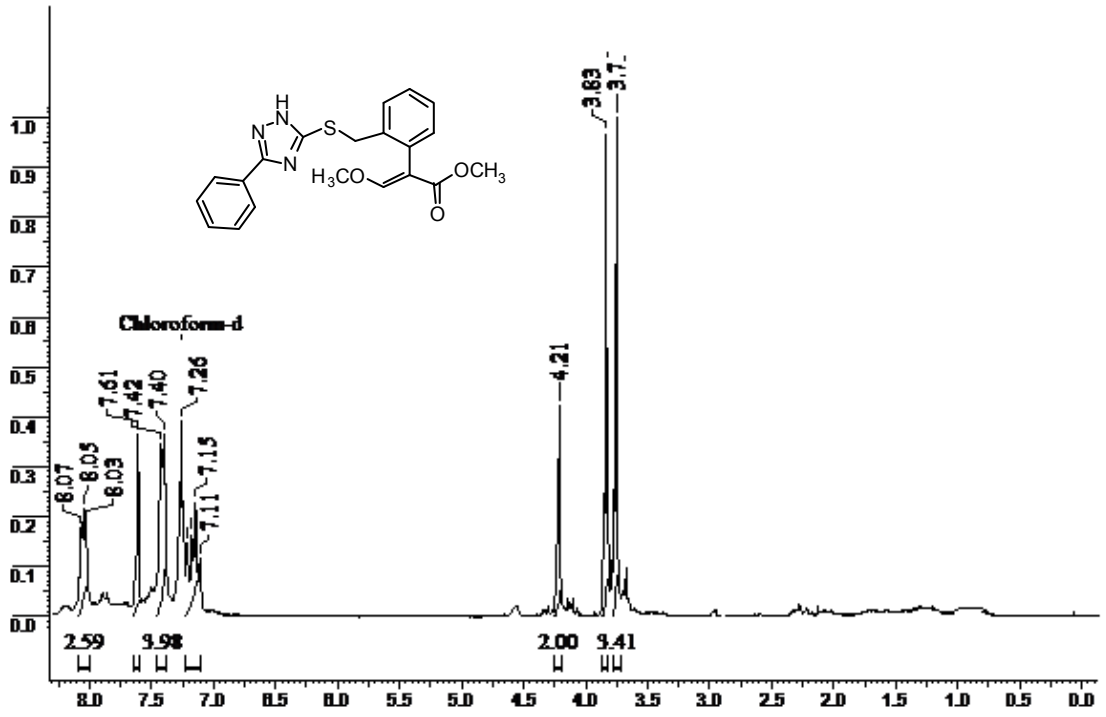
30c: ^{1}H NMR, 400 MHz, $CDCl_3$



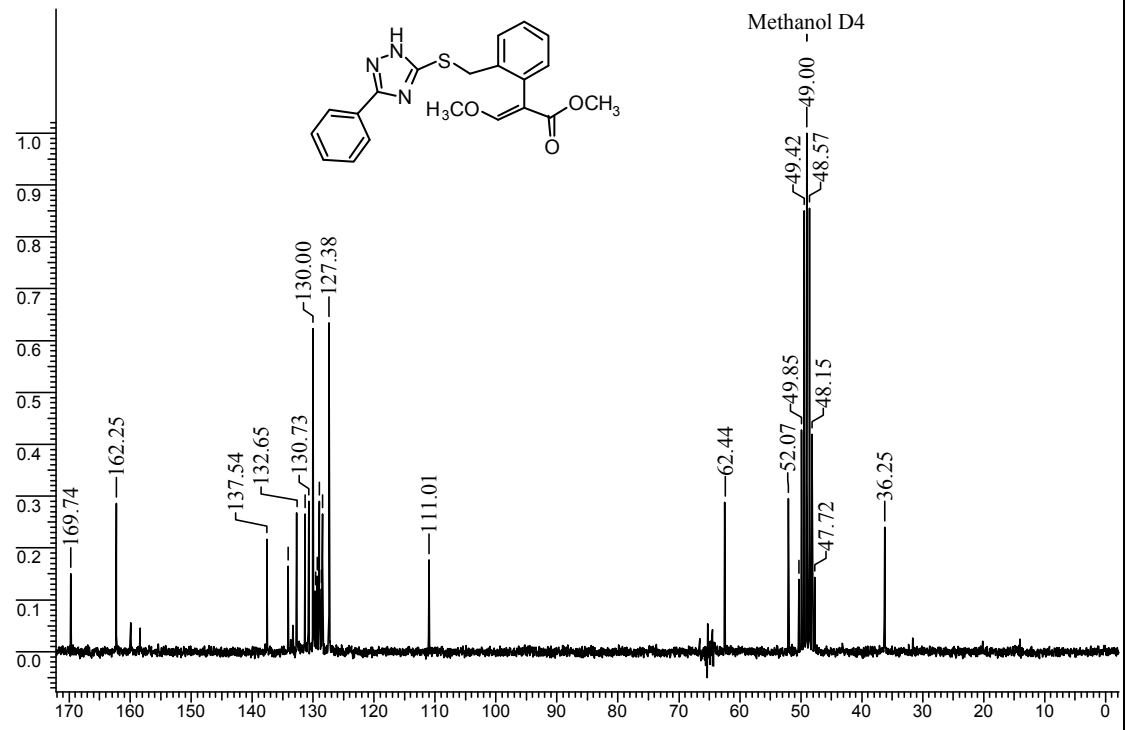
30c: ^{13}C NMR, 100 MHz, $CDCl_3$



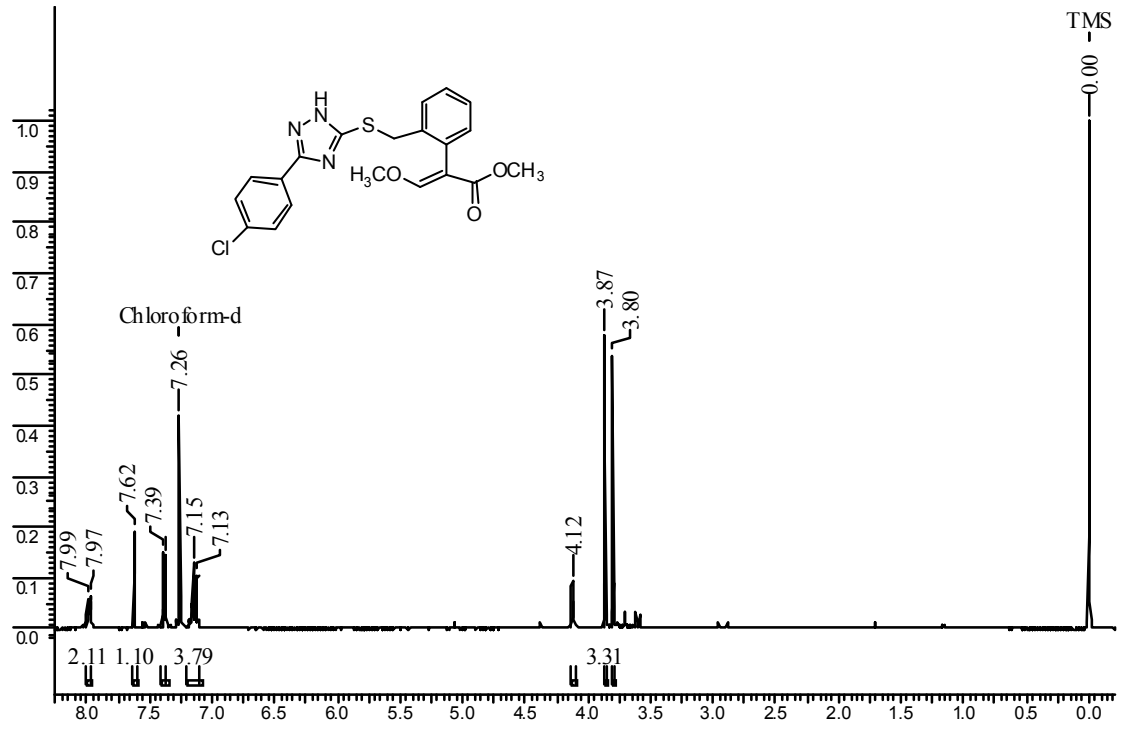
30d: ^{1}H NMR, 400 MHz, $CDCl_3$



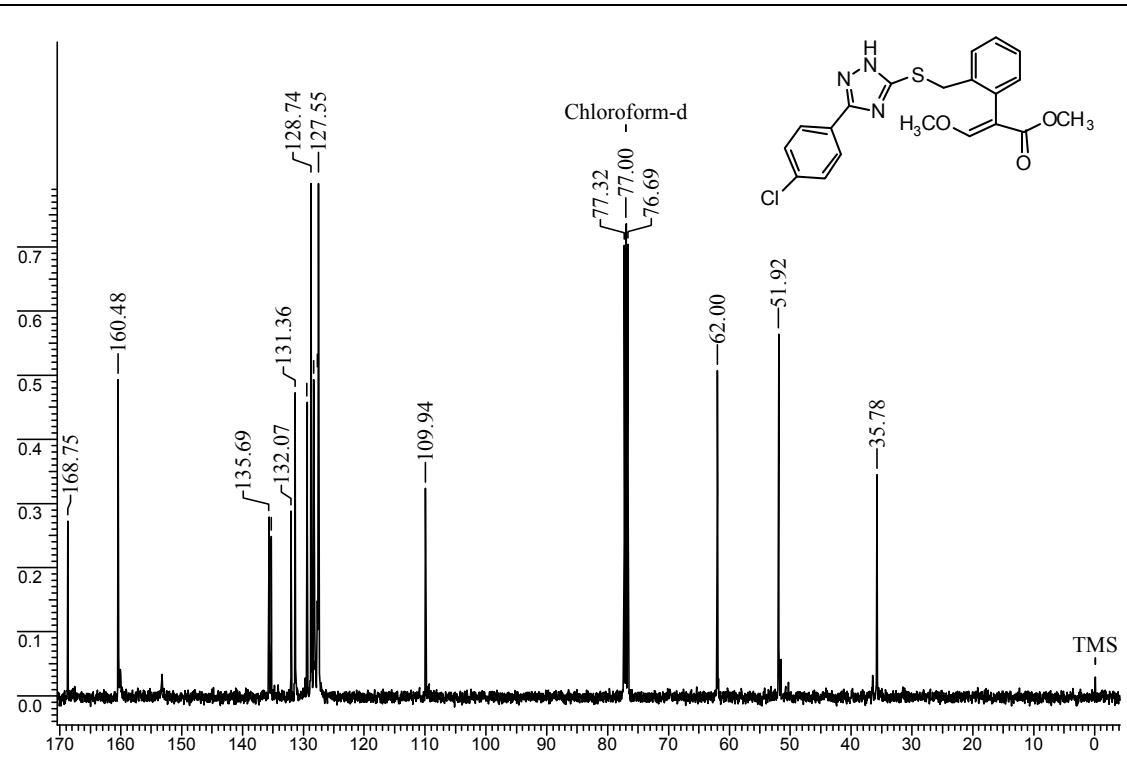
30d: ^{13}C NMR, 100 MHz, $CDCl_3$



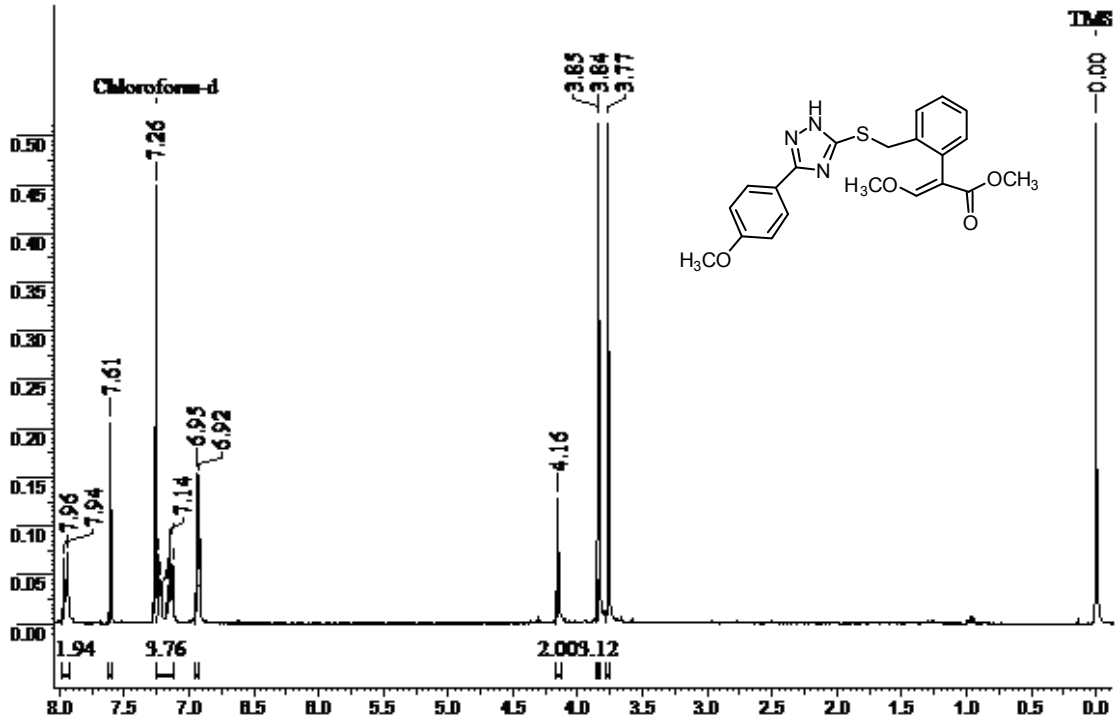
30e: ^{1}H NMR, 400 MHz, $CDCl_3$



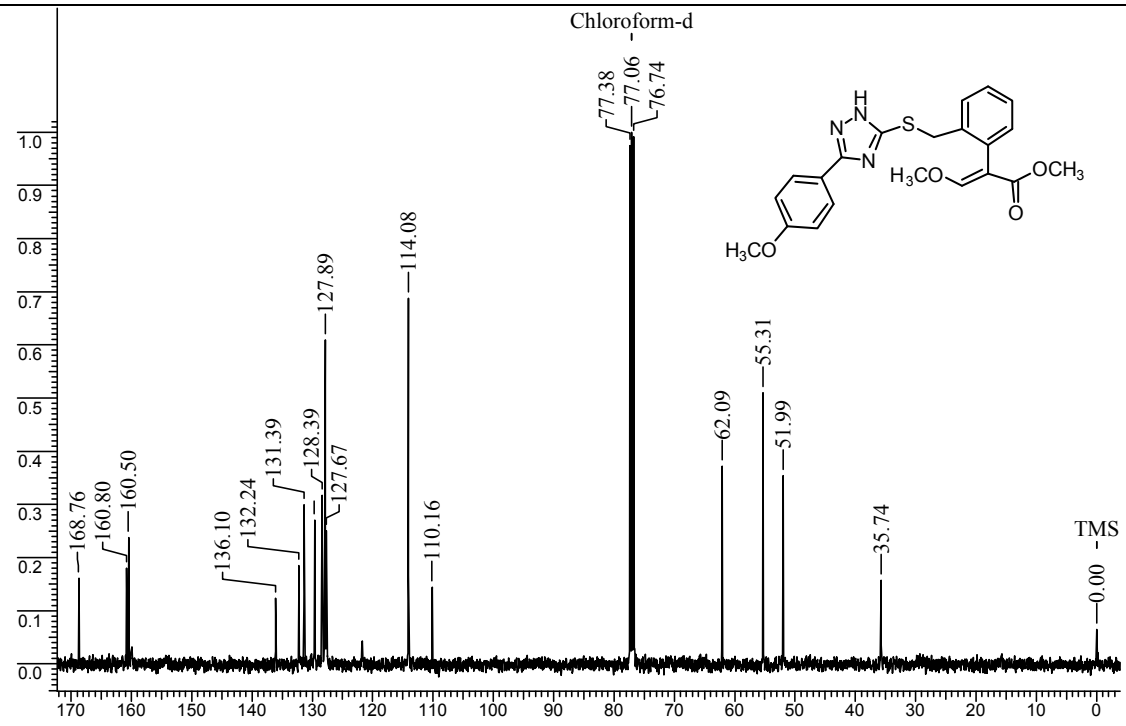
30e: ^{13}C NMR, 100 MHz, $CDCl_3$



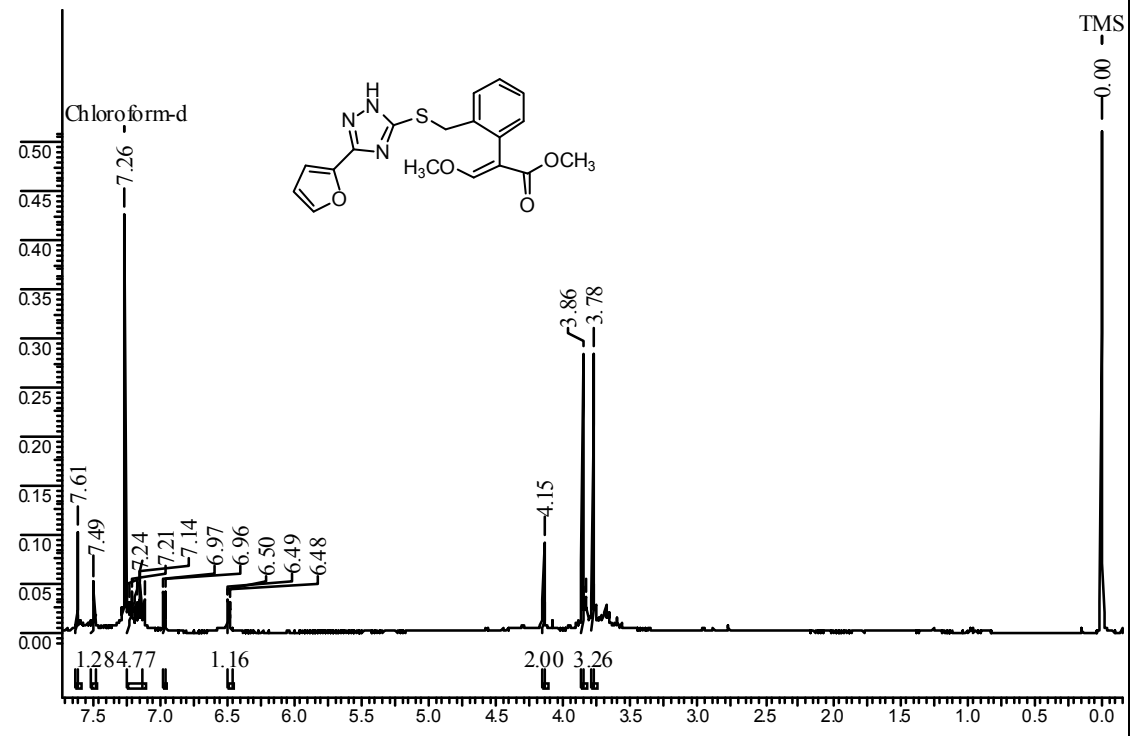
30f: ^{1}H NMR, 400 MHz, $CDCl_3$



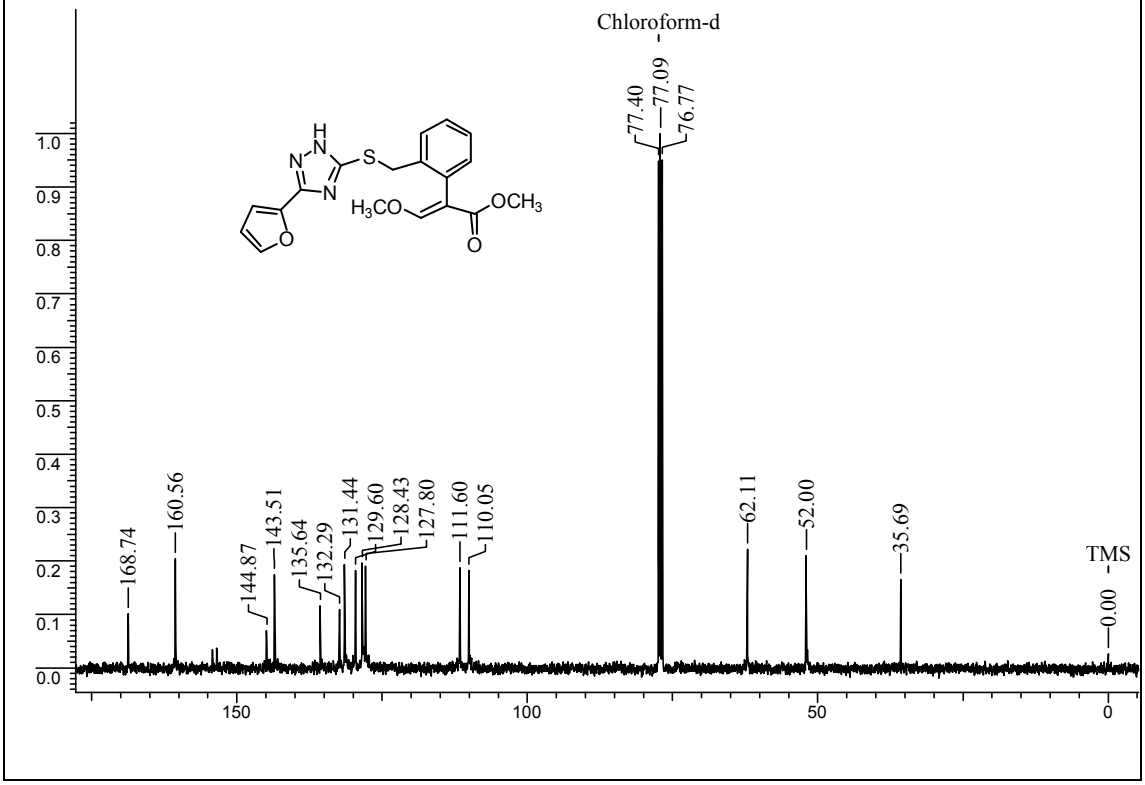
30f: ^{13}C NMR, 100 MHz, $CDCl_3$



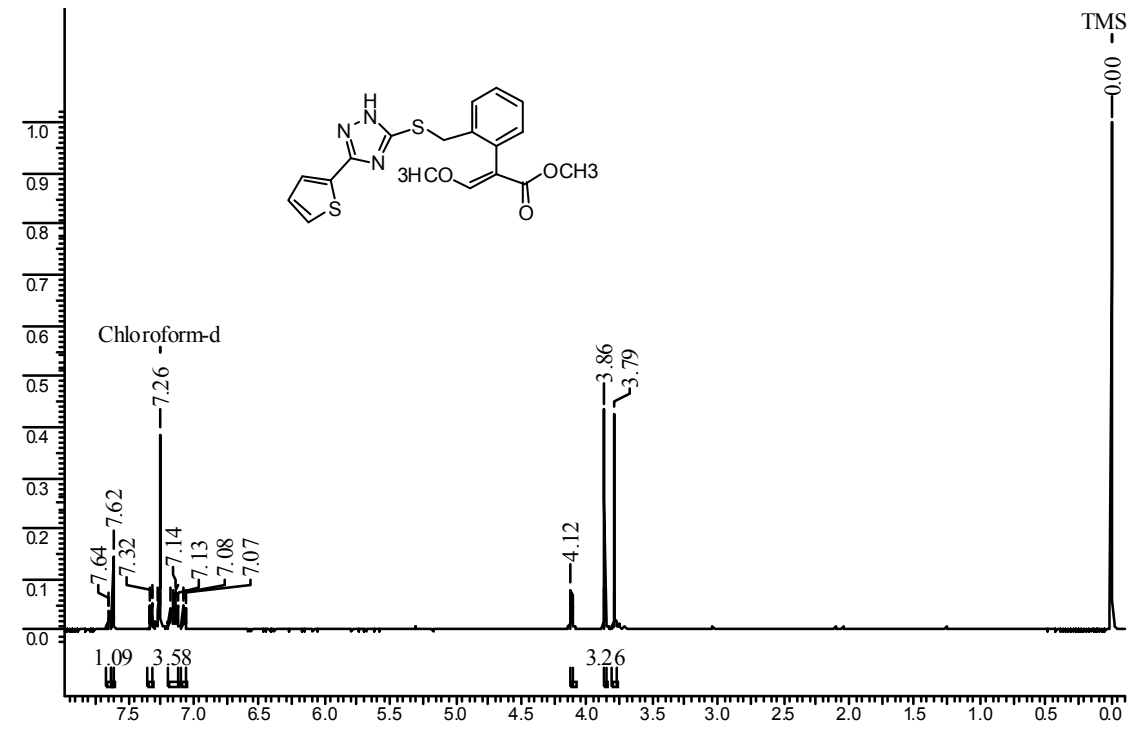
30g: ^{1}H NMR, 400 MHz, $CDCl_3$



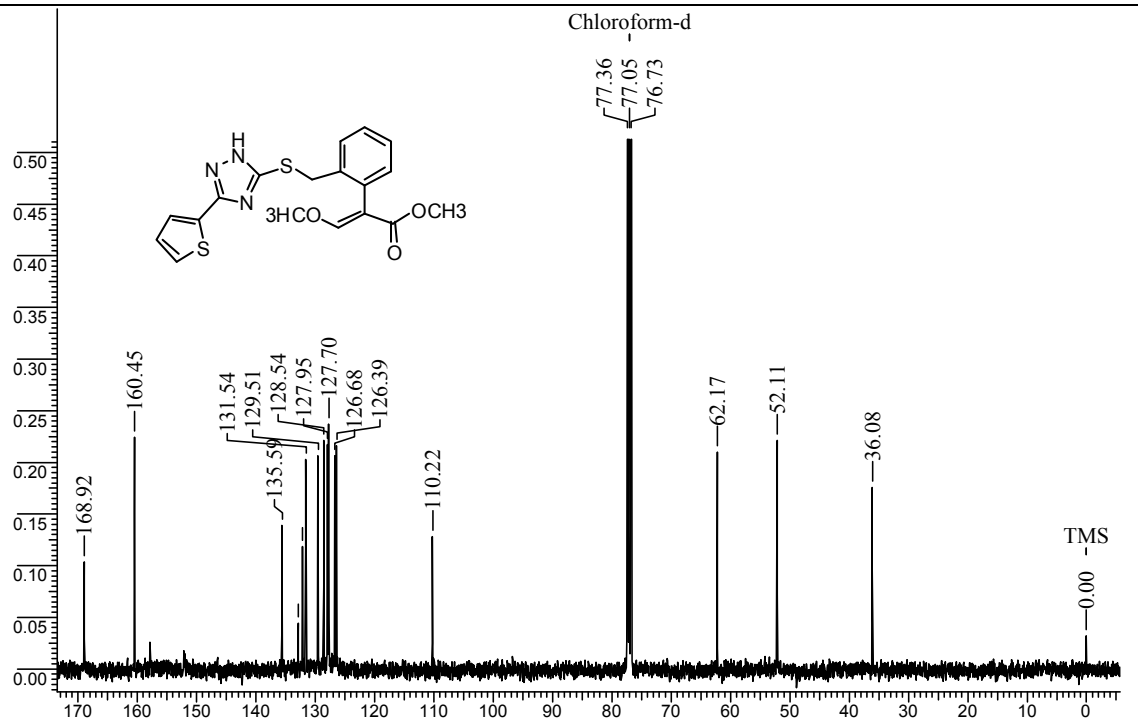
30g: ^{13}C NMR, 100 MHz, $CDCl_3$



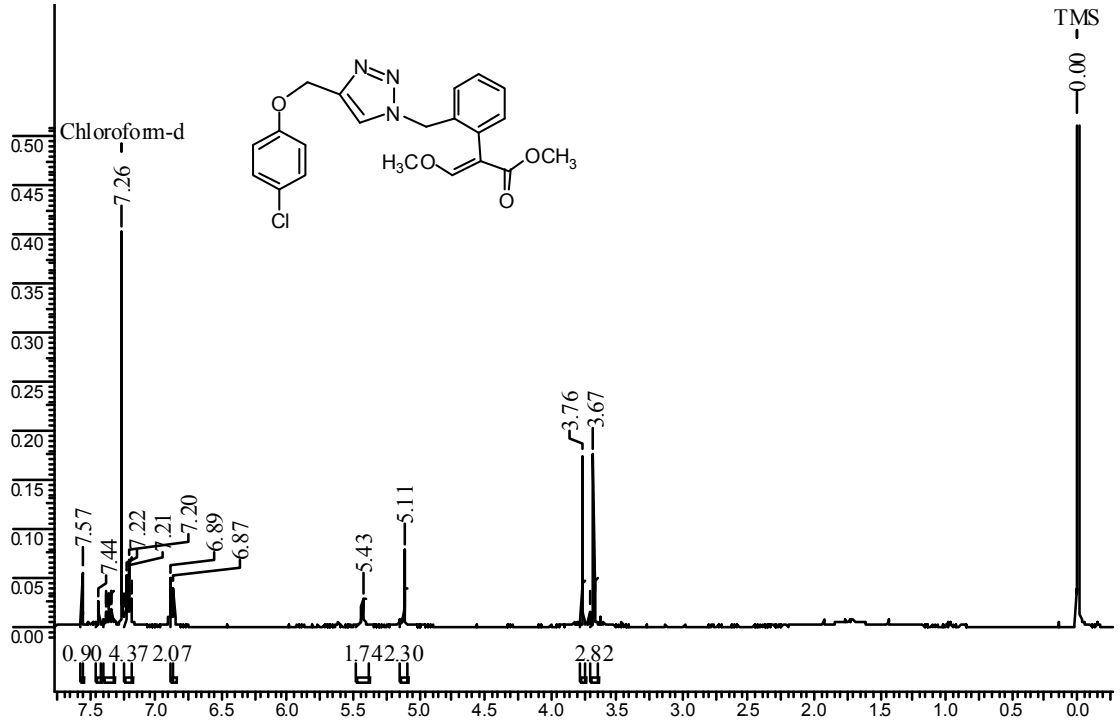
30h: ^{1}H NMR, 400 MHz, $CDCl_3$



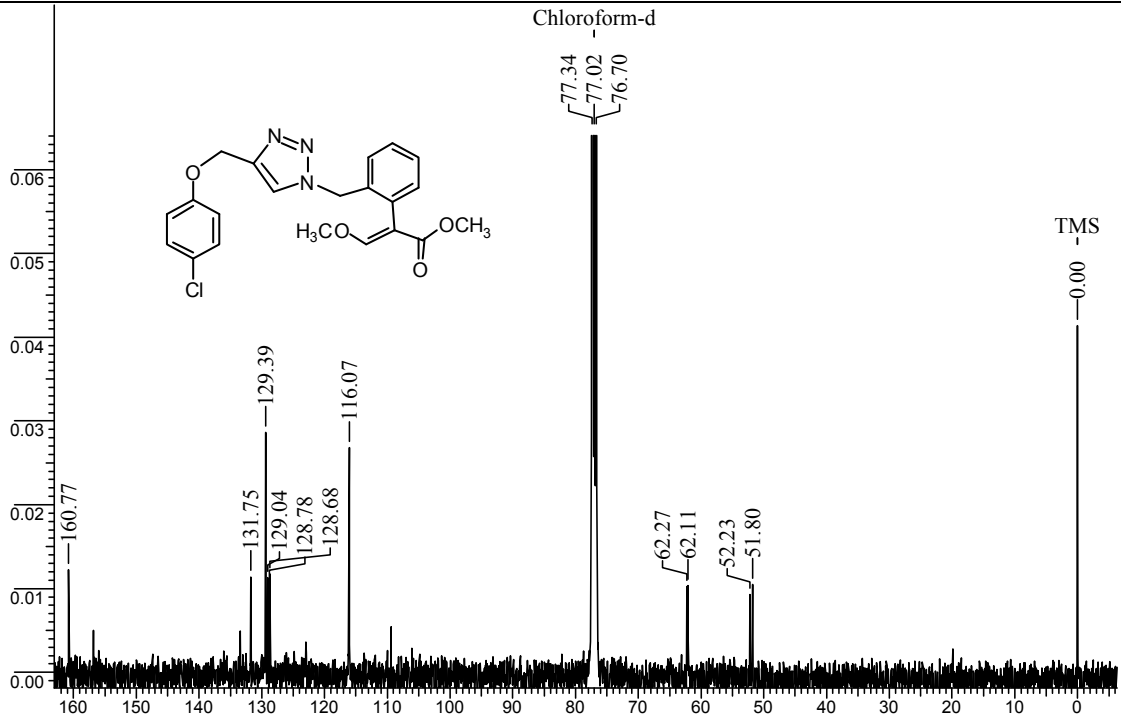
30h: ^{13}C NMR, 100 MHz, $CDCl_3$



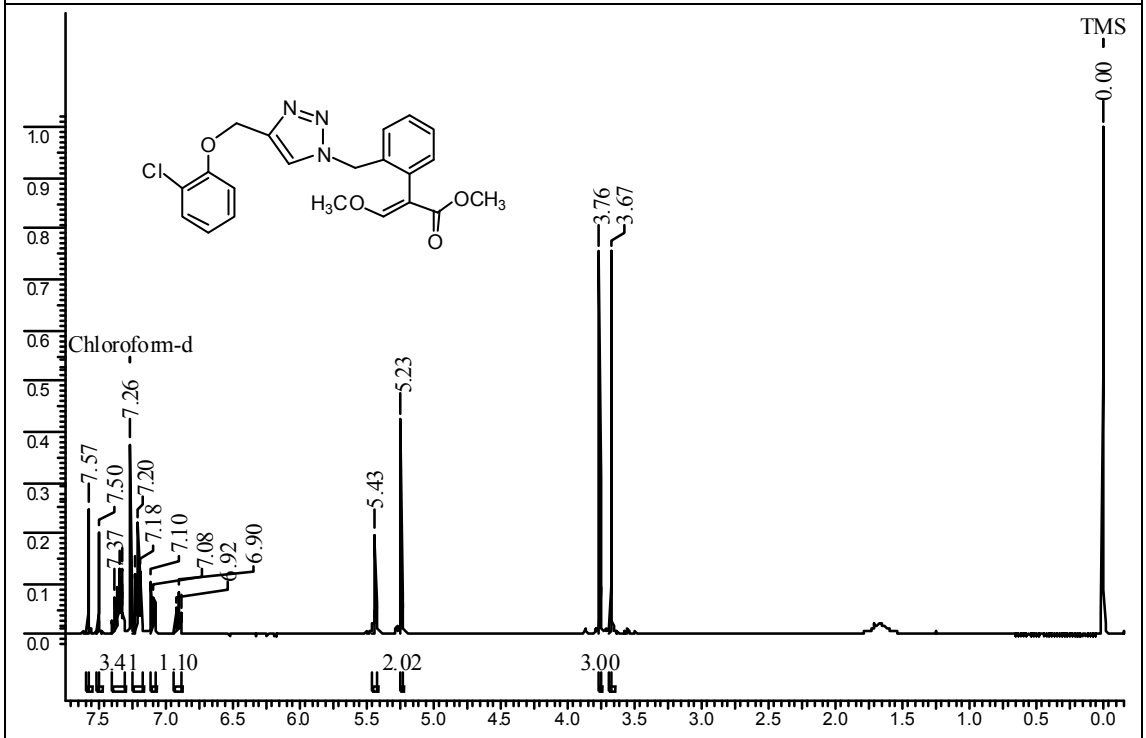
35a: ^{1}H NMR, 400 MHz, $CDCl_3$



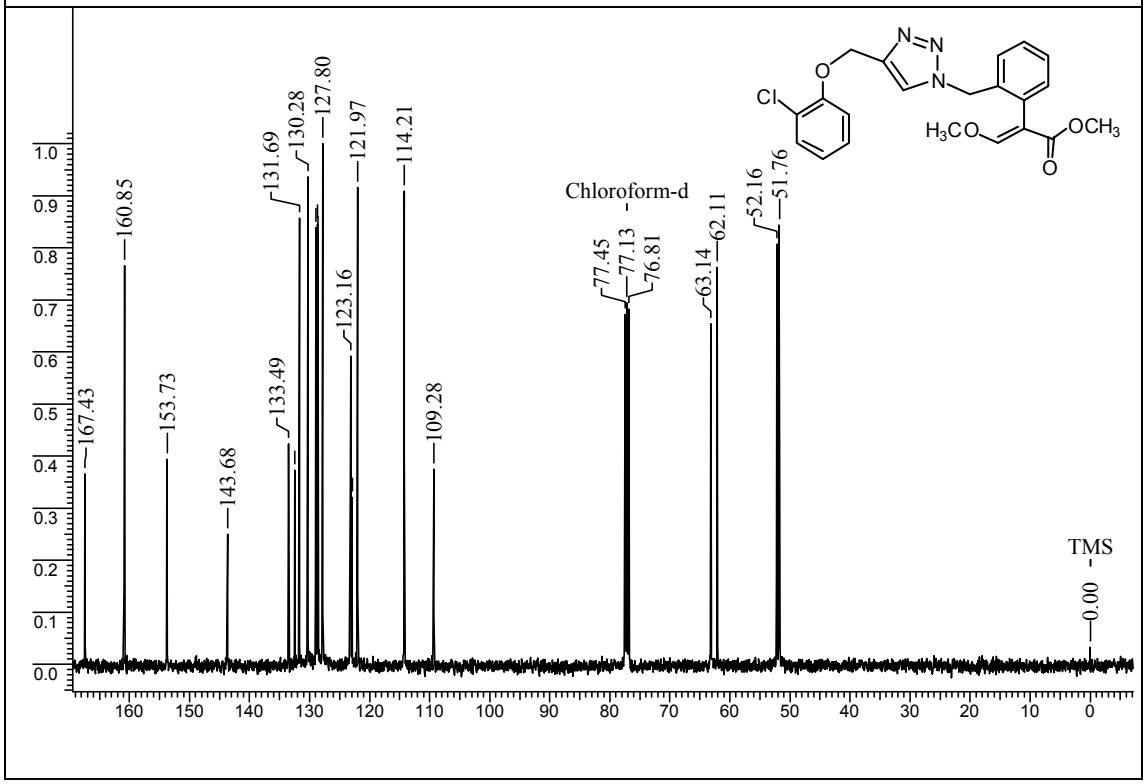
35a: ^{13}C NMR, 100 MHz, $CDCl_3$



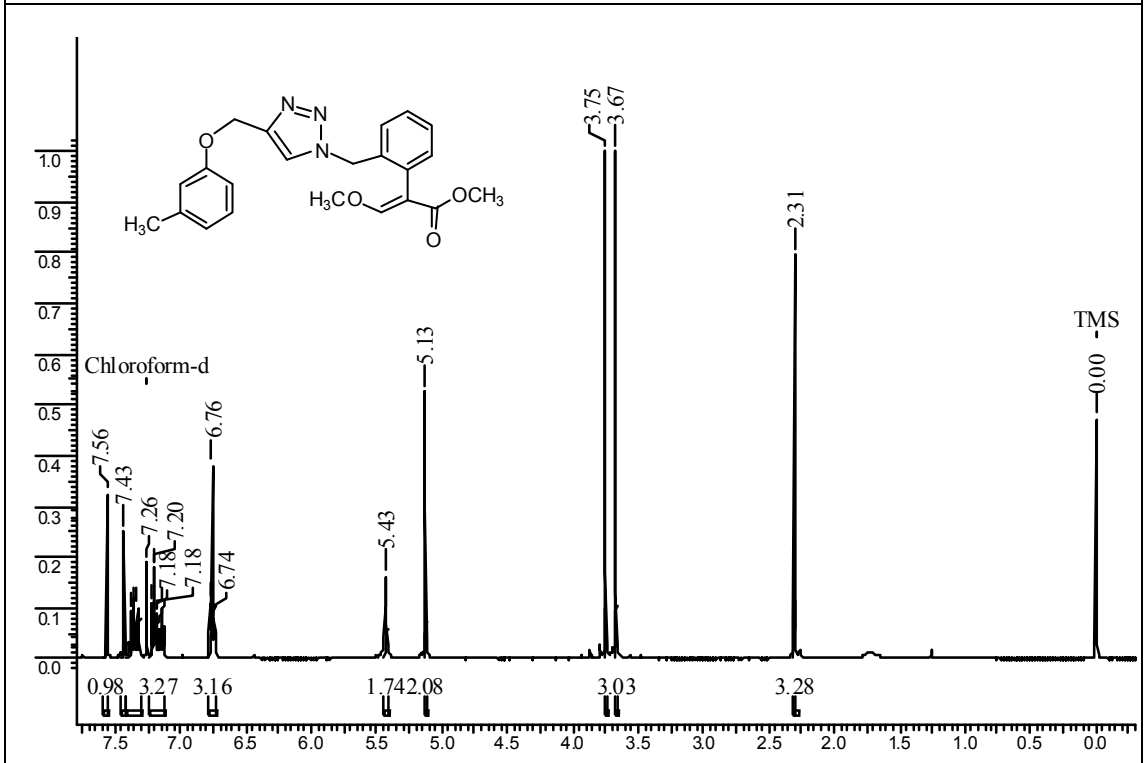
35b: ^{1}H NMR, 400 MHz, $CDCl_3$



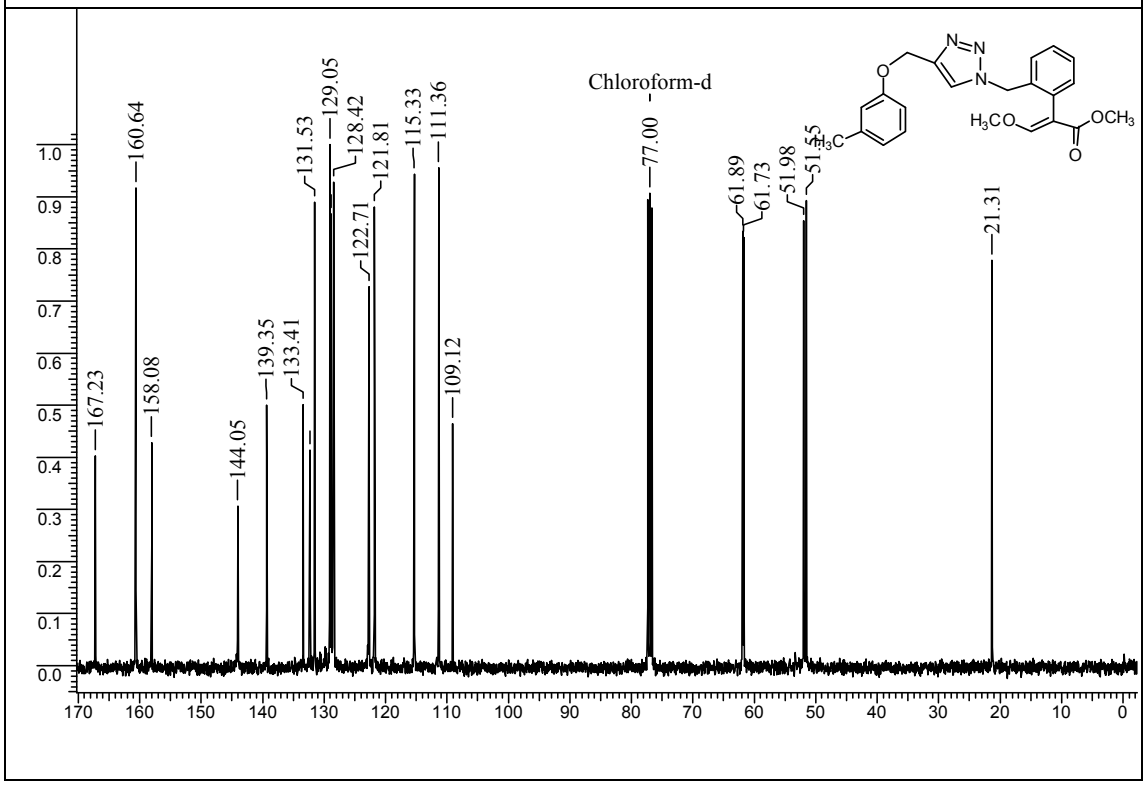
35b: ^{13}C NMR, 100 MHz, $CDCl_3$



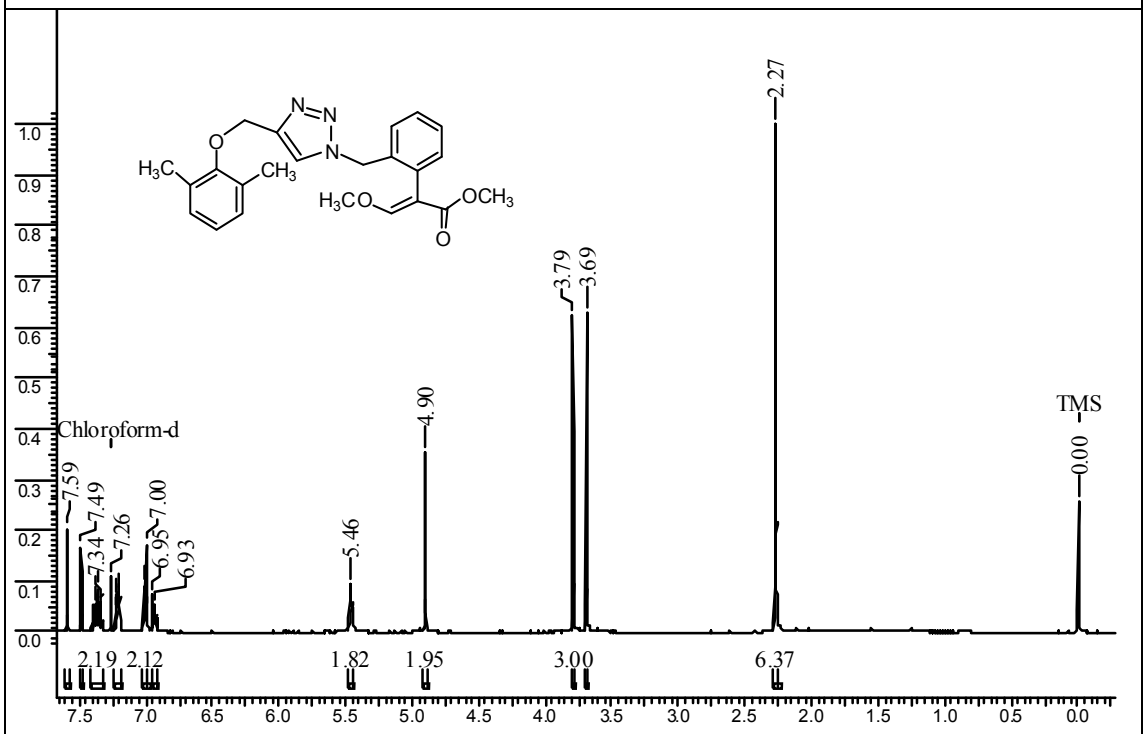
35d: ^{1}H NMR, 400 MHz, $CDCl_3$



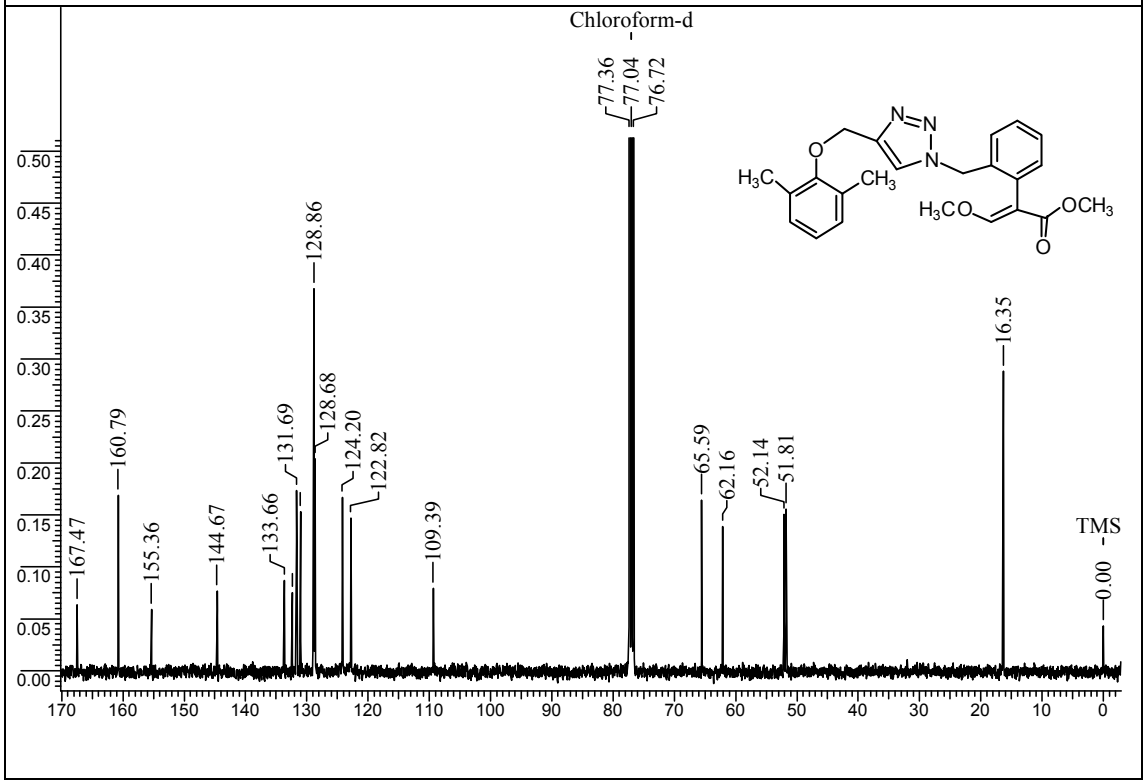
35d: ^{13}C NMR, 100 MHz, $CDCl_3$



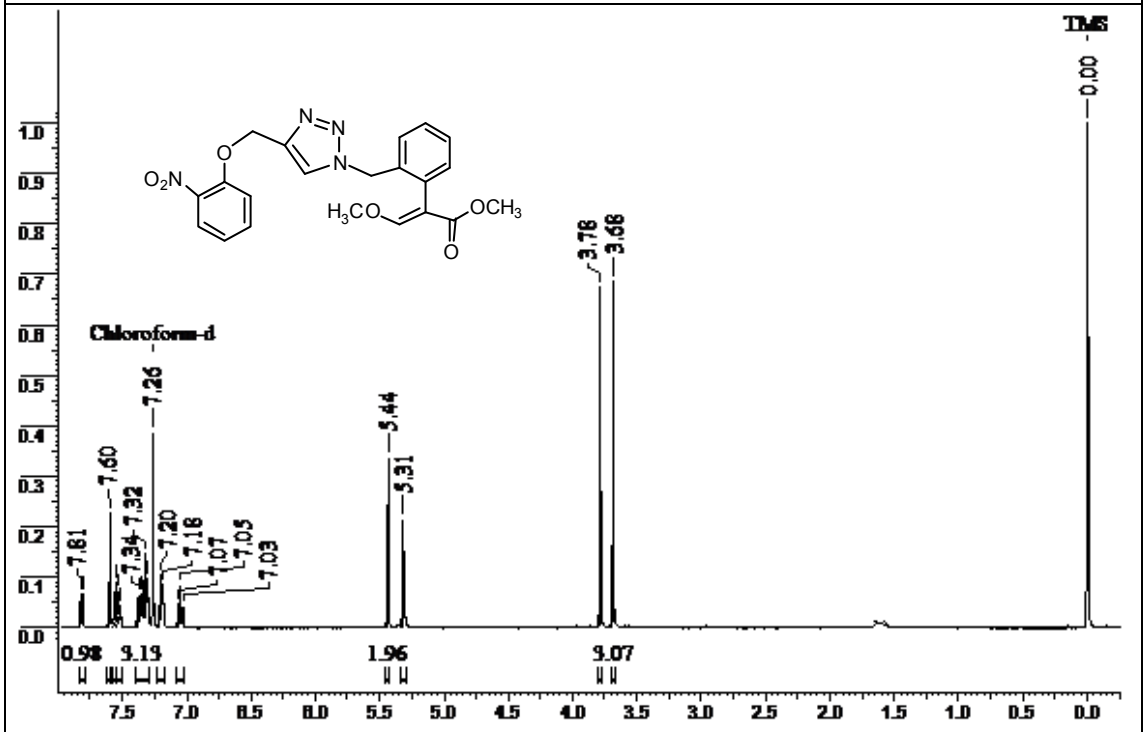
35e: ^{1}H NMR, 400 MHz, $CDCl_3$



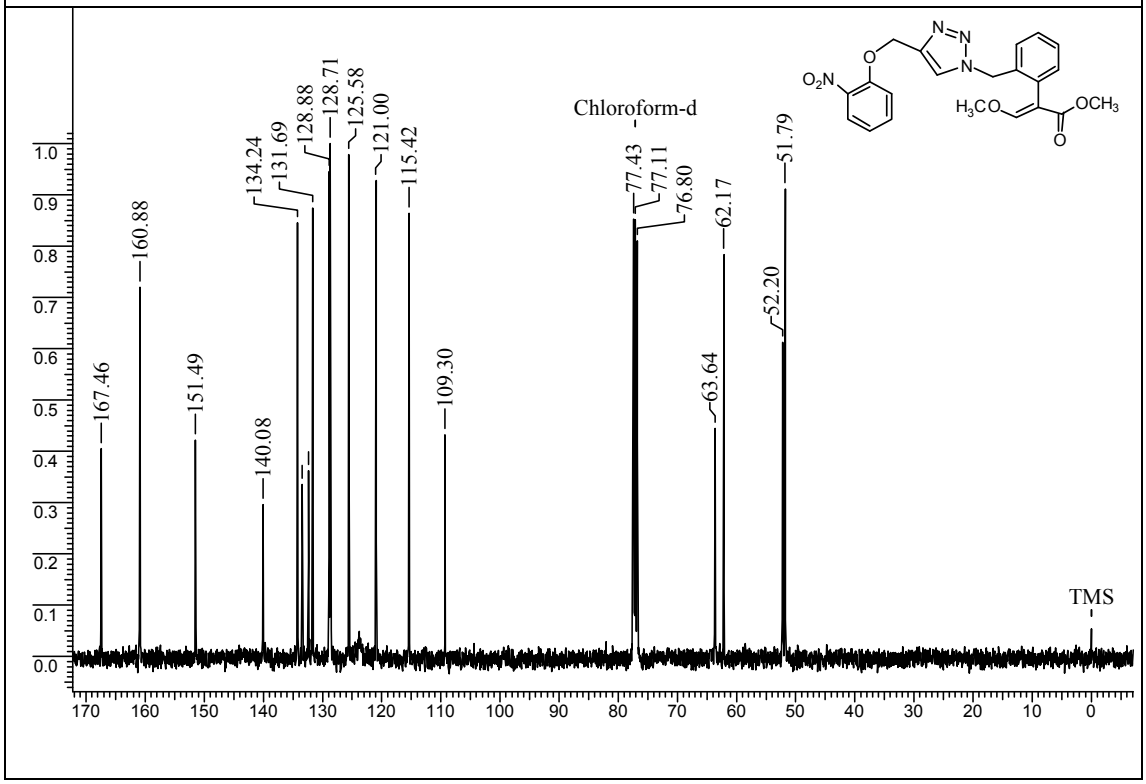
35e: ^{13}C NMR, 100 MHz, $CDCl_3$



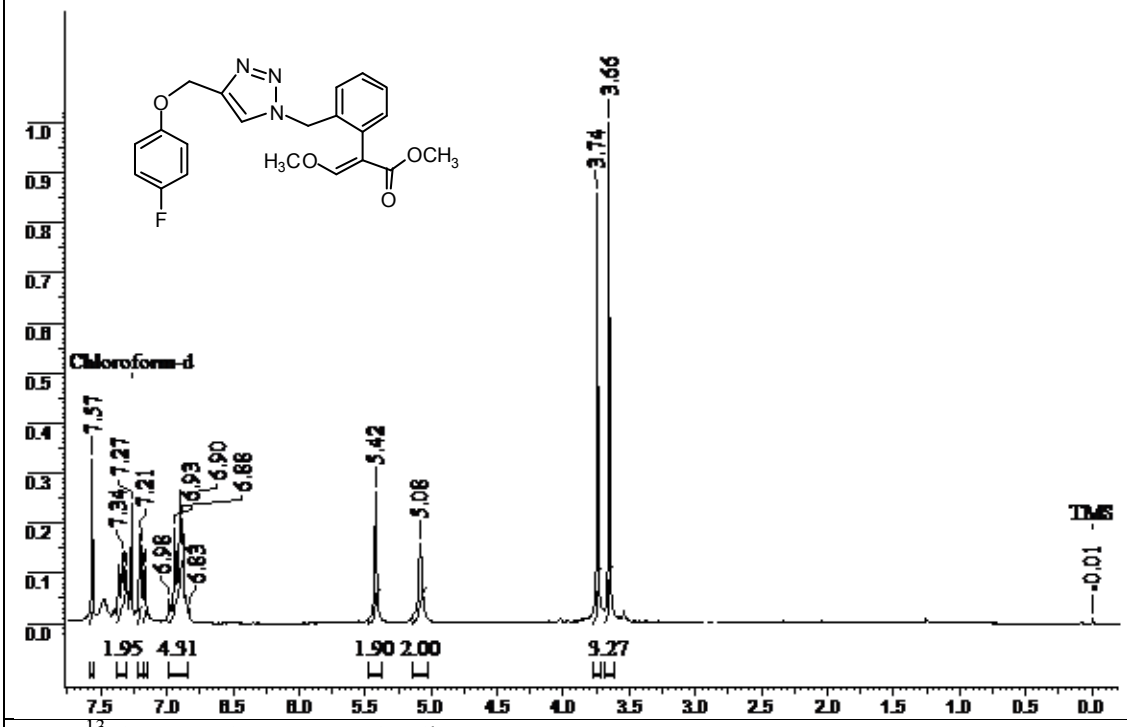
35f: ^{1}H NMR, 400 MHz, $CDCl_3$



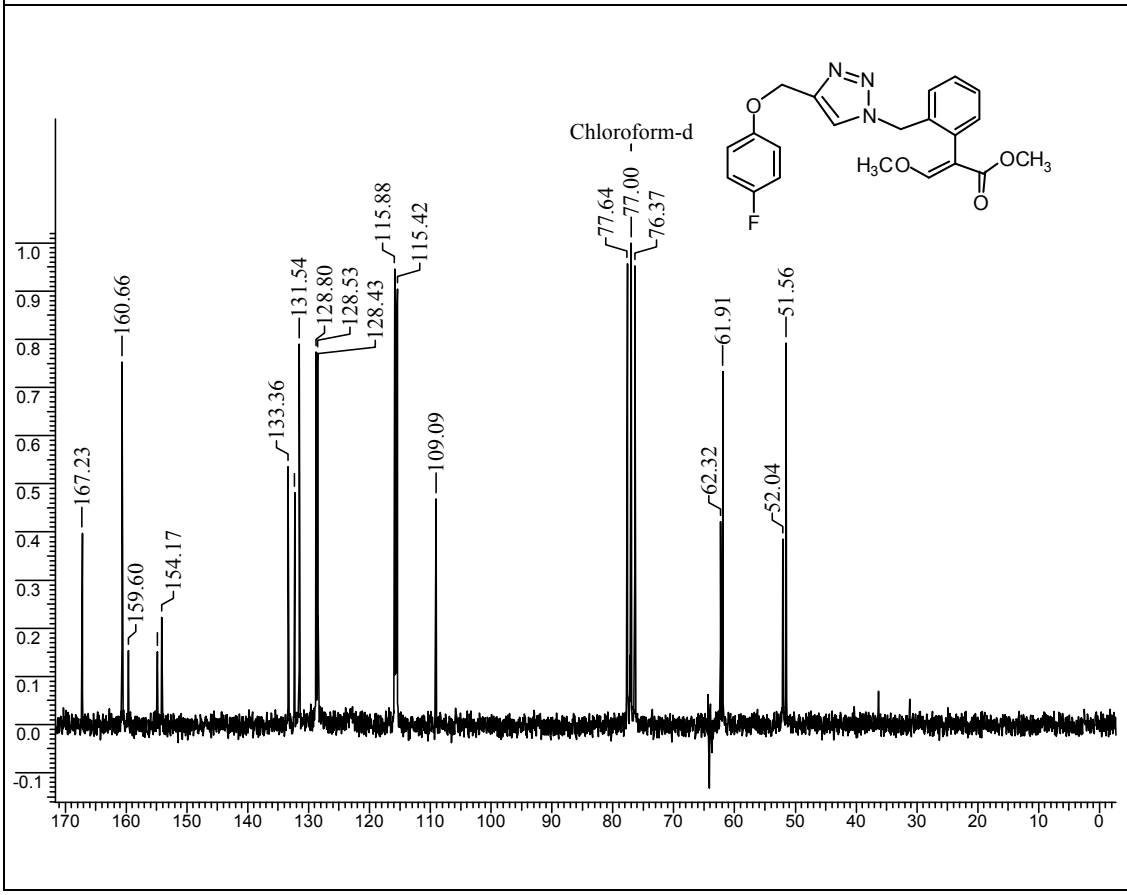
35f: ^{13}C NMR, 100 MHz, $CDCl_3$



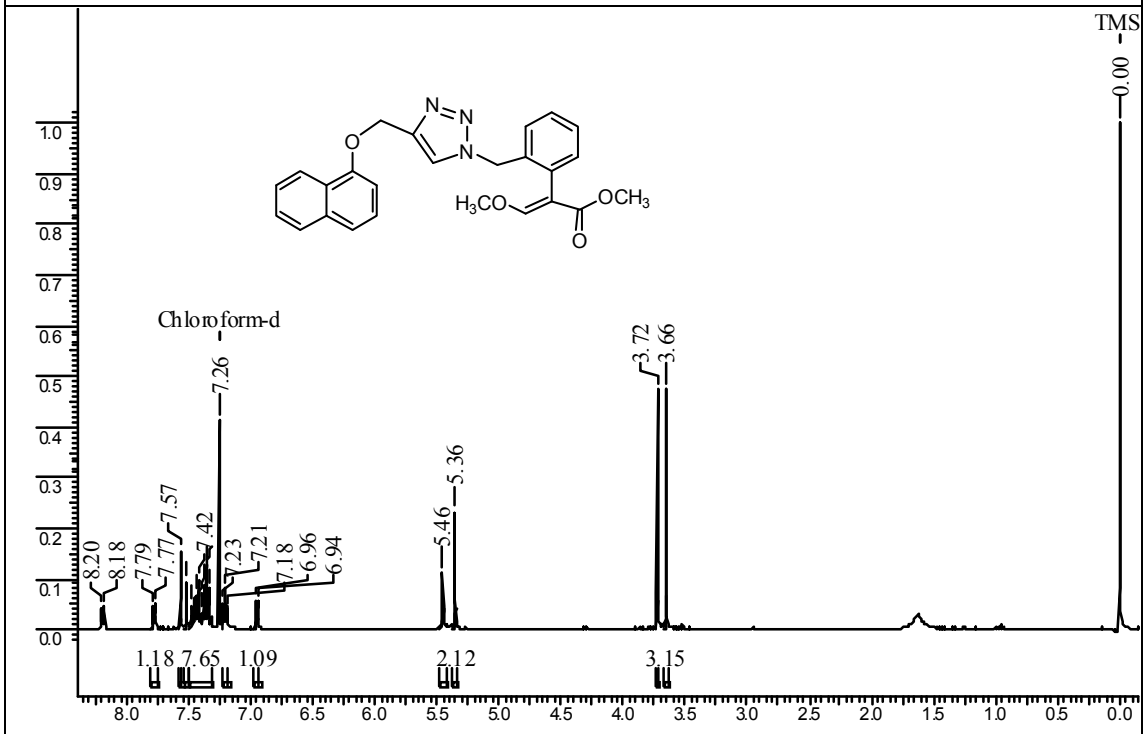
35g: ^{1}H NMR, 400 MHz, $CDCl_3$



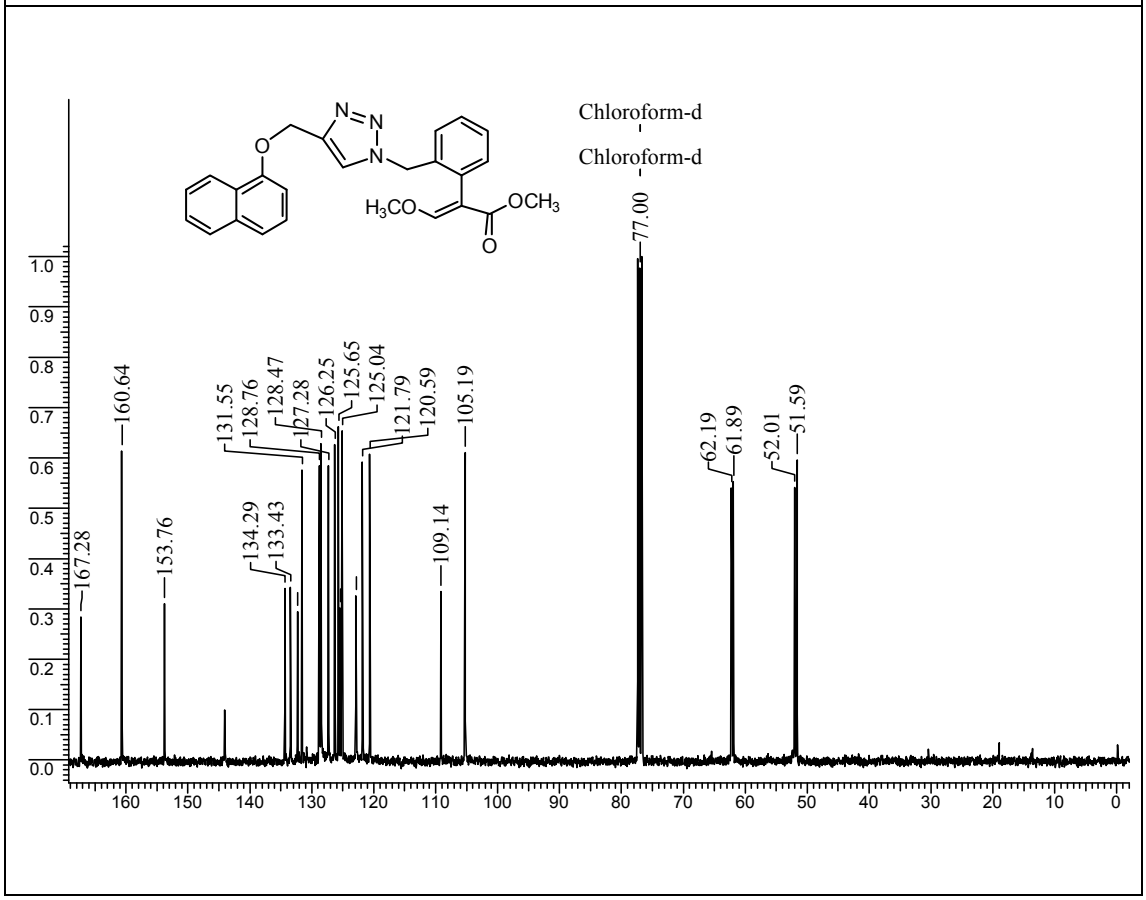
35g: ^{13}C NMR, 100 MHz, $CDCl_3$



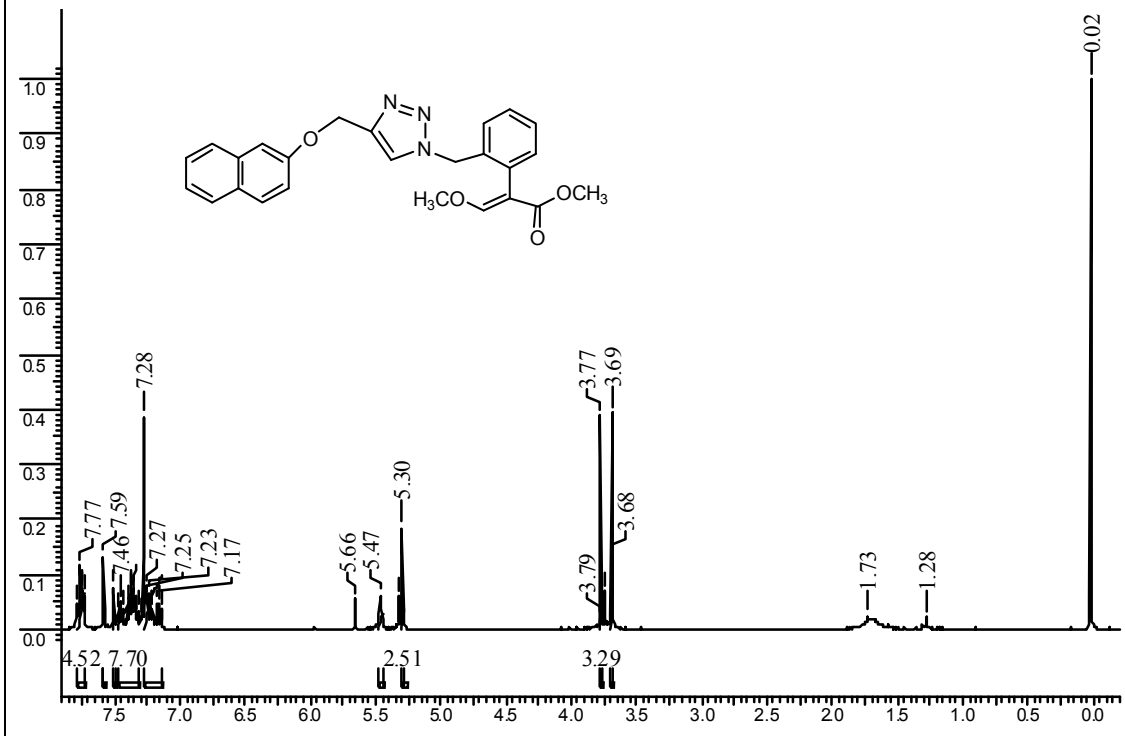
35h: ^{1}H NMR, 400 MHz, $CDCl_3$



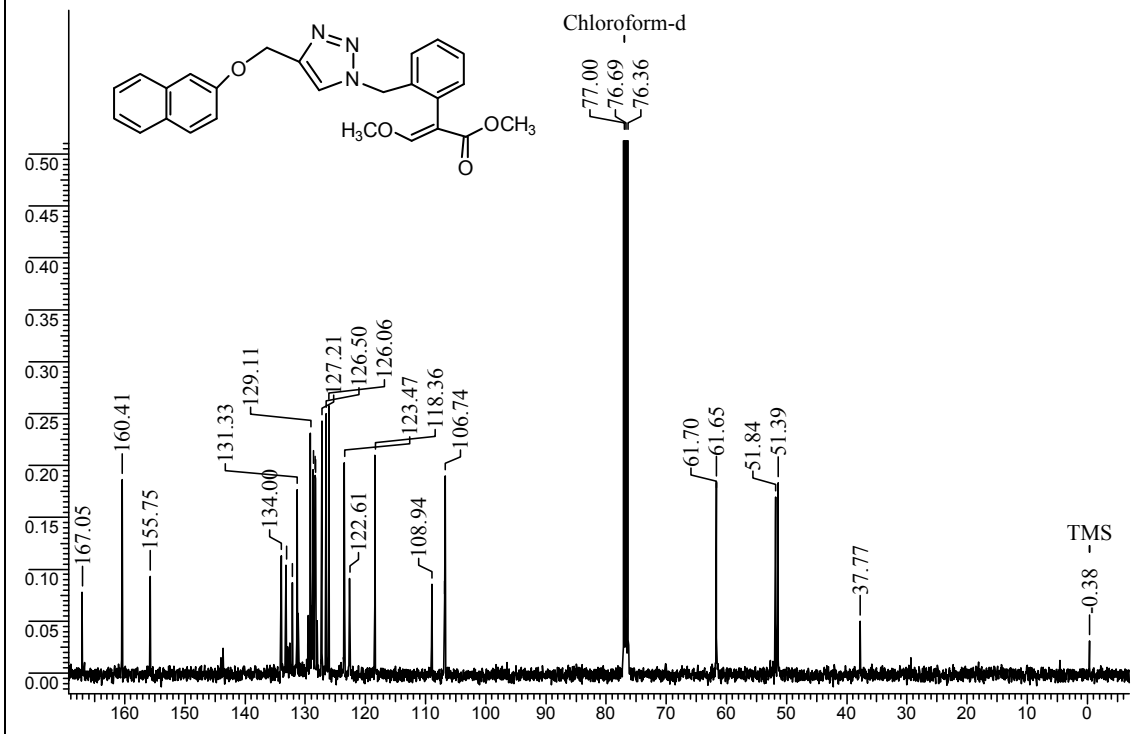
35h: ^{13}C NMR, 100 MHz, $CDCl_3$



35i: ^{1}H NMR, 400 MHz, $CDCl_3$



35i: ^{13}C NMR, 100 MHz, $CDCl_3$



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SUMMARY AND CONCLUSION

In recent years the risk of fungal diseases has increased significantly with increase in the population of the immunocompromised patients. Hence, there is a widely recognized need for new antifungal agents which will be target specific having no or minimum side effects; should target cellular processes for which there is no or little acquired resistance from the use of presently available antifungal agents. Chitin, a main structural component of fungal cell wall, is essential for growth of all fungi and is absent in plants and animals. It is formed from nucleotide UDP-GlcNAc by the action of chitin synthase (CS), a membrane bound enzyme. Therefore, targeting CS will be a promising strategy for the development of antifungal agent. Polyoxins and nikkomycins are peptidyl nucleoside CS inhibitors, isolated from the culture broth of *Streptomyces cacoi* and *Streptomyces tendae* respectively. Although polyoxins and nikkomycins exhibit activity against a variety of fungi, some polyoxins were rapidly degraded by intracellular peptidases and low uptake of nikkomycin Z by *C. albicans* cells make them unsuccessful for clinical applications. The structural features of these peptidyl nucleosides present several sites for modification. Main aim of the present study was the synthesis of CS inhibitors mainly through modification of nucleoside peptides at C5′ position by replacing peptide with 1,2,3-triazole, which may lead to safer and effective antifungals.

Substitution of 1,2,3-triazoles by replacing peptide group at 5′ position of uridine was decided as strategy for nikkomycin modification. The rationale behind use of 1,2,3-triazole was that they are present in many known antifungal agents, stable to intracellular hydrolysis and are readily taken up by the cells. For the synthesis of 1,2,3-triazole substituted derivatives Cu (I) catalyzed azide alkyne coupling was used. The alkyne part selected was different phenol propargyl ethers and propargyl esters of phenyl acetic acid. Total fourteen 1,4-substituted-1,2,3-triazolyl uridine derivatives were synthesized out of these seven were aryl ether linked 1,2,3-triazolyl uridine (**30 a-g**, chapter 2) and remaining seven were aryl ester linked 1,2,3-triazolyl uridine (**31a-f** and **32,** chapter 2). Synthesis of the derivatives **30a-g, 31a-f and 32** (chapter 2) was carried out using click chemistry approach in good yields. Structures of these derivatives were identified by IR, ^{1}H NMR, ^{13}C NMR and Mass Spectrometry. All synthesized 1,2,3-triazole linked uridine compounds **30a-g, 31a-f** and **32** were found to be active against all tested fungal human and plant pathogens with MIC values in the range of 8-128 μg/ml. Compound **31a** inhibited growth of all tested fungi with

MIC 32 μg/ml, indicating the equal sensitivity of yeast and filamentous fungi for **31a**. Compound **31d** showed MIC 32 μg/ml against *F. oxysporum* and *C. albicans*, while MIC 64 μg/ml against *C. neoformans*. Compounds **30c**, **30d**, **31a** and **31b** were found to be potent inhibitor of *C. albicans* with MIC 32 μg/ml compared to nikkomycin (64 μg/ml). The MIC values of all aryl ether linked 1,2,3-triazolyluridine derivatives **30a-g** were found to be lower than aryl ester linked 1,2,3-triazole uridine derivatives **31a-f** and **32**. This may be attributed to increase in the hydrophilicity of **31a-f** and **32** due to increase in the heteroatoms. Compounds **30b, 30c, 30g, 31a** and **31f** have profound inhibitory effect on yeast-hypha transition, exhibiting >70% inhibition at concentration 4 μg/ml. Compounds **30d, 30e, 30f** and **31f** exhibited >80% inhibition of CS activity comparable to that of nikkomycin at 4 μg/ml. Comparing the results, compounds **30a, 30b, 30d, 30f** and **31a** were identified as lead CS inhibitors for potential application in health care and agriculture.

Based on the alternating orientation of the GlcNAc residues within the chitin chain; it was proposed that CS possesses two active sites. So in such case, a bivalent inhibitor may show double/improved inhibition as compared to monomeric inhibitor. Dimeric CS inhibitors with carbamate and tartarate spacers have been previously synthesized and shown to exhibit 10-fold improvement in CS inhibition. We designed and synthesized dimeric inhibitors with aliphatic spacer using 1,2,3-triazole as a connecting unit. Total nine dimeric inhibitors (**15a-g, 17a** and **17b,** chapter 3) were synthesized using Cu (I) catalyzed azide alkyne coupling. Along with these compounds, monomeric inhibitor **22** was also synthesized. In the dimeric molecules, the spacer chain length was increased from two carbon atoms to twelve carbon atoms. These molecules failed to inhibit the growth of human and plant pathogenic fungi even at the highest concentration tested i.e. 512 μg/ml. All dimeric compounds **15a-g, 17a** and **17b** showed Y-H transition inhibition of *B. poitrasii* in the range 45-73% at 4 μg/ml concentration. For the dimeric inhibitors **15a, 15c, 15e** with 2, 4, and 6 methylene groups in the spacer chain, CS inhibition was 19-23%. Whereas, for dimers with odd number of spacer methylene groups CS inhibition was <10%. Maximum CS inhibition was observed for **15g** (29.46%) and **17b** (33.82%), wherein the spacer methylene chain length was 10 and 12 respectively (Table 3.2). This suggested the chain length and flexibility contributes to CS inhibitory activity. The CS inhibitory activity was very low for the dimers (assay concentration 250 μg/ml) as compared to

the aryl ether or aryl ester linked 1,2,3 triazole uridine derivatives (assay concentration 4 μg/ml). This loss of CS inhibitory activity on dimerization may be due to use of non polar spacer.

Monomeric inhibitor **22** (Chapter 3) showed potent growth inhibition of all *C. neoformans* strains (MIC 4-16 μg/ml) as compared to nikkomycin (MIC 32-512 μg/ml). **22** inhibited the growth of *C. albicans* strain 1 with MIC 64 μg/ml, which is comparable to that of nikkomycin (MIC value 64 μg/ml). Growth inhibition of *C. albicans* strains and *C. glabrata* by **22** was also better than nikkomycin. For **22**, CS inhibition was 57% at 250 μg/ml concentration. For phytopathogens *Magnaporthe grisae, F. oxysporum* and *Dreschlera oryzae* the observed MIC values of compound **22** were 32, 16 and 32 μg/ml, respectively. These results suggested that compound **22** in addition to CS inhibition may be acting by some other mechanism too. Propidium iodide, DCFH-DA and rhodamine 123 staining indicated that antifungal mode of action of monomeric inhibitor **22** was by generation of intracellular reactive oxygen species, disruption of fungal cell membrane and loss of mitochondrial membrane potential.

Total seventeen new strobilurin derivatives (**30a-h** and **35a-i,** Chapter 4) containing different 1,2,3-triazoles and 1,2,4-triazole thiol were synthesized with good yields. The methods used for the synthesis of the compounds were simpler. 1,2,4-Triazole thiol derivatives **30a-h** were found to inhibit the growth of different human pathogenic and phytopathogenic fungi. Compound **30c** with *t*-butyl substituent and **30e** with p-chlorophenyl substituent found to be the most potent inhibitors of transition with 75.5 and 66 % of Y-H transition inhibition respectively. 1,2,3-Triazole strobilurin derivatives **35a-i** showed 24-55% inhibition of yeast to hypha transition in *B. poitrasii* at 4 μg/ml concentration, however, no growth inhibition was observed for any of these compound even at 512 μg/ml. CS activity was inhibited in the range of 30-50 % by compounds **30a-f** at 64 μg/ml concentration. Compound **30c** with *t*-butyl substituent was found to inhibit CS activity most potently with 50.87% of inhibition. Compounds **35a**, **35d-i** showed CS inhibition in the range of 30-55%. Compound **35d** with methyl substituent at meta position in phenyl ring showed potent inhibition of CS activity (46.46%). Compound **30e** was identified as a potent inhibitor as it inhibited the growth of all tested fungi in the range of 15-62.5 μg/ml concentration along with the 38% CS inhibition at 62 μg/ml. Growth and CS activity inhibition

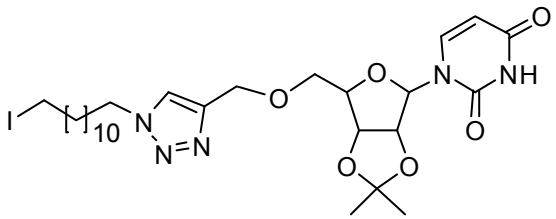
results indicated that CS inhibition is secondary mechanism for antifungal action of **30a-h**, and they may be acting primarily through respiratory inhibition similar to other strobilurins.

In conclusion, current endeavour enabled a practical, reliable and efficient synthesis of forty-one novel 1,4-disubstituted-1,2,3-tiazolyluridine, dimeric uridine derivatives and strobilurin derivatives by 'click chemistry' approach. Though dimeric uridine derivatives and 1,2,3-triazole strobilurin derivatives did not showed growth inhibition, exhibited potential for control of pathogenic fungi through inhibition of morphological transition. Most of the aryl ether and aryl ester linked 1,2,3-triazole uridine derivatives showed potent antifungal activity, CS inhibition and Y-H inhibition. Compounds **30a, 30b, 30d, 30f** and **31a** (Chapter 2) holds promise as antifungal agents with potential applications in medicine and agriculture for the control of pathogenic fungi.

Annexure I

MECHANISM OF ANTIFUNGAL ACTION OF MONOMERIC INHIBITOR (22) IN *CRYPTOCOCCUS NEOFORMANS*

Monomeric inhibitor **22** (chapter 3 and Figure I.1) showed growth inhibition of all *Cryptococcus neoformans* strains at MIC 4-16 μg/ml, whereas CS inhibition by **22** at 250 μg/ml was 57%. These results suggested towards CS inhibition as secondary effect with another mechanism as primary mode of action. To explore the mode of action of **22**, its effect on plasma membrane integrity, intracellular reactive oxygen species production and mitochondrial membrane potential we checked.



22

Figure I.1: Structure of monomeric inhibitor **22**

I.1 Results and discussion

I.1.1 Time kill kinetics

For time kill assay, *C. neoformans* cells (1×10^6 cells) were inoculated in YPG media containing monomeric inhibitor **22** (16 μg/ml). The tubes were incubated at 37 °C for 24 h. Samples were collected intermittently and CFU/ml was determined. It was observed that the number of the *C. neoformans* cells decreased rapidly after treatment with **22**. More than >99% killing of *C. neoformans* cells was observed within 6 h (Figure I.2).

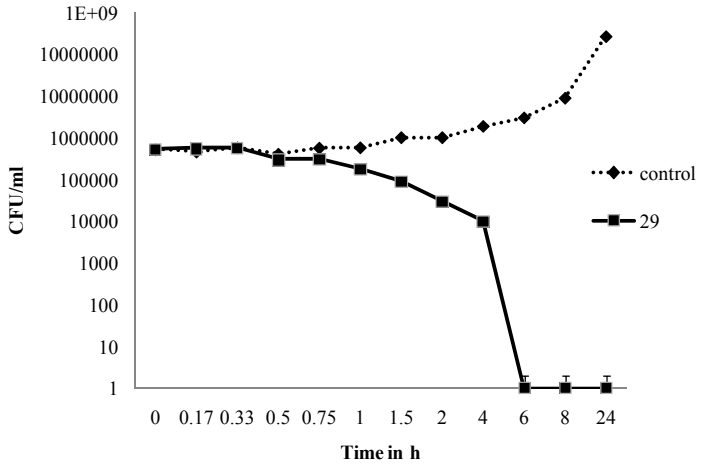


Figure I.2: Time kill assay of *Cryptococcus neoformans* by monomeric inhbitor **22**

I.1.2 Effect on plasma membrane permeability

As the compound **22** showed >99% killing of *C. neoformans* within 6 h, its effect on plasma membrane of *C. neoformans* cells was checked by propidium iodide (PI) staining. PI is a membrane impermeable, nucleic acid staining dye, which upon binding to double stranded nucleic acid gives red fluorescence (excitation at 480 nm). PI can only enter into those cells, which have permeable membrane. It was observed that with increase in the concentration of monomeric inhibitor **22**, number of PI stained cells increased (Figure I.3). No PI fluorescence was observed when *Cryptococcus* cells were incubated with PI alone.

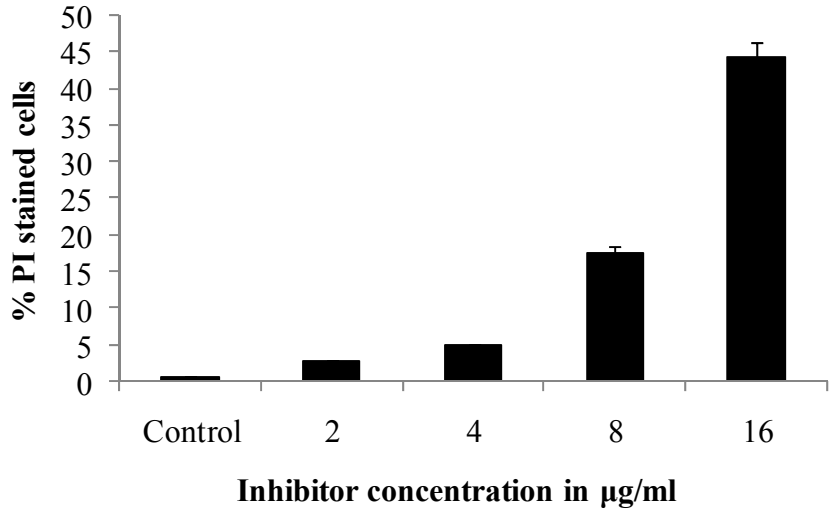


Figure I.3: Effect of monomeric inhibtior **22** on plasma membrane integrity

Rast *et al.* have reported that amphotericin B and nystatin (polyenes acting by disruption of plasma membrane) with MIC in the range of 1-4 µg/ml in *Mucor rouxii* inhibited its CS activity at 10 µg/ml concentration [1]. This inhibition of CS may be because of existence of binding sites specific to polyenes on the chitosome and these sites must be playing important role in the operation of CS. Compound **22** inhibited 57% of CS activity in *B. poitrasii* at 250 µg/ml concentration which is higher than its MIC (15.6 µg/ml), along with this compound **22** showed plasma membrane damage at 16 µg/ml.

I.1.3 Reactive oxygen species generation

Fluorescence based assays such as 2′,7′- dichlorofluorescein diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR123) staining were used to monitor the

generation reactive oxygen species (ROS) in the *Cryptococcus* cells after incubation with compound **22**. The cell-permeant dye DCFH-DA and DHR123 are oxidized by ascorbic acid, peroxinitrite and hydroxyl radicals (OH•) to yield the fluorescent molecule 2′,7′- dichlorofluorescein and rhodamine123.

C. neoformans cells were incubated with different concentrations of compound **22**, and then treated with DCFH-DA or DHR123 for 30 min. Fluorescence resulting from oxidation of the dye DCFH-DA and DHR123 were observed at 540 nm. It was found that with increasing concentration of compound 22, the relative fluorescence increased (Figure I.4 and I.5). This clearly indicated production of intracellular ROS.

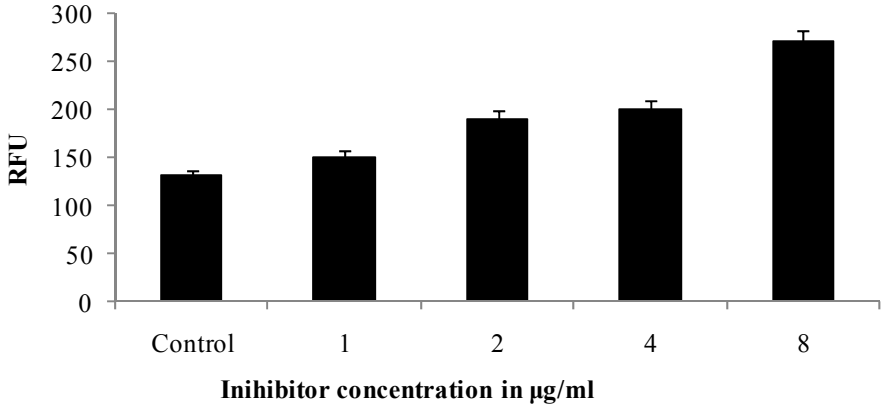


Figure I.4: Effect of monomeric inhibitor **22** on ROS production using DCFH-DA

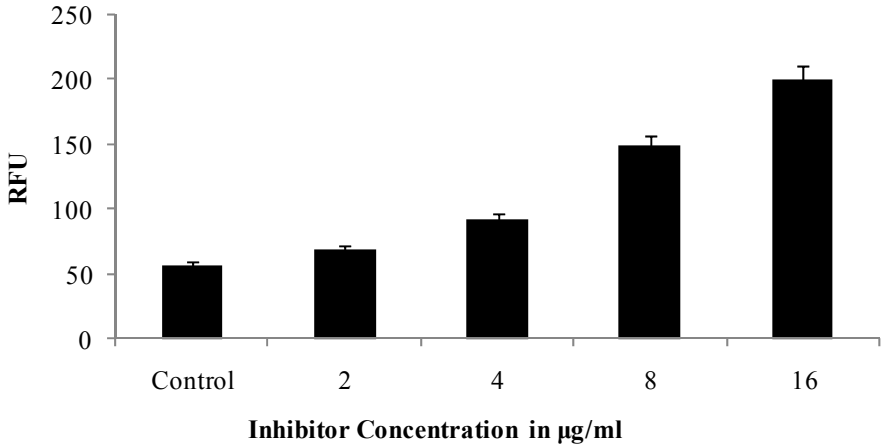


Figure I.5: Effect of monomeric inhibitor **22** on ROS production using DHR 123

Sangalli-Leite *et al.* reported that amphotericin B showed killing in *C. neoformans* by strong intracellular oxidtative burst before affecting plasma membrane [2]. These results suggested that amphotericin B showed its effect on *C. neoformans*

through different killing mechanisms, which indicates that gaining resistance to this agent is a difficult as it requires accumulation of several mutations in genome. This might be the reason for low clinical resistance seen to this antifungal drug. Kobayashi *et al.* reported that endogenous reactive oxygen species (ROS) production takes place when fungi were treated with miconazole [3]. They measured ROS production in *C. albicans* and found that the ROS production increased with increase in the concentration of miconazole. Same group has also reported that fluconazole induces ROS production in *C. albicans*. Miconazole showed actin mediated mitochondrial dysfunction leading to ROS generation in *S. cerevisae* [4]. Compound **22** also contains 1,2,3-triazole moiety which might be responsible for generation of intracellular ROS.

I.1.4 Effect on mitochondrial membrane potential

Effect of **22** on mitochondrial membrane potential was checked by rhodamine 1,2,3 staining [5]. It was observed that fluorescence increased with increase in the concentration of **22**, suggesting that **22** led to hyperpolarisation of mitochondrial membrane potential (Figure I.6).

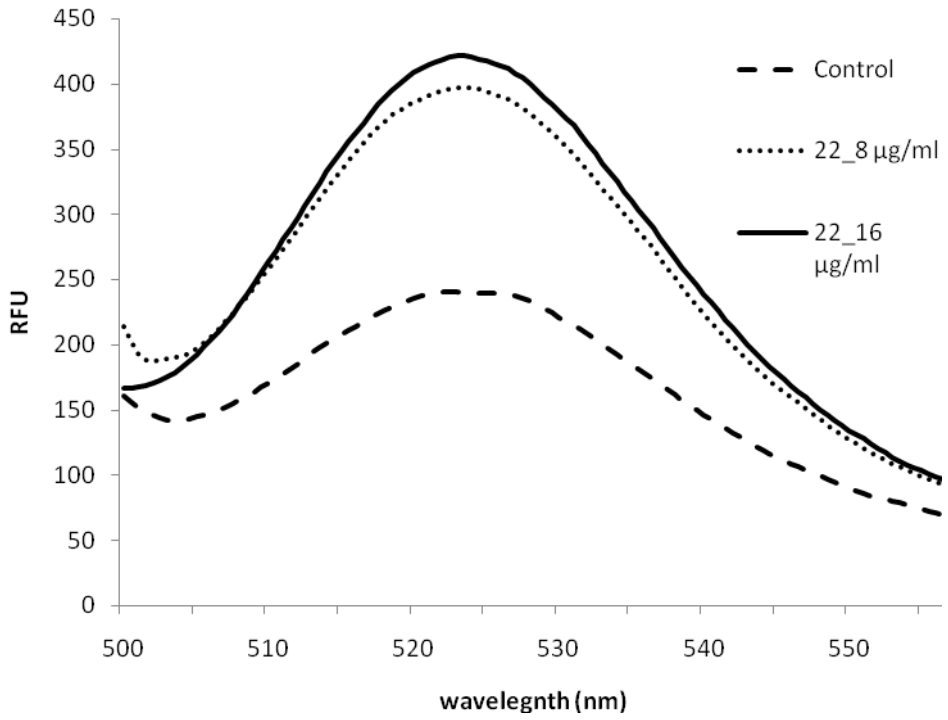


Figure I.6: Effect of monomeric inhibitor **22** on mitochondrial membrane potential

It is well known that accumulation of ROS causes oxidative damage of mitochondrial proteins and lead to the collapse of mitochondrial membrane potential. It has been reported that hydrogen peroxide causes strong oxidative burst inside the cell, which leads to disruption of mitochondrial membrane potential [6]. Helmerhorst *et al.* have reported that antifungal peptides Histatin 5 showed its strong antifungal action in *C. albicans* by perturbation of plasma membrane for internalization in the cells followed by disruption of mitochondrial membrane potential [7]. Similarly in case of compound **22,** perturbation of plasma membrane followed by generation of intracellular ROS, disruption of the mitochondrial potential led to killing of *C. neoformans* cells.

I.1.5 Haemolysis assay

Toxicity of compound **22** was assessed by using RBC haemolysis assay. The range of concentrations checked was 4-256 μg/ml. It was found that at 128 μg/ml concentration only 13% haemolysis was observed (Table 1).

Table I.1: Haemolysis assay for monomeric inhibitor **22.**

Concentration of inhibitor in μg/ml	**% Haemolysis**	
	Compound 22	**Amphotereicin B**
256	99.9	100
128	13	100
64	2.5	97.44
32	1.62	96.98
16	0.6	94.51
8	0.19	91.93
4	0	50.00

On the other hand, similar acting drug, amphotericin B at 8 μg/ml concentration showed 91.93% haemolysis. The fungicidal concentration of **22** was 4-16 μg/ml, at which no RBC haemolysis was observed.

I.2 Conclusions

The monomeric inhibitor **22** exhibited anticandidal and anticryptococcal activity. Antifungal mode of action of monomeric inhibitor **22** was by cell membrane

damage, generation of intracellular reactive oxygen species and loss of mitochondrial membrane potential, which was similar to polyenes and cationic amphipathic antimicrobial peptides such as melittin.

I.3 Experimental section

I.3.1 Time kill assay

C. neoformans cells (1×10^6 cells/ml) were inoculated in YPG medium containing inhibitor (4x MIC) and incubated at 37 °C. At pre-determined time points 0, 5, 10, 15, 30, 45 and 60 min, 100 µl aliquot was removed, serially diluted in saline and spread plated on YPG agar plates. Colony counts were determined after incubation at 37 °C for 24 h.

I.3.2 Membrane integrity assay

PI staining was used for checking of fungal plasma membrane integrity after inhibitor treatment. *C. neoformans* cells were harvested at the logarithmic phase and 1×10^6 cells/ml were added in phosphate buffer saline (PBS, 0.1 mM, pH 7.2) containing inhibitor. The tubes were incubated at 37 °C for 1 h. Cells were separated by centrifugation and washed with PBS. Cells were then incubated with 1.49 µM of PI for 10 min, harvested by centrifugation, washed (using PBS) and suspended in PBS. PI stained cells were counted using epifluorescence microscope (Leitz Labor Lux, Germany). The excitation and emission wavelengths used were 488 nm and 520 nm respectively.

I.3.3 Measurement of reactive oxygen species (ROS) production

I.3.3.1 Dihydrorhodamine (DHR123) staining

Dihydrorhodamine 123 is the reduced form of rhodamine 123, commonly used as a fluorescent mitochondrial dye. Dihydrorhodamine 123 itself is nonfluorescent, but it readily enters cells and gets oxidized by reactive oxygen species (ROS) to fluorescent rhodamine 123 that accumulates in mitochondrial membranes. Free intracellular radicals were detected with DHR123 (Sigma). The DHR 123 staining was carried out according to the reported procedure by Wysocki and Korn [8]

1×10^7 cells of *C. neoformans* were inoculated in PBS buffer containing different concentrations of inhibitor and incubated at 37 °C for 60 min. After

completion of incubation, cells were harvested by centrifugation and washed with PBS. 5 µg/ml DHR123 was added (from a 2.5 mg/ml stock solution in ethanol) to the cells resuspended in PBS and tubes were further incubated for 1 h. Cells were separated by centrifugation. Cell pellet was washed and resuspended in PBS. Cells were observed for fluorescence with excitation and emission wavelengths of 500 nm and 550 nm respectively.

I.3.3.2 2′,7′-dichlorofluorescein diacetate staining

Amount of ROS generated was measured by fluorometric assay with DCFH-DA as described earlier [3]. Briefly, $1X10^7$ cells of *C. neoformans* were inoculated in PBS containing inhibitor and incubated at 37 °C for 60 min. After completion of incubation, 10 µM of DCFH-DA was added and incubated for 30 min. Cells were separated by centrifugation. Cell pellet was washed with PBS and resuspended in PBS. The fluorescence intensities (excitation 485 nm and emission 540 nm respectively) of the resuspended cells were measured with a Spectrofluorometer.

I.3.4 Mitochondrial membrane potential by rhodamine 123 staining

Assay of the electrochemical potential was carried out as reported previously [9]. *C. neoformans* cells ($1X10^7$/ml) were inoculated in PBs buffer containing inhibitor and incubated at 37 °C for 1 h. Cells were harvested by centrifugation and washed with PBS. To the suspension of the cells in PBS, 2 µM Rh123 (rhodamine 123) was added and incubated for 30 min. Cells were washed and then resuspended in PBS. Mitochondrial electrochemical potential was expressed as the fluorescence intensity of Rh123, which was read through excitation at 480 nm and emission at 530 nm.

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List of Publications based on Ph. D. work

Research papers:

1. **Chaudhary P. M.**, Tupe S. G. and Deshpande M. V. (2012) Chitin synthase inhibitors as antifungal agents. Accepted for publication in *Mini-rev. Med. Chem.*
2. **Chaudhary P.M.**, Chavan S.R., Shirazi F., Razdan M., Nimkar P., Maybhate S.P., Likhite A.P., Gonnade R., Hazara B.G., Deshpande M.V and Deshpande S.R (2009) Exploration of click reaction for the synthesis of modified nucleosides as chitin synthas inhibitors. *Bioorg. Med. Chem.* 17:2433-2440.

Poster presentations

1. **Chaudhary P. M.**, Tupe S. G. and Deshpande M. V. Synthesis of an antifungal triazolyl uridine (TU) derivative: Study of it's mode of action in *Cryptococcus neoformans.* Poster was presented in International Conference on Yeast Biology held at IIT Bombay during December 10-13, 2011.
2. **Chaudhary P. M.**, Deshpande S. R., Natu A. A. and Deshpande M. V. Design and Synthesis of New Dimeric Chitin Synthase Inhibitors. The poster was presented in AMI conference 'The Third Golden Era of Microbiology' held at National Chemical Laboratory, Pune during December 15-18, 2009.
3. **Chaudhary P. M.,** Chavan S., Likhite A. P., Maybhate S. P. and Deshpande S. R. Search for novel heteryl substituted polyoxin fungicides. Poster was presented in Research scholar meet held at National Chemical Laboratory, Pune during February 27-28, 2006.

Other research papers published:

1. Pete U., Zade C., Bhosale J., Tupe S. G., **Chaudhary P. M**, Dikundwar A., Bendre R. (2012) Hybrid Molecules of Carvacrol and Benzoyl Urea/Thiourea with Potential Applications in Agriculture and Medicine. Accepted for publication in *Bioorg. Med. Chem. Lett.*
2. Maurya I. K., Pathak S., Sharma M., Sanwala H., **Chaudhary P. M.**, Tupe S. G., Deshpande M. V., Chauhan V. S. and Prasad R. (2011) Antifungal activity of novel synthetic peptides by accumulation of reactive oxygen species (ROS) and disruption of cell wall against *Candida albicans*. *Peptides* 32:1732–1740.

3. Deshpande S.R., Maybhate S.P., Likhite A.P. and **Chaudhary P.M.** (2010) A facile synthesis of *N*-substituted maleimides. *Indian J. Chem.* 49B: 487-488.

4. **Chaudhary P. M.**, Chavan S. R., Kavitha M., Maybhate S. P., Deshpande S. R., Likhite A. P. and Rajamohanan P. R. (2008) Structural elucidation of propargylated products of 3-substituted-1,2,4-triazole-5-thiols by NMR techniques. *Magn. Reson. Chem.* 46: 1168–1174.

Patent

1. Deshpande S. R., Deshpande M. V., Shirazi F., **Chaudhary P. M.**, Sharma M. B., Nath N., Rao N. M., Kumar B. A., Kaliannan G., Sanjoy P., Raj K., Rao B. V., Bodhanrao G. B., Reddy V. V. N, and Singh Y. J. (2010**)** Substituted 1,4-dioxa-8-azaspiro[4,5]decanes useful as fungicides and a process for the preparation thereof. International publication number WO 2010/109299 A2.

Poster presentations

1. **Chaudhary P. M.**, Shirazi F., Tupe S. G. and Deshpande M. V. Antifungal β-lactam–bile acid conjugates linked via triazole inhibits ornithine decarboxylase enzyme and induce apoptosis in *Benjaminiella poitrasii.* Poster was presented in Research scholar meet held at National Chemical Laboratory, Pune during February 28-29, 2012.

2. Likhite A. P., **Chaudhary P. M.**, Chavan S. R., Deshpande S. R., Jogdand G., Maybhate S. P. and Rajamohanan P. R. Synthesis & characterization of bitriazolyl methyl compounds by NMR studies. Poster was presented in 11th CRSI National Symposium in Chemistry held at National Chemical Laboratory, Pune during February 6-8, 2009.

3. Likhite A. P., Maybhate S. P., **Chaudhary P. M.**, Chavan S. R. and Deshpande S. R. Study of regioisomerism in propargylation of 1,2,4-triazol-3-thiols and their conversion to 1,2,3-triazole derivatives by click chemistry. Poster was presented in National Seminar on Frontiers in Organic Chemistry held at University of Calicut during January 11-12, 2007.

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Exploration of click reaction for the synthesis of modified nucleosides as chitin synthase inhibitors

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ABSTRACT

Click reaction approach toward the synthesis of two sets of novel 1,2,3-triazolyl linked uridine derivatives **19a–19g** and **21a–21g** was achieved by Cu(I)-catalyzed 1,3-dipolar cycloaddition of 5′-azido-5′-deoxy-2′,3′-*O*-(1-methylethylidene)uridine (**17**) with propargylated ether of phenols **18a–18g** and propargylated esters **20a–20g**. Structure of one of the representative compound **19d** was unambiguously confirmed by X-ray crystallography. Chitin synthase inhibition study of all these compounds **19a–19g** and **21a–21g** was carried out to develop antifungal strategy. Compounds **19d, 19e, 19f**, and **21f** were identified as potent chitin synthase inhibitors by comparing with nikkomycin. Compounds **19a, 19b, 19c, 19d, 21a**, and **21b** showed good antifungal activity against human and plant pathogens. Compounds **19a, 19b, 19f, 21c, 21f**, and **21g** were identified as lead chitin synthase inhibitors for further modifications by comparing results of inhibition of growth, % germ tube formation and chitin synthase activity.

Recently Zhou et al. have reported the synthesis of 1,2,3-triazole functionalized thymidines **1**, **2**, and **3** (Fig. 1), which were found to be antiviral.[1] Synthesis of triazole linked coumarin nucleoside conjugates **4** and **5** (Fig. 1) was documented by Kosiova et al.[2] These materials have wide applications as fluorescent probes and signaling units. Solid phase synthesis of 1,2,3-triazolyl uridines **6** (Fig. 1) from 5′-azido uridine and mono substituted and carboxy-substituted alkyne components was reported by Epple et al.[3] The synthesis of 4-substituted triazolyl-nucleosides **7** and **8** (Fig. 1) showing cytostatic activity on CEM tumor cell lines was reported by Akri et al.[4]

The chitin, β-1,4-linked *N*-acetylglucosamine polymer, is the main structural component of the fungal cell wall. As it is absent in plants and mammals, targeting the enzyme chitin synthase involved in the synthesis of chitin could be one of the eco-friendly strategies for the control of plant pathogenic fungi in agriculture.[5] Discovery of polyoxins **9** (Fig. 2) which are peptidyl nucleoside antibiotics, isolated from an actinomycete, *Streptomyces cacaoi* in the sixties by Isono and co-workers[6,7] has opened a new era in the field of development of novel fungicides.[8–10] Naturally occurring antifungal uridine nucleosides such as polyoxins **9**, nikkomycin Z (NZ) **10** (Fig. 2) and uridine di-phosphate (UDP)[11] are known to be chitin synthase inhibitors. The inhibitory activity of these molecules is due to their structural similarity to UDP-*N*-acetyl-D-glucosamine, a substrate for chitin synthase. Therefore most of these compounds possess uridine as a basic constituent with peptide side chain. Modifications for enhanced biological activities at uracil moiety, ribose ring and/or phosphate chain, as well as in the peptide side chain of NZ have been reported in the literature.[12,13]

Phenols and phenyl acetic acids are present in a large number of biologically active compounds. Phenolic ether linkages are present in many naturally occurring bioactive molecules like exophilic acid **11** inhibiting HIV-integrase,[14] methyl-4-[[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy]-3-hydroxybenzoate **12** (Fig. 3) which is antitumoral, antimicrobial and antioxidative too.[15] Along with these some synthesized compounds like C_3-symmetric triantennary *N*-acetyl-(1–6)-β-D-glucosamine octadecaoligosaccharide derivatives **13** (Fig. 3) containing phenolic ether linkage showed good antitumor activity.[16]

Compounds containing phenyl acetic acid ester linkages such as methyl-2-acetyl-3,5-dihydroxy phenyl acetate **14** possesses antibacterial activity,[17] some comb sugar polymers **15** (Fig. 3) are useful in protein–carbohydrate interaction study.[18]

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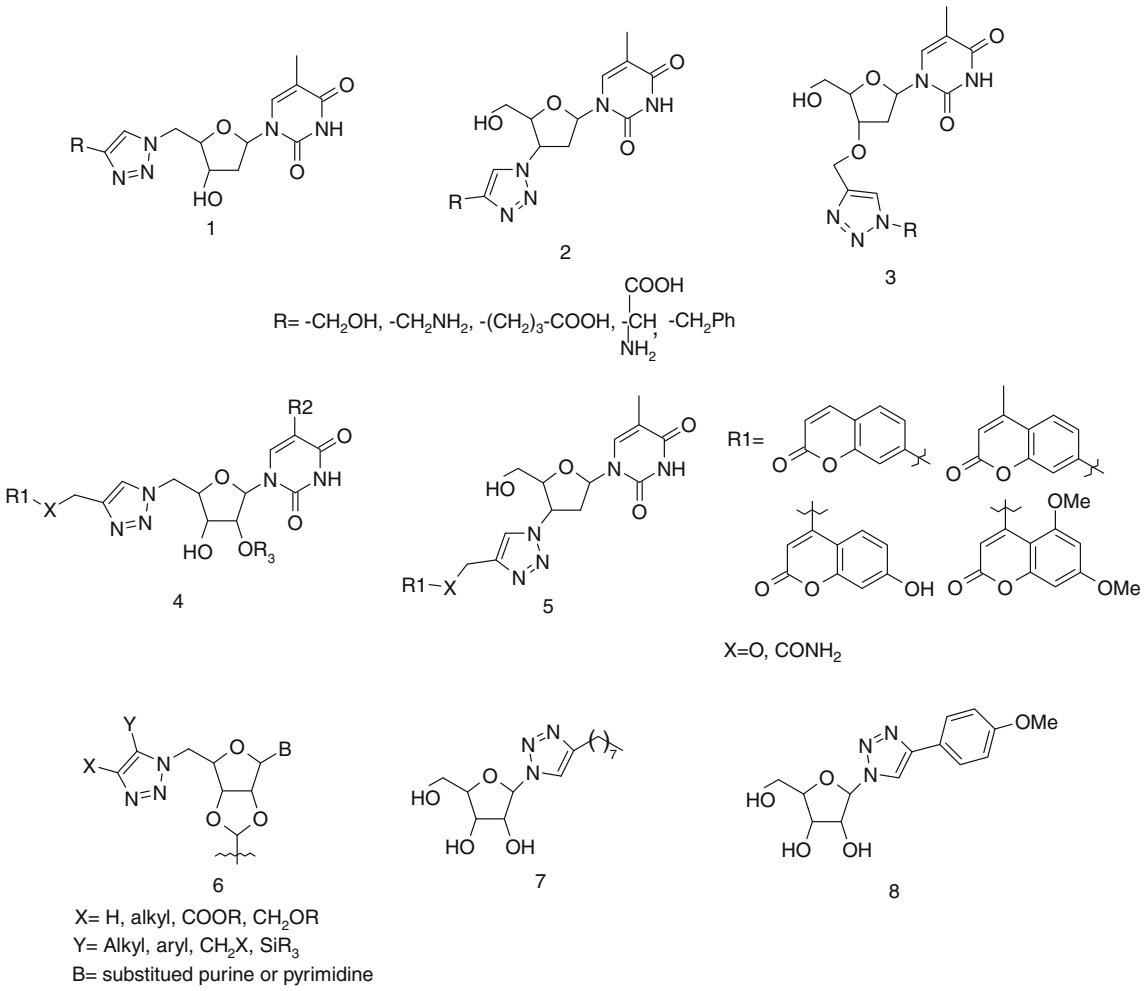


Figure 1. Synthetic nucleoside analogues.

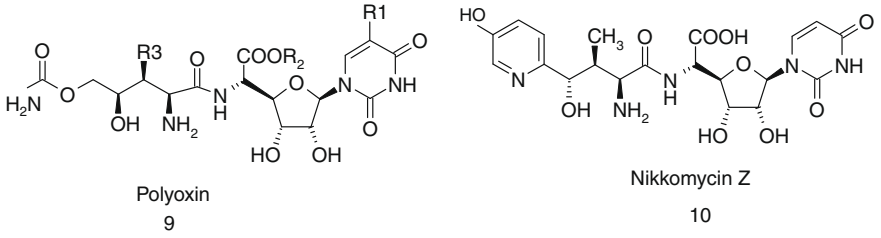


Figure 2. Structure of naturally occurring antifungal uridine nucleosides.

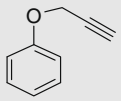
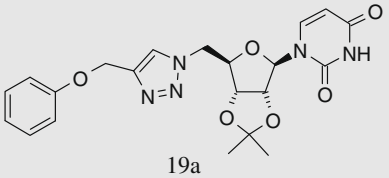
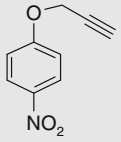
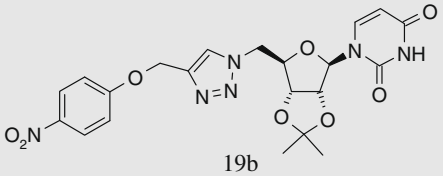
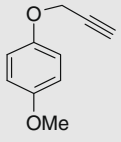
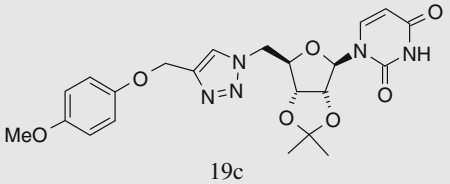
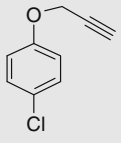
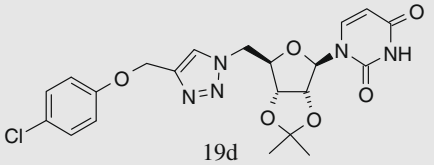
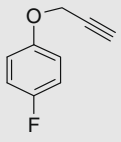
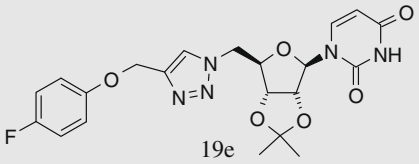
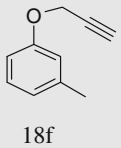
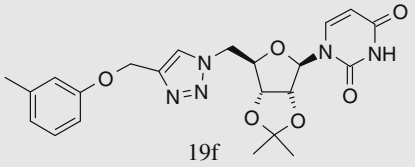
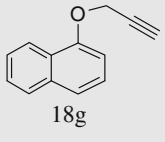
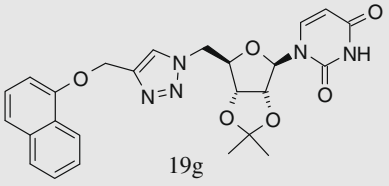
In the present investigations modification in uridine nucleosides with 1,2,3-triazoles substituted at 5′ position of uridine was chosen. The design for this target was based on replacing the peptide bond of nikkomycin **10** with triazole moiety. 1,2,3-Triazole unit may be considered as a surrogate for a peptide bond as these triazoles have atom placement and electronic properties similar to the peptide bond.[19] It was also established that 1,2,3-triazole cores interact strongly with biological targets through hydrogen bonding to nitrogen atoms and their large dipole moments.[20] The construction of 1,2,3-triazole from substituted azide and alkyne derivative in presence of Cu(I) catalyst using click chemistry proved to be powerful tool in a biomedical research, ranging from combinatorial chemistry and target-template in situ chemistry, for lead discovery, to bioconjugation strategies for proteomics and DNA research.[21]

Synthesis of targeted 1,2,3-triazole substituted uridine derivatives **19a**–**19g** and **21a**–**21g** was achieved using Cu(I)-catalyzed Sharpless click chemistry[22] approach from 5′-azidouridine **17** and propargyl derivatives of phenols **18a**–**18g** (Table 1) and propargyl derivatives of acids **20a**–**20g** (Table 2). The addition of Cu(I) catalyst strongly activates terminal acetylenes toward 1,3-dipole in organic azides, exclusively forming the 1,4-disubstituted regio-isomer.

Thus compound **17** was obtained from compound **16** by using reported procedure with a 95% yield (Scheme 1).[23]

Propargyl ethers of phenol were prepared by the reaction of phenols with propargyl bromide and K_2CO_3 in DMF. Propargyl esters were synthesized in excellent yield by heating acids in excess of propargyl alcohol using catalytic amount of *p*-toluenesulfonic acid (*p*-TSA) at 80 °C. These propargylated compounds were then reacted with 5′-azidouridine **17** in the presence of copper sulfate

Table 1
Synthesis of 1,2,3-triazole derivatives of uridine by click reaction between 5′-azidouridine **17** and propargyl ethers **18a–18g**

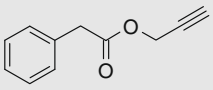
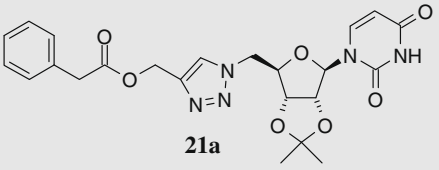
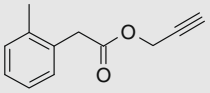
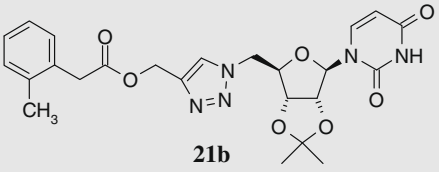
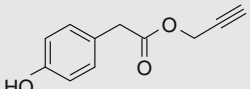
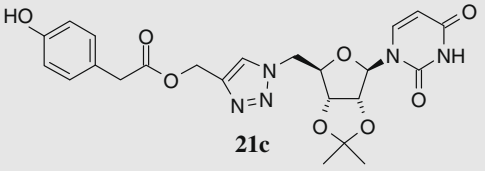
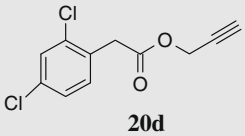
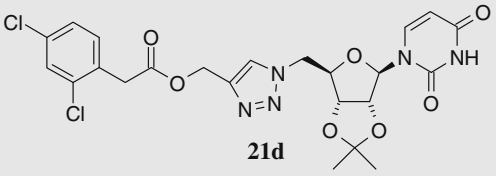
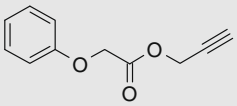
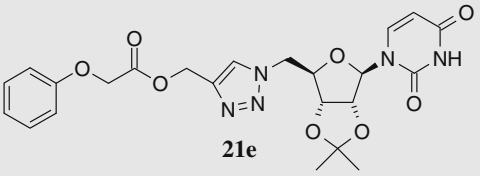
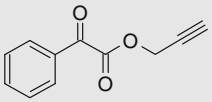
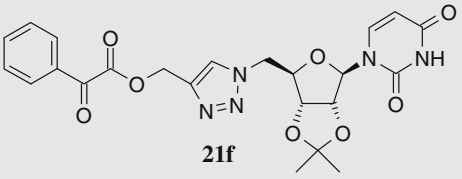
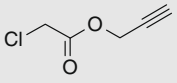
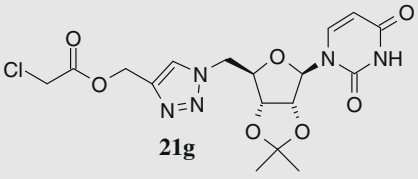
Sr. No	ArO	Product	Yield (%)
1	 18a	 19a	56
2	 18b	 19b	69
3	 18c	 19c	71
4	 18d	 19d	65
5	 18e	 19e	70
6	 18f	 19f	73
7	 18g	 19g	83

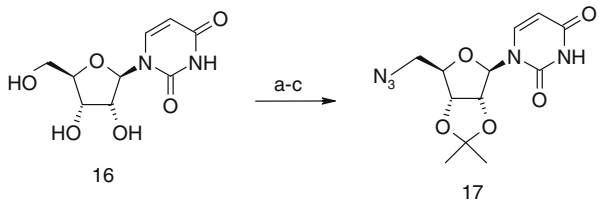
and sodium ascorbate in *t*-butanol/water (8:2) to yield exclusively 1,4-disubstituted-1,2,3-triazoles at 5′ position of uridine (Schemes 2 and 3 and Tables 1 and 2).[33] The structures of final compounds **19a–19g** and **21a–21g** were confirmed by IR, ^{1}H NMR, ^{13}C NMR, MS spectra and elemental analysis. [34–36]

To get single crystal an ethyl acetate solution of nucleoside **19d** was subjected to slow evaporation. The molecular structure of **19d** was unambiguously confirmed (ORTEP diagram, Fig 4). [37]

Screening of all the synthesized compounds was carried out using NCL isolates. The fungal strains *Candida albicans* and *Cryptococcus neoformans* (human pathogens), *Benjaminiella poitrasii* were maintained on YPG (yeast extract, 0.3%; peptone, 0.5%; and glucose, 1%) agar slants, and *Fusarium oxysporum* (plant pathogen) was maintained on PDA (potato, 20%; dextrose, 2%) slants at 28 °C as described earlier.[24,25] MIC (minimum inhibitory concentration for 90% inhibition of growth) and IC_{50} (50% inhibition of

Table 2
Synthesis of 1,2,3-triazole derivatives of uridine by click reaction between 5′-azidouridine **17** and propargyl esters **20a–20g**

Sr. No.	RCOO	Product	Yield (%)
1	 **20a**	 **21a**	60
2	 **20b**	 **21b**	74
3	 **20c**	 **21c**	50
4	 **20d**	 **21d**	57
5	 **20e**	 **21e**	55
6	 **20f**	 **21f**	53
7	 **20g**	 **21g**	60

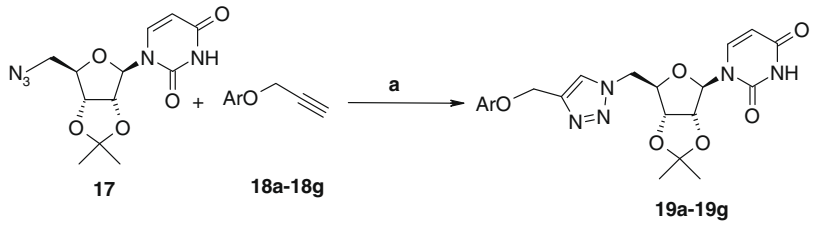


16 17

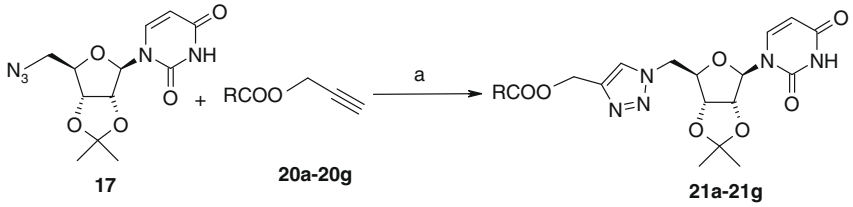
Scheme 1. Reagents and conditions: (a) dimethoxy propane/acetone, *p*-TSA, 28 °C; (b) *p*-TsCl, pyridine, 28 °C; (c) NaN_3, DMF, 60 °C.

growth) of the synthesized compounds were determined according to standard broth microdilution technique as per NCCLS guidelines.[26–28] Compounds were solubilized in DMSO (2.5% v/v), and stock solutions of 1.28 mg/mL were prepared to find out MIC and IC_{50}. Nikkomycin, amphotericin B, and fluconazole (stock solutions of 1.28 mg/mL), known reference antifungal agents were also dissolved in DMSO (2.5% v/v), and were used as a positive control.

As seen in Table 3 synthesized 1,2,3-triazolyl linked uridine compounds **19a–19g** and **21a–21g** were tested for their antifungal activity. It was observed that aryl ether 1,2,3-triazolyl



Scheme 2. Reagents and conditions: (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, *t*-BuOH/H_2O, 28 °C.



Scheme 3. Reagents and conditions: (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, *t*-BuOH/H_2O, 28 °C.

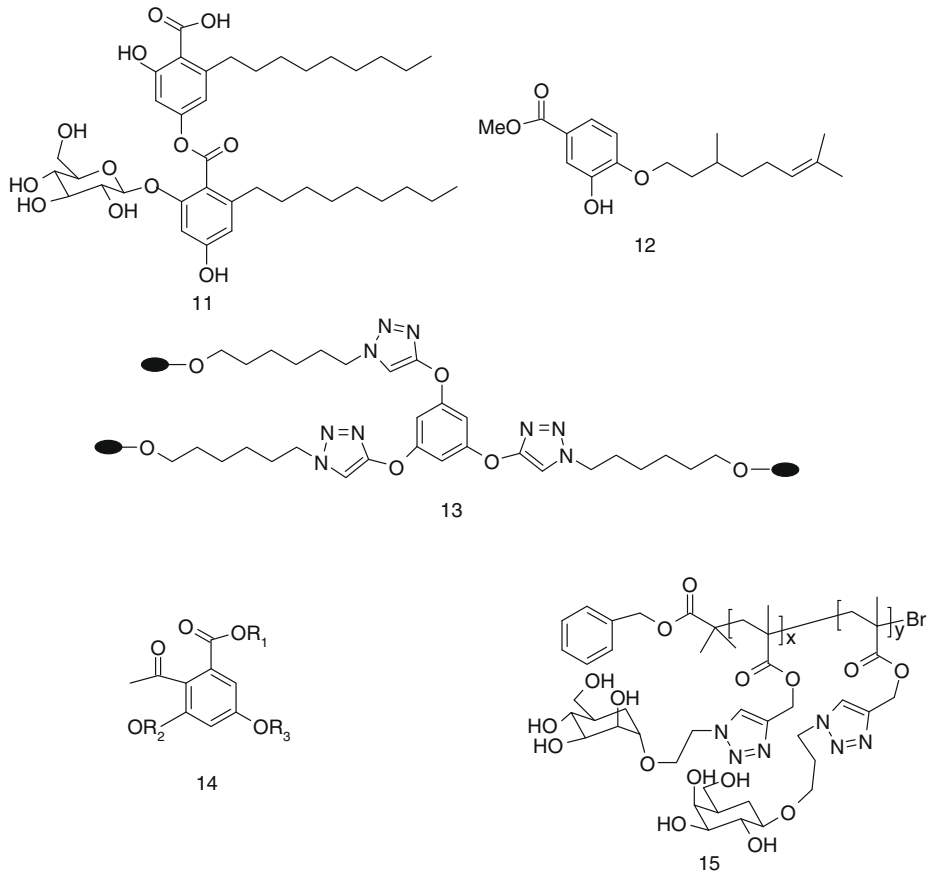


Figure 3. Compounds containing ether and ester linkage.

linked uridine compounds **19a–19g** were found to be active against all tested fungal strains with MIC values 8–128 μg/mL (0.003–0.305 μmol). The activity of compounds **19b–19d** was found to be higher than that of standard nikkomycin and fluconazole against *C. neoformans* and *C. albicans* with MIC of 32 and 24 μg/mL (0.066 and 0.136 μmol). Compound **19a** found to be most potent with lowest MIC of 8 μg/mL (0.018 μmol) against *C. neoformans* as compared to all standards used.

Aryl ester 1,2,3-triazolyl linked uridine compounds **21a–21g** also found to be active against all tested fungal strains with MIC of values 16–128 μg/mL (0.033–0.257 μmol). Compounds **21a, 21b** showed MIC of 31 and 32 μg/mL (0.064 μmol) against human pathogens *C. neoformans* and *C. albicans,* respectively which was comparable to nikkomycin and fluconazole. Compound **21g** showed MIC of 66 μg/mL (0.149 μmol) against *C. albicans* which was comparable to nikkomycin, while it showed

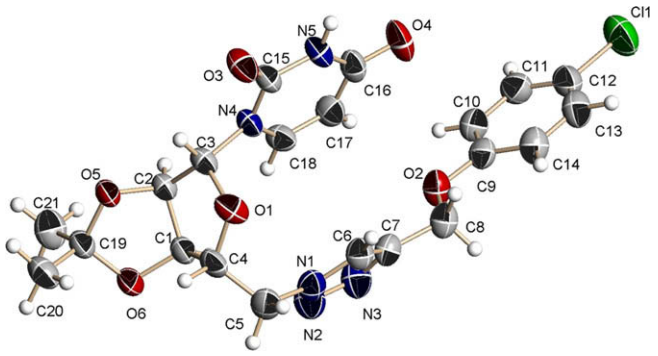


Figure 4. ORTEP diagram of compound **19d**.

Table 3
Antifungal activity detection by using micro broth dilution method

Compound No.	Growth inhibitory concentration in μg/mL					
	F. oxysporum		*C. neoformans*		*C. albicans*	
	MIC[a]	IC_{50}[b]	MIC[a]	IC_{50}[b]	MIC[a]	IC_{50}[b]
19a	>128	128	8	4	>128	68
19b	64	32	32	14	>128	>128
19c	124	70	49	35	24	16
19d	32	16	57	30	32	18
19e	>128	128	128	64	>128	9
19f	64	32	64	8	128	34
19g	64	32	64	32	>128	34
21a	32	16	31	18	32	18
21b	>128	64	64	32	32	18
21c	57	35	>128	128	>128	>128
21d	>128	>128	64	32	>128	32
21e	33	16	>128	128	>128	64
21f	106	55	>128	16	>128	>128
21g	16	8	>128	125	66	32
Nikkomycin	8	6	30	16	64	32
Fluconazole	8	4	32	16	32	14
Amphotericin B	16	8	16	8	2	0.5

Positive control, Amphotericin B, Fluconazole, and Nikkomycin.
Negative control, DMSO, No inhibition.
[a] Minimum inhibitory concentration for 90% inhibition of growth.
[b] 50% Inhibition of growth.

potent inhibition of *F. oxysporum* with MIC value 16 μg/mL (0.036 μmol).

Most of the pathogenic fungi change their morphology reversibly between viz. unicellular yeast or filamentous hypha for survival and proliferation in the host. In other words, it is a change from a nonpathogenic/saprophytic to pathogenic form. Therefore, the compounds which retard this dimorphic transition can have a potential as an antifungal drug. A nonpathogenic dimorphic fungus *B. poitrasii* was used as a model[29] to check the effect of compounds on yeast to hypha transition. Yeast inoculum was grown in YPG (glucose 1%) medium at 37 °C for 24 h and the transition to the hypha form was studied in YP medium at 28 °C. The yeast cells were inoculated in YP broth (with and without compounds) at 28 °C for 6 h and the percentage of cells forming germ tubes was assessed as described earlier.[30] Nikkomycin Z (chitin synthase inhibitor) was used as a positive control in the yeast-hypha transition experiment (Table 4). It was observed that compounds**19b, 19c, 19g, 21a**, and **21f** exhibited >70% of inhibition while **19e, 19f, 21b, 21c, 21g** showed >60% of inhibition at 4 μg/mL. While remaining compounds showed moderate activity as compared to nikkomycin Z.

Chitin synthase activity of *B. poitrasii* cells was estimated with and without compounds (4 μg/mL) using a non-radioactive chitin synthase assay, according to Lucero et al.[31] The assay involved binding of synthesized chitin to WGA-coated surface followed by detection of polymer with horseradish peroxidase-WGA conjugate. Activity of horseradish peroxidase was determined by measuring the absorbance at 430 nm. It was observed that compounds **19d, 19e, 19f**, and **21f** inhibited 80–95% of chitin synthase activity. Compounds **19a, 19b, 19g, 21a, 21c, 21e**, and **21g** exhibited 70–80% inhibition of chitin synthase activity. All other compounds exhibited inhibition in the range of 60–70% except **21b.** (Table 4).

For the screening of antifungal compounds, the inhibition of spore germination or yeast to hypha differentiation assays are more sensitive than the growth inhibition assay.[29] Moreover, the number of pathogens exhibit differentiation (yeast or hypha) for the survival and then proliferation in the host.[29] The comparative evaluation of compounds using in vitro inhibition of chitin synthase activity, inhibition of yeast to hypha transition (measured as% germ tube formation) and inhibition of yeast growth can be used to identify target specificity, hydrophobicity, and toxicity, in general. Results of inhibition of growth, germ tube formation and chitin

Table 4
Comparative analysis of all compounds using whole cell based assay, yeast to hypha transition and chitin synthase inhibition against *Benjaminiella poitrasii*

Group	Compound	% Inhibition		
		Chitin synthase activity[a]	% Germ tube formation[b]	Growth[c]
I	**Nikkomycin**	96.56	>95	45.45
	19d	95.08	58.13	34.65
	19e	84.06	64.70	9.43
	19f	82.14	69.23	34.43
	21f	80.04	72.00	51.52
II	**19a**	77.15	45.09	21.51
	19b	77.78	78.20	17.45
	19c	68.48	71.42	29.30
	19g	76.86	72.72	32.44
	21a	71.00	75.00	31.88
	21c	73.39	61.53	28.21
	21d	67.00	57.14	38.64
	21e	74.00	52.50	56.25
	21g	75.71	60.00	30.21
III	**21b**	36.38	67.21	27.28

Concentration of compounds 4 μg/mL.
Inhibition of chitin synthase activity—Group I, >80%; Group II, 60–70%; Group III, <60%.
[a] Chitin synthase assay. Activity in control 14.86×10^{-2} nmol GlcNAc/mg of protein/min.
[b] Germ tube formation inhibition at 28 °C for 6 h. Control DMSO (2.5%, v/v).
[c] Standard broth microdilution technique, control DMSO (2.5%, v/v).

synthase activity at 4 ppm concentration against *B. poitrasii* were compared (Table 4). With nikkomycin, for instance, inhibition of chitin synthase activity and% germ tube formation were >95% while that of growth was 45% (Table 4). This indicated that nikkomycin uptake by *B. poitrasii* was high enough to show antifungal activity, unlike earlier report of low nikkomycin uptake by *C. albicans*.[32] Based on the in vitro inhibition of chitin synthase activity all the compounds were divided in 3 groups (Table 4). From group I however **19e** did not show growth inhibition comparable to other two parameters which could be attributed to less uptake by the cells. While compounds **19c** and **21a** showed higher inhibition of germ tube formation as compared to chitin synthase activity. These compounds may be acting on some additional targets. The compound **21b** must be acting on some other target since it showed lesser inhibition of chitin synthase activity as compared to other two assays.

Obi et al. reported that in case of synthesized nikkomycin analogues anti-chitin synthase activity was found to be enhanced by introducing β-dimethyl group as β-methyl group of nikkomycin.[12] Furthermore, introduction of hydroxyl group in terminal aryl moiety resulted in significantly enhanced anti-chitin synthase activity. In present investigation introduction of methyl group at *meta* position of benzene ring of compound **19f** showed higher anti-chitin synthase activity. Whereas in compound **21b** decrease in anti-chitin synthase activity was observed, which may be attributed to *ortho* position of methyl group on benzene ring. Unlike reported by Obi et al. in case of compound **21c** introduction of hydroxyl group in benzene ring did not show significant increase in anti-chitin synthase activity.

In conclusion the current endeavor enables a practical, reliable and efficient synthesis of several novel 1,4-disubstituted-1,2,3-triazolyluridine derivatives by 'click chemistry' approach, most of which showed significant antifungal activity. Compound **19a** showed potent antifungal activity as compared to all three standards used against *C. neoformans* with MIC of 8 μg/mL (0.018 μmol). Compounds **19c, 19d, 21a**, and **21b** demonstrated potent antifungal activity in comparison to nikkomycin with MIC value of 24–32 μg/mL (0.048–0.067 μmol) against *C. albicans.* Compounds **19b, 19c, 19g, 21a**, and **21f** have profound suppressive effect on yeast-hypha transition, exhibiting >70% inhibition at concentration 4 μg/mL. Compounds **19d, 19e, 19f**, and **21f** exhibited >80% inhibition of chitin synthase activity comparable to that of nikkomycin at a concentration 4 μg/mL. Comparing the results of all three types of assays, compounds **19a, 19b, 19f, 21c, 21f**, and **21g** can be identified as lead chitin synthase inhibitors for further modifications to increase their antifungal as well as application potential in health care and in agriculture.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.019.

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33. *General experimental protocol for the synthesis of 1,2,3-triazoles **19a–19g** and **21a–21g***: To the stirred solution of **17** (1 mmol) and propargyl ethers **18a–18g** (1 mmol) or propargyl esters **20a–20g** (1 mmol) in 10 mL of tertiary butanol/water (8:2) was added copper sulfate (24 mg, 5 mol %) and sodium ascorbate (40 mg, 10 mol %). Reaction mixture was stirred at 28 °C for 4–12 h. Completion of the reaction was monitored by TLC. Tertiary butanol was removed under reduced pressure, reaction mixture was partitioned between ethyl acetate and water. Organic phase was washed successively with water, brine, and dried over sodium sulfate and concentrated to furnish compounds **19a–19g** or **21a–21g**, yields ranging from 50% to 83%.
34. *Spectral data and elemental analysis for 5′-deoxy-2′,3′-O-(methylethylidene)-5′-{4-[(3-methoxyphenoxy)methyl]-1,2,3-triazol-1-yl}uridine (**19c**)*: ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.82 (s, 1H), 7.61 (s, 1H), 7.05 (d, 1H, J = 8 Hz), 6.84 (q, 4H, J = 8 Hz), 5.17 (d, 1H, J = 8 Hz), 5.50 (s, 1H), 5.12 (s, 2H), 5.03 (dd, 1H, J = 1.6 Hz), 4.98 (m, 1H), 4.67 (br s, 2H), 4.48 (m, 1H), 3.74 (s, 3H), 1.52 (s, 3H), 1.32 (s, 3H). ^{13}C ($CDCl_3$, 50 MHz): 174.9, 163.8, 154.2, 152.3, 150.3, 144.3, 143.4, 125.0, 115.8, 114.7, 102.8, 96.1, 86.2, 84.2, 81.8, 62.6, 55.6, 51.8, 27.0, 25.2, 20.7 ppm. MS m/z: (MH^+) 472.10; IR ($CHCl_3$) ν: 1714, 1693 cm^{-1}. Anal. Calcd for $C_{22}H_{25}N_5O_7$: C, 56.05**;** H, 5.35; N, 14.85. Found: C, 56.00**;** H, 5.25; N, 14.55.
35. *Spectral data and elemental analysis for 5′-{4-[(4-hydroxyphenylacetoxy)methyl)]--1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-O-(methylethylidene)uridine (**21c**)*: ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.88. (s, 1H), 7.41 (s, 1H), 7.05 (d, 1H, J = 8 Hz), 7.01 (d, 1H, J = 6 Hz), 6.73 (d, 2H, J = 6 Hz), 5.07 (d, 1H, J = 8 Hz), 5.47(s, 1H), 5.20 (s, 2H), 5.04 (dd, 1H, J = 1.6 Hz), 4.89 (m, 1H), 4.64 (m, 2H), 4.45 (m, 1H), 3.63 (s, 2H), 1.53 (s, 3H), 1.33 (s, 3H). ^{13}C ($CDCl_3$, 50MHz): 176.2, 168.2, 159.8, 154.2, 147.6, 146.6, 134.2, 129.0, 128.4, 119.3, 118.7, 106.5, 100.0, 90.0, 88.1, 85.7, 61.5, 55.8, 44.0, 30.7, 28.9 ppm. MS m/z: (MH^+) 501.03; IR ($CHCl_3$) ν: 3338, 1683, 1693 cm^{-1}. Anal. Calcd for $C_{23}H_{25}N_5O_8$: C, 55.31; H, 5.05; N, 14.02. Found: C, 55.00**;** H, 4.99; N, 14.00.
36. Spectral data and elemental analysis data for the characterization of compounds **19a**, **19b**, **19d–19g** and **21a**, **21b**, **21d–21g** have been incorporated as Supplementary data.
37. *Crystallographic data for 5′-{4-[(4-chlorophenoxy)methyl]-1,2,3-triazol-1-yl}-5′-deoxy-2′,3′-O-(methylethylidene)uridine (**19d**)*: The single-crystal diffraction data were collected on a Bruker AXS Smart Apex CCD diffractometer at 297(2) K. The X-ray generator was operated at 50 kV and 30 mA using graphite-monochromatized (Mo K_α = 0.71073 Å) radiation. Data were collected with ω scan width of 0.3° and with four different settings of φ (0°, 90°, 180° and 270°) keeping the sample-to-detector distance fixed at 6.145 cm and the detector position (2θ) fixed at −28°. $C_{21}H_{22}Cl_1N_5O_6$: M = 475.89, crystal dimensions 0.62 × 0.38 × 0.19 mm^3, T = 297(2) K,

monoclinic, space group $P2_1$, $a = 11.4808(15)$, $b = 5.6022(7)$, $c = 17.836(2)$ Å, $\beta = 107.144(2)^\circ$; $V = 1096.2(2)$ Å^3; $Z = 2$; $\rho_{calcd} = 1.442$ g cm^{-3}, μ (Mo K_α) = 0.224 mm^{-1}, $F(000) = 496$, $2\theta_{max} = 52.00^\circ$, 11,494 reflections collected, 4274 unique, 4141 observed ($I > 2\sigma(I)$) reflections, 300 refined parameters, R value 0.0324, $wR_2 = 0.0835$ (all data $R = 0.0334$, $wR_2 = 0.0844$), $S = 1.036$, minimum and maximum transmission 0.8737 and 0.9587; maximum and minimum residual electron densities +0.194 and −0.185 eÅ^{-3}.

All the data were corrected for Lorentzian, polarization and absorption effects using Bruker's SAINT and SADABS programs. SHELX-97 (G. M. Sheldrick, SHELX-97 program for crystal structure solution and refinement, University of Gottingen, Germany, 1997) was used for structure solution and full-matrix least-squares refinement on F^2. All non-hydrogen atoms were refined anisotropically while all hydrogen atoms were refined with appropriate geometric constraints.

Crystallographic data (excluding structure factors) for the structure **19d** in this paper have been deposited with Cambridge crystallographic data center as supplementary publication number CCDC 709791.