Design and Synthesis of Tumor Targeted Agents from Biologically Active Natural Products (Neocarzinostatin, Xanthine)

A dissertation presented

by

Dong Ma

to

The Department of Chemistry and Chemical Biology

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the field of

Chemistry

Northeastern University

Boston, Massachusetts

July 2008
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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate School of Arts and Sciences of Northeastern University, July, 2008
ABSTRACT

Recently, the subject of tumor targeted therapy has drawn more and more attention as a result of increasing knowledge of biology and physiology. A tumor specific agent with high therapeutic index, good bioavailability and easy preparation is highly preferred. To achieve this goal, rational design and development of synthetic lead compounds based on active natural products remain as the most successful strategy in cancer chemotherapy. It serves as both the objective and the philosophy of this thesis and is demonstrated in the following two areas of research.

Bulges are unpaired nucleotides on one strand of the DNA double helix. They have been linked to many biomolecular processes including cancer. Agents with high specificity to bulges would be of considerable utility to the biological research community for the discovery of active anti-tumor entities. NCSi-gb is a natural metabolite of neocarzinostatin chromophore (NCS chrom), an anti-tumor antibiotic. It was found to bind specifically to bulged DNA at nanomolar concentration due to its unique wedge-shaped spirocyclic structure. Modeled on the molecule, mimics of NCSi-gb were rationally designed and a synthetic route was devised and executed to prepare them. These analogues showed nanomolar binding affinity and two-base bulge specificity.

In a second area of my research, tumor targeting is investigated using a prodrug approach based on the A2A receptor mediated tumor escape mechanism in T-cells. A prodrug of KW6002, an 8-substituted xanthine, was designed and synthesized. The proof-of-principle study validated the design for enzyme activated drug release. A major obstacle to accessibility of this class of compounds is the tedious two-step cyclization for
the formation of xanthine’s five-membered rings. Therefore, a novel one-pot methodology for the preparation of 8-substituted xanthines was developed. Using this methodology, the key intermediate for the preparation of the tumor targeting prodrug was more efficiently synthesized, along with a number of analogues.
This work described herein is dedicated to my wife, Weixiang Yang. From day one she has been standing by my side without hesitation, offering nothing but encouragement and support. She is, and will forever be, the love and inspiration of my life.
ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my research advisor, Professor Graham B. Jones, for his guidance, encouragement, and mentoring during the course of this work. Professor Jones is a brilliant and remarkably gifted chemist as well as an admirable person and it has been a honor and a pleasure to work for him.

I also want to sincerely thank Professor Michail Sitkovsky of Pharmaceutical Science and his research group. I have learned so much from him. I owe much for his continuing support to my research. I thank Dr. Akio Ohta for his nice suggestions and help with biological studies. It was my pleasure to work with them.

I am also indebted to the Jones group for their help and suggestions over the last four years of my PhD study.

I acknowledge gratefully our collaborator Professor Irving Goldberg and his group members at Harvard Medical School for helping me conduct the biological studies on bulge selective agents. I would like to warmly thank Professor Eriks Rozners and his group members for giving me daily help, discussion and useful inputs. I also want to thank Professor Paul Vouros and James Glick for providing help on HPLC and mass spectrometry studies. They not only helped me with instrumentation but broadened my experience and knowledge. I would like to thank Dr. Hongning Fu of Fluoropharma Inc., Dr. Roger Kautz, Professor Penny Beuning, J. Adam Hendricks and Dr. Jianxin Guo for their kind help and discussion.

Finally, and most importantly, I want to send my most heartfelt thanks to my parents. They have provided the most supportive environment conceivable and are solely
responsible for making it possible for me to walk the path that I chose. They are very special people and have always been there for me no matter how far away they are.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A2AR</td>
<td>A2A adenosine receptor</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>AIBN</td>
<td>2, 2'-azobisisobutyronitrile</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Alloc</td>
<td>Allyloxycarbonyl</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BDMS</td>
<td>bromodimethylsulfonium bromide</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Bz</td>
<td>benzoyl</td>
</tr>
<tr>
<td>Boc</td>
<td>t-butoxycarbonyl</td>
</tr>
<tr>
<td>n-Bu</td>
<td>n-butyl</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cbz</td>
<td>benzyloxycarbonyl</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CD8</td>
<td>cluster of differentiation 8 (a co-receptor for the T cell receptor)</td>
</tr>
<tr>
<td>gCOSY</td>
<td>gradient-selected correlation spectroscopy</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte (T-cell)</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-Diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>DIAD</td>
<td>Diisopropyl azodicarboxylate.</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide,</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DT-diaphorase</td>
<td>quinone oxidoreductase</td>
</tr>
<tr>
<td>ESI</td>
<td>electron spray ionization</td>
</tr>
<tr>
<td>EDCA</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment, antigen binding</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9H-fluoren-9-ylmethoxycarbonyl)</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HOBt</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrum</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration of a drug that is required for 50% inhibition &lt;i&gt;in vitro&lt;/i&gt;</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma or macrophage-activating factor</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal injection</td>
</tr>
</tbody>
</table>
**i.v.**  intravenous

ms  molecular sieves

Ms  mesylate

mU  micromoles/min/mg (activity of an enzyme per milligram of total protein)

N₂  nitrogen

NBS  N-Bromosuccinimide

NCS  neocarzinostatin

nM  nanomolar

NMO  N-methylmorpholine N-oxide

NMM  N-methylmorpholine

NMR  Nuclear magnetic resonance

NOE  Nuclear Overhauser Effect

OMe  Methoxy

Ph  phenyl

Py  pyridine

TCR  T cell receptor

TES  triethylsilyl

TFA  triflic acid

THF  tetrahydrofuran

TLC  thin layer chromatography
PREFACE

Cancer is a disease of worldwide importance. It is comprised of more than 100 different types of malignancies and it can affect every organ in a human body. Statistics show that one in three people will develop cancer and its mortality occupies the second rank as a cause of death\(^1\). In 2005, about 1.4 million new cases of cancer were diagnosed. A recent study showed that 20,000 people die of cancer daily and it is the second leading cause of death in the USA (22.8% of deaths)\(^1\).

The treatment of cancer is based on surgery, radiotherapy and drug therapy. However, only about half of the cancer patients can be cured by one of the above methods or their combinations. Many of the patients have only prolonged survivals and suffer severe side effects. One of the major reasons is the high toxicity and the lack of selectivity of drugs towards tumors. Consequently, the development of targeted anti-cancer drug therapy has expanded dramatically in the past decades.

The first clinically effective cancer chemotherapeutic agents were nitrogen mustards-- a landmark of the beginning of the modern era of cancer chemotherapy. Mechlorethamine (nitrogen mustard, HN\(_2\)), was the first clinical cancer chemotherapeutical agent. It was an analogue of mustard gas which was used in chemical weapons and is still in application today\(^2\). (Figure P.1.) The mechanism of nitrogen mustard's cytotoxicity can be explained by its ability to block DNA replication in tumor cells by cross-linking DNA. Later, many agents were discovered to react with DNA with therapeutic effects and were categorized as a class of compounds called DNA-interactive agents.
One of the most famous DNA interactive drugs is mitomycin C. It is reductively activated followed by two N-alkylations. Both alkylations are sequence specific for a guanine nucleoside in the sequence 5'-CpG-3'. (Scheme P.1.).

Some DNA-interactive drugs react with DNA in a way that they first intercalate into DNA and then are metabolically activated to generate radicals. These radicals typically abstract hydrogen atoms from the DNA sugar-phosphate backbone or from the DNA bases, resulting in DNA strand scission. A well known example is neocarzinostatin (NCS-chrom), the oldest known member of the enediyne family of antibiotics. It has been approved for clinical use in Japan for a variety of cancers (Figure P.2.). Mechanistic studies show that the naphthoate ester moiety intercalates in to DNA and positions the
epoxybicyclo[7.3.0.]dodecadienediyne portion of the chromophore in the minor groove\textsuperscript{4}. Upon activation, it undergoes a Bergman rearrangement to a bi-radical intermediate which is highly reactive and abstracts hydrogen atoms of DNA and causes DNA strand scission\textsuperscript{5,6}. (Scheme P.2.)

Goldberg found that in the presence of base, neocarzinostatin degredates to a spiro cyclic compound NCSI-gb. Its unique wedged spiro-helix shape matches the cavity of two base bulge of DNA so well that it binds to the bulged DNA with nanomolar affinity\textsuperscript{7} (Figure P.2.). Although its instability makes it impossible for practical application, it pointed to the possibility for targeted antitumor antibiotics. Based on this discovery, Jones initiated a program for the development of synthetic DNA bulge selective chemotherapeutic agents. A spirocyclic template was rationally designed to mimic the core structure of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig_p2.png}
\caption{Neocarzinostatin chromophore (NCS-chrom)}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme_p2.png}
\caption{Degradation of NCS-chrom to bulged DNA specific agent NCSI-gb}
\end{figure}
NCSi-gb. It was reasoned to be stable and could be synthesized readily\textsuperscript{8}. Recently, a series of NCSi-gb mimics were made based on this design and displayed nano-molar affinity for DNA bulge binding with excellent sequence selectivity\textsuperscript{9-11}. (Figure P.3.) A platform was thus established for preparation of bulged DNA targeted drugs by modification of synthetic lead compounds.

![Figure P.3](image.png)

**Figure P.3.** Development of DNA sequence specific chemotherapeutic agents

Antimetabolites are another group of agents for cancer chemotherapy. Many of them have been approved by the FDA for treatment of cancer. As analogues of DNA's components, they interfere with the formation of normal cellular metabolites in a way of either inhibiting enzymes involved in the *de novo* synthesis of DNA or incorporating into DNA. A famous antimetabolite is 5-fluorouracil (5-FU) (Figure P.4.), designed and synthesized by Heidelberger and co-workers\textsuperscript{12,13}.
It principally acts as a thymidylate synthase inhibitor to block synthesis of the pyrimidine thymidine which is a nucleotide required for DNA replication\textsuperscript{14}. In a cell, it is firstly converted to the active species such as FdUMP by enzymes which then inhibit thymidylate synthase and incorporate into DNA and RNA. As a result, the DNA synthesis of the cell is stopped by the induced cell-cycle arrest and apoptosis. (Figure P.4., Scheme P.3.)
A major challenge of cancer research is to circumvent the disadvantages of 5-FU such as high toxicity and non-selectivity. A prodrug of 5-FU called CAP or Xeloda® was developed in Japan in 1990's to improve its tumor specificity.

After oral administration, CAP crosses the gastrointestinal barrier intact and is rapidly absorbed\(^\text{16,17}\). It is subsequently converted into FU in a three-stage mechanism (Scheme P.4). In the first step, it is metabolized into 5'-deoxy-5-fluorocytidine (5'dFCR) by the hepatic carboxylesterase. Then, 5'dFCR is deaminated into 5'dFUrd by the cytidine deaminase mainly localized in liver and tumor tissues. In the last step, 5'dFUrd is transformed into FU under the action of TP, an enzyme with higher activity in tumors.

than in normal tissues. Higher levels of FU are produced within tumors with minimal exposure of healthy tissues to FU\textsuperscript{18}. Therefore, the drug concentration in healthy tissues is decreased and consequently, its systemic toxicity is reduced. Clinical trial showed good toleration and higher therapeutic index. CAP has been approved by the US Food and Drug Administration for use in patients with metastatic breast cancer and is now commercialized in Europe as a monotherapy for the first-line treatment of metastatic colorectal cancer.

The above two classes of agents – DNA interactive agents and antimetabolites – comprise by far the largest number of the compounds that have been approved by the FDA for the treatment of cancer.

Another class of anti-cancer chemotherapeutic agents are topoisomerase inhibitors. Changes in DNA structure are controlled by topoisomerase I and II through catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle. The growth of tumor cells can then be interrupted or stopped by inhibiting the enzymes. 20(S)-Camptothecin (CPT) (Figure P.5.) is a potent inhibitor of DNA topoisomerase I. It was first isolated by Wall and coworkers from *Camptotheca acuminata*\textsuperscript{19,20}. CPT has been identified to stabilize covalent binding of topoisomerase I to DNA, resulting in irreversible and lethal strand breaks in DNA during its replication\textsuperscript{21}. However, the clinical application of CPT to cancer treatment was suspended because of its unfavorable
properties such as non-specific toxicity and low water solubility\textsuperscript{22}. Many efforts have been applied for development of CPT analogues which have acceptable therapeutic index and elevated bioavailability.

A successful example is CPT-11 (irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxyacamptothecin). It has reduced toxicity in normal tissues, but it can be converted by carboxylesterases in tumor tissues to SN-38, a potent inhibitor of topoisomerase I with IC\textsubscript{50} values in the low nM range\textsuperscript{23}. (Scheme P.5.) CPT-11 has been approved by FDA in US and is marketed by Pfizer as Camptosar\textsuperscript{®}.

Approaches such as utilizing bioreductase excessively expressed in hypoxic tumor regions have also been applied to development of tumor targeted agents. CPT4 is a water-soluble prodrug of CPT and can be bioreductively activated in hypoxic tumor tissues. (Scheme P.6.) In the presence of DT-diaphorase, it is quickly reduced to eliminate the conjugated indolequinone moiety and produce CPT3, which then effectively undergoes an intramolecular cyclization to release CPT\textsuperscript{24}. \textit{In vitro} studies shows that it has at least one magnitude of lower cytotoxicity than CPT, indicating the tumor selective cytotoxicity\textsuperscript{24}. 

\begin{center}
\textbf{Scheme P.5.} Activation of CPT-11 in tumor tissue
\end{center}
Tumor growth can also be inhibited at the phase of cell division, *i.e.* mitosis, during which the duplicated chromosomes of a cell are separated into the two identical sets before it is divided into two daughter cells. Microtubules have been important targets for anti-cancer drugs because they are key structural components which cells use to pull apart the sister chromatid of each chromosome in mitosis. Drugs binding to microtubule proteins with high affinity classified as antimitotic agents and a famous example is

**Scheme P.6.** Mechanism of CPT4 activation by a reductase of DT-diaphorase

Tumor growth can also be inhibited at the phase of cell division, *i.e.* mitosis, during which the duplicated chromosomes of a cell are separated into the two identical sets before it is divided into two daughter cells. Microtubules have been important targets for anti-cancer drugs because they are key structural components which cells use to pull apart the sister chromatid of each chromosome in mitosis. Drugs binding to microtubule proteins with high affinity classified as antimitotic agents and a famous example is
Paclitaxel (Taxol®). (Figure P.6.) It was discovered in 1967 and first isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. It has been approved for the treatment of lung, ovarian, breast, head and neck cancer. However, due to its toxicity and the need for organic solvents for i.v. injection, severe side effects often result. Chemical composition, formulation and delivery have been investigated to improve tumor specificity and bioavailability.

![Figure P.6. Taxol (Paclitaxel)](image)

In January 2005, FDA approved Abraxane® (a protein-bound Paclitaxel), developed by Abraxis BioScience, for the treatment of breast cancer. It is an injectable form of Paclitaxel in which Paclitaxel is bound to albumin, a nano-sized natural protein carrier of lipophilic molecules, as the delivery agent. (Figure P.7.)

![Figure P. 7. Formulation of paclitaxel by bonding to albumin](image)
These drug enclosed nanoparticles are transported in the blood stream and accumulate in tumors at higher concentration from leaky junctions of the blood vessel and the tumor interstitium. They then bind to the albumin specific receptors around cell surfaces, which transport Paclitaxel into tumor cells\textsuperscript{25}.

In another case, Paclitaxel was conjugated to docosahexaenoic acid (DHA) through an ester bond at the paclitaxel 2’-oxygen\textsuperscript{26,27} (Figure P.8.). DHA is a natural fatty acid which is taken up readily by tumor cells for use as biochemical precursors and energy sources. The resulting Paclitaxel-fatty-acid conjugate (DHA-Paclitaxel) does not inhibit microtubules and is non-toxic. In the M109 mouse tumor model, DHA-Paclitaxel was less toxic than Paclitaxel and cured 10/10 tumor-growing animals, whereas Paclitaxel cures 0/10\textsuperscript{27}. One explanation for its greater therapeutic index is that the fatty acid alters the pharmacokinetics of the drug to increase its Area Under the drug concentration–time Curve (AUC) in tumors and decrease its AUC in normal cells\textsuperscript{26} (Figure P.9.). More importantly, the half-life of DHA-Paclitaxel in tumors is much longer than Paclitaxel. It is now under going phase I/II clinical trial\textsuperscript{27}.

![Figure P.8. Structure of DHA-Paclitaxel](image-url)
Figure P. 9. Number of surviving mice bearing M109 lung carcinomas after treatment with paclitaxel and DHA-paclitaxel.

It has been a long held dream that humans can be protected from cancer by immune surveillance. Thus, it is the goal of cancer immunotherapy to stimulate the patient's immune system to attack malignant cells. However, established and metastasized tumors are difficult to be eliminated because the clinically detectable tumors have already evolved in a way to become insensitive to their hosts' immune environments. It has been explained by several ‘tumor immune-escape’ mechanisms in which tumors have inhibitory effects either directly or indirectly on the effector cells of the immune system\textsuperscript{28-30}. In the past two decades, the accumulation of detailed knowledge of immunologic processes and characterization of many cancer antigens on the cellular and molecular level has established a theoretical framework for development of targeted approaches to cancer immunotherapy.
Monoclonal antibody (mAb) techniques have emerged as a new generation of therapeutic delivery methods. A mAb binds only to cancer cell-specific antigens and induces an immunological response against the targeting cancer cells. A range of anti-tumor entities such as toxins, radioisotopes, cytokines or other active conjugates can be attached to such mAb to be delivered to tumor cells. It is also possible to design bispecific antibodies that can bind with their Fab regions both to an antigen and to a conjugate or an effector cell\textsuperscript{31}. (Figure P.10.)

**Figure P.10.**\textsuperscript{31} Tumor targeted immunotherapy through monoclonal antibodies that bind only to cancer cell-specific antigens. ADEPT, antibody directed enzyme prodrug therapy; ADCC, antibody dependent cell-mediated cytotoxicity; CDC, complement dependent cytotoxicity; MAb, monoclonal antibody; scFv, single-chain Fv fragment.

Development of drugs based on immune escape mechanisms represent an important new approach currently extensive research. Sitkovsky, et al found that tumors can create a “hostile” immunosuppressive microenvironment that prevents them from being destructed by anti-tumor T cells\textsuperscript{32-35}. Within the hypoxic tumor environment, excessive
adenosine is produced. They bind to the A2A receptors on T-cells’ surfaces and trigger the down-regulation signal via cAMP which inhibits the secretion of anti-tumor cytokines by T-cells such as IFN-γ\(^35\). (Figure P.11.) Accordingly, the tumor survives due to lack of inflammation.

**Figure P.11.** Model of A2AR mediated adenosine protection of tumors from T-cells

This mechanism provides the rationale for development of tumor targeted drugs by the antagonism of A2A receptors of T-cells. Proof-of-concept studies have shown that the combination of commercially available antagonists Caffeine or ZM241385 with T-cells strongly enhances the anti-tumor activities of mice\(^35\). KW6002, a potent A2A receptor antagonist, results in regression of the lung tumors of mice\(^36\). (Figure P.12.)
Based on those promising results, we began to develop potent antagonists as potential drug candidates for A2A receptor mediated anti-tumor immunotherapy.

The above discussion is not intended to give a comprehensive review of contemporary anti-cancer chemotherapy. It intends to set up a framework in which the contents of this thesis can be understandably placed for a common objective. Our common goal is to develop tumor specific agents based on anti-tumoral natural products or their derivatives. This is demonstrated in the following two areas of research:

Firstly, the design and preparation of mimics of the natural product NCSi-gb for targeting bulges on DNA. The bioassays for the analogues synthesized herein provides critical information on how changes in the drug stereostructures influence the binding affinity and the selectivity to the bulged sequences;

Secondly, we report the development of novel A2AR antagonists which target tumor tissues. KW6002 was synthesized and its anti-tumor effects were evaluated. Based on these result, the design and synthesis of a prodrug of KW6002 is described to
demonstrate the possibility of tumor targeted immunotherapy. Meanwhile, a novel synthetic methodology was developed for efficient preparation of 8-substituted xanthine antagonists.
References


36. Unpublished results shown in chapter 3.
CHAPTER 1

DESIGN AND SYNTHESIS OF NCSi-gb MIMICS TO TARGET ON BULGED NUCLEIC ACIDS
1.1. **Introduction**

1.1.1. **Biological significances of bulged nucleic acids in cancer therapy**

There are various conformations in nucleic acids such as hairpins, knots, pseudoknots, triple helices, loops, helical junctions, and bulges\(^1\) (Figure 1.1). Bulges are unpaired bases looping out of double helical nucleic acid on only one strand, creating a unique micro-environment that usually results from two sequentially mismatched base pairs that are incorporated into either DNA (single stranded or duplex) or RNA\(^2\). Bulges are related to many biological processes as intermediates, including RNA splicing, frame-shift mutagenesis, intercalator induced mutagenesis, imperfect homologous recombination, in viral replication, and in the ribosomal synthesizing machinery\(^3\). For example, bulges are binding motifs for regulatory proteins involved with viral replication in the TAR region of HIV-1, the single-stranded RNA genome of human immunodeficiency virus type 1\(^4\)\(^-\)\(^6\).

There are two primary gene regulatory proteins, *tat* and *rev*, in the virus which bind to hairpin stem-loop RNA conformations TAR (trans-acting responsive region, as showed in Figure 1.2.) and RRE (rev responsive element)\(^7\). They control the replication cycle of

![Figure 1.1. Assorted Nucleic Acid conformations](image-url)

```text
5' 3' 5'
duplex

3' 5'
hairpin

5' 3' 5'
three stranded triple helix

5' 3'
single, multiple base bulges
```
The \textit{tat} protein binds to the three-base bulge in TAR along with cellular proteins that bind at adjacent regions of TAR to control gene expression in HIV-1 via formation of an antitermination complex (Figure 1.3.)\textsuperscript{13-15}.

\textbf{Figure 1.2.} The Tar RNA region of HIV-1

\textbf{Figure 1.3.} Formation of antitermination complex by Tat binding to bulge in TAR RNA

Cancer inherently is consequence of instability and inconsistencies of genetic translation. One of the reasons is believed to be the defective damaged DNA enzyme-repair mechanism for bulged polynucleotides, in which genetic codes are lost or mistranslated during the slipped synthesis of DNA strands\textsuperscript{16}. It has been observed that bulges are key intermediates in the unstable extension of triplet repeats in colon cancer cells\textsuperscript{16,17}. Those observations have been attributed to reiterative synthesis due to slippage and bulge formation in the newly formed DNA strand\textsuperscript{18-20}. During DNA replication, the polymerase sometimes slips from the template strand and causes the new strand to unpair (release) from the template strand. It is this event, called polymerase slippage, that many researchers believe holds the key to codon expansions. If the slip occurs at the template’s codon (such as CAG) repeat region, then when the new strand tries to reattach to the template strand, it will have many identical copies of the codon to choose from. With so many identical codon copies to reattach to, the new strand may reattach to the template at
the wrong site, usually one more distant than the copy that was adjacent to the polymerase before it slipped. As a result of this misplacement, the new strand forms a bubble of unpaired bases, which represents the expansion of the new strand (Figure 1.4a., 1.4b.). Once DNA replication is complete, an unknown mechanism allows the template strand to realign with the new strand and bring the bases from the bubble back into line with the template strand. The bases are then paired with their corresponding partner bases. In the end, the brand new double helix of DNA contains more CAGs in the repeat

**Figure 1.4a.** Replication of CAG repeat regions

**Figure 1.4b.** Formation of bulge

**Figure 1.4c.** Induction of slippage
region than existed before. Polymerase slippage has thus caused expansion (Figure 1.4c.) 21. Many facts remain unknown, however, regarding the mechanics of the slippage process and the intervention of ligase and polymerase enzymes in this key process. On thermodynamic grounds, formation of the bulged intermediates appears unfavorable. However the possibility that hairpin like structures are involved, where internal hydrogen bonding can help mitigate thermodynamic obstacles, has been proposed 22-23. As such, compounds capable of binding to bulges should be useful probes to study the slippage process, the architecture of bulges and hairpins, and the involvement of enzymes by bulge-agent-enzyme ternary complexes. More importantly, appropriately functionalized agents may be applied to inhibit bulge formation or exclude the downstream effects of the bulge through covalent or non-covalent interactions and therefore, mitigate the impact of the bulged sites.

1.1.2. Synthetic agents with affinity for bulges

Despite the importance of bulges in cancer, systematic studies of synthetic bulge binders have not been reported. There are only few natural anti-cancer drugs reported to have affinity for bulges, such as duocarmycin 24a, nogalamycin 24b and actinomycin D 24c, but they also bind to DNA-duplexes and their sequence selectivity is low.

There has been considerable interest in the design of compounds capable of recognizing the Tat binding domain of RNA for the search of potential anti HIV-1 candidates. The development of such agents has been based on the following design philosophy (a) an aromatic core capable of pi-stacking to a base in the bulge (b) A cationic (amine) unit
which interacts with the nucleic acid phosphate backbone and (c) A linker moiety to join the above two parts. A couple of examples are shown in Figure 1.5. Though diaminopurine-acridine conjugate 1-1 only showed moderate binding, a related compound CGP 40336A (1-2) was studied in depth and was found to block HIV expression through Tat with an IC\(_{50}\) of 1.2 \(\mu\)m.

![Figure 1.5. Polyamine-acridine-based Tat-antagonists](image)

For development of a bulge specific agent, the challenge was to identify a template capable of forming stable complexes in the pocket of the bulged site wherein the critical binding criteria can be thoroughly explored.

1.1.3. Enediyne metabolites targeting bulged microenvironments
The enediyne family of antitumor antibiotics has received considerable attention because of their excellent biological profiles, unique mode of action, and proven clinical efficacy\textsuperscript{28}. More than twenty natural enediynes have been found to have bio-activity; two of them are currently in clinical use; one has been approved by FDA\textsuperscript{29-31}. The unique mode of action is believed to be derived from their inherent ability to generate a 1, 4 diradical \textit{via} the 3-hexen-1, 5- diyne functionality, which subsequently abstracts a hydrogen atom from DNA. This abstraction process produces a new radical and finally results in cell lysis through a cascade type mechanism\textsuperscript{32}. Although extensive studies have been performed on these agents, high toxicity and non-selectivity remain as the major limitation for their application.

One of the most thoroughly studied natural enediynes is neocarzinostatin chromophore 1-3 (NCS-chrom). Its unique structure is distinguished by the unsaturated epoxy bicycle-[7.3.0]dodecadienediyne core. (Figure 1.6.)

\textbf{Figure 1.6. Neocarzinostatin chromophore (1-3)}

The nine-membered ring is highly strained. The average \( \text{C} - \text{C} = \text{C} \) bond angle (161.5 ± 1.2\textdegree) is significantly distorted from linearity\textsuperscript{33}. It is probably the reason why it is so labile
in solution. The chromophore becomes highly unstable, once it is separated from its carrier protein. The half-life is only about 30s at pH 8.0 (0 °C).34.

In the early nineties, Goldberg found that NCS-chrom undergoes cellular thiol addition to generate the cumulene intermediate 1-5, followed by cyclization to form the indacene diradical 1-6 with radical centers at C-2 and C-6.35,36 (Scheme 1.1.) The diradical then abstracts hydrogens from DNA to form the reduced chromophore 1-7 and results in the strand scission. This process explains its biological activity.35 However, in the absence of thiols, the enolate 1-8b is formed as a resonance form of the naphtholate anion 1-8a of NCS-Chrom under basic conditions (pH about 9). Then, it undergoes an unusual 5-exo-trig intramolecular Michael addition in concert with an epoxide ring opening to generate

**Scheme 1.1.** Proposed thiol-dependent mechanism of NCS-Chrom induced cleavage of duplex DNA
the cumulene intermediate 1-9. This intermediate then undergoes a Bergman-type rearrangement to the 2, 6-biradical 1-10, which, in the absence of bulge DNA, abstracts hydrogens from solvent and converts to NCSi-gb 1-4 (Scheme 1.2.)\textsuperscript{36}.

\begin{center}
\begin{tikzpicture}

\t\node[draw,rectangle,inner sep=5pt] (1) at (0,0) {1-8a};
\t\node[draw,rectangle,inner sep=5pt] (2) at (2.5,0) {NCS-Chrom};
\t\node[draw,rectangle,inner sep=5pt] (3) at (5,0) {1-8b};
\t\node[draw,rectangle,inner sep=5pt] (4) at (7.5,0) {1-9};
\t\node[draw,rectangle,inner sep=5pt] (5) at (8.5,0) {1-10};

\t\node[draw,rectangle,inner sep=5pt] (6) at (10,0) {NCSi-gb (1-4)};

\t\node[draw,rectangle,inner sep=5pt] (7) at (12,0) {solvent};

\t\draw[->,thick] (1) -- (2);
\t\draw[->,thick] (2) -- (3);
\t\draw[->,thick] (3) -- (4);
\t\draw[->,thick] (4) -- (5);
\t\draw[->,thick] (5) -- (6);
\t\draw[->,thick] (6) -- (7);

\end{tikzpicture}
\end{center}

\textbf{Scheme 1.2.} Proposed thiol-independent mechanism of action of NCS-Chrom

It was found that NCSi-gb recognizes bulged nucleic acid sequences with high affinity (up to 33 nM) and selectivity, especially for the sequences containing a GT bulge\textsuperscript{37,38}. It was able to induce bulge formation in otherwise unstructured regions of DNA\textsuperscript{39}. The NMR solution structure of the complex formed between NCSi-gb and an oligonucleotide containing the known binding-cleavage site (Figure 1.7.) has been determined in order to understand the molecular basis for the recognition of the bulged structure\textsuperscript{40}. It is clear that the recognition of the bulged site by NCSi-gb is from the major groove. The two aromatic rings of NCSi-gb are rigidly held and transposed approximately 60° by the spirolactone ring to form a molecular wedge that penetrates the binding pocket and immobilizes the
otherwise flexible bulged A and T residues\(^{37}\) (Figure 1.8., A, B and C). The ring systems stack with the DNA base pairs above and below, and mimic the geometry of the helical bases with a right hand twist angle of \(\sim 35^\circ\) (measured by the long axes). It is, most likely, the \(\pi\)-stacking interactions among the aromatic moieties that stabilize the NCSi-gb complex. Unlike most aminosugars of other DNA binding drugs that usually recognize the sugar phosphate backbone through the minor groove, the N-methyl fucosamine moiety of NCSi-gb sits in the center of the major groove and is responsible for the major groove recognition. In summary, the following key features are responsible for the two-base bulge binding. Firstly, a spirocyclic ring junction that offsets the systems by 30-40 \(^\circ\) matches the prism like bulge pocket and allows the molecule to fit

\[
\begin{align*}
&3'-G_{18}G_{17}G_{16}C_{15}T_{14} \quad A_{11} \quad C_{10} \quad G_{9} \\
&5'-C_{1} \quad C_{2} \quad C_{3} \quad G_{4} \quad A_{5} \quad T_{6} \quad G_{7} \quad G_{8}
\end{align*}
\]

**Figure 1.7.** Proposed sequence of the bulged DNA construct

**Figure 1.8.** (A) Corey-Pauling-Kolton (CPK) drawing of the complex viewed into the minor groove with DNA backbond in purple, the bases in gray, and NCSi-gb in green. (B) The triangular prism binding pocket in the DNA duplex shown in a CPK drawing, view from major groove. (C) Drawing shown the binding and the cleavage site from the minor groove.
into it. Secondly, the two independent aromatic systems provide the stacking interactions and the stabilization. Thirdly, an aminosugar moiety enhances binding affinity and is responsible for the major groove recognition.

The remarkable affinity (up to 33 nM) of NCSi-gb for bulged nucleic acids prompted our efforts to design and synthesize its mimics that might evolve into potential drug candidates. One option would be to use this natural metabolite as a template for the development of lead compounds. However, it is impractical for the following two reasons. Firstly, it is difficult to be prepared as it requires complex total synthesis. Secondly, the spirolactone ring is unstable, the half–life is only about 5 h at pH 8.2 at room temperature. Therefore, a stable and readily available agent is required for research and development. Based on the studies of NCSi-gb, we started the program for design and preparation of a readily available family of bulge specific agents.

1.2. Design and synthesis of NCSi-gb mimics as bulged DNA binder

1.2.1. Design philosophy for synthetic NCSi-gb mimics

First of all, a synthetically accessible template must be identified which mimics the unique shape of NCSi-gb but avoids the complexity of synthesis. It should contain neither the unstable spirolactone nor the cyclic carbonate moiety which is detrimental for binding. Based on molecular modeling studies (Figure 1.9.) of the core element (1-11) of NCSi-gb, the structure 1-12 was designed as the template (Figure 1.10.). The key difference between them is that the spirolactone of 1-11 is replaced with a spiroketoalcohol in 1-12. The spiro alcohol can be produced readily through an
intramolecular Aldol reaction and the wedged helical feature of NCSi-gb is preserved in the designed template.

**Figure 1.9** PM3 molecular modeling of NCS core and its proposed analog

![Proposed Analog vs NCS Core](image)

**Figure 1.10.** Design philosophy for NCSi-gb mimics

1.2.2. Synthesis of the spirocyclic model compound

Directed by rational design and a modeling study, a model compound 1-20 was synthesized by our previous group members.\(^{41}\) (Scheme 1.3.).

The cycloaddition of tetrabromoxylene (1-13) and cyclopentenone followed by the bromination at benzylic position of compound (1-14) and the elimination of hydrogen
bromide formed the dienophile (1-15). The commercially available tetralone (1-16) was first reduced by sodium borohydride and then eliminated to make the diene. The Diels-Alder reaction of the diene (1-17) and the dienophile provided the cycloadduct (1-18). The dihydroxylation followed by oxidative cleavage by sodium periodate converted the compound (1-18) into the aldehyde (1-19), which then formed the spiroalcohol model compound (1-20) by an intramolecular aldol reaction.

X-ray crystallographic study\textsuperscript{38} was immediately applied to confirm the structural similarity between 1-20 and NCSi-gb (Figure 1.11.) after the spiro model compound was made. The data showed that the two aromatic ring systems are clearly offset with a $\sim 35^\circ$C helical twist and a wedge with a cone angle of $\sim 32^\circ$C by virtue of the spirocyclic

\[ \text{Scheme 1.3. Preparation of the spirocyclic model compound} \]
junction is also established. Superimposing the structure of 1-20 upon the NMR solution structure of NCSi-gb\textsuperscript{38} (data not shown) confirmed the high similarity of the model compound and the core of NCSi-gb. Hence, the design of a synthetically accessible spiro template to mimic the spiro lactone of NCSi-gb was validated. Now, the stage was set for preparation of NCSi-gb mimics.

\textbf{Figure 1.11.} ORTEP of spiroalcohol 1-20
1.2.3. Refined design criteria

The following criteria are desired for mimics of NCSi-gb: Firstly, the components causing instability must be removed. Hence, neither the lactone of NCSi-gb nor the hydroxyl group at spiro-ring junction in the model compound (1-20) should remain. It was expected that the transformation of the hydroxy group to a spiro alkene could enhance stability and rigidity. Secondly, it has been shown that the bulky groups on NCSi-gb jeopardize bulge binding$^{40}$, so the cyclocarbonate, the hydroxy group and the methyl group should be removed from the synthetic mimics. Thirdly, the methoxy group 

\textit{para} to the enone group may be retained to maximize the conjugation of the enone and the benzene ring, which enhances the compound’s fluorescence. The binding affinity can then be measured conveniently by fluorescence. (Figure 1.12.)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure12.png}
\caption{Refined design criteria}
\end{figure}

There is no doubt that the amino sugar pendent to the natural product NCSi-gb plays a critical role in bulge binding. However, how the relative space geometry, \textit{i.e.} $\alpha$ or $\beta$
anomeric linkage, between sugar (glycone) and spirocycle (aglycone) influences the binding mode remains unknown. Does “Nature” give us the best one? If N-methyl fucosamine is stereoselectively linked to the spiroalcohol, two glycosic forms are possible. The optimal structure can then be identified by measuring its binding affinity to bulges. All in all, the desired NCSi-gb mimics should be stable, fluorescent, and have the pendant aminosugar moiety with an appropriate spatial direction.

1.2.4. Retrosynthetic analysis (Scheme 1.4.)

The proposed NCSi-gb mimics can be constructed by the stereoselective glycosylation of the glycone (the N-methyl fucosamine) and the aglycone (the spiroalcohol). The hydroxy group at the spiro ring junction should be eliminated before the glycosylation. The spiro alcohol (i) in which the benzylic alcohol is properly protected can be formed by the intramolecular aldol reaction from the intermediate (ii), and the required ketone and aldehyde functional groups can be generated by the oxidative cleavage of the alkene (iii). Accordingly, the diene (iv) and the dienophile (v) are needed as the key intermediates for the preparation of alkene (iii) via a Diels-Alder reaction. Both the diene (iv) and the dienophile (v) can be easily prepared from commercially available starting materials.
1.3. Synthesis of the NCSI-gb mimic as the bulged DNA binding agents

1.3.1. Synthesis of the spirocyclic aglycone

Based on the above analysis of the design criteria and the retrosynthesis, the spirocyclic alcohol of the NCSI-gb mimic was synthesized as follows. The diene (1-23) was prepared from the commercially available 7-methoxy tetralone. The Diels-Alder reaction of the dienophile (1-20) and the diene (1-23) provided the cycloadduct (1-24). Then, it
was subjected to the reduction and the Mitsunobu reaction to generate the chiral center at the benzylic carbon same as the one in NCSi-gb. The resulted benzoate ester was subjected to dihydroxylation, oxidative cleavage of resulting diol and intramolecular aldol reaction to produce the spiro alcohol (1-26). Compound 1-26 was firstly mesylated and then eliminated to form a stable spiro alkene (1-27). Alpha carbon bromination of the spiro alkene and the elimination with DBU were applied to form the enone which was
then subjected to saponification to obtain the spirocyclic alcohol (1-29). The overall yield was 3.6%.

1.3.2. Synthesis of the protected N-methyl fucosamine

The 3- and 4-hydroxy group of N-methyl fucosamine had to be properly protected prior to the coupling with aglycone and only 1-hydroxyl group was left free for the glycosylation reaction. Harsh conditions for the deprotection such as strong acid, strong base and hydrogenation need to be avoided as they may result in the decomposition of the spirocycle. Therefore, trimethylsilyl (TES) was chosen as the protective group because it can be easily installed and after the glycosylation, it can be removed by a weak acid.

The synthetic route started from the commercially available galactosamine hydrochloride (1-30), the primary amine was firstly protected with the benzylloxycarbonyl (Cbz) group. The anomeric hydroxy group was then protected with the benzyl (Bn) group. The 6-hydroxy group of the compound 1-31 was transformed into the tosylate 1-32, followed by tosyl-iodide exchange to afford compound 1-33. The remaining 3, 4-hydroxy groups were then protected by the TES groups and the generated bis-TES ether 1-34 was reduced by tributyltin hydride to form compound 1-35. N-methylation followed by the hydrogenation of both Cbz and Bn groups then formed the protected N-methyl fucosamine 1-37. The overall yield was 26%. (Scheme 1.6.)
1.3.3. Strategies for stereoselective glycosylation

The alpha stereoselective glycosylation of the aglycone (1-29) with \(N\)-methyl fucosamine was completed by the previous group member\(^\text{42}\). It was based on the method that Mayer’s group developed\(^\text{43}\) (Scheme 1.7.) in which a glycosyl acceptor is directed to the \(\alpha\)-face of the oxonium ion by interaction with the amino group (possibly via hydrogen bonding). Hence, the alpha glycosylated mimics were produced by coupling of 2-methylamino-fucosyltrichloroimidate (1-38) and the aglycon (1-29). (Scheme 1.8.)
9-Fluorenylmethyl carbamate (Fmoc) can be introduced as a participating group to the secondary amine to instead induce beta glycosylation of the aglycone 1-37. Activated by a Lewis acid, an intramolecular substitution occurs and the oxazolindine forms as an intermediate which occupies the α-face of the anomeric carbon and forces the aglycone to approach to the carbon from its β-face. (Scheme 1.9.)
The Fmoc group can be easily removed by a mild base after the glycosylation reaction.

1.3.4. Synthesis of NCSI-gb mimics via β-stereoselective glycosylation with N-methyl fucosamine

Based on the above discussion, an Fmoc group was firstly introduced to the secondary amine of the intermediate 1-37. (Scheme 1.10.) Different bases were examined to promote the reaction for the preparation of Fmoc-N-methyl fucosamine trichloroimidate (1-41). The best results were obtained when 0.8 equivalents of sodium hydride were used. Other bases such as DBU and potassium carbonate were unsuccessful, either causing the severe deprotection of the Fmoc group or resulting in a slow reaction.
Due to the unstable nature of trichloroimidate (1-41), the crude product was used in the glycosylation step without further purification. After the coupling of the glycosyl donor 1-41 and the aglycone 1-29 followed by the deprotection of the Fmoc group with DBU, the generated two diastereomers (1-42a and 1-42b) were separated by preparative TLC. Each of them was then subjected to the deprotection of the TES groups by hydrofluoric acid in pyridine to provide the final products 1-43a and 1-43b. (Scheme 1.11)

The handedness of the aglycone portion of both analogues (1-43a and 1-43b) was then assigned by circular dichroism (CD) spectroscopy. The β-configuration at anomeric carbon was determined by \(^1\)H NMR and gCOSY\(^{44}\) spectroscopy of both compounds. Compound 1-43a exhibited an anomeric resonance at δ 4.95 ppm, with coupling constant J = 8.0 Hz, while compound 1-43b had the corresponding resonance at δ 4.81 ppm with coupling constant J = 8.5 Hz.

**Scheme 1.10.** Synthesis of Fmoc protected trichloroimidate 1-41 as glycosyl donor
Scheme 1.11. Synthesis of the NCSi-gb mimics 1-43a and 1-43b

Figure 1.13. Purification of the final products with HPLC.
Before the final compounds were subjected to bio-assays, they were further purified via HPLC to ensure the removal of impurities and silica gel. (Picture 1.13.)

1.3.5. Binding affinity study

The β glycosylated analogues 1-43a and 1-43b were screened against a panel of bulge–containing synthetic oligodeoxynucleotides (Table 1.1).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Code</th>
<th>Confirmation</th>
<th>NCSi-gb Right Hand 1-3</th>
<th>Diastereomers of α Glycosylation Right Hand 1-39a</th>
<th>Diastereomers of β Glycosylation Right Hand 1-43b</th>
<th>Glycosylation Left Hand 1-43a</th>
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</thead>
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<tr>
<td>1</td>
<td>DA12:DAe12</td>
<td>5’-GTCGGATGGCGT 3’-CAGGCTACGACT</td>
<td>307</td>
<td>NAB</td>
<td>NAB</td>
<td>NAB</td>
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<tr>
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<td>H314AT</td>
<td>5’-GTCGGATGGCGT 3’-CAGGCTACGACT</td>
<td>10</td>
<td>10</td>
<td>13</td>
<td>9.2</td>
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<td>3</td>
<td>DA12:BA14</td>
<td>5’-GTCGGATGGCGT 3’-CAGGCTACGACT</td>
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<td>0.6</td>
<td>0.8</td>
<td>0.43</td>
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<td>4</td>
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<td>5’-GTCGGATGGCGT 3’-CAGGCTACGACT</td>
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<td>0.08</td>
<td>0.13</td>
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<td>1.7</td>
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<td>0.026</td>
<td>0.11</td>
<td>0.24</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 1.1. Comparison of Kd of NCSi-gb, 1-39a, 1-39b, 1-43a and 1-43b. Fluorescence quenching studies conducted using a SPEX Fluoromax-3 at 4 °C in phosphate buffer (10 mmol, pH 7.0). Dissociation constants (μM) of DNA binding by the drugs determined via emission spectra (λexc 360 nm; λemm 480 nm for 1-43a and 1-43b) 4°C. Dissociation constant (Kd) was derived from curve-fitting (Kaleidagraph). Data for NCSi-gb, 1-39a and 1-39b are cited from reference 45.

In table 1.1., the binding affinity of the different analogs was compared in terms of the dissociation constants (Kd). The newly prepared analogs 1-43a and 1-43b showed good binding affinity and in some cases (entries 3, 5 and 7) results were better than the reported binding of NCSi-gb to the bulged sequences. In comparison with NCSi-gb, 1-
39a shows similar binding ability to GT pair bulge sequence, and 1-43a shows about 10 fold lower binding affinity to a GT bulge. The logical conclusion can be made that a drug having an α-glycosylation linked fucosamine offers the best bulge binding affinity. The new analogs 1-43a and 1-43b proved another important impact from the introduction of the fucosamine into the bulge binding spirocycle. In contrast to the NCSi-gb in which strong binding affinity to the two-base bulged DNA was provided by the right-handed diastereomers, the left-handed diastereomers of the synthetic mimics (1-39a and 1-43a) bind tighter in the most of the cases.

A recent study on the solution structure of two-base bulged DNA with the right-handed analog 1-39b proved that although NCSi-gb and 1-39b resemble each other closely, when they bind to the bulges, they differ in the important local environment\textsuperscript{46}. The synthetic spirocyclic aglycon has a better hand-in glove fit at the bulge site due to the lack of the bulky cyclocarbonate of NCSi-gb. The 2-N-methylfucosamine moiety, which resides in the major groove, of 1-39b stays closer to the sugar-phosphate backbone of the bulge-containing strand. The binding data of new analogs 1-43a and 1-43b proved that DNA bulged structures prefer the α-glycosylated fucosamine. However, variation of the subtle structure and space geometry of the sugar and the aglycone might alter the binding pattern at a bulge site.
1.4. Conclusion

- New analogs of NCSi-gb 1-43a and 1-43b have been synthesized with exclusive β stereoselective glycosylation of the fucosamine.

- The binding affinity of analogs 1-43a and 1-43b was measured and showed strong binding affinity and selectivity to the two-base bulged DNA.

- The impact of the carbohydrate moiety to the NCSi-gb mimics as bulge binding agents was highlighted by the newly synthesized analogs. The space geometry clearly places a critical role in better binding to bulge sites.

- By the synthesis of compound 1-43a and 1-43b, we supported the role of the α-glycosylated fucosamines for nanomolar affinity. Analog 1-39a showed best binding affinity among other synthetic mimics of NCSi-gb, and is a lead compound for the development of bulge specific chemotherapeutical drugs.
1.5. References


44. For the enlarged 2D gCOSY spectrum analysis, please refer to the page 221-222 for compound 1-43a and the page 224-226 for compound 1-43b.


Chapter 2

DEVELOPMENT OF A NOVEL RING CLOSURE METHODOLOGY FOR
PREPARATION OF 8-SUBSTITUTED XANTHINES
2.1. Clinical significance of 8-substituted xanthines

Xanthines represent an important class of agents. Observed pharmacological actions include stimulation of the central nervous system (CNS)\(^1\), relaxation of muscle constrictions of the smaller bronchi and other smooth muscles\(^{11}\), dilation of the small pulmonary arteries\(^{14}\), stimulation of cardiac muscles with increased cardiac output and the promotion of mild diuresis\(^8\).

![Diagram of purine and xanthine](image)

The therapeutic targets to which xanthines are directly correlated are summarized as the following:

1) Alzheimer’s disease – A2A and A2B adenosine receptor antagonists\(^2\);
2) Asthma – anti-inflammatory: A1, A2A, and A2B adenosine receptor antagonists and phosphodiesterase inhibitors\(^3\);
3) Behavioral targets – antidepressant, anxiolytic, cognitive enhancement, neuroprotection; adenosine receptor antagonists and phosphodiesterase inhibitors\(^{1,4}\);
4) Cancer – G2 checkpoint inhibitors\(^5\); A2A adenosine receptor antagonists\(^6\);
5) Diabetes – A2B adenosine receptor antagonists\(^7\); phosphoenolpyruvate carboxykinase inhibitors\(^8\); dipeptidyl peptidase IV inhibitors\(^9\);
6) Pain – A2A and A2B adenosine receptor antagonists, phosphodiesterase inhibitors\(^{10,11}\).
7) Parkinson’s disease – A2A adenosine receptor antagonists, phosphodiesterase inhibitors;\(^\text{12}\)

8) Renal effects – diuretics: A1 and A2A adenosine receptor antagonists;\(^\text{13}\)

9) Respiratory targets – antitussives, apnea, chronic obstructive pulmonary disease, cystic fibrosis: adenosine receptor antagonists;\(^\text{14-17}\)

**Figure 2.1.** Samples of xanthines as therapeutic agents

- BG9928 – congestive heart failure, A1 receptor antagonist
- Fenetylline – central stimulant
- CH13584 – antitussive
- 8-piperidinyl-xanthine – DPP-IV inhibitor, anti-diabetic
- KW6002 – Parkinson’s disease, A2A receptor antagonist
- Arylpiperazine-xanthine – Anxiolytic
In the above therapeutic areas, the core structure of xanthine (3, 7-dihydro-1H-purine-2, 6-dione) provides a versatile scaffold for the development of lead compounds. A number of derivatives have been screened for selective ligands for specific targets. (Figure 2.1.) Among all the xanthines under investigation, the 8-substituted xanthines demonstrated to be an important class of compounds for the identification of potent A1, A2A and A2B adenosine receptors antagonists as drug candidates. Some examples are shown in Figure 2.2. During the past 20 years, a number of potent antagonists for the above three A-

**Figure 2.2.** 8-substituted xanthines as potent antagonists of adenosine receptors

subtype receptors have been developed. Among them, the 8-styryl substituted xanthines represents potent A2A receptor antagonists\(^\text{18}\). Xanthine derivatives having substituted
phenyl groups at the 8-carbon have been suggested potent A2B receptor antagonists\textsuperscript{19}. Also there are many examples of potent A1 receptor antagonists bearing bulky lipophilic substitutes at the 8-carbon of 1, 3-dipropylxanthines\textsuperscript{20-23}.

2.2. Formation of xanthine’s five-member ring

Despite the widespread application of 8-substituted xanthines, formation of the xanthine’s five-membered ring represents a major challenge for preparation of this class of compounds. The most commonly used methods can be summarized as the following:

\textbf{(Scheme 2.1.)}

a) Coupling reaction of a 5, 6-diaminouracil \textbf{1} with a carboxylic acid or its derivative to form an amide bond, followed by an intramolecular ring closure of aminoacylamine \textbf{2}.

b) Condensation of a 5, 6-diaminouracil \textbf{1} with an aldehyde to form a 5-iminouracil intermediate \textbf{3}, followed by a strong oxidative ring closure reaction.

In route a, expensive coupling reagents such as EDC are needed for the formation of the amide bond. Reflux is required for the ring closure reactions. Yields usually are less satisfactory due to the side reactions of activated carboxylic groups and the competition of intermolecular reactions.

In route b, the formation of the imine bond from 5, 6-diaminouracil and an aldehyde is relatively easy. However, strong oxidants and elevated temperature such as I\textsubscript{2}/DME/50\degree C\textsuperscript{26}, DEAD/DMF/100\degree C\textsuperscript{27}, SOCl\textsubscript{2}/reflux\textsuperscript{28}, Fe\textsubscript{3}Cl\textsubscript{3}/EtOH/reflux\textsuperscript{29}, Fe\textsubscript{3}Cl\textsubscript{3}/AcOH\textsuperscript{30}, m-CPBA/MeCN\textsuperscript{31} or NBS/CHCl\textsubscript{3}/reflux\textsuperscript{32} are required in the ring
closure-step. Also, the scope of application is limited because many substrates cannot be affected by these conditions\textsuperscript{31}.

\textbf{Scheme 2.1.} The most commonly used methods for the formation of xanthine’s five-membered ring

In summary, the existing methods are either less efficient or limited by scope of application. There is lack of a mild and efficient methodology for preparation of the 8-substituted xanthines.
2.3. Bromodimethylsulfonyl bromide (BDMS) mediated ring closure reactions of 5, 6-diaminouracils and aldehydes

Bromodimethylsulfonyl bromide (BDMS) (Figure 2.3.) is a yellow crystalline solid which can be readily obtained from bromine and dimethyl sulfide\textsuperscript{33}. BDMS is considered convenient storage for the bromonium ion, just as hypobromite, N-bromosuccinimide or bromoazide. It was discovered by Meerwein in 1965\textsuperscript{34}. Since then, it has been used in wide range of reactions either as a reagent or a catalyst\textsuperscript{35–41} due to its ease of handling and low cost.

Furukawa, \textit{et. al.} first used this reagent to transform alcohols to bromides with the inversion of chiral carbon configuration\textsuperscript{42} (scheme 2.2.).

\[
\begin{array}{c}
R\text{-OH} \quad \text{BDMS} \quad 4-5h \\
\end{array}
\]

**Scheme 2.2.** Preparation of alkyl bromides from corresponding alcohols

BDMS was also used to prepare α-bromo enones from enones\textsuperscript{43} (Scheme 2.3.). It first reacts with conjugated enones and then is treated with a mild base such as potassium carbonate to give α-bromo enones in excellent yields.

\[
\begin{array}{c}
\text{O} \quad \text{1.BDMS} \quad \text{2.K}_2\text{CO}_3 \\
\end{array}
\]

**Scheme 2.3.** Synthesis of α-bromoenones from enones
BDMS was also used as an efficient regioselective brominating agent for activated aromatics such as phenols, anisole, diphenyl ether and N-alkyl anilines\(^{44}\) (Scheme 2.4.). Highly para selective products were produced.

**Scheme 2.4.** Regioselective para-halogenation of activated arenes

The application of BDMS has been increasingly exploited within the last fifteen years. Khan, et al. successfully used BDMS as an regioselective bromination reagent to convert 1,3-diketones and \(\beta\)-keto esters to \(\alpha\)- brominated products\(^{45}\) (Scheme 2.5.).

**Scheme 2.5.** Regioselective bromination of 1, 3-diketones

Biswanas, et al. applied a catalytic amount of BDMS and synthesized \(\alpha\)- bromonitrile in a one-pot three component condensation of carbonyl compounds, amines and trimethylsilyl cyanide at room temperature\(^{46}\) (Scheme 2.6.).

**Scheme 2.6.** BDMS catalyzed one-pot synthesis of alpha-aminonitriles

It was also used as a highly efficient catalyst for protection of aldehydes and ketones in the form of 1.3-oxathiolanes under solvent free condition (Scheme 2.7.). It is a very
economic and compatible method for protection of carbonyl groups in the presence of other protective groups.\(^{37}\)

**Scheme 2.7.** Protection of ketone and aldehyde with 1,3-oxathiolane

More recently, Das, *et al.* successfully used BDMS to make benzimidazole from 1,2-phenylenediamine and an aldehyde in high yield at room temperature\(^{47}\) (Scheme 2.8.).

**Scheme 2.8.** BDMS mediated formation of benzimidazole

Stimulated by the above reports, we hypothesized that the formation of the xanthine’s five-member ring from 5, 6-diaminouracil and an aldehyde could be promoted by BDMS because of the structural similarity of benzimidazole and xanthine.

The BDMS promoted ring closure reaction was presumably expected to proceed in two stages. (Scheme 2.9. and 2.11.) Firstly, an intermolecular nucleophilic reaction of 5, 6-diaminouracil and an aldehyde is promoted by the sulfur cation and the generated hydrobromide. Due to the stronger nucleophilicity of the amine at the 5-carbon, imine (a) is formed.

Imine (a) is then activated by hydrobromide through protonation of the nitrogen and undergoes the intramolecular nucleophilic attack of the free amine to the sp2 carbon to
Scheme 2.9. BDMS catalyzed imine formation

form the five-membered ring. In the presence of BDMS and other intermediates regenerated in situ (Scheme 2.10.\textsuperscript{48}, the secondary amine is then brominated, followed by elimination to form the double bond (Scheme 2.11.).

Scheme 2.10. Reaction pathway for in situ generation of BDMS
Scheme 2.11. BDMS catalyzed intramolecular cyclization

Based the above assumption, a ring closure reaction of 5, 6-aminouracil and benzaldehyde with a catalytical amount of BDMS was conducted. Unfortunately, the reaction was extremely slow. It was possibly because the \textit{in situ} generation of BDMS was slow and the by-product water further slowed the formation of BDMS\(^{48}\) (Scheme 2.10.). Therefore, additional BDMS was used to speed up the reaction.

Scheme 2.12. Reaction with a catalytical amount of BDMS

Optimal results were obtained when 0.5 equivalents of BDMS are used. The reaction was completed in 4 hours and the yield of the desired product 70\%. (Scheme 2.13.)
Scheme 2.13. Formation of 1, 3-diethyl-8-phenylxanthine

Other solvents such as tetrahydrofuran and methylene chloride were investigated, but the reactions proved too slow to be practical.

Following his initial success, a series of aldehyde substrates were subjected to the same reaction condition for development of the methodology. (Scheme 2.14.) They were chosen based on the following criteria:

Firstly, they should represent a broad range of substrates and contain different substituents;

Secondly, the aldehyde itself should not contain any strong nucleophilic group;

Thirdly, in the case of substituted benzaldehydes, both electron donating and electron withdrawing groups should be included;

Additionally, the aldehydes should be inexpensive and commercially available to minimize cost. The results are summarized in table 2.1.49

Scheme 2.14. BDMS mediated xanthine five membered ring closure reactions
<table>
<thead>
<tr>
<th>Entry</th>
<th>Aldehyde</th>
<th>Product</th>
<th>Time (h)</th>
<th>Isolated yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OHC-苯</td>
<td><img src="2-1" alt="Image" /></td>
<td>4</td>
<td>70%</td>
</tr>
<tr>
<td>2</td>
<td>OHC-苯-Br</td>
<td><img src="2-2" alt="Image" /></td>
<td>2</td>
<td>79%</td>
</tr>
<tr>
<td>3</td>
<td>OHC-苯-F</td>
<td><img src="2-3" alt="Image" /></td>
<td>6</td>
<td>70%</td>
</tr>
<tr>
<td>4</td>
<td>OHC-苯-NO2</td>
<td><img src="2-4" alt="Image" /></td>
<td>3</td>
<td>70%</td>
</tr>
<tr>
<td>5</td>
<td>OHC-苯-NO2</td>
<td><img src="2-5" alt="Image" /></td>
<td>6</td>
<td>72%</td>
</tr>
<tr>
<td>6</td>
<td>OHC-苯-OCH3</td>
<td><img src="2-6" alt="Image" /></td>
<td>5</td>
<td>71%</td>
</tr>
<tr>
<td>7</td>
<td>OHC-苯-N</td>
<td><img src="2-7" alt="Image" /></td>
<td>4</td>
<td>66%</td>
</tr>
<tr>
<td>8</td>
<td>OHC-苯-OCH3</td>
<td><img src="2-8" alt="Image" /></td>
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<td>63%</td>
</tr>
<tr>
<td></td>
<td>Structure 1</td>
<td>Structure 2</td>
<td>Yield (%)</td>
<td></td>
</tr>
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<td>-------------</td>
<td>-----------</td>
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<td>5</td>
<td>66%</td>
</tr>
<tr>
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<td><img src="image12-2.png" alt="Structure 12-2" /></td>
<td>5</td>
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<tr>
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<td><img src="image13-2.png" alt="Structure 13-2" /></td>
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<td>60%</td>
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<td>68%</td>
</tr>
<tr>
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<td><img src="image16.png" alt="Structure 16" /></td>
<td><img src="image16-2.png" alt="Structure 16-2" /></td>
<td>8</td>
<td>70%</td>
</tr>
</tbody>
</table>

**Table 2.1.** Synthesis of 8-substituted xanthines using bromodimethylsulfonium bromide (BDMS)
The methodology developed herein covered a broad range of aldehyde substrates including benzaldehydes substituted with electron donating and electron withdrawing groups, heterocycles, aliphatic aldehydes, etc. All substrates were commercially available and inexpensive. In addition, the reaction tolerates the presences of mild nucleophilic groups such as a hydroxy and a carboxylic acid on the substrate aldehydes. The yields of the reactions were good. The workup and purification is straightforward, products generally form as precipitates. Pure products were obtained by filtration followed by recrystallization.

2.4. Conclusion

A novel methodology for preparation of the 8-substituted xanthines has been developed. It is superior to traditional, two-step ring closure reactions and has the following advantages:

- a one-pot reaction
- a mild reaction condition
- an inexpensive reagent (BDMS)
- a broad range of substrates
- easy workup and purification

The method was immediately applied to the development of potent A2AR antagonists. For example, compound 2-10 shown in table 2.1. is the key intermediate of the prodrug of KW6002 in chapter 3.
2.5. References


Chapter 3

A PRODRUG APPROACH FOR A2A RECEPTOR MEDIATED ANTI-TUMOR IMMUNOTHERAPY: DESIGN, SYNTHESIS AND PROOF-OF-PRINCIPLE STUDY
3.1 A2AR antagonists as anti-tumor immunotherapeutic agents

For some time, we have sought an explanation for the co-existence of tumors and anti-tumor immune cells in cancer patients. The growth of tumors continues even after the large number of anti-tumor T-cells are injected in the patient\textsuperscript{1,2}. It has been arguable that cancerous cells are able to escape from specific T-cell mediated immunity\textsuperscript{1-3}. Very recently, experimental evidence has been obtained from mice and humans\textsuperscript{4}. It was found that the A2A receptors of T-cells play a vital role in down-regulation of anti-tumor signals in tumor microenvironment\textsuperscript{4-8}. The growth of established tumors was inhibited in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{diagram.png}
\caption{Diagram of physiological loop 1. Inflammatory stimuli $\implies$ 2. Local tissue hypoxia $\implies$ \{Hypoxia–induced transcription factors (HIF-1a, HIF-2)\}$\implies$ 3. Accumulation of extracellular adenosine $\implies$ 4. signaling through G protein-coupled receptors on immune cells $\implies$ 5. OFF signaling to stop secretion of pro–inflammatory cytokines $\implies$ 6. Inhibited anti-tumor T-cell response.}
\end{figure}
60 percent in mice whose A2A receptors had been genetically deleted while no rejection was observed in control wild-type (WT) mice\(^9\).

The co-existence of tumors and anti-tumor T-cells can be explained by the A2AR mediated tumor escape mechanism. (Figure 3.1.) Tumors are protected from T-cells by an immunosuppressive signaling pathway, hypoxia=>adenosine=>A2AR, via T-cell A2A adenosine receptors\(^{10-12}\) activated by extracellular adenosines which are excessively produced in hypoxic tumor environments. In other words, the unique hypoxia feature in tumors triggers the excessive expression of adenosine, which in turn terminates the inflammatory signals of T cells\(^{13,14}\), so tumors survive for lack of inflammation.

A therapeutic strategy for tumor destruction was then proposed based on this mechanism. It was expected that by introducing antagonists of the A2A receptor, the hypoxia => adenosine => A2A receptor signaling could be weakened and hence, antitumor T-cells made less susceptible to inhibition by tumor-produced extracellular adenosines.

Caffeine, a natural A2AR antagonist, and ZM241385, a commercially available A2AR antagonist, were chosen for proof of concept studies (Figure 3.2.).

![Caffeine](image1.png) ![ZM241385](image2.png)

**Figure 3.2.** Selected A2AR antagonists for treatment of the lung tumor of mice

It was shown that Caffeine and ZM241385 significantly delayed CL8-1 tumor growth in WT mice, which developed anti-CL8-1 CD8\(^+\) T cells even if injections of ZM241385
started after tumors reached a relatively large size\(^4\), but they did not affect tumor growth in a control group of nude mice without anti-CL8-1 CD8\(^+\) T cells\(^4\) (data not shown). In addition, the inhibition of tumor growth was only observed when antagonists were given in combination with anti-CD8\(^+\) T cells\(^4\). (Figure 3.3. and Figure 3.4.)

Even though both tested antagonists significantly delayed the onset of fast tumor growth, there was no survival during the course of the treatment in any tested mode. This was mainly because of the short lifetime of the tested antagonists (t\(_{1/2}\)=30-50min) \textit{in vivo}\(^4\).

![Figure 3.3. Improved destruction of CMS4 lung metastasis by antitumor CD8\(^+\) T cells combined with Caffeine (i) and ZM241385 (ii).](image)

Figure 3.3. Improved destruction of CMS4 lung metastasis by antitumor CD8\(^+\) T cells combined with Caffeine (i) and ZM241385 (ii).
Figure 3.4. Treatment of mice with the A2AR antagonist enhanced destruction of established tumors by tumor antigen-specific CD8+ T cells. (a) The antagonist ZM241385 improved destruction of CMS4 lung metastasis by antitumor CD8+ T cells (CTL). (b) Enhancement of destruction of CMS4 lung metastasis by adoptively transferred CD8+ T cells in mice that consumed Caffeine in drinking water. Based on the aggregation of the data points in the control "only tumor" and "Caffeine alone" groups, an arbitrary cutoff value (a more than =20% decrease in the number of metastatic nodules) was assigned to illustrate a potential therapeutic efficacy threshold (shown as the percentage of mice with decreased number of metastases) for those groups of mice that were treated with both CD8+ T cells and A2 receptor antagonists. (c) ZM241385, an antagonist of A2AR, enhances CD8+ T cell-mediated antitumor immune response in mice with established s.c. solid CL8-1 melanoma. Data represent mean ± SEM.
KW6002 (Kyowa Hakko Kogyo Co., Ltd. JP), an 8-substituted xanthine, is a potent and highly selective A2A receptor antagonist (rA2A=13nM)\textsuperscript{15}. It is currently under phase II clinical trial for Parkinson’s disease in US and Japan\textsuperscript{16a,16b}. It has a much longer \textit{in vivo} half life (\(t_{1/2} \approx 8\) hour). It was anticipated that the tumor suppressive effects of KW6002 would be superior to Caffeine and ZM241385.

3.2 Synthesis of KW6002 and its anti-tumor effect

Synthesis of KW6002 was carried out as shown in Scheme 3.1. Firstly, 1,3-diethyl-6-aminouracil (3-1) was produced by the cyclization of N, N’-diethylurea and cyanoacetic acid in acetic anhydride followed with treatment of 10% aqueous sodium hydroxide. Compound 3-1 was nitrosated to yield 1, 3-diethyl-6-amino-5-nitrosouracil (3-2) which was then reduced to afford 1, 3-diethyl-5, 6-amino-uracil (3-3). The yield for the above four steps was 65%. Compound 3-3 was coupled with 3, 4-dimethoxycecinic acid to provide acrylamide (3-4) which undergoes intramolecular cyclization under reflux to give compound 3-5. This two step ring closure reaction afforded a 30.6% yield. Finally, KW6002 was generated by the methylation of the secondary amine. The total yield was 19%. 

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{structure_kw6002.png}
\caption{Structure of KW6002}
\end{figure}
Scheme 3.1. Synthesis of KW6002

The anti-tumor effects of KW6002 were tested in pulmonary metastases in mice (Figure 3.6). KW6002 itself did not confer tumor inhibitive effects. However, when it was combined with immune cells, e.g. Cytotoxic T lymphocytes (CTL), 100% tumor regression resulted\(^\text{17}\). The growth of lung tumors in mice was halted by T-cells in combination with KW6002.
**Figure 3.6.** Demonstration of the dramatic improvement of adoptive tumor immunotherapy by long-lived *in vivo* A2A receptor antagonist KW6002 T cells were prepared from tumor draining lymph nodes isolated from lymphnodes 12 days after s.c. inoculation with 1.5×10^5 MCA205 fibro sarcoma. After 2 days anti-CD3 activation and additional 3 days IL-2 expansion, these T cells were injected into mice with 10 days established pulmonary metastases (1.5×10^5 MCA205 cells). After the adoptive transfer, the mice received daily i.p. injection of 20mg/kg of A2AR antagonist KW6002. The pulmonary metastases were examined by day 21 after tumor inoculation.

Although the results for KW6002 were promising, its application for *in vivo* studies on larger animals and human beings is limited due to its extremely poor water solubility (0.16µg/mL)^18 due to the hydrophobic and highly saturated heterocyclic structure.
A certain degree of water-solubility is a prerequisite for in vivo activity of a drug. Bruns and Fergus have postulated that the ratio of solubility over adenosine receptor affinity of a compound (the so-called Bruns±Fergus or BF index) has to be more than 100 in order to exhibit good in vivo activity. For experimental studies, it is often desirable that compounds are highly water-soluble (e.g. in order to be allowed for parenteral application).

Another major concern for KW6002 is its potential side effects. It was originally developed as a highly lipophilic stimulant for the central nervous system (CNS). Studies in rats show that it accumulates in the striate body of the brain. Therefore, treatment of tumors with KW6002 may result in side effects such as insomnia and dizziness.

3.3. Prodrug approach to tumor targeted antagonists

3.3.1. Design philosophy

The concept of prodrugs was first introduced by Albert and Harper in the late 1950’s. As a derivative of an active molecule, an ideal prodrug has the following characteristics:

i. It enhances desired physicochemical and pharmacokinetic properties;

ii. It is pharmacologically inactive;

iii. It can be converted to an active molecule by chemical or enzymatic transformations upon administration.

Clearly the prodrug approach might be able to provide a less cytotoxic chemotherapeutic agent which has improved solubility and can convert to an active A2AR antagonist within or in the vicinity of a tumor mass.
A prodrug is composed of an active molecule or a drug and a carrier which are connected by a covalent bond\textsuperscript{25}. (Figure 3.7.) A drug is released through chemical or enzymatic reactions either at or near the site of action\textsuperscript{25}. Hence, its design is critically based on the mechanism of how a drug is activated in the targeting region.

For example, the prodrug approach was applied to MSX-2, a potent A2AR antagonist, to increase its water solubility. MSX-3, a phosphoric acid ester, was developed as the prodrug and it is transformed to MSX-2 \textit{in vivo} by phosphatases\textsuperscript{26} (Scheme 3.2.). MSX-3 is very soluble in water and can even be used for local intracerebral injection\textsuperscript{27-29}. However, its application is restricted by its potential side effects and toxicity to other tissue as phosphatases are ubiquitous.
Legumain, the only asparaginyl endopeptidase of the mammalian genome, is highly expressed by neoplastic, stromal, and endothelial cells in solid tumors\textsuperscript{30-33}. It is present extracellularly in tumors, associated with matrix and cell surfaces and it functions locally in the reduced pH of the tumor microenvironment\textsuperscript{33}. Therefore, a Legumain specified peptide substrate can potentially be used as a carrier which is linked to an active molecule.

Previous studies for xanthine type A2AR antagonists have shown that a highly hydrophobic core structure is required for strong binding affinity\textsuperscript{34}. Considering that a hydrophilic residue would be left on the active molecule after a prodrug is hydrolyzed by Legumain, the peptide carrier should be connected to the aromatic ring of KW6002 as shown in Figure 3.8.

It was expected that by attaching KW6002 to a peptide carrier, the water solubility could be greatly increased. At the same time, the binding affinity of the prodrug could be minimized because steric hindrance of the carrier. Accordingly, the side effects might be alleviated since a more hydrophilic prodrug is less likely to pass the blood brain barrier.
If this prodrug approach were successful, a highly tumor targeted agent becomes a possibility e.g. using a biodegradable polymer carrier for the drug delivery. (Figure 3.8.)

![Figure 3.8](image)

**Figure 3.8.** Design philosophy for Legumain activated anti-tumor prodrug

3.3.2. Synthesis of the model compound

Based on the above discussion, a model compound was synthesized as the prodrug of KW6002 for proof of principle.

The synthesis of the active part of the prodrug (3-9) is shown in Scheme 3.3. Using the one-pot methodology described in Chapter 2 for preparation of the 8-substituted xanthine key intermediate 3-7 was prepared from 5, 6-diaminouracil and 4-methoxy
cinnamaldehyde. The yield was 63%, superior to the commonly used two-step cyclization shown in Scheme 3.3.

![Scheme 3.3](image)

**Scheme 3.3.** Synthesis of the active binding domain of the prodrug

Methylation of the secondary amine in compound 3-7 provided 7-methylxanthine (3-8) which was then demethylated by bromide to generate the compound 3-9. The overall yield was 54%.

A tetrapeptide Ala-Asn-Ala-Ala(Boc) was designed as the carrier, which contains Asparagine, the amino acid needed for the hydrolysis by Legumain. It was synthesized by the following steps: (Scheme 3.4.) Firstly, the intermediate 3-11 was synthesized from
Scheme 3.4. Synthesis of the peptide carrier of the prodrug
Scheme 3.5. Synthesis of the prodrug (Boc)Asn and Ala-OMe by amide bond formation followed by deprotection of the Boc group. Compound 3-12 was prepared from the commercially available Ala-Ala by the protection of primary amine with a Boc group. The coupling reaction between (Boc)Ala-Ala (3-12) and Asn-Ala-OMe (3-11) followed by the removal of the OMe group then produced the peptide carrier (3-14).

Finally, prodrug (3-15) was synthesized by coupling compound 3-9 and 3-14 in 65% yield. (Scheme 3.5.) It should be pointed out that the formation of the ester bond was mediated by HBTU, NMM and DMAP. The previously used conditions such as DCC and Et₃N failed presumably due to the weaker nucleophilicity of the phenol group in 3-9.
3.3.3. Proof-of-principle study for the prodrug

Before the model compound 3-15 was subjected to anti-tumor studies, it had to be confirmed that the active binding molecule could be released by Legumain. Therefore, the enzyme digestion experiments were carried out for compound 3-15. (Scheme 3.6.)

Scheme 3.6. Hydrolysis of the prodrug by Legumain

The active molecule 3-16 was chemically synthesized from compound 3-9 and (Boc)Ala (Scheme 3.7.). It was used only for identifying the product of the enzymatic hydrolysis of the prodrug (3-15).

Scheme 3.7. Synthesis of the active molecule
The incubation experiment of the prodrug with Legumain was conducted under the reported conditions, and it was monitored by reversed-phase HPLC. The prodrug was completely hydrolyzed within 2.5 hours incubation and the active molecule (3-16) was released. The result proved that the design of the Legumain activated prodrug was a viable approach for development of tumor targeted A2AR antagonists.

Figure 3.9. HPLC analysis for the enzyme digestion of the prodrug 3-15. Peak a is the active molecule; Peak b is the prodrug. (i) the active molecule only (10mM) in buffer; (ii) compound 3-15 only (10mM) in buffer (slightly degraded); (iii) compound 3-15 (10 nmol) is digested with Legumain (0.2mU) at 37°C for 2.5h in 100μl buffer; (iv) buffer blank. The buffer is 20mM sodium acetate, pH 5.5, containing 1mM EDTA and 10mM dithiothreitol.

*In vitro* anti-tumor effect of this prodrug is currently under the investigation.
3.4. **Conclusion**

- KW6002, a potent A2A receptor antagonist, was synthesized. It results in 100% tumor inhibition of lung tumor in mice.
- A prodrug was designed and synthesized to address the solubility issue of KW6002 and aims to improve its tumor specificity.
- Preliminary results show that the peptide carrier of the prodrug can be completely hydrolyzed by Legumain, a tumor specific enzyme. It points to the possibility of development of tumor targeted A2AR antagonists.
3.5. References

17. Unpublished data.


CHAPTER 4

EXPERIMENTAL PROCEDURES
4.1 General information

All reactions were carried out in dry glassware unless otherwise noted. Dry glassware was obtained either by heating in a laboratory oven (150°C) for 12 h and upon retrieval, immediately sealed with a virgin rubber septum, septum-glass boundary sealed with Teflon tape, placed under a steady stream of dry N₂ via a 19.5 gauge needle, and allowed to cool to room temperature, or vessels were sealed with a virgin rubber septum, septum-glass boundary sealed with Teflon tape, and flame dried using repeated cycles. Reaction paraphernalia (luer lock connectors, needles, cannulas, etc.) as well as accessory glassware (condensers, syringes, vacuum adapters, etc.) were stored in a 150°C oven (minimum 12 h) and cooled under a blanket of dry N₂ immediately prior to use. Syringes possessing Teflon or rubber seals/plungers were dried (24 h) using an evacuated Abderhalden drying apparatus at 56°C (acetone) with potassium hydroxide as the desiccant.

All reactions were carried out under anhydrous, inert atmosphere (nitrogen or argon) with dry, freshly distilled solvents unless otherwise noted. Diethyl ether and THF were distilled from sodium-benzophenone ketyl immediately prior to use. Dichloromethane, triethylamine, benzene, toluene were distilled from calcium hydride immediately prior to use. DMF was stirred for 6 h with calcium oxide, allowed to settle overnight, and then decanted onto activated neutral alumina. It was then distilled under reduced pressure onto activated 4 Å molecular sieves. Methanol was dried by refluxing with magnesium and iodine and then distilled onto activated 4 Å molecular sieves. Unless otherwise stated, all
reagents were purchased from either the Sigma Aldrich Chemical Company or Fisher Scientific and used as supplied.

Cold baths at -78°C were achieved using a slurry of frozen acetone (acetone/dry ice), while cold baths at -50°C and -20°C were prepared using acetonitrile/dry ice and saturated aqueous calcium chloride/dry ice, respectively.

NMR spectra were obtained on either a Varian Mercury 300 (300 MHz) or a Varian Inova 500 (500 MHz) spectrometer and are reported in parts per million (ppm). Multiplicity, coupling constant (Hz), and proton count follow each peak assignment.

Mass spectra were obtained either on a Micromass LCT mass spectrometer (UIUC Mass Spectrometry Facility). Analytical thin layer chromatography (TLC) was performed using silica gel 60 F524 precotated plates (Scientific Adsorbents, Inc.) and then visualized using a 254 nm / 366 nm UV lamp, phosphomolybdic acid, Ninhydrin or an iodine/silica gel mixture. Preparative thin layer chromatography was carried out using Silica Gel GF plates (Analtech, Inc.). Flash chromatography was performed using silica gel 60 (230-400 Mesh, Whatman Inc.). High performance liquid chromatography was performed using Waters 510 pump and gradient controller equipped with Waters 486 tunable UV absorbance detector and 474 scanning fluorescence detectors.
4.2 Experimental procedures for chapter 1

Synthesis of 6-methoxy-3-vinyl-1, 2-dihydronaphthalene 1-23:

Sodium borohydride (7.5 g, 198.3 mmol) was added to a solution of ethanol and water (450 mL, 2:1) containing 2-acetyl-7-methoxy-1-tetralone 1-22 (7 g, 32.1 mmol) over a period of 30 min. The solution was refluxed for 3 h and then the solvent was removed by evaporation and the resulting mixture was extracted with chloroform (3×50 mL). The combined organic layer was dried over MgSO₄ and concentrated in vacuo to give a mixture of the diols (7 g) which was used in the next step without purification.

A mixture of the diols (7 g), dry pyridine (600 mL) and phosphorus oxychloride (5.6 mL) was refluxed for 3 h under nitrogen. The solvent was evaporated under vacuum and the residue was poured into ice-cold 10% H₂SO₄ solution and extracted with ethyl ether (3×200 mL). The organic extracts were washed with sodium bicarbonate solution (100 mL) and brine (100 mL), dried over MgSO₄ and evaporated under reduced pressure to give a residue. The crude product was purified by silica gel column chromatography to afford the title compound 1-23 as a colorless oil (4g, 67%).

TLC (hexanes: ethyl acetate = 19:1): Rf 0.46
$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 2.49 (t, $J = 8.1$ Hz, 2H), 2.83 (t, $J = 8.1$ Hz, 2H), 3.82 (s, 3H), 5.19 (d, $J = 10.8$ Hz, 1H), 5.39 (d, $J = 18$ Hz, 1H), 6.44 (s, 1H), 6.59 (dd, $J = 10.8$, 17.7 Hz, 1H), 6.67-6.73 (m, 2H), 7.07 (d, $J = 8.1$ Hz, 1H).

$^{13}$C NMR (300 MHz, CDCl$_3$): $\delta$ 22.95, 27.10, 55.54, 112.32, 112.49, 128.14, 128.21, 128.50, 135.78, 138.54, 138.71, 158.61.

Data are similar with the one reported in our group’s paper: *Org. Lett.* **2005**, 7, 71.
Synthesis of Diels-Alder adduct 1-24:

Dienophile 1-20 (0.75 g, 4.17 mmol) and diene 1-23 (1.16 g, 6.26 mmol) were dissolved in dry CCl₄ (20 mL) and the solution was refluxed for 72 h. The solvent was then removed in vacuo. The crude product was purified by flash chromatography to afford the title compound 1-24 as a pale yellow solid (0.85 g, 53%).

TLC (hexanes: ethyl acetate = 9:1): Rf 0.25

¹H NMR (300 MHz, CDCl₃): δ 1.05 (m, 1H), 2.01-2.15 (m, 3H), 2.20-2.33 (m, 1H), 2.94 (ddd, J = 7.2, 7.2, 1.8 Hz, 1H), 3.11 (dt, J = 8.1, 1.8 Hz, 1H), 3.84 (d, J = 6.6 Hz, 1H), 3.92 (s, 3H), 4.09 ( t, J = 7.2 Hz, 1H), 5.59-5.62 (m, 1H), 6.15 (s, 1H), 6.90 (s, 2H), 7.12 (s, 1H), 7.31-7.39 (m, 3H), 7.82 ( d, J = 8.7 Hz, 1H), 8.13 (s, 1H).

¹³C NMR (300 MHz, CDCl₃): δ 25.60 , 28.41 , 30.79 , 42.74 , 46.03 , 48.87 , 55.63 , 112.40 , 114.28 , 120.67 , 122.86 , 126.23 , 126.38 , 128.24 , 128.32 , 129.21 , 130.21 , 132.36 , 133.48 , 136.42 , 136.73 , 138.39 , 139.99 , 147.64 , 158.96 , 210.05.

HRMS (ESI), m/z (M+H)^+ : calcd 367.1698, obsd 367.1693.

Data are similar with the one reported in our group’s paper: Org. Lett. 2005, 7, 71.
Synthesis of benzoate ester 1-25:

To a solution of ketone 1-24 (570 mg, 1.56 mmol) in THF (16 mL) and methanol (8 mL) was added sodium borohydride (235 mg, 6.24 mmol) in methanol (8 mL) at 0 °C. The reaction mixture was stirred and allowed to warm to room temperature for 4 h. The solution was diluted with EtOAc (250 mL) and washed with water (2× 100 mL), brine (50 mL) and dried over MgSO$_4$. The solution was concentrated to dryness and the crude product was used in the next step without purification.

to a solution of the above product (630 mg, 1.71 mmol), benzoic acid (420 mg, 3.42 mmol) and triphenylphosphine (898 mg, 3.42 mmol) in THF (30 mL) was added dropwise diisopropyl azodicarboxylate (0.75 mL, 3.42 mmol) at -20 °C under nitrogen. The resulting yellow solution was allowed to warm to room temperature and stirred for overnight. The reaction mixture was then concentrated and the crude product was purified by flash chromatography to provide the title compound 1-25 as a pale yellow solid (615 mg, 76%).

TLC (hexanes: ethyl acetate = 19:1): Rf 0.26

$^1$H NMR (300 MHz, CDCl$_3$): δ 1.90-2.05 (m, 1H), 2.20-2.58 (m, 5H), 3.18-3.26 (m, 1H), 3.88 (s, 3H), 3.97 (br d, J = 4.5 Hz, 1H), 4.39 (t, J = 6.3 Hz, 1H), 5.57 (d, J = 1.5 Hz, 1H), 6.27 (d, J = 2.4 Hz, 1H), 6.29 (s, 1H), 6.89 (dd, J = 8.7, 2.7 Hz, 1H), 7.06 (d, J =
8.7 Hz, 1H), 7.12 (d, J = 3 Hz, 1H), 7.30-7.58 (m, 6H), 7.74 (d, J = 7.5 Hz, 1H), 7.86 (s, 1H), 8.06 (d, J = 7.2 Hz, 2H).

$^{13}$C NMR (300 MHz, CDCl$_3$): δ 26.47, 29.42, 31.96, 41.23, 45.57, 48.93, 55.64, 83.58, 112.04, 114.01, 120.21, 124.27, 125.23, 125.49, 125.89, 128.24, 128.55, 129.11, 129.39, 129.97, 130.71, 130.81, 132.65, 132.95, 133.17, 134.07, 139.07, 139.18, 140.70, 143.14, 158.87, 166.97.

Data are similar with the one reported in our group’s paper: Org. Lett. 2005, 7, 71.
Synthesis of spiroalcohol 1-26:

Osmium tetraoxide (0.3 mL, 4 wt% in water) was added to a solution of ester 1-25 (570 mg, 1.21 mmol) and 4-methylmorpholine N-oxide (156 mg, 1.33 mmol) in THF (24 mL), acetone (3 mL), water (3 mL) and t-butyl alcohol (0.6 mL). The resulting mixture was stirred for 12h at room temperature. The reaction was quenched with sodium dithionite (1 g) in water. Then the reaction mixture was diluted with EtOAc, washed with water (2×50 mL), dried over MgSO$_4$ and condensed in vacuo to give a residual solid (610 mg), which was used in the next step directly.

The crude product (610 mg, 1.20 mmol) and sodium periodate (590 mg, 2.76 mmol) were dissolved in THF (34 mL). H$_2$SO$_4$ (0.84 mL, 5 wt% in water) was added and the reaction mixture was stirred at room temperature for 2h. The reaction mixture was diluted with EtOAc, washed with water (2×50 mL), dried over Na$_2$SO$_4$ and condensed in vacuo to afford a solid (607 mg). The crude aldehyde product was used in the next step without purification.

Potassium carbonate (500 mg, 3.61 mmol) was added to a solution cooled to of aldehyde (607 mg, 1.20 mmol) in dry DMF (25 mL) at 0 ºC. The resulting mixture was stirred at 0ºC for 30 min. The reaction solution was diluted with ethyl ether (50mL), washed with
water (2×100mL) and brine (100 mL) and dried over MgSO$_4$. The solution was dried *in vacuo* and purified by flash chromatography to afford the title compound **1-26** as a white solid (377 mg, 62% for 3 steps).

TLC (hexanes: ethyl acetate = 7:3): Rf 0.28  mp =133-136 °C

$^1$H NMR (500 MHz, CDCl$_3$): δ 2.34-2.41 (m, 1H), 2.65-2.76(m, 2H), 2.92-3.12 (m, 3H), 3.22-3.28 (m, 1H), 3.40 (s, 3H), 3.44-3.52 (m, 1H),  4.38 (br s, 1H), 4.58 (d, J =8 Hz, 1H), 6.20 (s, 1H), 6.41 (d, J = 3 Hz, 1H), 6.42 (d, J = 2 Hz, 1H), 6.85 (dd, J = 8, 2.5 Hz, 1H), 7.25 (d, J = 8.5 Hz, 1H), 7.32-7.40 (m, 5H), 7.48-7.52 (m, 1H), 7.76 (d, J = 8 Hz, 1H), 7.97-8.20 (m, 2H).

$^{13}$C NMR (300 MHz, CDCl$_3$): δ 28.00, 36.73, 38.26, 46.32, 50.47, 55.52, 65.92, 75.83, 84.04, 113.88, 114.56, 125.61, 126.08, 126.47, 128.18, 128.33, 128.58, 129.12, 129.94, 130.41, 130.82, 133.31, 134.13, 136.93, 140.20, 158.83, 166.70, 214.20.

HRMS (ESI), m/z (M+Na)$^+$: calcld 527.1834, obsd 527.1838.

Data are similar with the one reported in our group’s paper: *Org. Lett.* **2005**, 7, 71.
Synthesis of mesylate 1-26’:

TEA (120 μL, 0.86 mmol) and methanesulfonyl chloride (60 μL, 0.72 mmol) were added sequentially to a solution of alcohol 1-26 (145 mg, 0.29 mmol) in dry CH₂Cl₂ (3 mL) at 0 °C. The reaction mixture was stirred for 2 h and diluted with CH₂Cl₂ (25 mL), washed with saturated NaHCO₃ (5 mL), brine (5 mL) and dried over MgSO₄. The solution was concentrated in vacuo to give a crude oil which was purified by flash chromatography to afford the title compound as a yellow oil (145 mg, 87%).

TLC (hexane: ethyl acetate = 7: 3): Rf 0.28

¹H NMR (500 MHz, CDCl₃): δ 2.56-2.61 (m, 1H), 2.71-2.81 (m, 2H), 2.88 (m, 3H), 2.90-2.95 (m, 1H), 3.06-3.17 (m, 2H), 3.23 (s, 3H), 3.28-3.35 (m, 1H), 3.43-3.48 (m, 1H), 4.55 (d, J = 8.5 Hz, 1H), 5.31 (t, J = 8.5 Hz, 1H), 6.15 (d, J = 3 Hz, 1H), 6.24 (s, 1H), 6.48(d, J = 2.5 Hz, 1H), 6.82 (dd, J = 8.5, 2.5 Hz, 1H), 7.24 (d, J = 8.5 Hz, 1H), 7.36-7.43 (m, 5H), 7.55 (t, J = 7.5 Hz, 1H), 7.80 (d, J = 8.5 Hz, 1H), 7.98-8.03 (m, 2H).

¹³C NMR (300 MHz, CDCl₃): δ 27.95, 34.50, 39.06, 39.76, 47.56, 51.85, 55.33, 65.13, 83.70, 84.17, 125.70, 126.23, 126.26, 126.63, 128.08, 128.34, 128.62, 129.49, 129.96, 130.26, 130.58, 133.40, 133.45, 134.09, 135.46, 139.32, 140.02, 158.40, 166.72, 209.59.
Synthesis of alkene 1-27:

Lithium bromide (108 mg, 1.24 mmol) and lithium carbonate (110 mg, 1.49 mmol) were added to a solution of mesylate 1-26' (145 mg, 0.25 mmol) in dry DMF (20 mL). The reaction mixture was refluxed for 1 h at 150 °C. The solution was cooled to room temperature and diluted with ethyl ether (200 mL), washed with 5% HCl (20 mL), saturated NaHCO$_3$ (20 mL) and brine (20 mL). The solution was concentrated *in vacuo* to give a crude oil which was purified by flash chromatography to afford the title compound 1-27 as a yellow solid (63 mg, 52%).

TLC (hexanes: ethyl acetate = 4:1): R$_f$ 0.26  
mp = 85-87 °C

$^1$H NMR (500 MHz, CDCl$_3$): δ 2.75-2.82 (m, 1H), 3.08-3.23 (m, 2H), 3.26 (s, 3H), 3.44-3.51 (m, 1H), 3.98-4.00 (m, 1H), 4.55 (d, J =7 Hz, 1H), 5.68 (dd, J = 5.5, 2.5 Hz, 1H), 5.98 (d, J = 2.5 Hz, 1H), 6.44 (dd, J = 5.5, 2.5 Hz, 1H), 6.57 (s, 1H), 6.79 (dd, J = 8.5, 2.5 Hz, 1H), 7.28-7.42 (m, 6H), 7.52-7.55 (m, 1H), 7.77 (d, J =8.5 Hz, 1H), 8.00-8.04 (m, 3H).

$^{13}$C NMR (300 MHz, CDCl$_3$): δ 27.84, 38.39, 54.45, 55.39, 58.42, 69.96, 80.21, 113.83, 115.66, 125.80, 125.94, 126.23, 128.10, 128.19, 128.54, 128.61, 129.34, 129.92, 130.47, 133.17, 133.24, 133.46, 134.04, 135.79, 138.71, 139.57, 144.56, 158.52, 166.71, 211.32.

HRMS (ESI), m/z (M+Na)$^+$: calcd 509.1729, obsd 509.1722.
Synthesis of enone 1-28:

Pyridinium tribromide (43.6 mg, 0.13 mmol) was added to a solution of alkene 1-27 (63 mg, 0.13 mmol) in THF (3mL). The reaction mixture was stirred at room temperature for 15 min. The reaction was quenched by adding a mixture of saturated NaHCO$_3$ and 0.1 N Na$_2$S$_2$O$_3$ solutions (1:1, 0.8 mL). The resulting mixture was diluted with ethyl ether (3mL), washed with water (5 mL), brine (5 mL) and dried over MgSO$_4$. The reaction was dried *in vacuo* to give a solid (70 mg), which was used in the next step without purification.

DBU (74 µL, 0.49 mmol) was added to a 5 mL round bottom flask containing the crude product (70 mg, 0.12 mmol) in CH$_2$Cl$_2$ (1.5 mL). The reaction mixture was stirred at room temperature for 1h and then was diluted with CH$_2$Cl$_2$, washed with 5% HCl (5 mL), brine (5 mL) and dried over MgSO$_4$. The solvent was removed *in vacuo*. The crude product was purified by flash chromatography to give the title compound 1-28 as a yellow solid (51 mg, 88% for 2 steps).

TLC (hexanes: ethyl acetate = 4: 1): Rf 0.23

$^1$H NMR (500 MHz, CDCl$_3$): δ 3.20 (s, 3H), 4.04-4.07 (m, 1H), 4.60 (dd, J =8, 1 Hz, 1H), 5.58 (dd, J = 5.5, 2 Hz, 1H), 5.93 (d, J = 3 Hz, 1H), 6.27 (d, J = 9.5 Hz, 1H), 6.42 (s, 1H), 6.56 (d, J = 2 Hz, 1H), 6.63-6.66 (m, 2H), 7.28 (d, J = 8 Hz, 1H), 7.34-7.43 (m,
5H), 7.52-7.56 (m, 1H), 7.59 (d, J = 9.5 Hz, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.93 (s, 1H),
8.02-8.05 (m, 2H).

$^{13}$C NMR (300 MHz, CDCl$_3$): $\delta$ 55.23, 58.53, 58.91, 69.72, 80.79, 113.59, 116.40, 122.13,
123.59, 125.38, 128.83, 125.90, 126.26, 127.92, 128.24, 128.58, 129.94, 130.35, 130.68,
132.49, 133.26, 133.31, 133.89, 137.23, 139.71, 140.29, 143.45, 145.80, 160.69, 166.83,
202.59.

Data are similar with the one reported in our group’s paper: *Org. Lett.* **2005**, 7, 71.
Synthesis of the aglycon 1-29:

Aqueous sodium hydroxide (0.5 M, 1.2 mL) was added to a solution of enone 1-28 (53 mg, 0.11 mmol) in THF and MeOH (2.4 mL, 1:1). The resulting mixture was stirred at room temperature for 1 h. It was diluted with ethyl ether (5 mL), washed with water (10 mL), brine (10 mL) and dried over MgSO$_4$. The solvent was removed in vacuo and the crude product was purified by flash chromatography to give the title compound 1-29 as a pale yellow solid (41 mg, 100%).

TLC (hexanes: ethyl acetate = 1: 1): Rf 0.42 mp = 105-106°C

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 3.15 (s, 3H), 3.74-3.80 (m, 1H), 4.42 (d, J = 7.5 Hz, 1H), 5.27 (d, J = 2.4 Hz, 1H), 5.46 (dd, J = 5.7, 2.4 Hz, 1H), 5.82 (d, J = 2.7 Hz, 1H), 6.19 (d, J = 10.2 Hz, 1H), 6.33 (s, 1H), 6.47 (dd, J = 5.7, 2.4 Hz, 1H), 6.57 (dd, J = 8.7, 2.4 Hz, 1H), 7.21 (d, J = 8.7 Hz, 1H), 7.28-7.38 (m, 3H), 7.51 (d, J = 10.2 Hz, 1H), 7.71 (d, J = 7.2 Hz, 1H), 7.76 (s, 1H).

$^{13}$C NMR (300 MHz, CDCl$_3$): $\delta$ 55.17, 58.74, 61.35, 69.56, 79.01, 113.30, 116.32, 122.10, 123.60, 123.86, 125.70, 125.91, 127.94, 128.02, 130.62, 131.83, 133.39, 133.71, 137.66, 139.51, 143.61, 143.78, 145.77, 160.58, 202.94.

HRMS (ESI), m/z (M+H)$^+$: calcd 381.1490, obsd 381.1485.

Data are similar with the one reported in our group’s paper: Org. Lett. 2005, 7, 71.
Synthesis of Toluene-4-sulfonic acid 6-benzyloxy-5-benzyloxy carbonylamino-3, 4-dihydroxy-tetrahydro-pyran-2-ylmethyl ester 1-32:

4(dimethylaminopyridine (8 mg, 0.065 mmol) and p-toluenesulfonyl chloride (270 mg, 1.42 mmol) were added sequentially to a solution of compound 1-31 (460 mg, 1.14 mmol) in anhydrous pyridine (8.5 mL) at 0 °C. The reaction mixture was allowed to stand at room temperature overnight. The reaction mixture was diluted with ethyl acetate (100 mL) and washed with 3 N aqueous hydrochloric acid (3×10 mL) and saturated sodium chloride (20 mL). The organic layer was dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography to afford the title compound 1-32 as a white solid. (439 mg, 69%)

TLC (hexanes: ethyl acetate = 1: 2): Rf 0.53

1H NMR (500 MHz, CDCl₃): δ 2.46 (s, 3H), 2.81 (bs, 1H), 3.65 (br s, 1H), 3.80 (d, J = 10 Hz, 1H), 3.91 (s, 1H), 4.00-4.08 (m, 2H), 4.22 (t, J = 10 Hz, 1H), 4.28 (dd, J = 10, 5 Hz, 1H), 4.42 (d, J = 11.5 Hz, 1H), 4.68 (d, J = 11.5 Hz, 1H), 4.90 (d, J = 4 Hz, 1H), 5.07-5.17 (m, 3H), 7.27-7.39 (m, 12H), 7.82 (d, J = 8.5 Hz, 2H).
Synthesis of (2-Benzylxylo-4, 5-dihydroxy-6-iodomethyl-tetrahydro-pyran-3yl)-
carbamic acid benzyl ester 1-33:

Sodium iodide (970 mg, 6.5 mmol) was added to a solution of compound 1-32 (360 mg, 0.65 mmol) in 1, 2-dimethoxyethane (7 mL). The reaction mixture was stirred at 85 °C for 12 h. The reaction mixture was cooled to room temperature, diluted by ethyl acetate (150 mL) and washed with brine (50 mL). The organic layer was dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by flash chromatography (first, hexanes: ethyl acetate = 1: 1, then 1: 2) to afford the title compound 1-33 as a white solid. (315 mg, 95%)

TLC (hexanes: ethyl acetate = 1: 2): Rf 0.71

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 3.22 (s, 1H), 3.37-3.39 (m, 1H), 3.81-3.84 (m, 1H), 3.98 (t, J = 7 Hz, 1H), 4.06-4.09 (m, 2H), 4.52 (d, J = 12 Hz, 1H), 4.84 (d, J = 11 Hz, 1H), 4.95 (d, J = 4 Hz, 1H), 5.08-5.12 (m, 2H), 5.22 (d, J = 9.5 Hz, 1 H), 7.39 (m, 10 H).

$^{13}$C NMR (300 MHz, CDCl$_3$): $\delta$ 3.49, 51.74, 67.73, 69.76, 69.90, 71.14, 71.47, 96.89, 128.43, 128.50, 128.60, 128.83, 136.10, 136.91, 158.03.
Synthesis of (2-Benzylxoy-6-iodomethyl-4, 5-bis-triethylsilanyloxy-tetrahydro-pyran-3-yl)-carbamic acid benzyl ester 1-34:

To a solution of compound 1-33 (300 mg, 0.59 mmol) in dry DCM (25 mL) at -78°C was added 2, 6-lutidine (417 μL, 3.51 mmol) and triethylsilyl trifluoromethanesulfonate (535 μL, 2.34 mmol) sequentially. The resultant solution was stirred at -78°C for 30 min and then allowed to warm to 0°C over 4 h. Excess triethylsilyl trifluoromethanesulfonate was quenched with methanol (0.5 mL) and the solvent was evaporated in vacuo. The crude product was purified by flash chromatography (hexanes: ethyl acetate = 20: 1) to afford the title compound 1-34 as a colorless oil. (390 mg, 90%)

TLC (hexanes: ethyl acetate = 9: 1): Rf 0.47

$^1$H NMR (500 MHz, CDCl$_3$): δ 0.60-0.80 (m, 12H), 0.93-1.05 (m, 18H), 3.23-3.27 (m, 2H), 3.79 (d, J = 10 Hz, 1H), 3.90 (t, J = 7 Hz, 1H), 4.09 (s, 1H), 4.29(t, J = 18 Hz, 1H), 4.56 (d, J = 12 H, 1H), 4.81(d, J = 11.5 Hz, 2H), 4.90 (d, J = 3.5 Hz, 1H), 5.11(s, 2H), 7.34-7.40 (m, 10 H).

$^{13}$C NMR (300 MHz, CDCl$_3$): δ 4.43, 5.17, 5.53, 7.10, 7.26, 51.00, 67.02, 69.67, 71.25, 73.03, 73.33, 97.70, 128.17, 128.28, 128.35, 128.47, 128.74, 136.73, 137.46, 155.97.

HRMS (ESI), m/z (M+Na)$^+$: calcd 742.2456, obsd 742.2476
Synthesis of (2-Benzylxylo-6-methyl-4, 5-bis-triethylsilanyloxy-tetrahydro-pyran-3-yl)-carbamic acid benzyl ester 1-35:

Tributyltin hydride (426 μL, 1.58mmol) and 2, 2’-azobisisobutyronitrile (14 mg, 0.104 mmol) were added to a solution of compound 1-34 (390 mg, 0.53 mmol) in 1, 2-dimethoxyethane (10 mL) sequentially. The reaction mixture was stirred at 85 °C for 4 h. The reaction mixture was cooled to room temperature and diluted with 1 : 2 ethyl acetate/hexanes (150 mL). The organic layer was washed with water (50 mL) and brine (50 mL) and dried over magnesium sulfate. The solution was concentrated in vacuo and the crude residue was purified by flash chromatography (hexanes grading to 4% ethyl acetate in hexanes) to afford the title compound 1-35 as a colorless oil. (307 mg, 95%)

TLC (hexanes: ethyl acetate = 9: 1): Rf 0.42

$^1$H NMR (500 MHz, CDCl$_3$): δ 0.62-0.76 (m, 12H), 0.94-1.06 (m, 18H), 3.71-3.73 (m, 1H), 3.81-3.89 (m, 2H), 4.32 (dt, J = 11, 7 Hz, 1H), 4.50 (d, J = 12 Hz, 1H), 4.70 (d, J = 12.5 Hz, 1H), 4.83 (d, J = 10.5 Hz, 1H), 4.88-4.91 (m, 1H), 5.11 (d, J = 2.5 Hz, 2H), 7.24-7.41 (m, 10H).

$^{13}$C NMR (300 MHz, CDCl$_3$): δ 5.20, 5.51, 7.11, 7.22, 17.20, 51.16, 66.91, 68.14, 69.48, 71.51, 75.10, 98.26, 127.95, 128.28, 128.45, 128.66, 136.85, 137.98, 156.02.
Synthesis of (2-Benzylxy-6-methyl-4, 5-bis-triethylsilanyloxy-tetrahydro-pyran-3-yl)-methyl-carbamic acid benzyl ester 1-36:

To a solution of 1-35 (304 mg, 0.49 mmol) in dry THF (6 mL) was added NaH (60% dispersion in mineral oil, 200 mg, 4.9 mmol), followed by iodomethane (0.93 mL, 14.7 mmol). The reaction mixture was stirred at 40 °C for 30 min and then cooled to room temperature. It was then diluted with ethyl acetate and hexanes (120 mL, 1: 2). The organic layer was washed with phosphat buffer (0.5 M, pH 7.0, 2×25 mL), dried over MgSO₄ and concentrated in vacuo. The residue was purified by flash chromatography (hexanes: ethyl acetate = 20: 1) to afford the title compound 1-36 as a colorless oil. (290 mg, 93%)

TLC (hexanes: ethyl acetate = 9: 1): Rf 0.42

¹H NMR (500 MHz, CDCl₃): N-diastereomeric mixture (rotamers) as shown.

¹³C NMR (300 MHz, CDCl₃): N-diastereomeric mixture (rotamers) as shown.

HRMS (ESI), m/z (M+Na)⁺: calcd 652.3466, obsd 652.3457.
Synthesis of 6-methyl-3-methylamino-4, 5-bis-triethylsilyloxy-tetrahydro-pyran-2-ol 1-37:

Palladium hydroxide on activated carbon (300 mg, 20% w/w Pd) was added to a solution of 1-36 (290 mg, 0.46 mmol) in dry ethyl acetate (10 mL). The flask was flushed with argon before a hydrogen balloon was mounted to the top of the flask. The flask was briefly evacuated and filled with hydrogen, and this process was repeated three times. The mixture was stirred at room temperature for 12 h and then filtered through a plug of Celite®. The filtrate was concentrated in vacuo and the crude product was purified by flash chromatography (hexanes: ethyl acetate = 7: 3 with 4% (v/v) triethylamine) to afford the title compound 1-37 as a colorless oil. (166 mg, 89%)

TLC (hexanes: ethyl acetate = 7: 3 with 4% v/v triethylamine): Rf 0.26

$^1$H NMR (500 MHz, CDCl$_3$): 0.63-0.71 (m, 12H), 0.96-1.01 (m, 18H), 1.19 (d, J = 7 Hz, 3H), 2.41 (s, 3H), 2.89 (dd, J = 10, 3 Hz, 1H), 3.60 (d, J = 1 Hz, 1H), 3.74 (dd, J = 9.5, 2.5 Hz, 1H), 4.01 (q, J = 6.5 Hz, 1H), 5.26 (d, J = 3.5 Hz, 1H).

HRMS (ESI), m/z (M+Na)$^+$: calcd 406.2809, obsd 406.2805.

Data are similar with the one reported by Myers, et al. *JACS*, 2002, 124(19), 5380.
Synthesis of N-fmoc-N-methylaminolactol 1-40:

K$_2$CO$_3$ (63 mg, 0.46 mmol) and Fmoc-Cl (59 mg, 0.21 mmol) were added sequentially to a solution of compound 1-37 (92 mg, 0.21 mmol) in THF and water (2 mL, 2:1). The reaction mixture was stirred at room temperature for 30 min. The solution was diluted with water (1 mL) and extracted with diethyl ether (3×20 mL). The organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by flash chromatography (hexanes: ethyl acetate = 6:1 to 7:3) to afford the title compound 1-40 as a white solid. (122 mg, 99%)

TLC (hexanes: ethyl acetate = 7:3): Rf 0.28

$^1$H NMR (500 MHz, CDCl$_3$): N-diastereomeric mixture (rotamers) as shown.

$^{13}$C NMR (300 MHz, CDCl$_3$): N-diastereomeric mixture (rotamers) as shown.

HRMS (ESI), m/z (M+Na)$^+$: calcd 628.3490, obsd 628.3498.
**Synthesis of N-fmoc-N-methylaninolactol trichloroacetimidate:**

A flame dried 10 mL round bottom flask was charged into the drybox with NaH (95%, 4 mg, 0.156 mmol), brought out, and cooled to 0°C. A solution of the aminolactol 1-40 (122 mg, 0.195 mmol) in dry dichloromethane (1 mL) was added. The resultant suspension was held at 0°C for 10 min. Trichloroacetonitrile (563 mg, 3.9 mmol) was added, the reaction mixture was allowed to warm to 23°C and held for 30 min. The reaction mixture was filtered through a layer of Celite®, and the filtrate was concentrated *in vacuo* to afford a white solid (140 mg) which was used in the next step without purification.
Synthesis of glycoside 1-42a & 1-42b:

A heterogeneous mixture of glycosyl acceptor 1-29 (10 mg, 0.026 mmol) and crude trichloroimidate 1-41 (140 mg, ~0.156 mmol) in anhydrous toluene (1.5 mL) with 3 Å molecular sieves (60 mg) was stirred at room temperature for 30 min and then was cooled to –30 °C. A solution of boron trifluoride etherate (12 μL, 0.104 mmol) in dry toluene (600 μL) was added in 6 portions at 10 min intervals. The reaction was stirred at –30 °C for 4 h then quenched by solid sodium bicarbonate (25 mg). The reaction mixture was filtered through a plug of cotton. The filtrate was concentrated in vacuo to dryness and the crude product was used for next step without purification.

DBU (16 μL, 0.104 mmol) was added to a solution of the crude product in dry CH₂Cl₂ (3.2 mL) and the resulting solution was stirred at room temperature for 1h. The reaction was quenched with phosphate buffer (pH = 5.5, 0.2M, 0.41 mL). The solution was diluted with CH₂Cl₂ (10 mL) and organic layer was washed with brine (2.5 mL) and concentrated
in vacuo to give a residue which was purified by preparative TLC (hexanes: ethyl acetate = 3:1) to afford compound 1-42a (7.6 mg, 38% for 2 steps) and 1-42b (7 mg, 35% for 2 steps) as yellow oils.

Compound 1-42a:

TLC (hexanes: ethyl acetate = 1 : 3): Rf 0.13.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 0.62-0.72 (m, 12H), 0.94-1.06 (m, 18H), 1.34 (d, J = 6 Hz, 3H), 2.02 (m, 1H), 2.83 (s, 3H), 3.17 (s, 3H), 3.63-3.81 (m, 3H), 4.11 (d, J = 7.5 Hz, 2H), 4.16 (dd, J = 10.5, 2.5 Hz, 1H), 4.49 (d, J = 8 Hz, 1H), 5.41 (s, 1H), 5.47 (dd, J = 8, 2 Hz, 1H), 5.81 (s, 1H), 6.22 (d, J = 9.5 Hz, 1H), 6.46-6.51 (m, 3H), 7.15 (d, J = 8.5 Hz, 1H), 7.28-7.35 (m, 4H), 7.53 (d, J = 9.5 Hz, 1H), 7.83 (d, J = 5 Hz, 1H), 8.32 (s, 1H).

MS (ESI), m/z (M+Na)$^+$: calcd 790.39, obsd 790.39.

Compound 1-42b:

TLC (hexanes: ethyl acetate = 1 : 3): Rf 0.32.

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 0.62-0.72 (m, 12H), 0.94-1.06 (m, 18H), 1.29 (d, J = 6 Hz, 3H), 2.53 (s, 3H), 2.79 (m, 1H), 3.19 (s, 3H), 3.55-3.66 (m, 4H), 4.03 (dd, J = 11, 2.5 Hz, 1H), 4.48 (d, J = 8 Hz, 1H), 5.43 (d, J = 2 Hz, 1H), 5.47 (dd, J = 8, 2 Hz, 1H), 5.81 (d, J = 2 Hz, 1H), 6.22 (d, J = 10 Hz, 1H), 6.44 (s, 1H), 6.53 (dd, J = 11, 3 Hz, 2H), 7.19 (d, J = 8 Hz, 1H), 7.24-7.34 (m, 4H), 7.53 (d, J = 10 Hz, 1H), 7.70 (d, J = 7 Hz, 1H), 7.93 (s, 1H).

MS (ESI), m/z (M+Na)$^+$: calcd 790.39, obsd 790.39.
Synthesis of glycoside 1-43a:

A 10 mL flask was charged with a solution of glycoside 1-42b (7.6 mg, 0.01 mmol) in dry THF (0.7 mL). Hydrogen fluoride- pyridine (65%, 152 μL) was added to the solution. The reaction was held at room temperature for 1 h. The excess acid was quenched with solid sodium bicarbonate (200 mg). The reaction mixture was filtered through a plug of Celite® and concentrated. The residue was purified by preparative TLC plates (ethyl acetate: methanol: water = 9: 2: 1) to give the title compound 1-43a as a colorless oil. (5.3 mg, 100%) TLC (ethyl acetate : methanol : water = 9 : 2 : 1): Rf 0.48

^1^H NMR (500 MHz, CD₃OD): δ 1.45 (d, J = 6 Hz, 3H), 2.54 (s, 3H), 3.04-3.08 (m, 1H), 3.17 (s, 3H), 3.62-3.67 (m, 3H), 4.02 (dd, J = 10.5, 2.5 Hz, 1H), 4.45 (d, J = 8 Hz, 1H), 4.60 (s, 1H), 4.95 (d, J = 8Hz, 1H), 5.41 (d, J = 2Hz, 1H), 5.45 (dd, J = 8, 2 Hz, 1H), 5.80 (d, J = 2.5Hz, 1H), 6.19 (d, J = 9.5 Hz, 1H), 6.43 (s, 1H), 6.49 (dd, J = 7.5, 2.5Hz, 1H), 6.65 (dd, J = 10.5, 2.5Hz, 1H), 7.34-7.40 (m, 4H), 7.75 (d, J = 10Hz, 1H), 7.82 (d, J = 7.5Hz, 1H), 7.91 (s, 1H).

HRMS (ESI), m/z (M+H)^+: calcd 540.2381, obsd 540.2386.
Synthesis of glycoside 1-43b:

A 10 mL flask was charged with a solution of glycoside 1-42b (7 mg, 0.009 mmol) in THF (0.6 mL). Then hydrogen fluoride-pyridine (65%, 140 μL) was added. The reaction mixture was held at room temperature for 1 h. The excess acid was quenched with solid sodium bicarbonate (200mg). The reaction mixture was filtered through a plug of Celite® and concentrated. The residue was purified by preparative TLC plates (ethyl acetate: methanol: water = 9: 2: 1) to give the title compound 1-43b as a colorless oil. (4.8 mg, 100%) TLC (ethyl acetate : methanol : water = 9 : 2 : 1): Rf 0.55

¹H NMR (500 MHz, CD₃OD): δ 1.37 (d, J = 6 Hz, 3H), 2.62 (s, 3H), 2.82-2.86 (m, 1H), 3.20 (s, 3H), 3.64-3.71 (m, 3H), 3.98 (dd, J = 10, 2.5 Hz, 1H), 4.48 (d, J = 7 Hz, 1H), 4.60 (s, 1H), 4.80 (d, J = 8.5Hz, 1H), 5.48 (dd, J = 8, 2 Hz, 1H), 5.53 (d, J = 2.5Hz, 1H), 5.86 (d, J = 2.5Hz, 1H), 6.19 (d, J = 9.5 Hz, 1H), 6.43 (s, 1H), 6.49 (dd, J = 8, 2.5Hz, 1H), 6.64 (dd, J = 11.5, 2.5Hz, 1H), 7.31-7.37 (m, 4H), 7.74 (d, J = 7Hz, 1H), 7.75 (d, J = 10Hz, 1H), 7.90 (s, 1H).

HRMS (ESI), m/z (M+H)⁺: calcd 540.2381, obsd 540.2379.
4.3 Experimental procedures for chapter 2

**Synthesis of Bromodimethylsulfonium Bromide (BDMS)**:

Dimethyl sulfide (1.83 mL, 25 mmol) was dissolved in 5 mL of dry DCM in an 100 mL flask. A solution of bromine (1.2mL, 25 mmol) in dry DCM (5 mL) and added slowly into the above solution at 0°C over 5 min. After the addition of bromine was completed, the orange precipitate was collected by vacuum filtration. The yellow solid was then washed with dry hexane and dried *in vacuo* to give the title compound (4.3 g, 77%).

mp 80-83 °C.

*The procedure used herein is same as the one reported in *J. Org. Chem.* 2006, 71, 8961.*
Synthesis of 1,3-diethyl-8-phenyl-1H-purine-2,6(3H,7H)-dione 2-1:

BDMS (0.25 mmol) was added to a solution of benzaldehyde (53 mg, 0.25 mmol) and 1, 3-diethy-5, 6-aminouracil (25 mg, 0.25 mmol) in dry acetonitrile (5 mL). The reaction mixture was stirred at room temperature. The product formed as precipitate and the reaction was monitored by TLC. After completion of the reaction, the precipitate was filtered, washed with ethyl acetate and then was dried in vacuo. The crude solid was purified by recrystallization with DMSO and water to give the title compound 2-1. (50 mg, 70%)

mp > 300 °C;

$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 1.16 (t, $J = 7$Hz, 3H), 1.26 (t, $J = 7$Hz, 3H), 3.97 (q, $J = 7$Hz, 2H), 4.10 (q, $J = 7$Hz, 2H), 7.44-56 (m, 3H), 8.14 (d, $J = 8$Hz, 2H), 13.90 (br, 1H).

HRMS (ESI), m/z (M+H)$^+$: calcld 284.1273, obsd 284.1277
Synthesis of 8-(4-bromophenyl)-1,3-diethyl-1H-purine-2,6(3H,7H)-dione 2-2:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as yellow solid. (72 mg, 79%)

mp > 300 ºC;

$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 1.13 (t, J = 7Hz, 3H), 1.26 (t, J = 7Hz, 3H), 3.94 (q, J = 7Hz, 2H), 4.08 (q, J = 7Hz, 2H), 7.01 (d, J = 8.5Hz, 2H), 8.05 (d, J = 8.5Hz, 2H), 13.89 (br, 1H).

HRMS (ESI), m/z (M+H)$^+$: calcd 362.0378, obsd 362.0369.
Synthesis of 1,3-diethyl-8-(4-fluorophenyl)-1H-purine-2,6(3H,7H)-dione 2-3:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a white solid. (53 mg, 70%)

mp > 300 °C;

$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 1.14 (t, $J = 7$Hz, 3H), 1.27 (t, $J = 7$Hz, 3H), 3.95 (q, $J = 7.5$Hz, 2H), 4.08 (q, $J = 7$Hz, 2H), 7.37 (m, 2H), 8.18 (m, 2H), 13.85 (br, 1H).

HRMS (ESI), m/z (M+H)$^+$: calcd 302.1179, obsd 302.1159.
Synthesis of 1,3-diethyl-8-(4-nitrophenyl)-1H-purine-2,6(3H,7H)-dione 2-4:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid. (57 mg, 79%)

mp > 300 °C;

$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 1.15 (t, J = 7Hz, 3H), 1.29 (t, J = 7Hz, 3H), 3.96 (q, J = 7.5Hz, 2H), 4.11 (q, J = 7Hz, 2H), 8.37 (s, 2H)

HRMS (ESI), m/z (M+H)$^+$: calcd 329.1124, obsd 329.1124
Synthesis of 1,3-diethyl-8-(2-nitrophenyl)-1H-purine-2,6(3H,7H)-dione 2-5:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid. (59 mg, 72%)

mp > 300 °C;

$^1$H NMR (500 MHz, DMSO-d6): δ 1.15 (t, J = 7Hz, 3H), 1.21 (t, J = 7Hz, 3H), 3.95 (q, J = 7.5Hz, 2H), 3.99 (q, J = 7.5Hz, 2H), 7.74-7.76 (m, 1H), 7.83-7.86 (m, 1H), 7.93 (d, J = 7.5Hz, 1H), 8.03 (d, J = 8Hz, 1H)

HRMS (ESI), m/z (M+H)$^+$: calcd 329.1124, obsd 329.1111.
Synthesis of 1,3-diethyl-8-(4-methoxyphenyl)-1H-purine-2,6(3H,7H)-dione 2-6:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The product was afforded as a yellow solid. (56 mg, 71%)

mp > 300 °C;

\(^1\)H NMR (500 MHz, DMSO-d6): \(\delta 1.14 (t, J = 7\text{Hz}, 3\text{H}), 1.27 (t, J = 7\text{Hz}, 3\text{H}), 3.82 (s, 1\text{H}), 3.95 (q, J = 7\text{Hz}, 2\text{H}), 4.09 (q, J = 7.5\text{Hz}, 2\text{H}), 7.06 (d, J = 9\text{Hz}, 1\text{H}), 8.08 (d, J = 9\text{Hz}, 1\text{H}), 13.61 (br, 1\text{H})\)

HRMS (ESI), m/z (M+H)\(^+\): calcd 314.1379, obsd 314.1392.
Synthesis of 8-(4-(dimethylamino) phenyl)-1,3-diethyl-1H-purine-2,6(3H,7H)-dione 2-7:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid (53 mg, 66%).

mp > 300 °C

$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 1.14 (t, J = 7Hz, 3H), 1.27 (t, J = 7Hz, 3H), 2.98 (s, 3H), 3.33 (s, 3H), 3.94 (q, J = 7Hz, 2H), 4.08 (q, J = 7.5Hz, 2H), 6.77 (d, J = 9.5Hz, 1H), 7.96 (d, J = 9.5Hz, 1H), 13.35 (br, 1H)

HRMS (ESI), m/z (M+H)$^+$: calcd 327.1695, obsd 327.1678.
Synthesis of 8-(3,4-dimethoxyphenyl)-1,3-diethyl-1H-purine-2,6(3H,7H)-dione 2-8:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid (54mg, 63%).

mp > 300 ºC ;

$^1$H NMR (500 MHz, DMSO-d6): δ 1.14 (t, $J = 7.5$Hz, 3H), 1.27 (t, $J = 7.5$Hz, 3H), 3.82 (s, 3H), 3.84 (s, 3H), 3.95 (q, $J = 7.5$Hz, 2H), 4.09 (q, $J = 7$Hz, 2H), 7.08 (d, $J = 8$Hz, 1H), 7.71-7.74 (m, 2H)

HRMS (ESI), m/z (M+H)$^+$: calcd 344.1485, obsd 344.1475.
Synthesis of 1,3-diethyl-8-(naphthalen-2-yl)-1H-purine-2,6(3H,7H)-dione 2-9:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid (61mg, 74%).

mp > 300 °C ;

$^1$H NMR (500 MHz, DMSO-d6): δ 1.16 (t, J = 7.5Hz, 3H), 1.31 (t, J = 7.5Hz, 3H), 3.97 (q, J = 6.5Hz, 2H), 4.14 (q, J = 7Hz, 2H), 7.08 (d, J = 8Hz, 1H), 7.58-7.61 (m, 2H), 7.96-7.99 (m, 1H), 8.01-8.06 (m, 2H), 8.25 (dd, J = 10, 2Hz, 1H), 8.73 (s, 1H), 14.01 (br, 1H)

HRMS (ESI), m/z (M+H)$^+$: calcd 334.1430, obsd 334.1449.
Synthesis of (E)-1,3-diethyl-8-(4-methoxystyryl)-1H-purine-2,6(3H,7H)-dione 2-10:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid (54mg, 63%).

mp > 300 ºC; 

$^1$H NMR (500 MHz, DMSO-d6): δ 1.13 (t, J = 7.5Hz, 3H), 1.25 (t, J = 7.5Hz, 3H), 3.79 (s, 1H), 3.93 (q, J = 7Hz, 2H), 4.06 (q, J = 7Hz, 2H), 6.89 (d, J = 17Hz, 1H), 6.98 (d, J = 9Hz, 1H), 7.56-7.62 (m, 2H), 13.46 (br, 1H)

HRMS (ESI), m/z (M+H)$^+$: calcd 340.1535, obsd 340.1521.
Synthesis of 1,3-diethyl-8-(2-hydroxyphenyl)-1H-purine-2,6(3H,7H)-dione 2-11:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid (50mg, 66%).

mp > 300 °C ;

$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 1.14 (t, J = 7Hz, 3H), 1.27 (t, J = 7Hz, 3H), 3.95 (q, J = 7Hz, 2H), 4.08 (q, J = 7Hz, 2H), 6.62-6.98 (m, 2H), 7.30-7.33(m, 1H), 8.06 (d, J = 7.5Hz, 1H).

HRMS (ESI), m/z (M+H)$^+$: calcd 300.1222, obsd 300.1220.
Synthesis of 2-(1,3-diethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)benzoic acid 2-12:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a yellow solid (56mg, 68%).

mp > 300 ºC;

$^1$H NMR (300 MHz, DMSO-d6): $\delta$ 1.07 (t, J = 7Hz, 3H), 1.14 (t, J = 7Hz, 3H), 3.80 (q, J = 7.5Hz, 2H), 3.92 (q, J = 7Hz, 2H), 7.32 (d, J = 7.2Hz, 1H), 7.41-7.54 (m, 2H), 8.86 (d, J = 7.2Hz, 1H), 8.80 (s, 1H)

HRMS (ESI), m/z (M+H)$^+$: calcd 328.1172, obsd 328.1188.
Synthesis of 1,3-diethyl-8-(furan-2-yl)-1H-purine-2,6(3H,7H)-dione 2-13:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a white solid (41mg, 60%).

mp > 300 °C;

$^1$H NMR (500 MHz, CDCl$_3$): δ 1.32 (t, J = 7Hz, 3H), 1.40 (t, J = 7Hz, 3H), 4.18 (q, J = 7Hz, 2H), 4.26 (q, J = 7Hz, 2H), 6.59-6.60 (m, 1H), 7.24-7.26 (m, 1H), 7.58-7.59 (m, 1H), 11.58 (br, 1H)

HRMS (ESI), m/z (M+H)$^+$: calcd 274.1066, obsd 274.1085.
Synthesis of 8-cyclohexyl-1,3-diethyl-1H-purine-2,6(3H,7H)-dione 2-14:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a white solid (49mg, 68%).

mp > 300 °C;

\(^1\)H NMR (500 MHz, DMSO-d6): δ 1.10 (t, J = 7Hz, 3H), 1.20 (t, J = 7Hz, 3H), 1.27-1.36 (m, 2H), 1.50-1.58 (m, 2H), 1.62-1.68 (m, 2H), 1.72-1.78 (m, 2H), 1.86-1.90 (m, 2H), 2.69-2.75 (m, 1H), 3.90 (q, J = 7Hz, 2H), 3.99 (q, J = 7Hz, 2H), 13.05 (br, 1H)

HRMS (ESI), m/z (M+H)^+: calcd 290.1740, obsd 290.1755.
Synthesis of 1,3-diethyl-8-pentyl-1H-purine-2,6(3H,7H)-dione 2-15:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a white solid (47mg, 68%).

mp > 300 °C;

$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 0.85 (t, J = 7Hz, 3H), 1.10 (t, J = 7.5Hz, 3H), 1.20 (t, J = 7Hz, 3H), 1.21-1.31 (m, 4H), 1.64-1.70 (m, 2H), 2.65 (t, J = 7.5Hz, 2H), 3.90 (q, J = 7Hz, 2H), 4.00 (q, J = 7Hz, 2H)

HRMS (ESI), m/z (M+H)$^+$: calcd 278.1743, obsd 278.1744.
Synthesis of (E)-1,3-diethyl-8-(pent-1-enyl)-1H-purine-2,6(3H,7H)-dione 2-16:

The experimental procedure was the same as the one used for the synthesis of compound 2-1. The title compound was afforded as a white solid (48mg, 70%).

mp > 300 °C;

$^1$H NMR (500 MHz, DMSO-d6): δ 0.91 (t, $J = 7$Hz, 3H), 1.11 (t, $J = 7.5$Hz, 3H), 1.21 (t, $J = 7$Hz, 3H), 1.40-1.52 (m, 2H), 1.17-1.24 (m, 2H), 3.91 (q, $J = 7$Hz, 2H), 4.01 (q, $J = 7$Hz, 2H), 6.28 (d, $J = 15.9$Hz, 1H), 6.76-6.87 (m, 1H)

HRMS (ESI), m/z (M+H)$^+$: calcd 276.1586, obsd 276.1602.
4.4 Experimental procedures for chapter 3

Synthesis of 1,3-diethyl-6-aminouracil 3-1:

Cyanoacetic acid (8.2 g, 0.095 mol) was added to a solution of acetic anhydride (25 mL) containing N, N-diethylurea (10.3 g, 0.086 mol). The solution was stirred at 70°C for 2 h. The excess acetic anhydride was distilled off under reduced pressure to give an oily residue. The residue was treated with 10% aqueous sodium hydroxide (370 mL) and stirred at room temperature for 1 hour, then at 0°C for 2 hours. The precipitate was collected via vacuum filtration, washed thoroughly with cold water, and dried in vacuo.

The crude product which was purified by recrystallization from ethyl acetate to give the title compound 3-1 as a white solid. (14.9 g, 97%)

mp = 134-136°C;

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 1.19 (t, J = 7Hz, 3H), 1.31 (t, J = 7Hz, 3H), 3.91-4.00 (m, 4H), 4.97 (s, 1H), 5.20 (br, 2H)

$^{13}$C NMR (300 MHz, CD$_3$OD): $\delta$ 12.09, 12.37, 35.82, 37.72, 75.69, 151.54, 155.53, 163.96.
Synthesis of 1,3-diethyl-6-amino-5-nitrosoouracil 3-2:

NaNO₂ (3.04g, 0.044mol) was added in small portions to a solution of 1,3-diisobutyl-4-aminouracil 3-1 (7.3g, 0.04mol) in water (90 mL) and acetic acid (4.85 mL) at 5°C. The reaction mixture was stirred at room temperature for 2h. The mixture was cooled to 0°C and stirred for an additional hour. The precipitate was collected by vacuum filtration, washed with cold water and dried in vacuo to give the title compound as a deep purple solid (7.6g, 95%) which was used in the next step without further purification.

mp = 205-208 °C;

¹H NMR (300 MHz, DMSO-d6): δ 1.12-1.22 (m, 6H), 3.87-4.00 (m, 4H), 9.21 (br, 2H)

¹³C NMR (300 MHz, DMSO-d6): δ 12.68, 13.63, 36.73, 37.33, 139.69, 145.95, 149.27, 160.43.
Synthesis of 1,3-diethyl-5, 6-diaminouracil 3-3:

Sodium dithionite (14.34g, 0.07mol) was added in small portions to a solution of 1,3-diisobutyl-4-amino-5-nitrosouracil 3-2 (4.24g, 0.02mol) in 25% aqueous ammonia (30 mL). The reaction mixture was stirred at 50°C for 1 hour and then allowed to stand overnight at room temperature. The reaction mixture was cooled in an ice-bath and the formed precipitate was collected via vacuum filtration, washed with cold water and dried in vacuo. The crude product was recrystallized from CH₂Cl₂ and diethyl ether to give the title compound as a white solid. (3.23g, 85%)

mp = 101-104 °C;

¹H NMR (300 MHz, CDCl₃): δ 1.05-1.17 (m, 6H), 3.80-3.95 (m, 4H), 2.95 (br, 2H), 6.25 (br, 2H)

¹³C NMR (300 MHz, CDCl₃): δ 13.91, 13.99, 36.01, 38.02, 96.62, 144.70, 149.65, 159.23.

HRMS (ESI), m/z (M+H)⁺: calcd 198.1117, obsd 198.1119.
Synthesis of \( (E) \)-N-(6-Amino-1, 3-diethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-3-(3,4-dimethoxyphenyl) acrylamide 3-4:

\( (E) \)-3,4-dimethoxycinnamic acid (6.1 g, 0.03 mol) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (7.8 g, 0.04 mol) were added to a solution of 1,3-diethyl-5, 6-diaminouracil (6g, 0.03mol) in dioxane (90 mL) and water (180 mL). The reaction mixture was stirred at room temperature for 2 hours at pH 5. The mixture was extracted with chloroform (3×250 mL) and the organic extracts were washed with brine (100 mL), dried with MgSO\(_4\) and evaporated \textit{in vacuo}. The resultant residue was purified via flash column chromatography to afford the title compound 3-4 as a yellow solid (5.9g, 51%).

TLC (MeOH: CH\(_2\)Cl\(_2\) = 1:9): Rf 0.43

\(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta \) 1.16 (t, \( J = 7.25\)Hz, 3H), 1.28 (t, \( J = 7.25\)Hz, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 3.94 (m, 4H), 5.72 (s, 2H), 6.54 (d, \( J = 15.4\)Hz, 1H), 6.79 (d, \( J = 8.5\)Hz, 1H), 6.97 (d, \( J = 1.6\)Hz, 1H), 7.02 (dd, \( J = 8.5,1.6\)Hz, 1H), 7.51 (d, \( J = 15.4\)Hz, 1H), 8.07 (s, 1H)

\(^13\)C NMR (300 MHz, CDCl\(_3\)): \( \delta \) 13.1, 13.3, 36.9, 38.3, 55.8, 55.9, 92.4, 109.7, 111.0, 117.6, 122.2, 127.5, 141.9, 149.1, 149.7, 150.8, 159.8, 165.7.

HRMS (ESI), m/z (M+H)+: calcd 388.1747, obsd 388.1766
Synthesis of (E)-8-[2-(3, 4-Dimethoxyphenyl) vinyl]-1, 3-diethyl-3, 7-dihydropurine-2, 6-Dione 3-5:

An aqueous solution of NaOH (1N, 75 mL) was added to a solution of compound 3-4 (5.76 g, 15 mol) in dioxane (50 mL). The clear yellowish solution was refluxed for 1 hour and then acidified to pH 2 by the dropwise addition of concentrated HCl. The reaction was extracted with chloroform (3×200 mL) and the organic extracts were washed with water (100 mL) and brine (100 mL), dried with MgSO₄ and evaporated in vacuo. The residue was purified via flash chromatography to provide the title compound 3-5 as a yellow solid. (3.39g, 60%)

mp=268-269 °C; TLC (MeOH: CH₂Cl₂ = 05:9.5): Rf 0.63

¹H NMR (500 MHz, CDCl₃): δ 1.30 (t, J = 7.3 Hz, 3H), 1.39 (t, J = 7.3 Hz, 3H), 3.91 (s, 6H), 4.15-4.34 (m, 4H), 6.94 (d, J = 16.2 Hz, 1H), 6.86-7.12 (m, 3H), 7.73 (d, J = 16.2 Hz, 1H), 13.00 (s, 1H)

¹³C NMR (300 MHz, CDCl₃): δ 13.4, 13.5, 36.9, 39.0, 55.8, 56.0, 107.3, 109.1, 111.2, 113.4, 121.2, 128.6, 136.8, 149.3, 149.6, 150.4, 150.5, 151.7, 155.6.

HRMS (ESI), m/z (M+H)+: calcd 370.1641, obsd 370.1647.
Synthesis of (E)-8-[2-(3, 4-Dimethoxyphenyl) vinyl]-1, 3-diethyl-7-methyl-3, 7 dihydropurine-2,6-dione (KW 6002).

K₂CO₃ (1.35g, 9.43mmol) was added to a solution of compound 3-5 (2.02g, 5.45mmol) in dry DMF (27mL). Iodomethane (0.68mL, 10.77mmol) was added and the reaction mixture was stirred at room temperature for 1h. The formed precipitate was filtered off. The filtrate was diluted with water (30 mL) and the resulting mixture was extracted with chloroform (3×100 mL). The organic extracts were washed with water (100 mL) and brine (100 mL), dried with MgSO₄ and evaporated in vacuo. The residue was purified via flash chromatography to give the title compound as a yellow solid. (2.0g, 95%)

mp=191-195 °C; TLC (hexanes: ethyl acetate = 3: 2): Rf 0.22

¹H NMR (500 MHz, CDCl₃): δ 1.23 (t, J = 7 Hz, 3H), 1.36 (t, J = 7 Hz, 3H), 3.90 (s, 3H), 3.93 (s, 3H), 4.03 (s, 3H), 4.07 (q, J = 7 Hz, 2H), 4.18 (q, J = 7 Hz, 2H), 6.74 (d, J = 15.8 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 7.06 (d, J = 1.9 Hz, 1H), 7.15 (dd, J = 8.2, 1.9 Hz, 1H), 7.70 (d, J = 15.8 Hz, 1H)

¹³C NMR (300 MHz, CDCl₃): δ 13.3, 13.4, 31.5, 36.3, 38.4, 55.9, 56.0, 108.0, 109.3, 109.5, 111.2, 121.2, 128.6, 138.1, 148.2, 149.2, 150.2, 150.4, 150.7, 155.0

HRMS (ESI), m/z (M+H)⁺: calcld 384.1798, obsd 384.1789.
Elemental Analysis: (C\textsubscript{20}H\textsubscript{24}N\textsubscript{4}O\textsubscript{4}) calcd (%): C, 62.48; H, 6.29; N, 14.57; found (%): C, 62.45; H, 6.39; N, 14.55

Data are similar with the one reported in the patent, EP 0590919.
Synthesis of \((E)-N-(6\text{-Amino-1, 3-diethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl})-3\text{-}(4\text{-dimethoxyphenyl})\text{ acrylamide }3\text{-7}':\)

\((E)-4\text{-methoxycinnamic acid (1.47 mg, 8.25 mmol) and } N\text{-}(3\text{-Dimethylaminopropyl})-N'\text{-ethylcarbodiimide hydrochloride (1.91 mg, 9.79 mmol) were added to a solution of 1,3-diethyl-5, 6-diaminouracil }3\text{-3 (6 mg, 7.5 mmol) in dioxane (22 mL) and water (45 mL) containing. The reaction mixture was stirred at room temperature for 2 hours. The solution was neutralized with 1M aqueous sodium hydroxide (pH 7) and extracted with chloroform (3×80 mL). The organic extracts were washed with brine (50 mL), dried with MgSO}_4 and evaporated in vacuo. The residue was purified via flash chromatography to provide the title compound }3\text{-7}’ \text{ as a yellow solid. (1.62g, 60%)}

TLC (MeOH: CH\(_2\)Cl\(_2 = 0.5:9.5\)): Rf 0.21

\(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta \) 1.13 (t, \(J = 7\)Hz, 3H), 1.21 (t, \(J = 7\)Hz, 3H), 3.77 (s, 3H), 3.83-3.94 (m, 4H), 5.90 (s, 2H), 6.59 (d, \(J = 15\)Hz, 1H), 6.77 (d, \(J = 8\)Hz, 1H), 7.33 (d, \(J = 8\)Hz, 1H), 7.49 (d, \(J = 15\)Hz, 1H), 8.12 (s, 1H)

\(^{13}\)C NMR (300 MHz, CDCl\(_3\)): \(\delta \) 13.4, 13.5, 37.2, 38.7, 55.6, 91.9, 114.6, 111.0, 117.8, 127.5, 129.9, 142.0, 149.1, 145.0, 160.4, 161.5, 166.8.

HRMS (ESI), m/z (M+H): calcd 358.1641, obsd 358.1658.
Synthesis of (E)-8-[2-(4-Dimethoxyphenyl) vinyl]-1, 3-diethyl-3, 7-dihydropurine-2, 6-Dione 3-7:

An aqueous solution of NaOH (1M, 15 mL) was added to a solution of compound 3-7’ (323 mg, 0.9 mol) in dioxane (11 mL). The clear yellowish solution was refluxed for 1 hour and acidified (pH 2) by dropwise addition of concentrated HCl. The mixture was extracted with chloroform (3×30 mL). The extracts were washed with water (100 mL) and brine (100 mL), dried with MgSO₄ and evaporated *in vacuo*. The residue was purified via flash chromatography to provide the title compound 3-7 as a yellow solid (189mg, 62%).

mp=244-246°C ; TLC (MeOH: CH₂Cl₂ = 0.5:9.5): Rf 0.43

1H NMR (300 MHz, DMSO-d₆): δ 1.17 (t, J = 7 Hz, 3H), 1.29 (t, J = 7 Hz, 3H), 3.84 (s, 3H), 3.97 (q, J = 7 Hz, 2H), 4.10 (q, J = 7 Hz, 2H), 5.79(s, 1H), 6.94 (d, J = 16.2 Hz, 1H), 7.03(d, J = 9Hz, 2H), 7.61 (d, J = 9Hz, 2H), 7.65 (d, J = 16.2 Hz, 1H)

13C NMR (300 MHz, DMSO-d₆): δ 14.1, 36.5, 38.9, 39.6, 56.1, 107.9, 114.2, 115.3, 128.9, 129.5, 128.6, 135.8, 148.9, 149.6, 150.9, 151.1, 154.5, 161.0.

HRMS (ESI), m/z (M+H)⁺: calcd 340.1535, obsd 340.1551.
Synthesis of (E)-8-[2-(4-Dimethoxyphenyl) vinyl]-1, 3-diethyl-7-methyl-3, 7 dihydropurine-2,6-dione 3-8:

K₂CO₃ (135 mg, 0.98 mmol) was added into a solution of compound 3-7 (194 mg, 0.57 mmol) in dry DMF (2.8 mL). Iodomethane (71 μl, 1.14 mmol) was added and the reaction mixture was stirred at 50°C for 1h. The precipitate was filtered off, and the filtrate was diluted with water (3 mL). The mixture was extracted with chloroform (3×15 mL) and the organic extracts were washed with water (10 mL), brine (10 mL), dried over MgSO₄ and concentrated in vacuo. The residue was purified via flash chromatography to afford the title compound 3-8 as a yellow solid. (182mg, 95%)

mp=269-273 °C; TLC (MeOH: CH₂Cl₂ = 0.5:9.5): Rf 0.45

¹H NMR (300 MHz, CDCl₃): δ 1.26 (t, J = 7 Hz, 3H), 1.38 (t, J = 7 Hz, 3H), 3.85 (s, 3H), 4.03 (s, 3H), 4.08 (q, J = 7 Hz, 2H), 4.20 (q, J = 7 Hz, 2H), 6.76 (d, J = 15.9 Hz, 1H), 6.92 (d, J = 9 Hz, 2H), 7.52 (d, J = 8.5 Hz, 2H), 7.74 (d, J = 15.9 Hz, 1H)

¹³C NMR (300 MHz, CDCl₃): δ 13.8, 31.7, 36.6, 38.7, 55.7, 109.4, 114.7, 128.7, 129.2, 138.2, 148.5, 150.7, 150.8, 151.1, 155.4,161.1

HRMS (ESI), m/z (M+H)+: calcd 354.1692, obsd 354.1681.
**Synthesis of (E)-1,3-diethyl-8-(4-hydroxystyryl)-7-methyl-1H-purine-2,6(3H,7H)-dione 3-9:**

A 1M BBr$_3$ in CH$_2$Cl$_2$ (170 μl, 0.85 mmol) was added to a solution of compound 3-8 (100 mg, 0.28 mmol) in dry CH$_2$Cl$_2$ (0.35 mL). The reaction mixture was stirred at room temperature for 3 hours and diluted with water (0.3 mL). The formed precipitate collected via vacuum filtration, washed with excess water and dried *in vacuo* to give the title compound 3-9 as a yellow solid. (90mg, 95%)

mp > 300 °C; TLC (MeOH: CH$_2$Cl$_2$ = 1: 9): Rf 0.2

$^1$H NMR (500 MHz, DMSO-d$_6$): δ 1.12 (t, $J = 7$ Hz, 3H), 1.25 (t, $J = 7$ Hz, 3H), 3.91 (q, $J = 7.5$ Hz, 2H), 3.99 (s, 3H), 4.06 (q, $J = 7.5$ Hz, 2H), 6.80 (d, $J = 9$ Hz, 2H), 7.11 (d, $J = 16$ Hz, 1H), 7.58 (d, $J = 16$ Hz, 1H), 7.62 (d, $J = 9$ Hz, 2H).

$^{13}$C NMR (300 MHz, DMSO-d$_6$): δ 51.1, 69.2, 73.3, 75.6, 144.9, 147.1, 153.6, 164.6, 167.3, 174.8, 185.4, 187.9, 188.3, 191.8, 196.7

HRMS (ESI), m/z (M+H)$^+$: calcd 340.1535, obsd 340.1549
Synthesis of Asparaginyl-Alanine-OMe 3-11:

N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (290 mg, 1.51 mmol) was added to a solution of Boc-Asn (338 mg, 1.45 mmol), Ala-OMe (210 mg, 1.51 mmol) and 1-hydroxybenzotriazole hydrate (HOBt) (220 mg, 1.64 mmol) in dry CH₂Cl₂ (7 mL) at 0°C. The reaction mixture was stirred at 0°C for 1h and then was allowed to stand at room temperature overnight. The solvent was evaporated under reduced pressure. The residue was diluted with ethyl acetate (25 mL). The solution was washed with 1N hydrochloric acid (10 mL), saturated sodium bicarbonate solution (10 mL) and brine (10 mL) sequentially. The organic layer was dried over MgSO₄ and evaporated in vacuo to give the crude product as a white solid (358mg).

Trifluoroacetic acid (TFA) (8.5 mL) was added into a flask containing the above crude product and the reaction mixture was stirred at room temperature for 1h. The excess TFA was then removes in vacuo. The oily residue was diluted with water (20 mL) and washed with diethyl ether (3×10 mL). The aqueous layer was freeze dried to afford the title compound 3-11 as a white solid. (387mg, 70% two steps)

TLC (MeOH: CH₂Cl₂ = 1: 4): Rf 0.15

¹H NMR (300 MHz, CD₃OD): δ 1.45 (d, J = 7.2 Hz, 3H), 2.73-3.01 (m, 2H), 3.75 (s, 3H), 4.21-4.26 (dd, J = 13.2, 3.9Hz, 1H), 4.49 (q, J = 7.2Hz, 1H)
$^{13}$C NMR (300 MHz, CD$_3$OD): $\delta$ 18.09, 36.97, 53.01, 53.75, 170.23, 174.25, 174.98

HRMS (ESI), m/z (M+H)$^+$: calcd 217.1063, obsd 217.1080.
Synthesis of Boc-Alaninyl-Alaninyl-Asparaginyl-Alanine-OMe:

Triethylamine (0.28 mL, 1.978mmol) was added into a solution of compound 3-11 (596mg, 1.806mmol), 3-12* (470mg, 1.806mmol) and 1-Hydroxybenzotriazole hydrate (HOBt) (300mg, 2.162mmol) in DMF (9.5 mL). The solution was cooled to 0°C and N,N'-dicyclohexylcarbodiimide (DCC) (452mg, 2.17mmol) was added. The reaction mixture was stirred for 1 h at 0°C and then allowed to stand overnight at room temperature. After the reaction was completed, the solvent was removed under reduced pressure. Ethyl acetate (15 mL) was added to the residue and the precipitate was collected via vacuum filtration. The precipitate was washed with ethyl acetate and dried in vacuo to give the title compound 3-13 as a white solid (520mg, 65%).

TLC (MeOH: CH₂Cl₂ = 1: 9): Rf 0.45 mp=188-190°C

¹H NMR (500 MHz, DMSO-d6): δ 1.16 (d, J = 7 Hz, 3H), 1.25 (d, J = 6.5 Hz, 3H), 1.26 (d, J = 7.5 Hz, 3H), 1.37 (s, 9H), 2.40-2.48 (m, 2H), 3.98-3.98 (m, 1H), 4.18-4.25 (m, 1H), 4.50 (q, J = 7Hz, 1H), 6.90-6.93 (m, 2H), 7.32 (s, 1H), 7.95 (d, J = 6.5Hz, 1H), 7.98 (d, J = 6.5Hz, 1H), 8.04 (d, J = 8Hz, 1H)
$^{13}$C NMR (300 MHz, DMSO-d6): $\delta$ 17.85, 18.95, 25.44, 29.17, 37.67, 48.68, 49.29, 50.25, 50.51, 52.86, 79.13, 156.09, 171.79, 172.47, 172.89, 173.72, 173.83

HRMS (ESI), m/z (M+H)$^+$: calcld 459.2329, obsd 459.2311.

* The compound 3-12 was synthesized according to the reference: Rella, M. R., Williard, P. G. *J. Org. Chem.* 2007, 72, 525.
Synthesis of Boc-Alaninyl-Alaninyl-Asparaginyl-Alanine 3-14:

Lithium hydroxide (50mg, 2.094mmol) was added to the solution of compound 3-13 (802mg, 1.745mmol) in THF/MeOH/H₂O (20 mL, 3:1:1). The reaction mixture was stirred at room temperature for 5h. The pH of the solution was adjusted pH 3 with 1N KHSO₄. The mixture was extracted with ethyl acetate (3×150 mL) and the organic extracts were dried with MgSO₄, concentrated *in vacuo*. The residue was purified via flash chromatography to give the title compound 3-14 as a white solid (655mg, 90%).

TLC (MeOH: CH₂Cl₂ = 1: 1): Rf 0.5 mp= 155-159°C

¹H NMR (300 MHz, CD₃OD): δ 1.32 (d, J = 6.9Hz, 3H), 1.37 (d, J = 7.2Hz, 3H), 1.41 (d, J = 6.9Hz, 3H), 1.43 (s, 9H), 2.70-2.74 (m, 2H), 3.29-3.34 (m, 1H), 4.02-4.07 (m, 1H), 4.68-4.72 (m, 1H)

¹³C NMR (300 MHz, CD₃OD): δ 18.55, 19.09, 26.91, 29.58, 25.60, 38.44, 51.65, 52.14, 52.41, 81.54, 158.71, 173.45, 175.59, 175.86, 176.56, 176.92.

HRMS (ESI), m/z (M+H)⁺: calcd 445.2173, obsd 445.2155.
Synthesis of the prodrug 3-15:

4-Dimethylaminopyridine (DMAP) (11 mg, 0.088 mmol) was added to a flask containing compound 3-9 (30 mg, 0.088 mmol) in dry DMF (1.2 mL). The solution was stirred at room temperature for 30 min. It was transferred into a flask containing a solution of compound 3-14 (41 mg, 0.088 mmol), N-methyl morpholine (NMM) (23 μl, 0.211 mmol) and O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (59 mg, 0.152 mmol) in DMF (1.2 mL) at 0°C. The light sensitive reaction mixture was stirred in the dark at room temperature for 6 hours. After the reaction was completed, the solvent was removed in vacuo and ethyl acetate (20 mL) was added to the residue. The resulting precipitate was collected via vacuum filtration, washed with ethyl acetate and dried in vacuo to give the title compound 3-15 as a light yellow solid (44 mg, 65%).

TLC (MeOH: CH$_2$Cl$_2$ = 1: 9): Rf 0.41  mp=208-212°C

$^1$H NMR (500 MHz, DMSO-d$_6$): δ 1.13 (t, $J = 7.5$Hz, 3H), 1.16-1.21 (m, 6H), 1.26 (t, $J = 7.5$ Hz, 3H), 1.37 (s, 9H), 1.44 (d, $J = 6$Hz, 3H), 2.50 (m, 2H), 3.93 (q, $J = 6$Hz, 2H), 3.98 (m, 1H), 4.04 (s, 3H), 4.07 (q, $J = 6$Hz, 2H), 4.21 (m, 1H), 4.40-4.45 (m, 1H), 4.57(m, 1H), 6.90-6.96 (m, 2H), 7.16 (d, $J = 7$Hz, 2H), 7.34 (s, 1H), 7.36 (d, $J = 15.5$Hz, 1H), 7.67 (d, $J = 15.5$Hz, 1H), 7.85 (d, $J = 6.5$Hz, 1H), 7.95-8.01 (m, 1H), 8.08-8.13(m, 1H), 8.25 (dd, $J = 25.5$, 6.5Hz, 1H)
\[^{13}\text{C} \text{ NMR (300 MHz, DMSO-d6)}: \delta \ 14.15, 17.49, 18.93, 19.14, 36.46, 37.61, 38.66, 42.43, 42.71, 49.13, 50.23, 50.47, 79.03, 108.40, 113.95, 122.99, 129.71, 134.32, 136.46, 148.40, 150.58, 150.93, 151.99, 154.95, 156.04, 171.90, 172.09, 172.30, 172.86, 173.74.\]

HRMS (ESI), m/z (M+H)^+: calcd 767.3602, obsd 767.3612.
Synthesis of the active molecule (3-16):

4-dimethylaminopyridine (DMAP) (36 mg, 0.29 mmol) was added to a flask containing compound 3-9 (100 mg, 0.29 mmol) in DMF (3.5 mL). The solution was stirred at room temperature for 30 min. Then, it was transferred into a flask containing a solution of (Boc)Ala (54 mg, 0.29 mmol), N-methyl morpholine (NMM) (76 μl, 0.696 mmol) and O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (195 mg, 0.501 mmol) in DMF (3.5 mL) at 0°C. The light sensitive reaction mixture was stirred in the dark at room temperature for 6 hours. After reaction was complete, the solvent was removed in vacuo and ethyl acetate (50 mL) was added to the residue. The resulting precipitated was collected via vacuum filtration, washed with ethyl acetate and dried in vacuo to give the title compound 3-15 as a light yellow solid.

TFA (4 mL) was added to a flask containing the above product and the reaction mixture was stirred at room temperature for 1 hour. The excess TFA was then removed under reduced pressure. The residue was dissolved in water (20 mL) and washed with diethyl ether (3×10 mL). The aqueous layer was freeze dried to afford the title compound 3-11 as a white solid (128 mg, 80% two steps).

TLC (MeOH: CH₂Cl₂ = 1: 9): Rf 0.41 mp=277-281°C
$^1$H NMR (500 MHz, DMSO-d6): $\delta$ 1.13 (t, $J = 7$Hz, 3H), 1.26 (t, $J = 7.5$ Hz, 3H), 1.58 (d, $J = 6$Hz, 3H), 2.50 (m, 2H), 3.92 (q, $J = 6$Hz, 2H), 4.05 (s, 3H), 4.07 (q, $J = 6$Hz, 2H), 4.21 (m, 1H), 4.44 (m, 1H), 7.26 (d, $J = 8.5$Hz, 2H), 7.39 (d, $J = 15.5$Hz, 1H), 7.69 (d, $J = 16$Hz, 1H), 7.93 (d, $J = 9$Hz, 2H), 8.48 (br, 2H)

$^{13}$C NMR (300 MHz, DMSO-d6): $\delta$ 14.42, 17.02, 32.88, 38.16, 40.31, 110.24, 114.45, 123.72, 130.69, 136.58, 138.65, 150.14, 152.62, 152.93, 153.04, 156.98, 170.67

HRMS (ESI), m/z (M+H)+: calcd 411.1907, obsd 411.1922.
APPENDIX

SPECTRAL DATA
Appendix 1: CD spectra

CD Spectrum of the beta-glycosylated analogues 1-43a and 1-43b

Experimental method:
CD spectra were recorded on a Jasc-710 spectropolarimeter using 1cm cuvettes. Data were recorded at a bandwidth of 1.0nm over a range of 200-400 nm for compounds in methanol at 25°C.
Appendix 2: Data of the fluorescence quenching study for compound 1-43a and 1-43b.

Fluorescence quenching studies were carried out using SPEX Fluoro Max-3 at 5 °C in a 10 mM phosphate buffer (pH 7.5). Dissociation constant $K_d$ was derived from curve-fitting with Kaleidagraph, using the equation $i/i_0 = 1 + (\Delta i/2i_0)\times([T_0] + [\text{DNA}] + K_d - ([T_0] + [\text{DNA}] + K_d)^2 - 4*[T_0][\text{DNA}])^{1/2}$, wherein $[T_0]$ is the initial concentration of the fluorescent probe, $i$ is the intensity of the sample, $i_0$ is the initial intensity of the sample, $[\text{DNA}]$ is the concentration of the DNA in micromolar ($\mu$M), and $\Delta i$ is the total change in intensity per drug unit from the free state to the total binding state.
\[ \frac{i}{i_0} \]

\[ y = 1 + m2^* (1.78 + m0 + m1 - \sqrt{1 \ldots}) \]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>8.2476</td>
<td>2.4243</td>
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<tr>
<td>m2</td>
<td>0.65345</td>
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<tr>
<td>Chisq</td>
<td>0.0051096</td>
<td>NA</td>
</tr>
<tr>
<td>R</td>
<td>0.99663</td>
<td>NA</td>
</tr>
</tbody>
</table>
1-43a - BA14

\[
y = \frac{1}{1 + m_2^2 \left( 3.69 + m_0 + m_1 \right) - \sqrt{\left( 3.69 + m_0 + m_1 \right)^2 - 4 \cdot 3.69 \cdot m_0}}
\]

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>0.43156</td>
<td>0.08718</td>
</tr>
<tr>
<td>m2</td>
<td>0.11523</td>
<td>0.00206356</td>
</tr>
<tr>
<td>Chiaq</td>
<td>0.0040591</td>
<td>NA</td>
</tr>
<tr>
<td>R</td>
<td>0.99905</td>
<td>NA</td>
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</tbody>
</table>
1-43a -HT3AGTT

\[ y = 1 + m_2(3.35 + m_0 + m_1 - \sqrt{3...}) \]

<table>
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<tbody>
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<td>m1</td>
<td>0.20014</td>
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<tr>
<td>m2</td>
<td>0.062364</td>
</tr>
<tr>
<td>Chisq</td>
<td>0.00038498</td>
</tr>
<tr>
<td>R</td>
<td>0.99907</td>
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</table>

i /i0
\[ y = 1 + m_2 \cdot (1.45 + m_0 + m_1 - \sqrt{(1.45 + m_0 + m_1)^2 - 2 \cdot 1.45 \cdot m_0}) \]

<table>
<thead>
<tr>
<th></th>
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<th>Error</th>
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<tbody>
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<td>( m_1 )</td>
<td>14.037</td>
<td>2.1537</td>
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<td>( m_2 )</td>
<td>5.882</td>
<td>0.57997</td>
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<tr>
<td>( \text{Chi} )sq</td>
<td>0.059964</td>
<td>NA</td>
</tr>
<tr>
<td>( R )</td>
<td>0.99918</td>
<td>NA</td>
</tr>
</tbody>
</table>
\[ y = 1 + m_2 \times (2.95 + m_0 + m_1 - \sqrt{(2.95 + m_0 + m_1)^2 - 4 \times 2.95 \times m_0}) \]

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>( m_1 )</td>
<td>1.8063</td>
<td>0.6411</td>
</tr>
<tr>
<td>( m_2 )</td>
<td>0.10502</td>
<td>0.0094423</td>
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<tr>
<td>Chi( sq )</td>
<td>0.0027091</td>
<td>NA</td>
</tr>
<tr>
<td>( R )</td>
<td>0.9937</td>
<td>NA</td>
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</tbody>
</table>
1-43a - HT3AGTA

\[ y = 1 + m_2^{0.45 \cdot m_0^{0.5}} - \text{sqrt}(2) \]

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</thead>
<tbody>
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<td>0.26381</td>
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<td>m2</td>
<td>0.42541</td>
<td>0.03548</td>
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<tr>
<td>Chisq</td>
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<tr>
<td>R</td>
<td>0.98875</td>
<td>NA</td>
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</table>
\[ y = 1 + m2 \times (2.91 + m0 + m1 - \sqrt{(2... \right) \]

<table>
<thead>
<tr>
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<th>Error</th>
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<tbody>
<tr>
<td>m1</td>
<td>0.18779</td>
<td>0.36844</td>
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<tr>
<td>m2</td>
<td>0.10075</td>
<td>0.014853</td>
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<tr>
<td>Chisq</td>
<td>0.016614</td>
<td>NA</td>
</tr>
<tr>
<td>R</td>
<td>0.96057</td>
<td>NA</td>
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</table>
1-43b - HT3AT

\[ \frac{i}{i_0} \]

[DNA]

\[
y = 1 + m_2^2 (1.84 + m_0 + m_1 - \sqrt{1 - \sqrt{1 - \frac{m_1}{m_0} - m_3}})
\]

<table>
<thead>
<tr>
<th>Value</th>
<th>Error</th>
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<tbody>
<tr>
<td>(m_1)</td>
<td>9.2051</td>
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<tr>
<td>(m_2)</td>
<td>1.1665</td>
</tr>
<tr>
<td>Chisq</td>
<td>0.0090738</td>
</tr>
<tr>
<td>R</td>
<td>0.99815</td>
</tr>
</tbody>
</table>
\[ y = 1 + m_2 \cdot (3.69 \cdot m_0 + m_1 - \sqrt{(3.69 \cdot m_0 + m_1)^2 - 2 \cdot 4 \cdot 3.69 \cdot m_0}) \]

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>(m_1)</td>
<td>0.43156</td>
<td>0.087718</td>
</tr>
<tr>
<td>(m_2)</td>
<td>0.11523</td>
<td>0.00206356</td>
</tr>
<tr>
<td>Chisq</td>
<td>0.0040591</td>
<td>NA</td>
</tr>
<tr>
<td>(R)</td>
<td>0.99995</td>
<td>NA</td>
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</tbody>
</table>
1-43b - HT3AGTT

\[
y = 1 + m2^2 \cdot (3.14 + m0 + m1 - \sqrt{3...})
\]

<table>
<thead>
<tr>
<th>Value</th>
<th>Error</th>
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<tbody>
<tr>
<td>m1</td>
<td>1.6611</td>
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<td>m2</td>
<td>0.094591</td>
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<td>Chisq</td>
<td>0.0019443</td>
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<td>R</td>
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</table>
1-43b - HT3AGT

\[ y = 1 + m2 \times ((1.84 + m0 + m1 - \sqrt{((1.84 + m0 + m1)^2 - 4 \times 1.84 \times m0)}) \]

<table>
<thead>
<tr>
<th></th>
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<th>Error</th>
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<tbody>
<tr>
<td>m1</td>
<td>5.4608</td>
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<td>m2</td>
<td>0.48623</td>
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<tr>
<td>R</td>
<td>0.99955</td>
<td>NA</td>
</tr>
</tbody>
</table>
1-43b - HT3AGCTT

\[
y = 1 + m2 \times (2.91 + m0 + m1 - \sqrt{(2.91 + m0 + m1)^2 - 4 \times 2.91 \times m0})
\]

<table>
<thead>
<tr>
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<th>Error</th>
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</thead>
<tbody>
<tr>
<td>m1</td>
<td>0.9527</td>
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<td>m2</td>
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<tr>
<td>Chsq</td>
<td>0.0028163</td>
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<tr>
<td>R</td>
<td>0.9995</td>
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</tbody>
</table>
The image contains a graph with the x-axis labeled "[DNA]" and the y-axis labeled "i/i_0". The graph shows a curve fitting equation:

\[ y = 1 + m2^2(2.44 + m0 + m1 - \sqrt{((2.44 + m0 + m1)^2 - 20^2(2.44 + m0))}) \]

The table below lists the values and errors for the parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
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<td>0.20571</td>
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<tr>
<td>m2</td>
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</tr>
<tr>
<td>Chisq</td>
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<td>NA</td>
</tr>
<tr>
<td>R</td>
<td>0.9944</td>
<td>NA</td>
</tr>
</tbody>
</table>
The graph shows a plot of $i/i_0$ against [DNA] for the sequence 1-43b-HT4AGTT. The equation for the curve is $y = 1 + m_2^2(2.95 + m_0 + m_1)^{-1} \cdot \sqrt{(2.95 + m_0 + m_1)^2 - 2.4^2 \cdot 2.95^2 \cdot m_0)}$. The table below lists the values and errors for $m_1$, $m_2$, $\text{Chsq}$, and $R$:

<table>
<thead>
<tr>
<th>Value</th>
<th>Error</th>
<th>Value</th>
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</tr>
</thead>
<tbody>
<tr>
<td>$m_1$</td>
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<td>$m_2$</td>
<td>0.18769</td>
</tr>
<tr>
<td>$\text{Chsq}$</td>
<td>0.0054666</td>
<td>$R$</td>
<td>0.99744</td>
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<tr>
<td>$\text{Chsq}$</td>
<td>NA</td>
<td>$R$</td>
<td>NA</td>
</tr>
</tbody>
</table>

The values and errors are given in the table.
Appendix 3: NMR spectra data