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# Synthesis and Spectroscopic Study of Anticancer agent A-007 Prodrugs and Progress Towards the Synthesis of Tetramic acid Antibiotics

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Synthesis and Spectroscopic Study of Anticancer agent A-007 Prodrugs
and
Progress Towards the Synthesis of Tetramic acid Antibiotics


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By

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Dedicated to:


My mother, Lakshmi

My father, Madhava Raju

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# Abstract

4,4'-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (A-007) has recently completed a phase-I clinical trial, and objective responses were seen in advanced breast cancer, lung cancer, ovarian cancer, melanoma, skin cancer and non-Hodgkin's lymphoma.  Despite the promising results in the clinical trials, the major disadvantage to using A-007 as a broad-scale therapeutic is its poor water solubility. To make use of this promising anticancer drug either orally or intravenously, the short-term obstacle must be to overcome the limited solubility of A-007 in water. There are several approaches to overcome this obstacle. The first approach is to make hydrolysable prodrugs of A-007. The second approach is to make an A-007 complex with a water soluble host, such as cyclodextrin. We used a combination of these two previously described methods, i.e. transforming A-007 into a more water soluble prodrugs and then further increasing the prodrug water solubility by making their cyclodextrin inclusion complexes.  Our syntheses and spectroscopic explorations of A-007 prodrugs are presented in this dissertation. Tetramic acid (2, 4 pyrrolidine-2,4-dione ring system) containing compounds have been found to display a remarkable diversity of biological activities and have attracted the interest of medicinal and synthetic chemists.  Magnesidin (1-acetyl-3-octanoyl-5-ethylidene tetramic acid) has strong antimicrobial activity against bacteria that cause gingivitis and dental plaque.  Current efforts toward the synthesis of Magnesidin are discussed along with the plans for the completion of synthesis.


Keywords: Cancer; Cyclodextrin; Inclusion complexes; A-007 prodrugs; Molecular aggregation; NOESY; ES mass spectroscopy; azlactone condensation; azlactone ring opening; oxazolones; tetramic acids; Magnesidin.

# Chapter I.  Introduction

## Cancer

From an immunological point of view, cancer cells can be defined as the cells that escaped the regulatory mechanisms that govern normal cell growth  to produce  non-functional cells.[1]  The proliferation of these non-functional cells give rise to clone cells, which continue the process of cell division and replication, leading to the development of a mass of non-functional, continuously replicating cells commonly known as a tumor.[1]  While the human immune system has cells that have many functions, including maintaining homeostasis of normal tissue by regulating cellular proliferation and cell death, it is clearly indicated by the number of annual cancer deaths worldwide that the level of effectiveness of the immune response to tumor cells is often inadequate or poorly expressed. Because of this, scientists have been increasing focus on the exploration of the immune system's response to the regulation and destruction of cancer cells, designing vaccines as well as molecular systems that enable the reactivation of human immune responses to cancer cells.[1]

## Overview of the Immune System

An effective immune response involves two cellular groups. These groups are known as lymphocytes and antigen-presenting cells. There are many types of lymphocytes, and these cells are produced as white blood cells from bone marrow.  Once the lymphocyte leaves the bone marrow, it circulates in the blood and the lymphatic systems, and finally resides in lymphoid organs in the body. Lymphocytes have antigen (foreign body) binding cell surface receptors that mediate immunological responses, such as specificity, diversity, memory, and self-nonself recognition.[1]

T-lymphocytes leave the bone marrow and travel to the thymus to mature. In the thymus, these cells differentiate to express a unique antigen binding molecule, known as the T-cell receptor, on its cell surface membrane. The T-cell receptor can only recognize antigens bound to cell membrane proteins, known as major histocompatibility complex (MHC) molecules. MHCs function in recognition, termed "antigen presentation" in which the recognition occurs between the molecule and glycoproteins found on cell membranes.[1]

Dendritic cells (DC) are antigen-presenting cells of the human immune system that are involved in the initiation of the immune response.[2a] DCs are responsible for the acquisition of antigens or cancer cells, and their subsequent transport to T- lymphocyte rich areas. They are present in lymphatic tissues and lymphoid organs. Once the DCs interact with antigens and become activated, they are able to derive specific immune responses. Secondary lymphoid organs, such as the skin, recruit both naive T- lymphocytes and antigen-stimulated DCs into T-cell rich lymphoid areas, and the co - localization of these early immune response constituents is representative of T-cell activation.[2a] Effective anti-tumor responses elicited from the immune system require the presence of both antigen presenting cells and T lymphocytes.[2b]

In order for a T-cell to become activated, which in turn initiates the immune response of antigen destruction, a T-cell activation signal is required. This signal is triggered by the recognition of the peptide - MHC molecular complex by the T-cell receptor as well as by a co-stimulatory signal. The co-stimulatory signal is usually triggered by an interaction between cell surface glycoproteins of the antigen presenting cell and the T-cell.[1] Because tumor cells express low levels of MHCs and lack necessary co-stimulatory molecules, both necessary to initiate the proliferation of T-cells, they are not effective modulators of antigen presenting cells.[7] Without

sufficient antigen presenting cells in the vicinity of a tumor, T-cells receive only partial activating signals, and tumors are allowed to proliferate.[1]

Immunotherapy is an approach in cancer treatment that attempts to supplement the natural immune defenses of the human body.[1] To that end, one compound that has been clinically shown to demonstrate significant anticancer activities is 4,4'- dihydroxybenzophenone - 2,4 - dinitrophenylhydrazone (**A-007** Figure I.1).[3] X-ray crystallography data revealed that **A-007** (monoclinic crystals) exists as two unique rotamers.[1] These rotamers differ only in the orientation of the bis-diphenylmethane group, where the rings are approximately perpendicular to each other and rotated approximately 90° from the orientation of the rings in each rotamer (Figure I.1).[4a] Both rotamers show strong intramolecular hydrogen bonds between the – NH of the – HN– N=C– moiety and an oxygen of the -nitro group. Examining the structure of **A-007**, one can see that there are three unique functional moieties present in **A-007** that may contribute to its overall biological activity. These three moieties are the dihydroxy-bis-diphenylmethane rings, the hydrazone moiety and the dinitrophenyl moiety. However, despite **A-007**'s high electrophilicity, it has no chemical reactivity with cell surface glycoproteins, making this compound and analogs of this compound promising new anti-cancer treatments.[4b]

Molecule 1    Molecule 2

(C-B)=14.5°    (C-B)=32.8°
(C-A)=117.6°    (C-A)=43.3°
(A-B)=105.2°    (A-B)=68.5°
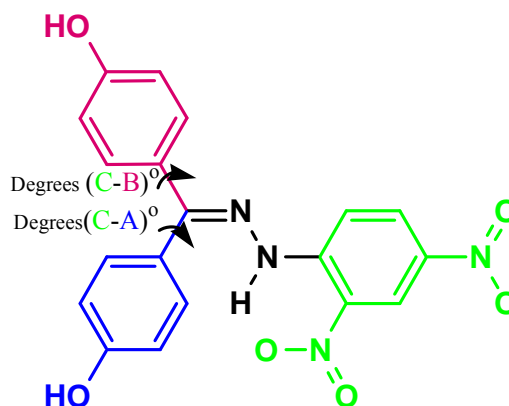
Figure I.1:  Structural crystallography characteristics of **A-007**.[4]

**A-007** and its structural analogs appear to act as T-cell activators *via* CD45+ surface

receptors on lymphoendothelial cells, and in particular with dendritic cells. Thus far, thirty-three

people have been treated with topical **A-007** (as a 0.25% gel) in the Phase I study, and of these

subjects, 31% objective remissions have been observed with two complete responses.[3] During

the Phase I study, it was discovered that **A-007** was not acting through a cytotoxic mechanism.

There was no local or systemic toxicity noted, and histochemical reviews of biopsies of human

skin topically treated with **A-007** revealed that increased infiltrates of T-lymphocytes containing

the membrane glycoproteins CD4+ (T-helper cells), CD3+(T-cytotoxic cells), CD8+(T-cytotoxic

cells), and CD45+ had occurred after treatment.[3] Increased skin infiltrates of CD11c+ dendritic

cells (function as antigen presenters to T-helper cells) were also observed in treated areas.[4]

Further immunohistochemical studies suggested that immune modulation had occurred *in vitro*

and *in vivo* following exposure to **A-007**.[3,5]

    **A-007** is a simple organic molecule that appears to act as an antigen, possibly due to the

unique electronic properties associated with this molecule. It has been hypothesized by Morgan,

*et al.*[1] that up-regulation of the CD45+ receptor is an initiation site for the **A-007**-induced

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immune modulations that are being observed in patients with cancer.[5] Our hypothesis is that if this is in fact the initiation site for up-regulation of receptor glycoproteins involved in immune modulation, then other water soluble analogs of **A-007** should be able to elicit the same, or greater responses.

CD45+ is expressed on dendritic cells, lymphocytes, monocytes, and leukocytes, as well as some neoplastic cells, as a protein tyrosine phosphatase (PTP), which together with other members of the PTPs, are responsible for phosphorylating tyrosine residues.[2d] Blocking the CD45+ sites with anti-CD45 antibodies has been shown to inhibit T-cell activation and prevent mitogen (lectin) activation of naïve T-cells[6a] CD45+ receptor surfaces contain the amino acid residues of arginine, serine/threonine, and cysteine, and these residues can bind to or transfer natural ligands to the surface of antigen presenting cells and hydrolyze tyrosyl phosphates.[6b] Morgan *et al.* hypothesized that **A-007** does not inhibit or block CD45+, but up-regulates lymphocytes and dendritic cells (to antigen presenting cells) *via* electrostatic and/or non-covalent binding with the Arg, Cys, Ser/Threo residues, as depicted in Figure I.2.[5] Furthermore, **A-007**-activated DCs are capable of initiating mitotic events with naïve human blood peripheral mononuclear cells (PBMC) and up-regulating both CD45+ and CD11c+ receptors in human peripheral dendritic cells,[6c] all exemplifying the fact that **A-007** is not an inhibitor of CD45+, but rather an up-regulator or modulator of the molecular sites (Figure I.2). The influence that functional group substitutions may have on **A-007**'s intra-/inter-molecular hydrogen bonding and electrostatic interactions is presented below (This figure illustrates several possible interactions, and is not meant to illustrate the fact that all interactions occur).
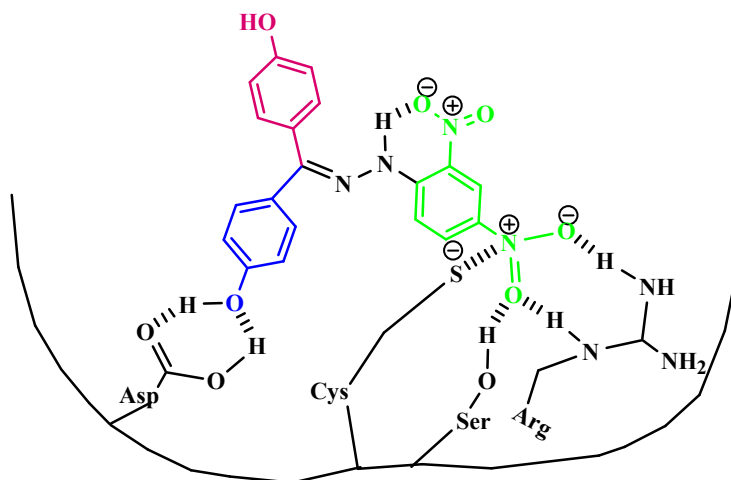
Figure I.2: Postulated interactions of **A-007** with the CD45 receptor (modified from Ref. 2d).

To improve the activity of **A-007**, functional group modifications to three functional moieties present in **A-007** that could influence biological activity have been performed.[7] Analogues of **A-007** were prepared to introduce rigidity in the diphenylmethane ring system, as well as to influence electrostatic properties. Compounds that have evolved by fusing the diphenyl ring system into polyaryl coplanar rings not only improved anticancer activity but also introduced potential problems from cardiac and bone marrow toxicities[7]. Substitutions of electron withdrawing groups into the dinitrophenyl ring system influenced activity. **A-007** and some of the simple analogues have intramolecular hydrogen bonding between the dinitrophenyl ring system and the N-NH of the hydrazone group, as well as between the hydrazone moiety and the hydroxyls of the diphenylmethane ring, all of which had an impact on biological activity.[7] Fresh human cancer tissue were studied as a test system because of the natural heterogeneity that exists in a tumor nodule or mass, i.e., cancer cells, lymphocytes, fibroblasts, endothelial cells, and natural extracellular connective tissue matrices.

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Considering the fact that to date, no analog of **A-007** with improved immune modulating

activity (apoptosis without direct cytotoxicity) has been identified, it was important that **A-007**

itself should be brought into a form that could be used as broad scale therapeutic.  Despite

promising results in the clinical trials with **A-007**, the major disadvantage to using **A-007** as a

broad scale therapeutic was its low water solubility.  In the current formulation, **A-007** is used

only as a topically applied 0.25% gel, making the uses of this very potent immune modulating

compound limited.[7] To further advance the use of **A-007**as an anticancer therapeutic that can be

used orally or intravenously, the short-term obstacle to overcome is the limited solubility of **A-**

**007** in water.[8]   Prodrug design is an approach that can be effectively used to overcome obstacles

such as solubility in promising drug candidates.


**The Prodrug Concept**

Albert and coworkers were the first to suggest the concept of the prodrug approach in

increasing the efficiency of drugs in 1950.[9] They described prodrugs as pharmacologically

inactive chemical derivatives that could be used to alter the physicochemical properties of drugs,

in a temporary manner, to increase their usefulness and/or to decrease associated toxicity[9].

Subsequently, such drug-derivatives have also been called 'latentiated drugs', 'bioreversible

derivatives', and 'congeners', but 'prodrug' is now the most commonly accepted term.[9] Thus,

prodrugs can be defined as a drug derivative that undergoes biotransformations, either

enzymatically or non-enzymatically, inside the body before exhibiting its therapeutic effect.

Ideally, a prodrug is converted to the original drug as soon as the derivative reaches the site of

action, followed by rapid elimination of the released derivatizing group without causing side

effects in the process. The definition of the prodrug indicates that the derivatizing group is

covalently linked to the drug molecule. However, the term prodrug has also been used to describe salts formed by the drug molecules[9]. A derivative of a known, active drug can be capped to furnish a prodrug. The prodrug enhances delivery characteristics and/or therapeutic value of the drug by transforming into the active drug via an enzymatic or a chemical process to remove the cap at the site of action to regenerate.
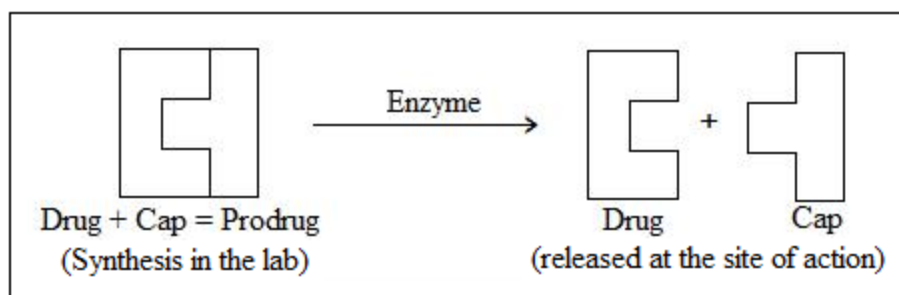


Scheme I.1: Capping an active drug to enhance delivery characteristics and/or therapeutic value.[9]

**Characteristics of a Prodrug**

In recent years numerous prodrugs have been designed and developed to overcome barriers to drug utilization, such as low oral absorption properties, lack of site specificity, chemical instability, toxicity, bad taste, odor, pain at application site, etc.[9] It has been suggested that an ideal drug carrier must:[9]

1) Protect the drug until it is at the site of action.

2) Localize the drug at the site of action.

3) Allow for release of the drug chemically or enzymatically.

4) Minimize host toxicity.

5) Be biodegradable, biochemically inert, and non-immunogenic.

6) Be easily prepared inexpensively.

7) Be chemically and biochemically stable in its dosage form.

**Development of Prodrugs**

Specific prodrugs, which were developed by taking explicit administration properties into consideration, are utilized for the following:

*1. To improve membrane transport:*[10a] Barbiturates (Figure I.3) are a group of compounds responsible for profound sedative-hypnotic effect[10b]. They are weakly acidic in nature and are converted to the corresponding sodium salt in aqueous sodium hydroxide. The sodium salt is extensively employed for intravenous anesthetic properties. Barbituric acid is the parent member of this group of compounds. Various barbiturates differ in the time required for the onset of sleep and in the duration of their effect. Hexobarbitone was found to be an effective drug but its membrane permeability was found to be low. However, *N*-methylhexobarbitone a simple barbituric acid derivative of the parent drug, was found to have better permeability characteristics. After intake, the *N*-methyl group is cleaved in the liver to release the physiologically active drug.



Barbituric acid      Hexobarbitone      *N*-Methylhexobarbitone
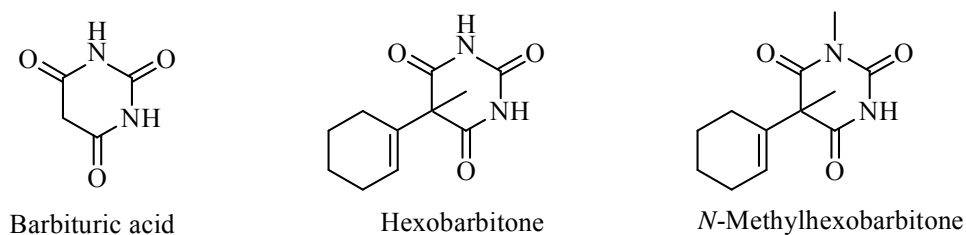
Figure I.3: Hypnotic and sedative agents.

Similarly, membrane transportation characteristics of the neurotransmitter dopamine (Figure I.4) used for the treatment of Parkinson's disease can be improved by administering its prodrug *L*-3,4 dihydroxyphenylalanine (Levo-DOPA, Figure I.4). This derivative has better

blood-brain permeation characteristics since it uses amino acid channels for transportation. Once

inside the cell, decarboxylase enzyme removes the acid group to generate dopamine.
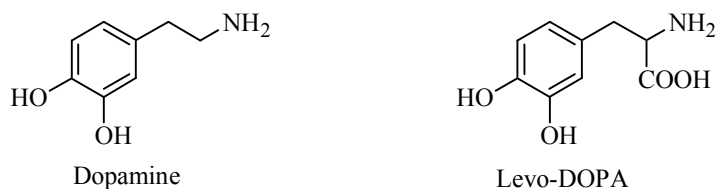
Figure I.4: Dopamine and Levo-Dopa.

**2. To prolong the activity**[10a] Nordazapam (Figure I.5) is a drug used for sedation, particularly as

an anxiolytic. It is also used as a muscle relaxant. However, it loses activity too quickly due to

metabolism and excretion. A prodrug introduced to improve the retention characteristics is

valium (Figure I.5). Due to presence of *N*-methyl group the prodrug resists quick degradation.

Slow release of the nordazapam in the liver by demethylation prolongs body retention

characteristics.[10b]
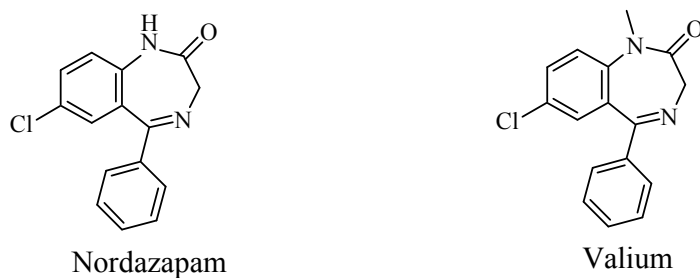
Figure I.5: Nordazapam and Valium.

Decreased or lack of secretion of the enzyme insulin in pancreas leads to diabetes.[9]

Insulin is responsible for degradation of carbohydrate molecules to smaller units and is important

in the catabolic process. Chronic diabetic patients take bovine insulin supplement through

intravenous injections. Retention time of insulin in the blood is about six hours. So, patients need

to administer required dose of insulin frequently. It is desirable to increase the retention

characteristics of the enzyme so as to make it effective for prolonged periods. It was found that

9- fluorenylmethoxycarbonyl (Fmoc) protection (Figure I.6) of the hydroxy/amino groups of the

enzyme makes it inactive and also increase its retention in blood for prolonged periods.[10c]  The

Fmoc group binds to the enzyme covalently and in the process makes it inactive as well as

reduces its rapid degradation by natural body process.  However, at the pH of about 7.4 prevalent

in the blood serum the protected enzyme gets hydrolyzed slowly and irreversibly back to the

enzyme and Fmoc protecting group. The hydrolysis process was found to be slow and constant,

which means that the release of enzyme is also slow and regulated. The hydrolysis rate can be

fine tuned by selecting derivatives of Fmoc protecting group or number of Fmoc groups. It was

shown that insulin having two Fmoc protecting groups was ten times more stable and more

effective than the parent enzyme. It should be noted that the hydrolysis of the protecting group

takes place in the blood without mediation from other enzymes.
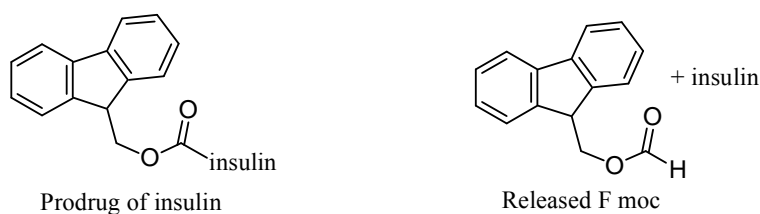


Figure I.6: Fmoc protected prodrug of insulin.

*3. Masking from other Enzymes*:[11a] Sometimes drugs are highly toxic when administered

directly. Suitable modification of the drug molecule to an inactive agent reduces toxicity. For

example propiolaldehyde (Figure I.7) is used in the aversion therapy on patients addicted to

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alcohol. However, it is a highly irritating chemical and causes allergic reactions. As an alternative, closely related compound, pargylene (Figure I.7), which is converted to propiolaldehyde by oxidative enzymes only in liver, is used for alcohol addiction.[11b]



Propiolaldehyde

Pargyline

Figure I.7: Prodrug for anti-alcoholic addiction.

*4. Prodrugs to Encourage Patience Acceptance:* A fundamental tenet in medicine is that in order for a drug to be effective, the patient has to take it. Painful injections and unpleasant taste or odor are the most common reasons for the lack of patient acceptance of a drug. An excellent example of how a prodrug can increase the potential for patient acceptance is related to the antibacterial drug clindamycin. Whereas clindamycin causes pain on injection, the prodrug clindamycin phosphate (trade name Dalacin) is well tolerated; hydrolysis of the prodrug *in vivo* occurs with a $t_{1/2}$ of approximately 10 min.[12c] Also clindamycin has a bitter taste, so it is not well accepted orally by children. However, it was found that by increasing the chain length of 2-acyl esters of clindamycin the taste improved from bitter (acetate ester) to no bitter taste palmitate ester.[12d] Bitter taste results from a compound dissolving in the saliva and interacting with taste receptor in the mouth. Esterification with long-chain fatty acids makes the drug less water soluble and unable to dissolve in the saliva. It also may alter the interactions of the compound with the taste receptor.

Clindamycin                  Clindamycin phosphate              Clindamycin palmitate
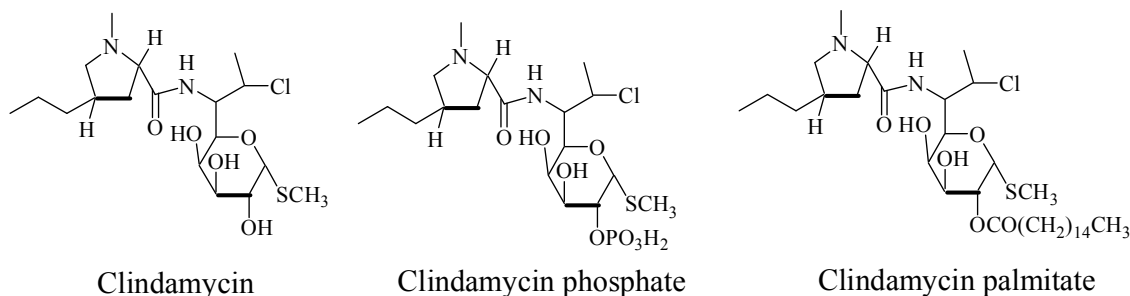
Figure I.8: Clindamycin and its prodrugs.

**5. *Prodrug Design Based on Site-specific Conditions*:**[13] A variety of conditions such as pH and oxygen content, which are site specific and are different from other parts of the body, can be effectively utilized for prodrug design.[13a] This aspect is an important feature in the research on prodrugs targeted at cancer cells. The cancer cell grows at a much faster rate than normal cells. Tumor cells associated with cancer can be differentiated from normal cells. The blood vessels in the tumor tissue often lack regularity and systematic connectivity leaving unvascularized zones, especially in the interior areas, which leads to unstable blood flow. Cells that do not have adequate blood supply die as a result of lack of oxygen. The intermediate regions also get a deficient supply of oxygen. This area is called the hypoxia region. Lack of oxygen in hypoxia cells or the bio-reductive conditions prevalent in them can be utilized for specific prodrug development where the active drug can be selectively released under bio-reductive conditions. The bio reductive enzymes present in the cell perform one electron reduction. In normal cells, oxygen reverses this reduction process. However, in hypoxia cells, due to near absence of oxygen, further reduction takes place to generate the active drug from the prodrug moiety.[13a, b] For example, Tyrapazamine (Figure I.9) has been developed as a cytotoxic agent. It has two *N*-oxide moieties, which upon one electron reduction twice gets converted to highly reactive diradical. The diradicals are responsible for cleavage of DNA. Even though such diradicles are

generated in normal cells, they get reconverted to *N*-oxides due to the presence of oxygen. In hypoxia cells the diradicles have a longer lifetime to interact with DNA molecules and further cleave them.  Such a cleavage of single or double stranded DNA leads to the destruction of cells. Thus, the *N*-oxide prodrug was found to be highly effective in hypoxia cells.

Tyrapazamine
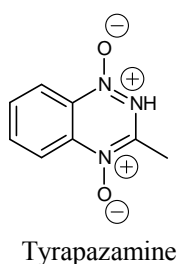
Figure I.9:  Prodrug directed to hypoxia cells.

*6. Enzyme Specific Prodrug Design:* Certain enzymes express predominantly in the cancer cells. Prodrug development can take advantage of this aspect so that over expressed enzymes can be exploited to facilitate the release the drug at the site of action. This condition is of particular importance in targeting cancer cells. Due to differing physiological conditions, enzyme groups such as glucuronidases[12], proteases-2 show abnormal activity in cancer cells compared to normal cells. Several prodrugs have been developed by taking advantage of the excessive activity of the above enzymes in tumor tissues.

Scheeren and colleagues[13, 14a] demonstrated that the cytotoxic activity of important antitumor drugs could be enhanced and restricted to tumor-affected tissues by making peptide derivatives.  They prepared derivatives of paclitaxel (Figure I.10) and doxorubicin (Figure I.11) wherein active sites are blocked by strategically attaching suitable polypeptide to the drug but separated by a spacer. The spacer was used to expose the polypeptide chain open for plasmin activity. Both the prodrugs were found to be inactive and stable under biological pH conditions

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but they were readily cleaved with the release of parent drugs in the presence of plasmin

enzymes present in tumor cells. These prodrugs were synthesized by blocking important

functional groups in the molecule with a polypeptide-capping agent to make them inactive. The

spacer group was designed to self eliminate after hydrolysis of the polypeptide chain by the
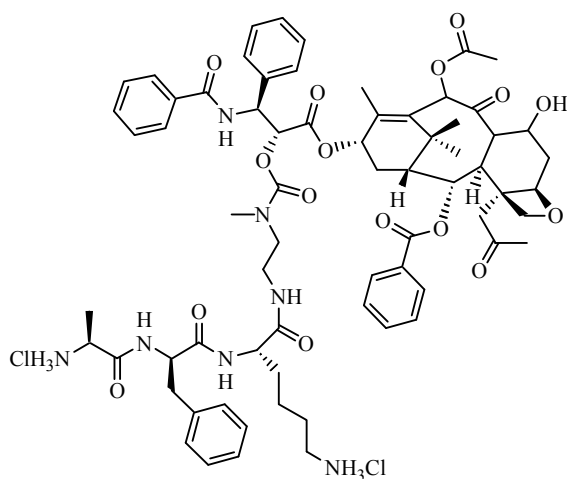
enzyme.



Plasmin targeted paclitaxel derived prodrug       Paclitaxel
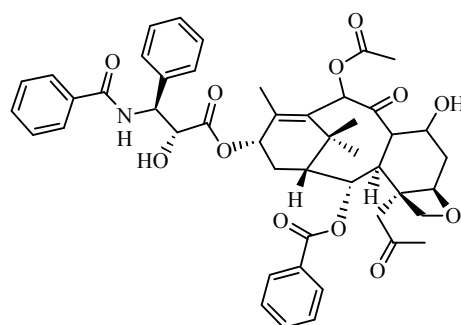
Figure I.10: Plasmin targeted anticancer prodrug and its parent drug.


Protease enzymes are responsible for the breakdown of proteins. They scissor the protein

chain at amide bonds linking specific amino acids. It was found that even though protease

enzymes are ubiquitous in all cells and blood serum, their activity is excessively associated with

tumor metastases. [13,14a,b] Primary malignant cells are encapsulated in extra cellular matrix made

of proteins.  In order to proliferate and to reach metastases the primary tumor has to break

through the matrix through protease activity. It was shown that serine protease plasmin plays an

important role in tumor invasion to other areas. In blood circulation plasmin activity is restricted

by the presence of anti-plasmin enzymes. [14a,c] Plasmin targeted doxorubicin based prodrug having a spacer Doxorubicin group was designed to self eliminate after hydrolysis of the polypeptide chain by the enzyme.



Plasmin targeted doxorubicin based
prodrug having a spacer

Doxorubicin
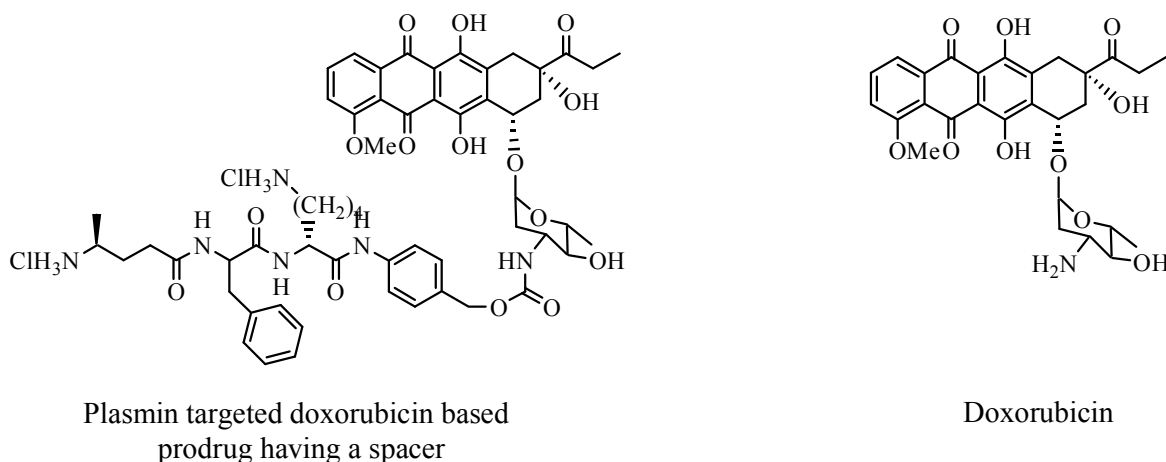
Figure I.11: Prodrug directed to hypoxia cells.

**7. *Prodrug Design for Increased Water Solubility:*** Many drugs are poorly water-soluble.[9, 15a] To permit aqueous injections or ophthalmic delivery of these drugs, they must be converted into water-soluble forms. However, there are two considerations one must take into account in the choice of a solublilizing group: The bond made should be stable enough in aqueous solution so that a ready-to-inject solution has a reasonably long shelf life, but it must be hydrolyzed *in vivo* with a reasonably short half-life after administration (less than 10 minutes). One example of this is dilemma is the water-soluble prodrug form of methylprednisolone, which is in medical use as methyl-prednisolone sodium succinate Solu-Medrol.[15b]

methyl-prednisolone          Solu-Medrol
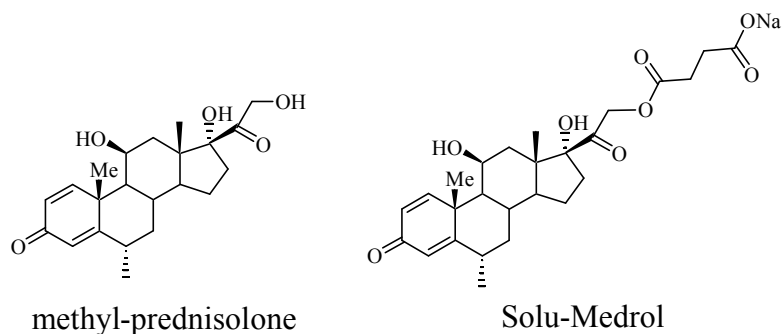
Figure I.12(a):  Prednisolone prodrugs.

However, the *in vitro* stability is low, probably due to intramolecular catalysis;

consequently, it is distributed as a lyophilized (freeze-dried) powder that must be reconstituted

with water and then used within 48 hours.  The lyophilization process adds to the cost of the drug

and makes its use less convenient.  To overcome the shortfalls associated with using this

compound, a series of more stable water-soluble methylprednisolone esters were synthesized,

and several of the analogs were shown to have shelf lives in solution of greater than 2 years at

room temperature.[15b]

Another example of  poor water solubility affecting the use of anticancer compounds is

associated with the antitumor drug etoposide ( R=H; Vepesid), which has to be formulated with

the detergent Tween 80, polyethylene glycol, and ethanol, all of which have been shown to be

toxic.[15c]  Conversion to the corresponding phosphate ester, etoposide phosphate (Figure I.12b),

allows the drug to be delivered in a more concentrated form over a much shorter period of time

without the detrimental vehicle.[15d]

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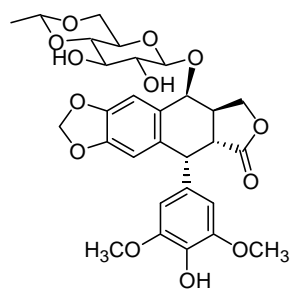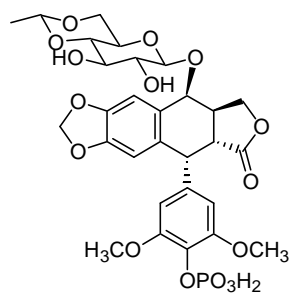
Etoposide (R=H)                    Etoposide phosphate (R=PO₃H₂)

Figure I.12(b):  Anticancer agent Etoposide and Etposide phosphate.

## Cyclodextrins as Drug Carrier

Cyclodextrins are a family of cyclic oligosaccharides[16a] with a hydrophilic outer surface and a lipophilic central cavity. Cyclodextrin molecules are relatively large with a number of hydrogen donors and acceptors and thus, in general, they do not permeate lipophilic membranes. Cyclodextrins have been used in the pharmaceutical industry as complexing agents to increase aqueous solubility of poorly soluble drugs, and to increase their bioavailability and stability. Studies in both humans and animals have shown that cyclodextrins can be used to improve drug delivery from almost any type of drug formulation. Currently there are ~ 30 different pharmaceutical products worldwide containing drug/cyclodextrin complexes on the market.[16b]

Reduction of drug crystalinity on complexation or solid dispersion with CDs also contributes to the CD increased apparent drug solubility and dissolution rate.[17a] CDs as a result of their ability to form *in situ* inclusion complexes in dissolution medium, can enhance drug dissolution even when there is no complexation in the solid state. CDs can also act as release enhancers, for example β-CD enhanced the release rate of poorly soluble naproxen and injection of the drug in a BALB/c mouse model.[17b] Further CD entrapment of drugs at the molecular level prevents their direct contact with biological membranes and thus reduces their side effects (by decreasing drug entry into the cells of nontargeted tissues) and local irritation with no drastic loss of therapeutic benefits. Inclusion complexation with HP-β-CD reduced the side effects of 2-ethyl hexyl-*p*-dimethyl aminobenzoate (a UV filter) by limiting the interaction of the UV filter with skin.[17b]

Applications of CDs in oral drug delivery include improvement of drug bioavailability due to increased drug solubility, improvement of rate and extent of dissolution, and/or stability of the drug at the absorption site, e.g., the gastrointestinal tract or in formulation, reduction of drug

induced irritation, and taste masking.[17b] CD complexation was found to decrease local drug irritation and also modify the time of drug release during GI transit. An itraconazole oral preparation containing 40% (wt/vol) of HP-β-CD (with reduced drug irritation) has been commercialized.[17c]

The principal advantages of using natural cyclodextrins as drug carriers are the following[18]: (1) well-defined chemical structure, yielding many potential sites for chemical modification or conjugation; (2) availability of cyclodextrins of different cavity size; (3) low toxicity and low pharmacological activity; (4) certain water solubility; (5) protection of included/conjugated drugs from biodegradation.

## Tetramic Acids

The term tetramic acid was coined in 1901 to refer to the heterocyclic system 1,5-dihydro-4-hydroxy-2H-pyrrol-2-one , a tautomer of 2,4-pyrrolidinedione which is the predominant species in solution.[19] Although tetramic acid (Figure I.13) was apparently synthesized only in 1972, a number of natural substances had been identified as derivatives of tetramic acids well before that time.[20] Secondary metabolites containing the tetramic acid (2,4-pyrrolidine-2,4-dione) ring system have been known for almost half a century, and well before the parent system was synthesized. Although the first naturally occurring tetramic acids were identified because of their activity as antibiotics and/or mycotoxins, more recently tetramic acid containing compounds have been found to display a remarkable diversity of biological activities. The often unusual and intricate

substituents modifying the tetramic acid structural unit make the synthesis of these

metabolites a challenging target.[21, 22]
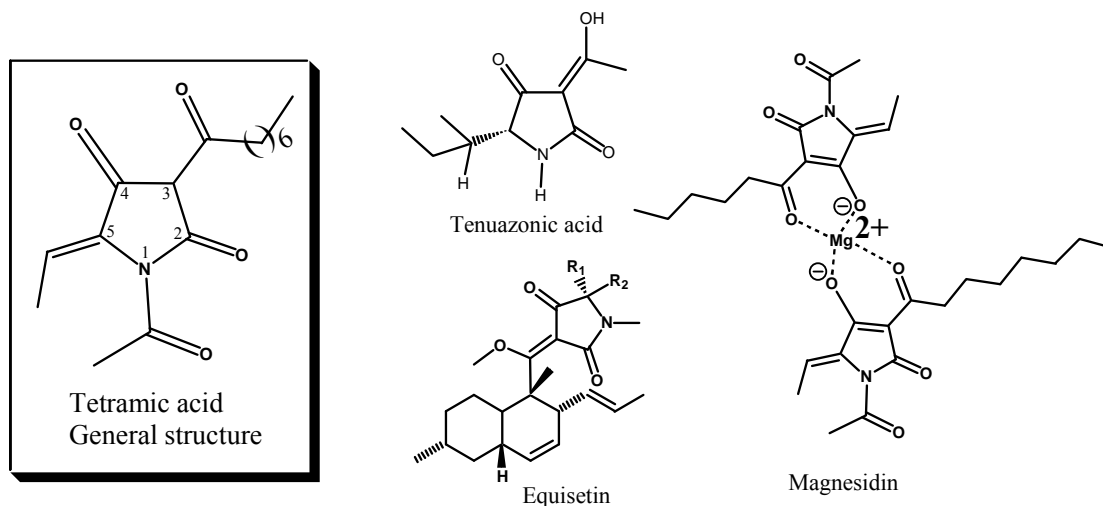


Figure I.13: General structure and some commercially important tetramic acids.

Recent studies have confirmed that tetramic acid metabolites have a wide distribution and

play a significant role in ecological interactions. They have been isolated from marine mollusks,

sponges and cyanobacteria, terrestrial and marine microorganisms, particularly endophytic fungi.

Tetramic acid is a much weaker acid than its oxygen counterpart and exists mainly in 2,4-

diketone form. The presence of an acyl group at C3 results in an increase in acidity (pKa 3.0-

3.5) and proton NMR spectra indicate complete enolisation and the presence of tautomeric

forms. As shown in Figure I.14 these can be separated into two sets of internal tautomers, which

are rapidly interconverting, and two pairs of external isomers that interconvert slowly on the

NMR time scale and often give rise to separate NMR signals. ^{13}C-NMR spectroscopy is more

useful in determining the predominant tautomeric form and it indicates that the one represented

by D (Figure I.14) is more important.
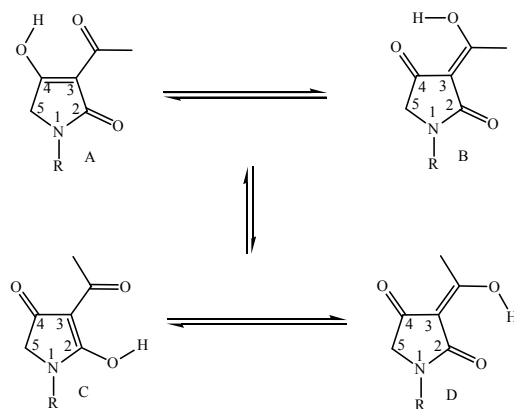
Figure I.14:  Tetramic acid Tautomeric forms.

From biosynthetic perspective naturally occurring tetramic acids can be regarded to arise from the assembly of an aminoacid and an activated acyl entity[11, 23] derived from an acetyl group or a more complex activated ester.

Compounds containing this structural unit (Figure I.14), sometimes intriguingly [23-28] camouflaged, exhibit a diverse range of biological activities and have attracted the interest not only of natural products chemists, but also of chemical ecologists, medicinal and synthetic chemists.

Magnesidin is an inseparable mixture of the magnesium chelates of the 3-hexanoyl and 3-octanoyl tetramic acid derivatives.[23]  It was the first recorded example of a naturally occurring tetramic acid magnesium salt.  It was isolated from a new Pseudomonas species, P. magnesiorubra, obtained from the surface washings of the marine green alga *Caulerpa peltata.*[24] Magnesidin inhibits gram-positive bacteria (MIC 2-7 µg/mL),[25] particularly spore bearers, and prevents the decay of foodstuff caused by spore germinating organisms.  An important application of Magnesidin is it is effective against gingivitis and reducing dental plaque.

22

To date, it appears that only chemicals that demonstrate potent anti-plaque and anti-cariogenic activity are fluoride and chlorhexidine, both of which are halogenated.[26a] Gingivitis is a concern for a large number of people. Epidemiological surveys indicate that an average of 50% of the adult population of the United States (US) have gingivitis. Gingivitis is characterized by gingival inflammation and/or bleeding and is caused by plaque at and under the gingival margins. Most people brush their teeth; however, toothbrushes cannot effectively remove plaque at or under the gum line. Floss is effective at removing plaque in difficult to reach locations; however only about 20% of the US population use floss. Inconvenience is a commonly cited reason for not flossing.[26b] Since gingivitis is caused by plaque and plaque is composed of various kinds of bacteria, in theory anti-microbial agents should be effective against gingivitis.

There are a number of anti-microbial agents formulated in toothpaste or rinses on the market. The most effective of these agents is chlorhexidine digluconate (CHG).[27] CHG reduced gingivitis by 50-80% in clinical trials. However, CHG is available in the US by prescription only and is generally used on a short-term basis (2-4 weeks only). Patient compliance is generally poor due to the unpleasant side effects associated with the use of CHG, which include staining of the teeth, interference with taste function, and enhanced calculus formation. Two products available on the over-the-counter (OTC) market have shown marginal effectiveness in clinical trials, Total® and Listerine®. Total, a toothpaste containing triclosan, reduced gingivitis by 20-25% in clinical trials. Listerine reduced gingivitis by 20-35% in clinical trials. Neither triclosan nor Listerine are substantive agents, thus the anti-microbial effect is lost quickly.[28] The remaining anti-microbial agents available in OTC products have failed to show effectiveness in clinical trials. Thus, there is currently no truly efficacious anti-gingivitis product that is both convenient to use and appealing to the consumer. It appears that the only chemicals that have

been shown to have potent anti-plaque and anti-cariogenic activity are fluoride and chlorhexidine, both of which are halogenated. Other commonly used compounds including the phenolics are not as effective as plaque- and caries-control agents.[29-31]

Considering the importance of healthy teeth and gums in general human health, and the lack of availability of a proper remedy, it is very important to develop an industrially viable synthetic approach for Magnesidin. In addition the synthetic method should be a general method such that it can be extrapolated to develop other tetramic acids of medicinal significance.

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# Chapter II.  Synthesis and NMR spectroscopic study of cyclodextrin inclusion complexes with A-007 prodrugs

## Abstract

One- and two-dimensional NMR spectroscopy was used to demonstrate the formation of inclusion cyclodextrin complexes with several **A-007** prodrugs. These complexes are comprised from the encapsulation of the two phenol moieties of the **A-007** prodrugs within the cyclodextrin cavity. Considering the size of the two phenol moieties of the **A-007** prodrugs compared to the sizes of α-, β-, and γ-cyclodextrin cavities, we observed complementary binding of the **A-007** prodrug with only β-cyclodextrin, which was also demonstrated spectroscopically. The β-cyclodextrin inclusion complexes increased the prodrug solubility and modified the prodrug half-life in water. Therefore, β-cyclodextrin inclusion complexes can be used as an essential form of **A-007** prodrug delivery.

## Introduction

4,4'-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (**A-007**, Figure 2.1) has recently completed a phase I clinical trial, where it was used as a therapeutic with the targeted treatment of advanced cancer, with minimal toxicity.[1] It was speculated that **A-007**'s activity is a result of complementary binding to lymphocyte receptors. This speculation was supported by experimental findings that indicate that **A-007** interacts, presumably through the electron-rich (phenol) and the electron-poor (dinitrophenyl) moieties, with the CD45 receptor through complementary interactions.[2]
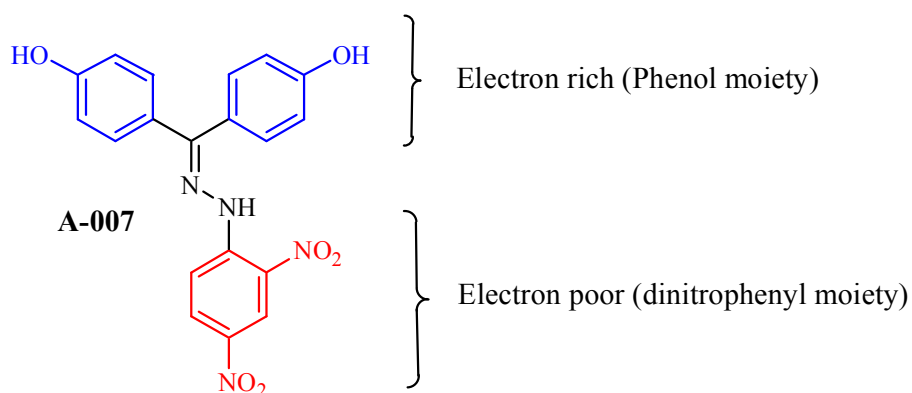
Figure 2.1. Chemical structure of the anticancer compound **A-007**

Despite promising results in the clinical trials, there is a major disadvantage to using **A-007** as a broad scale therapeutic. **A-007** has low water solubility and in its current formulation, is used only as a topically applied 0.25% gel.[3] To make use of this promising anticancer drug orally or intravenously, the short-term obstacle must be to overcome the limited solubility of **A-007** in water. Two possibilities exist to overcome the water solubility problem: the first is to make a hydrolyzable prodrug; and the second is to make an **A-007** complex with a water soluble host, such as cyclodextrin.[4,5] Considering the complex structure of **A-007**, we hypothesized using a combination of these two previously described methods, which would utilize the chemical transformation of **A-007** into a more water soluble prodrug and then further increase the water solubility of this newly formed prodrug through the formation of cyclodextrin inclusion complexes.

The application of cyclodextrins in drug delivery is well documented in the literature.[5,6] For example, the toxicity and compatibility of using cyclodextrins in drug delivery has been previously explored and utilized for several commercialized drugs on the market today.[6,7] Our

intention was to synthesize water soluble prodrugs of **A-007** by covalently attaching small

hydrophilic moieties and then spectroscopically explore both the stability of these prodrugs in

aqueous solution, as well as the ability of the prodrugs to form cyclodextrin inclusion complexes.

## Results and Discussion

**Synthesis of A-007 prodrugs**

To explore cyclodextrin inclusion complexes with **A-007** prodrugs, we prepared neutral,

anionic, and cationic prodrugs.  Scheme 2.1shows the prodrugs synthesized.
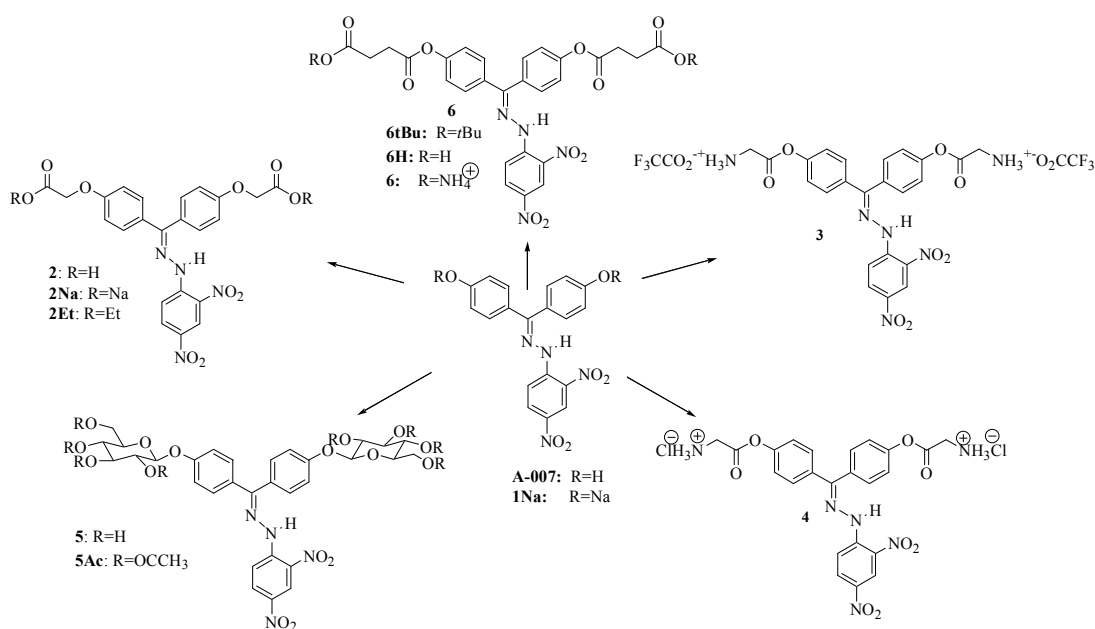


Scheme 2.1.  **A-007** prodrugs synthesized.

*Synthesis of Negative A-007 prodrugs*

The synthesis of starts with preparation of **A-007**.  It is made from commercially available

starting materials by the reaction of equivalent amounts of the 2, 4, dinitro phenyl hydrazine and

4, 4' dihydroxy benzophenone in methanol or ethanol with sulfuric acid as a catalyst.

Purification is done by recrystallization from ethanol or glacial acetic acid.[1,3]
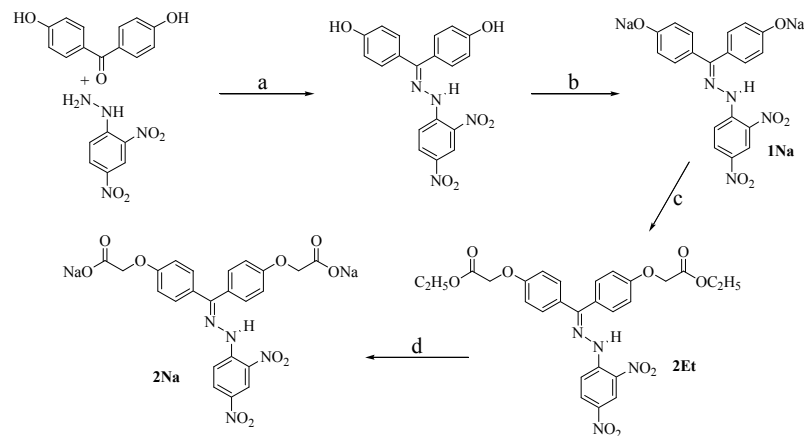


a. $H_2SO_4$, $CH_3OH$, 50°C, 78%  b. NaOH/$CH_3OH$, 99%  c. $BrCH_2CO_2C_2H_5$/$CH_3OH$, 94%  d. NaOH/$H_2O$-$CH_3OH$, 95%

Scheme 2.2.  Synthesis of negative **A-007** prodrugs

Acetic acid derivative **2Na** was prepared by treatment of ethyl 2-bromoacetate with **A-007** sodium salt.  The resulting ester is hydrolyzed, followed by acidification to yield prodrug **2Na** according to previously reported methods of preparation for similar phenol derivatives.[8] Prodrug **6** is a succenic acid derivative.  It is made by coupling **A-007** with mono-Boc protected succenic acid **8** under DCC coupling conditions, followed by trifluoroacetic acid deprotection of Boc group in dichloromethane.  The succenic acid prodrug is used in the form of its ammonium salt.  The salt is made by passing ammonia gas generated from a mixture of ammonium hydroxide and sodium hydroxide into a dichloromethane solution of **6H**.  The synthesis is shown in scheme 2.3.
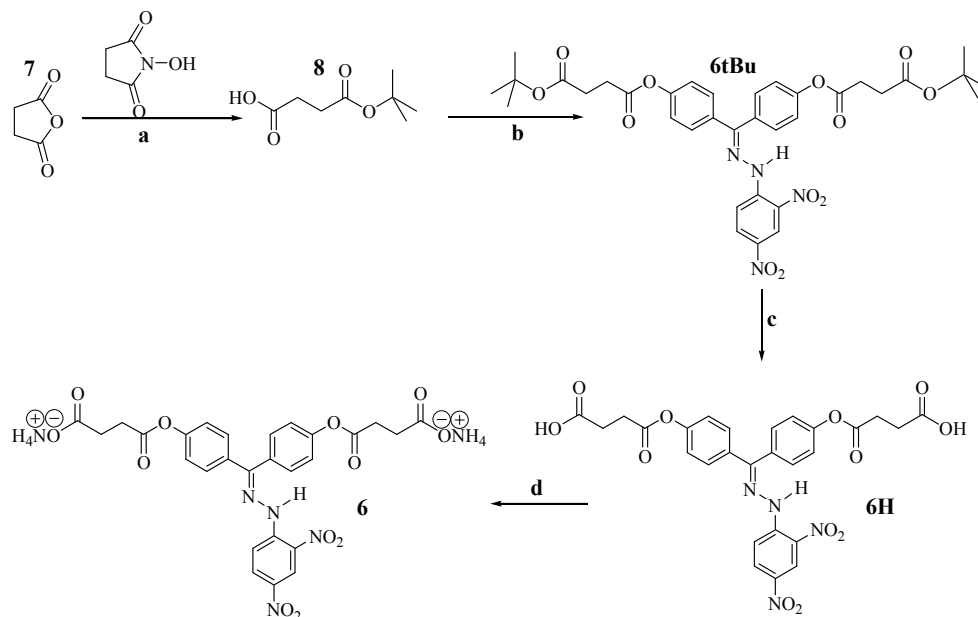
a. DMAP/Toluene, t-Bu-OH, 78%  b. A-007, DCC/CH$_2$Cl$_2$, 50%  c. CF$_3$COOH,CH$_2$Cl$_2$, 0°C , 98%  d. DCM/ NaOH+NH$_4$OH, 98%

Scheme 2.3.  Synthesis of prodrug 6

## Synthesis of Neutral A-007 prodrugs

The neutral **A-007** prodrug we synthesized was the glycoside **5**(Scheme 2.4).  This compound

was synthesized with the intention to use it for Enzyme Specific Prodrug Therapy(ESPT).  Under

this approach, excessive expression of glycosidase enzymes in the cancer cells is exploited to

break the glycosidic linkage in the prodrug.  This would set the active drug (**A-007**) free at the

site of action.  For this purpose the sugar needs to be linked to **A-007** at the anomeric position

regioselectively.  We chose to use trichloroacetimidate method of sugar action to carry out this

glycosidation.  This method utilizes BF$_3$ as a lewis acid catalyst which is mild enough not to

disturb the shiff base moiety of **A-007**.  D-glucose is completely protected and activated to tetra-

*O*-acetyl-a-D-glucopyranosyl trichloroacetimidate in three steps in 48% overall yield, using

literature procedures with minor modifications (Scheme 2.4).[10] The first step of prodrug

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synthesis involved Schmidt's glucosylation[10] of **A-007** with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate,[11] followed by sodium hydroxide hydrolysis in mixture of dichloromethane and methanol.
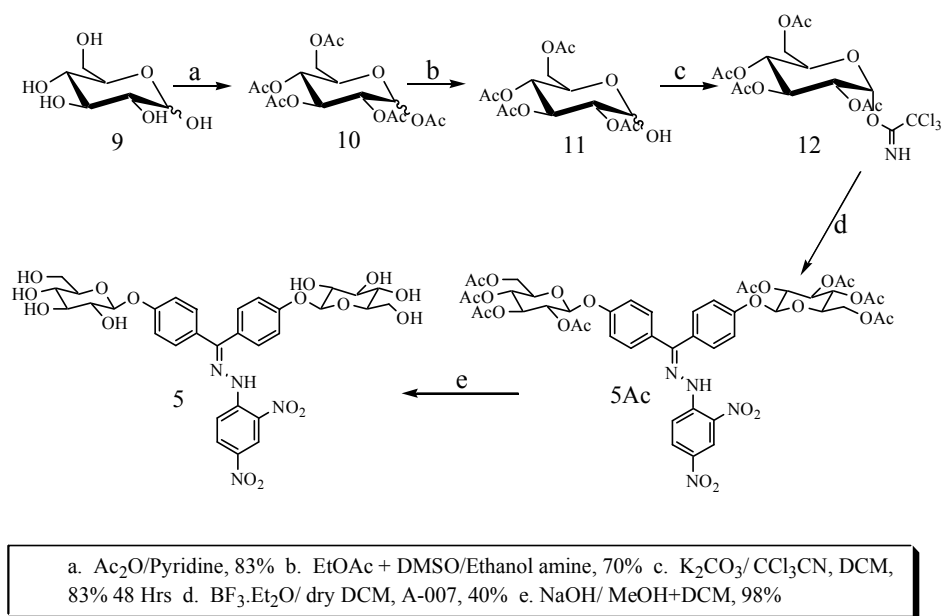


Scheme 2.4.  Synthesis of prodrug 5

*Synthesis of Positive A-007 prodrugs*

Amino acid glycine derivatives have been synthesized as the positive **A-007** prodrugs.  The trifluoroacetic acid salt **3** was prepared by the acylation of **A-007** with Boc-glycine in the presence of dicyclohexylcarbodiimide, followed by trifluoroacetic acid deprotection in dichloro methane.[9] The hydrochloric acid salt **4** was prepared in a similar manner as the trifluoroacetic acid salt, except that the Boc-deprotection was carried out with methanolic HCl instead of trifluoroacetic acid (Scheme 2.5).
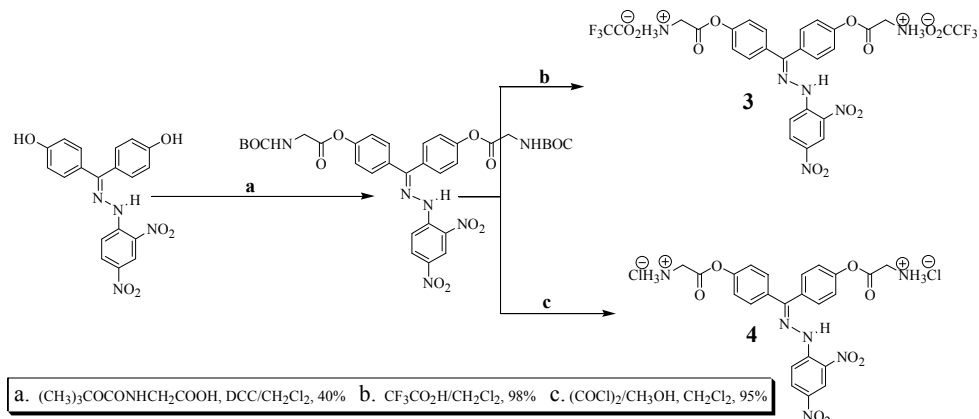
Scheme 2.5. Synthesis of positive **A-007** prodrugs

## Spectroscopic study of A-007 prodrugs

The simplest possible prodrug of **A-007** is its sodium salt (**1Na**). This compound will be
converted into free **A-007** under physiological conditions. Unfortunately, even this double
sodium salt is not soluble in water in high concentrations. While at an elevated temperature
(50°C) 10 mM water solution can be formed, upon cooling, a red colored precipitate forms at
room temperature. It is reasonable to speculate that because **A-007** is a surfactant by nature,
molecular aggregates could form in aqueous media. This should be reflected in chemical shift of
the aromatic protons of **1Na** versus its concentration. However, our spectroscopic studies
revealed no noticeable difference in chemical shift of 1 mM and 10 mM concentration of 1Na in
water media (Fig. 2), suggesting that **A-007** does not form molecular aggregates, such as
micelles.[13]    The formation of micelles in aqueous media is a desirable property for these
prodrugs, because micellar stability can be a function of concentration. Ideally, a prodrug should
be stable at higher aqueous concentrations, above the critical micellar concentration (CMC) and

it hydrolyzes easily at lower or below CMC concentrations. In this way, the prodrug can be

stored at higher concentrations and upon administration it slowly hydrolyzes in the blood stream.
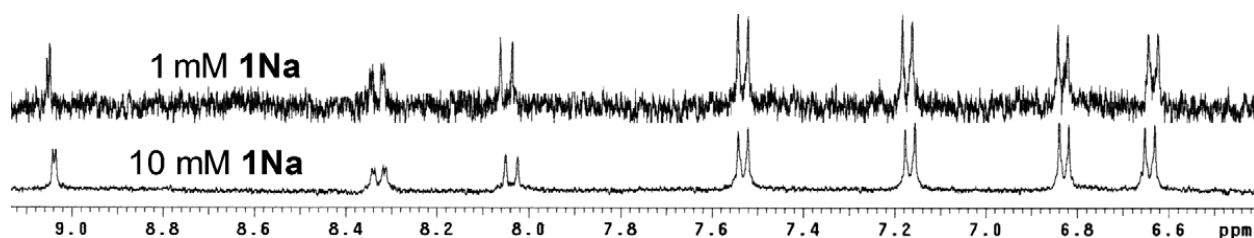


Figure 2.2. $^1$H NMR spectra of aqueous 1Na.

Ideally, compounds to be used as prodrugs should be water soluble or have the ability to

form cyclodextrin inclusion complexes. To test the capability of **1Na** to be used as a prodrug of

**A-007**, we analyzed the capacity of **1Na** to form a cyclodextrin inclusion complex with α–, β–,

and γ-cyclodextrins. We prepared a 10 mM solution of **1Na** with β-cyclodextrin, and observed

that the solution stayed clear for several days, a dramatic improvement considering that the

precipitate forms in approximately 30 min from 10 mM **1Na** without β-cyclodextrin. (A 10 mM

solution was made by mixing together **A-007** with sodium hydroxide in D$_2$O and sonicated at

 50°C for 5 min. A solution that is 0.1 mM is the highest water concentration of **1Na** that remain

stable for several days.) Table 2.1 gives a list of solubilities of **A-007** prodrugs and their β-

cyclodextrin inclusion complexes.

Table 2.1: List of solubilities of **A-007** prodrugs and their β-cyclodextrin inclusion complexes.

| Entry # | Prodrug | Max. Aqueous solubility | Solubility with saturated aqueous β-CD | Clog P |
|---------|---------|-------------------------|----------------------------------------|--------|
| 1 | **1Na** | 0.1mM | 10mM | 5.03 +/- 0.57 |
| 2 | **2Na** | 0.2mM | 12mM | 4.51 +/- 0.61 |
| 3 | **3** | 10mM | >25mM | 3.46 +/- 0.62 |
| 4 | **4** | 10mM | >22mM | 3.46 +/- 0.62 |
| 5 | **5** | 2.8mM | 14mM | 0.49 +/- 0.60 |
| 6 | **6** | 0.5mM | 21mM | 4.55 +/- 0.6 |

To determine whether a strong cyclodextrin inclusion complex is formed, it is necessary to observe changes in the spectroscopic pattern of the compound/complex, where these changes should be reflected by changes in the NMR chemical shifts. In our analyses, we found no noticeable change in chemical shifts of aromatic protons of **1Na** in combination with α- or γ-cyclodextrins.  This can be explained by the fact that the α-cyclodextrin cavity is too small to encapsulate the phenol moieties of **1Na**, while the γ– cyclodextrin cavity is too large to forms strong inclusion complexes with **1Na**. On the other hand it is well documented that β-cyclodextrin forms a strong inclusion complex with phenol groups,[14] and this observation corresponds to our spectroscopic data of **1Na** and β-cyclodextrin. We observed a significant chemical shift in the NMR spectra of free **1Na** and **1Na** in aqueous β-CD (Figures. 2.3 & 2.4). The chemical shift changes for **1Na** reached saturation at 2:1 ratio β-CD and **1Na**. This implies the possibility of a ternary cyclodextrin complex formation between one molecule of **1Na** and two molecules of β-CD.[24]
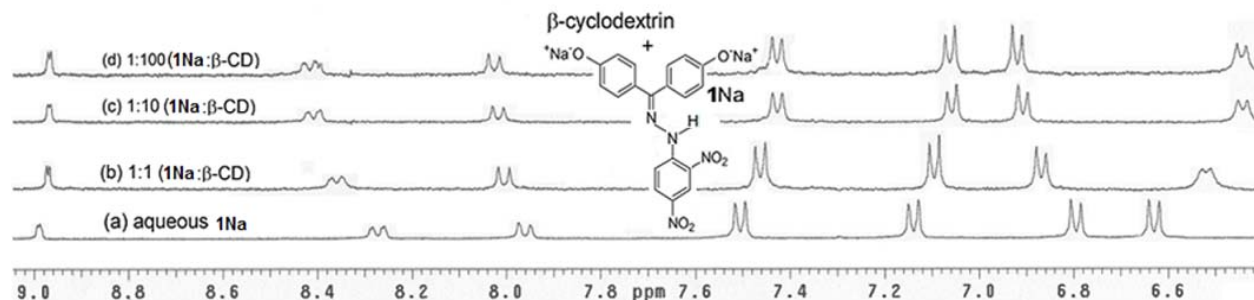
Figure 2.3. $^1$H NMR spectra of aqueous β-cyclodextrin of 1Na (10 mM).
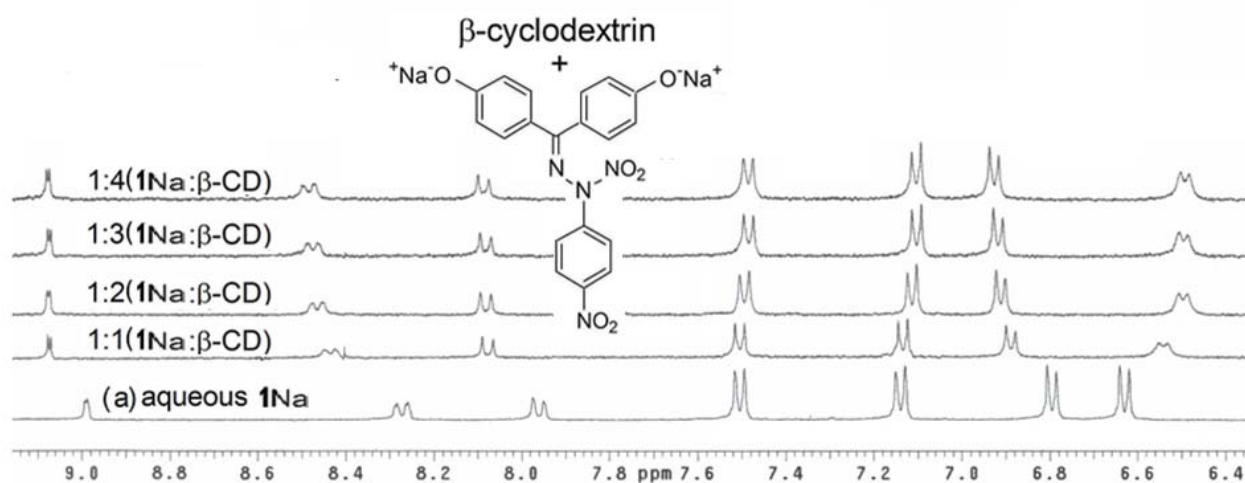


Figure 2.4. $^1$H NMR spectra of aqueous β-cyclodextrin of 1Na (10 mM).

Because our one-dimensional NMR spectroscopic study of β-CD complexation with **1Na** is not conclusive, additional spectroscopic investigation is essential. There are two additional spectroscopic methods that can shed more light on the nature of the complex, electron spray mass spectroscopy (ES-MS) and the two-dimensional nuclear Overhauser spectroscopy (NOESY). Electron spray mass spectroscopy (ES-MS)[15] is an important tool used for the characterization of non-covalent binding and using this spectroscopic technique, the ternary complex signal at 1331.1 *m/z* was observed in the β-cyclodextrin and **1Na** water solution. This signal corresponds to the molecular mass of two cyclodextrins and one **1Na** (Figure 2.5).

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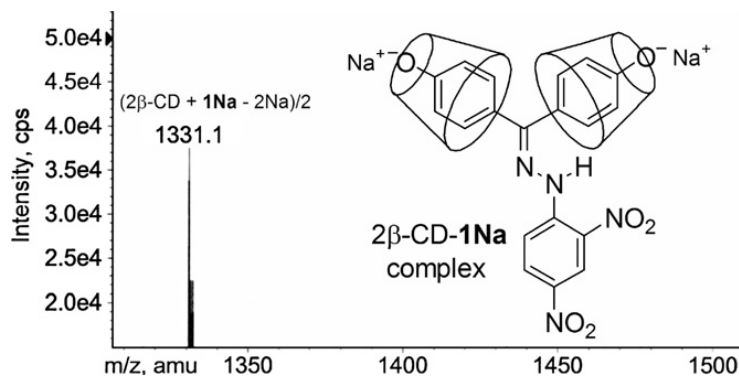
Figure 2.5. Negative ES-MS spectra of 1Na (0.01 mM) and β-cyclodextrin (0.03 mM).

Two-dimensional NOESY (Nuclear Overhauser Spectroscopy) can be used to demonstrate that two protons or groups of protons are in proximity, as the protons must be within 3.5Å of each other.[16] This spectroscopic method is a valuable tool to study β-cyclodextrin inclusion complexes with **A-007** prodrugs. Using NOESY, we found that the 2D NOESY spectra of our β-cyclodextrin complex with **1Na** showed intense cross peaks indicating interactions between β-cyclodextrin and phenol moiety of **1Na** (Figure. 2.6). These two cross peaks indicate the presence of these groups in the vicinity of 2–5 Å. However there are no cross coupling signals between β-cyclodextrin hydrogens and the hydrogens of the 2,4-dinitrophenyl moiety of **1Na**, suggesting that this part of the **1Na** molecule is not encapsulated in the β-cyclodextrin cavity. In summary, all spectroscopic studies, including the 1D NMR, 2D NOESY, and ES-MS, indicate that there is the formation of a ternary inclusion complex that substantially increases the water solubility of the simple **A-007** prodrug, **1Na**.
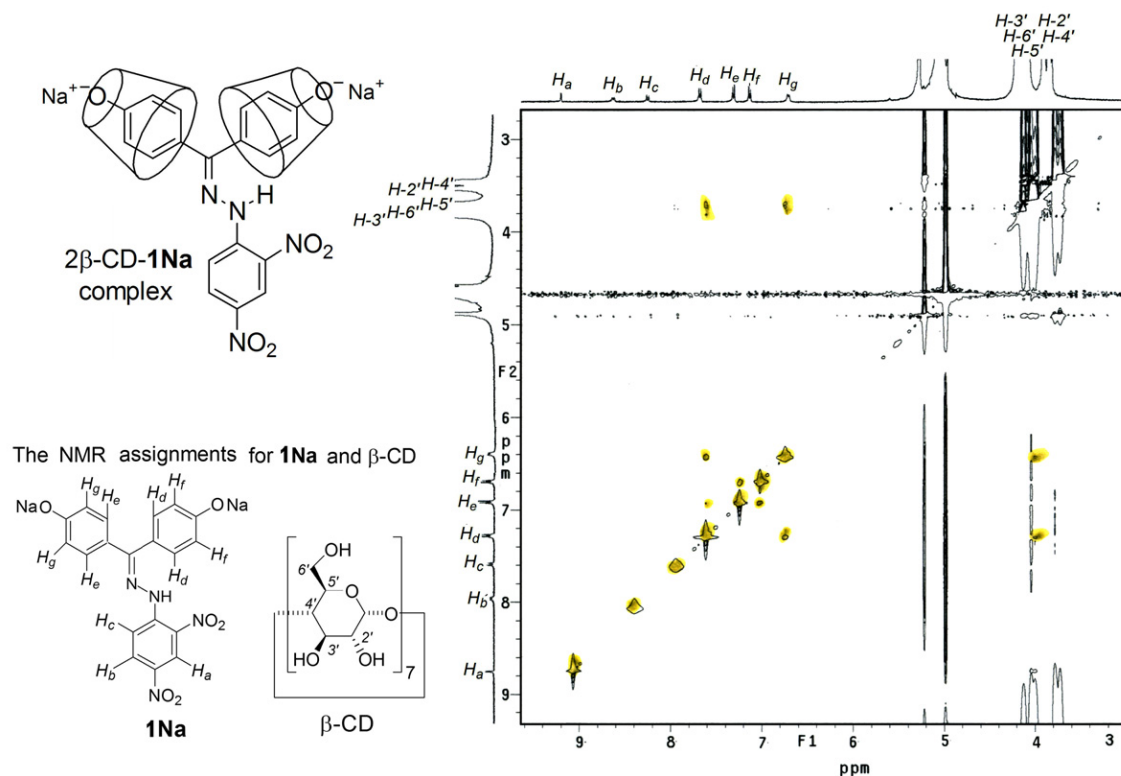
Figure 2.6. 2D NOESY spectra of **1Na** (10 mM) in aqueous β-cyclodextrin (30 mM).
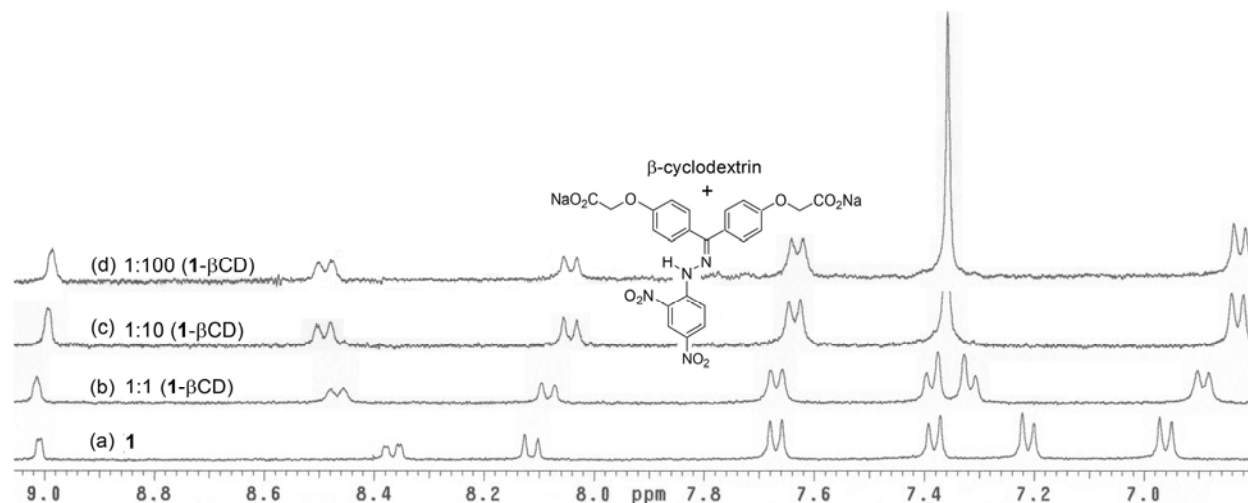


Figure 2.7. $^1$H NMR spectra of **2Na** (10 mM) in aqueous β-cyclodextrin.

Our second compound, the sodium salt **2Na**, does not strictly belong to the group of

hydrolysable **A-007** prodrugs because it cannot be directly converted into **A-007** itself.

Nevertheless, we chose to include this compound in our study due to the fact that phenol ethers metabolize via oxidative *O*-dealkylation, resulting in the formation of a free phenol group, or in our case to **A-007**.[23] In addition, the corresponding acid **2** also has anticancer activity that is similar to the original **A-007**. Unfortunately, the low solubility of this acid also hampers its oral or intravenous administration. Using carboxylic acid salts as a polar group of **2Na**, we expected this compound to possess physical properties similar to surfactants, lending it capable of forming molecular assemblies similar to taxol prodrugs reported by Nicolaou and coworkers.[17] First, we explored the change of the NMR chemical shift of **2Na** versus its concentration in pure water. There was a substantial downfield chemical shift observed in the spectra when the concentration of **2Na** increased from 1 mM to 10 mM, while the saturation was achieved after the 10 mM concentration (after CMC).
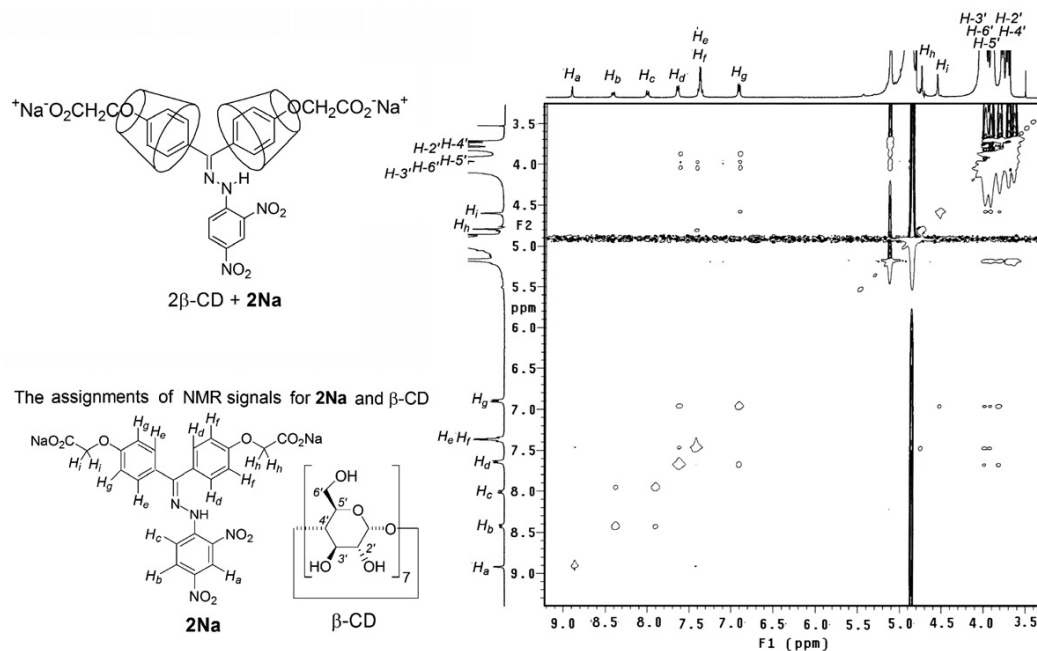


Figure 2.8. 2D NOESY of **2Na** (10 mM) in aqueous β-cyclodextrin (30 mM).

In the aqueous cyclodextrin solution of **2Na** we expected to observe the appropriate NMR chemical shift changes when the cyclodextrin inclusion complex is formed. There were no

noticeable changes when either α-cyclodextrin or γ-cyclodextrin was added to aqueous **2Na** (10 mM). This observation did not come as a surprise as the α-cyclodextrin cavity is too small (4.7–5.3 Å) while γ-cyclodextrin cavity is too big (7.5–8.3 Å) to form strong inclusion complexes with **2Na**.[18] However, there was a substantial difference with β-cyclodextrin (Figure. 2.7). This difference is profound on the chemical shift of the phenol moiety of the prodrug, indicating that this part of the molecule interacts with β-cyclodextrin. As was the case with **1Na**, the β-cyclodextrin titration of 10 mM solution of **2Na** suggests that a ternary (one molecule of **2Na** and two molecules of β-cyclodextrin) complex is formed. This was based on the fact that after a ratio of 1:2 was reached, further increases of β-cyclodextrin did not produce noticeable chemical shift changes in the NMR spectra of **2Na**.

Two-dimensional NOESY spectra clearly support our assumption that the ternary β-cyclodextrin inclusion complex with **2Na** is formed (Figure. 2.8). There are several NOE cross couplings between the two phenol moieties of prodrug **2Na** and the cyclodextrin hydrogens. However, we observed no cross coupling between hydrogens from the 2,4-dinitrophenyl moiety of **2Na** and the β-cyclodextrin hydrogens. Furthermore, cross coupling between the methylene hydrogens of the acetic acid moieties and β-cylcodextrin hydrogens was observed as well. All of these cross couplings strongly suggest that the formation of a ternary β-cyclodextrin inclusion complex with **2Na** in water media as demonstrated in Figure 2.8.

Another prodrug which behaves exactly similar to **1Na** and **2Na** is prodrug **6**. This is the succenic acid derivative of **A-007** used as its ammonium salt which is more stable and more water soluble than the corresponding free acid. The behavior of **6** towards different cyclodextrins is similar to the other negative prodrugs **1Na** and **2Na**, in that there were no noticeable changes when either α-cyclodextrin or γ-cyclodextrin was added into aqueous **6** (10

mM). However, there was a substantial difference with β-cyclodextrin (Figure. 2.9). This difference is profound on the chemical shift of the phenol moiety of the prodrug, indicating that this part of the molecule interacts with β-cyclodextrin.
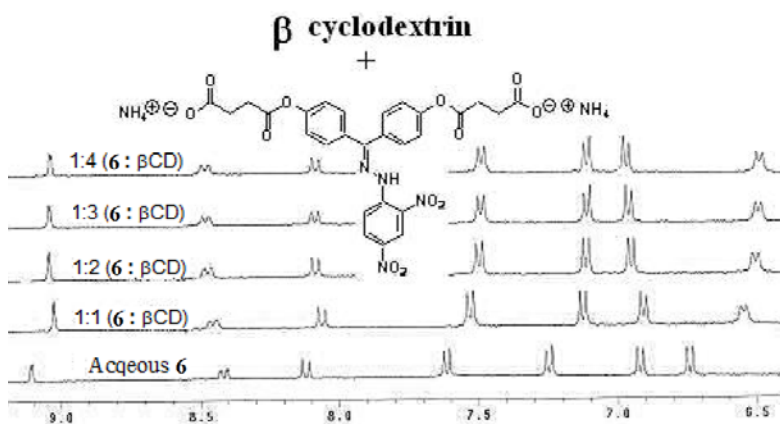


Figure 2.9. $^1$H NMR spectra of aqueous β-cyclodextrin of **6** (10 mM).

Two-dimensional NOESY spectra of **6** clearly supported our assumption that the ternary β-cyclodextrin inclusion complex is formed (Figure. 2.10). There are several NOE cross couplings between the two phenol moieties of prodrug **6** and the cyclodextrin hydrogen. However, we observed no cross coupling between hydrogens from the 2,4-dinitrophenyl moiety of **6** and the β-cyclodextrin hydrogens. Furthermore, cross coupling between the methylene hydrogens of the succenic acid moieties and β-cylcodextrin hydrogens was observed as well. All of these cross couplings strongly suggest that the formation of a ternary β-cyclodextrin inclusion complex with **6** as demonstrated in Figure 2.10.
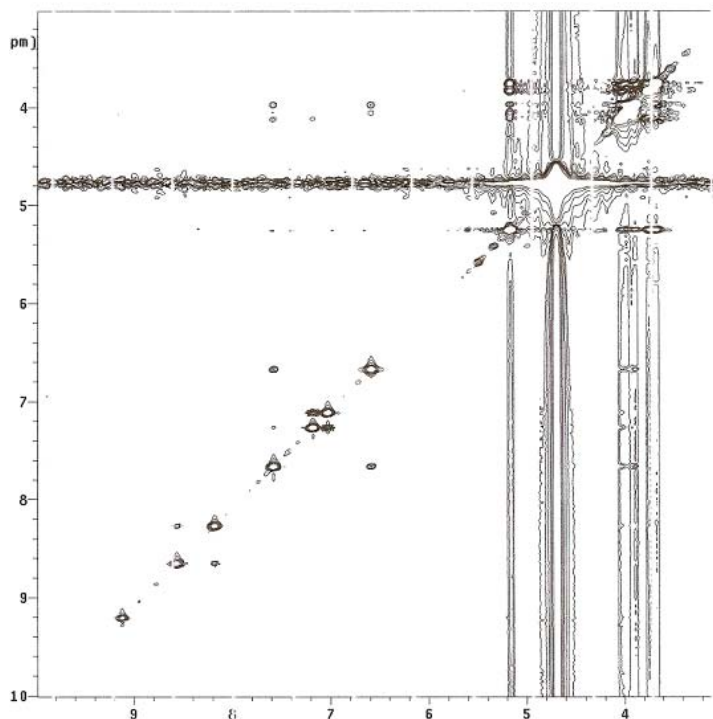
Figure 2.10. 2D NOESY of **6**(1 mM) in aqueous β-cyclodextrin (3 mM).

The previously described prodrugs (**1Na, 2Na** and **6**) are salts and therefore are negatively charged organic molecules that revert to the corresponding target low water soluble drugs upon acidification. At physiological pH (7.4) these prodrugs must be used in combination with β-cyclodextrin. To ensure a proper drug delivery using prodrugs, it is of crucial importance to explore 'neutral' (less pH sensitive) **A-007** prodrugs. A second very important point in the design of the **A-007** prodrug is its timely release to the targeted area. Therefore, the half-life of drug released from the prodrug is an important factor in drug design.[19] The ideal prodrug is the one that is instantly transformed into the target drug at the site in which biological activity is desired.

Glucosides of active biological compounds are very often used in ESPT (Enzyme specific prodrug therapy) strategy.[20] Due to differing physiological conditions enzyme groups such as

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glucoronidases show activity in excess in cancer cells compared to normal cells. Several

prodrugs have been developed taking advantage of the excessive activity of these enzymes in

tumor tissues.  This strategy provides the means to selectively deliver an active drug to its

intended target from an inactive glycoside prodrug. This condition is of particular importance in

targeting cancer cells.
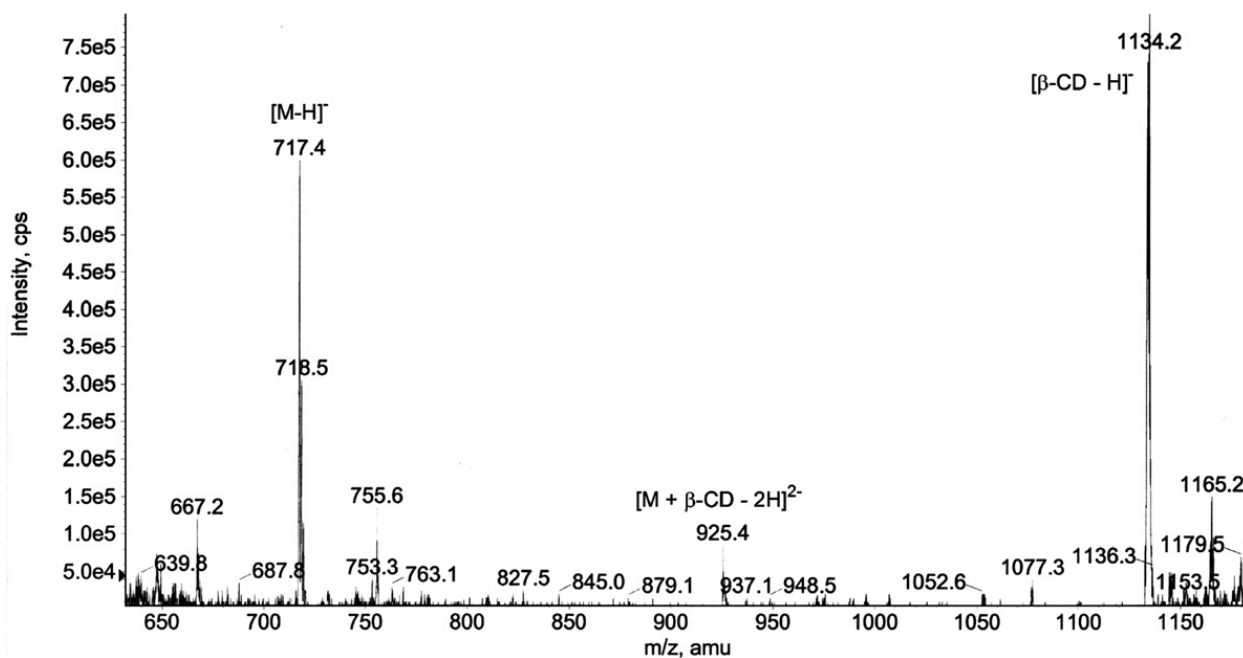


Figure 2.11. Negative ES-MS of diglucoside 5 (0.01 mM) and aqueous β-CD (0.03 mM).

Considering this, our glucoside **5** should be an ideal prodrug for **A-007**. After delivery of

the glucoside **5** to the vicinity of the cancer cell, the free drug **A-007** should be delivered by

enzymatic glycoside bond cleavage. To further support this hypothesis, it was also determined in

vitro that prodrug **5** has slightly higher anticancer activity than the original **A-007**.  However, the

solubility of **5** is still relatively low (~2 mg/1 mL of water). The solubility of this compound is

fivefold higher in the presence of β-cyclodextrin (from ~2 mg/1mL of water to 10 mg/1 mL of

aqueous β-CD). Even so, we could not observe substantial change of aromatic NMR chemical shift of **5** with any of the three cyclodextrins. However, in the ES-MS spectra of 5 in aqueous β-cyclodextrin, there is a weak signal that corresponds to the complex between one β-CD and one molecule of **5** (Figure. 2.11). The only plausible explanation is that inter-molecular interactions between **5** and β-cyclodextrin occurred between glucose moieties of **5** and the outside wall of β-cyclodextrin. That will increase the solubility of **5** in β-CD, show the molecular associate signal in ES-MS, but have no influence on the chemical shift of aromatic signals that are relatively far away from the molecular interactions.
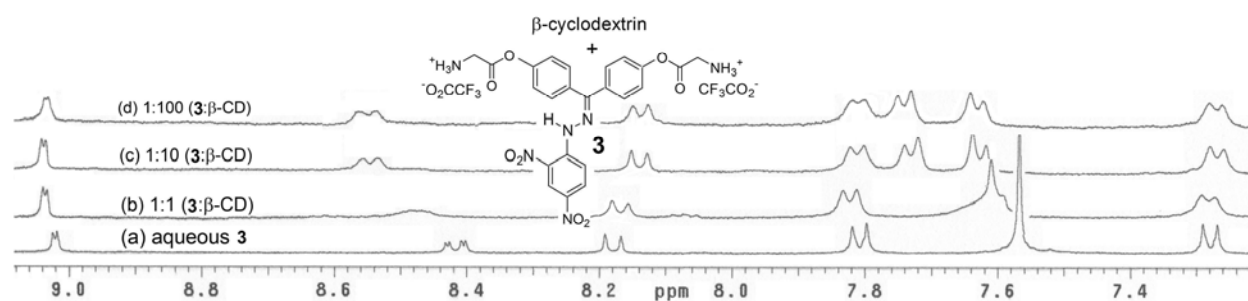


Figure 2.12. $^1$H NMR spectra of aqueous β-cyclodextrin of **3** (10 mM).
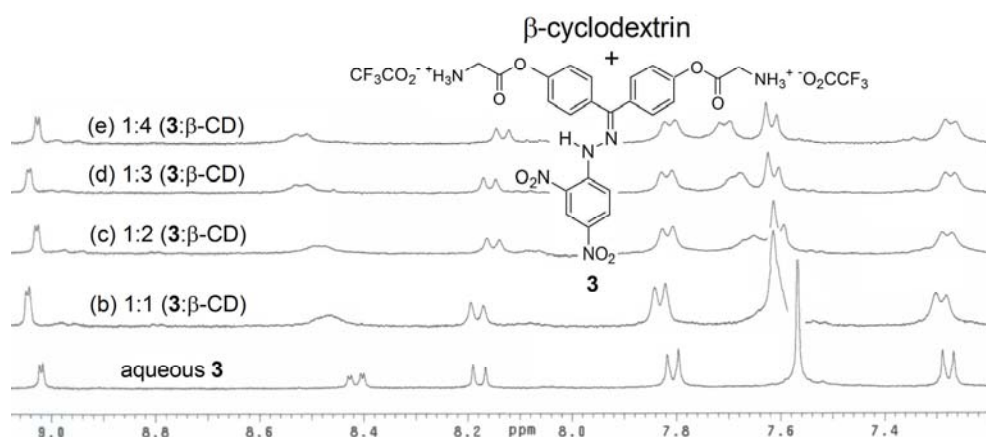


Figure 2.13. $^1$H NMR spectra different concentration of aqueous β-cyclodextrin of **3**.

To explore the influence of cationic **A-007** prodrugs on delivery (half-life), solubility, and anticancer activity, we prepared and studied the physical properties of glycine derived prodrugs **3** and **4**. The NMRs indicated the formation of β-cyclodextrin inclusion complexes (Figure 2.12 and 2.13). These compounds are hydrolysable prodrugs sensitive to the moisture and their stability in aqueous media might be questionable, based on the shelf-life of the compounds.[21] Considering that these compounds have surfactant-like properties, it is reasonable to expect that they might be able to form molecular aggregates similar to micelles in aqueous media. Aggregation was nicely demonstrated by following the NMR chemical shift for aromatic protons versus concentration of **4** in water media (Figure. 2.14). We observed a substantial downfield chemical shift with increasing concentration, with the maximal effect observed at 10 mM. At this concentration, aqueous **4** is stable at room temperature for long periods (at least three months). We assume that at this concentration, **4** forms molecular aggregates similar to micelles (Figure. 2.14), which slow down the hydrolysis of the ester group.
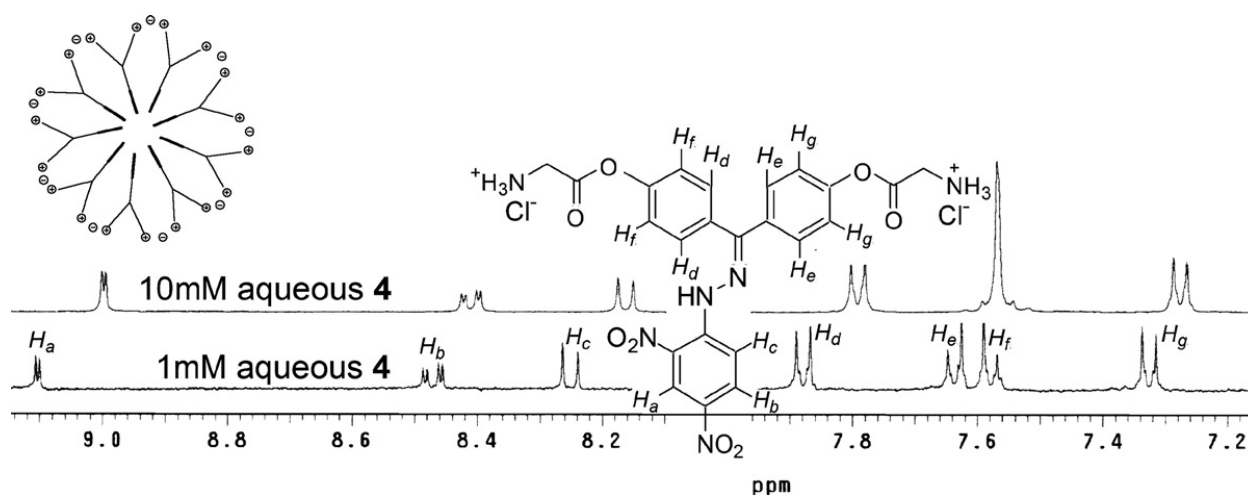


Figure 2.14. Dependence of concentration of 4 on the chemical shift.
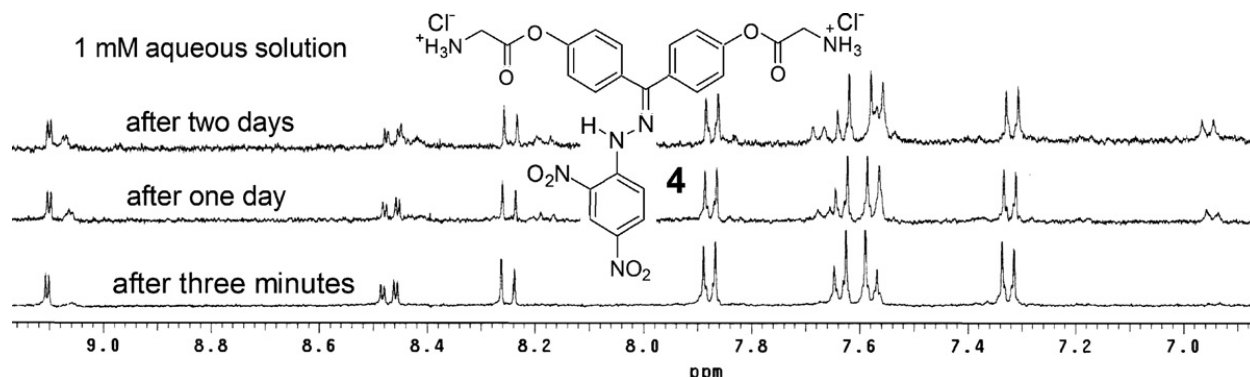
Figure 2.15. $^1$H NMR showing hydrolysis of **4**.

To confirm this assumption, we also explored the water stability of **4** at 1 mM concentration (Figure. 2.14, 2.15). Almost instantly, hydrolysis of the two ester groups of **4** started and the precipitation of **A-007** could be observed. Due to the exceptionally low water solubility of **A-007**, we were not able to obtain the NMR spectra of pure **A-007** in water, and the additional signals in the NMR spectra of **4** after one and two days (fig 2.15) correspond to the mono ester of **A-007** (partially hydrolyzed product of **4**). The NMR spectra of **4** with progress of time were used for computing the half-life of this. The half-life was determined by evaluating the decline of prodrug peak intensity (integration) versus time. It was determined that the half-life of **4** as a 1 mM concentration at room temperature was around 24 hours. As mentioned above, at a 10 mM concentration the formation of the precipitate was not observed even after a few months at 5°C. The comparative stabilities of **4** after 1, 2 and 3 days is shown in figure.2.16.

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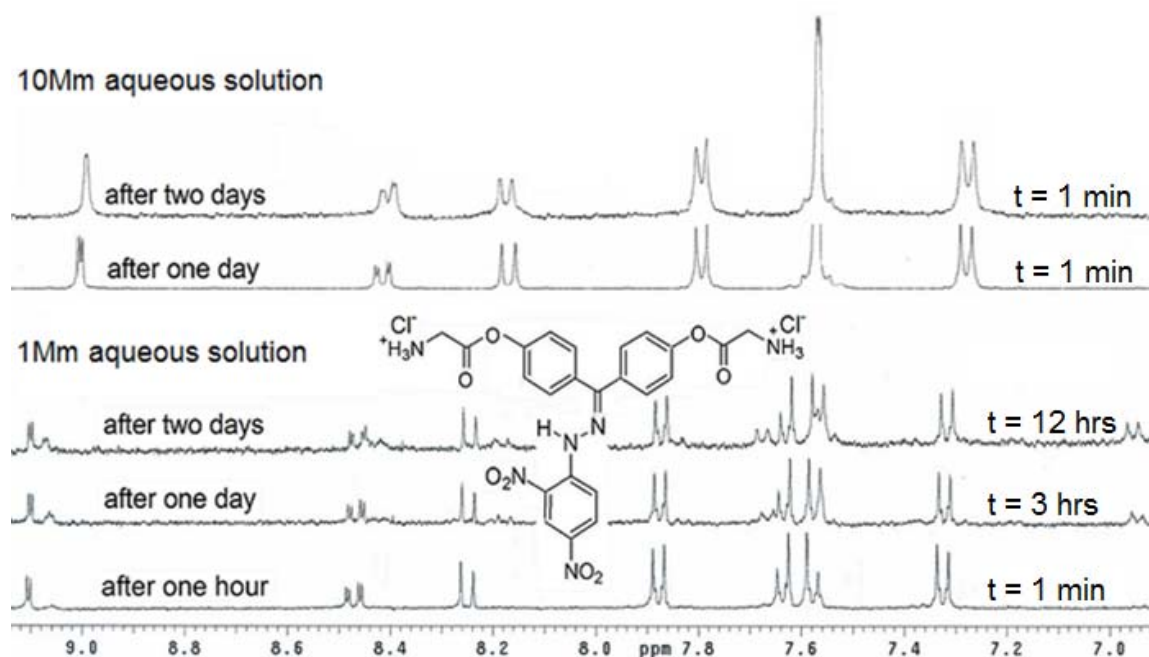
Figure 2.16. $^1$H NMR depicting concentration dependence of stability of **4**.

Prodrug **3** behaves similar to prodrug **4** in that there is a noticeable difference in the chemical shift of the **3** protons in aqueous β-cyclodextrin, indicating strong interactions between β-cyclodextrin and **3**. Unfortunately, we were not able to record a reliable two-dimensional NOESY NMR spectra due to fact that **3** hydrolyzed in water media. In the NMR sample there are three components (**3**, monoester of **A-007**, and **A-007**) in complex with β-cyclodextrin. The molar ratio of these three components varies with the progression of the NMR recording time. This is clearly demonstrated on the one-dimensional spectrum of **3** in water and β-cyclodextrin (Figure. 2.17). Even for the freshly prepared sample of **3** in water, the NMR spectra (acquired after 10 min) shows the presence of partially hydrolyzed **3**. Full hydrolysis of **3** generates **A-007** that immediately precipitates from the solution.

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Regardless of the fact that the characterization of the β-cyclodextrin inclusion complex with **3** is hard to evaluate by two-dimensional NMR spectroscopy, we believe that the ^{1}H NMR spectra supports the formation of the β-cyclodextrin inclusion complex. Furthermore, this prodrug half-life (~4.5 hours in aqueous β-cyclodextrin) seems to be ideal for pharmacological study of this prodrug [3].

## Conclusion

Several interesting **A-007** prodrugs were prepared through ester, ether, and acetal linkages of polar molecules to **A-007**. All the prodrugs have significantly higher water solubility as compared to original **A-007**. To further increase their water solubility, alter their water half-life, and biodelivery, cyclodextrin inclusion complexes were studied. Through one-dimensional NMR, two-dimensional NOESY, and negative ES-MS spectroscopy studies, it was demonstrated that inclusion complexes with β-cyclodextrin were formed for all but **A-007** glucoside **5**. It seems that the α-cyclodextrin cavity is too small and the γ-cyclodextrin cavity is too large to form stable inclusion complexes with these studied **A-007** prodrugs. It was postulated that β-cyclodextrin forms a 2:1 inclusion complex with the studied **A-007** prodrugs. None of the three studied cyclodextrins form inclusion complexes with the diglucoside of **A-007**, although they slightly increase the prodrug water solubility. This prodrug was prepared for targeted cancer therapy (ESPT).

## Experimental

Melting points were taken on an Electrothermal IA 9000 Digital Melting Point Apparatus and are uncorrected. The $^{1}$H and $^{13}$C NMR spectra were run on Varian 300 MHz Gemini2000, Varian 400 MHz Unity, Varian 500 MHz Unity and in CDCl$_3$, DMSO-d6 as solvent and internal standard. When D$_2$O is used as solvent then DMSO in D$_2$O (in a sealed capillary inside the NMR tube) is used as the internal standard (2.5 ppm). Two-dimensional NOESY spectra were recorded on Varian INOVA 500MHz spectrophotometer with D$_2$O as a solvent and internal standard (4.80 ppm). The mass spectra were recorded on a Micromass Quattro 2 Triple Quadropole Mass Spectrometer. The prepared ammonium **A-007** prodrugs are hydroscopic and decompose by ester hydrolysis. However under dry conditions they can be stored for at least several months. All compounds have melting points higher than 200°C and decompose before melting. For ammonium compounds **3** and **4** carbon NMR spectra were not reported because they decompose in solution during NMR acquisition time. For these compounds $^{1}$H NMR samples were prepared immediately before recording the spectrum.

### 4, 4' Dihydroxybenzophenone 2, 4 dinitrophenyl hydrazone (1)

MeOH suspensions (300 mL) of substituted 2,4 dinitrophenylhydrazine (29.3g, 0.148 mol) and concentrated sulfuric acid (20 mL) were stirred at 50°C. After the hydrazine dissolved, a MeOH solution (300 mL) of the 4, 4' dihydroxybenzophenone (0.1 mol) was added to the hydrazine and the resulting reaction mixture was stirred at 50°Cfor additional 30 min. The reaction mixture was concentrated to 1/4 of its original volume under vacuum and diluted with water (500 mL). The precipitates were separated by filtration and washed with 3% aqueous NaHCO$_3$ (3 x 100 mL) and water (3 x 50 mL). Products were recrystallized from MeOH, EtOH, or glacial AcOH. The

product was obtained as deep-red crystals in 78% yield. M.p. 270-272 °C. IR: 3506 (N-H), 3288 (N-H), 2921 (Ar-H), 1614 (C=N), 1592, 1503, 1417, 1336, 1311, and 1139 cm-1. ^{1}H NMR (DMSO-*d*6, 300 MHz): δ 11.13 (1H, s), 10.05 (1H, s), 9.99 (1H, s), 8.78 (1H, d, *J*= 2.7 Hz), 8.36 (1H, dd, *J*1 = 9.6 Hz, *J*2 =2.7 Hz), 8.12 (1H, d, *J*= 9.6 Hz), 7.47 (2H, d, *J*= 8.7 Hz), 7.22 (2H, d, *J*= 8.4 Hz), 7.01 (2H, d, *J*= 8.1 Hz), 6.81 (2H, d, *J*= 8.4 Hz). ^{13}C NMR (DMSO-*d*6, 100 MHz): δ 156.19, 155.31, 152.33, 140.47, 133.27, 126.50, 126.35, 126.02, 125.50, 124.07, 119.46, 118.19, 112.97, 112.90, and 111.91 ppm. Anal. Calcd for $C_{19}H_{14}N_4O_6$: C, 57.87; H, 3.58; N, 14.21. Found: C, 57.42; H, 3.81; N, 13.88.

**Ethyl {4-[[(2,4-dinitrophenyl)hydrazono]-( 4-ethoxycarbonyl methoxyphenyl)-methyl]-phenoxy}- ethanoate (2Et)**

An acetone (500 mL) suspension of **A-007** (3.94 g, 0.01 mol), potassium carbonate monohydrate (3.12 g, 0.01 mol), and ethyl 2-bromoacetate (3.34 g, 0.02 mol) was sonicated at room temperature for 2 h and heated at reflux overnight. The solvent from dark red suspension was evaporated to the solid residue. The solid residue was mixed with $CH_2Cl_2$ (200 mL), stirred at room temperature for 2 h, and the insoluble material was separated by filtration. The filtrate was washed with 10% potassium carbonate, dried over anhydrous magnesium sulfate and evaporated to deep red solid residue (5.3 g, 94%). According to the NMR spectroscopy product was more than 96% pure and was used in next step without further purification.

^{1}H NMR (300 MHz, CDCl₃) δ 11.30 (1H, s), 9.09 (1H, d, *J* = 2.4 Hz), 8.35 (1H, dd, *J*1 = 9.3, *J*2 = 2.4 Hz), 8.18 (1H, d, *J* = 9.3 Hz), 7,64 (2H, d, *J* = 9 Hz), 7.32 (2H, d, *J* = 8.4 Hz), 7.30 (2H, d, *J* = 8.7 Hz); 7.17 (2H, d, *J* = 8.4 Hz), 6.94 (2H, d, *J* = 9.0 Hz), 4.76 (2H, s), 4.69 (2H, s), 4.37 (2H, q, *J* = 6.9 Hz), 4.31 (2H, q, *J* = 6.9 Hz), 1.36 (3H, t, *J* = 6.9 Hz), 1.34 (3H, t, *J* = 6.9 Hz);

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) d 168.7, 159.8, 159.4, 155.2, 144.7, 138.0, 130.6, 130.1, 129.9, 129.6, 124.9, 123.7, 116.8, 116.3, 114.8, 65.6, 65.5, 14.4. Anal. Calcd for $C_{27}H_{26}N_4O_{10}$ (MW 566.52): C, 57.24; H, 4.63; N, 9.89. Found: C, 57.08; H 4.77, N 9.72.

**(4-{(4-carboxymethoxy-phenyl)-[(2,4- dinitro-phenyl)-hydrazono]-methyl}-phenoxy)-acetic acid (2)**

Water (150 mL), sodium hydroxide (1.6 g, 0.04 mol), and MeOH (150 mL) were mixed with $CH_2Cl_2$ (150 mL) solution of crude **A-007** ester (5.3 g, 9.3 mmol). This solution was stirred at room temperature for 4 h. The reaction mixture was concentrated to 1/3 of its original volume at 40°C and reduced pressure, diluted with water (100 mL), and neutralized with concentrated hydrochloric acid (10 mL). The solid material was separated by filtration, washed with water (3 x 100 mL), and dried at 110°C for a few hours to afford a product that was pure by NMR spectroscopy (4.5 g, 95%).

<sup>1</sup>H NMR (DMSO-d6, 400 MHz) d 11.12 (1H, s), 8.79 (1H, d, $J$ = 2.4 Hz), 8.34 (1H, dd, $J1$ = 9.6 Hz, $J2$ = 2.4 Hz), 8.17 (1H, d, $J$ = 9.6 Hz), 7.54 (2H, d, $J$ = 8.8 Hz), 7.38 (2H, d, $J$ = 8.4 Hz), 7.20 (2H, d, $J$ = 8.4 Hz), 6.99 (2H, d, $J$ = 8.4 Hz), 4.81 (2H, s), 4.73 (2H, s); <sup>13</sup>C NMR (DMSO-d6, 100 MHz) d 169.93, 169.90, 159.6, 159.0, 154.6, 144.1, 137.1, 130.1, 130.0, 129.6, 129.3, 123.8, 122.9, 116.6, 115.8, 114.7, 64.7, 64.6. Anal. Calcd for $C_{23}H_{18}N_4O_{10}$ (MW 510.41): C, 54.12; H, 3.55; N, 10.98. Found: C, 53.98; H, 3.63; N, 10.87.

**tert-butoxycarbonylamino-acetic acid 4-{[4-(2-tert-butoxycarbonylamino-acetoxy)-phenyl]- [(2,4-dinitro-phenyl)-hydrazono]-methyl}-phenyl ester (3BOC)**

tert-Butoxycarbonylamino-acetic acid (175 mg, 1 mmol), **A-007** (197 mg, 0.5 mmol), and dicyclohexylcarbodiimide (248 mg, 1.2 mmol) were taken in 20 mL of dry $CH_2Cl_2$. The reaction mixture was stirred at 0°C for 1 h and then at room temperature overnight. The progress of the reaction was monitored by proton NMR spectroscopy. The reaction mixture was concentrated to half of the original volume and filtered. The white residue was discarded and the filtrate was further concentrated and subjected to column chromatography on a silica gel column with $CH_2Cl_2$ and EtOAc (10:1) producing pure product (140 mg; 40%). ^{1}H NMR (CDCl$_3$, 400 MHz) 11.20 (1H, s), 9.01 (1H, d, $J$ = 2.8 Hz), 8.33 (1H, dd, $J1$ = 9.6 Hz, $J2$ = 2.0 Hz), 8.14 (2H, d, $J$ = 9.6 Hz), 7.43 (2H, d, $J$ = 8.8 Hz), 7.38 (2H, d, $J$ = 8.4 Hz), 7.15 (2H, d, $J$ = 8.8 Hz), 5.28 (2H, m), 4.22 (2H, d, $J$ = 4.8 Hz), 4.18 (2H, d, $J$ = 4.4 Hz), 1.48 (9H, s), and 1.47 (9H, s); ^{13}C NMR (CDCl$_3$, 100 MHz) 169.1, 168.9, 155.9, 153.6, 152.2, 152.0, 144.5, 136.4, 134.3, 130.1, 129.9, 129.8, 129.3, 123.5, 121.8, 116.7, 80.5, 42.8, 28.4. Anal. Calcd for $C_{33}H_{36}N_6O_{12}$ (MW 708.67): C, 55.93; H, 5.12; N, 11.86. Found: C, 55.73; H, 5.12; N, 11.77.

**3,3'-(4,4'-((2-(2,4-dinitrophenyl)- hydrazono)methylene)bis(4,1-phenylene)bis(oxy))bis- (2-oxoethylammonium trifluoroacetate) (3)**

A mixture of $CH_2Cl_2$–trifluoroacetic acid (9 mL:1 mL) was cooled to 0°C and **3BOC** was added (354 mg, 0.5 mmol). The reaction mixture was stirred at 0°C for 1 h and solvent was evaporated under a stream of nitrogen resulting in red crystalline product (361 mg; 98%). ^{1}H NMR (DMSO-d6, 400 MHz) d 11.04 (1H, s), 8.83 (1H, d, $J$ = 2.4 Hz), 8.48 (3H, m), 8.83 (3H, m), 8.26 (2H, d, $J$ = 9.3 Hz), 7.78 (2H, d, $J$ = 8.8 Hz), 7.66 (2H, d, $J$ = 8.4 Hz), 7.55 (2H, d, $J$ = 8.4

Hz), 7.32 (2H, d, $J$ = 8.8 Hz), 4.21 (2H, m), 4.16 (2H, m). Anal. Calcd for $C_{27}H_{22}F_6N_6O_{12}.2H_2O$ (MW 772.52): C, 41.98; H, 3.39; N, 10.88. Found: C, 42.02; H, 3.37; N, 10.75.

**3,3'-(4,4'-((2-(2,4-dinitrophenyl)- hydrazono)methylene)bis(4,1-phenylene)bis(oxy))bis- (2-oxoethylammonium chloride) (4)**

A $CH_2Cl_2$ (0.2 mL) solution of **3BOC** (45 mg; 0.06 mmol) was injected into an ice cold MeOH (2 mL) solution of hydrochloric acid (made by the addition of 0.2 mL of oxalyl chloride). A red hygroscopic precipitate was immediately formed. The product was separated by filtration, washed with $CH_2Cl_2$ (3 x 0.5 mL), and dried under nitrogen to produce a product that was pure by NMR spectroscopy (33 mg, 95%). ^{1}H NMR (DMSO-d6, 400 MHz) d 11.04 (1H, s), 8.33 (1H, d, $J$ = 2 Hz), 8.45 (1H, dd, $J1$ = 9.6 Hz, $J2$ = 2.0 Hz), 8.26 (1H, d, $J$ = 9.6 Hz), 7.77 (2H, d, $J$ = 8.4 Hz), 7.64 (2H, d, $J$ = 8.0 Hz), 7.56 (2H, d, $J$ = 8.4 Hz), 7.32 (2H, d, $J$ = 8.4 Hz), 4.18 (2H, s), 4.13 (2H, s). Negative ES-MS $m/z$ 507.4 (M-2HCl-H^{+}), 450.3 (M-3HCl-COCH_2NH_2[+]), 393.4 (M-2HCl- 2COCH_2NH_2-H^{+}); Anal. Calcd for $C_{23}H_{22}Cl_2N_6O_8$. $2H_2O$ (MW 617.39): C, 44.74; H, 4.24; N, 13.61. Found: C, 44.56; H, 4.31; N, 13.45.

**Penta-*O*-acetyl-D-glucopyranose (10):** D-glucose (20 g, 110 mmol) was refluxed in pyridine (150 mL) for 1 hour. Acetic anhydride (100 mL) was added and the mixture was refluxed an additional 2 hours. The resulting solution was cooled to room temperature, poured over ice (300 mL) and stirred until the product solidified, about 2 hours. The solid was filtered, washed with water, and recrystallized from ethyl acetate/hexanes to give **10** as a white solid (36 g, 83 % yield). **10β**: D-glucose (5.0 g, 28 mmol), sodium acetate (4.0 g, 50 mmol), and acetic anhydride (30 mL) were stirred at 100°C for 3 hours. The mixture was cooled to room temperature, poured

over ice (150 mL) and stirred for 2 hours. The resulting white solid was filtered and washed with water and recrystallized from methanol to give 10β as a white solid (3.7 g, 38 % yield). $^1$H NMR of **10β** (400 MHZ, CDCl$_3$): δ 5.72 (1H, d, $J$ = 4.0 Hz), 5.26 (1H, t, $J$ = 9.2 Hz), 5.14 (1H, t, $J$ = 8.8 Hz), 5.13 (1H, t, $J$ = 10.0 Hz), 4.30 (1H,  dd, $J_1$ = 6.2 Hz, $J_2$ = 2.2 Hz), 4.12 (1H, dd, $J_1$ = 6.3 Hz, $J_2$ = 1.0 Hz), 3.84 (1H, m), 2.12 (3H, s), 2.09 (3H, s), 2.04 (6H, s), 2.01 (3H, s). $^{13}$C NMR of **10β** (100 MHz, CDCl$_3$): δ 170.7, 170.3, 169.8, 169.5, 168.9, 89.1, 69.9, 69.2, 67.9, 61.5, 20.9, 20.8, 20.7, 20.6, 20.5.


**2,3,4,6-Tetra-*O*-acetyl–D-glucopyranose(11):**  D-glucose penta acetate (10 g, 26 mmol) and 2-aminoethanol (3.9 g, 64 mmol) were dissolved in ethyl acetate (250 mL) and DMSO (2.5 mL) and stirred at room temperature overnight. The mixture was washed with water (3 x 150 mL), dried over sodium sulfate and concentrated to give **11** as colorless syrup (6.2 g, 70% yield). This product was used immediately in the further reactions without further purification. $^1$H NMR of **11α** (300 MHz, CDCl$_3$): δ 5.51 (1H, t, $J$ = 9.9 Hz), 5.45 (1H, d, $J$ = 1.5 Hz), 5.06 (1H, t, $J$ = 9.9 Hz), 4.89 (1H, dd, $J_1$ = 5.03 Hz, $J2$ = 1.8 Hz), 4.26 – 4.06 (3H, m), 2.07 (3H, s), 2.06, (3H, s), 2.01 (3H, s), 2.00 (3H, s). $^{13}$C NMR of **11α** (100 MHz, CDCl$_3$): δ 171.2, 170.5, 170.4, 169.9, 95.3, 73.0, 72.5, 71.9, 68.4, 68.2, 20.8, 20.7, 20.6, 20.6. $^{13}$C NMR of **11β** (100 MHz, CDCl3): δ 171.2, 170.5, 170.4, 169.9, 90.0, 71.2, 70.0, 68.6, 67.0, 62.0, 20.8, 20.7, 20.6, 20.6.

**Tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate (12):**

2,3,4,6-Tetra-*O*acetyl–D-glucopyranose(**11**) (2.0 g, 3.3 mmol), $K_2CO_3$ (1.1 g, 8.2 mmol), and trichloroacetonitrile (5 mL) were stirred at room temperature in dichloromethane (20 mL) for 48 hours. The resulting suspension was filtered through silica gel using a 1:1 mixture of $CH_2Cl_2$ and $Et_2O$ (150 mL). The filtrate was concentrated to give **12** as a pale yellow solid (2.0 g, 83 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.67 (1H, s), 6.52 (1H, d, *J* = 1.8 Hz), 5.53 (1H, t, *J* = 9.6 Hz), 5.15 (1H, t, *J* = 10.0 Hz), 5.10 (1H, dd, $J_1$ = 6.8 Hz, $J_2$ = 2.0 Hz), 4.24 (1H, dd, $J_1$ = 6.2 Hz, $J_2$ = 2.0 Hz), 4.18 (1H, m), 4.10 (1H, dd, $J_1$ = 6.0 Hz, $J_2$ = 1.0 Hz), 2.05 (3H, s), 2.02 (3H, s), 2.00 (3H, s), 1.99 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.9, 170.3, 170.2, 169.8, 161.0, 93.0, 70.2, 70.0, 69.9, 67.9, 61.5, 20.8, 20.8, 20.6.


**4,4'-dihydroxybenzophenone-2,4-dinitrophenylhydrazone bis(2,3,4,6-tetra-*O*-acetyl-β-Dglucopyranoside) (5Ac)**

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-trichloroacetimidate (370 mg, 0.75 mmol) and **A-007** (118 mg, 0.3 mmol) were stirred under an atmosphere of nitrogen in anhydrous $CH_2Cl_2$ (20 mL) with 3Å molecular sieves for 1 h. The solution was cooled in an ice-bath for 30 min before $BF_3$-$OEt_2$ (2 mL) was added and stirred for 1 min the ice-bath followed by 1 h at room temperature. The solution was added to an ice cold saturated aq $NaHCO_3$ solution (100 mL) with vigorous stirring and then extracted with ether (2 x 75 mL). The organic layers were combined and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography ($CH_2Cl_2$ –$CH_3CO_2Et$ 10:1) to give 5Ac as red crystalline solid (86 mg; 40%).

$^1$H NMR (CDCl$_3$, 400 MHz) δ 11.28 (1H, s), 9.09 (1H, d, *J* = 2.8 Hz), 8.37 (1H, dd, *J*1 = 2.8 Hz, *J*2 = 9.6 Hz), 8.17 (1H, d, *J* = 9.2 Hz), 7.61 (2H, d, *J* = 8.8 Hz), 7.31 (2H, d, *J* = 8.8 Hz), 7.25 (2H, d, *J* = 8.8 Hz), 5.40–5.10 (6H, m), 4.30 (2H, m), 4.20 (2H, t), 3.9 (2H, m), 2.13 (3H, s), 2.09 (3H, s), 2.08 (3H, s), 2.075 (6H, s), 2,071 (3H, s), and 2.065 (3H, s). Negative ES-MS 1011.8 [M-H-] ; positive ES-MS 1035.8 [M+Na]$^+$. Anal. Calcd for C$_{45}$H$_{48}$N$_2$O$_{23}$ (MW 1012.88): C, 53.36; H, 4.78; N, 5.53. Found: C, 53.25; H, 4.88; N, 5.42.

**4,4'-dihydroxybenzophenone-2,4-dinitrophenylhydrazone bis(β-D-glucopyranoside) (5)**

Octa-acetate **5Ac** (101 mg, 0.1 mol) was dissolved in 2:1 MeOH–CH$_2$Cl$_2$ (30 mL). Sodium hydroxide (1 M) was added to a pH 9–10. The solution was stirred at room temperature overnight, neutralized with acidic Dowex resin, and evaporated. The solid residue was dried under reduced pressure at room temperature to give 68 mg (95%) of red crystalline product. $^1$H NMR (DMSO-d6, 400 MHz) d 11.13 (1H, s), 8.80 (2H, d, *J* = 2.4 Hz), 8.40 (2H, dd, *J*1 = 9.6 Hz, *J*2 = 2.4 Hz), 8.19 (2H, d, *J* = 9.6 Hz), 5.54 (1H, d, *J* = 4.8 Hz), 5.48 (1H, d, *J* = 4.8 Hz), 5.18 (4H, m), 4.99 (1H, d, *J* = 7.2 Hz), 4.92 (1H, D, *J* = 7.4 Hz), 4.74 (1H, t, *J* = 5.6 Hz), 4.67 (1H, t, *J* = 5.6 Hz), 3.2 (4H, m); $^{13}$C NMR (DMSO-d6, 100 MHz) δ 159.4, 159.0, 154.9, 144.5, 137.6, 130.6, 130.3, 129.7, 129.6, 125.0, 123.4, 117.8, 117.0, 116.6, 100.6, 100.3, 77.3, 76.8, 73.6, 73.5, 70.0, 61.0. Positive ES-MS 731.5 [M+Na]$^+$; Negative ES-MS 717.4 [M-H]. Anal. Calcd for C$_{31}$H$_{34}$N$_4$O$_{16}$ (MW 718.62) C, 51.81; H, 4.77; N, 7.80. Found: C, 51.65; H, 4.88; N, 7.68.

**Succinic acid mono-t-butyl ester (8)**

To a mixture of succinic anhydride (3g, 30 mmol), *N*-hydroxysuccinimide(1g, 9 mmol) and DMAP (0.35 g, 2.86 mmol) in toluene (50 mL) were added tert-butyl alchohol (5 mL) and triethyl amine (9mmol, 1.25 mL).  The brownish solution was refluxed for 24 h.  The solution was cooled and diluted with ethyl acetate (50 mL) was washed with 10% citric acid (2 x 50 mL) and brine, dried over anhydrous $Na_2SO_4$ , and concentrated to give a brown oil, which on recrystallization from diethyl ether/petroleum ether (1:3) afforded pure **8** (4.1g, 78%).  ^{1}H NMR (300 MHz, CDCl$_3$) δ 2.65-2.50 (m, 4H), 1.45(s, 9h).  ESI-MS: *m/z* [M-H][-] =173.1. ^{13}C NMR (DMSO-d6, 100 MHz)d 150.3, 141.9, 117.4.

**Succinic acid 4-{[4-(3-tert-butoxycarbonyl-propionyloxy)-phenyl]-[(2,4-dinitro-phenyl)-hydrazono]-methyl}-phenyl ester tert-butyl ester (6tBu)**

Succinic acid mono-tert-butyl ester (**8**) (3.13g), **A-007** (2.5g), and dicyclohexylcarbodiimide (3.92g) were taken in 50 mL of dry $CH_2Cl_2$.   The reaction mixture was stirred at 25°C for 1 h and then at room temperature overnight. The progress of the reaction was monitored by proton NMR spectroscopy. The reaction mixture was concentrated to half of the original volume and filtered. The white residue was discarded and the filtrate was further concentrated and subjected to column chromatography on a silica gel column with $CH_2Cl_2$ and EtOAc (7:1) producing pure product (2.24 g; 50%). ^{1}H NMR (400 MHz, CDCl$_3$) δ 11.26 (s, 1H), 9.09 (d, *J* = 2.5, 1H), 8.38 (dd, *J* = 2.5, 9.6, 1H), 8.19 (d, *J* = 9.6, 1H), 7.71 (d, *J* = 8.7, 2H), 7.422 (q, 4H), 7.166(d,*J*=8.8, 2H), 2.80 (m, 8H), 1.45 (m, 18H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 171.4, 171.0, 170.9, 153.9, 152.6, 152.3, 144.6, 138.3, 134.1, 130.2, 129.9, 129.7, 129.3, 129.0, 123.6, 122.0, 116.7, 30.4, 30.3, 29.7, 29.7, 28.3.  Anal. Calcd for $C_{35}H_{38}N_4O_{12}$(MW 706.70) C, 59.48; H, 5.42; N, 7.93. Found:  C, 59.36; H, 5.49; N, 7.83.

**Succinic acid mono-(4-{[4-(3-carboxy-propionyloxy)-phenyl]-[(2,4-dinitro-phenyl)-hydrazono]-methyl}-phenyl) ester (6)**

A mixture of $CH_2Cl_2$–trifluoroacetic acid (9 mL:1 mL) was cooled to 0°C and **6tBu** was added (200 mg, 0.28 mmol). The reaction mixture was stirred at 0°C for 8 h and solvent was evaporated under a stream of nitrogen resulting in red amorphous product.  It is stirred in 5 mL of ice cold hexane at 0°C for 10 minutes.  Hexane is reduced to half its volume under a slow stream of nitrogen.  The product is filtered under vacuum to yield pure product as seen in NMR (160 mg; 98%).  ^{1}H NMR (400 MHz, D$_2$O) δ 10.43 (s, 1H), 9.09 (s, 1H), 8.42 (d, $J$ = 16, 1H), 8.11 (d, $J$ = 12.2, 1H), 7.63 (d, $J$ = 8, 2H), 7.23 (d, $J$ = 16.2, 2H), 6.92 (d, $J$ = 8.7, 2H), 6.72 (d, $J$ = 8.4, 2H), 2.61(m, 8H); ^{13}C NMR (100 MHz, D$_2$O) δ 177.4, 169.1, 169.3, 155.9, 155.8, 155.0, 146.1, 135.9, 131.0, 138.2, 121.6, 121.1, 128.3, 128.4, 128.1, 116.5, 116.1; Anal. Calcd for $C_{27}H_{22}N_4O_{12}$  (MW 594.48) C, 54.55; H, 3.73; N, 9.42;  Found:  C, 51.43; H, 4.16; N, 8.89.

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# Chapter III.  Chemoselective debenzylation by Catalytic Hydrogen Transfer Reactions in the presence of the Nitro and Imino Functionalities.

## Abstract

It is impossible to envision any synthetic task of preparation of complex natural products without protecting groups in the course of their preparation.  Removal of the protecting group is sometimes a very demanding process due to presence of functionalities in the molecules that are sensitive to the deprotection reaction conditions.   In an attempt to synthesize hydrophilic **A-007** prodrugs, we have had a similar problem.  To circumvent this problem, we explored chemoselective benzyl deprotection methodologies and determined several simple solutions with variety of chemoselectivities towards aliphatic/ aromatic benzyl groups, nitro groups and hydrazone moieties in good yields.   For debenzylation of **A-007** prodrugs, catalytic hydrogenation as well as use of strong acids was avoided in deference to the sensitivity of nitro group and the lability of the hydrazone and ester moieties.

## Introduction

Despite promising anticancer activity,[1-9] due to its very low solubility in water, **A-007** cannot be delivered either orally or intravenously.  To be able to use it orally or intravenously the drug should be labile-bound to nontoxic water soluble compounds called carrier molecules. Several options for carrier molecules include saccharides and amino acids.[10-13]  Target structures of these simple **A-007** prodrugs from each of these  families are presented in Scheme 3.1.

Prodrug **1** is a saccharide derivative and prodrug **2** is lactic acid saccharide derivative of **A-007**. Using conventional protection groups to connect saccharide and lactic acid moieties is hampered by the acid-base sensitivity of ester group of **2**, and the acid sensitivity of acetal and hydrazone group of **1** and **3**. Benzyl protection of the alcohol moiety would be ideal but 2,4-dinitrophenylhydrazone moiety of **A-007** cannot withstand reduction conditions required for benzyl group removal. Therefore, if we have the capability to selectively remove the benzyl protection group from **3, 4** and **5,** then a road to preparation of oligosaccharide and peptidosachharide derivatives of **A-007** will be established.
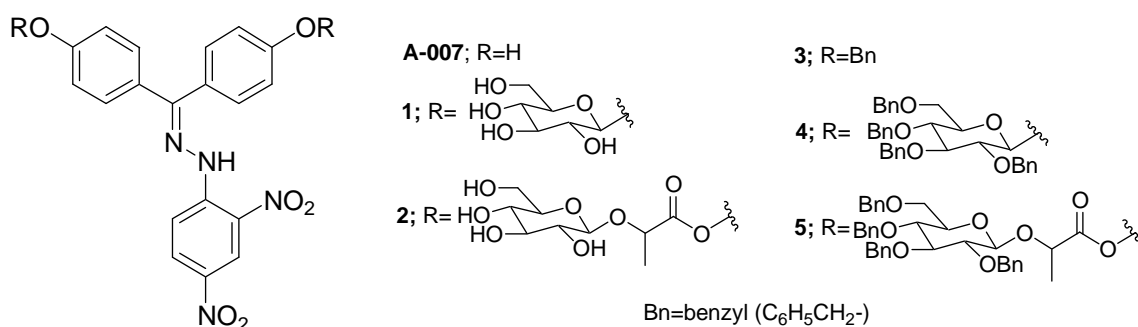


Figure 3.1. **A-007** saccharide and amino acid saccharide prodrugs

Considering the availability of both the 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate[14-18] and benzyl lactate[19], as well as availability of synthetic methodology for their coupling with **A-007**, one can assume that preparation of prodrugs **1** and **2** is a simple synthetic task (Figure 3.1). However, removal of the benzyl protection group from **4** and **5** is daunting task considering that the **A-007** moiety contains easy to reduce functionalities (nitro and hydrazine). Therefore, broadly used debenzylation methods such as catalytic hydrogenation[20, 21] cannot be applied in these cases. The presence of acetal, ester, amino, and hydrazine groups preclude the use of a strong acid as catalyst for dibenzylation.[22-25] There are a

few literature indications that benzyl protection can be removed through catalytic hydrogen transfer, but[26, 27] to the best of our knowledge a thorough study of benzyl deprotection in presence of easily reduced functionalities has not yet been performed.  For this reason, we explored benzyl deprotection methodologies.  The goal was to select a suitable catalyst and develop an optimized catalytic procedure in which the level of the nitro-reduced and hydrazone reduced product was minimized. A variety of palladium catalysts with different sources of hydrogen were employed to explore these possibilities. The effect of different variables on the activity and selectivity of the debenzylation were investigated attempting to establish a suitable one-pot reduction method for chemoselective debenzylation.  Our study indicated that with proper selection of the catalyst, hydrogen source and control of the reaction conditions, the selectivity for the desired debenzylation product can be achieved as high as 95%.  In addition, we also determined several simple solutions to obtain variety of other chemoselectivities (nitro reduction in preference to benzyl, aromatic debenzylation in preference to aliphatic debenzylation etc.) in good yields.
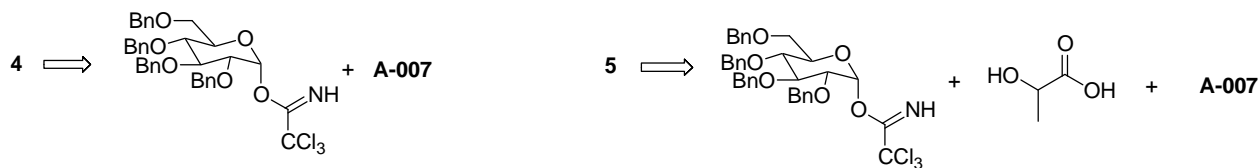


Scheme 3.1.  Retrosynthetic path for prodrugs **4** and **5**.

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# Results and discussion

In order to know the selectivity of different debenzylation conditions, we needed to first synthesize substrates **4** and **5**. At the start of this investigation, we did not have these compounds available and we needed to develop a method first before pursuing this synthetic pathway. Hence we needed model substrates which provided the required functionalities whose relative reducibility were to be determined. For this purpose, we designed the three model compounds shown in table **3.1**. Compound **6** can be used to test under a particular set of reduction conditions, the chemoselective reduction of nitro group over debenzylation or *vice versa* . Similarly, compound **8** can determine selectivity of the reducing agent towards the hydrazine functional group, in addition to the nitro group. The compound that we finally planned to perform the debenzylation, includes the benzyl group on a sugar moiety, hence analogues compound **7** is one of the model compounds included.
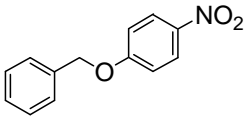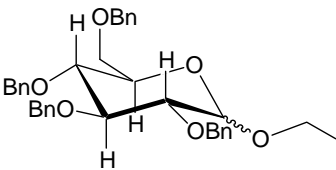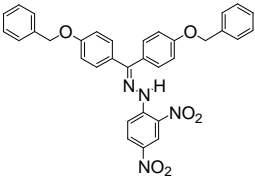


Table 3.1: Model compounds.

## *Transfer Hydrogenation with Formic Acid Analogues*

The first transfer hydrogenation reagent we choose was used formic acid as the hydrogen source.[26, 27] Transfer hydrogenation using formic acid as hydrogen donor is simple and is of

interest because it avoids some of the inconvenient aspects of conventional hydrogenation procedures. As compared to hydrogen, formic acid is very soluble in most common solvents. It reacts rapidly with palladium on charcoal at room temperature even without the presence of a hydrogen acceptor to give a hydrogenated palladium, $Pd_nH$, and carbon dioxide.[27] Transfer hydrogenation with formic acid[28] and palladium thus appears to proceed in two separate and distinct steps as follows:

$$HCOOH + 2n\ Pd \longrightarrow 2\ Pd_nH + CO_2$$
$$2\ Pd_nH + A \longrightarrow AH_2 + 2n\ Pd$$
$$\overline{HCOOH + A \qquad\qquad 2AH_2 + CO_2}$$
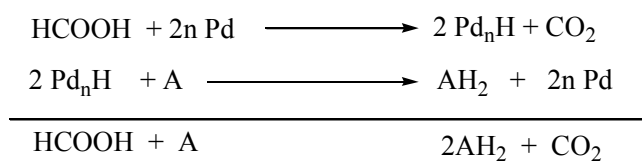
The simplicity of this procedure makes it very attractive considering no special hydrogenating equipment is necessary. We explored the novelty of this reaction on the three model compounds that were previously prepared (Table 3.1). Unfortunately, these conditions seemed too harsh for the reduction sensitive functionalities in our target substrates. Under these conditions, both the the nitro and benzyl groups were reduced in both model compounds **6** and **8**. Model compound **7** was completely debenzylated, indicating that while formic acid is a good debenzylating agent, it does not offer the required chemoselectivity with respect to the other reduction sensitive functionalities in question. We then attempted to see if chemoselectivity could be achieved by using lower molar equivalents of formic acid and by using shorter reaction times. These changes did not yield the required chemoselectivity either.

In an attempt to investigate the potential of ammonium formate[29,30a,b] as a milder derivative of formic acid, we performed the reactions on the model compounds with ammonium

formate[30b,31] as the hydrogen source.  Again, as in the case of formic acid, chemoselectivity could not be achieved by using lower molar equivalents or using shorter reaction times.  We finally succeeded in achieving the desired chemoselective debenzylation using ammonium acetate as the hydrogen source, in the case of aromatic benzyl ethers.  Under these conditions aliphatic benzyl ethers remain unaffected.  This was clear as under these conditions, compounds having benzyl group in the form of aromatic benzyl ethers namely **6** and **8** got completely debenzylated, while **7** remained unchanged.  The results of the hydrogen transfer chemoselective studies with formic acid analogues are shown in table **3.2**.  Compounds **6** and **8** having benzyl groups present in the form of aromatic benzyl ethers are completely reduced chemoselectively leaving nitro group unchanged.  On the other hand Compound **7**, which has benzyl groups protecting aliphatic alcohols are completely unaffected under similar conditions.

These results can be explained based on the fact that the aromatic ring lies flat on the metal surface for optimal coordination.[31, 32] In compounds where the substrate is an aromatic benzyl ether, the planar geometry facilitates the effective binding with the metal surface and hence the benzylic group in such compounds is preferentially cleaved.  On the other hand, aliphatic alcohols could have an adverse steric effect that would interfere with the planar geometry required for effective binding which would reduce its affinity for the metal surface as compared to its phenolic counterpart. Our experiments show that the affinity of the palladium metal to the substrate is proportional to the number of substrate occupied active sites on the palladium surface, which, in turn, determines the ease of the reduction process.  If this were to be true, one would anticipate that the 2-naphthylmethyl (NAP) group with its flat extended aromatic system should have a high affinity to the palladium surface and should be deprotected preferentially to the benzyl group[31,33]. Indeed literature indicates that the hydrogenolysis of ether

**9** shows that the NAP group can be selectively deprotected in the presence of a benzyl group (Scheme 3.3).
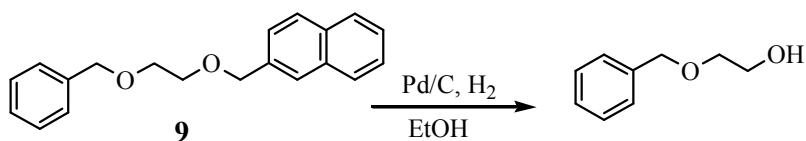


Scheme 3.3: Selective deprotection of NAP vs. Benzyl group.

The transfer hydrogenation reactions with ammonium acetate as the hydrogen source is very promising considering that it can not only be an extremely simple, mild chemoselective reduction method for benzyl reduction over nitro group, but it can also be used as a method of chemoselective debenzylation of aromatic benzyl ethers over aliphatic benzyl ethers. Unfortunately, the major disadvantage of this method is that the reaction must be carried out for relatively long times at refluxing temperature with potential damage particularly to other temperature sensitive functional groups. In order for this promising deprotection method to be of any practical significance, it is important to bring down the reaction time.

Table 3.2: Benzyl reductions with formic acid derivatives.

$$O\text{-Bn derivative} \xrightarrow[\substack{10\% \text{ Pd/C, Methanol, reflux under } N_2 \\ 80\text{-}85\%}]{\text{A formic acid derivative}} \text{Debenzylated product}$$

| Entry # | Substrate | Hydrogen source | Eq of Hydrogen source | Time (hours) | Result |
|---------|-----------|-----------------|------------------------|--------------|--------|
| 1 | 6 | $HCOONH_4$ | 5 Eq | 0.5 | Complete $-NO_2$ reduction and Debenzylation |
| 2 | 7 | $HCOONH_4$ | 5 Eq | 2.5 | Complete Debenzylation |
| 3 | 8 | $HCOONH_4$ | 5 Eq | 3.5 | Complete Debenzylation, $-NO_2$, $-C=N-$ reduction in progress |
| 4 | 6 | $HCOONH_4$ | 1 Eq | 5 | Parallel reduction of $-NO_2$ and $-Bn$ group |
| 5 | 7 | $HCOONH_4$ | 1 Eq | 56 | $-Bn$ group untouched |
| 6 | 8 | $HCOONH_4$ | 1 Eq | 15 | $-NO_2$ reduction started before Debenzylation and $-C=N-$ reduction |
| 7 | 6 | $CH_3COONH_4$ | 20 Eq | 120 | Complete Debenzylation, $-NO_2$ untouched |
| 8 | 7 | $CH_3COONH_4$ | 20 Eq | 120 | Benzyl group still present |
| 9 | 8 | $CH_3COONH_4$ | 20 Eq | 144 | Complete Debenzylation, $-NO_2$, $-C=N-$ untouched |

Recently there have been reports of microwave-induced organic reaction enhancement

(MORE) chemistry techniques[34] leading to reaction rapidity and improved yields. These reports

provoked us to test the feasibility of our debenzylation method under microwave irradiation. We

employed this deprotection procedure successfully to enhance reaction rapidity. The selectivity

is retained and the corresponding deprotection products are formed in up to 75% yield. The

reduction in overall reaction time is drastic, considering similar reactions done under reflux

conditions take several days as compared to reactions performed in microwave, which only take

few minutes.  There is a difference in using the microwave method. Methanol/ethanol solvent that is normally used in formic acid based transfer hydrogenation reactions do not yield good results. A simple modification of reaction conditions i.e. replacement of ethanol with high boiling solvents, such as ethylene glycol yielded good results.[35] The reaction is a one pot synthesis and purification involves a simple filtration followed by the aqueous workup.  The results are summarized in Table 3.3.  Unfortunately, in the target prodrugs **4** and **5** the benzyl groups are in the form of aliphatic benzyl ethers on a sugar moiety, hence this method cannot be applied to our target prodrugs.  Despite that, this transfer hydrogenation method is excellent for chemoselective aromatic ether debenzylations over aliphatic benzyl ethers and also over nitro reductions.

The above promising results encouraged us to look for low cost alternatives to perform this reaction.  The use of low-cost metals such as magnesium[36], zinc[37] and lead[38] used for deprotection of *N*-Bn groups is known in literature[39].  To our knowledge such methods for debenzylation of *O*-Bn groups is not known.  We decided to explore this avenue with the goal to select a suitable low-cost debenzylation catalyst that can retain the reactivity as well as selectivity of the Pd/C catalyst.

Table 3.3:  Benzyl reductions with ammonium acetate under microwave conditions.

$$O\text{-Bn derivative} \xrightarrow[\substack{10\% \text{ Pd/C, } 60\text{-}75\% \\ \text{microwave}}]{\text{Ammonium acetate, Alcohol}} \text{Debenzylated product}$$
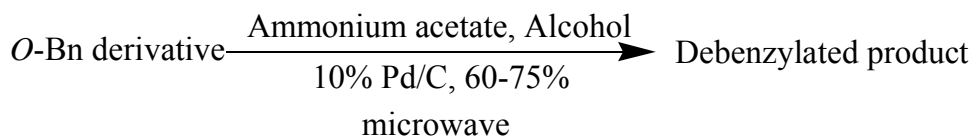
| Entry # | Substrate | Solvent | Result |
|---------|-----------|---------|--------|
| 1 | 6 | Methanol | No change |
| 2 | 7 | Methanol | No debenzylation |
| 3 | 6 | Ethanol | No change |
| 4 | 7 | Ethanol | No debenzylation |
| 5 | 6 | Isopropyl alcohol | No change |
| 6 | 7 | Isopropyl alcohol | No debenzylation |
| 7 | 6 | Ethylene glycol | Debenzylation, $-NO_2$ untouched |
| 8 | 7 | Ethylene glycol | No Debenzylation |
| 9 | 8 | Ethylene glycol | Debenzylation |

Initially, the reaction was performed under exactly similar conditions, except the catalyst Pd/C was replaced with Zn.  The results of this change were very interesting.  With Zn, the chemoselectivity was completely opposite to that of palladium/carbon.  The nitro group is very sensitive under these conditions and gets reduced completely to the amine while the benzyl group remains unchanged.  Even upon continuing the reaction for longer times, the benzyl group remains unchanged both from aliphatic and aromatic systems, indicating zinc is not good catalyst for debenzylations, at least under these conditions.   Zinc catalyzed reactions always seem to

reduce nitro groups in preference to benzyl groups. The same reaction can be performed with stronger reagents (sources of hydrogen) with much higher reaction rates even at room temperature. For instance, the reaction performed with zinc and pyridinium acetate is instantaneous even at room temperature. The reduction with zinc and acetic acid combination is very fast as well. We also tried to explore this reaction with other inexpensive metals namely, iron and nickel. These metals also gave similar results as zinc, except that the reactions of both these metals are much slower and needed extended periods of refluxing. All our attempts to carry out the reactions at room temperature with these metals (iron and nickel) failed. The results of the zinc, iron and nickel catalyzed reductions are summarized in Table 3.4.

In summary, transfer hydrogenation with formic acid derivatives and palladium is a good chemoselective debenzylation method for aromatic benzyl ethers over aliphatic benzyl ethers and nitro groups. The reaction rates have been successfully reduced employing the MORE[34] technique. It was also realized that chemoselective nitro reduction can be achieved by employing zinc and formic acid derivatives as the hydrogen source. Unfortunately, our target prodrugs **4** and **5** are aliphatic benzyl ethers, and this debenzylation method is not a good choice. Therefore, we continued to explore other transfer hydrogenation methods.

Table 3.4: Zinc, iron and nickel catalyzed reductions.

$$O\text{-Bn derivative} \xrightarrow[\substack{\text{Methanol, Reflux, >95\%} \\ \text{Metal catalyst}}]{\text{Ammonium acetate}} \text{Debenzylated product}$$
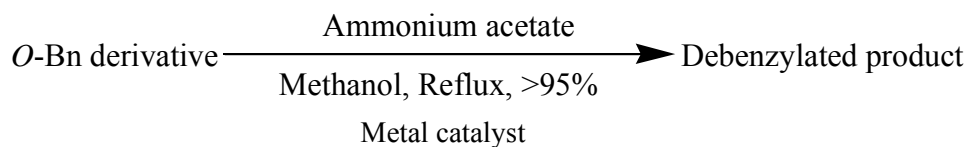
| Entry # | Reaction conditions | Effect on **6** | Time (hours) |
|---|---|---|---|
| 1 | Zn/CH$_3$COONH$_4$, CH$_3$OH, reflux | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 8 |
| 2 | Zn/pyridinium acetate, 25°C | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | Instantaneous |
| 3 | Zn/NH$_4$Cl, 25°C | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 7 |
| 4 | Zn/ HCOONH$_4$, 25°C | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 7 |
| 5 | Zn/CH$_3$COOH, 25°C | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 3 |
| 6 | Zn/CH$_3$COONa, CH$_3$OH, reflux | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 8 |
| 7 | Zn/ CH$_3$COONa , CH$_3$OH, 25°C | No change | >20 |
| 8 | Fe/ NH$_4$Cl, reflux | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 10 |
| 9 | Fe/ NH$_4$Cl, 25°C | No change | >20 |
| 10 | Fe/ HCOONH$_4$, reflux | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 12 |
| 11 | Fe/ HCOONH$_4$, 25°C | No change | >20 |
| 12 | Fe/ CH$_3$COONH$_4$, reflux | No change | >20 |
| 13 | Ni/ NH$_4$Cl, reflux | -NO$_2$ reduced to -NH$_2$, Benzyl group intact | 13 |
| 14 | Ni/ NH$_4$Cl, 25°C | No change | >20 |
| 15 | Ni/ HCOONH$_4$, 25°C | No change | >20 |
| 16 | Ni/ HCOONH$_4$, reflux | No change | >20 |

*Catalytic transfer hydrogenation using alcohols*

Heterogeneous, catalytic, transfer hydrogenolysis with alcohols is a known debenzylation procedure in literature. This method is used to deprotect benzyl ethers of poly-ols and has been utilized in carbohydrate chemistry as well.[40, 41] The reaction itself is very simple. The substrate is treated with 10% Pd/C and refluxed in 2-propanol. The reaction conditions are very mild and the reaction is very slow and very selective (Table 3.5).

We explored this reaction for its selectivity and the results indicate that the *O*-benzyl group is selectively removed in the presence of the nitro group, as observed in the model compounds. Under the specified conditions, aliphatic benzyl groups are removed as well. The reaction is very clean and go to completion in all the three model compounds as seen in entries **1, 2, 3** of Table 3.5. The nitro and hydrazone moieties remain untouched. However, long reaction times were necessary. To solve this problem, 3-methyl butanol was employed as a higher boiling solvent, with better results, in that the reaction times are reduced from over 48 hours to 14 hours. Attempts to further reduce the reaction time using cyclohexanol were derogatory in that under such high temperature, sugar derivative **2** degraded. Hence the choice of solvent is very important in order to tailor the reaction conditions for optimal results.

Table 3.5:  Transfer hydrogenation reactions with alcohols.

$$O\text{-Bn derivative} \xrightarrow[\text{10\% Pd/C, 70-75\%}]{\text{Alcohol, reflux}} \text{Debenzylated product}$$
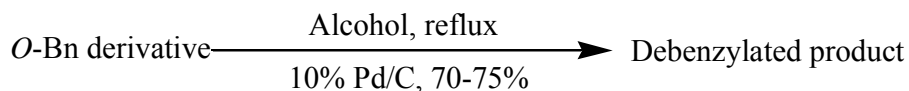
| Entry # | Substrate | Alcohol | Time | Result |
|---------|-----------|---------|------|--------|
| 1 | 6 | 2-propanol | 2 days | Complete Debenzylation, $-NO_2$ untouched |
| 2 | 7 | 2-propanol | 3 days | Complete Debenzylation |
| 3 | 8 | 2-propanol | 8 days | Complete Debenzylation, $-NO_2$ untouched |
| 4 | 6 | 3-methyl butanol | 14 hr | Complete Debenzylation, $-NO_2$ untouched |
| 5 | 7 | 3-methyl butanol | 14 hr | Complete Debenzylation |
| 6 | 8 | 3-methyl butanol | 14 hr | $-NO_2$ almost untouched, 50% Debenzylation |
| 7 | 7 | Cyclohexanol | 14 hr | Sugar degraded |

The kinetic, thermodynamic, and stereo chemical data on transfer hydrogenation reactions and their mechanisms are poorly understood.[42] There are many explanations regarding the mechanism of this reaction.  In general, heterogeneous catalysis can be difficult to interpret mechanistically, because of difficulties in preparing and reproducing uniform catalytic surfaces. In addition, the fact that hydrogen can form ionic and covalent hydrides and that it can also dissolve in palladium without there being any bond formation, complicates the mechanism of these reactions.

It is reported in literature,[40] that transfer hydrogenation with alcohols can take place between adsorbed species, and oxidative addition of the hydrogen donor to palladium, followed

by coordination of the substrate and transfer of hydrogen occurs in two steps, with the second

one formally involving a five-membered transition state (Scheme 3.4).
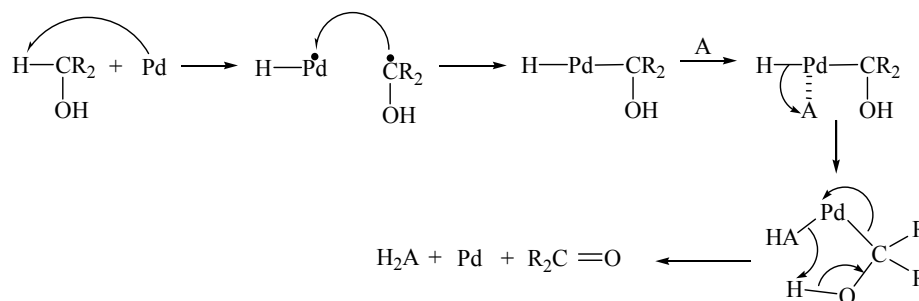


Scheme 3.4. Transfer hydrogenation with alcohols.[42]

If alcohol were to be the hydrogen donar in this reaction, then the by-product of the

reaction would be toluene and acetone, toluene being the reduced product of benzyl group, while

acetone being the oxidation product of 2-propanol. In practice however, we did not observe the

formation of these side products. Hence we concluded that this might not be the real mechanism.

An alternative explanation of mechanism of debenzylation with alcohols is believed to

occur via oxidation of benzyl group[43]. In this, the *O*-benzyl protecting group acts as hydrogen

donor.
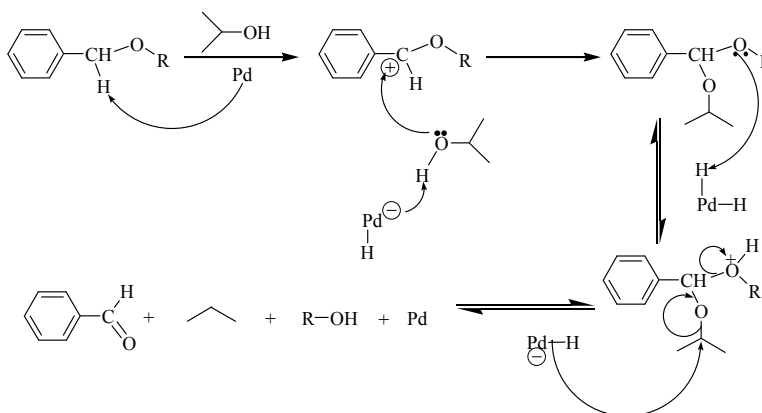


Scheme 3.5.: Transfer hydrogenation with alcohols via oxidation of benzyl group.[43]

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Initially the reaction was attempted in ethanol. There was a clear progress of the reaction with ethanol but the reaction was impractically slow. Replacing ethanol with 2-propanol gave good results. The improvement in the reaction rate could be due to one or both of two major factors that change with the replacement of the solvent. One factor is that 2-propanol is a higher boiling alcohol and under reflux conditions it offers higher reaction temperature which could result in accelerated reaction rate. The second factor is that 2-propanol, being a secondary alcohol, can form a more stable secondary radical (Scheme 3.4). If the reaction were to go through mechanism shown in Scheme 3.4, it must proceed faster in 2-propanol verses a primary alcohol of comparable boiling point. To determine whether this was the mechanism, the reaction was repeated with 3-methyl butanol, a higher boiling but primary alcohol. The reaction progressed much faster indicating that the reaction is not probably going through the mechanism one(Scheme 3.4), as mechanism one would have favored secondary alcohol 2-propanol over primary alcohol 3-methyl butanol. Further, benzaldehyde was isolated from the reaction mixture. This happens to be the by-product of reaction only when the reaction goes through mechanism two(Scheme 3.5) which involves oxidation of benzyl group to benzaldehyde. In summary it can be concluded that transfer hydrogenation with propanol follows oxidative debenzylation mechanism.

Another interesting mechanistic aspect worth noting is that, the planar geometry requirement does not seem to be as critical in these alcohol assisted debenzylation reactions as it is in ammonium acetate assisted debenzylation. This conclusion can be made based on the fact that alcohol assisted debenzylations proceed with equal ease in aliphatic compounds as well as aromatic systems, while this is not the case with ammonium acetate assisted debenzylations. Our experiments with *p*-methoxybenzyl protected nitrophenol further corroborated this point. Both

the above debenzylation methods have been tried on *p*-methoxybenzyl protected nitrophenol

with interesting results.
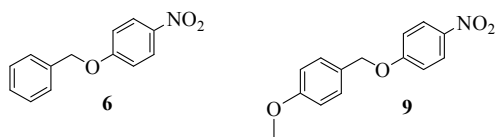


Figure 3.2. Model compounds **6** and **9**

Table 3.6. Relative reaction times for the debenzylation of model compounds in different

debenzylation methods.

| Entry # | Substrate | Debenzylation method | Time (hours) | Result |
|:---:|:---:|:---:|:---:|:---:|
| 1 | **6** | CH$_3$COONH$_4$ | 120 | Complete Debenzylation, $-$NO$_2$ untouched |
| 2 | **9** | CH$_3$COONH$_4$ | 170 | Complete Debenzylation, $-$NO$_2$ untouched |
| 3 | **6** | 2-propanol | 48 | Complete Debenzylation, $-$NO$_2$ untouched |
| 4 | **9** | 2-propanol | 6 | Complete Debenzylation, $-$NO$_2$ untouched |

The aromatic ring lies flat on the metal surface for optimal coordination[44,45]. It is possible

that substitution on the aromatic ring could have an adverse steric effect that would interfere with

the planar geometry required for effective binding and thus reduce its affinity for the metal

surface. This seems to be the case considering the time taken for debenzylation of **6** is much

more than for **9** as seen entries **1** and **2** of Table 3.6. This may explain why the least substituted

benzyl group, although not electronically favored, can still be preferentially cleaved.[44,45] This is

the true for ammonium acetate assisted debenzylation. The effect of this seems to be much

lower in alcohol assisted debenzylations.  In alcohol assisted debenzylations the electronic effect

seems to the deciding factor for the rate of debenzylation.

Unfortunately, again like in ammonium acetate debenzylations, the disadvantage with

this method is that the reaction must be carried out for relatively long times at reflux temperature

with potential damage particularly to other temperature sensitive functional groups.  Based on

the success we achieved in applying the microwave-induced organic reaction enhancement

(MORE) chemistry techniques,[34] we decided to attempt it for the alcohol assisted debenzylation

method as well.  The results are shown in Table 3.8.  We met with partial success in model

compounds **6** and **8** with 2-propanol, in that the reaction rates are increased dramatically.

Unfortunately, even before the reactions go to completion, degradation of the product starts.  The

degradation rate was so rapid in the higher alcohols 3-methyl butanol and cyclohexanol, that no

appreciable amounts of products were observed.  With compound **7** there was no product

formation.  Prolonged exposure of compound **8** only results in complete degradation of the

substrate.  The degradation was even faster with higher boiling alcohols.

Table 3.8.:  Microwave assisted transfer hydrogenation reactions in alcohols.

| Entry # | Substrate | Alcohol | Time | Result |
|---------|-----------|---------|------|--------|
| 1 | 6 | 2-propanol | 12 min | 60% Debenzylation, $-NO_2$ untouched Further microwaving degrades the substrate |
| 2 | 7 | 2-propanol | 55 min | No debenzylation Further microwaving degrades the substrate |
| 3 | 8 | 2-propanol | 32 min | 50%  Debenzylation Further microwaving degrades the substrate |

To summarize, alcohol assisted debenzylation can be successfully employed for debenzylation reactions. The reaction times were high with 2-propanol, but they can brought down drastically by choice of higher boiling point alcohol. The choice of alcohol is such that the boiling point should be high enough to increase the speed of the reaction while at the same time not so high that degradation of substrate and product occur. Among the alcohols tried 2-methyl butanol seemed to be a good choice, as the reaction time is reasonably low and products can be obtained in pure form with no degradation. Attempts to further speed up the reaction through microwave induced reaction techniques were not successful.

Among other transfer hydrogenations, the most important method is using cyclohexene as a hydrogen source. The major advantage of this debenzylation method is that the time required is much less than that for the usual catalytic hydrogenation and also the products formed are of greater purity. Unfortunately, information regarding the relative selectivity of this reduction method with respect to nitro reduction verses debenzylation is not available. Hence we decided to explore this method in detail.

*Transfer hydrogenation with Cyclohexene*

Transfer hydrogenation with cyclohexene is a simple and convenient method for removal of all protecting groups that are normally removed by catalytic hydrogenation.[46-48] The substrate is dissolved in a mixture of ethanol and cyclohexene (acetic acid may be added to assist dissolution) and the solution is refluxed with stirring in the presence of palladium-charcoal or palladium black.[49-51] The work up involves a simple filtration.

It is known in literature that the nitro group is reducible under these conditions.[52-56] We explored the possibility of using the same method under milder conditions. The various possibilities tried included lower temperature, lower catalyst loading, poisoned catalysts and limiting the concentration of hydrogen source. The results are indicated in Table 3.9.

We initially tried to determine if time based selective debenzylation is possible. It was noticed that under the specified conditions, the nitro reduction and debenzylation occurs simultaneously as can be concluded from entries 1, 2 and 3 of Table 3.9.

Next, we tried to carry out the reaction under milder temperature conditions. The reaction was attempted at room temperature, but at this temperature, there is absolutely no progress of the reaction. Hydrogen generation is supposed to occur through disproportionation of cyclohexene to benzene and cyclohexane. This reaction seems to have an activation energy barrier that cannot be reached under room temperature conditions. Hence there is no hydrogen available to carry out the reduction and neither the nitro group nor the benzyl group is reduced.

Ordinarily, cyclohexene transfer hydrogenations are carried out using cyclohexene alcohol mixtures as a solvent, where cyclohexene is used in large excess.[55] This is typical to many transfer hydrogenation reactions because, the hydrogen generated from the reagent used as hydrogen source is typically never used with 100% efficiency.[56,57] Nevertheless, we performed this reaction with stochiometric amount of cyclohexene, in order to understand the selectivity pattern of this transfer hydrogenation method. Under these conditions, it was noted that there is absolutely no reaction in any of the model compounds. We then increased the molar ratio to four equivalents. At this concentration of cyclohexene there was selectivity towards benzyl reduction. Model compound **6** is completely debenzylated before the unwanted nitro reduction starts. Additionally, compound **7**, the aliphatic system, is effectively debenzylated in a short

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period of time.  Entries **4, 5** and **6** (Table 3.9) indicate the fate of compounds **6, 7** and **8** respectively under these conditions. Unfortunately, the nitro reduction in compound **8** starts when debenzylation is only 70% complete.  Despite that, these results are promising and can be further improved, if there is a way to retard the nitro reduction.

We attempted to usethe poisoned palladium catalyst - Lindlar's catalyst, to determine if retardation of nitro reduction could occur.  Unfortunately, with this catalyst even the chemoselectivity proved to be different.  Nitro reduction precedes debenzylation in the case of aromatic debenzylations, as seen in entry 7 of Table 3.9.  Debenzylation of aliphatic benzyl ethers does not occur under these conditions. This is evidenced by model compound **7** entry **8** in Table 3.9.  In compound **6** there is complete reduction of nitro group even before debenzylation starts and in compound **8** the reductions occur simultaneously.

It is a known fact that activity and sometimes even the selectivity of surface catalysts depends on the surface topography of the catalyst used, which in turn depends on the method employed to make the catalyst.  The activity of the catalyst is structure specific.  We tried using Pd/C catalyst of different loadings and types to see if one of the methods could carry out complete debenzylation before the nitro reduction starts.   Several different catalysts were used but the one that ultimately gave the required chemoselectivity was the 5% Pd/C wet paste.  This catalyst has the required selectivity when used in 4 equivalents concentration versus the number of benzyl groups in the substrate.  The debenzylation is very fast and clean and is complete within half an hour with no competing reaction.  The results of the reduction method are shown in Table 3.9 entries 10 and 11.  This is the excellent method for chemoselective debenzylation considering the reaction is fast, simple, clean, and is a one pot synthesis with no special hydrogenation equipment needed.  Purification involves a simple filtration.

Table 3.9. Transfer hydrogenation reactions with cyclohexene.

$$\text{\textit{O}-Bn derivative} \xrightarrow[\text{Ethanol, reflux under } N_2, >95\%]{\text{4 Eq Cyclohexene, 5\% Pd/C (wet paste)}} \text{Debenzylated product}$$
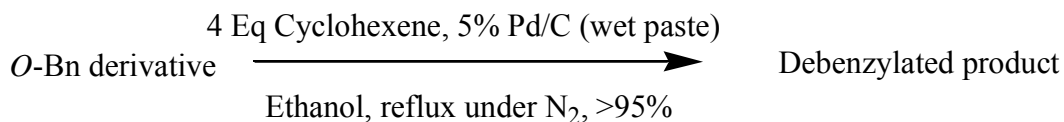
| Entry # | *O*-Bn derivative | Eq of Cyclohexene | Time (hours) | Catalyst | Result |
|---------|-------------------|-------------------|--------------|----------|--------|
| 1 | 6 | Large excess | 3 | 5% Pd/C - wet | Complete Debenzylation, $-NO_2$ reduced by 50% |
| 2 | 7 | Large excess | 1 | 5% Pd/C - wet | Complete Debenzylation |
| 3 | 8 | Large excess | 3 | 5% Pd/C - wet | 70% $-NO_2$ reduction, 50% C=N reduced while debenzylation was 50% |
| 4 | 6 | 4Eq | 2 | 5% Pd/C - wet | Complete Debenzylation, $-NO_2$ untouched |
| 5 | 7 | 4 Eq | 6 | 5% Pd/C - wet | 70% Debenzylation |
| 6 | 8 | 4 Eq | 6 | 5% Pd/C - wet | At 70% Debenzylation, nitro reduction starts |
| 7 | 6 | Large excess | 12 | 5% Lindlar's catalyst | Complete $-NO_2$ reduction, 5% Debenzylation |
| 8 | 7 | Large excess | 12 | 5% Lindlar's catalyst | No Debenzylation |
| 9 | 8 | Large excess | 15 | 5% Lindlar's catalyst | 50% Debenzylation, $-NO_2$ reduction starts |
| 10 | 7 | 4 Eq | ½ | 5% Pd/C – wet, paste | >95% Debenzylation |
| 11 | 8 | 4Eq | 1 | 5% Pd/C – wet paste | 95% Debenzylation with in ½ hr, within this time $-NO_2$ is untouched |
| 12 | 4 | 4Eq | ½ | 5% Pd/C – wet, paste | 95% Debenzylation |

## Conclusion

In the course of the preparation of ester-saccharide derivatives of **A-007** as anticancer drugs, we faced the problem of selective removal of benzyl groups without affecting either acidic or reduction sensitive functionalities present within the **A-007** molecule.  For this reason, we explored benzyl deprotection methodologies and determined several simple solutions which gave a variety of chemoselectivities in excellent yields.  Catalytic hydrogenation, as well as the use of strong acids, was avoided in deference to the sensitivity of the nitro group and the lability of the hydrazone and ester moieties. The selective deprotection methods developed include debenzylation of aromatic benzyl ethers in preference to aliphatic benzyl ethers, benzyl ethers in preference to nitro groups, and nitro groups in preference to benzyl ethers.  The selective deprotection methods developed are mild, simple, one pot methods with no special hydrogenation equipment needed.  In addition, the above methodologies developed are very general and can be applied to a variety of substrates.

## Experimental

Melting points were taken on an Electro thermal IA 9000 Digital Melting Point Apparatus and are uncorrected. The $^1$H and $^{13}$C NMR spectra were run on Varian 300 MHz Gemini2000 and on Varian 400 MHz Unity in CDCl$_3$, DMSO-d6, or D$_2$O as solvent and internal standards. A commercially available, domestic microwave oven (GE, model #: JES738WJ02) was used in all experiments. All the chemicals mentioned are from Aldrich chemical company unless otherwise mentioned. The catalyst that works best for cyclohexene reductions was Pd on 5% charcoal paste with 50-65% water content from Alfa Aeser. Cyclohexene is always freshly distilled before each reduction unless used from a freshly opened bottle. Pd/C used in transfer hydrogenation methods with formic acid analogues as hydrogen source can be reused at least three times with no loss in activity, while Pd/C used in alcohol and cyclohexene methods reused up to five times with no loss in activity.

**Preparation of *N*-[Bis-(4-benzyloxy-phenyl)-methylene]-N'-(2,4-dinitro-phenyl)-hydrazine (3):**

A mixture of A007 (1g, 2.5 m mol) and benzyl chloride (0.416g. 3.3 m mol ) is refluxed in a 1:1 mixture of methanol and 1.5% aqueous NaOH (50mL). The reaction can be monitored visually through change in color of the reaction mixture. At the beginning, the reaction mixture is a clear dark red solution. As the reaction progresses, bright red product is formed as precipitate and reaction mixture clears up until it completely becomes colorless. The product is a bright red precipitate which can be filtered out and washed with ice cold methanol. Pure product is obtained in 98% yield, no further purification is necessary.

Melting point:  The compound degrades beyond 200°C before it melts.  $^1$H NMR (400 MHz,

CDCl$_3$) d 11.3(1H, s), 9.09(1H, d, $J$ =4 Hz), 8.32(1H, dd, $J$=4, 2.4 Hz), 7.63(1H, d, $J$ =8 Hz),

7.49-7.22(14H, m), 5.18 (2H, s), 5.13(2H, s);  $^{13}$C NMR (100 MHz, CDCl$_3$) 160.9, 155.8, 144.8,

137.9, 136.6, 135.4, 130.1, 129.9, 128.98, 128.94, 128.5, 128.4, 127.9, 127.7, 124.2, 123.8,

116.8, 116.4, 115.0, 70.4.


**Preparation of 4,4'-dihydroxybenzophenone-2,4-dinitrophenylhydrazone bis( 2,3,4,6-tetra-**

**_O_-benzyl-β-D-glucopyranoside) (4):**

2,3,4,6-Tetra-_O_-benzoyl-α-D-glucopyranosyl)-trichloroacetimidate (**4a**)  (5.55g, 0.75 mmol) and

**A-007** (118 mg, 0.3 mmol) were stirred under an atmosphere of nitrogen in anhydrous CH$_2$Cl$_2$

(20 mL) with 3Å molecular sieves for 1 h. The solution was cooled in an ice-bath for 30 min

before BF$_3$-OEt$_2$ (2 mL) was added and stirred for 1 min the ice-bath followed by 1 h at room

temperature. The solution was added to an ice cold saturated aq NaHCO$_3$ solution (100 mL) with

vigorous stirring and then extracted with ether (2 x 75 mL). The organic layers were combined

and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure

and the residue was purified by flash column chromatography (with a solvent system - 20:1

dichloromethane –ethyl acetate ) to give **4** as red crystalline solid in 20% yield.  MP:  The

compound degrades at 200°C before it melts.  $^1$H NMR (DMSO-d6, 400 MHz) δ 7.82(2H, d, $J$

=6.4 Hz), 7.64-7.14(55H, m), 7.02(2H, d, $J$ =6.24 Hz), 5.18-3.56 (14H, m), 4.59(16H, s); $^{13}$C

NMR (DMSO-d6, 100 MHz), δ 160.9, 161.1, 155.5, 147.8, 139.6,

137.15, 137.16, 137.17, 135.8, 131.1, 128.3, 128.1, 137.16, 137.17, 135.8, 131.1, 128.3, 128.1, 1

27.15, 126.91, 126.94, 126.99, 97.1, 73.1, 71.3,

71.1, 66.9, 66.1.

**Preparation of 2,3,4,6-Tetra-*O*-benzoyl-α-D-glucopyranosyl)-trichloroacetimidate (4a):**

Tetrabenzoyl-D-glucopyranose (**4b**) (2.0 g, 3.3 mmol), $K_2CO_3$ (1.1 g, 8.2 mmol), and

trichloroacetonitrile (5 mL) were stirred at room temperature in dichloromethane (20 mL) for 48

hours. The resulting suspension was filtered through silica gel using a 1:1 mixture of $CH_2Cl_2$ and

$Et_2O$ (150 mL). The filtrate was concentrated to give **4a** as a pale yellow oil (2.0 g, 83

% yield). $^1$H NMR (300 MHz, CDCl3): δ 8.62 (1H, s) 8.02 (2H, d, $J$ = 3.9 Hz), 7.93 (4H, d, $J$ =

5.7 Hz), 7.85 (2H, d, $J$ = 4.2 Hz), 7.56 – 7.25 (12H, m), 6.82 (1H, d, $J$ = 2.0 Hz), 6.25 (1H, t, $J$ =

9.9 Hz), 5.79 (1H, t, J=9.9 Hz), 5.60 (1H, dd, $J_1$ = 5.1 Hz, $J_2$ = 1.8 Hz), 4.62 (2H, d, $J$ = 5.1 Hz),

4.46 (1H, dd, $J_1$ = 6.5 Hz, $J_2$ = 3.0 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.2, 165.8, 165.6,

165.4, 160.7, 133.7, 133.5, 133.4, 130.1, 129.91, 129.86, 129.7, 129.0, 128.73, 128.66, 128.60,

128.55, 128.51, 93.3, 90.9, 70.9, 70.3, 68.8,62.6.


**Preparation of 2,3,4,6-Tetra-*O*-benzoyl-D-glucopyranose (4b)**: Pentabenzoyl-β-D-

glucopyranose (**4c**) (10 g, 14 mmol) and 2-aminoethanol (2.0 g, 36 mmol) were stirred at room

temperature in ethyl acetate (150 mL) and DMSO (2 mL) overnight, during which time the initial

suspension formed a solution. The resulting solution was washed with water (3 x 150 mL) and

the organic layer was dried over sodium sulfate and concentrated. The residue was purified by

flash column chromatography ($CH_2Cl_2$ followed by $Et_2O$) to give **4b** as a white solid (6.0 g, 70

% yield). 100 mg of the product was recrystallized from diethyl ether/hexanes for $^1$H and $^{13}$C

NMR analysis. $^1$H NMR (300 MHz, CDCl$_3$) **4b-β**: δ 8.19- 7.82 (8H, m), 7.57-7.19 (12H, m),

6.23 (1H, t, $J$=9.9 Hz), 5.72 (2H, m), 5.30 (1H, dd, $J_1$=6.45 Hz, $J_2$=1.2 Hz), 4.66 (2H, m), 4.46

(1H, dd, $J_1$=8.6 Hz, $J_2$=2.4 Hz), 3.12 (1H, d, $J$=1.3 Hz). $^{13}$C NMR of **4b-β** (CDCl$_3$): δ 166.6,

166.13, 166.10, 165.5, 133.6, 133.5, 133.3, 130.1, 130.0, 129.8, 129.7, 129.6, 129.2, 129.1, 129.0, 128.9, 128.8, 128.6, 128.5, 128.46, 90.6, 72.5, 70.4, 69.6, 67.8, 63.0.

**Preparation of Penta-*O*-benzoyl-β-D-glucopyranose (4c):**

D-glucose (5.0 g, 28 mmol) was refluxed in pyridine (100 mL) for 1 hour. The solution was then added to benzoyl chloride (20mL) which had been heated to 65°C. After the mixture was cooled to room temperature, water (400 mL) was added and stirred until the product solidified, approximately 30 minutes. The solid was filtered and washed with water and recrystallized from ethyl acetate to give **4c** as a white solid (11 g, 56 % yield). ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.01 (4H, d, *J* = 4.0 Hz), 7.89 (4H, d, *J* = 3.8 Hz), 7.84 (2H, d, *J* = 3.9 Hz), 7.53 – 7.21 (15H, m), 6.28 (1H, d, *J*=3.9 Hz), 6.02 (1H, t, *J* = 9.3 Hz), 5.83 (2H, q, *J*=12.9 Hz), 4.64 (1H, dd, *J$_1$* = 6.0 Hz, *J2* = 1.4 Hz), 4.49 (1H, dd, *J$_1$* = 8.1 Hz, *J$_2$* = 2.1 Hz), 4.394 (1H, m). ^{13}C NMR (300 MHz, CDCl$_3$): δ 166.3, 165.8, 165.30, 165.28, 164.8, 134.0, 133.7, 133.6, 133.5, 133.3, 130.4, 130.0, 129.99, 129.96, 129.7, 128.9, 128.84, 128.82, 128.7, 128.62, 128.59, 128.5, 92.9, 73.3, 73.0, 71.0, 69.2, 62.8.

**Preparation of 1-Benzyloxy-4-nitro-benzene (6)**

A mixture of nitro phenol (13.9g, 0.1 mol) and benzyl chloride(5.75 mL, 0.05 mol) is refluxed in a 1:1 mixture of methanol and 1.5% aqueous NaOH. The reaction is stopped after 4 hours and product is extracted into methylene chloride. The organic layers are washed (x 3) with aqueous NaHCO$_3$ and dried with sodium sulfate. The product formed is 98% pure by NMR and hence used with no further purification. The product is obtained in 95% yield upon concentrated under vacuum .

MP: 107°C; $^{1}$H NMR (400 MHz, CDCl$_3$) δ 8.21(2H, d, $J$ =4.8 Hz), 7.43-7.37(m, 5H), 7.04(2H, d, $J$ =4.8 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) d 163.8, 141.8, 135.6, 129.0, 128.7, 128.7, 127.7, 127.2, 126.1, 115.0, 70.8.

**Synthesis of methyl 2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranoside (7)**

Methyl glycoside (25 g) is dissolved at 55°C in 300 mL of 33% aq NaOH solution. Tetrabutylammonium bromide(TBABr, 32 g) is added and benzyl chloride (77 mL) is added dropwise over 1h. The reaction mixture is stirred at 55°C for 4.5 h and then at room temperature overnight. 200 mL of toluene are added, the phases are separated and the organic phase is washed with water until it is neutral. It is concentrated *in vacuo* and purified by flash chromatography (toluene-ethyl acetate, 35:1) to yield 36 g (63 mmol, 75%) of the fully protected product as a colorless syrup. $^{1}$H NMR (400 MHz, CDCl$_3$) δ 7.8−7.0(20H, m), 5.39(1H, s), 5.01-3.39(17H, m); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 139.01, 138.75, 138.48, 138.38, 138.13, 134.67, 133.25, 130.29, 129.96, 129.92, 129.21, 128.62, 128.00, 127.84,104.91, 98.41, 84.84, 82.52, 82.33, 80.03, 78.07, 77.86, 75.95, 75.88, 75.22, 75.04, 74.94, 73.67, 73.58, 72.30, 70.24, 69.12, 68.66, 68.34, 66.88, 55.35.

**Preparation of *N*-[Bis-(4-benzyloxy-phenyl)-methylene]-N'-(2,4-dinitro-phenyl)-hydrazine (8):**

A mixture of A007 (1g, 2.5 m mol) and benzyl chloride (0.416g. 3.3 m mol ) is refluxed in a 1:1 mixture of methanol and 1.5% aqueous NaOH (50mL). The reaction can be monitored visually through change in color of the reaction mixture. At the beginning, the reaction mixture is a clear dark red solution. As the reaction progresses, bright red product is formed as precipitate and

reaction mixture clears up until it completely becomes colorless. The product is a bright red

precipitate which can be filtered out and washed with ice cold methanol. Pure product is

obtained in 98% yield, no further purification is necessary.

MP: The compound degrades beyond 200°C before it melts. $^1$H NMR (400 MHz, CDCl$_3$) δ

11.3(1H, s), 9.09(1H, d, $J$ =4 Hz), 8.32(1H, dd, $J$=4, 2.4 Hz), 7.63(1H, d, $J$ =8 Hz), 7.49-

7.22(14H, m), 5.18 (2H, s), 5.13(2H, s); $^{13}$C NMR (100 MHz, CDCl$_3$) 160.9, 155.8, 144.8,

137.9, 136.6, 135.4, 130.1, 129.9, 128.98, 128.94, 128.5, 128.4, 127.9, 127.7, 124.2, 123.8,

116.8, 116.4, 115.0, 70.4.


**Preparation of 1-(4-methoxy Benzyloxy)-4-nitro-benzene (9)**

$p$-methoxybenzyl alcohol(1.38g, 10mmol) is taken in 15 mL of anhydrous ether. To this mixture

thionyl chloride(1.5 mL, 20mmol) is added drop wise. The reaction mixture is stirred at room

temperature for 3 hours and then the solvent is evaporated under nitrogen. A dark purple residue

of $p$-methoxy benzyl chloride is formed. To this 20 mL of methanol is added. In a separate

container $p$-nitrophenol (1.39g, 10mmol) is dissolved in 20 mL of 1% aqueous NaOH. This

mixture is added dropwise to the methanol solution of $p$-methoxy benzyl chloride drop wise with

stirring. As the addition process is still in progress golden yellow flakes of product is formed.

The precipitate is filtered and then washed with ice cold water to give pure product in 96% yield.

MP : 125 ° C; $^1$H NMR (400 MHz, CDCl$_3$) d 8.21(2H, d, $J$ =9.2 Hz), 7.36(2H, d, $J$ =8.8 Hz),

7.02(2H, d, $J$ =9.2 Hz), 6.94(2H, d, $J$ =2.2 Hz), 5.09(2H, s), 3.83(3H, s); $^{13}$C NMR (100 MHz,

CDCl$_3$) d 164.0, 160.0, 142.1, 135.4, 129.5, 127.6, 126.1, 115.0, 114.4, 70.7, 55.5.

**General procedures for reductions**

The number of moles of substrate indicated is with respect to number of benzyl groups.  (Not the number of moles of substrate.)

**General procedure for reductions with ammonium salts:**

A suspension of substrate (0.5 m mol) and an equal weight of 10% Pd/C in dry methanol (15 mL) was added to anhydrous ammonium salt and then refluxed under nitrogen for the times indicated in Table 2.2.  The catalyst was filtered out and washed with methanol.  The combined washings and the filtrate are filtered through a celite pad (1 inch wide and 1 inch long) and concentrated under vacuum. The column was washed with a methanol-dichloromethane mixture and then the combined washings were evaporated under vacuum to obtain the product.  The number of moles of ammonium salt used and reaction times are indicated in Table 2.2.  The yields of the reduction products are in the range 80-85%.

**Microwave-Assisted reduction procedure with ammonium salts:**

 A suspension of substrate (0.5  mmol) and an equal weight of  10% Pd/C in ethylene glycol (15 mL)  and  ammonium salt (10 mmol) in an Erlenmeyer flask was allowed for microwave irradiation 160 power. A filter funnel was placed over the Erlenmeyer flask to prevent any accidental spillage. A "heat sink" was maintained to control the microwave energy input into the reaction mixture. After the reaction, the reaction mixture was cooled and then the catalyst was filtered out. The filtrate was diluted with water and extracted with ether or ethyl acetate, and the organic layer was washed twice with saturated brine solution and finally with water. The organic layer was dried over anhydrous sodium sulphate, filtered and then followed up with evaporation

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of the organic layer followed by purification either by preparative TLC or by column chromatography. The yields of the reduction products are in the range 60-75%.

**General procedure for reduction reactions using Zn/Fe/Ni metals:**

A suspension of substrate (10 mmol), ammonium salt (80 mmol), and metal dust (1g) in methanol (15 mL) was stirred at temperatures indicated in Table 3.4 until the completion of the reaction(monitored by TLC). After the completion of the reaction the reaction mixture was filtered through a celite pad, washed with solvent and then the combined filtrate and washings were evaporated under vacuum. The residue was taken into chloroform or ether, washed twice with saturated brine solution and finally with water. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporation of the organic yields the pure product in >95% yield.

**General procedure for debenzylation reactions with alcohols:**

A solution of *O*-benzyl derivative(1.5 mmol with respect to # of benzyl groups) in 10 mL of alcohol (indicated in Table 3.5) was added to a stirred suspension of 10% Pd/C (1 gm) in refluxing 2º alcohol (10 mL) and the mixture was boiled under reflux for the times indicated in Table 3.5. After the completion of the reaction (monitored by TLC), the catalyst was collected and washed with solvent alcohol. The combined filtrate and washings were evaporated under vacuum and then the residue was fractioned by column chromatography (7:5 hexane-ethyl acetate mixture) or preparative TLC to obtain the debenzylated product in 70-75% yield.

**General procedure for reductions with Cyclohexene:**

A mixture of *O*-benzyl derivative (0.5 m mol), 5% Pd/C wet paste (an equal weight of Pd/C is added per benzyl group), dry ethanol (20 mL) and cyclohexene (amount of cyclohexene used is as indicated in Table 3.9) were refluxed with stirring under air. At the end of times indicated (in Table 3.9), the catalyst was filtered off under suction to separate the catalyst, followed by filtration through a celite pad (1 inch wide and 0.25 inch long). The flash column is then washed with ethanol. The combined filtrate and washings were evaporated under vacuum to obtain pure products, which needed no further purification, in about 95% yield.

**Reduction products:**

***p*-aminophenol:**

MP: 248 ° C; ^{1}H NMR (400 MHz, CDCl$_3$) 6.97(2H, d, *J* =8.8 Hz), 6.77 (2H, d, *J* =8.8 Hz). ^{13}C NMR (100 MHz, CDCl$_3$) 164.1, 139.8, 126.3, 116.1.

**4, 4'-Dihydroxybenzophenone 2, 4,diaminophenyl hydrazone:**

The compound degrades beyond 200°C before it melts. ^{1}H NMR (400 MHz, CDCl$_3$) 7.62(1H, d, *J* =6 Hz), 7.54(1H, s), 7.42(1H, d, *J* =6.2 Hz), 7.34(2H, d, *J* =8 Hz), 7.14(2H, d, *J* =8 Hz), 6.92(2H, d, *J* =8 Hz), 6.72(2H, d, *J* =8 Hz); ^{13}C NMR (100 MHz, CDCl$_3$)158.5, 1 57.5, 136.9, 133.9, 131.4, 130.4, 123.6, 123.1, 116.8, 105.8, 101.1.

**methyl glycoside**: ^{1}H NMR (400 MHz, CDCl$_3$) 4.38(1H, d, *J* =4.38 Hz), 3.94-3.23(6H, m), 3.57(3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 99.41, 73.21, 71.70, 71.34, 69.67, 60.06, 55.13.

**p-nitrophenol:** MP: 115 º C; $^1$H NMR (400 MHz, CDCl$_3$)  8.182(2H, d, 9.2), 6.91(2H, d, 8.8); $^{13}$C NMR (100 MHz, CDCl$_3$) 164.1, 139.8, 126.3, 116.1.


**N-(4,4'-Dihydroxybenzhydrylidene)-N-(2,4-dinitrophenyl) hydrazine:**

MP: 270-272 °C. $^1$H NMR (DMSO-$d6$, 300 MHz): δ 11.13 (1H, s, NH), 10.05 (1H, s, OH), 9.99 (1H, s, OH), 8.78 (1H, d, $J$ = 2.7 Hz), 8.36 (1H, dd, $J1$= 9.6, $J2$ =2.7), 8.12 (1H, d, $J$= 9.6), 7.47 (2H, d, $J$ =8.7 Hz), 7.22 (2H, d, $J$= 8.4) , 7.01 (2H, d, $J$ =8.1),  6.81 (2H, d, $J$= 8.4), $^{13}$C NMR (DMSO-$d6$, 300 MHz): δ 156.19, 155.31, 152.33, 140.47, 133.27, 126.50, 126.35, 126.02, 125.50, 124.07, 119.46, 118.19, 112.97, 112.90, 111.91.


**p-benzyloxy aniline:**  MP:  55º C; $^1$H NMR (DMSO-$d6$, 400 MHz): δ 7.40–7.25(5H, m), 6.81(2H, d, $J$ =8.4 Hz), 6.64(2H, d, $J$ =8.8 Hz), 4.98(2H, s);  $^{13}$C NMR (DMSO-$d6$, 100 MHz): 140.36, 137.72, 128.73, 128.02, 127.72, 116.63, 116.40, 116.28, 70.97.


**4,4'-dihydroxybenzophenone-2,4-dinitrophenylhydrazone bis(β-D-glucopyranoside) (1)**

The compound degrades at 200°C before it melts.  $^1$H NMR (DMSO-d6, 400 MHz) δ 11.13 (1H, s), 8.80 (2H, d, J = 2.4 Hz), 8.40 (2H, dd, J1 = 9.6 Hz, J2 = 2.4 Hz), 8.19 (2H, d, J = 9.6 Hz), 5.54 (1H, d, J = 4.8 Hz), 5.48 (1H, d, J = 4.8 Hz), 5.18 (4H, m), 4.99 (1H, d, J = 7.2 Hz), 4.92 (1H, D, J = 7.4 Hz), 4.74 (1H, t, J = 5.6 Hz), 4.67(1H, t, J = 5.6 Hz), 3.2 (4H, m);

$^{13}$C NMR (DMSO-d6, 100 MHz) d 159.4, 159.0, 154.9, 144.5, 137.6, 130.6, 130.3, 129.7, 129.6, 125.0, 123.4, 117.8, 117.0, 116.6, 100.6, 100.3, 77.3, 76.8, 73.6, 73.5, 70.0, 61.0.

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# Chapter IV.  Electrospray Ionization Mass Spectroscopy Study of Cyclodextrin Complexes with A-007 Prodrugs.

## Abstract

Electrospray mass spectroscopic study of several **A-007** prodrugs in aqueous α-cyclodextrin (α–CD), β-cyclodextrin (β–CD), and γ-cyclodextrin (γ–CD) was performed. Acetic acid derivative of **A-007** should be metabolized *in vivo* before it becomes **A-007**, while on the other hand, glycine attached **A-007** prodrugs have surfactant like physical properties and they are slowly hydrolyzed in the aqueous cyclodextrins by releasing free **A-007**.  ESI-MS spectroscopic study gives insight into the process of prodrug hydrolysis in the presence of cyclodextrins and also on the influence of cyclodextrins on timely **A-007** prodrug release. Formation of various molecular aggregates and cyclodextrin inclusion complexes of **A-007** prodrugs and their hydrolyzed products was demonstrated by ESI-MS spectroscopy.

## Introduction

One of the challenges of utilizing **A-007** as potent anticancer drug is to overcome its low water solubility that limits the **A-007** anticancer application only to cancer cases where topical application is possible.[1] This problem can be solved with pharmacologically acceptable **A-007** prodrugs that have good water solubility and low toxicity.  Furthermore good prodrug should be slowly transformed under physiological conditions into free **A-007**.  This task is delicate and should be controlled due to fact that fast **A-007** release in blood plasma will cause its precipitation resulting in possible blood vessel blockage.  Recent studies on amino acid derived camptothecin derivatives[2] as potent anticancer prodrugs stimulated us to explore water soluble

**A-007** anticancer prodrugs.[3]  To further our knowledge on **A-007** prodrugs and influence of α-cyclodextrin (α–CD), β-cyclodextrin (β–CD), and γ-cyclodextrin (γ–CD) on prodrug stability Electrospray Mass Spectroscopic (ESI-MS) study was performed.  Structures of four **A-007** prodrugs that were selected for ESI-MS spectroscopic study are presented in Figure 4.1.

RO─⟨⟩─C(─⟨⟩─OR)=N–N(H)–(2,4-dinitrophenyl)  
**1** (A007); R=H  
**1Na**; R=Na

NaO₂C–CH₂–O─⟨⟩─C(─⟨⟩─O–CH₂–CO₂Na)=N–N(H)–(2,4-dinitrophenyl)  
**2**; R=H  
**2Na**; R=Na

+H₃N–CH₂–CO–O─⟨⟩─C(─⟨⟩─O–CO–CH₂–NH₃+)=N–N(H)–(2,4-dinitrophenyl)  F₃CCO₂⁻  
**3**

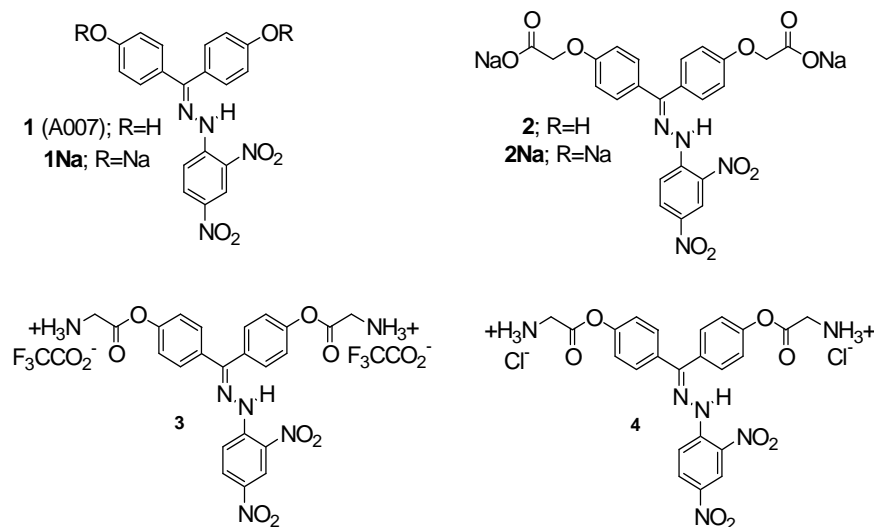+H₃N–CH₂–CO–O─⟨⟩─C(─⟨⟩─O–CO–CH₂–NH₃+)=N–N(H)–(2,4-dinitrophenyl)  Cl⁻  
**4**

Figure 4.1.  Structures of **A-007** and its prodrugs for ESI-MS spectroscopic study.


Acetic Acid derivative **2** is not in classical sense the **A-007** prodrug because it should be first metabolized by liver enzyme oxidation[4] into corresponding hemiacetal that upon hydrolysis will produce free **A-007**.  Neither **A-007** nor **2** have sufficient water solubility to be administrated alone as anticancer drug.  Therefore for successful administration of the **A-007** prodrugs additional water solubilizing agents must be added.  Besides increasing the prodrug solubility, these materials should also deliver the drug as well as control rate of the prodrug transformation into free **A-007** drug to avoid its precipitation in the blood vessels.  α-Cyclodextrin (α–CD), β-cyclodextrin (β–CD), and γ-cyclodextrin (γ–CD), are commonly used as host molecules to

increase solubility and deliver aromatic drugs and prodrugs.[5] We decided to use the same approach to alter physical properties of our prodrugs.

## Results and Discussion

It is well established that *p*-substituted phenols can form strong complexes with $\alpha$-cyclodextrin.[6] These complexes are well organized in solid crystalline state.[7] In mixtures with ratio of **A-007** and $\alpha-$CD 1:15, the negative ESI-MS shows mostly ions of free **A-007** and $\alpha-$CD, and molecular aggregates of $\alpha-$CD by itself (Figure 4.2). The most intensive signals are 485.6 $[\alpha-CD-2H]^{2-}$, 393.5 $[A007-H]^{-}$, and 972.1 $[\alpha-CD-H]^{-}$. However there is a small intensity signal (2.5%) at 1169.5 that corresponds to ternary complex, $[\textbf{A-007}+2\alpha CD-2H]^{2-}$, between A007 and two molecules of $\alpha-$CD. Interestingly this A007 inclusion complex with $\alpha-$CD is of lower intensity than the ternary complex of $\alpha-$CD self-complexation at 1458.6 indicating its very low concentration in the solution. If we assume that intensity of the cyclodextrin-complexed A007 and free A007 are proportional to their concentration in the gas phase then there is only 5% (2.5/55x100) A007 in the ternary $\alpha-$CD complex.
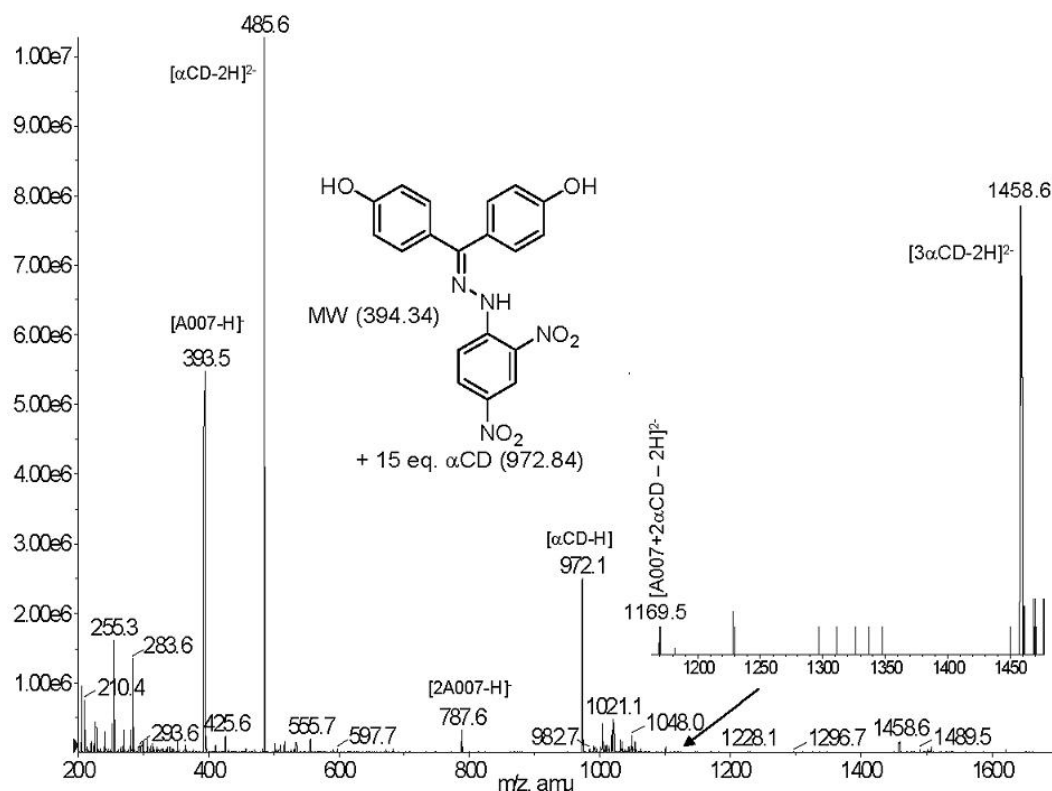
Figure 4.2. Negative ESI-MS spectra of aqueous A007 in presence of α-cyclodextrin

Similar pattern of molecular aggregation was observed in negative ESI-MS spectra of **A-007** in aqueous β-cyclodextrin (β–CD) (Figure 4.3) with noticeable difference in relative intensity of ternary complex of one molecule of **A-007** with two molecules of β–CD) in comparison with ESI-MS signal for complex between three molecules of β–CD. This indicates that β–CD forms stronger complex with **A-007** in comparison with α– CD. This finding is also in the agreement with our ^{1}H-NMR spectroscopic study of β–CD inclusion complexes with **A-007**. This study also reveals that bulk of A007 molecule still resides in water media rather than in cyclodextrin cavity.
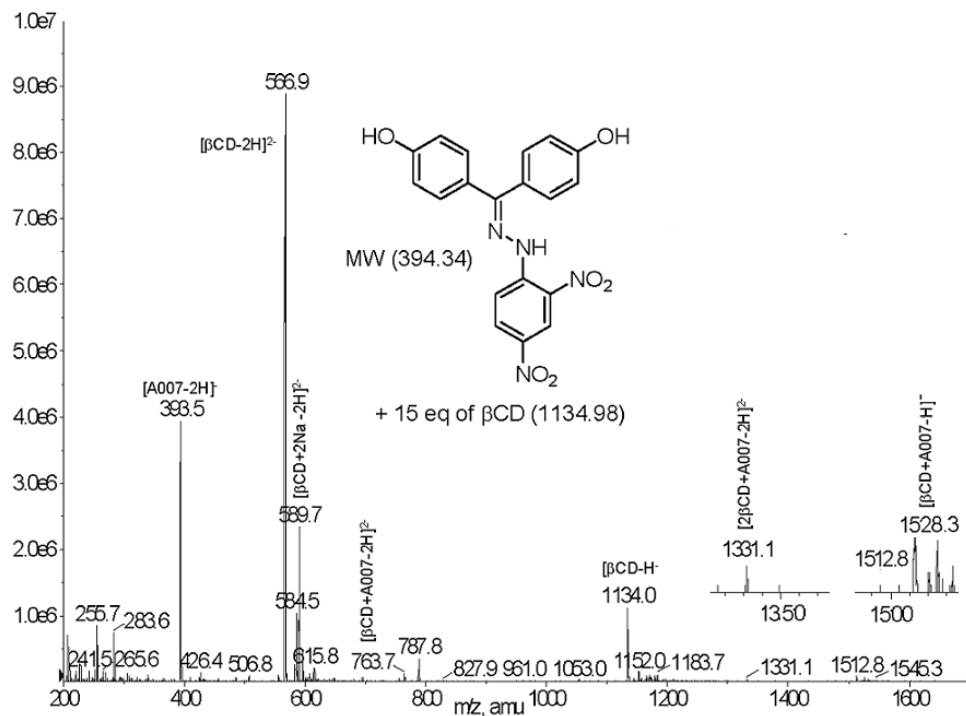
Figure 4.3.  Negative ESI-MS of aqueous A007 in β−CD.

As it is the case with α−CD and β−CD dominant signals in ESI-MS of **A-007** in aqueous γ−CD are for free A007 and γ−CD molecular ions (Figure 4.4).  However there are some spectroscopic patterns that distinguish this MS from other two.  One is the presence of MS signal that corresponds to mass of molecular complex between one **A-007** and three molecules of γ−CD.  This can be easily explained with the fact that γ−CD has the largest cavity in comparison with α−CD and β−CD and can easily accommodate dinitrophenyl moiety of **A-007**.  The ESI-MS spectroscopic study results can be very well related to solubility studies where the drug includes a tri-substituted phenyl moiety.  For instance similar approach with γ−CD was used by Kamigauchi and coworkers to extract some alkaloids containing tri-substituted phenyl moiety from their natural materials by aqueous cyclodextrins.[8] In this finding the best results were obtained with aqueous γ−CD due to alkaloid-cyclodextrin complementarily size.
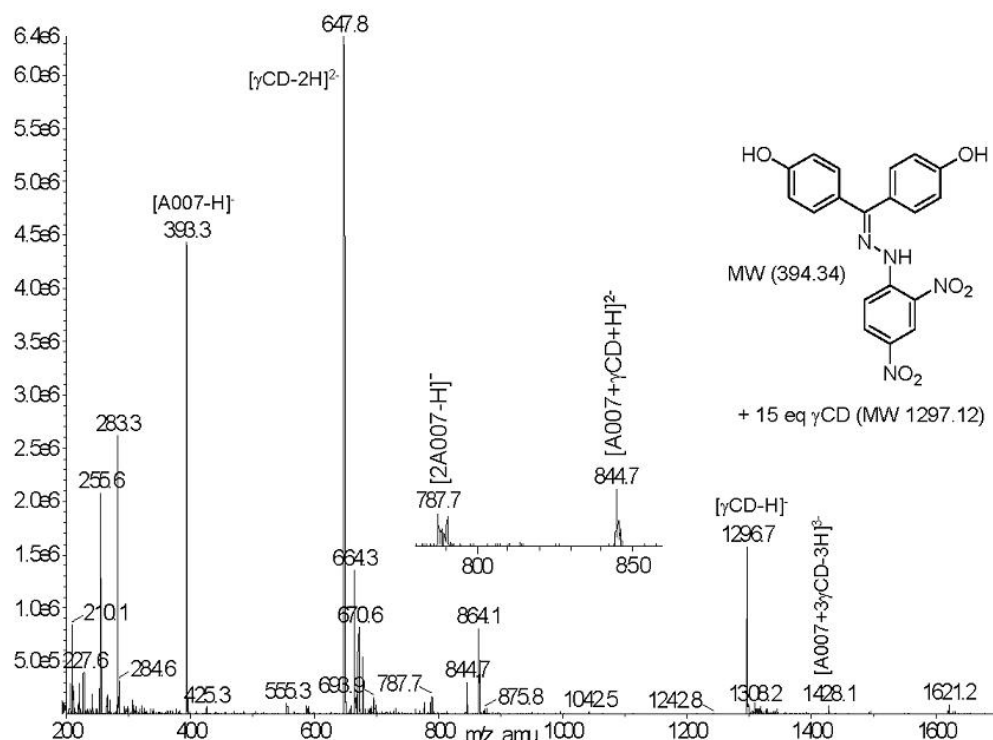
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Figure 4.4.  Negative ESI-MS spectra of A007 in γ–CD

As discussed previously compound **2** is an acetic acid derivative of A007 and it is prodrug that by itself has similar anticancer activity as **A-007**.  It can be administered as a aqueous sodium bicarbonate salt intravenously.  For an oral administration, cyclodextrins delivery approach should be utilized due to fact that in low pH stomach environment salt of **2** will become again free acid **2**.  It is also possible that it can be metabolized into its oxalic hemiacetal by the liver enzyme that can upon entering into blood stream hydrolyze into **A-007**.  The ESI-MS spectroscopic study of this compound in aqueous cyclodextrins indicates formation of inclusion complexes.  If we assume that intensity for the cyclodextrins complexed **2** and free **2** corresponds their molar ratio in gas phase then we can estimate the molar percentage

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cyclodextrins complexation of 2. There should be around 40% of **2** in 1:1 complex with α–CD (one molecule of A007 and two molecules of α–CD) and around 3% in 1:2 complex with α–CD (Figures 4.5). The signals that are used for the comparison are 254.5 $[M-2H]^{2-}$, 740.6 $[\alpha CD+M-2H]^{2-}$, and 1227.5 $[2\alpha CD+M-2H]^{2-}$ (Figure 4.5). Considering the size of the phenyl group and the cyclodextrins cavity, it seems that β–CD has a required complementarily to form inclusion complexes with polar 1,4-disubstituted benzene.[9] This is perfectly reflected in presence of signals for high order cyclodextrins complexing of **2** (Figure 4.6). Particularly presence of signals at 1304.4 for $[3\beta CD+M-3H]^{3-}$ and signal at 1389.2 $[2\beta CD+M-2H]^{2-}$. Possibility of formation high order complexes with **2** is even more emphasized in negative ESI-MS spectra of **2** in aqueous γ–CD (Figure 4.6). On the other hand there is no substantial complex formation between prodrug **2** with three molecules of α–CD however these kind of complex are observed between prodrug 2 and both β–CD and γ–CD due to larger cavities (Figure 4.6).



Figure 4.5a. Negative ESI-MS spectra of **2** in α-cyclodextrin
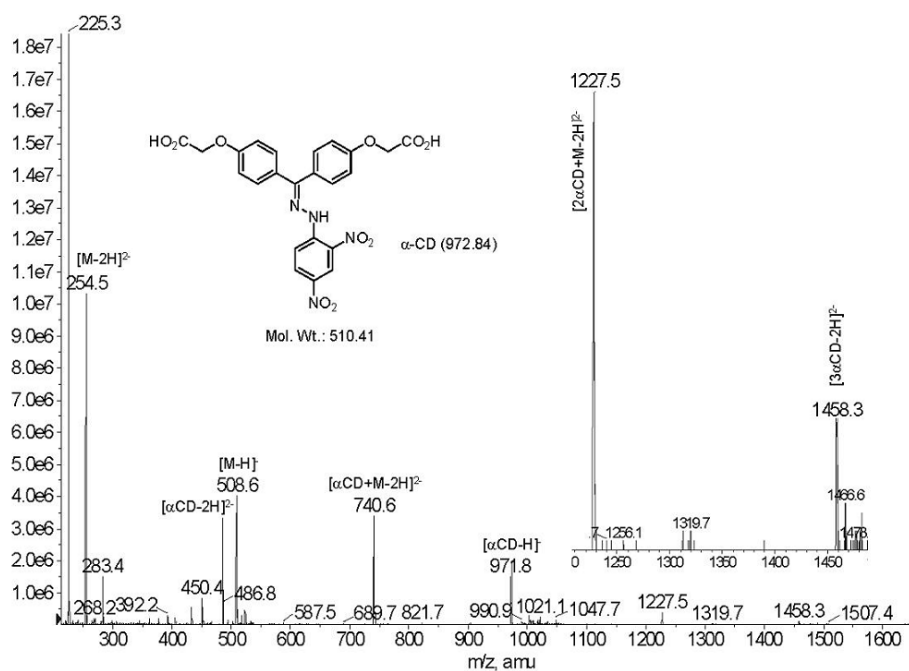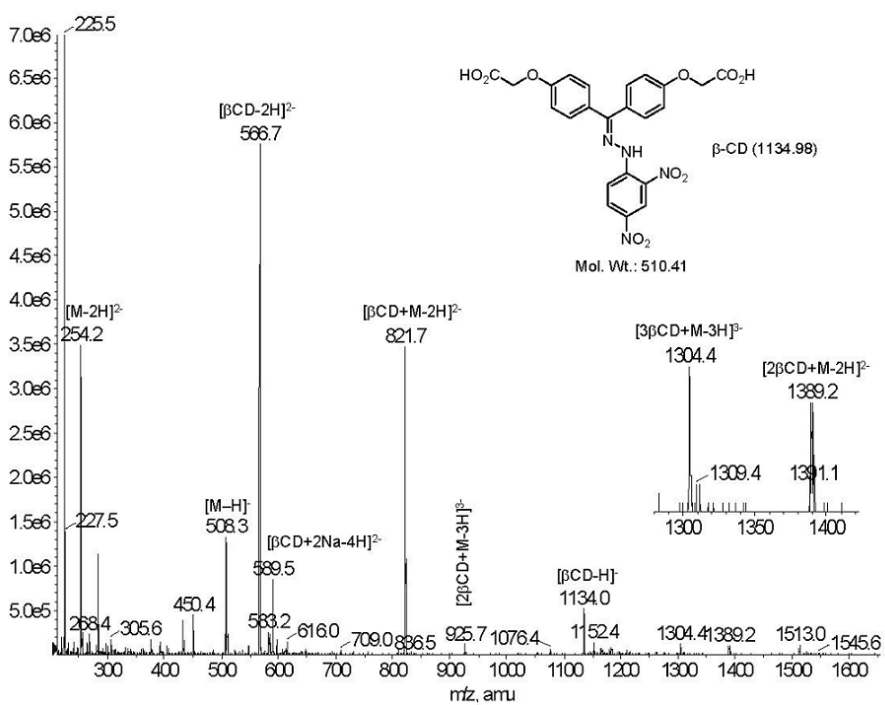
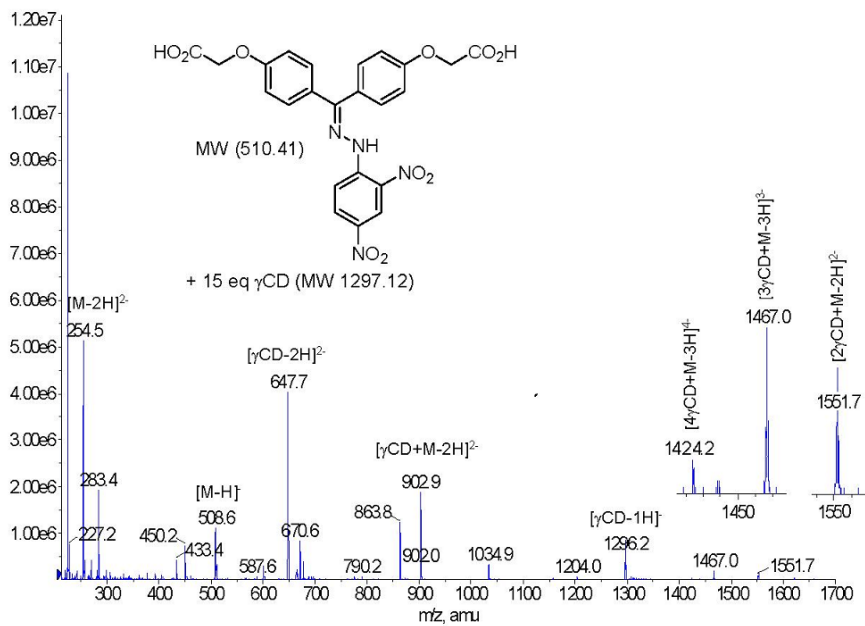Figure 4.5b. Negative ESI-MS spectra of **2** in β-cyclodextrin



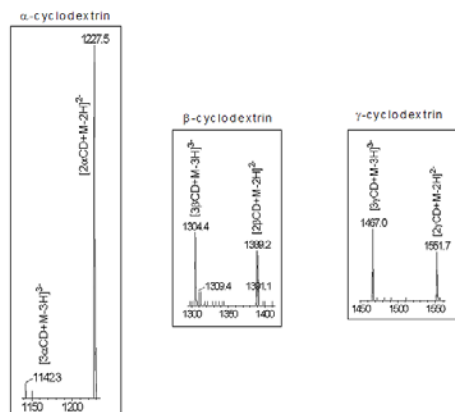Figure 4.5c. Negative ESI-MS spectra of **2** in γ-cyclodextrin

Figure 4.6. Relative intensity of ESI-MS signals of multi-cyclodextrin complexes with prodrug 2.

Compounds **3** and **4** are classical examples of cationic prodrugs. They both under physiological conditions metabolize into our **A-007** drug. They also have surfactant like properties, hydrophobic and hydrophilic parts of molecule. Physical properties of these kind of molecules are sensitive to their solution concentration. This is due to formation of micelle type molecular aggregates.[10] Our earlier $^1$H-NMR spectroscopic study demonstrates that stability of **3** and **4** in water media depends on its water concentration. At relatively high concentration (10 mM) of both these prodrugs are stable at room temperature for several months. However at lower concentration (below 0.1 mM) they both hydrolyze into **A-007** with half-life time of ~24 hour. Due to surfactant like properties of both **3** and **4** these can be stored as high concentrated water solutions for long time and administrated as such to patients via intravenous injection. Naturally after the injection, concentration will become much lower than critical micellar concentration and compounds **3** and **4** will slowly hydrolyze into our targeted **A-007** drug. Cyclodextrins can substantially alter both the prodrug solubility as well as prodrug half-

life time.  In our case, 0.1 mM solution of **3** in 1 mM β-CD has half-life about five hours

as determined by $^1$H-NMR spectroscopy.  However $^1$H-NMR spectroscopic study was

hampered by existence of many molecular species and subsequently even many more

molecular aggregates in aqueous solution of **3** and cyclodextrins.  Because the exchange

rate between many molecular aggregates is much faster than the NMR time scale an

average chemical shift was observed for all of these molecular aggregate.  On the other

hand EMI-MS spectroscopy is capable to give us signals for every molecular species

present in an aqueous media.[11]
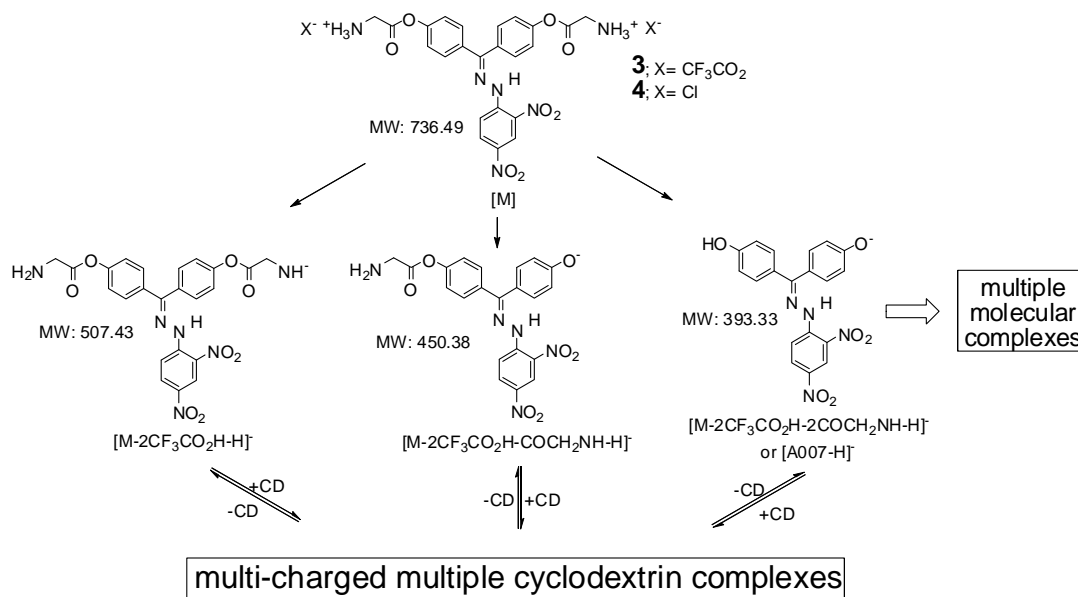


Figure 4.7.  Three molecular species originated from **3** and **4** that are present in aqueous

cyclodextrin

Considering the two step hydrolysis of **3** and **4** there are at least three molecular

species present in their negative ESI-MS of their cyclodextrin solutions (Figure 4.7).  It

also seems that cyclodextrins catalyze the hydrolysis of the ester groups which makes it

practically difficult to record ESI-MS of **3** and **4** and cyclodextrins without its hydrolyzed

products. Furthermore all of these three ionic species are capable to form self assembly

molecular aggregates, molecular aggregates with each other, and above all, they can form

inclusion complexes with cyclodextrins (Figure 4.7). This will all result in complex ESI-

MS spectra of **3** and **4** in aqueous cyclodextrins. A few structures of molecular

aggregates of **3** and **4** and their hydrolyzed products are presented in Figure 4.8.
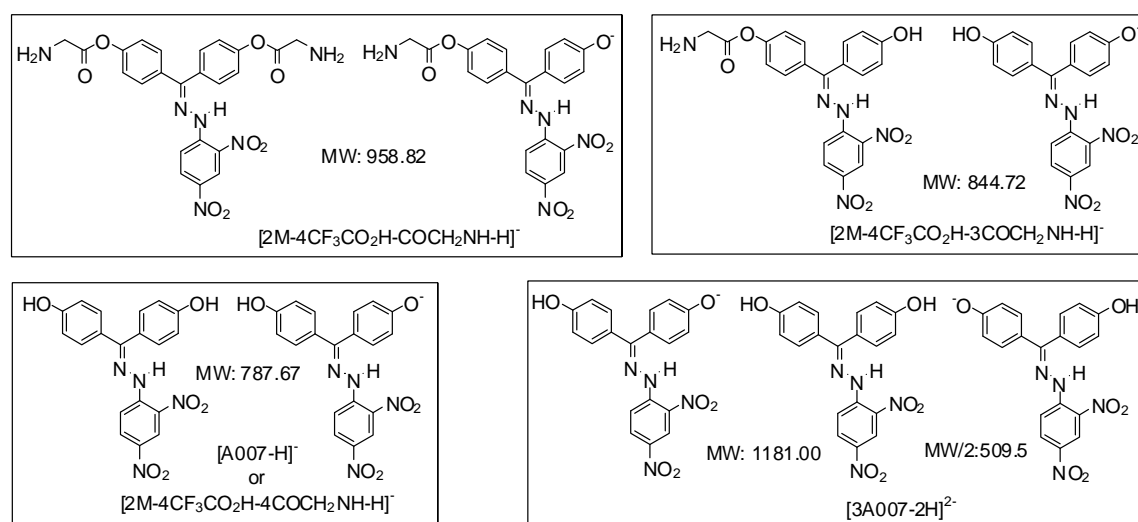


Figure 4.8. Structure of a few prodrug **3** and **4** multi-molecular complexes

Signals of three possible molecular species (molecular ion at 507.3, partially

hydrolyzed molecular ion at 450.2, and fully hydrolyzed molecular ion at 393.5) and their

molecular aggregates are present in negative ESI-MS spectra of **3** or **4** in aqueous $\alpha$-

cyclodextrin ($\alpha$–CD) (Figures 4.9). Recent spectroscopic studies on linear ammonium

threading molecules with $\alpha$-cyclodextrin show formation of cyclodextrin complexes with

formation of rotaxanes.[12] The attached two glycine moieties to the **A-007** forms

ammonium treading that can form inclusion complex with **A-007** as evidenced by signal

at 994.6 that corresponds to complex of α-CD with two molecular ions of ammonium

prodrugs **3** and **4** (Figure 4.9). Naturally presence of α−CD complex with fully

hydrolyzed **3** and 4 is observed as well (signal at 682.7 for complex of molecular double

ion of A007 with one α−CD). The negative ESI-MS spectra of 3 or 4 in aqueous β−CD

also represents dispersion of various molecular ions and molecular ion aggregates of

prodrugs **3** and **4** (Figure 4.9).
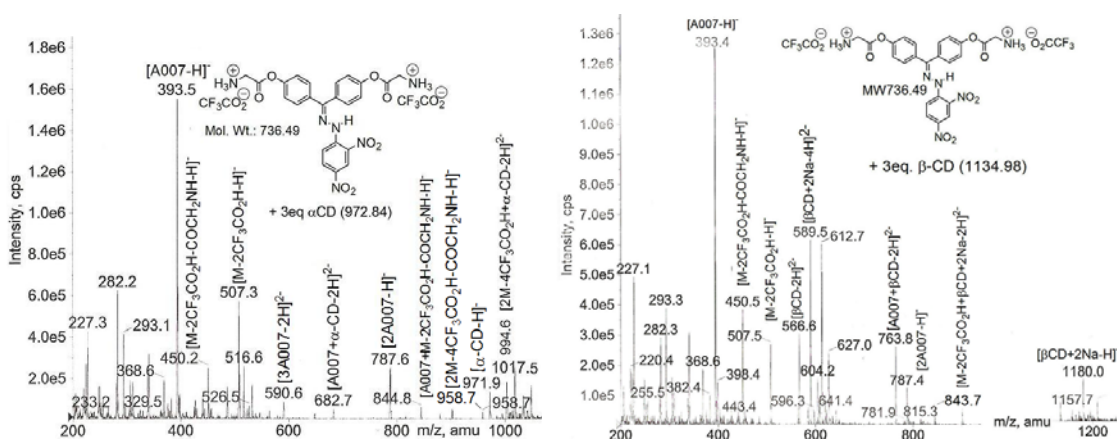


Figure 4.9. The negative ESI-MS spectra of aqueous **3** in α−CD and β−CD
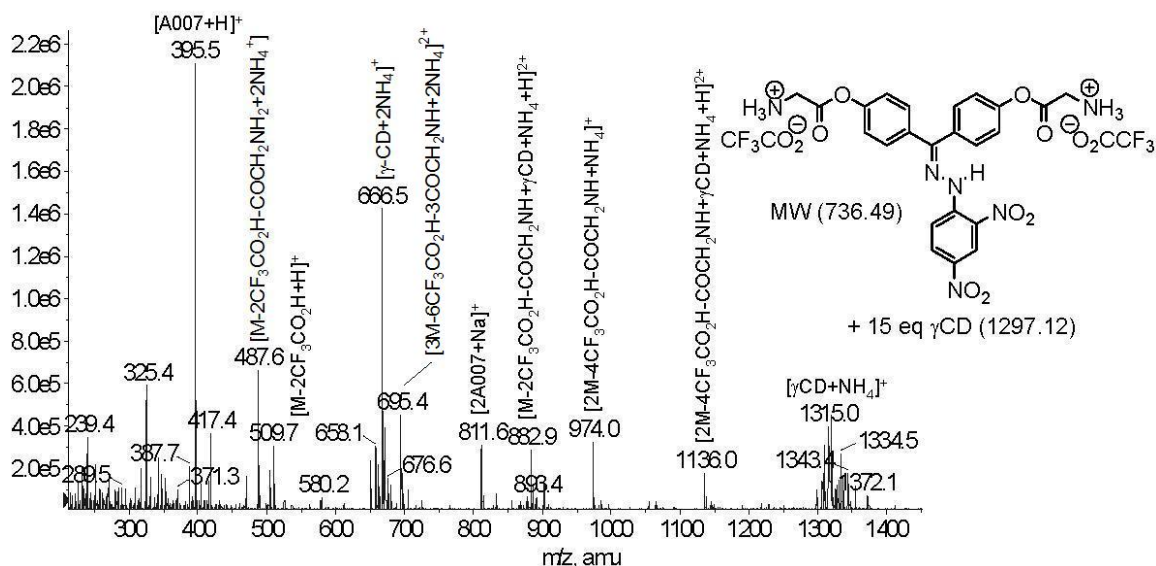
Figure 4.10.  Positive ESI-MS spectra of **3** in aqueous γ−CD

Interestingly positive ESI-MS spectra of aqueous cyclodextrin solutions of **3** and **4** are remarkably similar to negative ESI-MS spectra adjusted for molecular weight of proton, ammonium, and sodium ion.  That was perfectly demonstrated on positive ESI-MS spectra of **3** in aqueous γ−CD (Figure 4.10).  Noticeable are signals for γ−CD inclusion complex with two **3** at 1136.0 and γ−CD inclusion complex of partially hydrolyzed **3** at 882.9.  All the other signals corresponds either to molecule 3 (signal at 509.7), partially hydrolyzed **3** (signal at 487.6), fully hydrolyzed **3** (signal at 395.5) or their various molecular associates (Figure 4.10).

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**Conclusion**.

Water soluble **A-007** prodrug interactions with α−cyclodextrin, β−cyclodextrin, and γ−cyclodextrin were studied with ESI-MS spectroscopy. Cyclodextrins were used for several purposes; to solubilize the prodrug, change stability of the prodrug and prodrug carrier. It was demonstrated that in cyclodextrin water solution half-time of prodrug **3** was decreased to around 4-5 hours which is the ideal for *in vivo* drug release. It was also demonstrated that during prodrug hydrolysis various molecular associates were formed that can substantially alter drug absorption and release capability. Therefore cyclodextrins seems to be crucial for both drug delivery as well as controlling the drug release by altering the prodrug half-life time in blood plasma.

## Experimental

All cyclodextrins, solvents and chemicals were obtained from Sigma-Aldrich and are used without further purification.  Preparation procedures and characterization A007 prodrugs were reported previously.  A aqueous solution were sonicated for freshly prepared to eliminated large percentage of hydrolyzed A007 drugs and sonicated for a few minutes to insures homogeneity All the mass spectrometric analysis was performed on an Applied Biosystems/MDS SCIEX (Foster City, CA, USA) 3200 QTRAP hybrid quadrupole/linear ion trap mass spectrometer with direct infusion of each prodiginine sample in aqueous methanol (50/50) spiked with 0.1% formic acid at a flow rate of 4 μL/min. Positive-ion and negative mode ES-MS was used for the analysis, with the Turbo VTM("superscript") source settings for **A-007** or its derivatives optimized as follows: ionspray voltage 5.5 kV, declustering potential 60 V, source temperature 120°C, GS1 40, and curtain gas 10. Ultrahigh grade (99.999%) nitrogen was utilized as nebulizing gas and drying gas.

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9. for instance see Chan, L. W.; Kurup, T. R. R.; Muthaiah, A.; Thenmozhiyal, J. C. *Int. J. Pharmaceutics* **2000**, *195*, 71.

10. For instance see: Wong, T. C. "Micellar Systems: Nuclear Magnetic Resonance Spectroscopy" IN:" Encyclopedia of Surface and Colloid Science" Somasundaran, P; Hubbardlopdia, A. Editors, Second Edition, CRC Press, **2006**, pp 3738.

11. For instance see Beni, S.; Szakacs, Z.; Csernak, O.; Barcza, L.; Noszal, B. Europ. *J. Pharm. Science.* **2007**, *30*, 167.

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# Chapter V.  Attempts Towards the Synthesis of the Tetramic Acid Antibiotic - Magnesidin

## Abstract

Magnesidin, the tetramic acid natural product, is a drug candidate for gingivitis and other novel anti-bacterial applications.  The key step in the synthetic strategy of Magnesidin and other tetramic acids is the ring opening of an oxazolone ring with an activated ester. In this context, an efficient two-step synthetic procedure for the preparation of numerous variations of oxazolone rings from *N*-protected glycine and either aliphatic or aromatic aldehydes was developed. The major problem associated with Magnesidin synthesis is the volatile nature of key intermediate oxazolone.  This problem has been circumvented by the design and synthesis of a tag molecule, which serves as a synthetic equivalent of a resin/solid support with the aim of restoring homogeneous reaction conditions and anchoring the volatile molecule to the solution phase.

## Introduction

Attempts to synthesize Magnesidin and Magnesidin-like compounds by methods analogous to those reported in the literature for 3-acetyl tetramic acids[1,2], through the intermediate *N*-alkanoyl-acetylamino acid ethyl ester or the azlactone either met with failure or gave the desired compounds in very poor yields.  The presence of the olefhinic moiety at position 5 of the five membered Magnesidin tetramic acid ring **1**(Figure. 5.1) makes it aromatic in nature and synthetically inaccessible through the traditional methods of tetramic acid synthesis.  Our approach to the synthesis of Magnesidin involved a new route through the ring opening of advanced intermediate **5** (Scheme5.1) with β-keto ester.

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The β-keto ester is used as a multicoupling reagent[3], utilizing the both the electrophilic carbonyl and nucleophilic active methylene carbon to perform the required synthesis. Once the condensation product formed by the nucleophilic attack of active methylene carbon of β-keto ester with intermediate 5 is in place, we envisioned a possibility of intramolecular cyclization by the elimination of alcohol to make the Magnesidin ring.
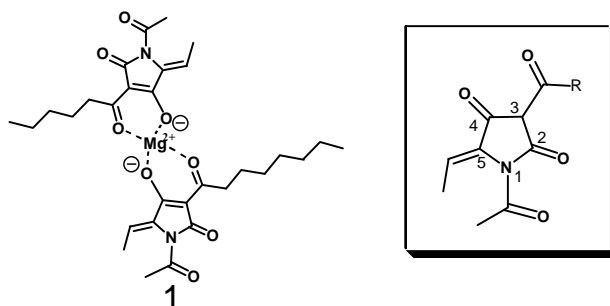
## Results and Discussion



Figure. 5.1.  Structure of Magnesidin.

### Initial Retrosynthetic Analysis of Magnesidin

The synthetic approach for Magnesidin can be seen in the retrosynthetic analysis depicted in Scheme 5.1.  It was envisioned that the synthesis of Magnesidin would commence from the straightforward reaction of the commercially available amino acid threonine[4] (Scheme 5.1).  As per the retrosynthetic analysis, the benzoylation of carboxylate[6] protected threonine would yield compound **6**.  Subsequent ring closure of compound **6** by intramolecular condensation under basic conditions would provide key oxazolone ring **5**.  Making the oxazolone ring sets the stage for incorporating the β-keto ester moiety to make the tetramic acid core.  The nucleophile generated by proton

abstraction from a β-keto ester can open the oxazolone ring to make the basic tetramic acid skeleton. A sequence of required substitutions (Scheme.5.1) would then make the tetramic acid Magnesidin.
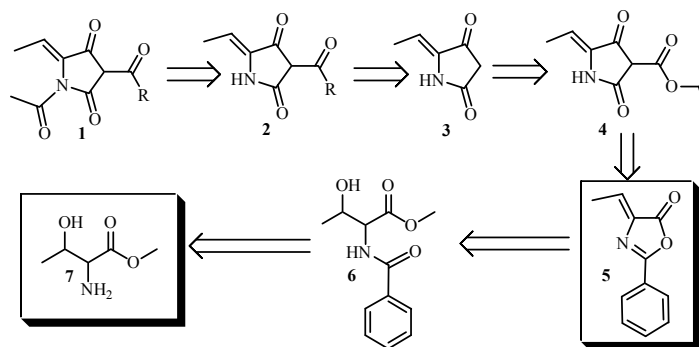


Scheme 5.1. Initial Retrosynthetic analysis of Magnesidin.

***First Attempt to Synthesize Magnesidin***

Substituted threonine **11** was prepared from commercially available amino acid **8** in 88% yield over three steps following literature procedures[4,5] with minor modifications (Scheme 5.2). In the first step, threonine was esterified to yield **9**. This compound is commercially available but it can be synthesized very cost effectively.

The esterification is carried out in order to protect the carboxylic acid from chemoselective benzoylation of the amine group in the following step to make compound **11**. The ester formed as its hydrochloride salt needs to be free-based for the subsequent benzoylation to occur. Ordinarily, a simple wash with a mild aqueous base like sodium bicarbonate would suffice, but considering that we have a hydroxyl moiety in **11,** the molecule is hydrophilic and is lost upon aqueous work up. Therefore, we decided to continue to the next step, namely benzoylation of amine, taking care to use two equivalents of the base triethylamine. The extra base scavenges the HCl from the

ammonium salt to make triethylamine hydrochloride which is insoluble in

dichloromethane and can be filtered out after crystallization. Benzoylation is done with

benzoyl chloride and triethyl amine on **11**. The reaction completes in two hours, and the

reaction mixture is subsequently left overnight, to crystallize out triethylamine

hydrochloride, which is easily filtered off. The aqueous work up is completed to obtain

the pure product. The ester deprotection was achieved under reflux conditions with ethyl

acetate as the solvent and a mixture of acetic acid/hydrochloric acid as the reagent. The

deprotection was initially attempted under milder conditions at room temperature and

with acetic acid as the reagent, but under these conditions the expected hydrolysis did not

occur. After a standard work up and purification, the product was obtained in 88% yield

over the 3 steps on a multigram scale. Unfortunately, as expected, we could not make

the oxazolone ring under dehydration conditions using acetic anhydride and sodium

acetate from compound **11**. Our subsequent attempts to carry out this transformation

using stronger conditions, by varying the temperature and base, did not bring about the

required transformation.



**a**. MeOH/HCl, 98% **b.** Benzoyl chloride, DCM,Triethyl amine, 98% **c**. AcOH/HCl, 95% **d**. Ac₂O/AcONa
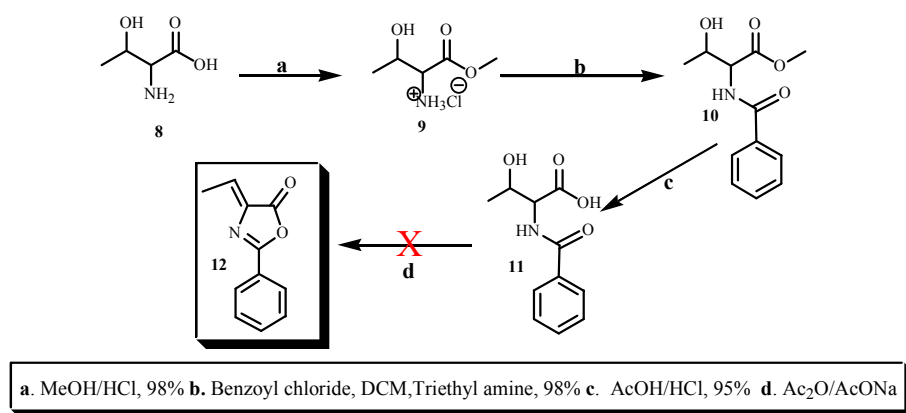
Scheme 5.2. Attempted synthesis of oxazolone ring.

To our surprise, we could not find in the literature a reliable general procedure for the preparation of oxazolone ring of this general structure. These oxazolone rings are important precursors in the synthesis of $\alpha,\beta$-unsaturated acids and various other valuable pharmaceuticals.[6,7] Therefore, it was important to develop an industrially viable synthetic procedure that was inexpensive and capable of producing the product in high yields. To meet these requirements, a simple, preferably one-pot synthesis should be developed using readily available starting materials. The procedure we developed adheres to these requirements.

*N*-Protected glycine and the corresponding aldehydes were chosen as the optimal starting reagents for these reactions. A suspension of *N*-protected glycine is stirred with sodium acetate and acetic anhydride at 0°C and into this suspension, the aldehyde is added. The reaction mixture is stirred at 0°C for two hours and then it is refluxed until the reaction goes to completion, which is monitored by NMR. The first step in this reaction is the formation of 2-R-4H-oxazol-5-one **14**, which readily condenses with the aldehyde to form the required oxazolone ring **15**.



a. $(CH_3CO)_2O/CH_3CO_2Na$   b. $R^2CHO$  80-95 %
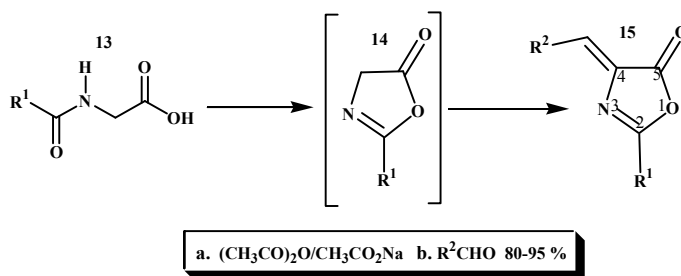
Scheme 5.3: Synthesis of oxazolone ring.

To fully understand and demonstrate the scope of this condensation, the reaction progression between *N*-acetylglycine and 1,4-benzenedicarbaldehyde was followed by

NMR spectroscopy[8] (Figure.5.2). The first step in the reaction is the formation of 2-methyl-4H-oxazol-5-one (**B**), which readily condenses with 1,4-benzenedicarbaldehyde (**C**) to form the monocondensation product (**D**). The reaction continues, and the final double condensation product (**E**) is formed. According to our NMR studies, the conversion appears to be nearly quantitative.  Similar reaction conditions were used for the preparation all the oxazolones listed in Table. 5.1.
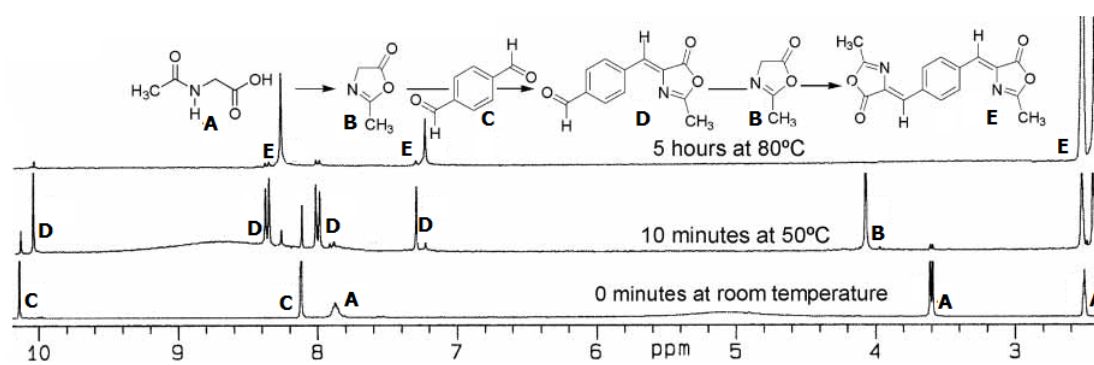


Figure 5.2.   HNMR data showing the reaction progression for the preparation of oxazolone ring.

With a general method for the synthesis of numerous variations of oxazolone rings in hand, the next step was to open the oxazolone ring with a β-keto ester to make the tetramic acid skeleton.  To have a better understanding of the nature and scope of the ring opening of the oxazolone ring with a nucleophile, we decided to study the behavior of oxazolone ring with hydroxyl and methoxy nucleophiles.[8]

Opening the oxazolone ring with hydroxy and alkoxy groups leads to the formation of *N*-protected α,β-unsaturated α-amino acids and esters, respectively.  As discussed earlier, these compounds are of high commercial importance.  A variety of

asymmetric synthetic methods for amino acid preparation have been developed and are available in literature.[9-15] One method of importance involves hydrogenation of an enamide using chiral transition-metal catalysts. Perhaps one of the best examples using enamides as a starting material[16, 17] is enantioselective synthesis of [(S)-2-amino-3-(3,4-dihyroxyphenyl)propanoic acid] (L-DOPA),[18] which involves a rhodium hydrogenation catalyst containing a chiral phosphorus ligand reacting with an enamide. Unfortunately, large-scale manufacturing of $\alpha,\beta$-unsaturated amino acids has proven to be problematic. Theoretically, amino acids are ideal starting materials for the preparation of these compounds. For instance, L-threonine methyl ester is an excellent starting material for the preparation of Z-2-(N-acetylamino) butenoate.[19] However, there is no general synthetic procedure using amino acids as precursors. Now that we have a general procedure to make oxazolone ring, it will not only make an excellent general method to make -$\alpha,\beta$-unsaturated acids and esters, but also it will give insight into behavior of oxazolone ring towards different nucleophiles.

The ring opening of oxazolone ring was achieved very easily. A methanol solution of oxazolone and sodium methoxide was stirred at room temperature for 15 min. The solvent was evaporated, and the residue was partitioned between 10% aqueous ammonium chloride (100 mL) and methylene chloride. After standard workup, the oily residue was recrystallized from petroleum ether to give the pure product N-protected $\alpha,\beta$–unsaturated $\alpha$-amino acids ester (Scheme 5.4). The corresponding N-protected $\alpha,\beta$–unsaturated $\alpha$–amino acids were prepared under similar conditions except, the solvent used was a 1:1 mixture of methanol and water and the base used was sodium hydroxide. To this end, our method of opening an oxazolone ring with an appropriate

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nucleophile, served as an excellent general method to synthesize α-acetylamino- and α-benzoylamido-α,β-unsaturated acids and esters. The starting materials are inexpensive, and the reaction procedures are very simple. The products are purified by simple recrystallization from methanol. Therefore, this method is applicable to large-scale (industrial) preparation of these valuable intermediates for use in the pharmaceutical industry.
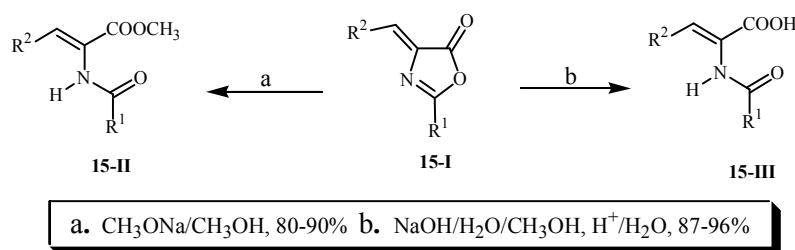


Scheme 5.4: Synthesis of α-acetylamino-, α-benzoylamido-α,β-unsaturated acids and esters.

To determine the stereochemistry of the double bond generated in the course of the condensation reaction, X-ray structural analysis was performed with 4-Ethylidene-2-phenyl-4H-oxazol-5-one (**15-I-g**) (Figure. 5.3). X-ray analysis proved that this isomer was the major isomer (more than 95%) of the condensation reaction. Our X-ray crystallographic study confirms that the stereochemistry of the major isomer formed during condensations of **14** with aldehyde has Z-configuration of the newly formed double bond of oxazolone. Therefore, in preparation of both esters and acids, which are prepared by ring opening of oxazolone, the stereochemistry of the double bond must be preserved.

Table 5.1.  Oxozolones (15-I),  α-acetylamino- and α-benzoylamino-α,β-unsaturated

acids (15-III) and esters (15-II) synthesized and their Isolated yields.

| $R_1$ | $R_2$ | 15-I | %Yield | 15-II | %Yield | 5-III | %Yield |
|---|---|---|---|---|---|---|---|
| $C_6H_5$ | $2,4,6\text{-}(MeO)_3C_6H_4$ | a | 92 | a | 80 | a | 87 |
| $CH_3$ | $C_6H_5$ | b | 90 | b | 90 | b | 93 |
| $C_6H_5$ | 2-thiophenyl | c | 92 | c | 82 | c | 96 |
| $CH_3$ | 2-thiophenyl | d | 93 | d | 90 | d | 88 |
| $CH_3$ | 1-naphthyl | e | 80 | e | 80 | e | 92 |
| $C_6H_5$ | 2-furyl | f | 90 | f | 89 | f | 96 |
| $C_6H_5$ | $CH_3$ | g | 95 | g | 90 | g | 91 |
| $C_6H_5$ | 1-naphthyl | h | 84 | h | 87 | h | 92 |
| $C_6H_5$ | $C_6H_5$ | i | 90 | i | 90 | i | 96 |
| $C_6H_5$ | $3,4,5\text{-}(MeO)_3C_6H_4$ | j | 80 | j | 80 | j | 93 |
| $C_6H_5$ | $4\text{-}(Me_2N)C_6H_4$ | k | 90 | k | 90 | k | 91 |
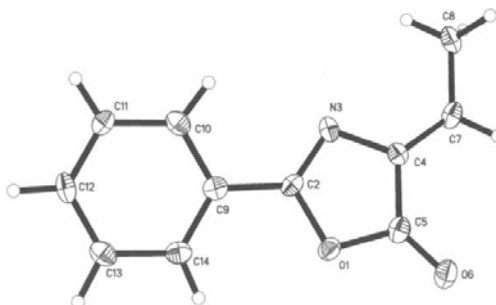
Figure 5.3. Ortep drawing of the X-ray determined structure of **15-I-g** (Table 5.1) (Courtesy of E.D. Stevens, University of New Orleans)

We established that it was possible to open oxazolone rings with nucleophiles under basic conditions. Our next step was to establish a method for opening the oxazolone ring with an activated ester to make the desired tetramic acid skeleton. (Figure 5.4)

In an attempt to open the oxazolone ring with the anion of β-keto ester, we stirred a mixture of 3-oxo-decanoic acid methyl ester in methonol and sodium methoxide, while 4-ethylidene-2-phenyl-4H-oxazol-5-one was added and stirring continued. After several hours there was no trace of our desired product formed. The reaction conditions were varied and checked for the progress of the reaction. Refluxing the reaction mixture, changing solvents, trying stronger base did not help to bring about the desired transformation.



Figure 5.4. Expected reaction of oxazolone with anion of β-ketoester.

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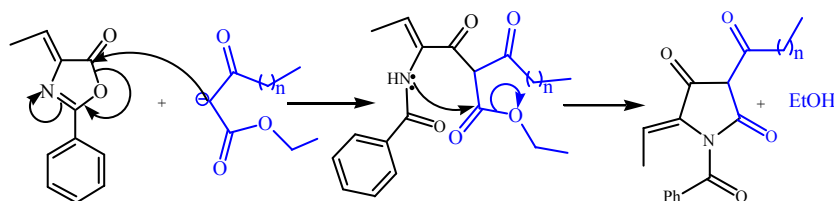We figured the electron withdrawing benzoyl group on amine nitrogen was deactivating the amine group and preventing its nucleophilic activity, consequently interfering with ring closure. We speculated that if the benzoyl group was replaced by an electron donating group or at least with a group that is low in electron withdrawing ability, this transformation should be feasible. Hence we decided to replace benzoyl group with ethoxycarbonyl group (Scheme.5.5, compound **18**). The rationale for the replacement benzoyl group was twofold. First, this replacement allowed for us to determine if it is indeed the benzoyl group that is preventing the ring closure by decreasing the nucleophilicity of amine nitrogen. Second, it allowed us to continue with the standardized oxazolone route with few steps.
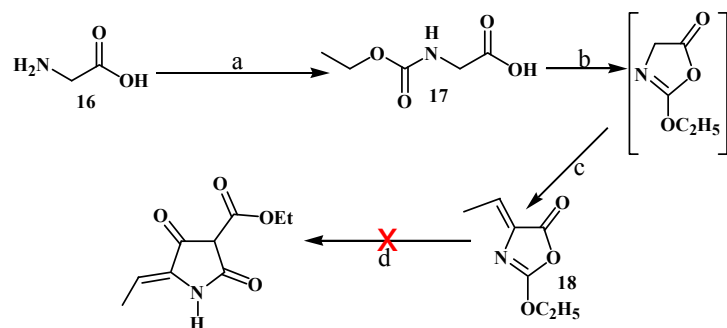


**a.** Ethyl chloroformate/ NaOH+NaOH, 70% **b.** Ac$_2$O, 99% **c.** Acetone, 99% **d.** Diethyl melonate/NaOEt

Scheme 5.5. Synthesis of ethoxy-substituted oxazolone ring.

To obtain the amino-substituted glycine **17** (Scheme 5.5.), a solution of the glycine in aqueous NaOH was stirred and ethyl chloroformate and aqueous NaOH was added dropwise. The mixture was stirred at 0°C and washed with ether.[20] After a standard workup the compound was directly used to make the required oxazolone ring **18**

with ethoxy substituent on the ring nitrogen.   A suspension of 2-Ethoxycarbonylamino-ethanoic acid, acetaldehyde, sodium acetate and acetic anhydride was stirred at 0° C first and then under reflux, essentially following the method developed to make oxazolone rings.   The reaction mixture was then mixed with water and stirred at room temperature. After the workup, the NMR of both the organic and aqueous layers were almost void of any product.  There was a  trace amount oxazolone **18**.  We then realized because of the very small size of oxazolone **18,** it was a very volatile molecule which vaporized even at room temperature.  A molecule so volatile is difficult to manipulate and hard to work with.  Use of higher temperatures, even room temperature is precluded because of the low molecular weight.  Hence it was determined that further attempts to carry out the required transformation via this route would most likely be unsuccessful.

### *Second Attempt to Synthesize Magnesidin*

We decided to start with an open chain threonine derivative and introduce an active methylene group containing alkyl side chain on amine.  This procedure serves two purposes.  First, the substituent at $3^{rd}$ position of the product Magnesidin ring can be introduced directly by choosing the appropriate alkyl side chain.  Second, the early introduction of the heavy alkyl chain increases the molecular weight of the intermediates, such that the intermediates are rendered non-volatile and are therefore easy to handle. This precludes the volatility problem encountered in the previous synthetic attempts and since the reaction involves fewer steps, it is expected that this synthetic route would be more efficient.
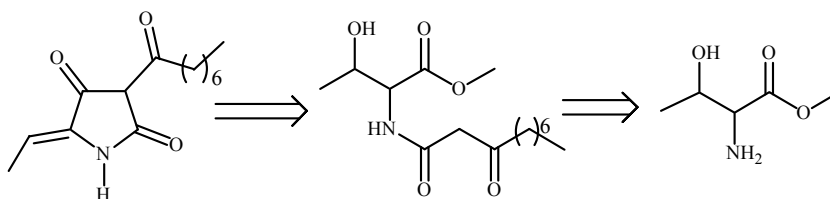
Scheme 5.6.  Third Retrosynthetic Analysis of the Magnesidin.


We started with the inexpensive amino acid threonine and made the

corresponding methyl ester by Fisher esterification.  The next step was to condense β-

keto-decanoic acid with threonine methyl ester under DCC coupling conditions.  The

intended next step was the dehydration to make the olefhinic moiety and subsequent ring

closure to make the tetramic acid core.  Unfortunately, the expected dehydration with

acetic anhydride and sodium acetate did not happen.   Instead the free hydroxyl group of

threonine was acetylated.  The ring closure did not happen either, as evident by the NMR.

Assuming the dehydration could be achieved at a later stage under high temperature

dehydration conditions, the compound was separated and the ring closure was tried with

the stronger base sodium methoxide.  Under these conditions instead of the expected ring

closure, the acetyl group was lost and compound **19** was regenerated.  At this stage, it

was realized even if we could protect the free hydroxyl group of threonine methyl ester in

the form of silyl ether, it would  not be successful as a synthetic scheme, considering mild

basic conditions, namely acetic anhydride and sodium was unable to accomplish ring

closure.  Using strong basic conditions might lead to ring closure but selectivity is lost

considering there are multiple acidic protons (Figure 5.5) resulting in the possibility for

multiple enolic forms and hence too many byproducts.  Therefore it is important to use a

method which avoids strong basic conditions and minimizes the possible enolic forms

and hence multiple product formation.  One possibility is carrying out dehydration before

using the base to close the ring.  This has been tried in Scheme 5.9.
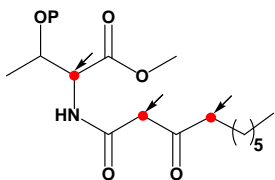


Figure 5.5.  Possible deprotonation sites upon using a strong base.
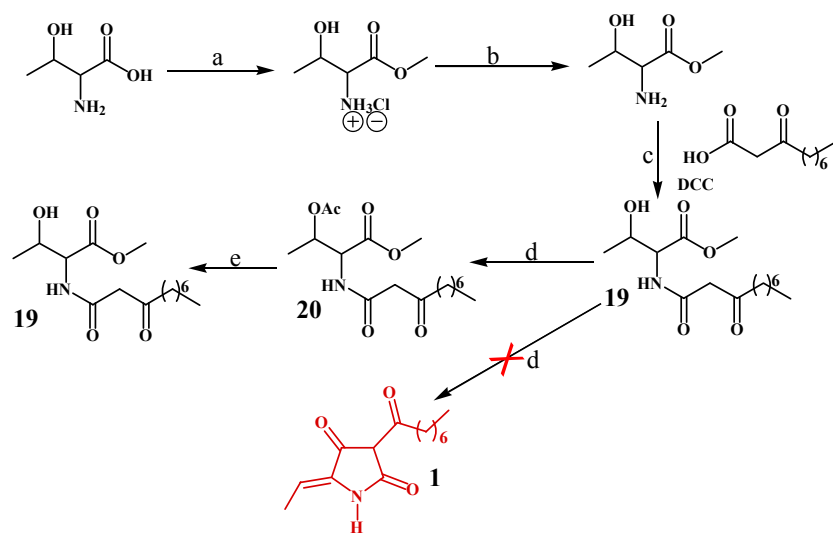


a.  Methanol/HCl, 98%   b. DCM/Na$_2$CO$_3$/H$_2$O  99%  c. 3-Oxo-decanoic acid, DCC, DCM  80%
d.  NaOAc/Ac$_2$O,  98%  e.  NaOMe/MeOH, 99%

Scheme 5.7:  Second Attempt to Synthesize Magnesidin

### Third Retrosynthetic Analysis of the Magnesidin

Our literature search indicated that ring closure to make tetramic acid under

extremely mild conditions can be achieved with Meldrum's acid[21] (2,2-dimethyl-1,3-

dioxane-4,6-dione, Scheme 5.8).  Meldrum's acid is an exceptionally acidic methylene

compound,[22] that has been used by Yonemitsu *et.al* as a versatile agent for various

syntheses which involve the reactions of nucleophiles generated from an active

methylene group under extremely mild conditions.[22-27]  For instance, β-keto esters were

obtained by acylation of meldrums acid using acyl chlorides in dichloromethane in the

presence of pyridine.    This gave the corresponding acyl meldrums acid almost

quantitatively.  Alcoholysis of acyl meldrums yields the corresponding  β-keto

esters(Scheme 5.8).  On the other hand a new process for the acylation of Meldrum's acid

with chiral *N*-protected amino acids was developed by Jouin et al[24].  In this process, the

*N*-protected amino acids were activated with isopreopenyl chloroformate and it was

shown that the acyl Meldrum's acids could be readily converted to the corresponding

chiral *N*-protected-substituted tetramic acids by heating in an organic solvent.  A variant

of this method was recently reported by Sandris *et,al*[20] which described a particularly

simple and convenient acylation reaction of Meldrum's acid via the imidazolides of *N*-

protected glycine **21** and the transformation of the acylated compounds **22** to the

corresponding *N*-protected tetramic acids **23.**
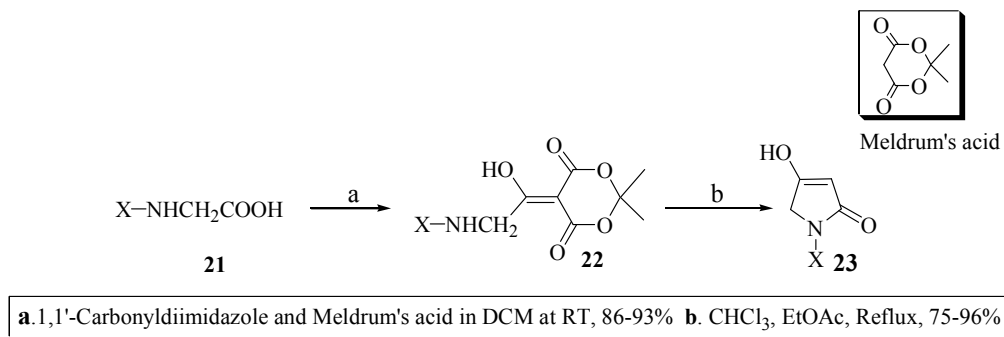


Scheme 5.8.  Meldrum's acid mediated synthesis of tetramic acids.

Based on the successful ring closure by Sandris *et al*[20] to make tetramic acid under mild basic conditions, and our requirement to avoid strong basic conditions, a new retrosynthetic analysis was designed. Considering the difficulties in the dehydration step we faced, it was also made sure that the synthetic scheme was designed such that olefhinic moiety was already in place, before condensation with Meldrum's acid was attempted.
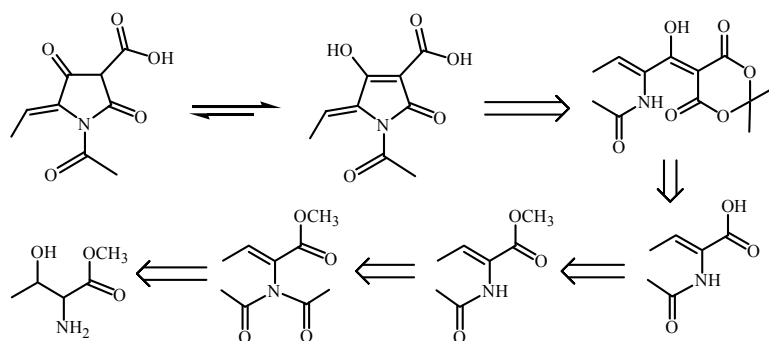


Scheme 5.9. Third Retrosynthetic Analysis of the Magnesidin.

The synthesis was started with the previously prepared threonine methyl ester. The diamide **24** (Scheme 5.10) was prepared successfully in 97% yield from the threonine methyl ester by simple reflux with acetic anhydride and sodium acetate.[28] Mono-amide **25** was made by hydrolysis of **24** using triethylamine as a base and methanol. Under these conditions exclusive mono-deacetylation occurs in 97% yield. The methyl ester **25** was hydrolyzed to give free acid **26** in 98% yield. Once we had the olephenic moiety in place, the stage is set for condensation with Meldrum's acid.
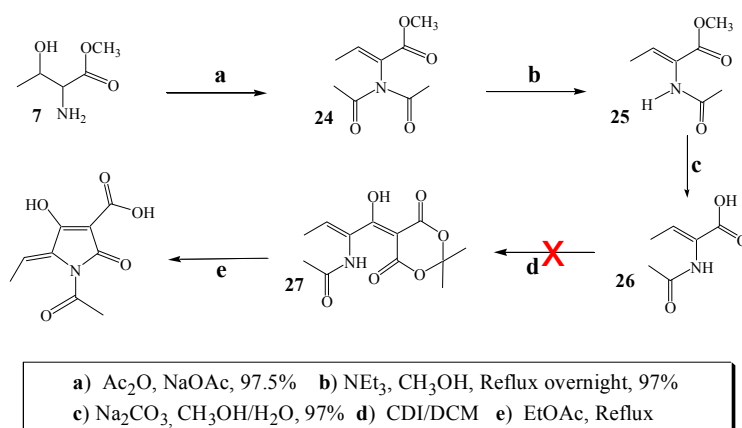
Scheme 5.10. Third Attempt to Synthesize Magnesidin.

Unfortunately, condensation of acid **26** with meldrums acid as described by Sandris *et al.* did not proceed as expected product **27** was observed. Surprisingly, there was no trace of starting acid **26**. Consequently, it was suspected that instead of the expected condensation with Meldrum's acid, the acid **26** self condensed to form 4-ethylidene-2-methyl-4H-oxazol-5-one **28** (Scheme 5.11). We reasoned that the presence of olefhinic moiety in the acid **26** could be the reason for the preferred self condensation over condensation with Meldrum's acid. The rationale for this reasoning was that due to the presence of olefhinic moiety in acid **26** the oxazolone **28** (scheme 5.11) is almost like aromatic furan system, considering each of the carbons involved in the ring is sp$^2$ hybridized, and all other criteria for aromaticity is met. So a possible aromatic ring formation by the self condensation process should be preventing the condensation reaction with Meldrum's acid.
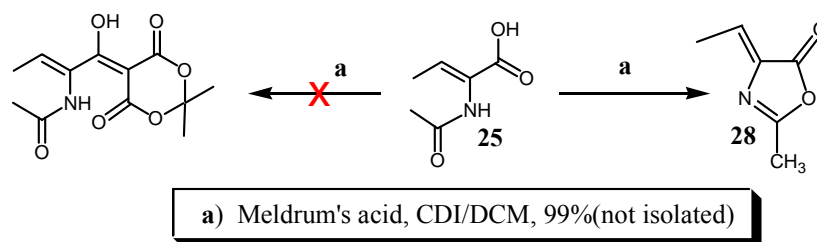
Scheme 5.11. Self condensation of **25.**

## *Fourth Retrosynthetic Analysis of the Magnesidin*

Based on the synthetic strategies tried thus far, it was deemed that the oxazolone method was very promising. For this method to work two things should be taken care of. First, for the successful ring opening of oxazolone ring with nucleophile generated from an active methylene group and subsequent ring closure to tetramic acid, it is important that the substituent on ring nitrogen is not very electron withdrawing in nature. Second, the oxazolone ring is heavy such that the molecule is non-volatile at room temperature.

Theoretically, this problem can be overcome by covalently attaching the volatile oxazolone ring to a solid support. However, despite the many benefits offered by solid phase approach, the heterogeneous nature of the insoluble polymers and reaction conditions often results in a series of problems including problems with loading capacity, inability to use conventional spectroscopic techniques for reaction monitoring, unequal distribution of and/or access to the reaction sites, solvation problems and inefficient coupling rate. Soluble polymer support such as polyethylene glycol polyvinyl alcohol and other ingenious variants of these polymers have limitations such as low loading capacity, limited solubility during the reaction processes, aqueous solubility, and insolubility in ether solvents etc. Hence we decided to develop an alternative methodology, a tag molecule, which serves as a synthetic equivalent of a resin/solid

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support with the aim of restoring homogeneous reaction conditions while at the same time anchoring the volatile molecule in the solution phase. Figure 5.6 shows the tag molecule which we developed, to be attached to the oxazolone ring. Our plan is to synthesize the tag molecule first and then attach the oxazolone ring to this tag, to solve the volatility problem.

The design of the molecular tag is done such that it can be used as soluble support for organic synthesis. Substrates anchored on molecular tag are expected to retain their reactivity, as in solution reactions, and allowed the use of conventional spectroscopic analysis during the synthetic process. Ideally, the constitution of molecular tag should be such that excess reagents and by-products in the multistep reactions can be removed easily by simple recrystallization. Also, the presence of huge aromatic moiety would make it easy to identify spectroscopically. Considering all the above factors the molecular tag has been designed as seen in Figure.5.6.
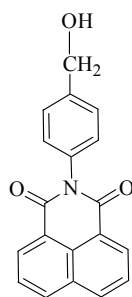


Tag molecule

Figure 5.6: Tag molecule.

# Fourth Retrosynthetic Analysis of the Magnesidin
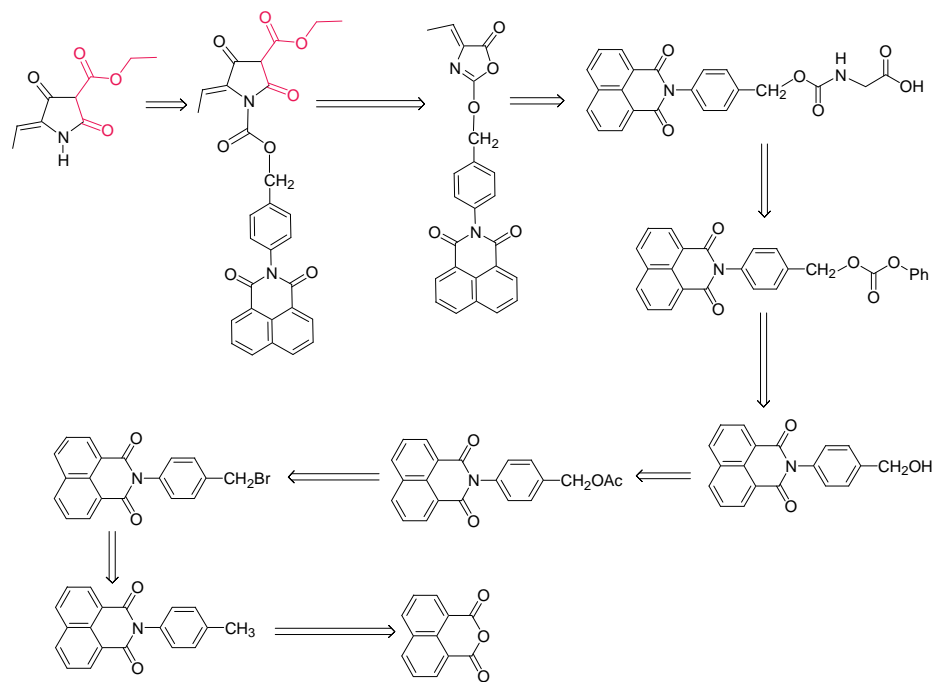


Scheme 5.12.  Retrosynthetic analysis of Magnesidin using tag molecular approach.

## Fourth Attempt to Synthesize Magnesidin
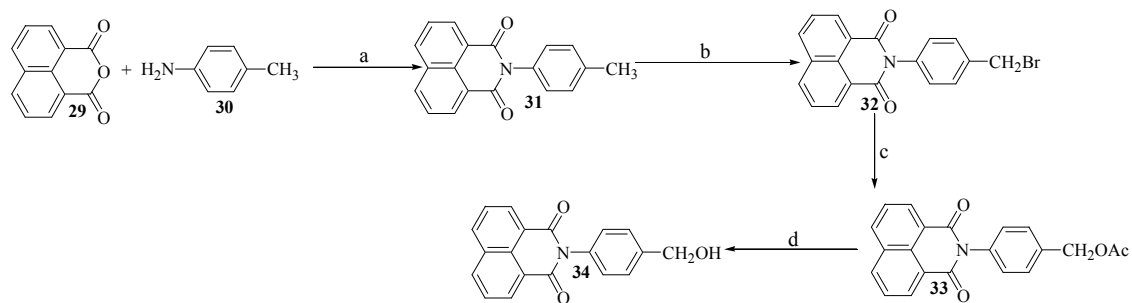


**a**) NEt$_3$,Toluene; 80%  **b**) benzoyl peroxide, CHCl$_3$,NBS; 85%  **c**) DMSO/NaOAc; 95%  **d**) MeOH/DCM, NaOH; 99%

Scheme 5.13.  Synthesis of tag molecule.

The synthesis of the tag molecule was started by condensation of commercially

available, inexpensive  Benzo[de]isochromene-1,3-dione (**29**) and *p*-amino toluene as

starting materials.  The product had physical properties that easily set it apart from both

starting materials.  In our spectral analyses, there were clear differences in the chemical

shift for the benzylic proton signals of the condensation product and the starting *p*-amino

toluene. The ratios of these signals were used to determine the percentage of reaction

conversion.  Through our experimentation, we determined that the best solvent for this

reaction is toluene and pyridine.  With toluene as solvent a non nucelophilic base such as

triethyl amine is required while with pyridine use of another basic reagent is precluded.

Compound **31** (Scheme 5.13) was brominated under benzylic bromination conditions

using NBS.  The dibromination product is a very common side product of this reaction.

The ratio of the mono and dibenzylation product and the extent of reaction strongly

varied with the nature of the solvent and temperature applied. The extent of formation of

dibenzylation product was dramatically reduced by using flash light instead of high

temperature conditions.  Furthermore, the cost of reaction is reduced by replacing the

carbon tetrachloride generally used for allylic bromination reactions, with chloroform.

Our attempts for direct hydrolysis of the **32**(Scheme 5.13) to alcohol under mild

conditions did not yield fruitful results.  It was important to avoid harsh conditions to

prevent hydrolysis of amide linkage.  Consequently, we avoided the direct hydrolysis of

the benzyl bromide **32,** and tried to circumvent the problem by generating an ester at the

benzylic position(compound **33**), which was hydrolyzed subsequently to yield alcohol **34.**

After thoroughly exploring different reaction conditions, acetylation has been achieved

with a simple sonication at room temperature with sodium acetate in dimethylsulfoxide as

solvent. Acetate **33** was hydrolyzed exclusively to obtain required alcohol (the tag molecule) in quantitative yield, with no side reactions. Thus we successfully achieved the synthesis of tag molecule.

Attaching the tag molecule synthesized to oxazolone ring, followed by ring opening of oxazolone with a β-keto ester should make the required tetramic acid Magnesidin. Since we already developed general methods to make oxazolone ring and also to perform the ring opening of oxazolone ring with a nucleophiles, we believe the synthetic attempts to make Magnesidin will be successful by this route.

## Conclusion

The synthesis of the Magnesidin is a very challenging endeavor. The structure of the molecule is very pH sensitive and exists in multiple tautomeric forms and it is almost impossible to lock into one particular form. This coupled with its very small size makes the synthesis complicated. Through this work, it has been determined that the most efficient approach to the synthesis of the Magnesidin is via the ring opening of corresponding oxazolone ring with an anion generated from active methylene of a β-ketoester. To this end, a simple one-pot synthesis to make oxazolone rings has been developed. The method can be used as a general synthetic procedure for making the synthons of a variety of tetramic acids. Definitive characterization of one of oxazolone rings, 4-ethylidene-2-phenyl-4H-oxazol-5-one was achieved by X-ray crystallography. Further the synthetic reaction developed was stereoselective with the major isomer formed during condensations being Z-isomer. The method is industrially viable method with readily available starting materials. Furthermore, an efficient two-step synthetic

procedure for the preparation of numerous variations of *N*-protected α,β-unsaturated α-amino acids and their corresponding esters from *N*-protected glycine and either aliphatic or aromatic aldehydes was developed. The reaction involved cyclization of the *N*-protected glycine into oxazolone, condensation with the aldehyde, and ring opening with a base.  All the products mentioned are produced in good to excellent yields.

Additionally, it was deemed that in order to successfully synthesize Magnesidin from an oxazolone ring, the major challenge is anchoring the volatile oxazolone ring to the solution phase.  Theoretically this problem can be overcome by covalently attaching the volatile oxazolone ring to a solid support.  However, considering many limitations this method offers such as low loading capacity, limited solubility during the reaction processes etc., we developed an alternative methodology, a tag molecule, which serves as a synthetic equivalent of a resin/solid support with the aim of restoring homogeneous reaction conditions while at the same time anchoring the volatile molecule in the solution phase.  In addition to above mentioned advantages, the tag molecule imparts crystalinity to the attached molecule, which intern simplifies the purification procedures.


## Experimental Section

All NMR reaction following experiments were performed on 500 MHz UNITY500 Varian NMR instrument by taking samples from the reaction mixture containing 0.02 mol *N*acetylglycine, 20 mL acetic anhydride, 3 g sodium acetate, and 0.01 mol of 1,4-benzenedicarbaldehyde. The NMR sample was prepared as DMSO-d6 solution. All DMSO-d6 samples were clear solutions. Elemental analysis was performed by Atlantic Microlab, Inc. Melting points were taken on an Electrothermal IA 9000 Digital Melting

Point Apparatus and are uncorrected.  X-ray structure determination was performed on Bruker SMART 1KCCD automated diffractometer. Crystals of compound 7g were obtained by slow crystallization from a diluted methanol solution of 7g.

**L-Threonine Methyl Ester Hydrochloride (9)**

Methanol(250 mL) was cooled at 0 °C, and thionyl chloride(18 mL; 0.25 mol) was added dropwise.  To the resultant solution of HCl in methanol was added L-threonine  (30 g; 0.25 mL), and the reaction mixture was heated under reflux for 1h.  The solvent was removed in vacuo, another 250mL of a solution of HCl in methanol, prepared in the same manner as before, was added, and the reaction mixture was heated under reflux for another 1h.  The solvent was removed in vacuo to yield the title compound (42g; 98%) as white solid:  MP160-163 °C. $^1$H-NMR (300 MHz, CD$_3$OD) δ 4.27( 1H, dq, $J$=6.5; 4.2 Hz), 3.92 (1 H, d, $J$=4.2 Hz), 3.84 (3H, s), 1.31(3H, d, $J$=6.5Hz); $^{13}$C-NMR (75 MHz, CD$_3$OD) δ 169.9, 66.3, 59.9, 53.7, 20.5.

***N*-Benzoyl L-Threonine Methyl Ester (10)**

A crude L-threonine methyl ester (39.49 g, 0.3 mol) was dissolved in 300 mL of methanol and then transferred to the round bottom flask equipped with magnetic stirrer and cooling ice bath. A solution was chilled to 0°C and a total of 64.1 g (0.63 mol, 3 eq.) was added to the flask. A total of 30.9 g (0.21 mol) of benzoyl chloride was added to the solution and resulting mixture stirred at 0°C for 1 hour. After this period of time the solvent was removed on rotavap to yield a viscous semi-solid. A total of 300 mL of cold

water was added to the residue and organic material was extracted with ethyl acetate (2.times.300 mL). The organic phase was separated, washed with brine (200 mL) and dried over sodium sulfate. The solvent was removed on rotavap to yield 52.02 g (98%) of clear yellow oil. Obtained oil was crystallized from 200 mL of ether to give a white crystalline solid, which was filtered off, washed with hexane (2 x 100 mL) and dried under suction. An additional drying in a vacuum desiccator yielded 42.35 g (84.7% yield) of clear syrup. ^{1}H-NMR (300 MHz, CDCl$_3$) δ: 7.82 (2H, d, $J$=7.3 Hz), 7.43 (1H, t, $J$=7.3 Hz), 7.40 (2H, t, $J$=7.3 Hz), 7.2 (1H, bd, $J_1$=9.0 Hz), 4.78 (1H, dd, $J$=2.4 Hz, $J$=8.7 Hz), 4.42 (1H, dq, $J$=2.6 Hz, $J$=6.3 Hz), 3.42 (1 H, s), 3.78 (3H, s),1.25 ( 3H, d, $J$=6.3 Hz); ^{13}C-NMR (75 MHz, CDCl$_3$) δ: 171.2, 167.1, 133.3, 131.4, 128.6, 127.5, 66.3, 63.1, 51.1, 17.4.


### *N*-Benzoyl L-Threonine (11)

*N*-Benzoyl Threonine Methyl Ester (10g, 45 mmol) was dissolved in 110 mL of 10:1 mixture of acetic acid and 30% hydrochloric acid and refluxed for 3 hours with stirring. After the methyl group completely disappeared in the proton NMR, the reaction was stopped. The solvent was removed *in vacuo* and washed with water (100 mL x 3). The residue was coevoporated with 100 mL of toluene (x 3). The toluene suspension was filtered under vacuum to give the 9.12 g of pure product as a white precipitate (95% yield). Mp 146°C, ^{1}H-NMR (300 MHz, DMSO-d6) δ 8.07(2H, d, $J$=8), 7.89(2H, d, $J$=8), 7.48-7.59(2H, m), 4.47(1H, dd, $J$=9,3), 4.28-4.21(1H, m), 1.17(3H, d, $J$=6); ^{13}C-NMR (300 MHz, DMSO-d6) 166.4, 171.8, 133.6, 131.2, 128, 127.1, 66.4, 58.6, 20.04.

*General Procedure for Preparation of Oxazolone (15-I):*

**Preparation of 2-Methyl-4-(3,4,5-trimethoxybenzylidene)-4H-oxazol-5-one (15-I-c)**

A suspension of *N*-acetylglycine (5.9 g; 0.05 mol), sodium acetate (4.1 g; 0.05 mol), and

acetic anhydride (30 mL) was stirred at room temperature for 30 min. Into the white

suspension, 3,4,5-trimethoxybenzaldehyde (8.3 g; 0.05 mol) was added. The resulting

suspension was stirred at room temperature for 1 h and then at 60°C for 5 h. The reaction

mixture became a brown solution that upon cooling to room temperature again became a

suspension. This suspension was mixed with water (1 L) and stirred at room temperature

for a half an hour. The insoluble material was separated by filtration, washed with water

(3 x 30 mL), and recrystallized from methanol (250 mL).

*General Procedure for Preparation of (15-II):*

**Preparation of Methyl (Z)-2-Benzoylaminobut-2-enoate (15-II-g)**

The methanol (20 mL) solution of 3g (1.87 g; 0.01 mol) and sodium methoxide in

methanol (2 mL of 25% $CH_3ONa$ in $CH_3OH$) was stirred at room temperature for 15 min.

Solvent was evaporated, and the residue was partitioned between 10% aqueous

ammonium chloride (100 mL) and methylene chloride (150 mL). The organic layer was

washed with water (3 x 100 mL), dried over anhydrous sodium sulfate, and evaporated to

an oily residue. The oily residue was dissolved in hot petroleum ether (500 mL) from

which white needles of product crystallized.

*General Procedure for Preparation of (15-III):*

**Preparation of 2-Acetylamino-3-phenylacrylic Acid (15-III-b)**

Into aqueous (200 mL) sodium hydroxide (4.0 g; 0.1 mol), a solution of methanol (200 mL) and 4-benzylidene-2-methyl-4H-oxazol-5-one (18.7g; 0.1 mol) was added. The reaction mixture was stirred at room temperature for 30 min, followed by methanol evaporation at reduced pressure. The remaining clear water solution was acidified with 10% hydrochloric acid to pH 3 and left at room temperature overnight. The formed crystalline product was separated by filtration, washed with cold water (3 x 50 mL) and dried at 110°C for 30 min to afford pure product.

**2-Phenyl-4-(2,4,6-trimethoxy-benzylidene)-4H-oxazol-5-one (15-Ia)**

Yield: 92%, product was obtained as orange amorphous solid.  MP:    170.5-171.5°C. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.07(2H, d, $J$ = 8 Hz),7.56-7.46(4H, m), 6.16(2H, s), 3.88(6H, s), 3.86(3H, s); $^{13}$C NMR (100 MHz, DMSO-d6) δ 187.9, 164.5, 160.9, 132.6, 128.9, 127.9, 126.6, 126.4, 105.5, 90.9, 55.9.

**2-Benzoylamino-3-(2,4,6-trimethoxy-phenyl)-acrylic acid methyl ester (15-IIa)**

Yield: 80%,  product  was obtained as orange crystalline solid.  MP:  135-136.5   °C. $^1$H NMR (400 MHz, CDCl$_3$) δ10.30(1H, s), 8.54(1H, s), 7.77 (2H, d, $J$ = 8 Hz); 7.47-7.28(4H, m), 6.12(2H, s), 3.81(6H, s), 3.76(3H, s); $^{13}$C NMR (100 MHz, DMSO-d6) δ 187.6, 165.6, 162.4, 158.5, 134.1, 131.7, 128.5, 127.2, 126.3, 121.4, 105.0, 91.1, 56.2.

**2-Benzoylamino-3-(2,4,6-trimethoxy-phenyl)-acrylic acid(15-IIIa)**

Yield: 87%, product was obtained as red crystalline solid.  MP:  138-139°C.

$^1$H NMR (400 MHz, CDCl$_3$) δ 10.25(1H, s), 8.57(1H, s), 7.74(2H, d, J=7.2 Hz), 7.43-7.33(4H, m), 6.08(2H, s), 3.77(6H, s), 3.71(3H, s); $^{13}$C NMR (100 MHz, DMSO-d6) δ 187.5, 165.5, 164.8, 163.9, 162.3, 158.3, 133.8, 131.5, 129.7, 128.4, 128.0, 127.0, 126.1, 121.5, 104.6, 90.9, 90.1, 56.0, 55.2, 52.1.

**4-Benzylidene-2-methyl-4H-oxazol-5-one (15-Ib)**

Yield:   90% product was obtained as off white amorphous solid.   MP:  148-150°C.  $^1$H NMR (400 MHz, CDCl$_3$) δ 7.32-8.20(5H, m), 7.16(1H, s), 2.45 (3H, s); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 133.3, 132.4, 131.7, 131.3, 129.1, 55.9, 15.8.

**Methyl (Z)-2-(N-acetylamino)-3-phenylpropenoate (15-IIb)**

Yield:   90% product was obtained as white amorphous solid.  MP:  121-122°C.

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.63-7.21(6H, m) , 7.12(1H, s), 3.84(3H, s), 2.11(3H, s); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 168.8, 165.7, 133.7, 132.3, 129.4, 129.6, 128.6, 124.2, 52.7, 23.3.

**2-Acetylamino-3-phenylacrylic Acid (15-IIIb)**

Yield: 93%, product was obtained as white amorphous solid.  MP:  190°C.

$^1$H NMR (DMSO-d6, 300MHz) δ 9.5 (1H, s, NH), 7.62 (2H, d, J = 7.2 Hz), 7.41 (2H, t, J = 6.0 Hz), 7.35 (1H, t, J = 7.2 Hz). 7.24 (1H, s), and 1.91 (3H, s); $^{13}$C NMR (DMSO-d6, 100 MHz) d 169.4, 166.5, 133.8, 131.2, 129.8, 129.2,128.6, 127.5, and 22.6 ppm. Anal. calcd. for C$_{11}$H$_{11}$NO$_3$ (MW 205.21): C, 64.38; H, 5.40; N, 6.83. Found: C, 64.26; H, 5.49; N, 6.89.

**2-Phenyl-4-thiophen-3-ylmethylene-4H-oxazol-5-one (15-Ic)**

Yield: 92%, product was obtained as yellow solid. MP: 176-177°C;

$^1$H NMR (400 MHz, DMSO-d6) δ 8.20-8.05(2H, m), (1H, d, $J$ = 4.5 Hz), 7.6(1H, d, $J$ =4 Hz), 7.55-7.45(3H, m), 7.44(1H, s), 7.12(1H, s); $^{13}$C NMR (100 MHz, DMSO-d6) δ 171.9, 160.0, 138.9, 138.8, 135.6, 135.4, 133.2, 129.8, 128.8, 128.5, 128.1, 120.3.

**2-Benzoylamino-3-thiophen-3-yl-acrylic acid methyl ester (15-IIc)**

Yield: 82%, product was obtained as pale yellow solid. MP: 187-188°C;

$^1$H NMR (400 MHz, DMSO-d6) δ 9.84(1H, s), 8.2-7.9(2H, m), 7.93(1H, s), 7.8-7.5(5H, m), 7.2-7.1(1H, m), 3.71(3H, s); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 165.2, 164.9, 133.1, 132.8, 132.0, 130.4, 129.9, 128.3, 127.1, 126.9, 127.1, 119.0, 114.8, 48.9.

**2-Benzoylamino-3-thiophen-3-yl-acrylic acid (15-IIIc)**

Yield: 96%, product was obtained as pale yellow solid. MP: 237-238°C; $^1$H NMR (400 MHz, DMSO-d6) δ 9.7(1H, s), 8.1-7.9(2H, m), 7.88(1H, s), 7.7-7.4(5H, m), 7.15-6.95(1H, m); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 171.2, 169.3, 135.2, 133.1, 132.2, 131.4, 129.6, 129.4, 129.1, 128.1, 127.0, 126.3, 121.1, 120.1.

**2-Methyl-4-thiophe*n*-3-ylmethylene-4H-oxazol-5-one (15-Id)**

Yield: 93%, product was obtained as yellow needles. MP: 132-133 °C;

$^1$H NMR (400 MHz, DMSO-d6) δ 7.65(1H, d, $J$ =4.5Hz), 7.55(1H, d, $J$ = 4 Hz), 7.35(1H, s), 7.12(1H, m), 2.4(3H, s); $^{13}$C NMR (100 MHz, CD$_3$OD) δ171.8, 168.9, 136.6, 133.0, 129.1, 128.4, 119.2, 19.1.

**2-Acetylamino-3-thiophen-3-yl-acrylic acid methyl ester (15-IId)**

Yield: 90%, product was obtained as yellow powder. MP: 118-119°C; ^{1}H NMR (400

MHz, DMSO-d6) δ 9.35(1H, s), 7.81(1H, D, *J*=5 Hz), 7.77(1H, s), 7.45(1H, d, *J* =4 Hz),

7.1-7.2(1H, m), 3.71(3H, s), 2.05(3H, s); ^{13}C NMR (100 MHz, CD$_3$OD) δ168.4, 163.1,

136.0, 134.9, 123.1, 122.9, 119.1, 116.5, 52.1, 20.9.


**2-Acetylamino-3-thiophen-3-yl-acrylic acid (15-IIId)**

Yield: 88%, product was obtained as yellow crystalline solid. MP: 230-231 °C; [1]H

NMR (400 MHz, DMSO-d6) δ 9.24(1H, s), 7.76(1H, s), 7.66(1H, d, *J*=4.5Hz), 7.43(1H,

d, *J*=4.5Hz), 7.07(1H, m), 2.02(3H, s); ^{13}C NMR (100 MHz, CD$_3$OD) δ 169.9, 169.1,

132.8, 132.1, 129.1, 128.0, 120.3, 120.1, 19.9.


**4-Naphthalen-2-ylmethylene-2-phenyl-4H-oxazol-5-one (15-Ie)**

Yield: 80%, product was obtained as white crystalline solid. MP: 159-160°C. ^{1}H NMR

(300 MHz, CDCl$_3$) δ 8.404 (s, 1H); 8.38-8.34 (m, 1H); 7.94-7.83 (m, 3H); 7.59- 7.50 (m,

2H); 7.307 (s, 1H); 2.449 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 168.0; 166.1; 134.5;

133.9; 133.3; 132.8; 131.6; 131.1; 129.3; 128.7; 128.2; 127.9; 127.8; 126.8; 15.8.


**Methyl 2-Acetamido-3-(2-naphthyl) propenoate (15-IIe)**

Yield: 80%, product was obtained as white crystalline solid. MP: 153-154°C.

$^1$H NMR (DMSO-*d*6) δ 9.773 (s, 1H), 8.170 (s, 1H), 7.95-7.91 (m, 3H), 7.779 (d, 1H, *J*= 8.79 Hz), 7.58-7.55 (m, 2H); 7.335 (s, 1H); 3.738 (s, 3H), 2.051 (s, 3H),$^{13}$C NMR (100 MHz, DMSO-*d*6) δ 169.5, 165.6, 133.0, 131.1, 130.9, 130.1, 128.4, 128.0, 127.5, 127.1, 126.9, 126.6, 126.4, 52.2, 22.5.

**2-Acetylamino-3-naphthalen-2-yl-acrylic acid (15-IIIe)**

Yield: 92%, product was obtained as white amorphous solid. MP: 184-186°C.
$^1$H NMR (100 MHz, DMSO-*d*6) δ 9.83(1H,s), 8.05-7.51(8H, m); 2.21(3H, s); $^{13}$C NMR (100 MHz, DMSO-*d*6) δ 166.5, 165.9, 134.5, 132.9, 133.1, 131.7, 131.0, 130.8, 129.9, 126.5, 126.1, 125.4, 124.1, 18.3.

**2-Phenyl-4-furon-3-ylmethylene-4H-oxazol-5-one (15-If)**

Yield: 90%, product was obtained as a white solid. MP: 170-171°C; $^1$H NMR (400 MHz, CDCl$_3$) δ 8.16 (d, 2H), 7.69-7.51 (5H, m), 7.19 (1H, s), 6.66(1H, dd, *J*1=0.4, *J*2=0.4); $^{13}$C NMR (CDCl$_3$, 100 MHz) d 167.3, 163.2, 150.7, 146.9, 133.4, 130.6, 129.1, 128.5, 125.8, 120.3, 118.5, 115.5, 114.0.

**2-Benzoylamino-3-furon-3-yl-acrylic acid methyl ester (15-IIf)**

Yield: 89%, MP: 136-137 °C. $^1$H NMR (CDCl$_3$, 300 MHz) d = 8.28(1H, s), 7.95-7.85(2H, m), 7.6-7.46(4H, m), 7.08(1H, s), 6.58(3H, d, *J* =3.6 Hz), 6.47(1H, dd, *J* =3.4Hz, *J* =1.6 Hz), 3.84(3H, s); $^{13}$C NMR (CDCl$_3$, 100 MHz) d 165.6, 165.4, 123.0, 116.2, 115.4, 133.5, 132.2, 128.7, 127.5, 149.9, 144.4, 112.3, 52.6.

**2-Benzoylamino-3-furon-3-yl-acrylic acid (15-IIIf)**

Yield: 96%, MP: 231-232 °C. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.74 (1H, s), 9.84 (1H, s), 8.00 (2H, d, $J$ = 7.5 Hz), 7.83 (1H, s), 7.56 (3H, m), 7.28 (1H, s), 6.83 (1H, d, $J$ = 3.4), 6.61 (1H, d, $J$ = 3.4); ^{13}C NMR (100 MHz, DMSO-d6) δ 165.7, 149.2, 145.2, 133.5, 131.6, 128.3, 127.5, 124.2, 120.6, 115.3, 112.4, 75.0.

**4-Benzylidene-2-methyl-4H-oxazol-5-one(15-Ig)**

Yield: 95%, product obtained as yellow needles. MP: 148-150°C; ^{1}H NMR (CDCl₃, 300 MHz) δ 7.32-8.20(5H, m), 7.16(1H, s), 2.45(3H, s); ^{13}C NMR (CDCl₃, 100 MHz) δ = 166.1, 137.3, 135.1, 133.3, 129.0, 128.2, 125.8, 14.8.

**2-Acetylamino-3-phenyl-acrylic acid methyl ester(15-IIg)**

Yield: 90%, product was obtained as white crystalline solid. MP: 79-80°C.

^{1}H NMR (CDCl₃, 400 MHz) δ 7.87 (2H, d, $J$ = 7.2 Hz), 7.63 (1H, broad s), 7.54 (1H, t, $J$ = 7.2 Hz), 7.46 (2H, t, $J$ = 7.2 Hz), 6.89 (1H, q, $J$ = 7.6 Hz), 3.78 (3H, s), and 1.84 ppm (2H, d, $J$ = 7.6 Hz). ^{13}C NMR (CDCl₃, 100 MHz) d 165.4, 165.1 133.9, 133.8, 132.0, 128.6, 127.4, 126.0, 52.4, 15.0.

**2-Benzoylamino-but-2-enoic acid (15-IIIg)**

Yield: 91%, white amorphous solid. MP: 135.5-137.5°C;

^{1}H NMR (400 MHz, DMSO-d6) δ 9.54(1H, s), 8.21(2H, d, $J$ =8.4 Hz), 7.98-7.44 (3H, m), 6.68(1H, q, $J$ =6.8 Hz), 1.72(3H, d, $J$ =6.8 Hz). ^{13}C NMR (100 MHz, DMSO-d6) δ 171.9, 165.7, 133.9, 133.8, 133.0, 131.6, 131.5, 128.3, 127.7, 127.6, 13.6.

**4-Benzylidene-2-naphtyl-4H-oxazol-5-one (15-Ih)**

Yield: 84%, product obtained as yellow needles. MP: 164-165°C.

^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.9(2H, d, *J* =8.4 Hz), 8.43(2H, d, *J* =8.4 Hz), 8.17-7.66 (9H, M); ^{13}C NMR (100 MHz, DMSO-*d*6) δ 201.1, 167.9, 164.2, 134.0, 133.8, 133.6, 132.6, 132.2, 132.1, 129.4, 129.3, 129.1, 128.6, 127.6, 127.1, 126.4, 125.9, 125.7, 122.9.

**2-Benzoylamino-3-naphtyl-acrylic acid methyl ester (15-IIh)**

Yield: 87%, product obtained as off white crystalline solid. MP: 118-118.5°C.

^{1}H NMR (400 MHz, CDCl₃) δ 8.78(1H, s), 8.04-7.37 (13H, m), 3.94(3H, s); ^{13}C NMR (100 MHz, CDCl₃) δ 165.9, 165.7, 133.8, 133.6, 132.3, 131.4, 131.1, 129.7, 129.0, 128.9, 128.8, 128.5, 128.2, 127.9, 127.6, 127.2, 127.0, 126.5, 125.2, 124.4, 53.0.

**2-Benzoylamino-3-naphtyl-acrylic acid (15-IIIh)**

Yield: 92%, product obtained as off-white crystalline solid. MP: 213.5-215°C. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 9.8(1H,S), 8.03-7.45(13H, m); ^{13}C NMR (100 MHz, DMSO-*d*6) d 166.7, 166.1, 134.8, 133.5, 133.1, 131.7, 131.0, 130.8, 129.9, 129.7, 128.5, 128.3, 127.6, 126.6, 126.5, 126.1, 125.4, 124.1.

**4-Benzylidene-2-phenyl-4H-oxazol-5-one(15-Ii)**

Yield: 90%, MP 160-161°C.

$^1$H NMR (300 MHz, CDCl$_3$) δ 8.12-7.44(10H, m), 7.24(1H, s); $^{13}$C NMR (100 MHz, DMSO-d6) δ 172.8, 161.1, 141.0, 140.6, 133.2, 132.9, 132.5, 130.3, 129.8, 128.9, 128.1, 126.2, 22.1.

**Methyl (Z)-2-(N-benzoylamino)-3-phenylpropenoate (15-IIi)**

Yield: 90%, MP 140.5-141.5°C.

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.88-7.82, 7.56-7.41 and 7.34-7.28(10H, m), 3.83(3H, s); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 165.8, 165.6, 133.8, 133.5, 132.1, 131.8, 129.6, 129.4, 128.7, 128.6, 127.4, 124.2, 52.7.

**2-Benzoylamino-3-phenyl-acrylic acid (15-IIIi)**

Yield: 96%, MP 233-235°C.

$^1$H NMR (300 MHz, DMSO-d6) δ 9.24(1H, s), 8.10-7.95, 7.75-7.25(12H, 2m); $^{13}$C NMR (100 MHz, DMSO-d6) δ 167.5, 162.4, 133.1, 132.4, 130.4, 128.3, 127.56, 127.50, 127.2, 127.3, 126.9, 126.0, 122.4, 119.3.

**2-Phenyl-4-(3,4,5-trimethoxy-benzylidene)-4H-oxazol-5-one (15-Ij)**

Yield: 80%, product obtained as Pale yellow crystalline solid. MP 152-153°C. $^1$H NMR (300 MHz, CD$_3$OD) δ 7.54 (2H, s), 7.09 (1H, s), 3.90 (6H, s), 3.85 (3H, s), 2.41 (3H, s); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 176.2, 161.7, 153.4, 152.9, 138.3, 133.4, 132.6, 131.9, 129.2, 129.1, 128.3, 127.3, 125.8, 125.6, 115.5, 110.0, 108.1, 95.6, 75.7, 73.3, 61.3, 61.0, 57.2, 56.4, 56.3.

**2-Benzoylamino-3-(3,4,5-trimethoxy-phenyl)-acrylic acid (15-IIj)**

Yield: 80%, product obtained as white crystalline solid, MP: 202-203°C;

^{1}H NMR (300 MHz, CD$_3$OD) δ 7.47 (1H, s), 6.95 (2H, s), 3.86 (6 H, s), 3.80 (3H, s), 2.13 (3H, s); ^{13}C NMR (CDCl3, 100 MHz) d, 165.9, 165.5, 152.6, 138.7, 133.9, 133.1, 132.0, 128.7, 128.5, 127.7, 127.5, 125.5, 107.6, 105.4, 60.1, 55.8, 55.6, 52.2, 51.9.

**2-Benzoylamino-3-(3,4,5-trimethoxy-phenyl)-acrylic acid (15-IIIj)**

Yield: 93%, product obtained as pale yellow amorphous solid. MP: Decomposes beyond 200°C before melting. ^{1}H NMR (300 MHz, CD$_3$OD) δ 9.93(1H, s), 8.0(2H, d, *J*=1.8), 7.59-7.44(3H, m), 7.05(1H, s), 6.69(1H, s), 3.65(3H, s), 3.64(6H, s); ^{13}C NMR (CDCl$_3$, 100 MHz) d, 154.1, 152.6, 128.5, 127.5, 114.9, 107.5, 60.0, 55.6.

**4-(4-Dimethylamino-benzylidene)-2-phenyl-4H-oxazol-5-one (15-Ik)**

Yield: 90%, product obtained as bright red crystalline solid; MP: 213-215 °C. ^{1}H NMR (CDCl$_3$, 400 MHz) δ, 8.16-8.13(3H, m), 7.56-7.50(5H, m), 7.21(1H, s), 6.75(2H, d, *J* =8.8 Hz), 3.11(6H, s); ^{13}C NMR (CDCl$_3$, 100 MHz) δ, 168.8, 162.0, 160.8, 152.4, 135.0, 135.0, 133.6, 132.5, 129.2, 129.0, 128.5, 128.0, 126.5, 121.9, 121.3, 112.0, 40.2.

**2-Benzoylamino-3-(4-dimethylamino-phenyl)-acrylic acid (15-IIk)**

Yield: 90%, product obtained as red crystalline solid. MP: 180-181°C.

^{1}H NMR (400 MHz, DMSO-d6) δ 9.86 (1H, s), 8.00 (2H, d, *J* = 7.2 Hz), 7.54 (m, 5H), 7.40(1H, s), 6.69 (2H, d, *J* = 8.9 Hz), 3.69 (3H, m), 2.93 (6H, s); ^{13}C NMR (100 MHz,

CDCl$_3$) 166.6, 151.3, 135.4, 134.5, 134.2, 132.1, 131.3, 129.0, 128.9, 127.7, 121.3, 119.3, 111.8, 95.6, 70.4, 52.6, 40.1

**2-Benzoylamino-3-(4-dimethylamino-phenyl)-acrylic acid methyl ester (15-IIIk)**

Yield: 91%, product obtained as red crystalline solid. MP: 216-218°C.

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.144(2H, m), 7.55-7.48(5H, m), 7.21(1H, s), 6.74(2H, d, $J$ = 8.8 Hz), 3.105(6H, s); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 168.7, 160.7, 152.4, 135.0, 133.6, 132.4, 128.9, 128.4, 127.9, 127.3, 126.5, 121.9, 111.9, 40.27, 40.24.

**Ethoxycarbonylamino-acetic acid (17)**

To a stirred solution of glycine (10.0 g, 0.13 mol)) in 2 M NaOH (1.0 equiv.) at 0°C were added simultaneously ethyl chloroformate (14.45g, 0.13 mol) and 2 M NaOH (1.0 equiv.) dropwise. The mixture was stirred at 0°C for 3 h then washed with ether (3x 20mL). The aqueous phase was acidified with 2 M HCl and extracted with ethyl acetate (3 × 50 mL). The combined organic phase was dried and the solvent evaporated to furnish the product as colorless solid in 70% yield. M.p. 67-69°C. $^1$H-NMR (400 MHz, DMSO-d6) 7.34(1H, t, $J$ =6 Hz), 4.05-3.97( 2H, m), 3.62(2H, d, $J$ =6.4 Hz), 1.15(3H, t, $J$ =6.8 Hz); $^{13}$C-NMR (100 MHz, DMSO-d6) δ 174.4, 173.7, 157.2, 43.2, 42.6, 62.4, 61.8, 14.6.

**3-Hydroxy-2-(3-oxo-decanoylamino)-butyric acid methyl ester (19)**

Threonine methyl ester (1g, 6 mmol) and 3-Oxo-decanoic acid were added to 40 mL of dichloromethane and stirred at room temperature. To this mixture dicyclohexylcarbodiimide (1.2g, 6 mmol) and sodium bicarbonate (0.5g, 6 mmol) were

added and stirring is continued. The reaction mixture looked turbid white with undissolved material. The reaction was stirred overnight at room temperature until the starting material completely disappeared under TLC. The mixture was then cooled to 0°C and filtered through filter paper and the residue was discarded. The filtrate was concentrated and filtered through a short silica gel column. The column is washed thoroughly with a mixture of 3:1 dichloromethane, ethyl acetate solvent system. The filtrate was left in an uncovered container overnight. Pure product crystallized out as white longitudinal crystals (1.4g, 80% yield) m.p. 210.3-211.5°C.

^{1}H-NMR (400 MHz, CDCl$_3$) δ: 6.29(1H, d, $J$=16 Hz), 4.63(1H, dd, $J$=3.2 Hz), 4.35(1H, dq, $J$=3.2 Hz), 3.77(3H, s), 2.28(2H, t, $J$=10 Hz), 1.62-1.68(2H, m), 1.45-1.21(10H, m), 0.86-0.88(3H, m); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 205.4, 172.7, 171.3, 66.2, 62.2, 51.7, 44.6, 41.9, 32.6, 29.8, 23.2, 23.5, 17.6, 13.9.

### 3-Acetoxy-2-(3-oxo-decanoylamino)-butyric acid methyl ester (20)

A mixture of 3-Hydroxy-2-(3-oxo-decanoylamino)-butyric acid methyl ester (1.4g, 4.6 mmol) and sodium acetate (0.95g, 11.6 mmol) was dissolved in 10 mL of acetic anhydride and refluxed overnight. The solvent is removed under vacuum, and then residue is washed with water and extracted into dichloromethane and concentrated to yield pure **20** as a colorless oil (1.56g, 98%) yield.

^{1}H-NMR (400 MHz, CDCl$_3$) δ: 6.33(1H, d, $J$=16 Hz), 5.43(1H, dd, $J$=3.2 Hz), 4.95(1H, dq, $J$=3.2 Hz), 3.73(3H, s), 2.28(2H, t, $J$=10 Hz), 2.07(3H, s),1.61-1.65(2H, m), 1.45-1.22(10H, m), 0.86-0.87(3H, m); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 205.4, 172.8, 171.2, 70.5, 62.2, 56.7, 44.6, 41.9, 32.6, 29.8, 23.2, 17.4, 17.8, 13.9.

**2-Diacetylamino-but-2-enoic acid methyl ester (24)**

A suspension of L-threonine methyl ester hydrochloride (34g; 0.2 mol), anhydrous

sodium acetate( 100g; 1.2 mol) in acetic anhydride (200 mL) was refluxed with stirring

for four hours.  The reaction mixture is continued to stir at room temperature for

additional five hours.  Solid material was separated by filtration and filtrate was

concentrated under vacuum and bath temperature of 80°C.  After distillation was stopped,

liquid residues was diluted with methanol (150 mL) and again concentrated at 80°C

under vacuum aspirator.  This procedure was repeated three more times.  The oily residue

was dissolved in methylene chloride (200mL) and washed with saturated $NaHCO_3$

(4x200mL).  Organic layer was dried under anhydrous sodium sulfate and solvent was

evaporated to yield pure product (39 g, 97.5%) yield as colorless oil.

$^1$H-NMR (300 MHz, CDCl$_3$) δ 7.19(1H, q, $J$ = 7.2 Hz), 3.78(3H, s), 2.32(6H, s), 1.77(3H,

d, $J$=7.2 Hz);$^{13}$C-NMR (300 MHz,CDCl$_3$) δ 172.26, 163.97, 140.84, 140.67, 131.08,

52.86, 52.75, 25.90, 25.86, 13.81, 13.73.


**Methyl 2-acetylamino-but-2-enoate (25)**

A solution of methyl 2(diacetylamino)but-2-enoate (14.45g, 72.54) and triethylamine (1.0

g, 10 mmol) in methanol (200 mL) was heated at reflux overnight.  The volatiles were

removed at reduced pressure to afford a yellow liquid (13.43 g)  which crystallized over a

course of several hours.  The product was titrated with hexane and dried in vacuum to

afford product (11.09 g; 97%) as an off-white solid, m.p. 53-54°C, of adequate purity.

The product was dissolved in ether (20 mL/g) and cooling the resulting solution to -25°C

to provide the product as snow white needles m.p. 59-60°C.  ^{1}H-NMR(300 MHz, CDCl$_3$)

δ 6.81(1H, q, *J* =8 Hz) , 3.76(s, 3H),   2.13(3H, s), 1.77(3H, d, *J* =8 Hz).  ^{13}C-NMR(300

MHz, CDCl$_3$) δ 168.75, 165.17, 134.27, 126.44, 52.27, 23.19, 14.52.


**2-Acetylamino-but-2-enoic acid (26)**

A solution of methyl 2-acetylamino-but-2-enoate  (1.57 g, 10 mmol) in methanol (25 mL)

was added to  sodium carbonate (2.8g, 25mmol) in water (25 mL).  The mixture was

refluxed for 2 hours and then brought to room temperature.  The reaction mixture was

acidified with 2N HCl to a pH 2 and was concentrated in vacuum aspirator and bath

temperature of 80°C.  After methanol was completely removed, the product precipitates

out as clear oil.  The precipitate was washed with ice cold 2N HCl.  The product was

dried first with a filter paper and then co-evaporated with toluene (50 mL x 4).  The

product obtained is pure in 97% yield as brownish oil.

^{1}H-NMR(300 MHz, CDCl$_3$)  δ 6.97(1H, q, *J* = 9.6 Hz), 3.40(3H, s), 1.82(3H, d, *J*=9.6

Hz);  ^{13}C-NMR(100 MHz, DMSO-d6) δ 168.32, 165.71, 131.13, 128.95, 22.50, 13.58.


**2-*p*-Tolyl-benzo[de]isoquinoline-1,3-dione (31):**

A suspension of 1,3-dioxo-1H,3H-benzo[de]isochromenium (0.76g, 7 mmol) and *p*-

amino toluene in 25 mL of pyridine was heated at 70°C for about 20 hours.  The excess

solvent was distilled off by increasing the temperature to reflux and setting up a collector

to collect the solvent.  The solvent was collected until enough solvent remained for

recrystallization.  The compound was obtained as white needles upon bringing the

reaction mixture to room temperature in 80% yield.  MP:  311°C.

$^1$H-NMR(400 MHz, CDCl$_3$) δ 8.64 (2H, dd, *J*=1.2, 1.2 Hz), 8.26(2H, dd, *J*=1.2, 1.2 Hz),

7.79(2H, dd, *J*=7.80, 7.80 Hz), 7.36(2H, d, *J*=8 Hz), 7.20(2H, d, *J*=8 Hz), 2.44(3H, s);

$^{13}$C-NMR(100 MHz, CDCl$_3$) δ 164.72, 138.85, 134.47, 132.94, 131.96, 131.83, 130.39,

128.76, 128.49, 127.24, 123.09, 55.91, 21.57.


**2-(4-Bromomethyl-phenyl)-benzo[de]isoquinoline-1,3-dione (32):**

To a solution of 2-*p*-Tolyl-benzo[de]isoquinoline-1,3-dione (**31**) (2.7g, 9.4 mmol) in 75

mL of chloroform was added *N*-bromo-succinimide (3.63, 20.3 mmol) and catalytic

amount of benzoyl peroxide (15 mg) at 0°C.  The solution was stirred under a flash light

for the reaction to occur.  The reaction was monitored by TLC for completion.  After

completion of the reaction the solvent was removed under vacuum and the residue was

subjected to column chromatography with 2:1 dichloromethane and hexane mixture in

85% yield as a white amorphous solid.  MP:  Starts decomposing before melting at

249.1°C. $^1$H-NMR(400 MHz, CDCl$_3$) δ 8.66(2H, dd, *J*=1.2, 1.2 Hz), 8.29(2H, dd, *J*=0.8,

0.8 Hz), 7.81(4H, t, *J*=7.6 Hz), 7.59(2H, d, *J*= 8.4 Hz), 3.31(2H, d, *J* = 8.4 Hz); $^{13}$C-

NMR(100 MHz, CDCl$_3$) δ 164.49, 138.30, 135.56, 134.61, 131.92, 130.32, 129.27,

127.27, 122.89, 32.84.


**Acetic acid 4-(1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-benzyl ester (33)**

A suspension of 2-(4-Bromomethyl-phenyl)-benzo[de]isoquinoline-1,3-dione (**32**) (3.5 g,

10 mmol)  and sodium acetate (1g, 12mmol)  in 10 mL of dimethylsulfoxide was

sonicated for 4 hours.   The reaction mixture was poured on to ice and left for 1 hour.

The product precipitated out as a white solid.  The solid was washed with a 100 mL water

(x 3) to give the crude product. The product was recrystallized from methanol to give

pure product in 95% yield.  MP:  Does not melt till 400°C.

$^1$H-NMR(400 MHz, CDCl$_3$) δ 8.65(2H, d, $J$ = 6.4 Hz), 8.27(2H, d, $J$ =7.6 Hz), 7.79(4H, t,

$J$=8 Hz), 7.54(2H, d, $J$ =8 Hz), 7.32(2H, d, 8 Hz), 5.19(2H, s), 2.16(3H, s).


## 4-(1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-benzyl alcohol (34)

To a solution of acetic acid 4-(1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-benzyl ester

(0.35g, 1mmol)  in a 15 mL of 1:1 methanol and dichloromethane was added powdered

NaOH (112 mg, 2.8 mmol) at 0°C and the reaction mixture was stirred for 2 hours and

the methanol was evaporated.  The residue was taken into 1N sodium bicarbonate

solution and the product was extracted into dichloromethane, which is dried under

MgSO$_4$.  The solvent is evaporated under vacuum to yield a yellowish solid. MP:  282°C.

$^1$H-NMR(400 MHz, CDCl$_3$) δ  8.66(2H, dd, $J$=0.8, 0.8 Hz), 8.29(2H, dd, $J$=1.2, 1.2 Hz),

7.81(4H, dd, $J$=7.6, 7.6 Hz), 7.57(2H, d, $J$=8.4 Hz), 7.33(2H, d, $J$=8.4 Hz), 4.81(2H, d,

$J$=6 Hz); $^{13}$C-NMR(100 MHz, CDCl$_3$) δ 163.25, 142.63, 134.49, 131.52, 130.81, 128.76,

127.90, 127.31, 126.90, 122.68, 75.15, 62.65, 55.25, 48.62.

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