ABSTRACT

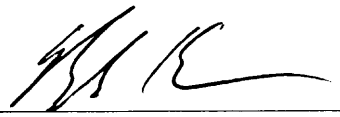
Synthesis of Sulfur and Amino 8-Substituted Adenine Derivatives as TLR7 Agonists

Rachel Petersen

Director: Robert R. Kane, Ph.D.

Toll-like receptors (TLRs) are a class of pattern recognition receptor (PRR) proteins, which play a vital role in the innate immune response. Upon recognition of microbial pathogens, TLRs initiate pathways which lead to the production of cytokines. For the past few years, TLR agonists have been used as adjuvants for vaccines against cancer, allergies, and viral infections. TLR7 agonists are of particular interest, as they can induce the production of interferons in plasmacytoid dendritic cells without stimulating the release of inflammatory cytokines. In this experiment, several sulfur and amino 8-substituted adenine molecules were synthesized for use as potential TLR7 agonists. Two of these compounds were tested for immunological activity at the Baylor Institute for Immunology Research in Dallas, and several others are still being developed.

APPROVED BY DIRECTOR OF HONORS THESIS:



Dr. Robert R. Kane, Department of Chemistry and Biochemistry

APPROVED BY THE HONORS PROGRAM:

_____

Dr. Andrew Wisely, Director

DATE: _____

SYNTHESIS OF SULFUR AND AMINO 8-SUBSTITUTED ADENINE DERIVATIVES AS TLR7 AGONISTS

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Rachel Petersen

Waco, Texas

December 2014

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

PRR. . . . . pattern recognition receptor

PAMP . . . . . pathogen-associated molecular pattern

APC. . . . . antigen presenting cell

MHC . . . . . major histocompatibility complex

CD . . . . . .cluster of differentiation

TLR. . . . . .toll-like receptor

IFN . . . . . interferon

TLC. . . . . thin layer chromatography

UV . . . . . ultraviolet

NMR . . . . . nuclear magnetic radiation

EAS . . . . . electrophilic aromatic substitution

K_2CO_3 . . . . . potassium carbonate

DMSO . . . . . dimethyl sulfoxide

MHz. . . . . megahertz

G . . . . . gram

Mg . . . . . milligram

Ng . . . . . nanogram

mmol . . . . . .millimol

mL . . . . . .milliliter

EtOAc . . . . . ethyl acetate

DCM. . . . . dichloromethane

BuOH . . . . . butanol

NaOH . . . . . sodium hydroxide

EtOH. . . . . ethanol

LCMS . . . . . liquid chromatography-mass spectrometry

OD . . . . . optical density

Boc . . . . . *tert*-butyloxycarbonyl

Cys . . . . . cysteine

ACKNOWLEDGMENTS

I would like to thank Dr. Kane for mentoring me throughout this project. I greatly appreciate the time and effort that you put into weekly meetings, emails, and revisions to help me through this process. I am grateful for the opportunity to explore research; working in this lab has been a formative part of my undergraduate experience. I would also like to thank Tope Akinbobuyi, Charles Chang, Zach Seifert, and Josh Horton for helping me in the lab, answering questions, sharing advice, and encouraging me as I continue on to pursue graduate school. I am glad to have had the opportunity to work with and learn from each of you, and I wish you all luck as you continue your research projects. Thank you also to Jeremy Quintana for being a fantastic lab partner. I have enjoyed working and learning with you in the lab, and I am glad to count you as a friend. Finally, thank you to my mom and dad, Drake Gates, Katie Rodriguez, Rachel Wells, and Roopali Sharma for encouraging and supporting me throughout my time at Baylor University. I am blessed to have each of you in my life; your support has motivated and strengthened me to finish the work that I started.

CHAPTER ONE:

Introduction

The human immune system acts in two distinct phases: the primary innate response and secondary adaptive response. The innate immune response occurs immediately when a pathogen enters the body[1]. Cell receptors called pattern recognition receptors (PRRs) recognize pathogen associated molecular patterns (PAMPs) associated with the pathogen, and numerous pathways are activated which result in the release of a flood of proinflammatory cytokines[2]. These cytokines summon antigen presenting cells (APCs) such as dendritic cells and macrophages, thus bridging to the secondary adaptive immune response. The APCs phagocytose and digest the pathogen and present peptide fragments from the pathogen on the cell surface as part of major histocompatibility complexes (MHCs). MHC-peptide complexes bind to and activate T cells, which differentiate CD4 T helper cells and CD8 cytotoxic T cells. CD4 T helper cells then activate naïve B cells, causing them to differentiate into plasma B cells and memory B cells. Plasma B cells produce high affinity antibodies against the invading pathogen, and memory B cells retain copies of the specialized antibody that can be quickly produced upon future encounter with the same pathogen. This adaptive process can take several days, and it is during this time that a person experiences symptoms of illness. However, once these highly pathogen-specific mature B cells, T cells, and antibodies are developed, they can quickly destroy all copies of the invading pathogen throughout the body. Furthermore, these cells and antibodies permanently circulate throughout the lymphatic

system, allowing the body to eliminate the pathogen upon any future contact before illness can occur[1].

However, many pathogens have developed evolutionary mechanisms that allow them to evade or overcome the immune system, often resulting in chronic illness or death for those who are infected. Vaccines have been demonstrated to be highly effective in combating such pathogens. First generation vaccines, including those against *Yersinia pestis, Bordetella pertussis,* and the variola virus (causes of the plague, pertussis, and smallpox, respectively), introduce to the immune system either dead or attenuated pathogens. Because the disabled pathogen cannot replicate at a normal rate, the immune system has time to develop a strong adaptive response to the pathogen without risk of contracting illness. The body is then equipped with mature B and T cells that can be quickly activated if the live pathogen is ever encountered, and the immune system can kill the pathogen before it has time to spread throughout the body and induce illness. Due to the success of these first generation vaccines, more vaccines were developed against the deadliest diseases affecting humans. The rise of second generation vaccines was marked by the increased use of live-attenuated pathogens to prevent illnesses such as polio, measles, rubella, mumps, and varicella. Eventually, however, it became clear that second generation vaccines are not effective for all pathogens; live-attenuated pathogens cannot be prepared for microbes that do not grow in vitro, those with antigenic hypervariability, or those which enter human cells[3]. Furthermore, even attenuated versions of some pathogens are still too dangerous to administer[4]. As a result, the scientific and medical communities began to search for alternative vaccine techniques.

Vaccines today, referred to as third generation vaccines, are based on the same theory behind first and second generation vaccines, but are more complex. Third generation vaccines contain three main components. The first component is the antigen. An antigen is a unique fragment, or synthetically prepared fragment, of a pathogen, most often a short peptide sequence. The second component is the adjuvant. Because the entire pathogen is not presented to the immune system, antigens are often not sufficient to induce a strong and long lasting immune response resulting in the release of proinflammatory cytokines and the maturation of B memory cells[5]. This necessitates the second component of a vaccine, called an adjuvant. Adjuvants trigger the innate immune response upon administration of the vaccine, often by activating PRRs[6]. This effectively enhances the immunogenicity of the antigen, leading to the desired strong and long lasting adaptive immune response which offers maximal protection against future infection[7]. Finally, a delivery system is required in order to deliver the vaccine to the proper location. A variety of delivery systems have been tested including mineral salts, synthetic microparticles, liposomes, and antibodies[2].

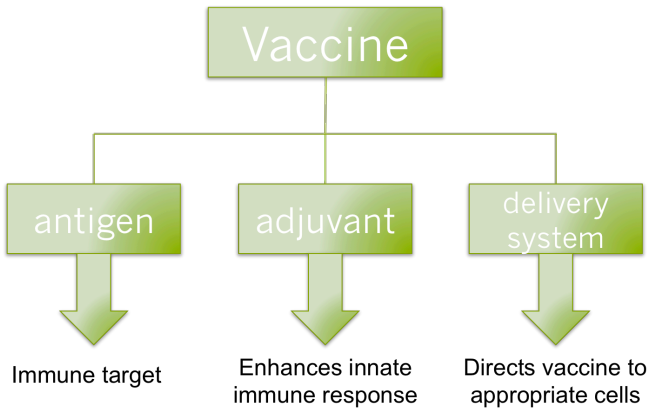


Figure 1. Essential Third Generation Vaccine Components and Their Functions

One type of PRR is a toll-like receptor (TLR). TLRs are type I transmembrane glycoproteins with leucine rich repeats[8], which are an integral component of the innate immune response. Upon activation, most TLRs initiate a cytokine cascade via the MyD88 pathway as shown in figure 2. Thus, TLR activation links the innate and adaptive immune responses[9].

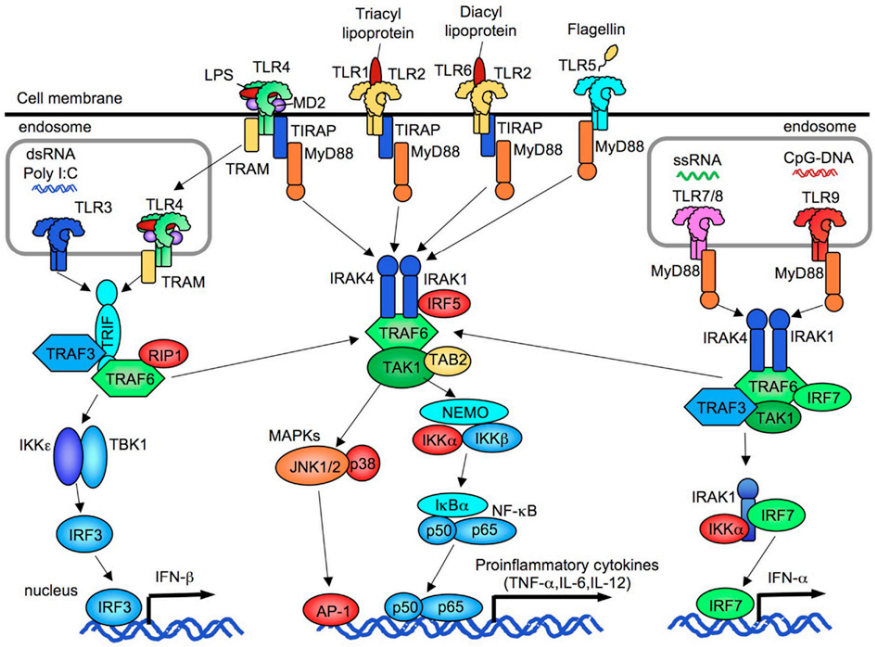


Figure 2. Toll Like Receptor Cytokine Cascade Pathways

Currently, 10 TLRs have been identified in humans. TLRs 1, 2, 4, 5, 6, and 10 are located on the outer cell membrane. These TLRs bind to PAMPs on extracellular pathogens. TLRs 3, 7, 8, and 9 are located inside of APCs on the endosomal membrane and bind to PAMPs on intercellular pathogens. TLRs are an attractive target for immune adjuvants as the activation of TLRs leads to the production of proinflammatory cytokines

and upregulation of MHC resulting in both a strong primary and anamnestic (enhanced secondary) response to pathogens[9].

For the past few years, TLR agonists have been used as adjuvants for vaccines against various types of cancer, allergies[10], and viral infections[11] including Hepatitis C and Human Papillomavirus. TLR7 agonists are of particular interest, as they can induce the production of type I interferons in plasmacytoid dendritic cells without stimulating the release of inflammatory cytokines[12]. Imidazoquinalines such as imiquimod (figure 3) and resiquimod (figure 4) are commonly cited TLR7 agonists which are highly successful at stimulating the production of interferons.

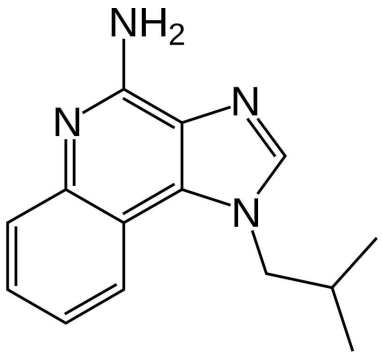


Figure 3. Structure of Imiquimod

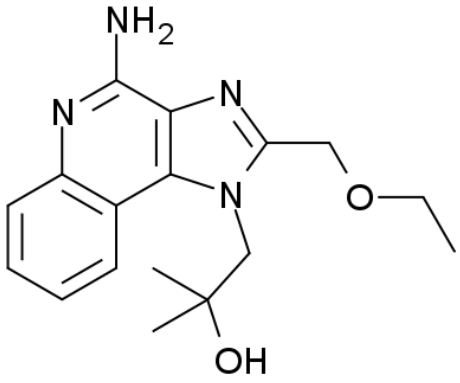


Figure 4. Structure of Resiquimod

Unfortunately, these compounds also were accompanied by unpleasant side effects including flu-like symptoms and hepatopathy, and the use of these compounds was discontinued[10].

More recently, it has been discovered that purine derivatives are also potent IFN inducers[11]. Adenine-based compounds in particular are potent TLR7 agonists. These compounds are most effective when they are substituted with an elongated alkyl chain[13] near the C2 region, a hydrophobic substituent near N9, a hydrogen bonding acceptor near C6, and a polarized group on the C8 carbon[14].

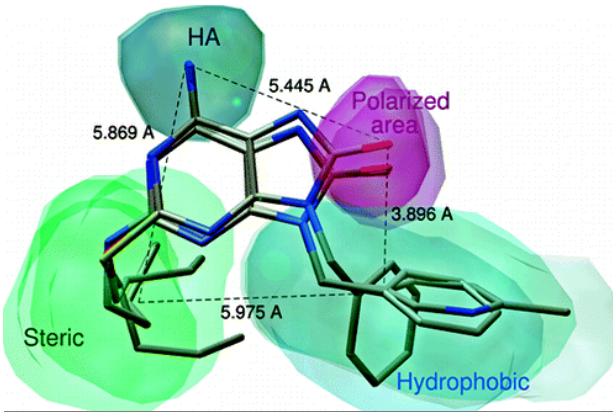


Figure 5. Ideal Components of a Maximally Effective Adenine-Based TLR7 Agonist[14]

In order to develop a TLR7 agonist for use as a potential vaccine adjuvant, a number of different molecules meeting the criteria summarized in figure 5 must be synthesized and tested for immune-stimulating activity. Many important organic synthesis techniques, including refluxing, microwave heating, thin-layer chromatography, liquid-liquid extraction, flash chromatography, crystallization, nuclear magnetic resonance, mass spectrometry, and x-ray crystallography are used in the production of these molecules.

Often, in order to synthesize a new compound, reactants need only to be stirred together until the reaction reaches completion. Sometimes, however, room temperature conditions are not sufficient to cause a reaction at a measurable rate, and elevated temperatures are used to increase the rate of reaction and improve product yield. A reaction heated at the boiling point of the solvent is said to "reflux." To set up a reflux reaction, reactants are first dissolved in a suitable solvent in a round bottom flask. A

magnetic stir bar is added to the flask, and the flask is placed in either an oil or sand bath on top of a stir plate. The flask is fitted with a condenser, which consists of concentric glass tubes. The oil or sand bath is heated to the boiling point of the solvent and cold water is flushed through the condenser and expelled in a waste container or drain. As the solvent boils, the vapors rise up into the hollow center of the condenser and condense due to the cooler temperature of the circulating water. Conducting the reaction at the boiling point of the solvent increases the kinetic energy of the reactants, maximizing the number of interactions between the reactants at any given time. Thus, the reaction reaches completion much sooner than it would have at room temperature.

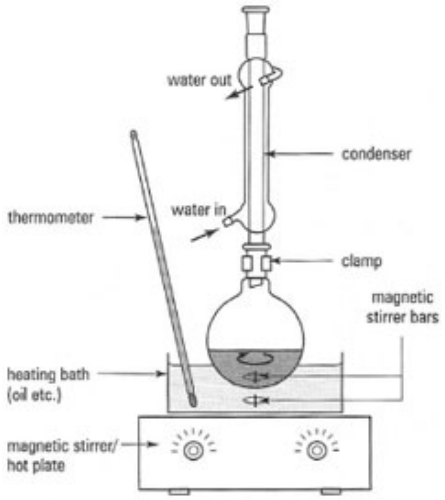


Figure 6. Reflux Setup[15]

A CM Discover Microwave is another tool that can be used to drastically increase the rate of a synthetic reaction. The reactants are measured into a glass microwave tube (10, 35, or 80 mL) and dissolved in a polar solvent. A magnetic stir bar is added to the tube and a plastic cap is used to seal the tube. The tube is placed into the microwave which simultaneously stirs, pressurizes, and heats the reaction mixture. Heating occurs due to excitation by electromagnetic radiation. Microwaves cause the polar solvent molecules to reorient their dipoles in line with the magnetic field. The kinetic movement of the molecules generates heat, increasing the temperature of the reaction mixture. The combination of magnetic stirring, pressure, and microwave heating often accelerates the reaction to a rate unachievable under normal laboratory conditions[16].

Thin layer chromatography (TLC) is an analytical technique used to monitor the progress of reactions by separating the components of a reaction mixture. A silica-coated piece of aluminum is trimmed to approximately 3" x 1". A light pencil line is drawn along the width of the silica, about 0.25" above the bottom edge. A small amount of each reactant in the reaction mixture is separately dissolved in an appropriate solvent. A capillary tube is then used to adsorb a dot of each reactant and of the combined reaction mixture evenly along the pencil line. The silica-coated aluminum (stationary phase) is then placed in a beaker containing running solvent (mobile phase) that lies below the adsorbed samples. A watchglass is placed on top of the beaker, and the running solvent is allowed to travel up the plate until it is about 0.25" from the top. The plate is carefully removed with tweezers, allowed to dry, and examined under ultraviolet (UV) light, which allows the detection of UV-absorbing compounds (most in the study). At least one spot

should appear above the initial adsorption spot at the base of the plate. The least polar organic molecules move highest up the plate, while the most polar molecules are closer to the bottom of the plate due to stronger interactions with the polar hydroxyl groups on the surface of the silica gel. Equation 1 can be used to calculate the R_f value of each spot, which is used as a relative measure of polarity.

$$R_f = \frac{distance\ travelled\ up\ plate\ by\ the\ spot}{distance\ travelled\ up\ plate\ by\ the\ solvent} \qquad \textbf{(Equation 1.)}$$

Many factors, including the concentration of sample, size of the plate, polarity of the mobile phase, and running time, can be manipulated to improve separation between spots on the plate. If the original spot adsorbed onto the stationary phase separates into more than one spot, this indicates that there are multiple organic components in the sample. Two spots with the same R_f value are generally assumed to be the same compound, unless two compounds being tested have very similar polarities. A reaction is assumed to have gone to completion if the reaction mixture does not present a spot with the R_f value of the starting material.

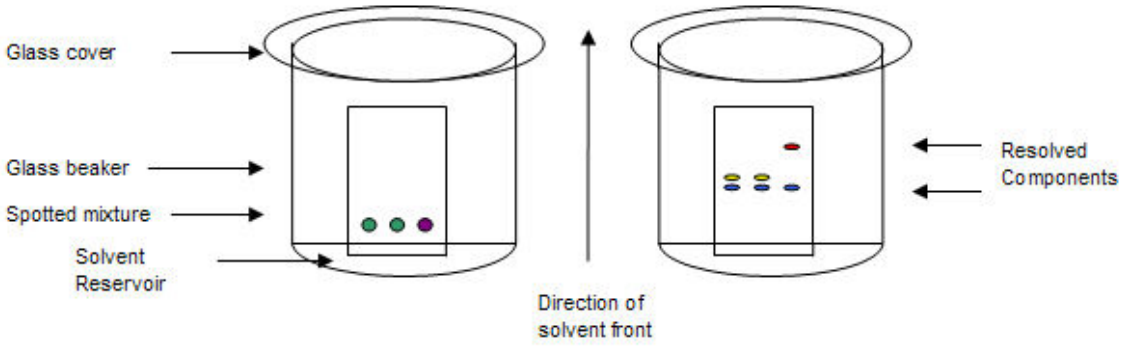


Figure 7. TLC Set Up and Results[17]

Once a reaction has gone to completion, the desired product must be isolated from the rest of the reaction mixture. If excess reagent or undesired side-products exist in the reaction mixture, a number of isolation techniques can be employed. Liquid-liquid extraction is one such technique, which partitions a reaction mixture based on the solubilities of the reaction components. To perform a liquid-liquid extraction, two immiscible solvents must be carefully selected. One of these solvents should be miscible with organic compounds and the other with other components of the reaction mixture. The reaction mixture and generous amounts of each of the two selected solvents are poured into a separation funnel. A stopper is inserted at the top of the funnel and the mixture is shaken vigorously. The pressure caused by built up gas is released, and the mixture is allowed to settle into two distinct layers. The organic products from the reaction mixture should be dissolved in the organic layer and the other reaction

components in the aqueous layer. Each layer is individually collected in a flask, effectively separating the organic-soluble from the aqueous-soluble reaction components.

Flash chromatography is a method used to separate the components of a mixture by polarity. To perform flash chromatography, a glass column is packed with dry silica gel to the desired level based on the amount of sample and R_f values of the components to be separated. A running solvent is then flushed through the silica gel until the gel is completely saturated with solvent and all air bubbles have been removed. The sample to be separated is applied to the top of the gel and allowed to soak into the column. More running buffer is then poured into the top of the column and an air hose is used to push the running solvent and sample through the column. The less polar sample moves through the gel faster while the more polar gel moves through the gel more slowly due to the attraction to the polar silica. Once sample reaches the bottom of the column, the eluent is collected in small fractions. Each fraction is spotted on a TLC plate to test for the presence of organic compounds. Fractions containing organic compounds of like polarities are combined.

To remove product from solution, one of two methods is commonly used. The solution can be transferred to a round bottom flask and attached to a rotary evaporator. This instrument simultaneously heats the sample, rotates the round bottom flask, and uses a vacuum to lower the vapor pressure in order to evaporate all of the solvent from the product. The solvent vapor travels through a glass tube until it reaches a condenser and condenses into a collection flask. Eventually only dry product is left in the round bottom

flask. This product can be further dried under a vacuum. If the desired product is not soluble in the reaction solvent, it can be readily isolated by filtering the reaction mixture. Alternatively, a solvent that the product is not soluble in can be added to the reaction mixture, causing the product to precipitate, at which point it can be filtered out. To filter a reaction mixture, the mixture is poured through a filter lined with porous filter paper. The solid product is caught on the paper while the solvent soaks through and drips into a collection flask. Often the filter is placed in the top of a filter flask, which has a side arm that can be connected to a vacuum tube to speed up the drying process. The solvent can be filtered multiple times to ensure maximum retrieval of product. This process can also be assisted by refrigerating the reaction to cause additional product to precipitate out of solution.

Once product from a reaction has been isolated, proton nuclear magnetic resonance (NMR) spectroscopy is used to tentatively confirm the identity of the product. A small amount of sample is dissolved in a deuterated solvent and transferred to an NMR tube. The tube is inserted into the NMR sample port. Electromagnetic energy is pulsed through the sample, realigning the nuclei of the atoms in the sample and recording the resonance frequency of the emitted electromagnetic radiation. From this information, the computer produces a plot showing a number of tall, narrow peaks, which represent different hydrogen atoms in the compound. On this plot, the x-axis represents the chemical shift (ppm), or resonance frequency of hydrogen nuclei. The chemical shift value for each peak indicates how close the hydrogen atom is to certain functional groups and electronegative elements.

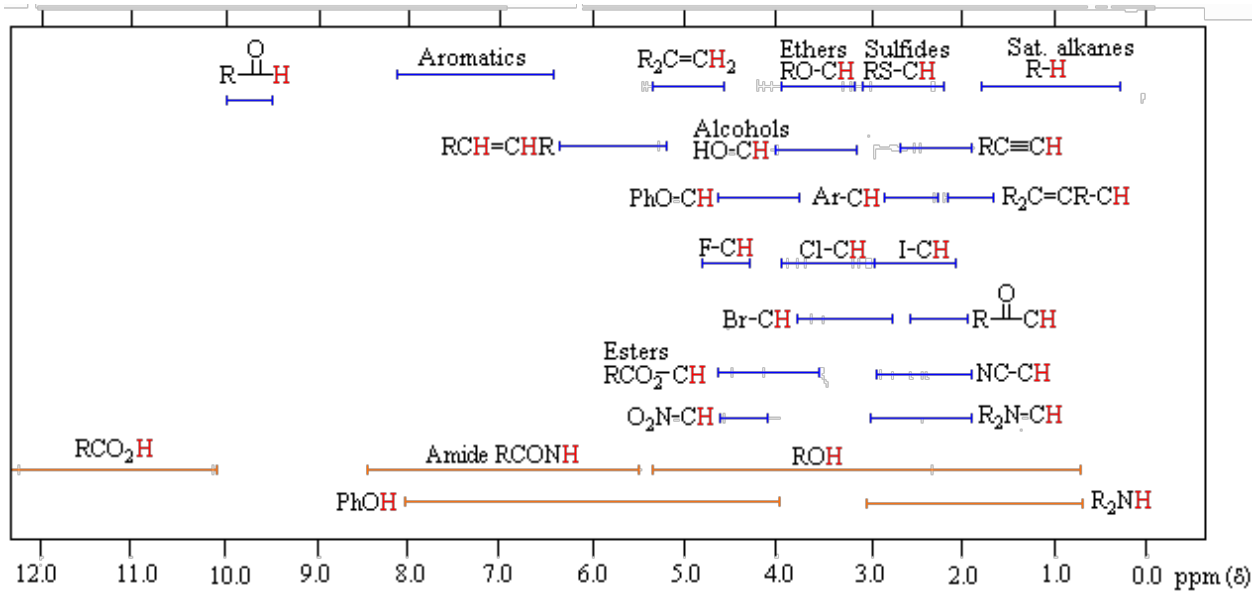


Figure 8. Summary of Neighboring Functional Groups Based on Chemical Shift[18]

The splitting pattern of each peak tells how many hydrogens neighbor the hydrogen represented by the peak. If the splitting pattern has four miniature peaks composing the large peak, then there are three neighboring hydrogens (number of peaks – 1) bound to other atoms. Finally, the integration of each peak tells how many hydrogens are located in that position on the molecule. The sum of the normalized integrals of the peaks tells how many hydrogens are located on the atom. This number should equal the expected number of hydrogens to be on the molecule. The experimental splitting patterns and chemical shifts can be compared to the theoretical results to determine if the identity of the compound matches what was expected.

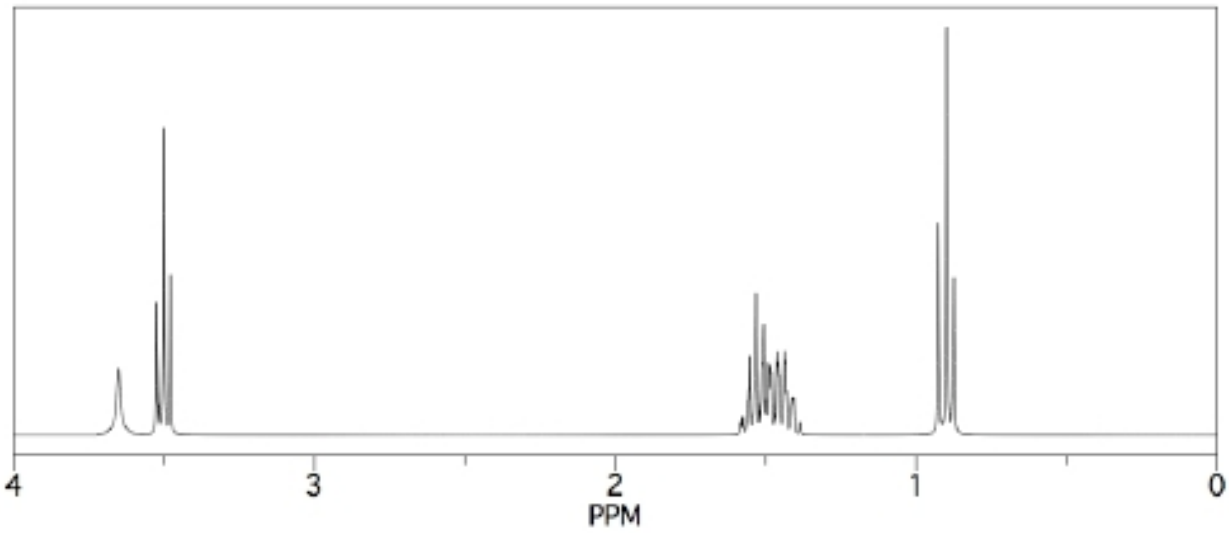


Figure 9. Proton NMR Spectrum

The goal of this research project is to synthesize a strong TLR7 agonist as a potential vaccine adjuvant. In particular, this experiment focuses on the development of three novel compounds with variation at the C8 carbon. Successful adjuvants, along with a desired antigen, can be conjugated to a monoclonal antibody as a delivery system in order to target[19] and promote uptake[20] into APCs and subsequent MHC processing, which hopefully will lead to a resilient adaptive immune response[5].

CHAPTER TWO:

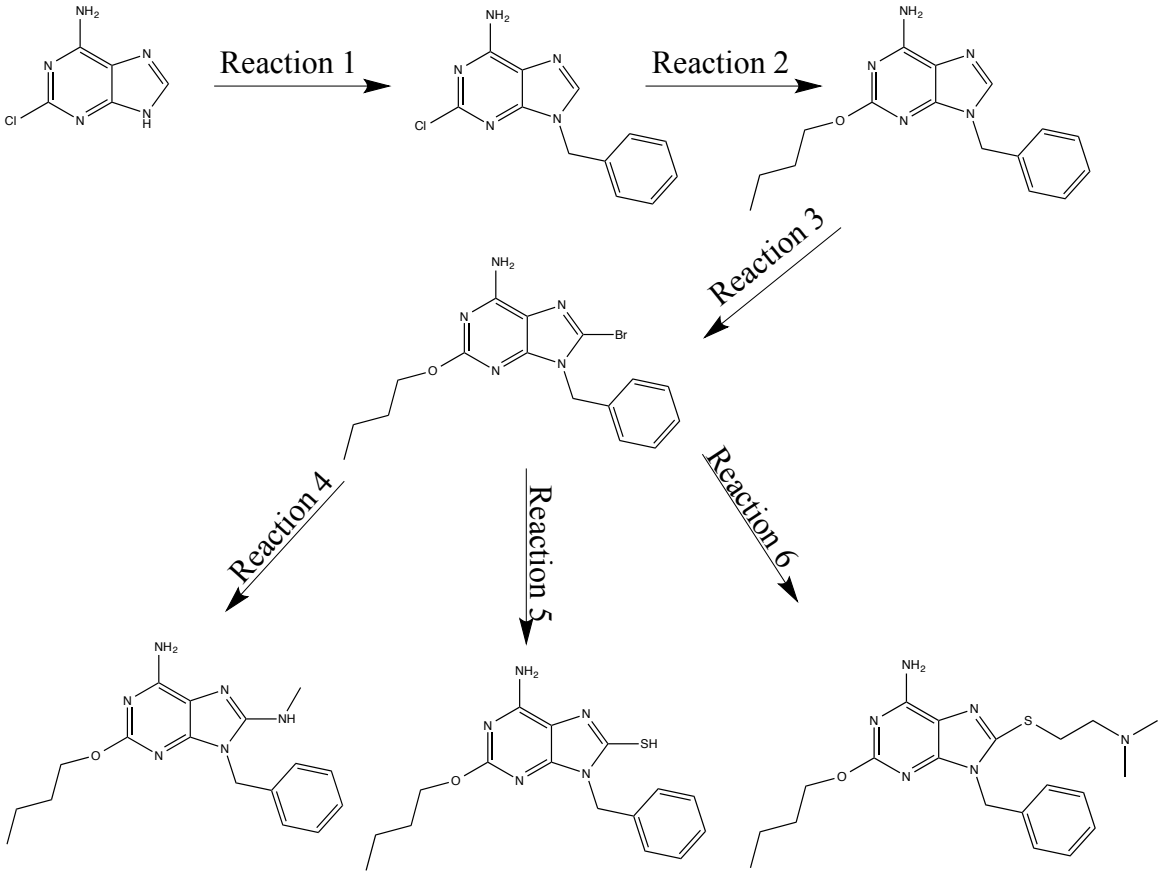
Materials and Methods

General Information

The reagents for these experiments were purchased from Sigma Aldrich, Pierce, Acros Organic, and Alfa Aesar. Solvents, including dichloromethane, ethanol, ethyl acetate, and butanol, were obtained from the Baylor Science Building stockroom. Several of the reactions were carried out in a CEM Discover microwave. All reactions were monitored by TLC (Sorbtech Silica G TLC Plates) with a running solvent composed of dichloromethane and methanol. The compound structures were confirmed by an Agilent Technologies 500 MHz NMR spectrometer, a Bruker 600 MHz NMR spectrometer, and a Thermo Scientific LTQ Orbitrap Discovery mass spectrometer. Proton NMR chemical shift values are reported in ppm (δ), and peaks are identified as either singlets (s), doublets (d), triplets (t), or multiplets (m).

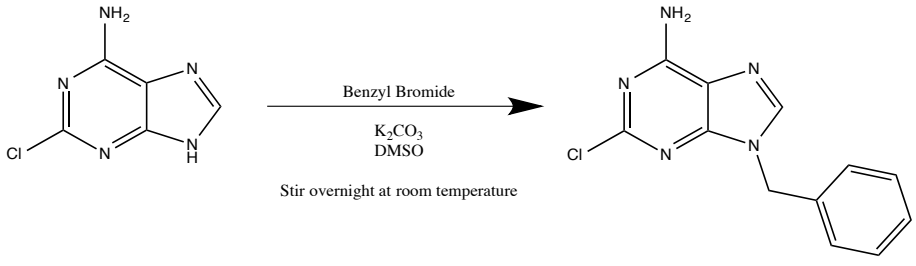
Synthesis Overview

The reaction scheme below summarizes the synthetic process of several sulfur and amino 8-substituted adenine compounds. In reaction 1, a benzene ring is substituted onto the N-9 position of the 2-chloroadenine starting material. In reaction 2, nucleophilic butoxide attacks the C-2 position, displacing the chlorine ion. In reaction 3, bromine replaces a hydrogen at the C-8 position via EAS. Once this brominated compound is achieved, the bromine can be replaced by a number of sulfur and amino substituents, as illustrated in reactions 4, 5, and 6.



Reaction Scheme Overview

Synthesis Procedures



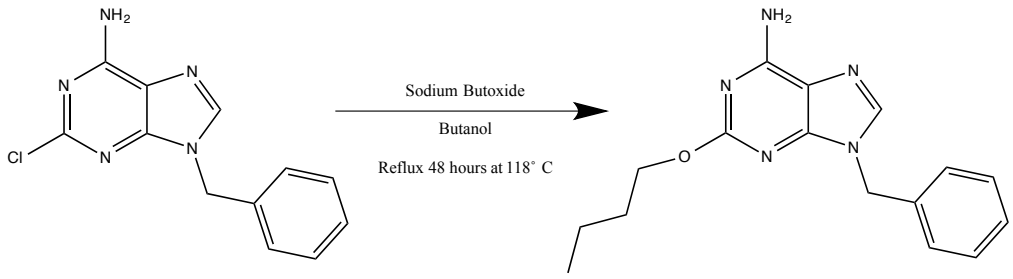
Scheme 1: Synthesis of 2-Chloro-9-Benzyl Adenine (Compound 1)

Method 1:

2-chloroadenine (2.00 g, 11.8 mmol) and K_2CO_3 (6.51 g, 47.1 mmol) were dissolved in DMSO (25 mL) and stirred for 10 minutes. Benzyl bromide (1.5 mL, 12.5 mmol) was added to the reaction mixture, which was then covered and allowed to stir overnight. The product was precipitated by refrigeration and dried by vacuum filtration. 96% yield was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.22 (s, 1H), 7.74 (s, 1H), 7.24 (s, 4H), 5.29 (s, 2H).

Method 2:

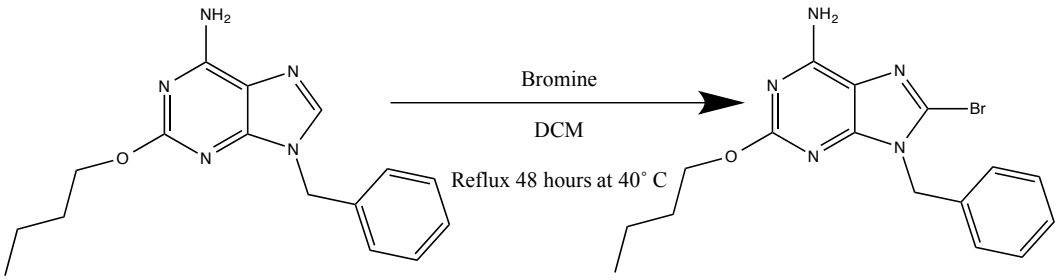
2-chloroadenine (4.01 g, 23.6 mmol) and K2CO3 (13.1 g, 94.2 mmol) were dissolved in DMSO (50 mL) and stirred for 10 minutes. Benzyl bromide (3.0 mL, 25 mmol) was added to the reaction mixture, which was then covered and allowed to stir overnight. The product was poured over cold water (100 mL), filtered, washed three times with EtOAc, and dried under vacuum. 59% yield was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.26 (s, 1H), 7.80 (s, 2H), 7.32 (d, J = 38.2 Hz, 5H), 5.33 (s, 2H).



Scheme 2: Synthesis of 2-Butoxy-9-Benzyl Adenine (Compound 2)

Method 1:

Sodium butoxide (45 mL) was prepared by adding sodium metal (0.4 g, 17.4 mmol) to n-butanol (45 mL) in a round-bottom flask under nitrogen gas. The sodium was allowed to react completely. Compound 1 (2.28 g, 8.46 mmol) was added to a second round-bottom flask containing n-butanol (80 mL) under nitrogen and stirred for 10 minutes. The sodium butoxide was poured into the round-bottom flask containing compound 1 and refluxed at 118˚ C for 48 hours. A rotary evaporator was used to remove half of the butanol from the reaction mixture. The product was filtered, rinsed with distilled water, and dried under vacuum for a 80% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.03 (s, 1H), 7.32 (s, 5H), 7.19 (s, 2H), 5.25 (s, 2H), 4.20 (s, 2H), 1.64 (s, 2H), 1.40 (s, 2H), 0.91 (s, 3H).



Scheme 3: Synthesis of 2-Butoxy-8-Bromo-9-Benzyl Adenine (Compound 3)

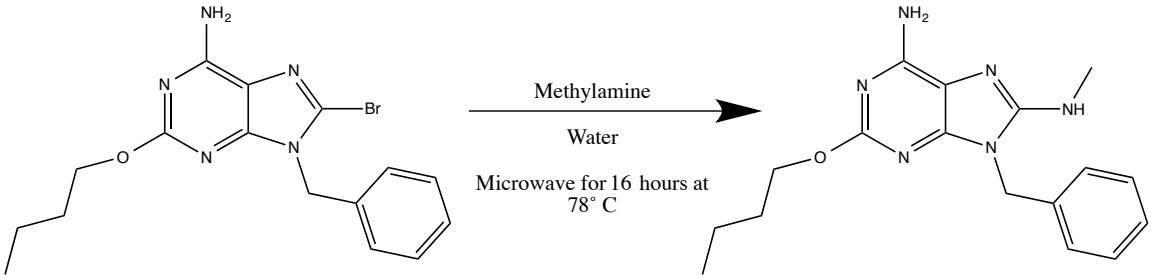
Method 1:

Compound 2 (0.94 g, 3.16 mmol) was dissolved in DCM (100 mL) in an Erlenmeyer flask. Bromine (8.4 mL, 160 mmol) was added to the reaction mixture and it

was refluxed for 48 hours at 40˚C. The product was extracted with DCM (50 mL) and sodium thiosulfate (200 mL) and washed with sodium bicarbonate (50 mL). During the extraction, the pressure inside of the separatory funnel blew off the glass stopper, and much of the product was lost. The organic layer was collected and dried over magnesium sulfate. The solvent was then evaporated with the rotary evaporator and the product was dried under vacuum for a 48% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.43 (s, 1H), 7.30 (d, J = 51.8 Hz, 5H), 5.25 (s, 2H), 4.21 (s, 2H), 1.64 (s, 2H), 1.39 (s, 2H), 0.91 (s, 3H).

Method 2:

Compound 2 (1.1 g, 3.7 mmol) was dissolved in DCM (100 mL). Bromine (9.5 mL, 190 mmol) was added to the reaction mixture, and the reaction was refluxed for 48 hours at 40˚ C. The reaction was transferred to a 500 mL beaker and DCM (100 mL) was added. The reaction was stirred for 5 minutes, and then sodium thiosulfate (250 mL) was added in 50 mL aliquots. The reaction was stirred violently for 15 minutes and then left to rest for 20 minutes. The aqueous layer was decanted, and the organic layer was transferred to a round-bottom flask. The organic layer solvent was removed using rotary evaporation. The product was filtered, washed with sodium bicarbonate and dried under vacuum overnight. ^{1}H NMR (600 MHz, Chloroform-d) δ 7.32 (s, 5H), 5.42 (s, 2H), 5.30 (s, 2H), 4.32 (s, 2H), 1.76 (s, 2H), 1.49 (s, 2H), 0.96 (s, 3H).



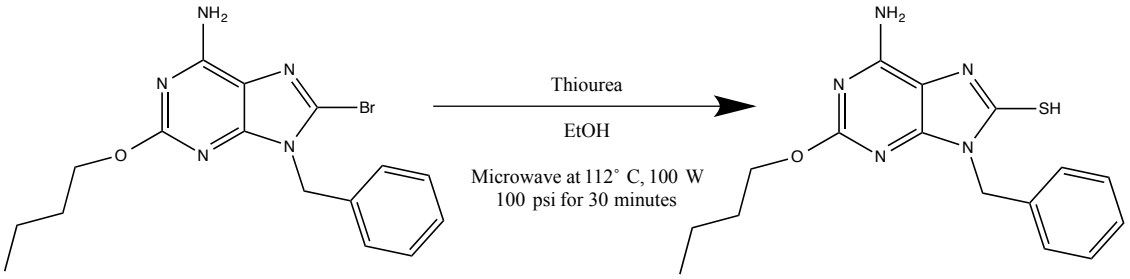
Scheme 4: Synthesis of 2-Butoxy-8-Methylamino-9-Benzyl Adenine (Compound 4)

Trial 1:

Compound 3 (0.10 g, 0.27 mmol) was added to methylamine (5 mL, 140 mmol) in a 10 mL CEM Discover microwave tube. The reaction was microwaved at 0˚ C, 250 W, 250 psi for one hour. The product was dried by rotary evaporation, but the TLC showed several components present in the reaction mixture. Flash chromatography was used to purify the desired product, but a clear NMR spectrum was not obtained.

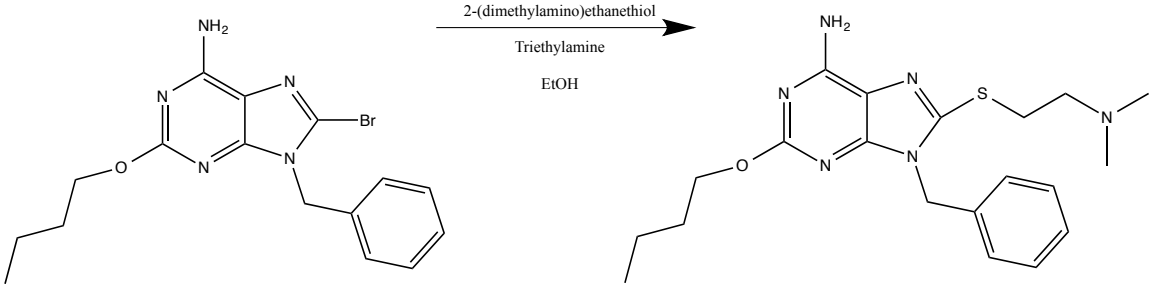
Trial 2:

Compound 3 (0.10 g, 0.27 mmol) was added to methylamine (2 mL, 58 mmol) and water (3 mL) in a 10 mL CEM Discover microwave tube. The reaction was microwaved at 120˚ C for 16 hours. The reaction was filtered and washed with water for a 35% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.31 (s, 5H), 6.87 (s, 2H), 5.26 (s, 2H), 4.17 (s, 3H), 2.08 (s, 3H), 1.63 (s, 2H), 1.38 (s, 3H), 0.90 (s, 3H).



Scheme 5: Synthesis of 2-Butoxy-8-Thio-9-Benzyl Adenine (Compound 5)

Compound 3 (0.5 g, 1.33 mmol), thiourea (0.2 g, 2.63 mmol), and ethanol (25 mL) were added to a 35 mL CEM Discover microwave tube. The reaction was microwaved for 30 minutes at 112˚ C, 100 W, 100 psi. The reaction was cooled, filtered, washed with distilled water, and dried under vacuum for a yield of 30%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.20 (s, 1H), 7.37 (s, 6H), 6.87 (s, 2H), 5.26 (s, 2H), 4.17 (s, 2H), 3.33 (s, 2H), 2.50 (s, 4H), 1.62 (s, 2H), 1.36 (s, 2H), 0.90 (s, 3H).



Scheme 6: Synthesis of 2-Butoxy-8-(Dimethylamino)thioethane-9-Benzyl Adenine (Compound 6)

Method 1:

Compound 3 (0.10 g, 0.27 mmol) was dissolved in ethanol (15 mL) along with 2-(dimethylamino)ethanethiol hydrochloride (0.08 g, 0.56 mmol) and triethylamine (0.2 mL, 1.43 mmol). The reaction was allowed to stir for 4 days at room temperature. The reaction was then refluxed for 48 hours at 78˚ C. All but 3 mL of the solvent was evaporated using a rotary evaporator. The remaining reaction mixture was added to 0.1 M NaOH (75 mL) in a separatory funnel. The product was extracted with DCM (150 mL), dried over magnesium sulfate, and filtered using gravity filtration. Rotary evaporation was used to remove the DCM solvent and the product was dried under vacuum overnight. TLC indicated that two products had been isolated. Flash chromatography was used to isolate the components, but these were determined to be compound 3 and 2(dimethylamino)ethanethiol starting material.

Method 2:

Sodium ethoxide was prepared by adding sodium metal (0.02 g, 0.87 mmol) to ethanol (15 mL) under nitrogen gas. 2(dimethylamino)ethanethiol (0.075 g, 0.71 mmol) and compound 3 (0.1 g, 0.27 mmol) were added to the sodium ethoxide and the reaction was allowed to stir for 12 days. Rotary evaporation was used to remove most of the solvent from the reaction. The product was extracted with DCM and 0.1 M sodium hydroxide. The organic layer was collected in a round bottom flask and dried using rotary evaporation. However, the isolated product appeared to be starting material.

CHAPTER THREE:

Results and Conclusions

Compound 1 was attempted and successfully synthesized 5 times. The greatest yield obtained was 96%, according to the optimized reaction procedures listed in the materials and methods chapter. The structure of compound 1 was confirmed with proton NMR and LCMS.

Compound 2 was attempted and successfully synthesized 5 times. The greatest yield obtained was 80% and the structure was confirmed with proton NMR and LCMS.

Compound 3 was attempted and successfully synthesized 5 times. The greatest yield obtained was 48%. For this reaction, method 1 produced purer product, but the high pressure inside the separatory funnel during extraction was problematic, resulting in the loss of product. The extraction technique in method 2 minimized the risk for product loss, but produced a product of lesser purity. The structure of compound 3 was confirmed by proton NMR and LCMS.

Compound 4 was attempted 3 times and successfully synthesized 1 time. The successful conditions for reaction are outlined in the materials and methods chapter, for a yield of 35%. The structure of this compound was confirmed by proton NMR and LCMS. This compound was conjugated to a monoclonal antibody and tested for immunological activity at the Baylor Institute for Immunology Research in Dallas (see results below).

Compound 5 was attempted 3 times and was successfully synthesized 1 time. The successful conditions for this reaction are outlined in the materials and methods

chapter, for a yield of 30%. The structure of this compound was confirmed by proton NMR and LCMS. This compound was conjugated to a monoclonal antibody and tested for immunological activity at the Baylor Institute for Immunology Research in Dallas (see results below).

Compound 6 was attempted 3 times, but successful synthesis of this compound has not yet been confirmed. Continuing attempts to synthesize this compound are outlined in the following chapter.

Compound 4 and compound 5 were sent to the Baylor Institute for Immunology Research, where an HEK-Blue TLR7 cell assay (InvivoGen) was used to test the immunological activity of these compounds. Each compound, as well as a positive control (resiquimod), was tested at concentrations of 1000 ng/mL, 500 ng/mL, 50 ng/mL, and 0 ng/mL. The optical density (OD) at each of these concentrations was recorded. Greater OD indicates an increased release of cytokines, triggered by TLR7 activation. Two trials were conducted. The results of these two trials are displayed in the table below.

Table 1. Optical Density of TLR7 Agonists Tested by HEK-Blue TLR7 Cell Assay at Varying Concentrations

Compound	**OD at 1000 ng/mL**		**OD at 500 ng/mL**		**OD at 50 ng/mL**		**OD at 0 ng/mL**	
Resiquimod	1.0549	0.8483	0.7192	0.7714	0.4544	0.4932	0.3623	0.3566
Compound 4	0.3718	0.5927	0.4847	0.5123	0.4276	0.4887	0.3623	0.3566
Compound 5	0.508	0.6914	0.7149	0.7242	0.5098	0.5655	0.3623	0.3566

The ODs for both trials were averaged and plotted in figure 9 and figure 10 below.

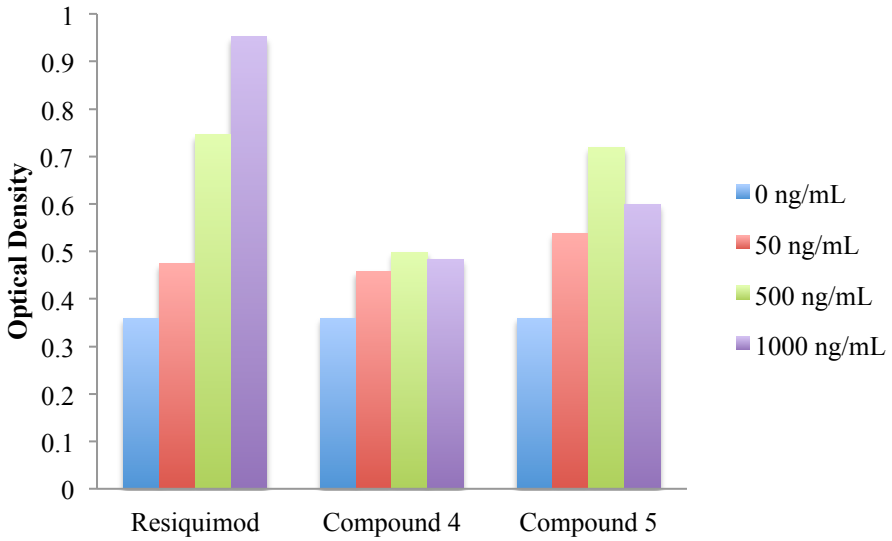


Figure 10. Average OD of Resiquimod, Compound 4, and Compound 5 at Varying Concentrations

Figure 9 demonstrates how dosage changes for each compound affect the immune response initiated by TLR7 stimulation. The general trend from this figure suggests that increasing the concentration of TLR7 agonist also increases the immunological response by the cell. However, both compound 4 and compound 5 show slightly decreased activity at a concentration of 1000 ng/mL. This decrease in OD at high agonist concentrations could indicate that compound 4 and compound 5 are fatally toxic to cells at 1000 ng/mL.

Figure 10 compares the potency of each agonist. At concentrations below ~500 ng/mL, compound 5 appears to induce a higher OD than does resiquimod. If a sufficient

immune response could be achieved at an OD less than ~0.7, then compound 5 may be a viable adjuvant as it could be administered in smaller doses than resiquimod and still achieve the same immunological response. Compound 4 showed little activity in comparison with resiquimod and compound 5 and showed little increase in activity with increasing concentration. Compound 4 could be a useful adjuvant for a vaccine that requires low levels of immune stimulation, and it would be easy to control because it is not highly sensitive to changes in concentration.

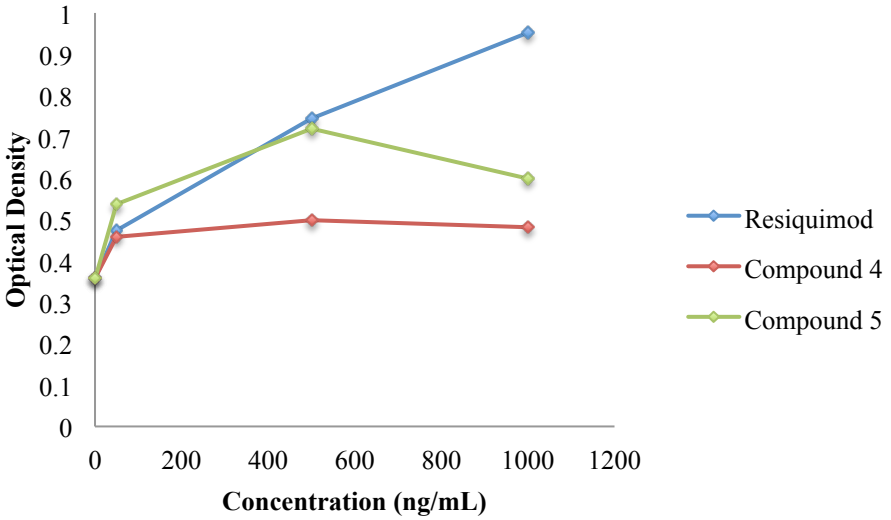
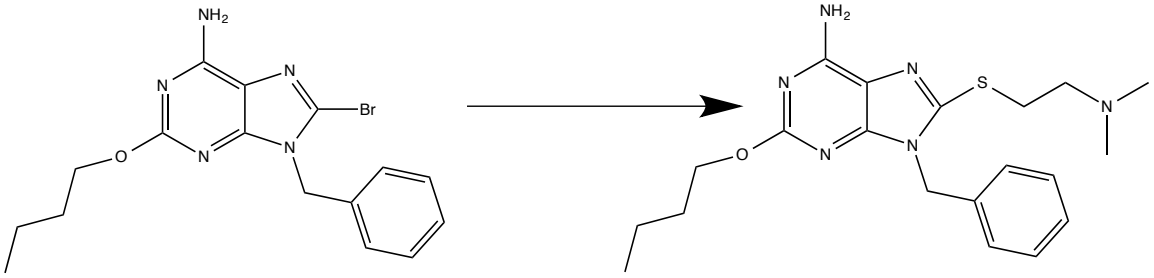


Figure 11. Optical Density Response Curve at Increasing Concentrations of Resiquimod, Compound 4, and Compound 5

Although both compound 4 and compound 5 may be possible candidates as vaccine adjuvants, it is advantageous to pursue the synthesis of more C8-substituted compounds, so that a panel of adjuvants, with various immunogenicities and potencies, can be available for inclusion in vaccine design.

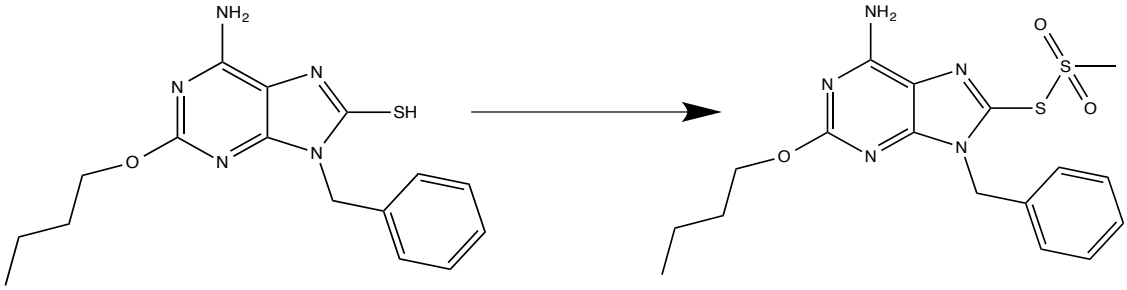
CHAPTER FOUR:

Future Work



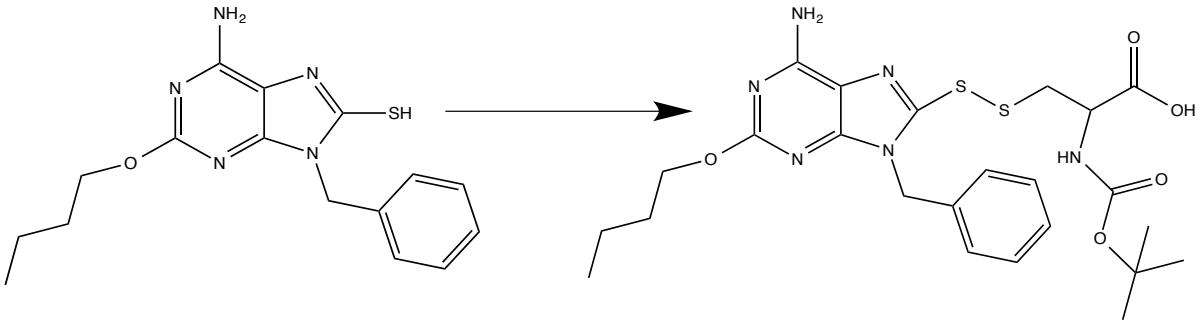
Scheme 6: Synthesis of 2-Butoxy-8-(Dimethylamino)thioethane-9-Benzyl Adenine (Compound 6)

In the preliminary attempts to synthesize compound 6, reaction did not occur despite being allowed to stir for an extended period of time. This reaction may be successful under high-heat and high-pressure conditions. Future trials will involve experimentation with the reaction microwave.



Scheme 7: Synthesis of 2-Butoxy-8-Methanesulfonyl-9-Benzyl Adenine (Compound 7)

It has been reported[21] that methanesulfonyl chloride will react with a thiol substituent on the C8 position of a purine ring to form a disulfide bond. The first trial for this reaction will be carried out according to the reaction conditions and workup reported in the paper. Compound 5 and methanesulfonyl chloride will be dissolved in DCM and pyridine and allowed to stir overnight at 4˚C. The reaction mixture will then be filtered, concentrated, and re-filtered, washed with EtOAc and dried. This reaction was reported to produce a 50% yield.



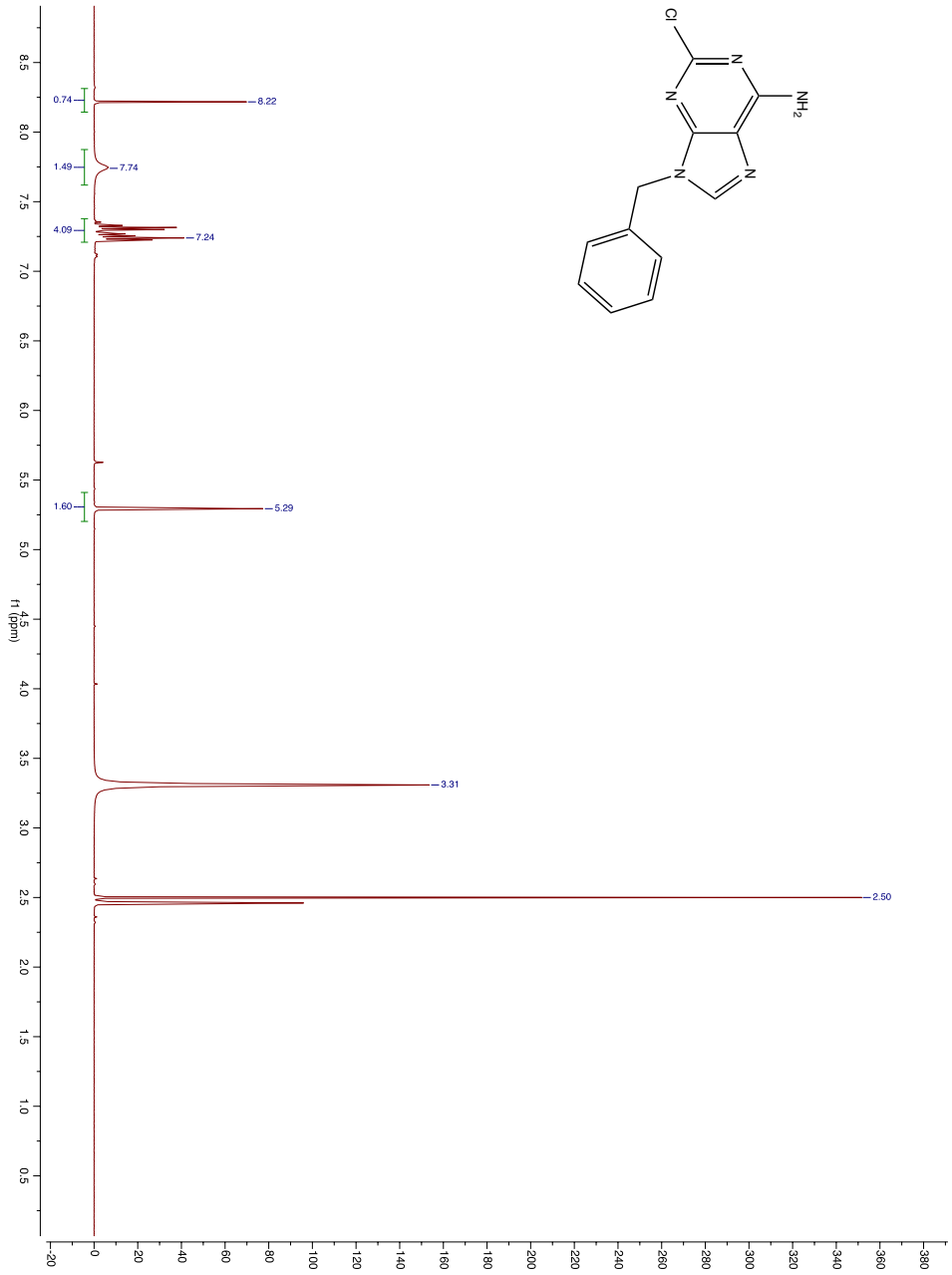
Scheme 8: Synthesis of 2-Butoxy-8-(Boc-Cys-OH)-9-Benzyl Adenine (Compound 8)

The oxidative formation of the disulfide bond between compound 5 and boc-cys-OH is an attempt at synthesizing a prodrug, which theoretically becomes active *in vivo* with the cyclization of the boc-cys-OH substituent. The first attempt at synthesizing this compound will be made simply by dissolving compound 5 (0.05 g, 0.15 mmol) in ethanol (20 mL) with $(boc\text{-}cys\text{-}OH)_2$ and stirring overnight at room temperature.

APPENDICES

APPENDIX A

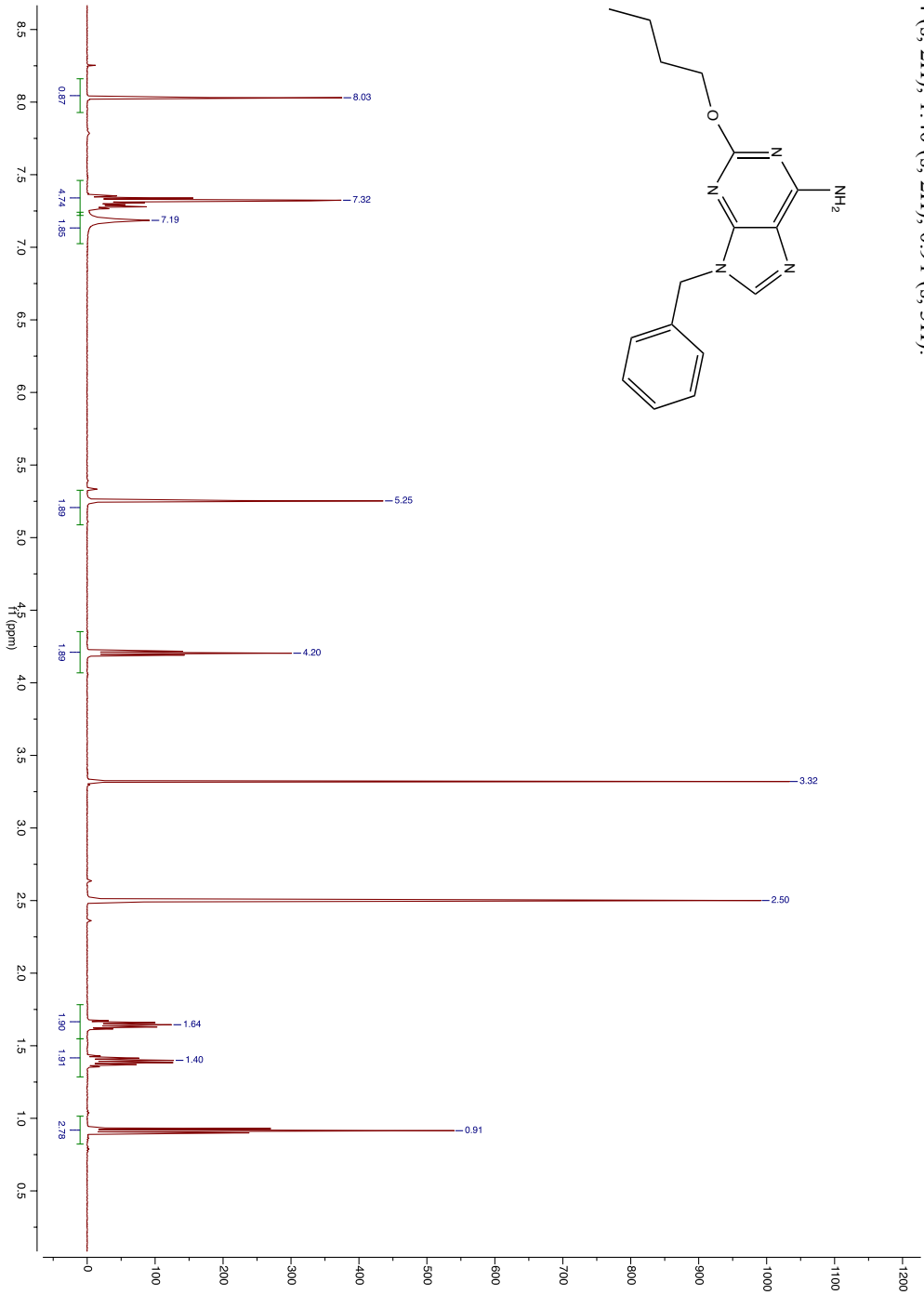
NMR of Compound 1



^{1}H NMR (500 MHz, DMSO-d_6) δ 8.22 (s, 1H), 7.74 (s, 1H), 7.24 (s, 4H), 5.29 (s,

APPENDIX B

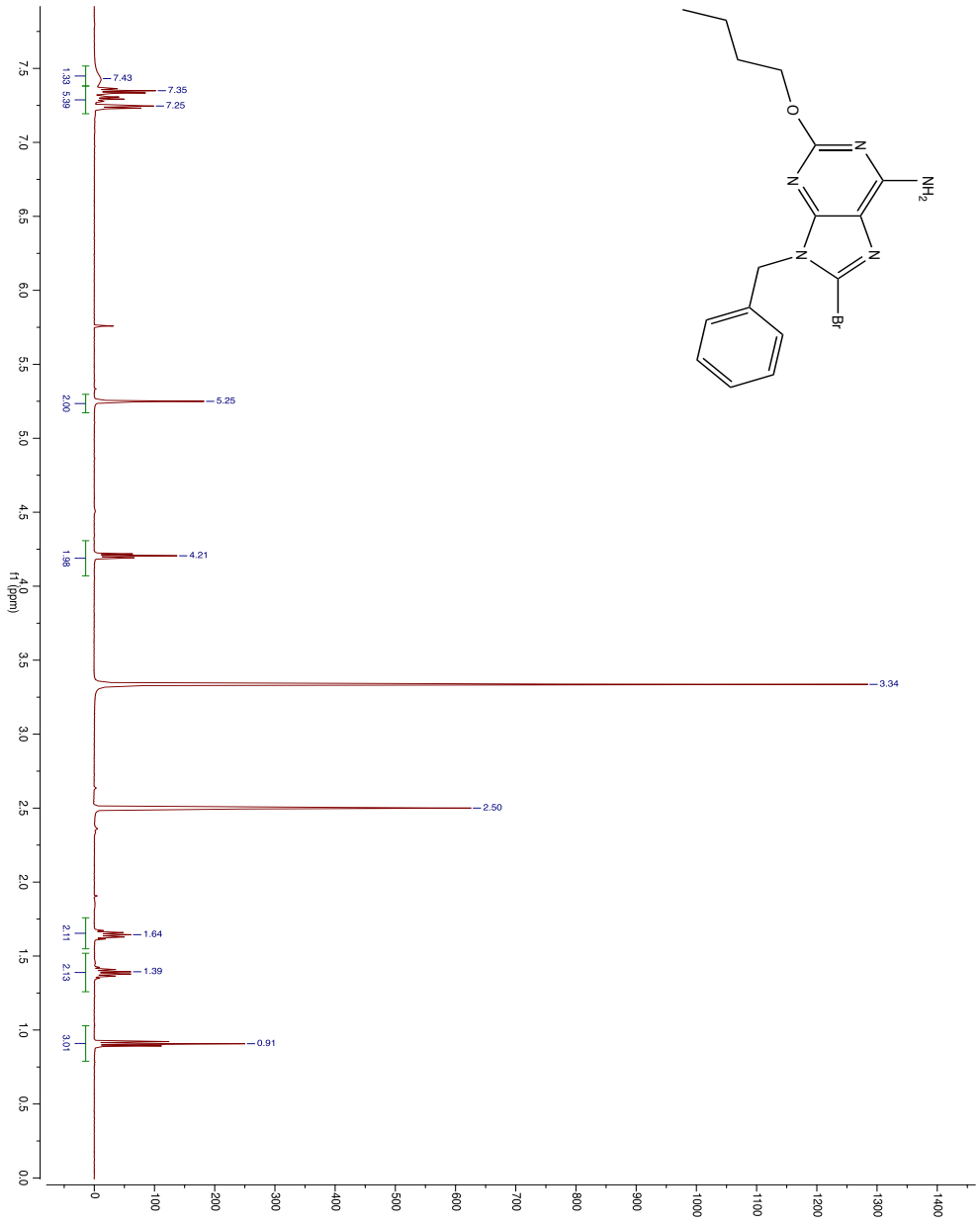
NMR of Compound 2



^{1}H NMR (500 MHz, DMSO-d_6) δ 8.03 (s, 1H), 7.32 (s, 5H), 7.19 (s, 2H), 5.25 (s, 2H), 4.20 (s, 2H), 1.64 (s, 2H), 1.40 (s, 2H), 0.91 (s, 3H).

APPENDIX C

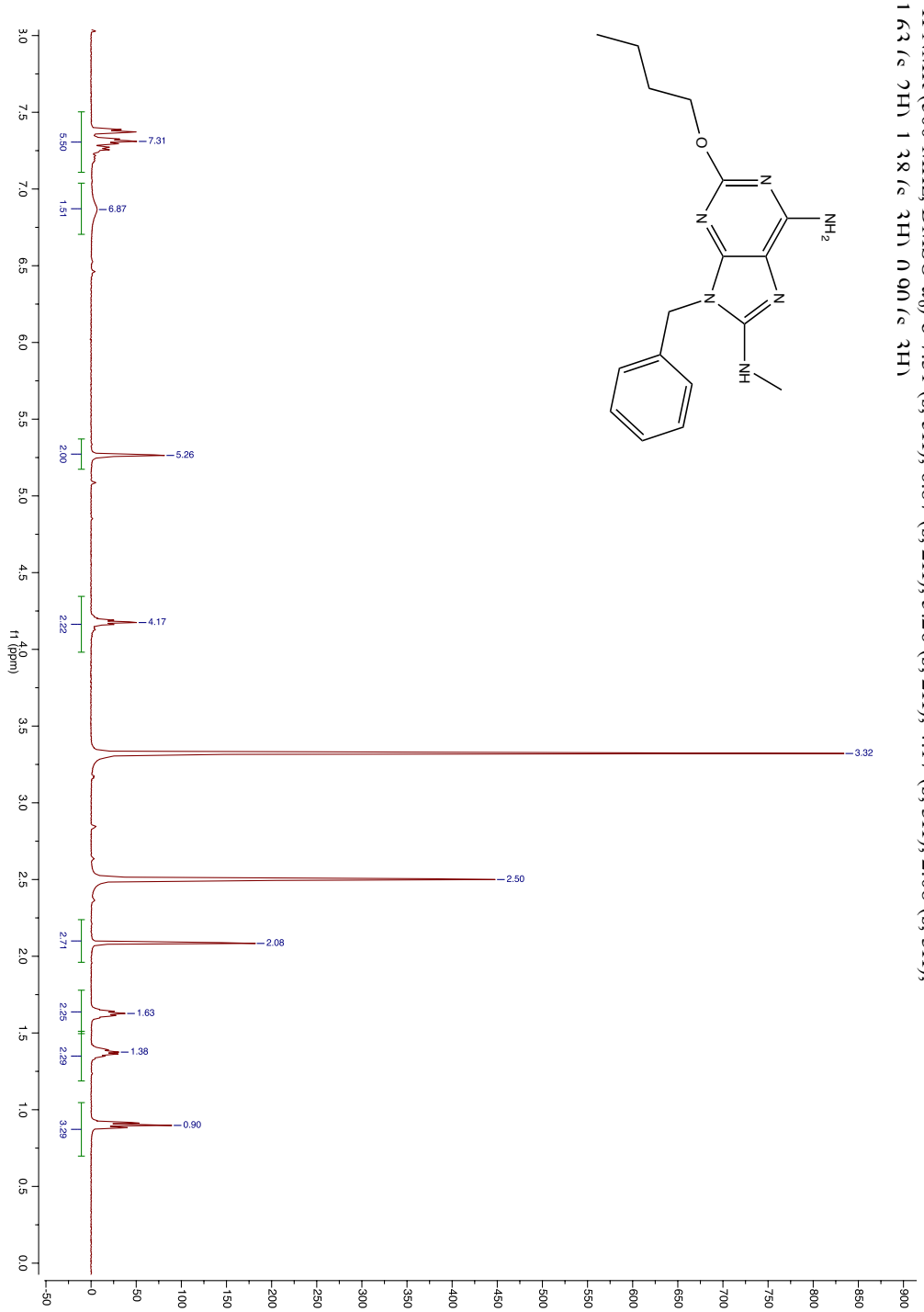
NMR of Compound 3



^{1}H NMR (500 MHz, DMSO-d_6) δ 7.43 (s, 1H), 7.30 (d, J = 51.8 Hz, 5H), 5.25 (s, 2H), 4.21 (s, 2H),

APPENDIX D

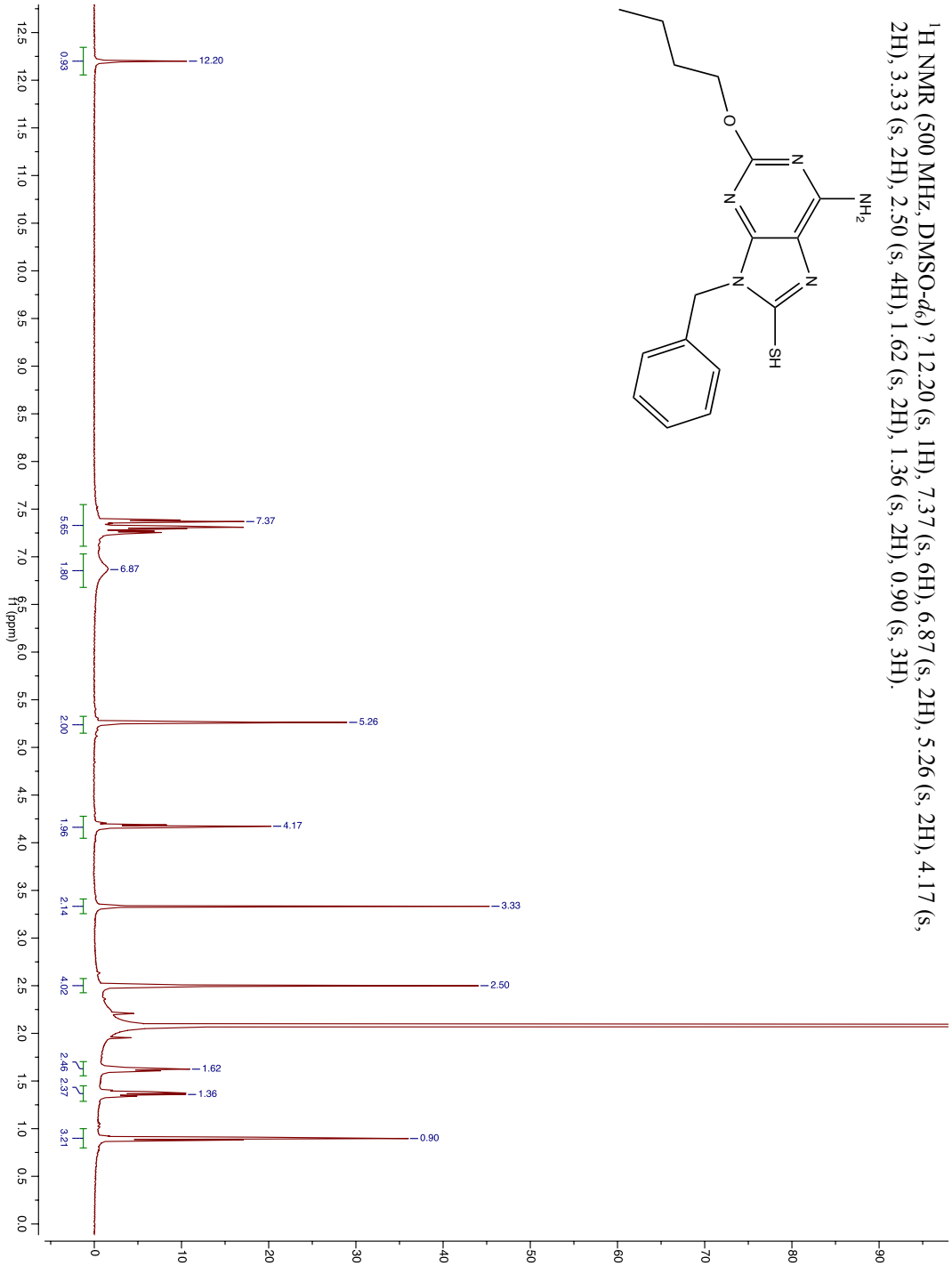
NMR of Compound 4



^{1}H NMR (500 MHz, DMSO-d_6) δ 7.31 (s, 5H), 6.87 (s, 2H), 5.26 (s, 2H), 4.17 (s, 3H), 2.08 (s, 3H), 1.63 (s, 2H), 1.38 (s, 3H), 0.90 (s, 3H)

APPENDIX E

NMR of Compound 5



^{1}H NMR (500 MHz, DMSO-d_6) ? 12.20 (s, 1H), 7.37 (s, 6H), 6.87 (s, 2H), 5.26 (s, 2H), 4.17 (s, 2H), 3.33 (s, 2H), 2.50 (s, 4H), 1.62 (s, 2H), 1.36 (s, 2H), 0.90 (s, 3H).

BIBLIOGRAPHY

(1) Parham, P. *The Immune System*; 3rd ed.; Garland Science, 2009.

(2) Pashine, A.; Valiante, N. M.; Ulmer, J. B. Targeting the Innate Immune Response with Improved Vaccine Adjuvants. *Nat Med* **2005**, *11*, S63–S68.

(3) Finco, O.; Rappuoli, R. Designing Vaccines for the Twenty-First Century Society. *Front. Immunol.* **2014**, *5*, 12.

(4) Bråve, A.; Ljungberg, K.; Wahren, B.; Liu, M. A. Vaccine Delivery Methods Using Viral Vectors. *Mol. Pharm.* **2007**, *4*, 18–32.

(5) Wilson, R. M.; Danishefsky, S. J. A Vision for Vaccines Built from Fully Synthetic Tumor-Associated Antigens: From the Laboratory to the Clinic. *J. Am. Chem. Soc.* **2013**, *135*, 14462–14472.

(6) Giannini, S. L.; Hanon, E.; Moris, P.; Van Mechelen, M.; Morel, S.; Dessy, F.; Fourneau, M. A.; Colau, B.; Suzich, J.; Losonksy, G.; Martin, M.-T.; Dubin, G.; Wettendorff, M. A. Enhanced Humoral and Memory B Cellular Immunity Using HPV16/18 L1 VLP Vaccine Formulated with the MPL/aluminium Salt Combination (AS04) Compared to Aluminium Salt Only. *Vaccine* **2006**, *24*, 5937–5949.

(7) Duthie, M. S.; Windish, H. P.; Fox, C. B.; Reed, S. G. Use of Defined TLR Ligands as Adjuvants within Human Vaccines. *Immunological Reviews* **2011**, *239*, 178–196.

(8) Vasselon, T.; Detmers, P. A. Toll Receptors: A Central Element in Innate Immune Responses. *Infect. Immun.* **2002**, *70*, 1033–1041.

(9) Shukla, N. M.; Salunke, D. B.; Balakrishna, R.; Mutz, C. A.; Malladi, S. S.; David, S. A. Potent Adjuvanticity of a Pure TLR7-Agonistic Imidazoquinoline Dendrimer. *PLoS ONE* **2012**, *7*, e43612.

(10) Kurimoto, A.; Hashimoto, K.; Nakamura, T.; Norimura, K.; Ogita, H.; Takaku, H.; Bonnert, R.; McInally, T.; Wada, H.; Isobe, Y. Synthesis and Biological Evaluation of 8-Oxoadenine Derivatives as Toll-like Receptor 7 Agonists Introducing the Antedrug Concept. *J. Med. Chem.* **2010**, *53*, 2964–2972.

(11) Hirota, K.; Kazaoka, K.; Niimoto, I.; Kumihara, H.; Sajiki, H.; Isobe, Y.; Takaku, H.; Tobe, M.; Ogita, H.; Ogino, T.; Ichii, S.; Kurimoto, A.; Kawakami, H.

Discovery of 8-Hydroxyadenines as a Novel Type of Interferon Inducer. *J. Med. Chem.* **2002**, *45*, 5419–5422.

(12) Nakamura, T.; Wada, H.; Kurebayashi, H.; McInally, T.; Bonnert, R.; Isobe, Y. Synthesis and Evaluation of 8-Oxoadenine Derivatives as Potent Toll-like Receptor 7 Agonists with High Water Solubility. *Bioorganic & Medicinal Chemistry Letters* **2013**, *23*, 669–672.

(13) Kurimoto, A.; Ogino, T.; Ichii, S.; Isobe, Y.; Tobe, M.; Ogita, H.; Takaku, H.; Sajiki, H.; Hirota, K.; Kawakami, H. Synthesis and structure–Activity Relationships of 2-Amino-8-Hydroxyadenines as Orally Active Interferon Inducing Agents. *Bioorganic & Medicinal Chemistry* **2003**, *11*, 5501–5508.

(14) Musmuca, I.; Simeoni, S.; Caroli, A.; Ragno, R. Small-Molecule Interferon Inducers. Toward the Comprehension of the Molecular Determinants through Ligand-Based Approaches†. *J. Chem. Inf. Model.* **2009**, *49*, 1777–1786.

(15) Reflux | Laboratory techniques http://www.eplantscience.com/index/dean/reflux.php (accessed Oct 15, 2014).

(16) Microwave Synthesis http://www.organic-chemistry.org/topics/microwave-synthesis.shtm (accessed Oct 15, 2014).

(17) Brewing and Microbiology | Faculty of Biological Sciences http://www.virtual-labs.leeds.ac.uk/brewing/TLCtheory.php (accessed Oct 15, 2014).

(18) Reusch, W. Proton NMR Table http://www.cem.msu.edu/~reusch/OrgPage/nmr.htm (accessed Oct 15, 2014).

(19) Proudfoot, O.; Apostolopoulos, V.; Pietersz, G. A. Receptor-Mediated Delivery of Antigens to Dendritic Cells: Anticancer Applications. *Mol. Pharm.* **2007**, *4*, 58–72.

(20) Wu, C. C. N.; Hayashi, T.; Takabayashi, K.; Sabet, M.; Smee, D. F.; Guiney, D. D.; Cottam, H. B.; Carson, D. A. Immunotherapeutic Activity of a Conjugate of a Toll-like Receptor 7 Ligand. *PNAS* **2007**, *104*, 3990–3995.

(21) Mahieu, J.-P.; Gosselet, M.; Sebille, B.; Beuzard, Y. Synthesis of New Thiosulfonates and Disulfides from Sulfonyl Chlorides and Thiols. *Synthetic Communications* **1986**, *16*, 1709–1722.

(22) Roh, Y. S.; Park, S.; Kim, J. W.; Lim, C. W.; Seki, E.; Kim, B. Toll-like Receptor 7-Mediated Type I Interferon Signaling Prevents Cholestasis- and Hepatotoxin-Induced Liver Fibrosis. *Hepatology* **2013**, n/a–n/a.

(23) Yang, L.; Seki, E. Toll-like Receptors in Liver Fibrosis: Cellular Crosstalk and Mechanisms. *Front. Physio.* **2012**, *3*, 138.

(24) TLR7 and TLR8: Key players in the antiviral response - Innate immunity - invivoGen http://www.invivogen.com/review-tlr7-tlr8 (accessed Apr 1, 2014).

(25) Petrovsky, N.; Aguilar, J. C. Vaccine Adjuvants: Current State and Future Trends. *Immunol Cell Biol* **2004**, *82*, 488–496.