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# Synthesis of a chemical probe for identification of Ger receptor proteins of Bacillus anthracis

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# SYNTHESIS OF A CHEMICAL PROBE FOR IDENTIFICATION OF GER RECEPTOR PROTEINS OF *BACILLUS ANTHRACIS*

by

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ABSTRACT

**Synthesis of a Chemical Probe for Identification of Ger**

**Receptor Proteins of *Bacillus Anthracis***

by

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*Bacillus anthracis*, a gram positive, endospore-forming, rod-shaped, aerobic bacterium, is the causative agent of anthrax. A key step in the pathogenic cycle is the spore germination, where the dormant spore transforms into a metabolically active cell. This allows *B. anthracis* to proliferate and secrete toxins. It is believed that spore germination is triggered when small molecules, known as germinants, are recognized by germination (Ger) receptor proteins located in the inner membrane of spores. Most Ger proteins are encoded by tricistronic operons, resulting in three distinct products, the A-, B-, C-subunits. Genomic sequencing showed that *B. anthracis* has seven ger receptor operons- six genomic ger receptor operons (gerA, gerH, gerK, gerL, gerS, and gerY) and one plasmidic ger receptor operon (gerX).

The most efficient germinant combination is mixtures of L-alanine and purine nucleosides. 6-thioguanosine (6-TG), an inosine analog, was shown to be a competitive inhibitor of inosine mediated *B. anthracis* spore germination. 6-TG is a mercaptopurine, can be activated by UV irradiation to crosslink with binding proteins. By utilizing the properties of this compound, we aimed to identify Ger receptor proteins of *B. anthracis*.

To investigate purine binding site of the Ger proteins, we used a chemical-biological strategy. With the help of synthetic chemistry, 5'-azido-5'-deoxy-6-thioguanosine ($N_3$-6-

TG) was prepared. Azide (-$N_3$) functionality is introduced as a bait for click reaction and a thiol group (-SH) allows crosslinking. This compound was subsequently UV irradiated for crosslinking *B. anthracis* spores. After decoating and lysis of the spores, "click chemistry" was involved, expecting efficient acivity-based protein profiling with biotinylated alkyne tags under Cu(I)-catalyzed conditions. SDS-PAGE and detection with streptavidin-horseradish peroxidase conjugate were carried out to isolate the cross-linked proteins conjugated with the biotin tag. The detected area of SDS-PAGE gel can be excised and characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

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# INTRODUCTION

## 1.1 *Bacillus Anthracis*

*Bacillus anthracis* is a gram positive, rod-shaped, and aerobic bacterium. It is the etiologic agent of anthrax, an infectious disease. The cells of *B. anthracis* produce endospores under nutrient depletion. Spores remain in a dormant state until they are exposed to a favorable environment where they germinate and return to vegetative growth. The resulting bacteria release toxins, initiating the pathogenic cycle. Because of the properties of forming endospores, *B. anthracis* spores can survive in harsh conditions and easily spread into mammalian systems through three different routes: skin lesions, inhalation, and ingestion (1).

Genetically *B. anthracis* is closely related to *B. cereus* and *B. thuringiensis*, a causative agent of foodborne illness and an insect pathogen, respectively. However, *B. anthracis* is distinguishable from those species by the fact that it contains two major virulence plasmids, pXO1 and pXO2. The pXO1 plasmid encodes a tripartite toxin complex, and the pXO2 is responsible for the synthesis of a poly-γ-D-glutamic acid capsule. Both plasmids are required for pathogenesis in humans (2).

## 1.2 Spore Germination

Spores germination is the first step in anthrax infection and it is the transformational process where the dormant spore grows into the vegetative form, an actively growing cell. The spores are metabolically dormant and resistant even in extreme conditions such as heat, radiation, desiccation, pH extremes, and toxic chemicals (3). However, the dormant

spores are also capable of monitoring their environment. Once the spore detects certain signaling molecules known as germinants, either nutrient or non-nutrient, it initiates germination process and goes through outgrowth. In *Bacillus* species, the most common nutrient germinants to trigger spore germination are combinations of amino acid and purine nucleoside (4, 5, 6).

The mechanism of spore germination is not clearly understood, but it is believed that the germinants bind to the germination receptors located in the inner membrane of the spore (5, 7, 8). Upon the activation of the germination receptors by the nutrients, it induces release of the components of spores and replacement by water. The process of germination after recognition of the germinants by the germination receptors can be summarized in five sequential steps (9). First, the spore releases ions including $H^+$, monovalent cations, and $Zn^{+2}$ from the spore core. Second, it releases dipicolinic acid (DPA) and its associated cations, mainly $Ca^{+2}$. Third, upon the replacement of DPA by water, initial increase in core hydration and loss of spore's wet-heat resistance occur. Fourth, the core hydration induces hydrolysis of the spore's peptidoglycan cortex. Fifth, further water uptake allows full core hydration, and ultimately the spore core begins to expand. After these five events, there is a successive period termed spore outgrowth. In this stage, the expanded spore core initiates spore metabolism and macromolecular synthesis. This entire process of spore germination occurs within minutes under optimal conditions (10).

## 1.3 Germination Receptors

Spore germination, as mentioned above, is initiated by recognition of germinants by the germination (Ger) receptors located in the inner membrane of the spore (5, 11, 12). It is believed that the Ger receptors are encoded by tricistronic operons, resulting in three distinct products, the A-, B-, C-subunits (13). For example, *gerA* operon in the genome of *B. subtilis* encodes GerA receptor which is composed of GerAA, GerAB, and GerAC (14). The GerAA protein has five or six predicted membrane-spanning segments, as well as large N- and C-terminal hydrophilic domains (9). The GerAB protein resembles an integral membrane protein with ten helices wound around the inner membrane (13). These A-, B-subunit proteins have transmembrane structure while the GerAC protein is predicted to have membrane-anchored feature with an N-terminally attached lipid moiety which sticks out of the membrane surface (15) (see Figure 1).

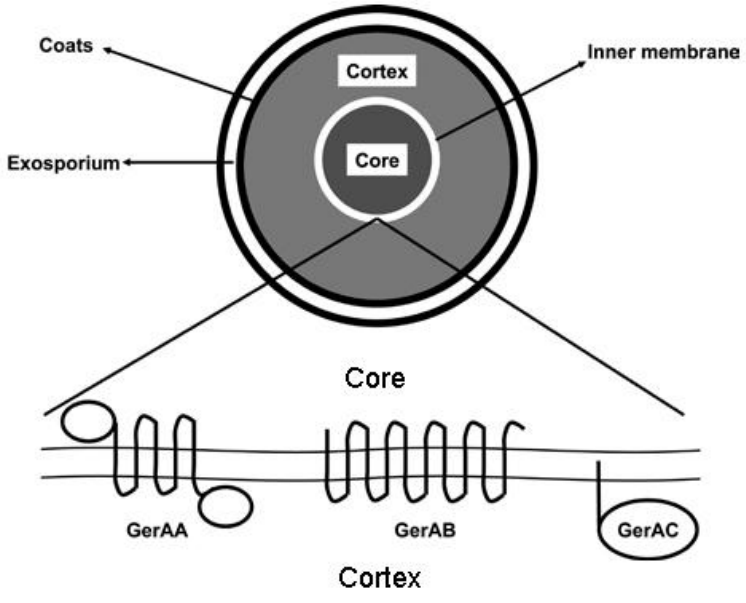



Figure 1. Typical model of the germination receptor GerA from *B. subtilis*. Subunits-A, B, C- are located in the inner membrane of the spores. (adapted from Moir et al., 2002)

Genome sequences of Ger receptors in *B. anthracis* have been characterized (16, 17). Genomic sequencing predicts that *B. anthracis* has seven ger receptor operons- six genomic operons (*gerA, gerH, gerK, gerL, gerS, and gerY*) located on the chromosome and one plasmidic operon (*gerX*) found on the pXO1 virulence plasmid (18). Among these ger operons, *gerA* and *gerY* were shown to have no effect on *in vitro* germination, but it is possible that they might encode non-functional receptors (17).

Initiation of germination is found to be triggered by several different pathways depending on germinant and Ger receptor interactions (19) (see Figure 2). In a mutant study of the germination pathway by combinations of amino acid and purine nucleoside germinants (17), it is reconfirmed that the GerH receptor is necessary for purine nucleoside-mediated germination. Meanwhile, the GerS receptor responds to aromatic amino acids. Both GerK and GerL receptors are activated by L-alanine (5). However, the ligands recognized by GerX, GerA, and GerY have not been identified. Overall, at least two distinct Ger receptors are required to direct the germination of *B. anthracis*, and they cooperate with each other (17).

A virulence study in an intratracheal mouse model showed that the presence of any one of GerH, GerK, GerL, or GerS receptors is sufficient enough to cause anthrax disease (66). This suggests that the site of germination after an intratracheal inoculation may contain many discrete germinant signals, and is able therefore to fulfill the broad specificities of these different receptors (66). They also concluded that only GerH receptor primarily facilitates fully virulent infection in a subcutaneous route. They speculated that as the ΔgerH strain travelled through the body, it eventually found a site rich in a germinant that either the GerK, GerL, GerS or GerX receptors recognized,

stimulating germination, and ultimately resulting in disease (66). These results indicate that each germinant receptor is active in different routes of infection.

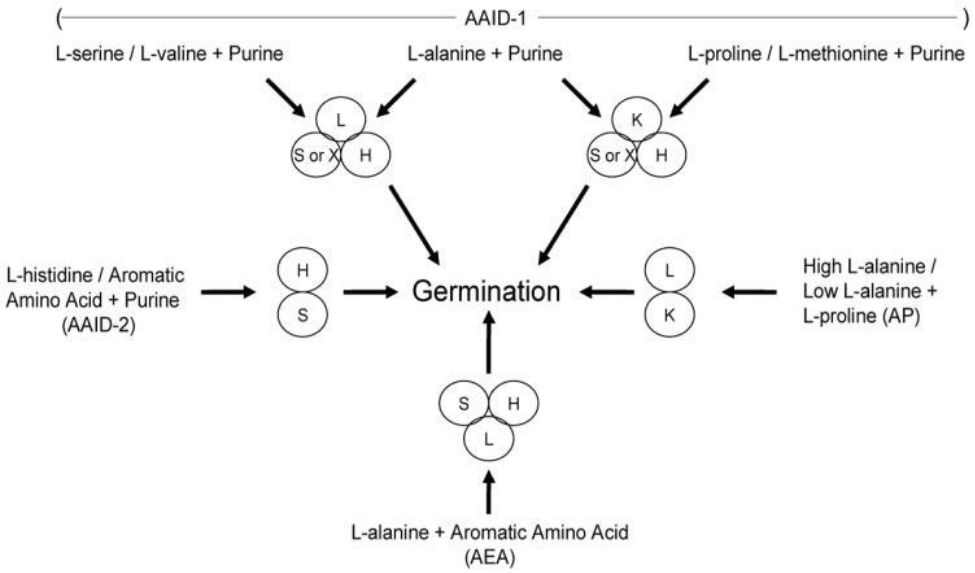



Figure 2. Diagram of *B. anthracis* germination pathways. Combinations of germination receptors are involved in different interacting pathways. (Fisher and Hanna, 2005)

## 1.4 Significance of 6-Thioguanosine

### 1.4.1 *In Vivo* Inhibitor of *B. Anthracis* Spore Germination

The most effective germinant set to induce spore germination of *B. anthracis* is inosine and L-alanine. 6-thioguanosine (6-TG), an inosine analog, appeared to have competitive inhibitory activities against *B. anthracis* spore germination. It kinetically competes with inosine for recognition by the spore (4). 6-TG also efficiently blocks spore germination in murine macrophages and protects cells from death when treated with *B.*

*anthracis* spores. Moreover, 6-TG is a weak germinant in the presence of high concentration of its cogerminant, L-alanine. Thus, 6-TG inhibits *B. anthracis* spore germination by acting as an alternative weak substrate. This overall information implies that 6-TG has certain interaction with a Ger receptor, which results in decrease in affinity of inosine to its cognate Ger receptor (4).

### 1.4.2 Protein Photocrosslinker

Photocrosslinking is a simple and powerful strategy for identification of protein-protein, as well as protein-ligand interactions in cellular environments (20). When a protein-ligand complex is irradiated with ultraviolet (UV) light, the photoactivatable small molecule forms covalent bonds with their interacting proteins (21). This technique has an advantage in providing precise knowledge of the attached proteins, while preserving the native structural and functional properties of the detected protein (22, 23, 24).

6-TG, an inhibitor of *B. anthracis* spore germination, can be activated by UV-light to crosslink with amino and sulphydryl groups of proteins (25). Specifically, ultraviolet A radiation (UVA) generates reactive oxygen species, which oxidize 6-thioguanine to guanine-6-sulfonate ($G^{SO3}$) (25). The photoproduct, $G^{SO3}$ is an important intermediate in crosslinking (26). Crosslinking occurs via covalent bond formations between the exocyclic sulfur atoms of 6-TG and residues of the target proteins (26).

## 1.5 Activity-Based Protein Profiling

Genome sequencing has established a complete inventory of the predicted proteins produced by eukaryotic and prokaryotic organisms (27). However, genomic sequencing

does not provide functional information of proteins. The necessity of understanding post-translational events that regulate functions and interactive relationships of these proteins has resulted in new methods. To overcome the challenge, synthetic chemistry has been incorporated into biological research, facilitating direct monitoring of the functional state of the proteome (28).

### 1.5.1 ABPP probe

Activity-based protein profiling (ABPP) has been introduced as a powerful chemical proteomic technology that utilizes active site-directed chemical probes with target specification in complex biological systems (28). The chemical probe is composed of a reactive group (RG), a reporter tag, and an optional spacer or binding group (BG). RG is designed to react with a specific site of proteins. Reporter tags facilitate target characterization. A variety of reporter tags can be used for enzyme visualization and enrichment, including fluorophores (e.g., rhodamine) and biotin, as well as "clickable" handles, such as azides and alkynes (see Figure 3).

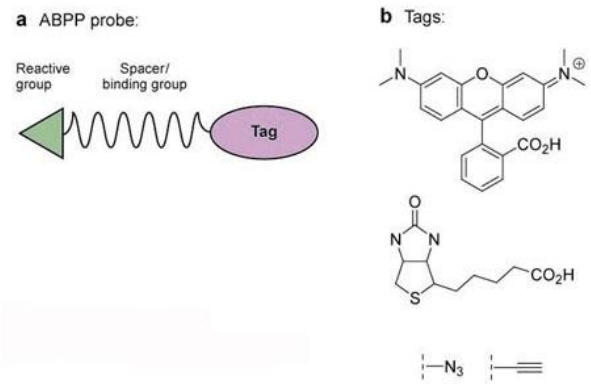



Figure 3. Representative structure of (a) an ABPP probe and (b) several reporter tags. (Cravatt et al., 2008)

### 1.5.2 Analytical Platforms for ABPP

A number of analytical platforms for ABPP have been developed. The analytical methods are well described by Cravatt et al. (27). Gel electrophoresis, the original method, is still widely used despite of limitation of poor resolution. It enables the rapid comparative analysis of large proteomes in parallel. In this approach, proteins are separated by one- (1D) or two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). Then, the probe-labeled proteomes are visualized by either fluorescence scanning or streptavidin blotting.

Liquid chromatography-mass spectrometry (LC-MS) platforms have emerged to overcome the resolution problems of gel-based method. LC-MS analysis can be carried out by either of two methods: (1) a fusion of activity-based protein profiling and multidimensional protein identification technology (ABPP-MudPIT) and (2) active-site peptide profiling (ASPP). These strategies differ in the order of performance between streptavidin enrichment and digestion of target protein with trypsin, and provide different information from LC-MS data analysis (see Figure 4). In ABPP-MudPIT, probe-labeled enzymes are first captured on streptavidin beads and then digested with trypsin, which ultimately provides peptide identification of the protein targets. This method does not offer a straightforward way to identify the probe-labeled peptides of enzyme targets. This problem can be solved by switching the streptavidin enrichment and trypsin digestion. In ASPP, probe-labeled peptides are selectively enriched for LC-MS analysis since trypsin digestion has already been preceded. The collected data of both ABPP-MudPIT and ASPP from LC-MS are analyzed by a tandem mass spectrometry data analysis program, SEQUEST to identify protein targets and their specific site of probe labeling.

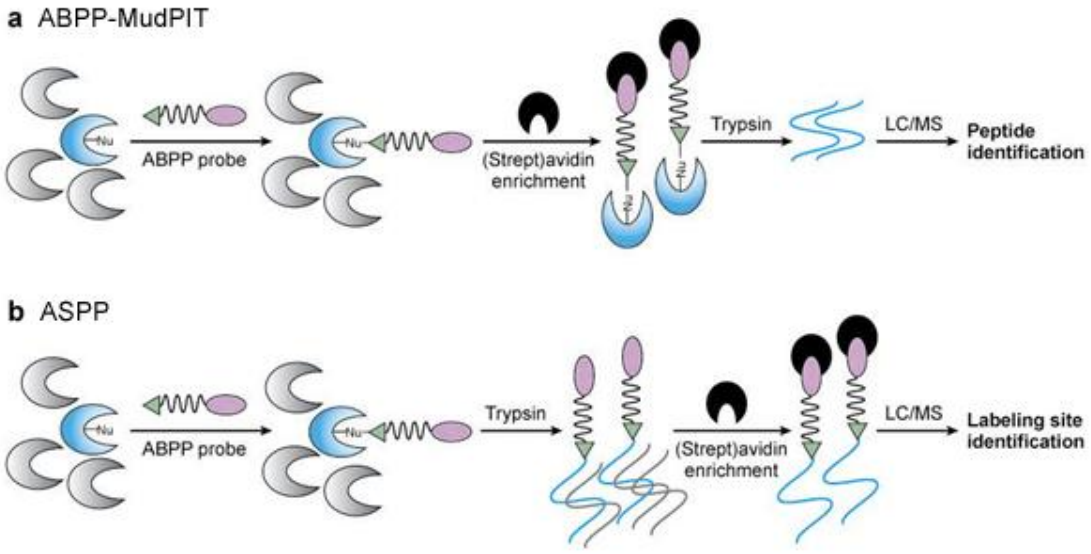



Figure 4. Gel-free, LC-MS based platforms for ABPP. Depending on the order of streptavidin purification and trypsin digestion, two methods above (a, b) provide different information from LC-MS data analysis. (Cravatt et al., 2008)

An advanced LC-MS based platform was also developed. Tandem-orthogonal proteolysis-activity-based protein profiling (TOP-ABPP) provides both sets of information acquired from ABPP-MudPIT and ASPP in a single experiment. This method introduces a Tobacco etch virus (TEV) protease cleavage site between the reactive group and biotin tag of ABPP probes by involvement of click chemistry methods. LC-MS approaches facilitated superior resolution and information content of ABPP. However, there are also drawbacks of LC-MS compared to gel-based methods. It requires larger quantity of samples, and is much slower than 1D-SDS-PAGE.

Most recently, two other platforms that combine the high throughput/high resolution and low sample demand have been developed: capillary electrophoresis-laser-induced fluorescence (CE-LIF) ABPP and ABPP microarrays (27).

## 1.6 Click Chemistry-Activity-Based Protein Profiling

The standard version of ABPP has limitation for *in vivo* application due to the bulkiness of the reporter tags. The size of tags can be up to 1000 daltons and this may cause impediment to the probe binding to enzymes (29). Thus, to circumvent the potential problem, a tag-free ABPP was introduced, whereby a reactive group of the probe binds to its recognizing site of proteins *in vivo* followed by *in vitro* conjugation with a reporter tag (see Figure 5).

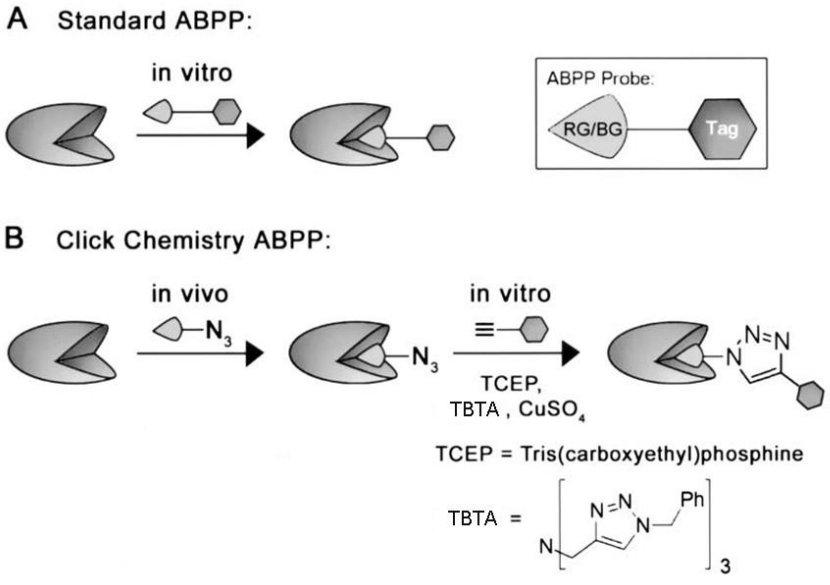



Figure 5. (A) Standard version of ABPP and (B) click chemistry mediated ABPP. (Speers and Cravatt, 2004)

The “Click reaction”, the simplified term to describe Cu(I)-catalyzed 1,3-dipolar cycloaddition (30), employs and allows the tag-free ABPP to be used. The reaction occurs between reporter tags and the probes bound to proteins of interest. The two main functionalities involved in the cycloaddition reaction are azide and alkyne groups.

The following subchapters are the descriptions of synthetic chemistry in azidation of nucleosides and Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction.

### 1.6.1 Azidation of Nucleosides

#### 1.6.1.1 Significance of Azide Compounds

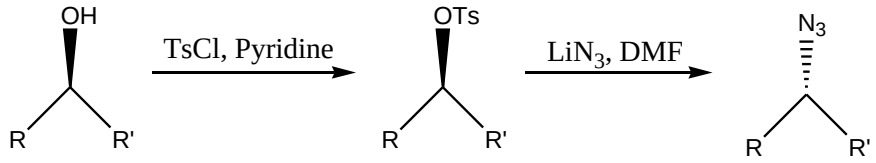
Organic azides can be simply depicted as R-$N_3$. The central nitrogen of the azide group carries a positive charge and a negative charge is delocalized between the terminal nitrogens, rendering the overall charge of the molecule zero.

The finding of 3’-azido-3’-deoxythymidine (AZT), a therapeutic agent for the treatment of human immunodeficiency virus (HIV) infections, has initiated explosive developments in synthetic chemistry of nucleosides (31). In specific, azidonucleosides have been spotlighted because of its reducibility in some cells to the corresponding aminonucleosides which possess potential therapeutic activity, causing DNA chain termination (32).

#### 1.6.1.2 Synthesis of Azide Compounds

In general, azidation is achieved by hydroxyl to azido functional group interconversion via nucleophilic substitution reaction. The traditional method to furnish an azido group is a “two-step” procedure: transformation of alcohols to halides, or sulfonyl groups such as mesylates, nosylates, tosylates, and triflates followed by nucleophilic substitution reaction with azide anion (see Figure 6). This indirect method

has problems of several purification steps and unexpected side reactions (63, 64, 65). In fact, azidation of nucleosides via sulfonyl intermediates has resulted in varied products depending on the kind of base and position of a hydroxyl group in nucleosides (33, 34, 35, 36, 37).




Ts: tosyl group

Figure 6. Two-step azidation via a sulfonyl intermediate.

The "one-pot" synthesis using *Mitsunobu* reaction condition is one of the straightforward methods to obtain the azide functionality. In this strategy, triphenylphosphine ($PPh_3$) combines with diethyl azodicarboxylate (DEAD) to generate a phosphonium intermediate, activating hydroxyl oxygen as a leaving group. Azide anion from hydrazoic acid ($HN_3$) displaces the leaving group to form an azido group (38) (see Figure 7).

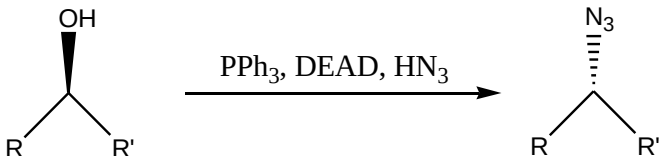



Figure 7. One-pot reaction of azidation using *Mitsunobu* condition.

Alternative methods of *Mitsunobu* condition, *Bose-Mitsunobu* and *Thomson's* procedures, were developed to circumvent the use of an explosive reagent, $HN_3$, and for easy work-up procedure, by using diphenyl phosphorazidate (DPPA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (39, 40). Phosphate chemisty also improved reaction efficiency, by incorporating Bis(2,4-dichlorophenyl) chlorophosphate, sodium azide ($NaN_3$), and 4- dimethylaminopyridine (DMAP) (41).

### 1.6.2 Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition

The approach "click chemistry" was firstly introduced by Sharpless and his colleagues to demonstrate the nature of reactions that are modular, wide in scope, stereospecific, give very high yield, and generate only inoffensive byproducts (42). In addition, one of the required characteristics to be categorized into click reactions is that the product must be stable under physiological conditions (42). Huisgen's 1,3-dipolar cycloaddition (30) has been regarded as "cream of the crop" of click chemistry. Under Cu (I)-catalyzed condition, the two components of the reaction, 1,3-dipole and a dipolarophile are readily united to afford a stable five-membered heterocycle, 1,2,3-triazole.

The Cu(I)-mediated process was found to be crucial to control regioselectivity of 1,4-disubstitued 1,2,3-trialzoles over 1,5 regioisomer (43) (see Figure 8). The Cu(I) source can be attained from direct Cu(I) salts, but in situ reduction from Cu(II) salts by reducing agents generates more stable and efficient source of Cu(I). In this regard, copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and sodium ascorbate are broadly used in 1,3-dipolar cycloaddition reactions. The proposed catalytic cycle for Cu(I)-catalyzed ligation is described in Figure 9.

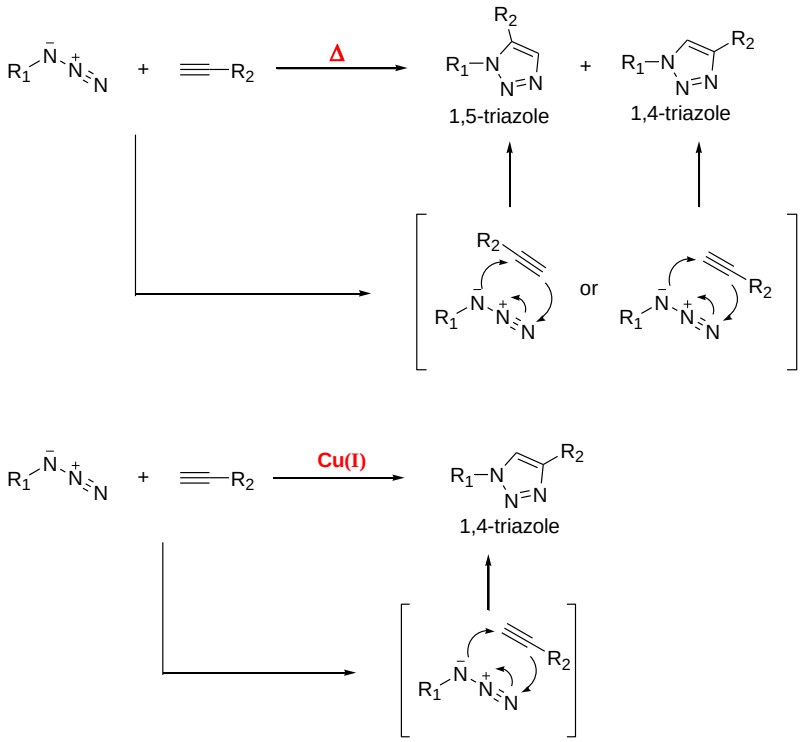



Figure 8. Regioselectivity of Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction.

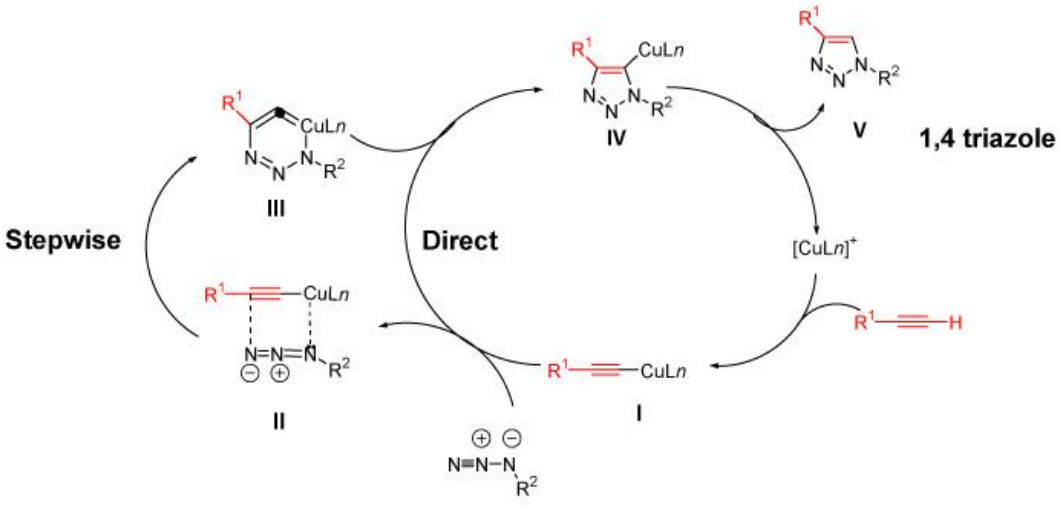



Figure 9. Proposed catalytic cycle for Cu(I)-catalyzed ligation. (adapted from Rostovtsev et al., 2002)

To apply the irreversible cycloaddition to investigation of complex biological environments, newly developed Cu(I)-catalyzed conditions were introduced (44). In this strategy, $CuSO_4$ was reduced by tris(2-carboxyethyl)phosphine (TCEP), a water-soluble agent which also protects cysteine residues in proteins from oxidative coupling. A ligand, tris-(benzyltriazolylmethyl)amine (TBTA), was also used to stabilize the Cu(I) oxidation state in water. This modified method provides wide usage of the intermolecular coupling reaction in living cells and organisms for drug discovery, activity-based protein profiling, and other biological applications.

## 1.7 Specific Aims and Hypothesis

Among the currently believed pathways of *B. anthracis* spore germination, we concentrated on the case where L-alanine and purine nucleosides simultaneously trigger the spore germination. Taking advantage of properties of 6-thioguanosine (6-TG), we aimed to synthesize a chemical probe for identification of the Ger receptor proteins of *B. anthracis*, which ultimately elucidate the mechanism occurring in the inner membrane of *B. anthracis* spores.

Herein, we created a synthetic probe, 5'-azido-5'deoxy-6-thioguanosine ($N_3$-6-TG), to isolate and characterize the target proteins that recognize inosine, an important germinant of *B. anthracis*. We firstly utilized $N_3$-6-TG as a crosslinker, making use of its exocyclic thiol group by activation with UV light. Also the azido group of the probe was introduced to provide a bait for click reaction to isolate the crosslinked proteins. We thus hypothesized that 6-TG will be recognized by a specific site of *B. anthracis* Ger receptor proteins during inhibition of spore germination.

## 1.8 Significance of the Study

The events after the spore activation, including DPA/$Ca^{+}$ release, spore core hydration, and expansion, are well-established. However, the mechanism of the spore activation has not been clearly understood. Also, the Ger receptor proteins have not been characterized in biochemical level. Thus, the significance of this study is that we utilized activity-based protein profiling technique which will allow identification of the unknown Ger receptor protein involved in inosine-mediated spore germination.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Bacterial Strains and Spore Preparation

The *Bacillus anthracis* Sterne 34F2 strain was generously provided by Dr. Arturo Casadevall from the Albert Einstein College of Medicine. The Sterne strain lacks the pXO2 plasmid for encapsulation but contains the pXO1 plasmid for the production of virulence factors for anthrax disease.

For sporulation, *B. anthracis* cells were plated in nutrient agar (20 g agar per liter; for 1 L preparation, 10 mL of 10 % KCl, 10 mL of 1.2 % $MgSO_4$, 1 mL of 1 M $Ca(NO_3)_2$, 1 mL of 0.01 M $MnCl_2$, and 1 mL of 1 mM $FeSO_4$) and incubated overnight at 37 $^oC$. Individual colonies were grown in LB (Luria-Bertani) medium for 3-4 h at 37 $^oC$ while shaking at 90 rpm. Cells were replated to obtain bacterial lawns (200 μL per plate) and incubated at 37 $^oC$ for 5 days. The resulting bacterial lawns were scraped from the plates and collected in ice-cold deionized water. Spores were pelleted by centrifugation at 8000 rpm at 4 $^oC$ for 5 min and resuspended in fresh deionized water. The washing step was repeated three times. The spores were separated from vegetative and partially sporulated forms by centrifugation at 11500 rpm at 4 $^oC$ for 35 min through a 20-50 % Histodenz gradient. Purified spores were washed five times with ice-cold deionized water and stored at 4 $^oC$ as a 1 mL aliquot in 1.5 mL centrifuge tubes.

## 2.2 Synthesis of Nucleoside Analogs

### 2.2.1 2’,3’-O-isopropylideneguanosine

To a suspension of guanosine (0.57 g, 2.0 mmol) in acetone (40 mL) under nitrogen, p-toluenesulfonic acid monohydrate (0.76 g, 4.0 mmol) and 2,2-dimethoxypropane (3 mL, 24 mmol) were added. After 2 h stirring at room temperature, triethylamine (0.79 mL, 6.0 mmol) was added, and the resulting solution was concentrated to give a white solid (0.59 g, 91 %). The resulting compound was pure based on TLC and its structure was verified by $^{1}$H NMR and FTIR. $^{1}$H NMR (DMSO-$d_6$, 400 MHz) δ 1.29 (s, 3 H), 1.49 (s, 3 H), 3.51 (d, *J* = 3.6 Hz, 2 H), 4.09 (bs, 1 H), 4.94 (m, 1 H), 5.01 (m, 1 H), 5.16(m, 1 H), 5.90 (d, *J* = 2.4 Hz, 1 H), 6.48 (bs, 2 H), 7.89 (s, 1H), and 10.64 (bs, 1 H); IR (neat) 3309, 3133, 2940, 2740, 1595, 1488, 1085, 884 $cm^{-1}$.

### 2.2.2 5'-Deoxy-5'-iodoguanosine

Iodine (14.2 g, 56 mmol) was added over 5 min to a magnetically stirred suspension of guanosine (5.0 g, 18 mmol), triphenylphosphine (15.3 g, 58 mmol), and imidazole (8.0 g, 117 mmol) in N-methyl-2pyrrolidinone (70 mL) at room temperature. During the addition complete dissolution occurred as the solution was warmed to 60 $^{o}$C. Once it reached 60 $^{o}$C, the solution was cooled back to room temperature and after 3h was diluted with dichloromethane (700 mL) and water (300 mL). A white crystalline solid was separated from solution and was collected by filtration, then dried out under reduced pressure to give 4.7 g (68 %) of the product. The resulting compound was pure based on TLC and its structure was verified by $^{1}$H NMR, FTIR, and MS. $^{1}$H NMR (DMSO-$d_6$, 400 MHz): δ 3.49 (dd, *J* = 10.2, 56.3 Hz, 2 H), 3.99 (m , 1 H), 4.06 (m, 1 H), 4.63 (m, 1 H), 5.37 (bs, 1 H), 5.54 (bs, 1 H), 5.71 (d, *J* = 6.4 Hz, 1 H), 6.51 (bs, 2 H), 7.93 (s, 1 H), and 10.70 (bs, 1 H); IR (neat) 3383, 3151, 2919, 1709, 1615, 1350, 1164, 1008, 825 $cm^{-1}$; MS m/z calcd for $C_{10}H_{12}IN_5O_4$ $[M+H]^+$: 393.9934.; found: 394.0009.

### 2.2.3 5’-O-methylguanosine

5’-deoxy-5’-iodoguanosine (140 mg, 0.36 mmol) was dissolved in a 1:9 mixture of anhydrous methanol (0.5 mL) and anhydrous DMSO (4.5 mL). Anhydrous potassium carbonate (0.5 g, excess) was added to the reaction flask over 5 min at room temperature. The stirred reaction mixture was refluxed under nitrogen for 3 h. Then, the solvent was evaporated *in vacuo* and the residue was resuspended in methanol (10 mL) to be stirred for 15 min. The precipitate was filtered out and washed with cold methanol to give a yellow powder (46 mg, 43 %). The resulting compound was pure based on TLC and its structure was verified by $^{1}$H NMR and FTIR. $^{1}$H NMR (DMSO-$d_6$, 400 MHz) δ 3.57 (dd, $J$ = 7.2, 46.8 Hz, 2 H), 3.87 (s, 3 H), 3.94 (m, 1 H), 4.03 (m, 1 H), 4.56 (m, 1 H), 5.29(bs, 1 H), 5.52 (bs, 1 H), 5.71 (d, $J$ = 6.0 Hz, 1 H), 6.68 (bs, 2 H), 7.89 (s, 1H), and 11.00 (bs, 1 H); IR (neat) 3336, 3120, 1685, 1620, 1543, 1477, 837 $cm^{-1}$.

### 2.2.4 Synthesis of 5’-Azido-5’-deoxyguanosine

#### 2.2.4.1 *Thomson’s* Method

A suspension of 2’,3’-O-isopropylideneguanosine (226 mg, 0.7 mmol) in toluene (15 mL) was cooled to 0 $^{o}$C. Diphenylphosphoryl azide (DPPA) (756 µL, 3.5 mmol) was added, followed by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (525 µL, 3.5 mmol). The reaction mixture was stirred at 0 $^{o}$C overnight. TLC showed 100 % conversion to a new single compound. The solution was then warmed to room temperature and added ethyl acetate (30 mL). The organic layer was washed with water (15 mL), saturated $NaHCO_3$ (45 mL), and brine (45 mL). The organic layer was dried over anhydrous $Na_2SO_4$. The solvent was evaporated *in vacuo* to give yellow oil. The crude product was purified by column chromatography (ethanol/dichloromethane, 1:9 to 3:7). The purified compound

was the phosphate triester intermediate (0.33 g, 90 %). The structure of the compound was characterized by $^{1}$H NMR and FT-IR.

#### 2.2.4.2 Phosphate Activation

To a suspension of 2',3'-O-isopropylideneguanosine (323 mg, 1 mmol) in anhydrous DMF (5 mL) were added sodium azide ($NaN_3$) (260 mg, 4 mmol), 4-dimethylaminopyridine (DMAP) (146 mg, 1.2 mmol), followed by bis(2,4-dichlorophenyl) chlorophosphate ($(Cl_2PhO)_2POCl$) (427 mg, 1.05 mmol). The reaction mixture was stirred overnight at 45 $^{o}$C. All the starting materials were consumed based on TLC. Ethyl acetate (100 mL) was added to the reaction mixture and washed with water (50 mL), 20 % NaOH (50 mL), and brine (50 mL). The organic layer was dried over anhydrous $MgSO_4$. The solvent was evaporated *in vacuo* to give yellow oil. The crude product was purified by column chromatography (ethanol/dichloromethane, 1:9 to 4:6). The purified compound was the phosphate intermediate (0.28 mg, 41 %) based on the characterization by $^{1}$H NMR and FT-IR.

#### 2.2.4.3 Sulfonylation

A solution of 2',3'-O-isopropylideneguanosine (323 mg, 1 mmol) in anhydrous DMF (10 mL) was cooled to 0 $^{o}$C. Pyridine (162 μL, 2 mmol) and *p*-toluenesulfonyl chloride (228 mg, 1.2 mmol) or triflic anhydride (380 mL, 2.3 mmol) dissolved in DMF (3 mL) were added in small portions with constant stirring. The reaction mixture was warmed to room temperature and stirred overnight. Based on the TLC, two unidentified products were obtained with the starting material. Ethyl acetate (50 mL) and water (50 mL) were added and the organic layer was washed successively with 1 N HCl, 5 % $NaHCO_3$, and

brine, and then dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo.* Due to the low yield of the reaction, no attempt was made to purify the two new products.

2.2.4.4 Iodination Followed by Azidation

A mixture of 5'-deoxy-5'-iodoguanosine (3.5 g, 9.0 mmol) and sodium azide (1.2 g, 18 mmol) in anhydrous DMF (25 mL) was stirred at 80 $^{o}$C under nitrogen for 20 h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was stirred in water (50 mL) for 30 min. The resultant solid was collected by filtration then washed successively with water (2 x 25 mL), cold ethanol (15 mL), and diethyl ether (10 mL) before drying *in vacuo* to give a light yellow powder (2.2 g, 79 %). The resulting compound was pure based on TLC and its structure was verified by $^{1}$H NMR, FTIR, and MS. $^{1}$H NMR (DMSO-$d_6$, 400 MHz) δ 3.75 (dd, *J* = 13.2, 58.4 Hz, 2 H), 3.98 (m, 1 H), 4.06 (m, 1 H), 4.57 (m, 1 H), 5.3–5.7 (bs, 2 H), 5.72 (d, *J* = 5.6 Hz, 1 H), 6.52 (bs, 2 H), 7.91 (s, 1 H), and 10.88 (bs, 1 H); IR (neat) 3447, 3112, 2929, 2105, 1684, 1175, 1118 $cm^{-1}$;MS m/z calcd for $C_{10}H_{12}N_8O_4$ $[M+H]^+$: 309.0982; found: 309.1056.

2.2.5 5'-Amino-5'-deoxyguanosine

A stirred solution of 5'-azido-5'-deoxyguanosine (134 mg, 0.43 mmol) in dry pyridine (2.2 mL) at 0 $^{o}$C was treated with triphenylphosphine (225 mg, 0.86 mmol). After stirring at room temperature for 3 h, the resulting thick suspension was re-cooled to 0 $^{o}$C, treated with ammonium hydroxide solution (28-30 % $NH_3$ basis, 625 μL) and water (2.2 mL), then stirred at room temperature for 18 h. The solvent was removed under reduced pressure, and the residue was suspended in ethyl acetate (15 mL), and stirred at room temperature for 15 min. The resultant solid was collected by filtration, washed with ethyl acetate (2.2 mL), cold ethyl acetate/methanol (1:1, 2.2 mL) and water (2.2 mL), and

then dried *in vacuo* to give a yellow powder (89 mg, 73 %). The resulting compound was pure based on TLC and its structure was verified by $^1$H NMR. $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 2.73 (dq, *J* = 13.2, 29.4 Hz, 2 H), 3.77 (m, 1 H), 4.06 (m, 1 H), 4.41 (m, 1 H), 4.5–5.5 (bs, 5 H), 5.64 (d, *J* = 6.0 Hz, 1 H), 6.52 (bs, 2 H), and 7.91 (s, 1 H).

### 2.2.6 5'-Deoxy-5'-iodo-6-thioguanosine

To prepare 5'-deoxy-5'-iodo-6-thioguanosine the same synthetic procedure was followed as for 5'-deoxy-5'-iodoguanosine. A yellow powder (Yield = 50 %). The resulting compound was pure based on TLC and its structure was verified by $^1$H NMR, FTIR, and MS. $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 3.50 (dd, *J* = 10.2, 54.9 Hz, 2 H), 3.95 (m, 1 H), 4.08 (m, 1 H), 4.65 (m, 1 H), 5.41 (bs, 1 H), 5.58 (bs, 1 H), 5.72 (d, *J* = 6.0 Hz, 1 H), 6.83 (bs, 2 H), 8.13 (s, 1 H), and 11.99 (bs, 1 H); IR (neat) 3425, 3340, 3174, 1627, 1577, 1419, 1195, 952 $cm^{-1}$; MS m/z calcd for $C_{10}H_{12}IN_5O_3S$ $[M+H]^+$: 409.9706; found: 410.0000.

### 2.2.7 5'-Azido-5'-deoxy-6-thioguanosine

To prepare 5'-azido-5'-deoxy-6-thioguanosine the same synthetic procedure was followed as for 5'-azido-5'-deoxyguanosine. A yellow powder (Yield = 29 %). The resulting compound was pure based on TLC and its structure was verified by $^1$H NMR, FTIR, and MS. $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 3.56 (dd, *J* = 3.6, 13 Hz, 2 H), 3.97 (m, 1 H), 4.06 (m, 1 H), 4.64 (m, 1 H), 5.2–5.6 (bs, 2 H), 5.80 (d, *J* = 29 Hz, 1 H), 6.72 (bs, 2 H), 8.14 (s, 1 H), and 11.96 (bs, 1 H); IR (neat) 3340, 3286, 3175, 2922, 2106, 1628, 1591, 1197, 956 $cm^{-1}$; MS m/z calcd for $C_{10}H_{12}N_8O_3S$ $[M+H]^+$: 325.0753; found: 325.0818.

## 2.3 Alkyl Spacer Synthesis in Guanosine

To a suspension of 2',3'-O-isopropylideneguanosine (0.5 g, 1.5 mmol) in anhydrous DMF (10 mL), sodium hydride (60 % dispersion in mineral oil; 74 mg, 1.8 mmol) was added. The mixture was stirred under nitrogen at room temperature for 30 min. Then, the solution was heated to 80 $^{o}$C while 4-(Boc-amino) butyl bromide (585 mg, 2.3 mmol) dissolved in anhydrous DMF (4 mL) was added by a syringe over a 20 min period. Once the temperature reached 80 $^{o}$C, the reaction mixture was stirred for 2 h, cooled to 50 $^{o}$C and then stirred overnight. A little conversion to the desired product had occurred based on TLC. Thus, additional amounts of sodium hydride (74 mg, 1.8 mmol) and 4-(Boc-amino) butyl bromide (585 mg, 2.3 mmol) were added five times over 5 days. Once significant conversion had occurred, the reaction was stopped and the solvent was evaporated *in vacuo.* The residue was dissolved in dichloromethane and washed several times with a great quantity of water to neutralize the excess of sodium hydride. Column chromatography (ethanol/dichloromethane, 1:9 to 3:7) afforded a yellow crystal (49 mg, 6 %) and a yellow powder (76 mg, 10 %), the desired 5'-O-alkylated compound and the unwanted 1-N-alkylated compound, respectively. The resulting compounds were pure based on TLC and the structures were verified by $^{1}$H NMR and Fourier transform infrared spectroscopy (FTIR).

## 2.4 Germination and Inhibition Assays

For kinetic studies of *B. anthracis* spore germination and germination inhibition, changes in optical density were monitored at 580nm. Spores scatter light strongly because of their highly dense structures. Once spore germination is initiated, the spore

core hydrates, which in turn reduces the intensity of light diffraction by spores. In the germination assays, wherein spores were treated with two germinants (L-alanine and one of the nucleosides or its derivatives), a decrease in optical density at 580 nm ($OD_{580}$) was observed. In contrast, in germination inhibition assays where spores were preincubated with 6-thioguanosine (6-TG) or its analogs for 15 min before the addition of L-alanine and inosine, a smaller decrease in $OD_{580}$ was observed compared to the typical L-alaine and inosine mediated germination. The smaller decrease in optical density strongly suggests inhibition of spore germination.

Before each experiment spores were washed twice with ice-cold deionized water and resuspended in 1 mL water followed by heat activation at 70 $^{o}$C for 30 min. The spores were resuspended in 0.5mL germination buffer (50 mM Tris-HCl [pH 7.5], 10 mM NaCl) and diluted to an $OD_{580}$ of ≈ 1. The spore suspension was monitored for auto-germination for 20 min and only non-auto germinated spores were used for experiments.

All germination and germination inhibition assays were carried out in a 96-well plate (final volume of 200 μL/well) using a Tecan Infinite M200 multimode microplate reader. Changes in optical density at 580 nm were monitored for 60 min in triplicates. The optical densities were normalized by dividing each data point by the initial optical density to report results as relative $OD_{580}$ values.

L-alanine stock solution was prepared in water and inosine, 6-thioguanosine, and other synthesized compound stock solutions were prepared in 220 mM NaOH solution due to the poor solubility. Negative control experiments were set up by treating spore suspension with water and/or 220 mM NaOH solution. For a positive control, spore

aliquots were supplemented with a specific concentration of L-alanine and inosine depending on each assay.

To verify the reactivity of synthesized compounds in spores, germination tests were carried out in various concentrations (0.05, 0.1, 0.5, 1, 5, 10 mM) of nucleosides (inosine, guanosine, 5'-azido-5'-deoxyguanosine, 5'-O-methylguanosine , 5'-amino-5'-deoxy-guanosine, 6-thioguanosine, and 5'-azido-5'-deoxy-6-thioguanosine) at a constant concentration (10 mM) of L-alanine. To test inhibitory activities of 6-thioguanosnie or its analogs, spore suspensions were individually supplemented with various concentrations (0.25, 0.5, 1, 5, 10 mM) of the compounds and incubated for 15 min at room temperature. To induce spore germination, spores preincubated with previously described compounds were next incubated with 250 μM inosine and 40 μM L-alanine (45). After running assays, germination was measured in selected samples by Schaeffer-Fulton staining, a technique used to identify endospores by staining any present endospores green and vegetative cells red.

## 2.5 MMPP Oxidation

50 mM of 6-TG (150 mg) was prepared in 10 mL of water. To the solution was added three equivalent of magnesium bis(monoperoxyphthalate) hexahydrate (MMPP) (741 mg). The pH of the solution was increased to pH 7.5 by adding concentrated NaOH solution. This stock solution (50 mM 6-TG, 150 mM MMPP) was serial diluted to prepare ten-fold lower solution (5 mM 6-TG, 15 mM MMPP). Germination inhibition assay was carried out by following the same protocol described above.

## 2.6 Synthesis of Biotinylated Alkyne Tag

### 2.6.1 N-Hydroxysuccinimido Biotin

To a solution of biotin (1.0 g, 4.0 mmol) and N-hydroxysuccinimide (510 mg, 4.4 mmol) in DMF (50 mL) was added N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (920 mg, 4.8 mmol). After being stirred for 24 h at room temperature, the reaction solution was concentrated to give a white solid. The white solid was washed by methanol several times, and the solvent was removed in vacuo to yield a white solid (0.93 g, 67 %), which was applied to the next step directly without further purification.

### 2.6.2 Biotinylated Alkyne

To a solution of the compound above (0.93 g, 2.7 mmol) dissolved in DMF (100 mL) were added propargyl amine (0.48 mL, 7.0 mmol) and triethylamine (1.4 mL, 10 mmol), and the solution was stirred at room temperature for 24 h. The reaction solution was then concentrated in vacuo and the resulting residues were resuspended in 200 mL of dichloromethane to remove excess unreacted reagents and wash out residue of DMF. Filtration followed by additional washing with diethyl ether gave a white solid (415 mg, 54 %). The resulting compound was pure based on TLC and its structure was verified by $^{1}$H NMR and FTIR. $^{1}$H NMR (DMSO-$d_6$, 400 MHz) δ 1.25 (m, 2 H), 1.3−1.7 (m, 4 H), 2.04 (t, *J* = 7.6 Hz, 2 H), 2.46 (m, 1 H), 2.56 (d, *J* = 12.8 Hz, 1 H), 2.78 (dd, *J* = 5.2, 6.2 Hz, 1 H), 3.03 (t, *J* = 2.4 Hz, 1 H), 3.05 (m, 1 H), 3.79 (dd, *J* = 2.4, 5.4 Hz, 2 H), 4.07 (m, 1 H), 4.25 (m, 1 H), 6.31 (s, 1 H), 6.38 (s, 1 H), and 8.17 (s, 1 H); IR (neat) 3278, 2930, 1700, 1646, 1539, 1462, 1264, 1146 $cm^{-1}$.

## 2.7 Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition Reaction

In a 1.5 mL microtube, 2.2 mM 5'-azido-5'-deoxyguanosine (440 μL, 5mM stock in 10 % 220mM NaOH solution/90 % germination buffer) and 4.4 mM propargyl amine (440 μL, 10 mM stock in DMSO) were allowed to react in the presence of 2 mM $CuSO_4$ (40 μL, 50mM stock in water), 2 mM tris(2-carboxyethyl)phosphine (TCEP) (40 μL, 50 mM stock in water), and 2 mM tris-(benzyltriazolylmethyl)amine (TBTA) (40 μL, 50 mM stock in DMSO). The reaction mixture was incubated for 1 h at room temperature on a shaker and the sample was submitted for mass spectrometry analysis. MS m/z calcd for $C_{13}H_{17}N_9O_4$ $[M+H]^+$: 364.1404; found:362.1991.

## 2.8 Gel-Based CC-ABPP

### 2.8.1 Protein Labeling

Heat-activated *B. anthracis* spore suspension in germination buffer (50 mM Tris-HCl [pH 7.5], 10 mM NaCl ) (45 mL of $OD_{580}$ = 1 or 3) was treated with 5 mM 5'-azido-5'-deoxy-6-thioguanosine ($N_3$-6-TG) (5 mL, 50 mM stock in 220mM NaOH solution), and the mixture was incubated for 1 h at room temperature. Another sample preparation in the same condition was designed with 6-thioguanosine (6-TG) as a control experiment.

The spore-compound mixtures were transferred to culture dish in 25 mL fractions and preincubated for 15 min on ice. The samples were then irradiated with 365 nm UV light by a UV cross-linker for 50 min at 10,000 uJ/$cm^2$/s on ice to give dose rates of 300 kJ/$m^2$. The mixtures were then washed three times with ice-cold water by centrifugation (8000 rpm, 4 $^oC$, 5 min).

### 2.8.2 Decoating of Spores

After cross-linking, the spores were decoated following established methods (46). Spores were incubated in decoating buffer (5 mM CHES buffer (2-(N-cyclohexylamino)ethanesulfonic acid) [pH 8.6], supplemented by 8 M urea, 70 mM dithiothreitol (DTT), and 1% (wt/vol) Triton X-100) for 90 min at 37 $^{o}$C on a shaker. The spores were then pelleted by centrifugation (11000 rpm, 4 $^{o}$C, 5 min) and the supernatant which contains coat fractions was stored for lysis and the click reaction. The pelleted spores were washed five times with ice-cold distilled water.

### 2.8.3 Lysis of Spores

The crosslinked decoated spores were resuspended in 10 mL of sonication buffer (10 % glycerol, 50 mM Tris-HCl [pH 8.0], 300 mM KCl). The spore suspension was then sonicated (twelve times of 5 sec on and 5 sec off sonication) and centrifuged at 18,000 rpm for 1 h at 4 $^{o}$C. The supernatant was discarded and the pellet was resuspended in 5 mL of 1 % Triton X-100 in sonication buffer. The second sonication was carried out as described above. It was predicted that the supernatant from centrifugation (18,000 rpm, 4 $^{o}$C, 30 min) at this point would contain the solubilized membrane fraction.

### 2.8.4 Removal of Nonspecific Binding Proteins

Before the click reaction with biotinylated alkyne, proteins that nonspecifically bind to streptavidin beads were removed. 1 mL of solubilized membranes and coat fraction were incubated with 100 μL of streptavidin agarose sedimented beads suspended in 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5, containing 2 mM sodium azide for 1 h at room temperature on a shaker. After 1 h, the samples were centrifuged using microcentrifuge (12,000 rpm, 1 min). The supernatant was saved for the click reaction.

### 2.8.5 The Click Reaction

855 μL of the bead-washed membrane and coat protein fraction was supplemented by 0.5 mM biotinylated alkyne (10 μL, 50 mM stock in DMSO), 0.5 mM tris-(benzyltriazolylmethyl)amine (TBTA) (10 μL, 50 mM stock in DMSO), 5 mM $CuSO_4$ (25 μL, 200 mM stock in water), and 5 mM tris(2-carboxyethyl)phosphine (TCEP) (100 μL, 50 mM stock in water). The mixture was incubated for 1 h at room temperature while rotating.

### 2.8.6 SDS-PAGE

Samples for SDS-PAGE were prepared by mixing the biotinylated membrane protein sample and Lemmli sample buffer as 1: 1 ratio. The mixed samples were boiled for 4 min at 95 $^{o}$C. Prepared samples were loaded in each assigned lane of Tris-HCl SDS gels along with a molecular weight marker. After loading the samples, the gel was run at 150 V for ~ 1 h in TGS running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS [pH 8.3]). The gels were stained with either Coomassie Blue or silver stain. For western blotting, the gels were not prestained.

### 2.8.7 Western Blots

After running the SDS-PAGE, the gels were quickly rinsed with deionized water and prewet in transfer buffer (25 mM Tris-base [pH 8.3], 192mM glycine, 20 % methanol) for 15 min. Fiber pads, blotting paper, and PVDF membrane were also prewet in transfer buffer. Protein transfer was carried out using Bio-Rad mini-gel box electrotransfer at constant 400 mA for 1~2 h in ice. The memebrane was blocked overnight at 4 $^{o}$C in blocking solution (5 % ECL blocking agent in TBS-T washing buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1 % Tween 20)). After washing steps (5 min x 2, 15 min x 1, 5 min x 2) with TBS-T washing buffer, the blot was incubated with streptavidin-

horseradish peroxidase conjugate (1:85000 dilution in blocking solution) for 1 h and washed as before. ECL Plus substrate was detection method and Typhoon 9410 was used for imaging chemifluorescent western blot.

## 2.9 Troubleshooting Click Chemistry

In a 1.5 mL microtube, 0.5 mM 5'-azido-5'-deoxyguanosine (100 μL, 5mM stock in 220mM NaOH solution) and 0.5 mM biotinylated alkyne (10 μL, 50 mM stock in DMSO) were dissolved in 755 μL of either 1 % Triton X-100 in sonication buffer (10 % glycerol, 50 mM Tris-HCl [pH 8.0], 300 mM KCl), or decoating buffer (1 % Triton X-100, 5 mM CHES [pH 8.6], 8 M urea, 70 mM dithiothreitol) to confirm the conditions that will be used in protein samples. To the mixture 5 mM $CuSO_4$ (25 μL, 200 mM stock in water), 5 mM tris(2-carboxyethyl)phosphine (TCEP) (100 μL, 50 mM stock in water), and 0.5 mM tris-(benzyltriazolylmethyl)amine (TBTA) (10 μL, 50 mM stock in DMSO) were added. The reaction mixture was incubated for 1 h at room temperature on a shaker and the sample was submitted for mass spectrometry analysis.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Activity-Based Protein Profiling

Completion of genomic sequencing of hundreds of organisms has established a comprehensive database. Genomic approaches such as chromosomal translocation, gene amplification, transcriptional profiling, and gene silencing provide only limited information about protein functions. Thus, proteomics has emerged as a next step in the study of biological systems to explore the structures and functions of proteins. Liquid-chromatography-mass spectrometry (LC-MS) platforms for shotgun analysis (47), yeast two-hybrid methods (48), and protein microarrays (49) have greatly enriched understanding of the expression patterns, protein interactions, and in vitro functional properties of proteins. However, more direct methods to assess protein activity were required for an accurate assessment of the functional state of proteins in cells and tissues. Thus, a chemical technology, referred to as activity-based protein profiling (ABPP), has emerged as a powerful chemical proteomic strategy.

The standard method of ABPP makes use of a chemical probe which includes three components- a reactive group, a reporter tag, a spacer. The reactive group is for binding and covalently labeling the active sites of particular target enzymes, while the reporter tag is for the detection and isolation of these labeled enzymes. The spacer between a reactive group and a tag gives the probe flexibility and space for the detection of the tag. As shown in Figure 10, the standard method makes use of one compound containing all three components and is suitable when the pocket of the active site in the target protein is fairly flexible and large enough to accommodate the entire molecule of the probe. However,

this method has a disadvantage that the bulkiness of reporter tags may decrease the reactivity of the probe in the cell environment. On the other hand, if the pocket is very tight and highly specialized to reactive molecules, click chemistry mediated ABPP (CC-ABPP) will be appropriate to utilize. Click chemistry relies on two compounds-one containing the reactive group and the second containing tag and spacer (see Figure 10). These two compounds react together becoming covalently attached to one another. This allows the small reactive group to bind to the target protein first and the reactive group is later tagged, thereby labeling the target protein. Because we did not know at priori whether our target active site was large enough to accommodate the complete chemical probe, we approached our objectives by both methods- standard ABPP and CC-ABPP, which will eventually provide same information.

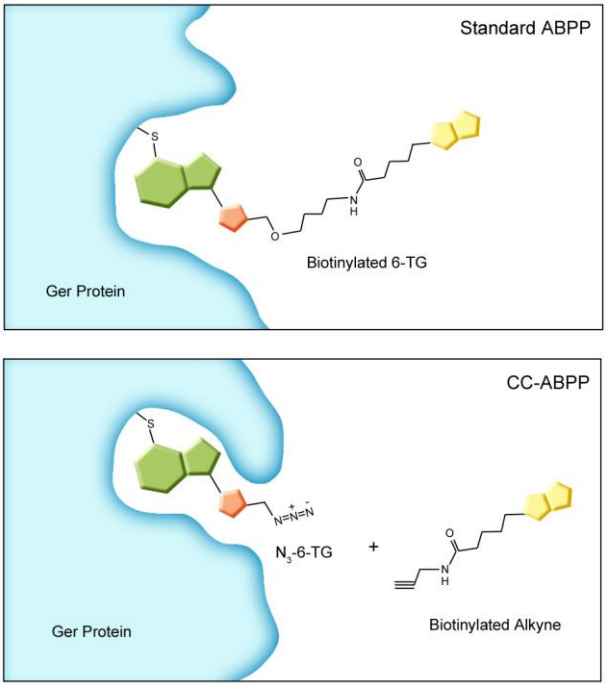



Figure 10. Standard and click chemistry ABPP.

### 3.1.1 Standard ABPP-based nucleosides

Although standard ABPP probes might be accompanied by unfavorable nonspecific interactions with proteins, we attempted to prepare entire molecules which contain a reactive group and a tag, 6-TG and biotin respectively, linked each other by a spacer. The reason for carrying out the standard ABPP method despite of its limitation is because we found that Ger receptors seem to have a flexible binding site from previous study (45). To determine if we could substitute the 5-hydroxyl group of guanosine without affecting binding to *B. anthracis* spores, we first methylated the 5'-OH position. Indeed, in germination test, we found that 5'-O-methylguanosine is able to act as a cogerminant with L-alanine (see Figure 11). This finding shows that the 5'-OH is not important for germinant recognition and can be used as a handle to introduce an affinity tag for ABPP.

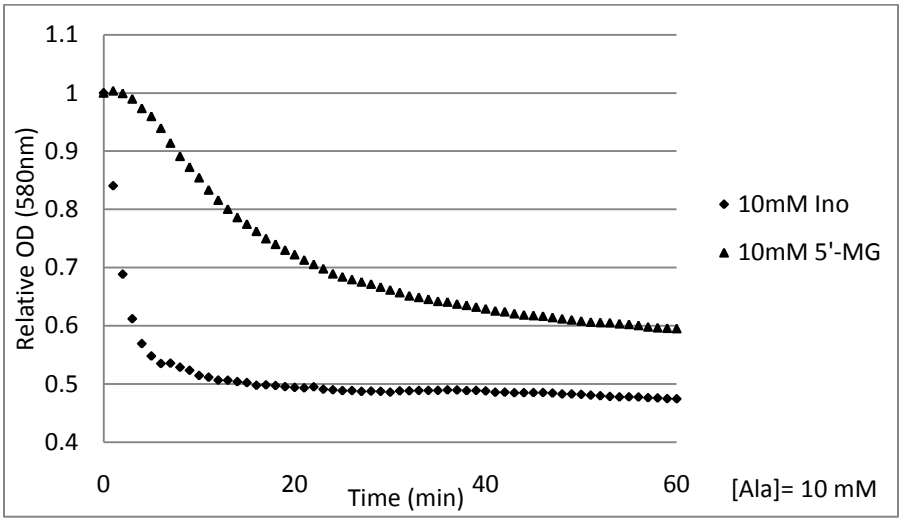



Figure 11. 5'-O-methylguanosine-mediated *B. anthracis* spore germination. (10 mM Ino: 10 mM inosine treated spores, 10 mM 5'-MG: 10 mM 5'-O-methylguanosine treated spores, concentreation of the cogerminant, L-alanie, was 10 mM)

Based on these results, we designed two different strategies to attach a biotin tag to the 5'-position of guanosine: (1) alkyl spacer synthesis (2) biotinylation (see Figure 12).

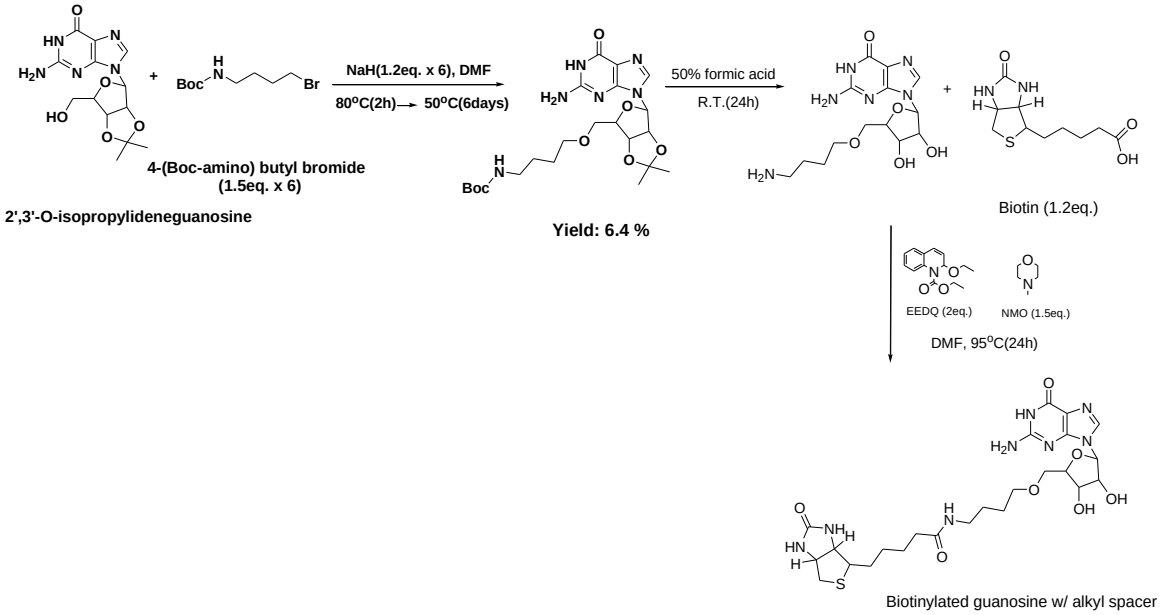



Figure 12. Schemes for synthesis of standard ABPP probe.

However, we encountered poor yield in the first step of the synthesis wherein protected guanosine (2',3'-O-isopropylideneguanosine) was treated with a bifunctional crosslinker (4-(boc-amino) butyl bromide) to create free alkyl chain to the reactive molecule. After 6 days of reaction and column chromatography purification, we have isolated the desired compound along with an unwanted product (see Figure 13). The structures were verified by $^{1}$H NMR, and FT-IR. Our desired compound was isolated

only 6 %. The side product was also isolated in 10 %, as a major product. Because of the relatively weak acidic nature of alcohols, a very strong base needed to be used to quantitatively convert the alcohol into the alkoxide anion. Therefore we chose sodium hydride (NaH) as a base in the reaction. However, due to the uncontrollable high reactivity, sodium hydride seemed to deprotonate not only 5'-OH but also 1-NH, resulting in substituted products on both positions. This side reaction lowered total yield of the desired compound, and it was not cost-effective to continue the following steps of the synthesis. For this reason, we decided to move onto the alternative method, tag-free version of ABPP, by utilizing click chemistry.

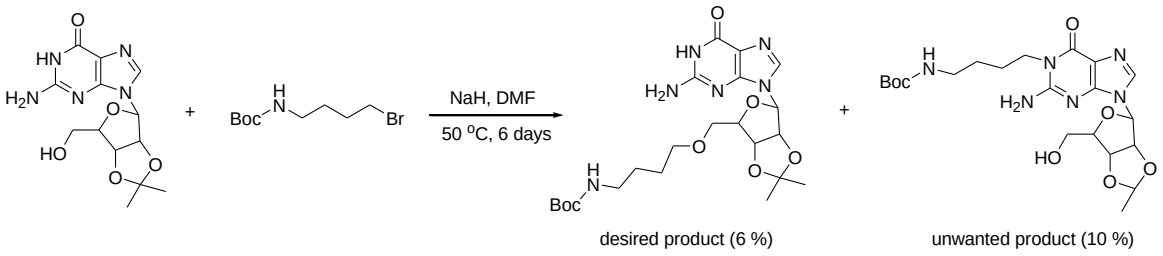



Figure 13. Unwanted product in standard ABPP probe synthesis.

### 3.1.2 Click Chemistry Mediated ABPP

Click chemistry has proven to be superior in satisfying many criteria (e.g., biocompatibility, selectivity, yield, stereospecificity, wide applicability, and inoffensive

byproducts) (50). It has been used in synthesis of potential therapeutic reagents in the field of drug discovery for several decades (51). Recently, this simple one-pot reaction has also improved target identification in activity-based protein profiling (52). There are several advantages of click reaction in the proteomic research. First, instead of a probe with bulky tags, small azide reactive molecules were used for protein isolation (see Figure 10). The small probes are more easily up taken by biological surroundings compared to the standard method. Second, the 1,3-dipolar cycloaddition can occur in water to be utilized in living systems. Third, there are no competing side reactions from normal metabolites because it is very selective reaction between azide and alkyne. Figure 14 demonstrates click reaction of our specific molecules. Both reactants are synthetic compounds, biotin-labeled alkyne and protein-cross-linked azide compound.

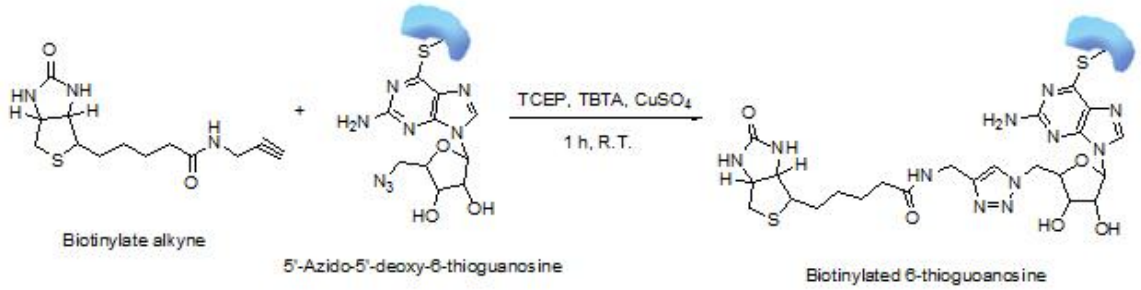



Figure 14. Proposed click reaction between biotinylated alkyne and protein-cross-linked 5’-azido-5’-deoxy-6-thioguanosine ($N_3$-6-TG).

## 3.2 Nucleoside Analogs Synthesis and Germination Studies

### 3.2.1 Synthesis of Nucleoside Analogs

Due to the cost associated with 6-thioguanosine, we developed our synthetic schemes with the common nucleoside, guanosine (G). Guanosine (G) is a weak germinant and closely resemble 6-thioguanosine in structure. Whereas 6-TG has an exocyclic sulphydryl group at position 6, guanosine has a carbonyl oxygen at the same position (Fig 15). Once the synthetic methods were confirmed with guanosine derivatives, we applied the same methods to synthesis of 6-thioguanosine analogs.

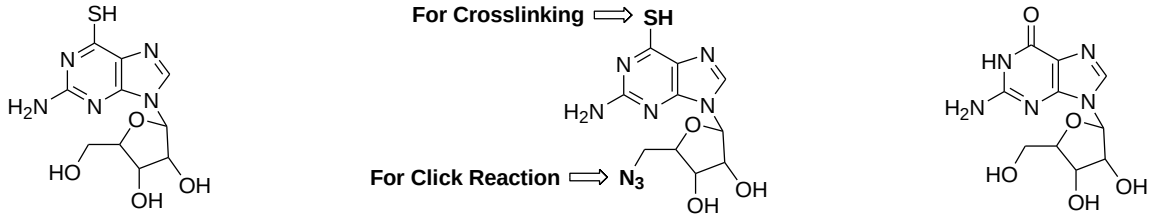



Figure 15. Structures of 6-TG, $N_3$-6-TG, G.

6-thioguanosine (6-TG) has been chosen as the structural base for this study because it inhibits *B. anthracis* spore germination of *B. anthracis* and can crosslink target proteins through its thiol group. We expect that these properties of 6-TG will play an important role in identifying nucleoside-binding proteins from *B. anthracis* spores. Therefore, the first step of this project was synthesis of 5'-azido-5'-deoxy-6-thioguanosine ($N_3$-6-TG), by functional group interconversion from the hydroxyl group of C-5' of 6-TG to an azido group. Since we had previously shown that the 5'-hydroxyl can be modified without

losing binding activity, we used this postion (and not the secondary 2' and 3'-hydroxyls) to introduce the azide functionality as a bait for click reaction (see Figure 15).

### 3.2.1.1 *Thomson's* Method

To avoid the inconvenience of multiple purification steps, we tested *Thomson's* "one-pot" procedures (53) to introduce the azide functionality on the C-5' position of guanosine. Figure 16 shows the reaction scheme. Before introducing azidation, the 2' and 3' hydroxyl groups of guanosine required an additional protection step by isopropylidene acetal formation. Guanosine was reacted with *p*-toluenesulfonic acid monohydrate and 2,2-dimethoxypropane to afford 2',3'-O-isopropylideneguanosine. The protected 2',3'-O--isopropylideneguanosine (PG) was then reacted with diphenylphosphoryl azide (DPPA) in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to give a phosphate triester intermediate. Surprisingly, the activated phosphate triester intermediate was not a good enough leaving group to facilitate azide displacement at C-5' position.

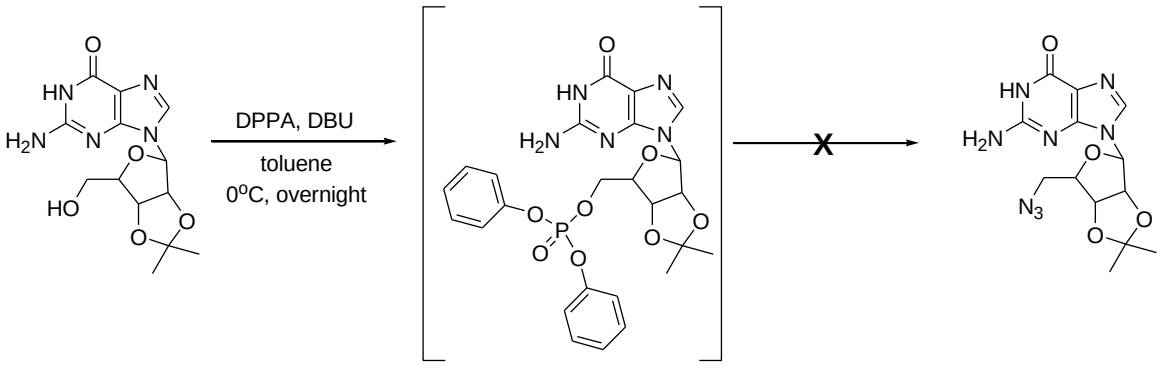



Figure 16. Reaction scheme of azidation using *Thomson's* method.

### 3.2.1.2 Phosphate Activation

The failure of *Thomson's* direct azidation procedure suggested that diphenyl phosphate was a poor leaving group to activate the alcohol to be displaced by azide. Therefore, we applied a modified-one-pot method that involves modification of phosphate group to create a better leaving group and introduction of a separate source of azide (54). In this alternative method, 2′,3′-O--isopropylideneguanosine was reacted with bis(2,4-dichlorophenyl) chlorophosphate ($(Cl_2PhO)_2POCl$) in the presence of sodium azide ($NaN_3$) and 4-dimethylaminopyridine (DMAP) (see Figure 17). Introduction of electron-withdrawing substituents on the phenyl ring was expected to make it a better leaving group and the resulting activated phosphate could be sufficiently reactive for displacement by azide ion. In addition, the azide ion was expected to be the driving force to convert the activated alcohol to the corresponding azide product. This is because the azide ion was the major nucleophile participating in the $S_N2$ displacement reactions (54). After overnight reaction, a complete conversion was observed by TLC. However, the isolated compound turned out to be a phosphate intermediate based on the characterization by $^1H$ NMR and FT-IR. This method also resulted in isolation of the phosphate intermediate with no further reaction to the azide product. Thus, substitution of the 5'-hydroxyl with aryl phosphates did not provide a direct route to the formation of the expected 5'-azide substitution.

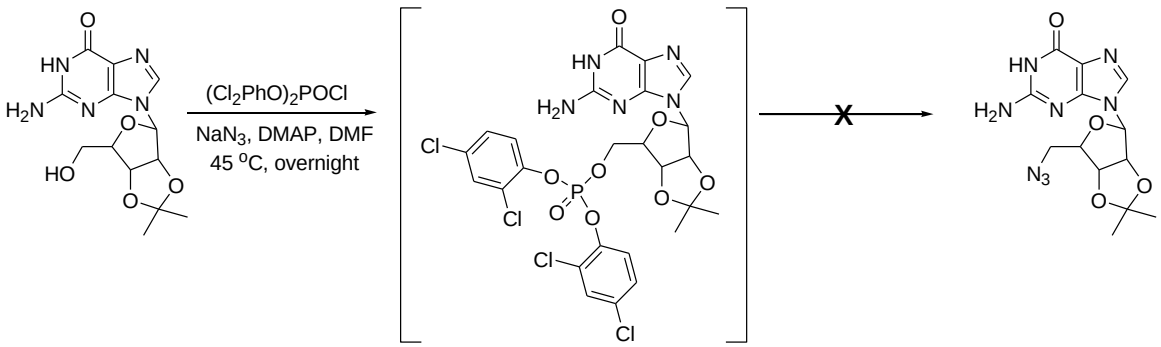



Figure 17. Reaction scheme of the simple one-pot azidation via phosphate activation.

### 3.2.1.3 Sulfonylation

Since the one-step methods for azidation of nucleosides failed, we decided to test a different set of leaving groups. This procedure involves the sulfonylation of the 5'-alcohol. After isolation of the activated nucleoside, the sulfonate group is displaced by azidation. The two most common and effective methods to transform an alcohol to the corresponding sulfonate ester are tosylation and triflation using *p*-toluenesulfonyl chloride or triflic anhydride, respectively. These reactions are carried out under the basic conditions usually with pyridine (see Figure 18).

2′,3′-O--isopropylideneguanosine was tosylated by *p*-toluenesulfonyl chloride in the basic condition. Based on the TLC (see Figure 19), two unidentified products were obtained at low yields even in the presence of excess concentration of *p*-toluenesulfonyl chloride. Due to the low yield of the reaction, no attempt was made to purify the two new products.

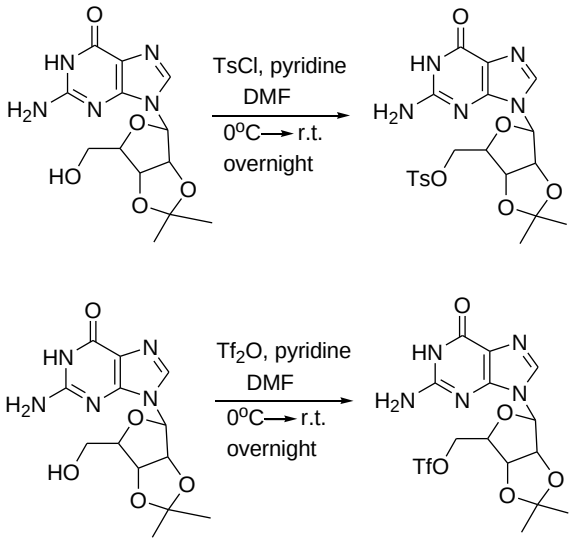



Figure 18. Reaction scheme of sulfonylation.

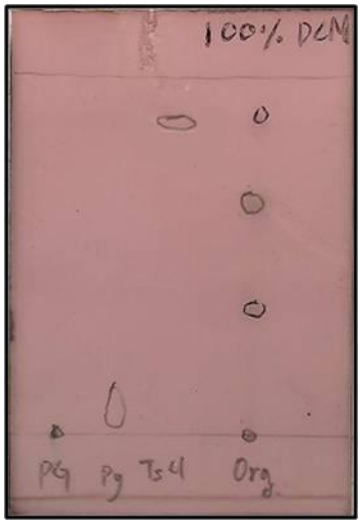



Figure 19. TLC results from tosylation of 2',3'-O-isopropylideneguanosine after overnight reaction. (PG: 2',3'-O-isopropylideneguanosine, Py: pyridine, TsCl: *p*-toluenesulfonyl chloride, Org: organic layer (ethyl acetate) after extraction, Eluent: 100 % dichloromethane)

Since tosylation of 2',3'-O-isopropylideneguanosine was too slow, we substituted *p*-toluenesulfonyl chloride for the stronger electrophile, triflic anhydride, expecting to see a faster reaction. The experiment was performed following the same procedure as for tosylation. However, TLC showed poor conversion after overnight reaction and the resulting products were not identified.

#### 3.2.1.4 Iodination

The failure of sulfonylation led us to test another method to activate the 5'-hydroxyl. Iodination on the 5' position of guanosine afforded 5'-deoxy-5'-iodoguanosine in 68 % yield (see Figure 20). Because the reaction was highly selective on the primary position, the 2' and 3' hydroxyl groups were not protected before iodination. This selectivity is attributed to the *in situ* protection of the 2' and 3'hydroxyls as a cyclic oxyphosphorane intermediate (55). Also, the use of N-methyl-2-pyrrolidinone as reaction solvent enhanced the reaction rate due to its good solvation properties (56).

When 5'-deoxy-5'-iodoguanosine was reacted with sodium azide ($NaN_3$), 5'-azido-5'-deoxyguanosine was obtained in 79 % yield. Azidation of the 5' position was confirmed by 21,00 $cm^{-1}$ asymmetric vibration of azides in IR-spectroscopy.

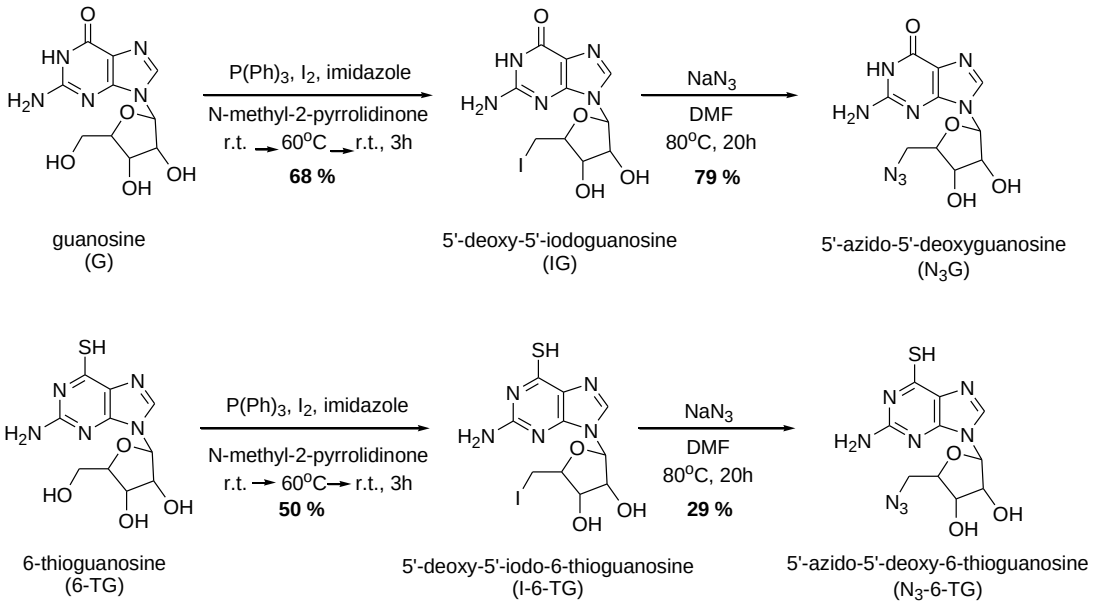



Figure 20. Synthesis of IG, $N_3G$, I-6-TG, and $N_3$-6-TG using an indirect method.

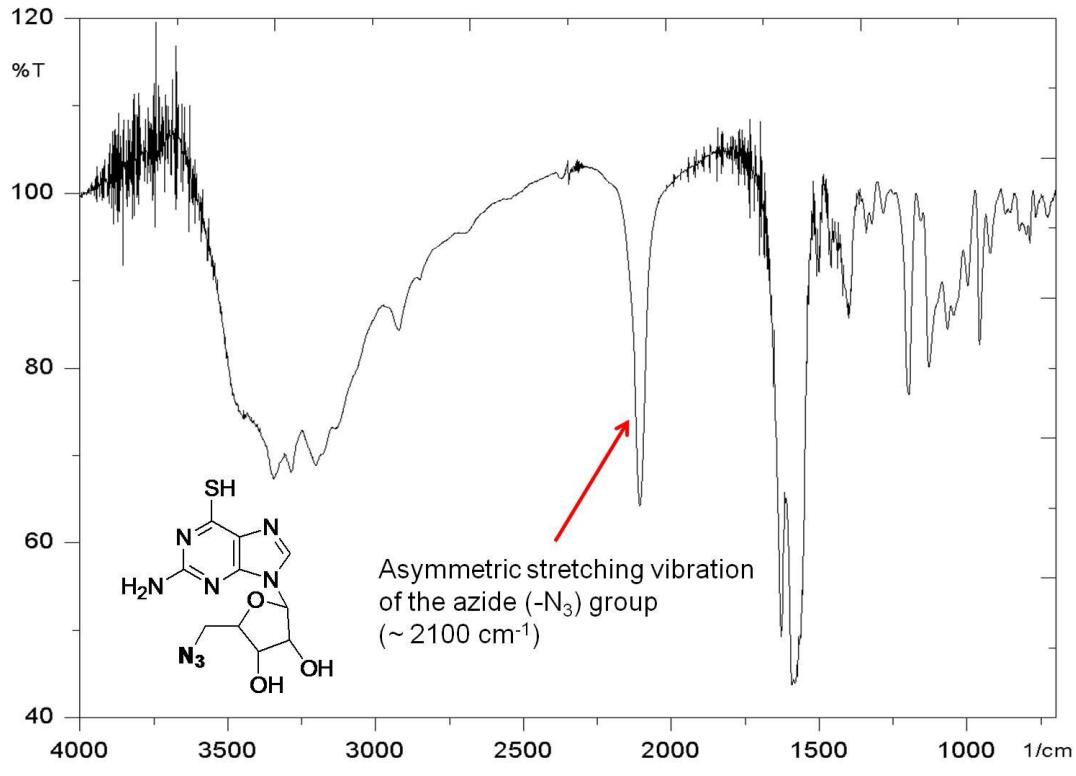



Figure 21. FT-IR spectrum of 5'-azido-5'-deoxy-6-thioguanosine.

After confirmation of the iodination and azidation methods, we proceeded with the synthesis of 6-thioguanosine analogs by following the same procedures used in the synthesis of 5'-deoxy-5'-iodoguanosine and 5-azido-5'-deoxyguanosine. The resulting compounds, 5'-deoxy-5'-iodo-6-thioguanosine and 5-azido-5'-deoxy-6-thioguanosine, were pure based on TLC and characterized by $^{1}$H NMR, FT-IR, and MS. Figure 21 shows a strong IR peak around 2100 $cm^{-1}$ which represents asymmetric stretching vibration of the azide group.

#### 3.2.1.5 Synthesis of 5-amino-5'-deoxyguanosine

5-amino-5'-deoxyguanosine was synthesized by reduction of 5-azido-5'-deoxy-guanosine with pyridine, triphenylphosphine, and ammonium hydroxide. This experiment was performed as an alternative method of click chemistry activity-based protein profiling, in an effort to make a biotinylated probe where the amino group of 5' position is linked directly with biotin through amide bond formation.

### 3.2.2 Germination and Germination Inhibition Studies

Synthesized inosine analogs were tested in *B. anthracis* spore germination and germination inhibition assays to determine the effects of modification on C-5' of the nucleosides. It is important to make sure that the structurally modified compounds still retain similar activities to the original germinant or inhibitor nucleosides before any further steps to be proceeded.

Firstly, we have tested both guanosine and 6-thioguanosine analogs in germination assays. *B. anthracis* spores were treated with varying concentrations (0.05, 0.1, 0.5, 1, 5, 10 mM) of guanosine analogs (inosine, 5'-azido-5'-deoxyguanosine, 5'-amino-5'-deoxyguanosine, 5'-O-methylguanosine) at constant concentration of L-alanine (10 mM).

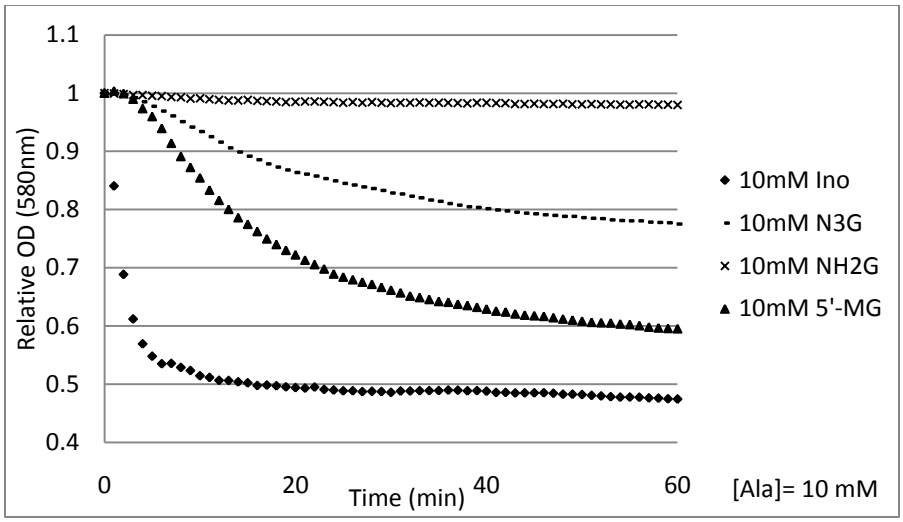
We tested varying concentrations of guanosine analogs to find the optimal concentration that induces the best germination and also to examine if any compound of high concentration forms a precipitate when added to spore suspension. As expected the highest concentration (10 mM) of guanosine analogs induced the best germination without precipitate formation. The data in Figure 22 (a) shows changes in relative optical density at 580 nm over time at 10 mM of guanosine analogs. 5'-azido-5'-deoxyguanosine and 5'-amino-5'-deoxyguanosine which were modified on 5'-C of guanosine by displacement with azido and amino groups, respectively, resulted in a decrease in germination activities. When the decrease in optical density of 10 mM inosine-treated spores after 1h incubation was set to represent 100 % germination, only 42 % and 4 % of germination in 5'-azido-5'-deoxyguanosine and 5'-amino-5'-deoxyguanosine-treated spores was observed, respectively. This observation implies that substitution of hydroxyl group with nitrogen containing groups in nucleoside germinants decreased spore germination. Because of the low germination activity of 5'-amino-5'-deoxyguanosine, this compound was not explored further. Interestingly, when the oxygen next to 5'-C of guanosine was retained in the structure the relative OD values of spores treated with the compound (5'-O-methylguanosine) showed 76 % of decrease compared to the inosine-treated spores (see Figure 22 (a) 10 mM 5'-MG). Even though 5'-O-methylguanosine seemed to be a better germianant than 5'-azido-5'-deoxyguanosine and 5'-amino-5'-deoxyguanosine based on the germination test, we could not pursue preparing an ether-linked probe (the standard version of ABPP probe) due to the synthetic problem as discussed in 3.1.1.

In germination assays for 6-thioguanosine analogs, *B. anthracis* spores were treated with varying concentrations (0.05, 0.1, 0.5, 1, 5, 10 mM) of inosine, 6-thioguanosine, 5'-azido-5'-deoxy-6-thioguanosine at constant concentration of L-alanine (10 mM). Like the guanosine analogs-mediated spore germination, the highest concentration (10 mM) of the 6-thioguanosine analogs also induced the best germination. Figure 22 (b) shows the data from 10 mM of nucleoside treated spore germination. 5'-azido-5'-deoxy-6-thioguanosine ($N_3$-6-TG) which will be utilized as a synthetic probe in profiling proteins has less activities in spore germination, showing 29 % of decrease in optical density compared to inosine-treated spores set to 100 %. (see Figures 22 (b) 10 mM N3-6-TG).

To determine if the synthesized compound, 5'-azido-5'-deoxy-6-thioguanosine ($N_3$-6-TG), still act as an inhibitor in *B. anthracis* spore germination, inhibition assays were carried out. *B. anthracis* spores were preincubated with varying concentrations (0.25, 0.5, 1, 5, 10 mM) of 6-TG or ($N_3$-6-TG) for 15 min, then added 40 μM L-alanine and 250 μM inosine to induce the germination. These concentrations of L-alanine and inosine have been found to be the lowest concentrations that can induce the maximal germination rate (45). At 10 mM, the highest concentration, of both 6-TG and $N_3$-6-TG, the best germination inhibition was observed. When the decrease in optical density of the control experiment after 1h incubation, where the spores were treated with L-alanine and inosine, was set to represent 100 % germination, 56 % and 81 % of spore germination occurred in 6-TG and $N_3$-6-TG preincubated spores, respectively (see Figures 23). This result implies that both 6-TG and $N_3$-6-TG associate with the inosine binding pockets of Ger receptors, impeding inosine to be recognized by the sites to induce spore germination. Based on the inhibition assay, 6-TG showed better inhibition activities compared to the modified

molecules, $N_3$-6-TG. However, $N_3$-6-TG still retains the inhibitory properties so that this compound can be used as a synthetic probe in protein profiling.

(a)




(b)

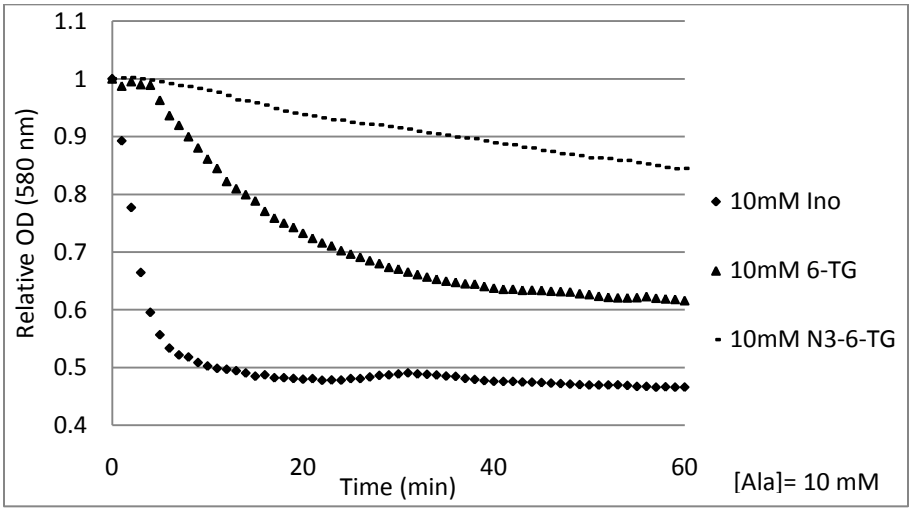



Figure 22. Inosine analogs-mediated *B. anthracis* spore germination. ( (a) 10 mM Ino: 10 mM inosine treated spores, 10 mM N3G: 10 mM 5'-azido-5'-deoxyguanosine treated spores, 10 mM NH2G: 10 mM 5'-amino-5'-deoxyguanosine treated spores, 10 mM 5'-MG: 10 mM 5'-O-methylguanosine treated

spores, (b) 10 mM Ino: 10 mM inosine treated spores, 10 mM 6-TG: 10 mM 6-thioguanosine treated spores, 10 mM N3-6-TG: 10 mM 5'-azido-5'-deoxy-6-thioguanosine treated spores, concentreation of the cogerminant, L-alanie, was 10 mM in every germination assay)

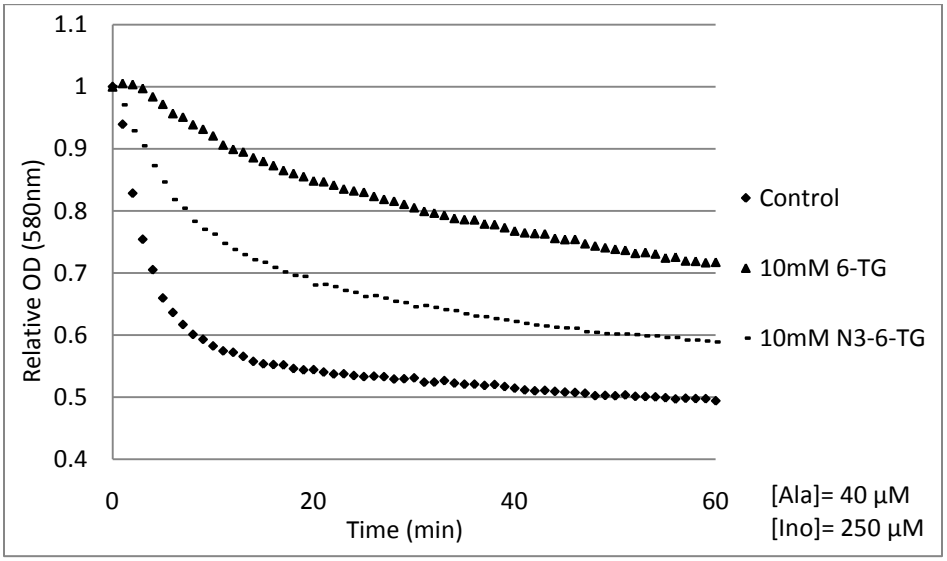



Figure 23. Inosine analogs-mediated *B. anthracis* spore germination inhibition. (Control: L-alanine and inosine treated spores without inhibitor preincubation, 10 mM 6-TG: 10 mM 6-TG preincubated spores , 10 mM N3-6-TG: 10 mM $N_3$-6-TG preincubated spores, every inhibitor preincubated spore was added L-alanine and inosine after 15 min to induce germination)

## 3.3 UV Crosslinking VS. MMPP Oxidation Methods

Oligonucleotides containing 6-thioguanine can be photochemically crosslinked to oligopeptides via peptide thiol and amino groups upon UV irradiation (25). In addition, when 6-thioguanine was oxidized with magnesium bis(monoperoxyphthalate) hexahydrate (MMPP), crosslinking was found to be significantly more efficient even in

the absence of UV irradiation (25, 26). We therefore tested the oxidation method using MMPP in germination inhibition assays to see if we can utilize the method instead of UV crosslinking in the ABPP experiment.

Inhibition assays were performed using the same protocol as described above. Preincubating spores with 6-TG in oxidizing conditions (6TG:MMPP=1:3) resulted in less germination after 1h incubation (see Figure 24). However, as shown in Figure 24, the oxidation with MMPP did not significantly improved germination inhibition to substitute UV crosslinking in the ABPP experiment. Therefore, we decided to return to general method of photocrosslinking using a maximum dose rate of UV (300 kJ/m$^2$).

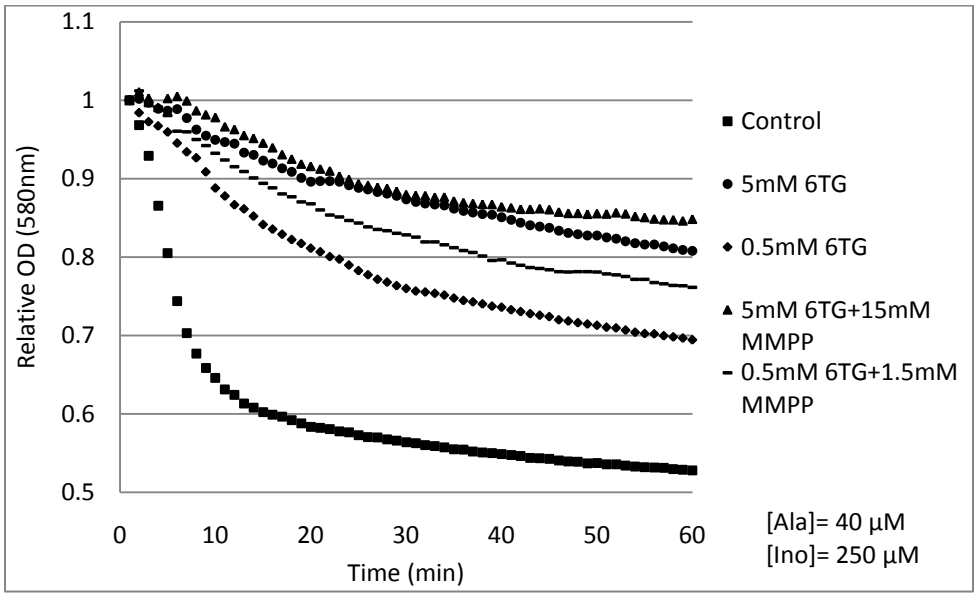



Figure 24. 6-TG mediated *B. anthracis* spore germination inhibition in oxidizing conditions. (Control: L-alanine and inosine treated spores without inhibitor preincubation, 5 mM 6-TG: 5 mM 6-TG preincubated spores , 0.5 mM 6-TG: 0.5 mM 6-TG preincubated spores, 5 mM 6-TG+15 mM MMPP: 5 mM 6-TG and 15 mM MMPP preincubated spores, 0.5 mM 6-TG+1.5 mM MMPP: 0.5 mM 6-

TG and 1.5 mM MMPP preincubated spores, every inhibitor preincubated spore was added L-alanine and inosine after 15 min to induce germination)

## 3.4 Gel-Based ABPP Analysis

For activity-based protein profiling (ABPP), with a chemical probe we employed photocrosslinking, click chemistry, and streptavidin purification. Figure 25 is a simplified illustration of the overall scheme.

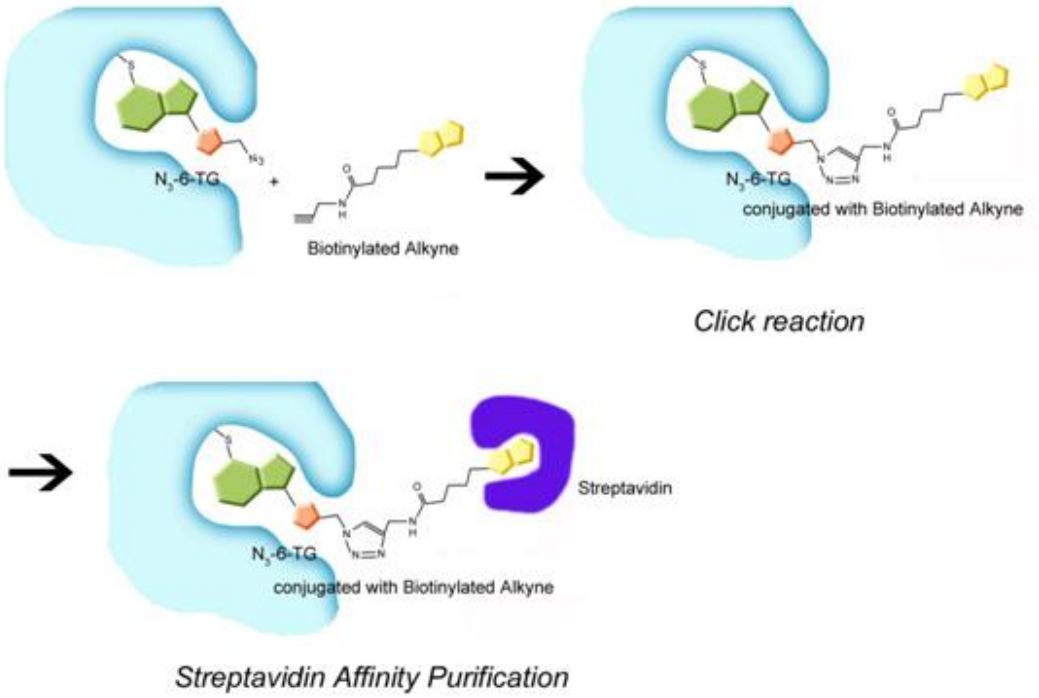



Figure 25. Protein profiling facilitated by a synthetic probe, click reaction, and streptavidin purification.

Even though liquid chromatography-mass spectrometry (LC-MS) platforms have superior properties of high resolution for the analysis of ABPP (27), we decide to pursue a gel-based method because SDS-PAGE and Western blot provide visually determinable information before mass spectrometry analysis. Also, SDS-PAGE allows examining whether there are any technical difficulties during each step of experiments. Figure 26 demonstrates the entire procedure, both gel-based and gel-free methods.

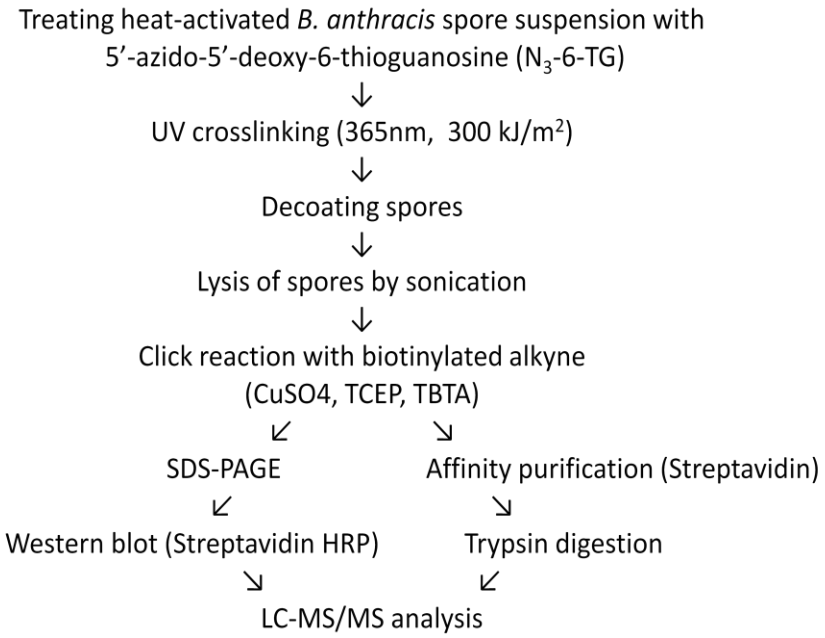



Figure 26. Gel-based (on the left) and gel-free (on the right) ABPP methods

### 3.4.1 Significance of Decoating

Bacterial endospores have a complex structure composed of several layers-spore core, inner membrane, cortex, outer membrane, spore coat, and exoporium (see Figure 1). Germination receptor proteins are found to be located in the inner membrane (57). In addition, altering the inner membrane has been seen to affect germination properties (58). The spore coat is a multilayered structure, has an important role as a permeability barrier by providing spores with resistance against exogenous lytic enzymes, organic solvents, and oxidative chemicals (59, 60). Since the Ger receptors are located in the inner membrane, decoating step was required to remove unnecessary parts of the spores and allow unrestricted access of the nucleoside probes to Ger receptors. In addition, decoating increases the efficiency of spore lysis that is required to obtain the membrane protein fractions.

To compare the efficiency of spore lysis with and without decoating step, *B. cereus* spores which has a similar structure with *B. anthracis* spores were tested. For the decoating samples, the spores were incubated in a decoating buffer (1 % Triton X-100, 5 mM CHES [pH 8.6], 8 M urea, 70 mM dithiothreitol) for 90 min at 37 $^{o}$C. Then, both normal spores and decoated spores were lysed by sonication (twelve times of 5 sec on and 5 sec off) in a sonication buffer (1 % Triton X-100, 10 % glycerol, 50 mM Tris-HCl [pH 8.0], 300 mM KCl). Figure 27 is the SDS-PAGE results. Compared to the normal spores (lane 1-3), decoated spores (lane 5-7) show efficient lysis, based on the number of bands appeared on the gel.

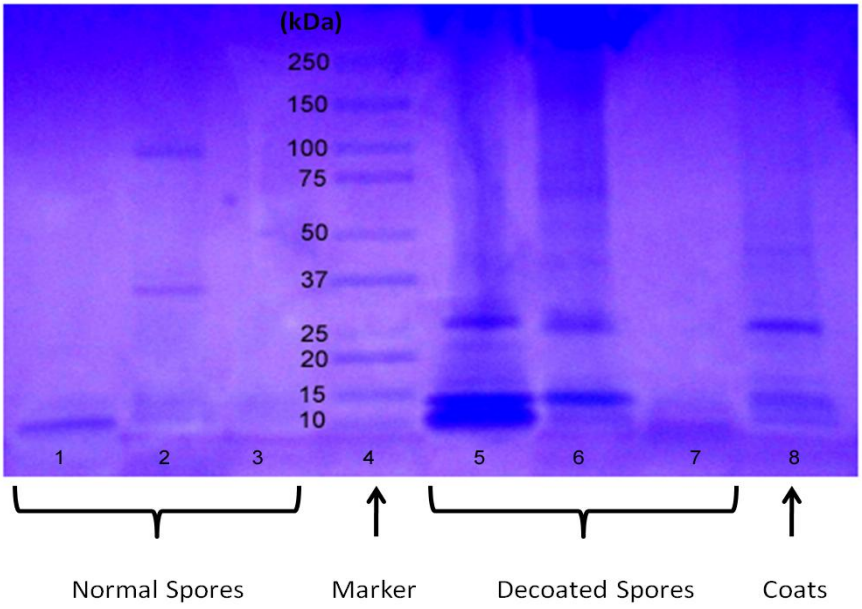



Figure 27. SDS-PAGE of lysed *B. cereus* spores with Coomassie Blue stanining. (Lane 1, 2, 3: lysed normal spore fractions, Lane 4: molecular weight marker, Lane 5, 6, 7: lysed decoated spore fractions, 8: coat proteins)

### 3.4.2 Lysis of Spores

Spore lysis was required for fractionation of the membrane proteins. The experiment was carried out with decoated spores by following the method described above. The result of spore lysis was shown in Figure 28. We confirmed the efficient lysis based on the SDS-PAGE gel results. The region from 30 to 90 kDa, particularly ~40 kDa area corresponds to the size of Ger receptor proteins of *B. anthracis* according to the protein sequences from *UniProt* (Universal Protein Resource).

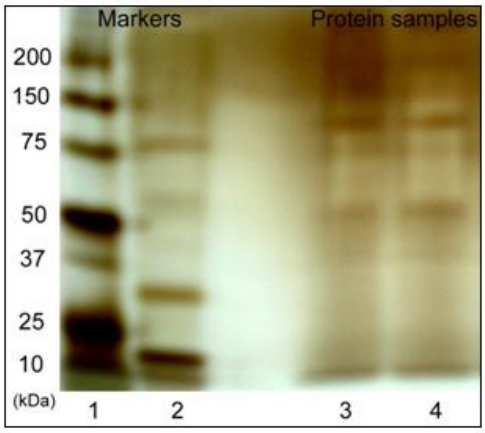



Figure 28. SDS-PAGE of *B. anthracis* lysed spores with silver staining. (Lane 1: precision plus protein westernC™ standard *(Bio-Rad)*, Lane 2: biotinylated molecular weight marker *(Sigma-Aldrich)*, Lane 3: 6-TG treated spores after lysis, Lane 4: $N_3$-6-TG treated spores after lysis)

### 3.4.3 Click Reaction

Click reaction between azide and alkyne needed to be confirmed before proceding with the reaction under biological conditions. We performed the cycloaddition reaction between 5'-azido-5'-deoxyguanosine and propargyl amine as a model reaction. Propargyl amine was chosen because it was the starting material for the synthesis of biotinylated alkyne. The reaction was carried out in a mixture of 220 mM NaOH, germination buffer, water, and DMSO.

5'-azido-5'-deoxyguanosine and propargyl amine were allowed to react in the presence of $CuSO_4$, tris(2-carboxyethyl)phosphine (TCEP), and tris-(benzyltriazolyl-methyl)amine (TBTA). The reaction mixture was submitted for mass spectrometry analysis.

The LC/MS data proved that the reaction was successful (see Figure 29). The cycloaddition product containing five-membered ring structure is shown at 362.19. Because the reaction condition was basic (pH 13), hydrogen from1-NH (pKa 9.50) and 2'-OH (pKa 12.5) of the product seems to be deprotonated, resulting a difference in mass. We can also reconfirm that the reaction completed by noticing the loss of every starting material; there are no peaks around 308.10 and 55.04 corresponding to the molecular weight of each reactant.

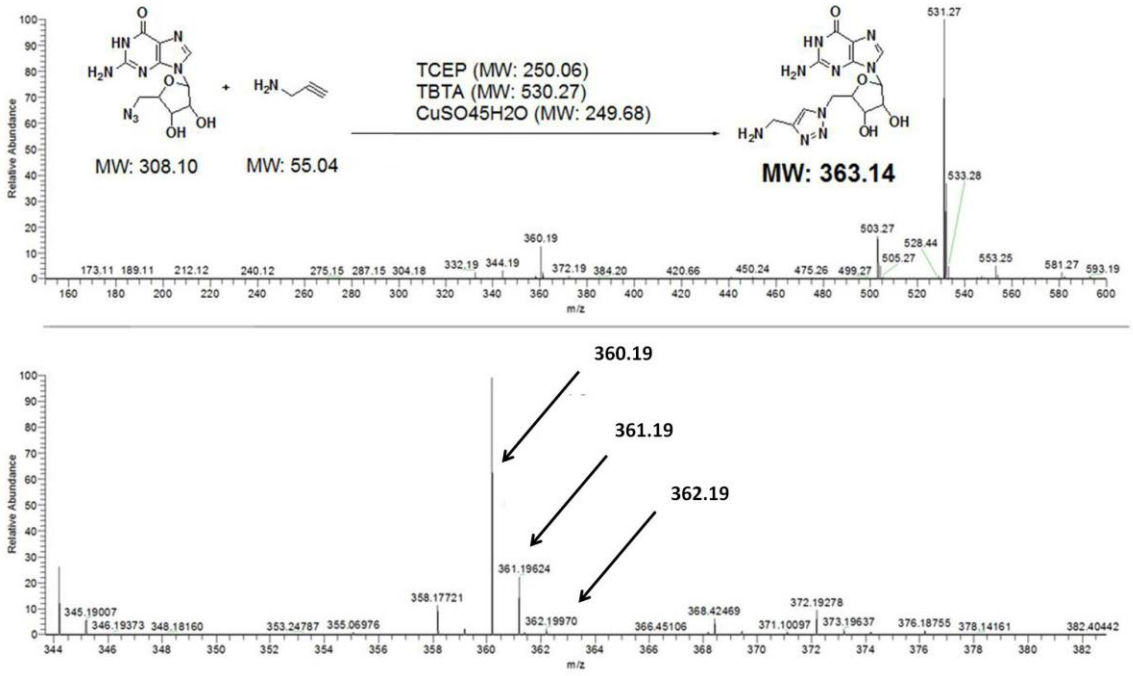



Figure 29. LC/MS result of click reaction between azide and alkyne.

### 3.4.4 Western Blot Detection

Because of a low yield of membrane proteins, large quantities of spores were required to be detected in the final step, western blot. Thus, we increased both the initial amount of spores and the concentrations of 5'-azio-5'-deoxy-6-thioguanosine ($N_3$-6-TG). 45 mL of spore suspension whose optical density at 580 nm was 3.0 was supplemented by 5 mL of 50 mM stock solutions of 6-TG or N3-6-TG to give a final concentration of 5 mM.

After the lysis step, the crosslinked protein samples needed to be washed with streptavidin agarose beads to remove nonspecific binding of proteins to the beads. Then click reaction was conducted with the bead-washed proteins. SDS-PAGE and western blotting were performed following a standard method. For immunoblotting detection, streptavidin-horseradish peroxidase conjugate was used to isolate biotinylated proteins with ECL Plus detection system.

The resulting immunoblot is shown in Figure 30. Lane 3 and 5 are the same protein samples that were crosslinked with 6-TG. Lane 4 and 6 are the same protein samples that were crosslinked with $N_3$-6-TG. The first four (lane 3, 4, 5, 6) samples are the membrane proteins. Lane 7 is 6-TG crosslinked coat proteins and lane 8 is $N_3$-6-TG treated coat proteins. In lane 4 and 6 (the membrane proteins treated with $N_3$-6-TG), there is no distinguishable unique band which would be suspected as the target protein, compared to lane 3 and 5 (the membrane proteins treated with 6-TG). The bands on the membrane seem to be a result from background labeling by the biotinylated akyne. This is probably due to the excessive amount of biotinylated alkyne added in click reaction.

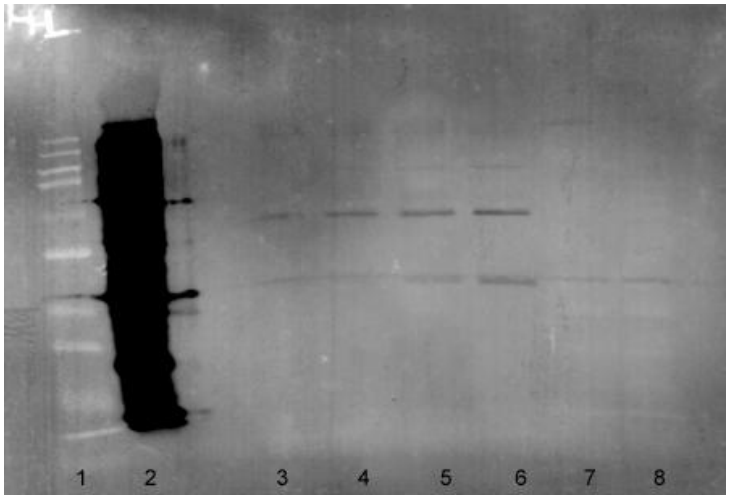



Figure 30. Western blot result. (Lane 1: precision plus protein western$C^{TM}$ standard *(Bio-Rad)*, Lane 2: biontinylated molecular weight marker *(Sigma-Aldrich)*, Lane 3, 5: 6-TG treated membrane proteins, Lane 4, 6: $N_3$-6-TG treated membrane proteins, Lane 7: 6-TG treated coat proteins, Lane 8: $N_3$-6-TG treated coat proteins)

## 3.5 Troubleshooting Click Chemistry

We investigated the effectiveness of 1,3-dipolar cycloaddition reaction between 5'-azido-5'-deoxyguanosine and biotinylated alkyne under the different conditions which will be applied to the actual protein samples. Those conditions include 1 % Triton X-100 of sonication buffer which membrane proteins will be suspended in and decoating buffer where removed coat proteins will stay.

The method was same as above except the reaction solvents- 1 % Triton X-100 of sonication buffer (10 % glycerol, 50 mM Tris-HCl [pH 8.0], 300 mM KCl), or decoating buffer (1 % Triton X-100, 5 mM CHES [pH 8.6], 8 M urea, 70 mM dithiothreitol).

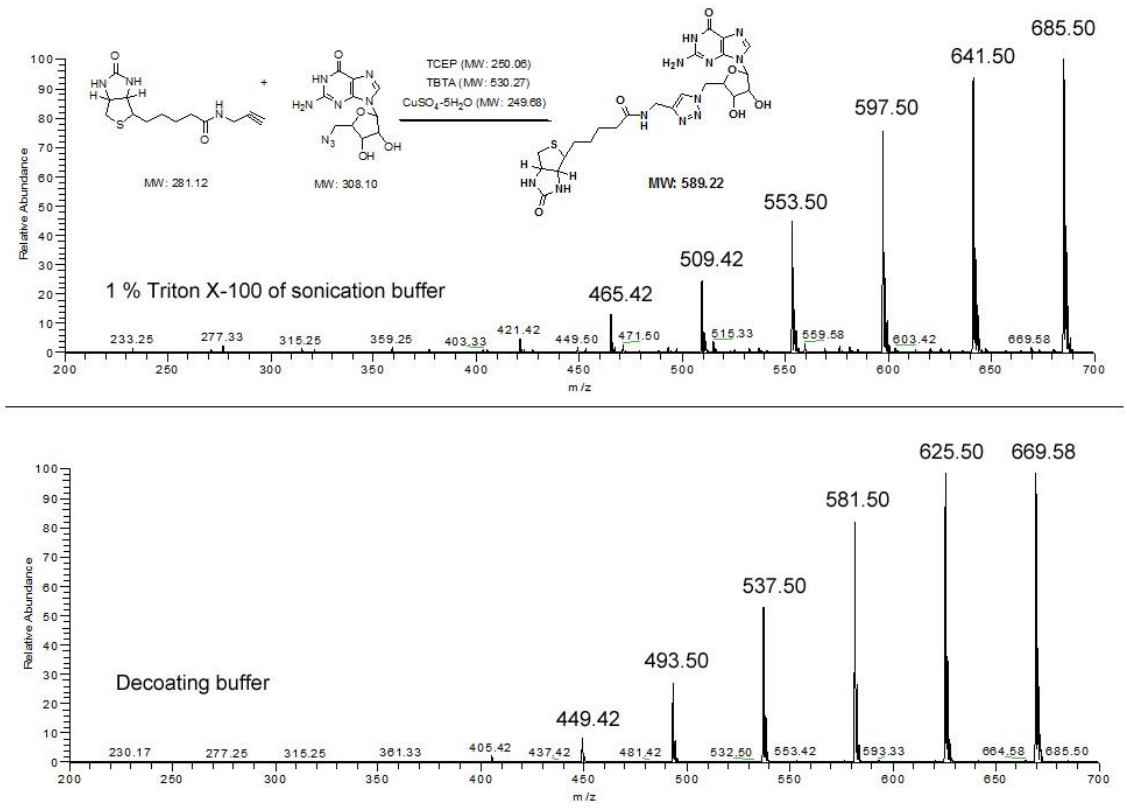



Figure 31. LC/MS result of click reaction in different conditions.

The LC/MS data in Figure 31 indicates that the 1,3-dipolar cycloaddition reaction did not produce the desired compound (MW: 589.22). It seems both reactants, biotinylated alkyne (MW: 281.12) and 5'-azido-5'-deoxyguanosine (308.10), have been reacted with reagents from 1 % Triton X-100 of sonication or decoating buffer. We still need to optimize the conditions for the click reaction to be utilized in ABPP.

# CHAPTER 4

# CONCLUSIONS AND FUTURE WORK

In conclusion, we tested different methods to synthesize the ABPP probe, 5'-azio-5'-deoxy-6-thioguanosine ($N_3$-6-TG). The compound was successively prepared by iodination followed by azidation. Other methods- *Thomson*'s method, phosphate activation, and sulfonylation- failed to make the desired compound due to isolation of intermediates and slow reaction. We found that $N_3$-6-TG still retains the inhibitory activities in inosine-mediated *B. anthracis* spore germination, enabling the compound to be used for the activity based protein profiling (ABPP).

The standard ABPP method was abandoned because of the synthetic problems to make an ether-linked biotin. Click chemistry was introduced as an alternative method to the standard ABPP. The 1,3-dipolar cycloaddition reaction was successful in the mixture of water, germination buffer, and DMSO verified by LC/MS analysis. However, in 1 % Triton X-100 of sonication and decoating buffer which are the conditions for the actual application to protein samples, the click reaction did not afford the desired product, 1,2,3-triazole.

We tested oxidation method with magnesium bis(monoperoxyphthalate) hexahydrate (MMPP) as an alternative of photocrosslinking. The germination inhibition assays with 6-TG and $N_3$-6-TG indicated the oxidation method did not significantly improve the inhibition of spore germination.

In gel-based ABPP, decoating step was found to be necessary for more efficient lysis of spores, and that was confirmed by SDS-PAGE. Spore lysis was required for

fractionation of membrane proteins and it was also confirmed by SDS-PAGE. There were no technical problems in SDS-PAGE and western blotting. However, because of the failure in click reaction the final results from immunodetection presented only background labeling of proteins by the excessive amount of biotinylated alkyne tag added in click reaction.

For future work, methods for click reaction under the conditions applied to the protein profiling need to be optimized. Also, reconfirmation is required to see if any of the chemical conditions from entire procedures has some factors that may cause dissociation of the crosslinking between proteins and probes. In addition, since the membrane proteins compose very small portion of total proteins of spores, we could also attempt to increase the initial amount of spores even more than we tested in this study.

In synthesis of chemical probes, switching the functionality of probe and reporter tag, azide to alkyne or vice versa, may reduce the background labeling as indicated in a literature (61). Also, making a longer spacer between probe and tag, by giving the probe more flexibility, would improve the detection of crosslinked proteins (62).

APPENDIX

SUPPLEMENTAL DATA

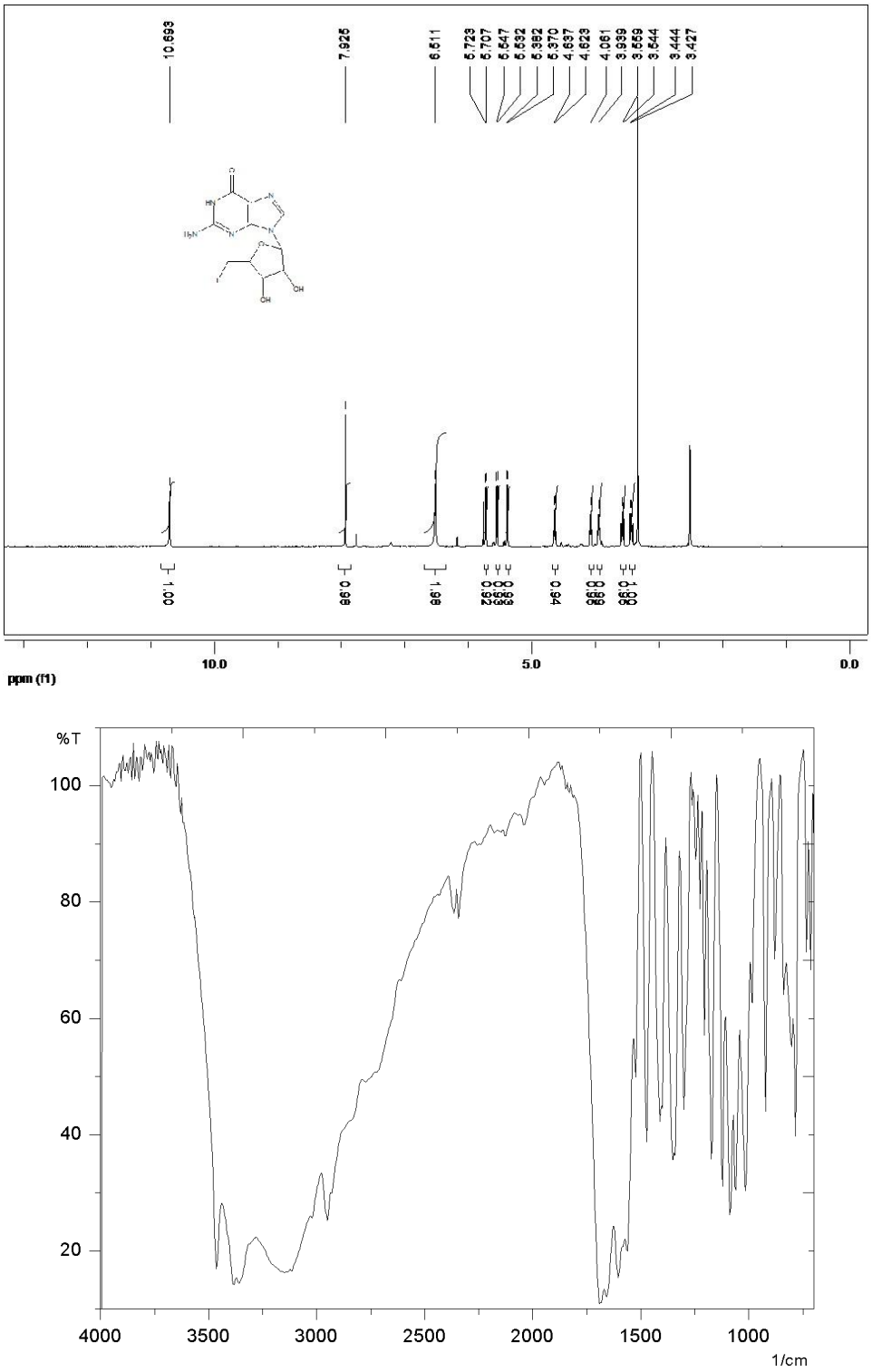
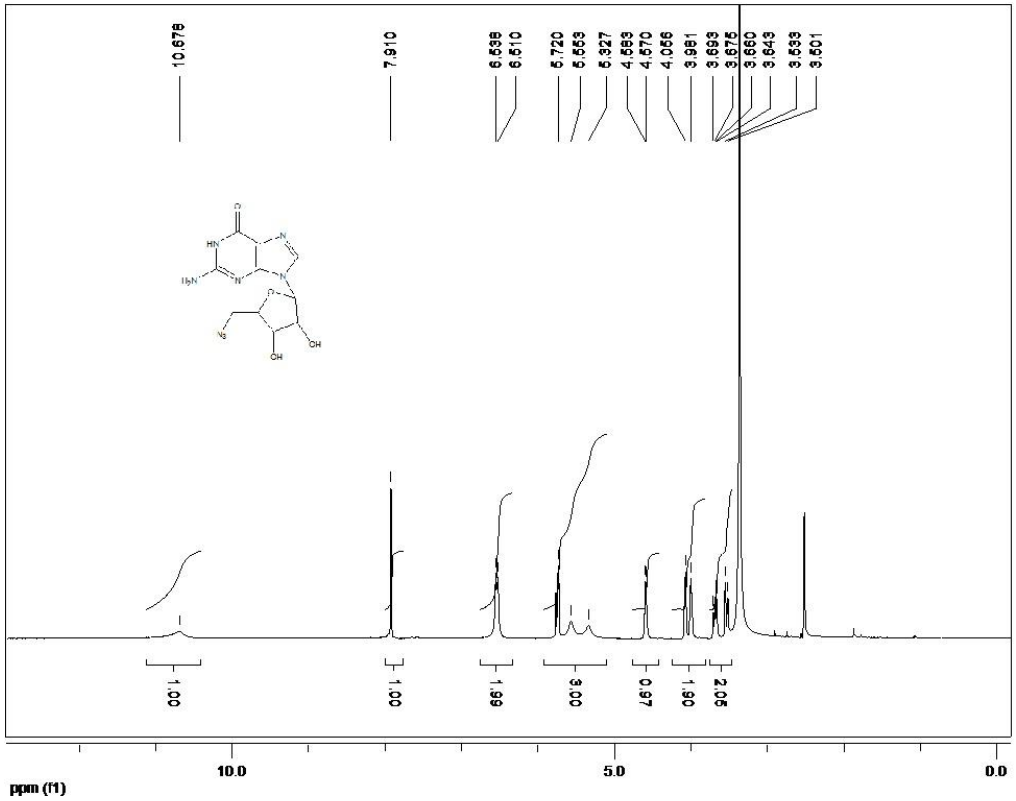



Figure S1. $^{1}$H NMR and FT-IR spectra of 5’-deoxy-5’-iodoguanosine.



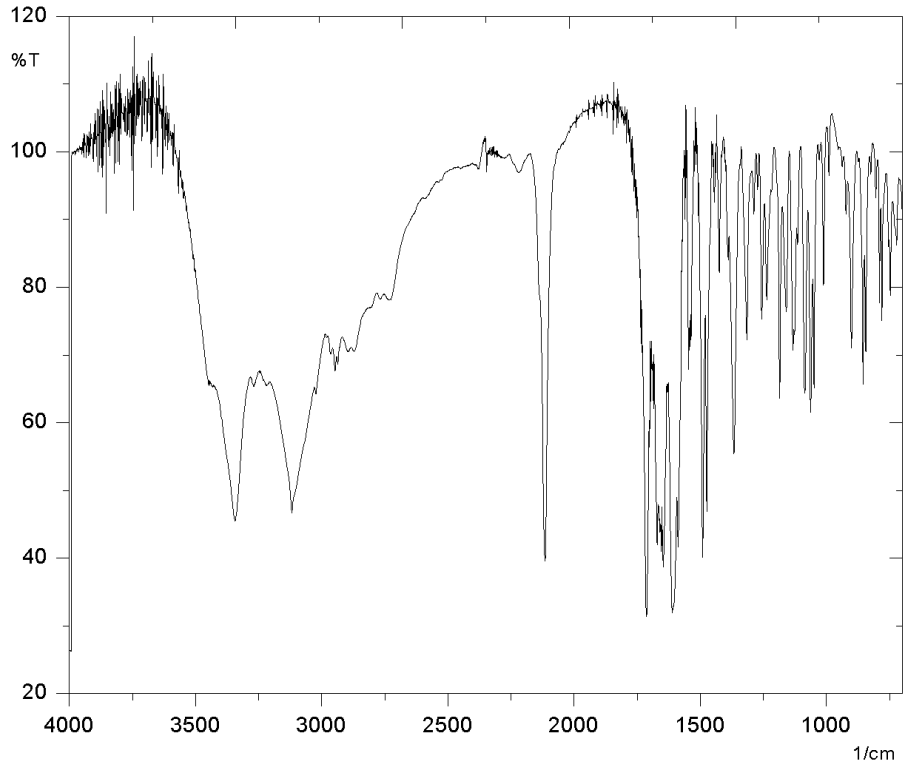
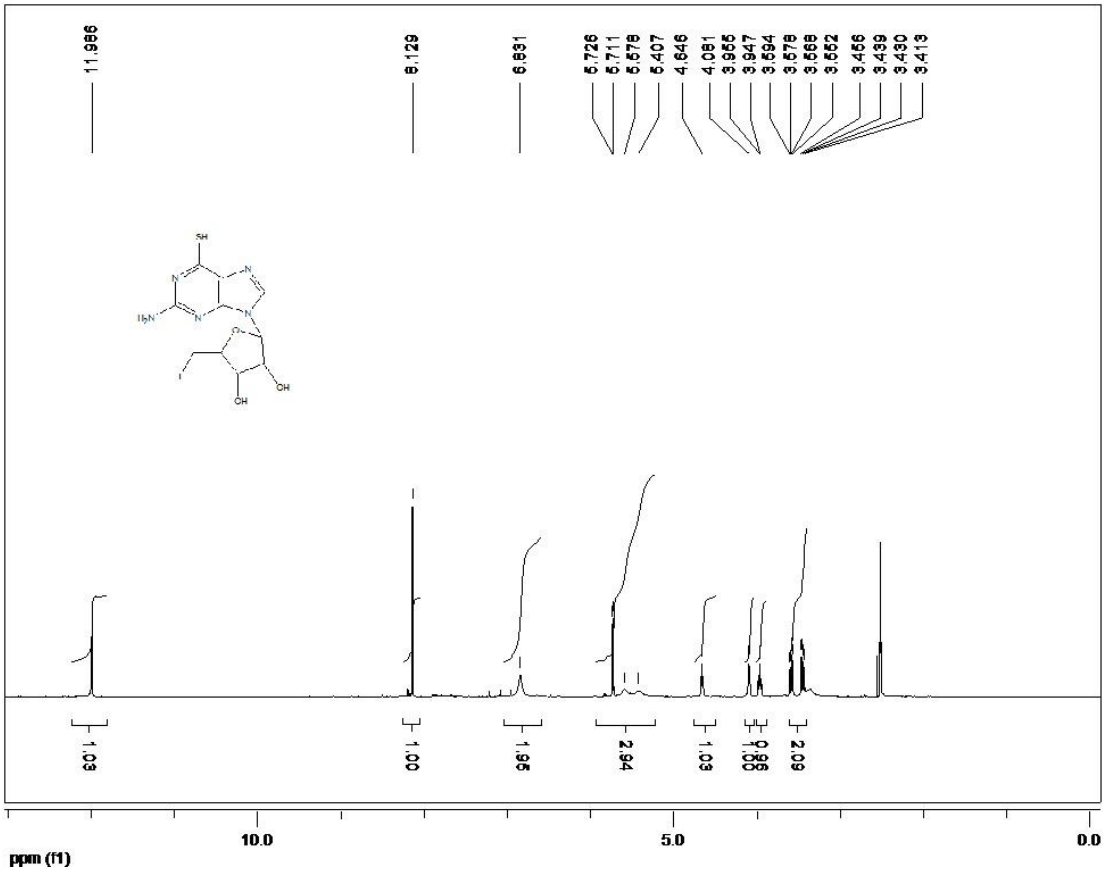




Figure S2. $^{1}$H NMR and FT-IR spectra of 5’-azido-5’-deoxyguanosine.



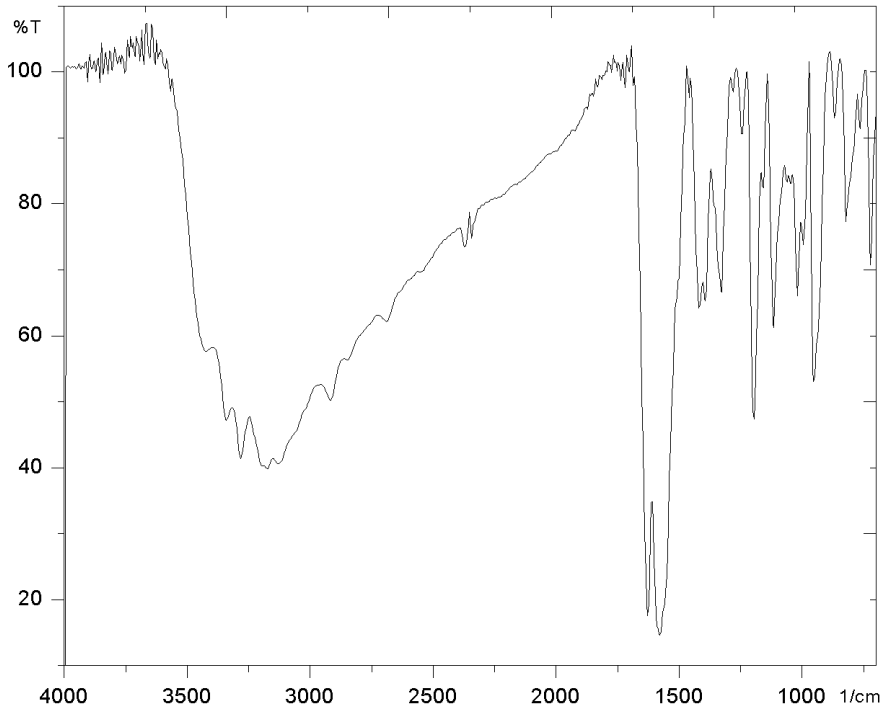
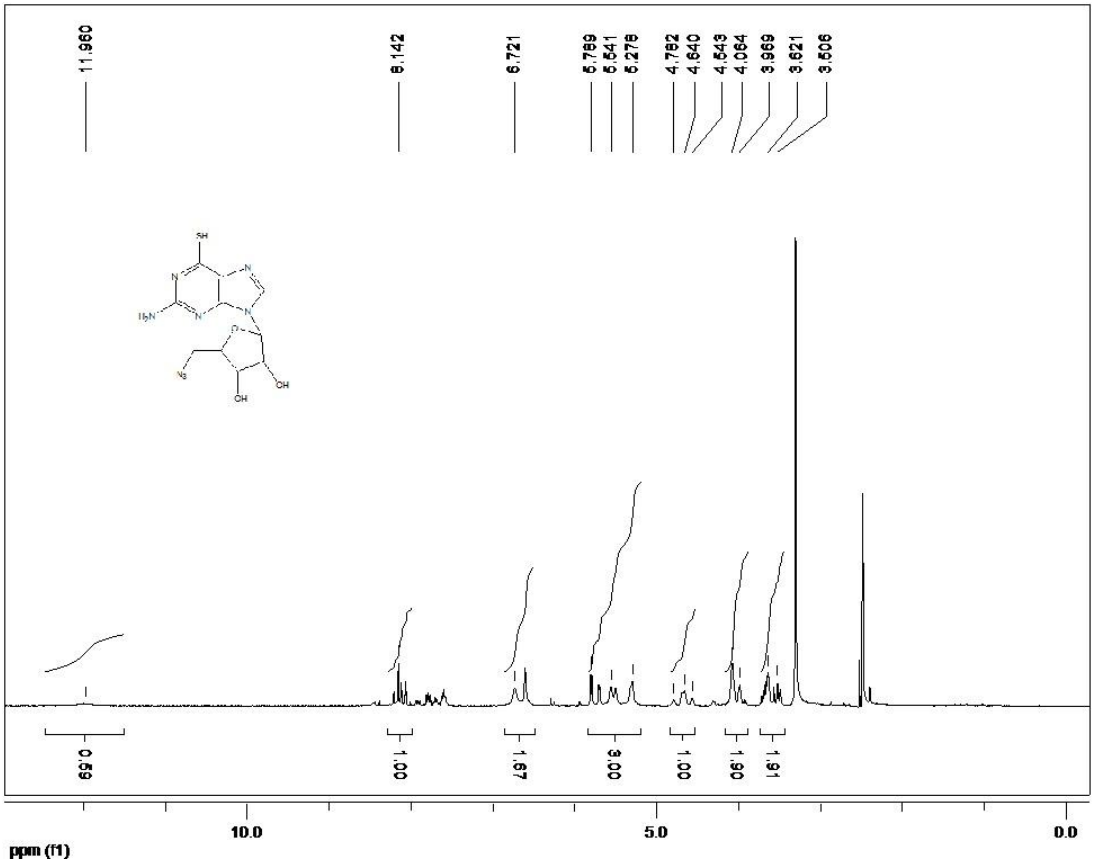




Figure S3. $^{1}$H NMR and FT-IR spectra of 5’-deoxy-5’-iodo-6-thioguaonisine.



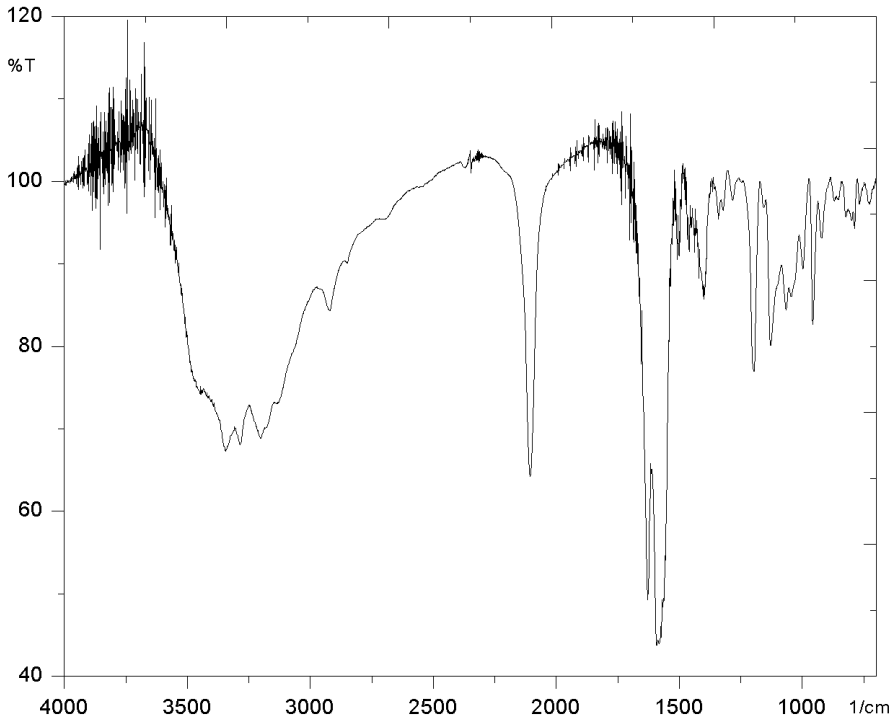
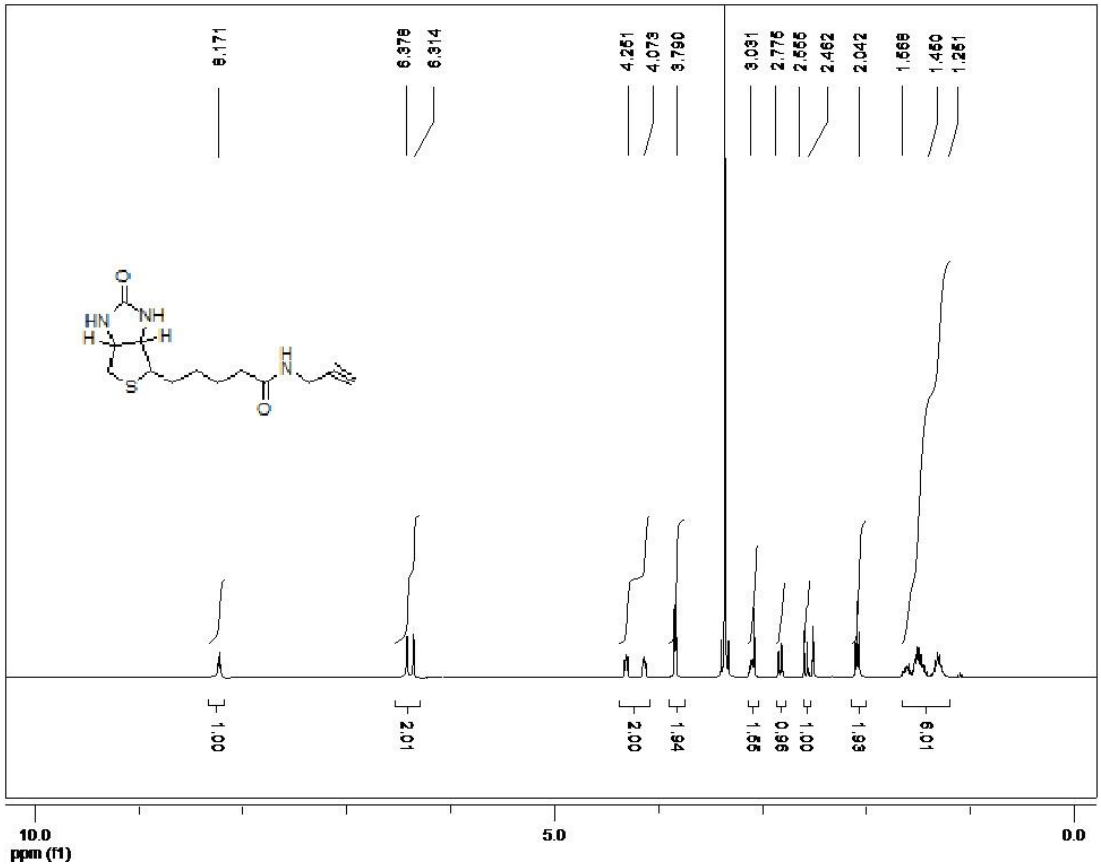




Figure S4. $^{1}$H NMR and FT-IR spectra of 5’-azido-5’-deoxy-6-thioguanosine.



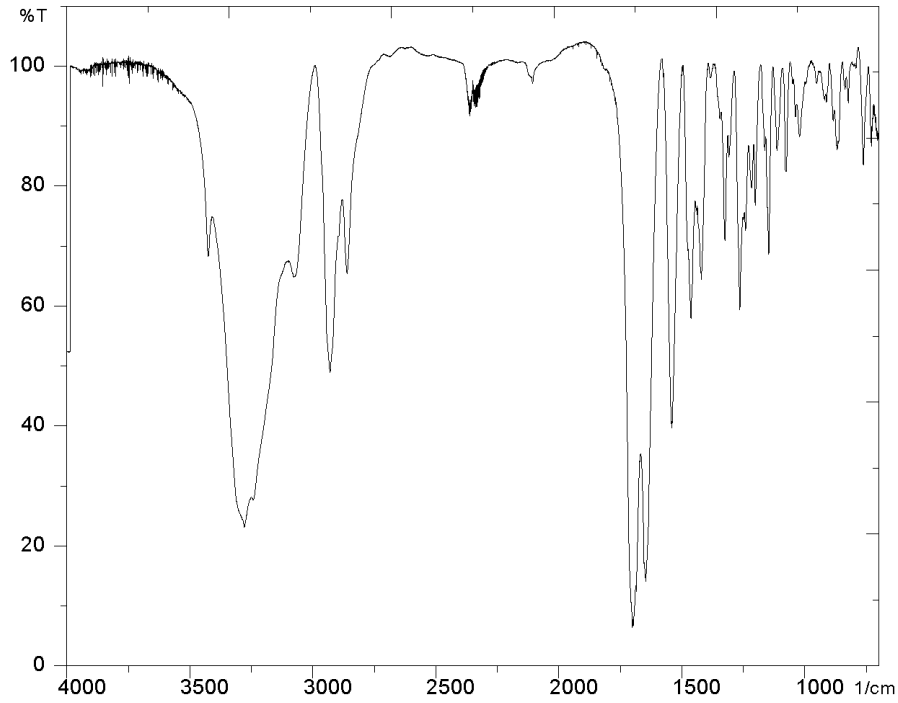




Figure S5. $^{1}$H NMR and FT-IR spectra of biotinylated alkyne.

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Thesis Title: Synthesis of a Chemical Probe for Identification of Ger Receptor Proteins of *Bacillus Anthracis.*

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