A Novel Synthesis Route for Acrylamide-Pendant Phos-tag™ Ligand

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Abstract

Phos-tag™ is a novel functional molecule that binds specifically with phosphorylated ions under neutral or slightly alkaline pH (7.2) to separate the phosphorylated proteins based on degree of phosphorylation. Protein phosphorylation is an important post-translational modification, playing an essential role in modifying the gene communication/ information transmission with living bodies and cellular function. The principal goal of this research is to develop a novel synthetic route for Phos-tag™ acrylamide ligand since this subject has insufficiently been touched on. Acrylamide-pendant phosphate-binding tag (Phos-tag™ ligand) is synthesized from commercially available starting materials via an economical route. Considering the important application on stable separation of proteins or ions with phosphate groups from the counterparts that are not, the synthesis of Phos-tag™ derivatives is desirable and will have a great meaning in scientific research.

Retrosynthetic analysis was primarily used in this research to design a synthesis route which involves multiple reductive amination reactions. The redesigned route avoids the difficult bromination process in previous modified method in the literature. In retrosynthetic analysis, synthesis of N-(2-aminoethyl)-6-(((3-(bis(pyridin-2-ylmethyl)amino)-2-hydroxypropyl)(pyridin-2-ylmethyl)amino)methyl)nicotinamide/ product **4** followed same procedure in literature, while other reaction conditions, substrates and schemes were proposed by the author. The final materials obtained from retrosynthetic analysis were cheap and accessible as illustrated in the rough pricing calculation, addressing the current high-price issues. The direct cost of making 1 mg of Phos-tag™ acrylamide was lowered from 1165 CNY/mg to approximately 2 CNY/mg. Procedures were uncomplicated and had high yield. The products were analyzed and identified by ^{1}H NMR and UP-LC. Phos-tag™ acrylamide was successfully synthesized from a 7-step reaction from 2-pyridine carboxaldehyde and 1,3-diaminopropan-2-ol. In this 7-step synthesis, 2 steps are one-pot reaction, which further shortens the preparation time and achieves a total yield of 42.1%.

During the experiments, major obstacles came from the difficulty in purifying the products and the sensitivity of substrates. Most of intermediates obtained have large polarity, thus during column chromatography, products partially stuck on the silica gel and lowered the yield in small scales experiment in the laboratory. Another striking phenomenon is that product **2** went corrupted quickly, so the experiment scheme 9 was repeated multiple times to prepare the freshly made starting material for the following reaction.

This thesis is arranged in following structure:
Chapter 1 introduces the background, importance, uniqueness and purposes of the research.
Chapter 2 introduces the defects of existing synthetic route and analyzes new ones.
Chapter 3 reports set up and the experiment procedure.
Chapter 4 discusses the effect of small amount production on yield rate and the route design.

Chapter 5 evaluates the benefits of this research outcome and draws a conclusion.

Key Words: Acrylamide-pendant Phos-tag™ ligand; Phosphorylated protein; Retrosynthetic analysis; Reductive amination; ^{1}H NMR.

Statement of Originality

The research process and result of this team are conducted and derived under the guidance of the instructor. Other than the referenced content and the acknowledged sources, this paper does not include any published findings by this group or any other researchers. If there is any inaccuracy, this team is accountable for all liabilities.

Signature:

Date:

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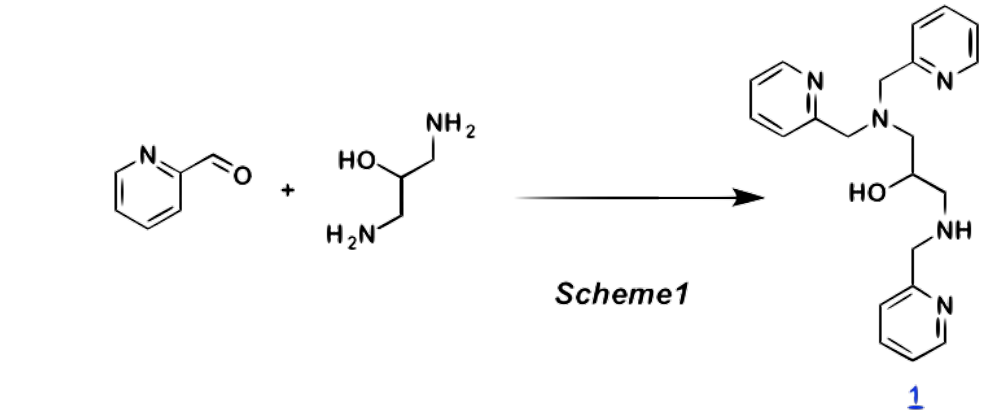
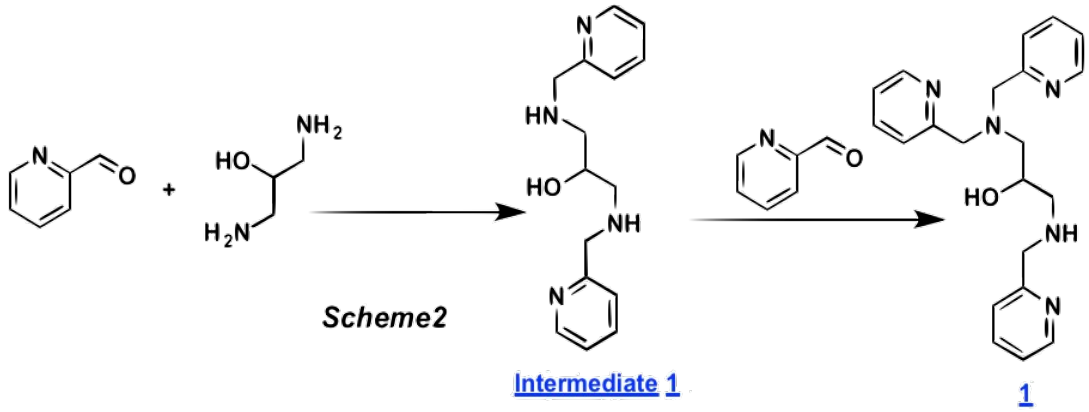


Scheme1

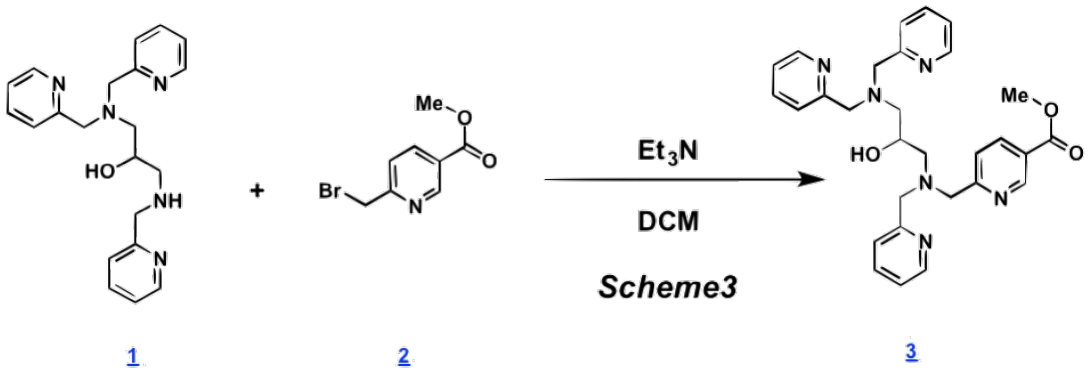
1



Scheme2

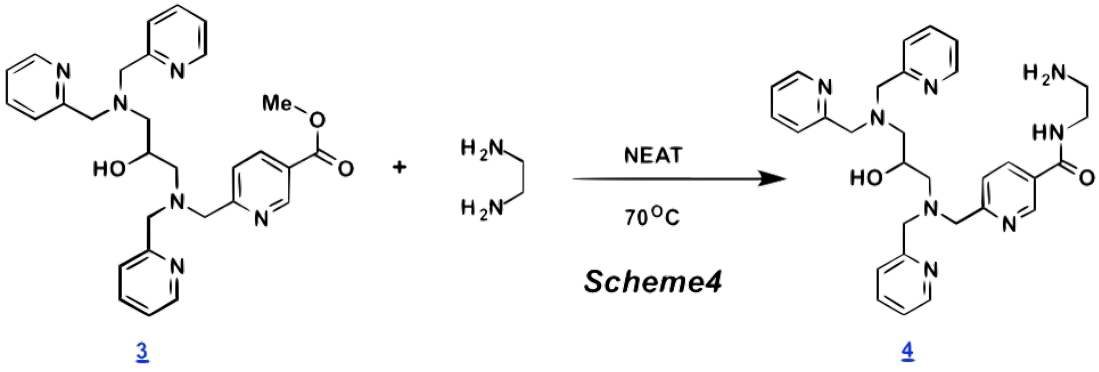
Intermediate 1

1



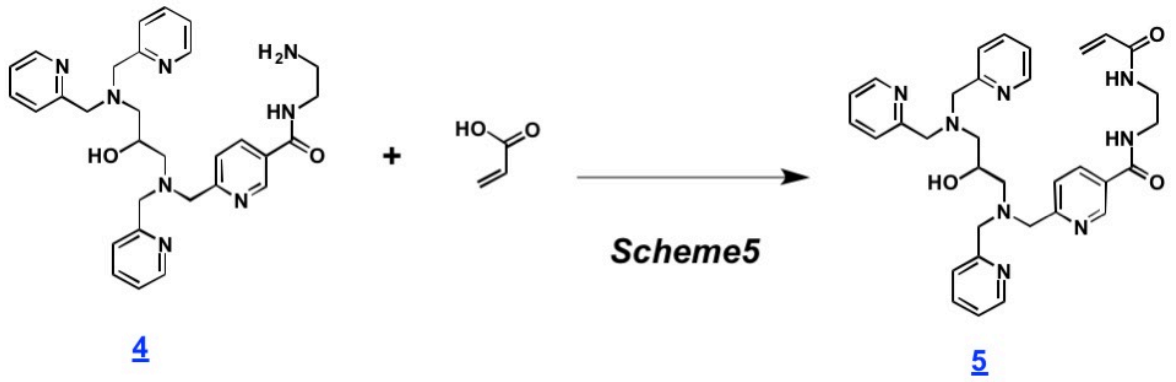
Scheme3

1 2 3



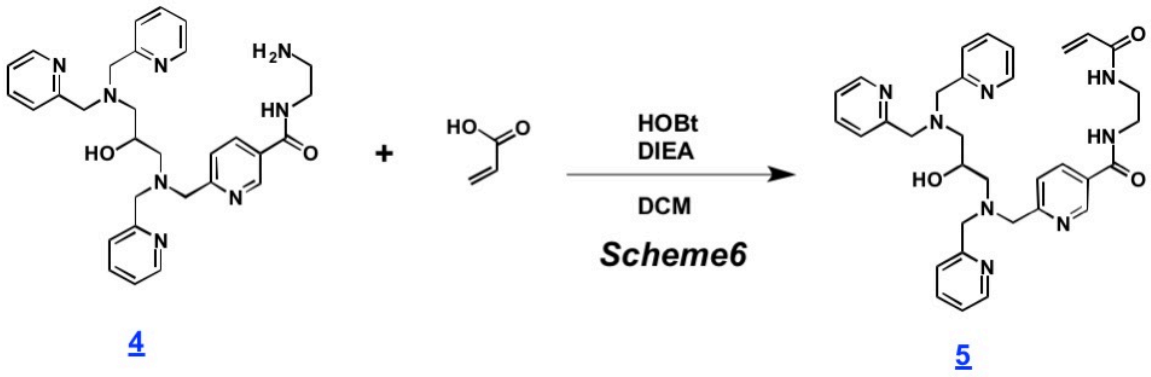
Scheme4

3 4



4 + → 5

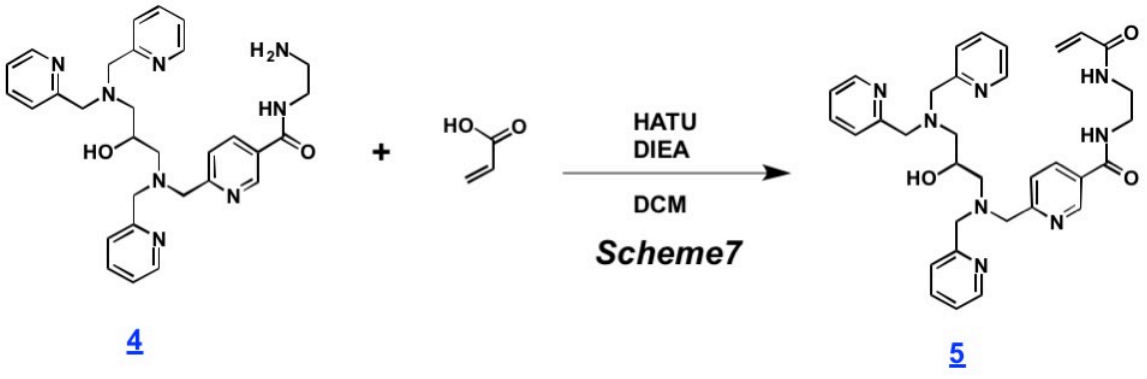
Scheme5



4 + → 5

HOBt
DIEA
DCM

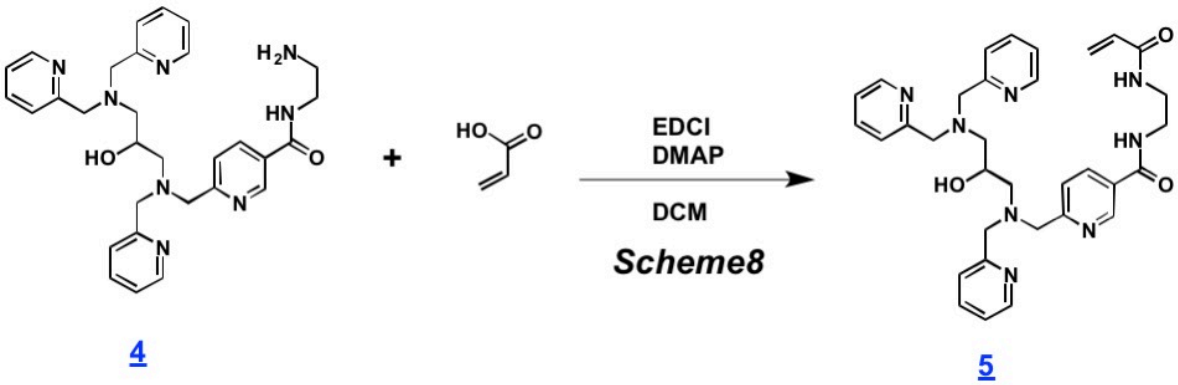
Scheme6



4 + → 5

HATU
DIEA
DCM

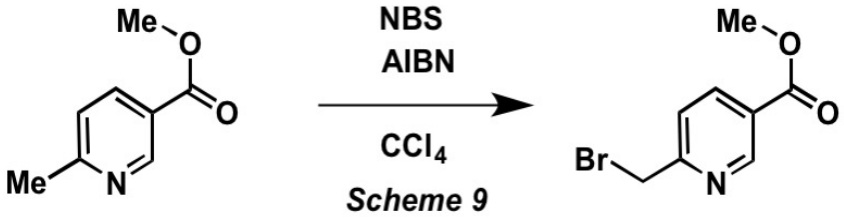
Scheme7



4 + → 5

EDCI
DMAP
DCM

Scheme8



Scheme 9

2

List of Abbreviations

Abbreviation	Name
Phos-tag™	Phosphate-binding tag
CCl_4	Tetrachloromethane
DIEA	N,N-Diisopropylethylamine
NBS	N-Bromosuccinimide
AIBN	Azobisisobutyronitrile
NH_4OH	Ammonia water
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid Hexafluoro phosphate
DMAP	4-Dimethylaminopyridine
EDCI	(3-dimethylaminopropyl)ethyl-carbodiimid monohydrochloride
DCM	Dichloromethane
HOBt	Hydroxybenzotriazole
MeOH	Methanol
PE	Petroleum ether
EA	Ethyl acetate
^{1}H NMR	^{1}H Nuclear Magnetic Resonance
^{13}C NMR	^{13}C Nuclear Magnetic Resonance
UP-LC	Ultra Performance- Liquid Chromatography
LC-MS	Liquid Chromatography- Mass Spectroscopy
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TLC[1]	Thin-layer chromatography
HPTLC	High Performance Thin-layer chromatography plate
PTM	Post-translational modification

Table 1: List of abbreviations of terminologies

[1] The purity of formed compounds was checked by TLC. Spots were either exposed to iodine vapor, Ultra violet, or the $KMnO_4$ solution.

1 INTRODUCTION

1.1 Introduction of Synthetic Chemistry and Retrosynthetic Analysis

Synthetic chemistry is building up complex molecules by uniting simple ones, which usually includes chemical synthesis without biosynthesis, purification/separation steps. The simple substances are always commercial available, such as alkenes, aldehydes, petroleum derivatives, and some natural products. The target of total synthesis is to synthesize natural molecules which may have potential bioactivities. Shorter steps, higher yield are always requested. Hence, modifying or improving the existing synthetic route is desirable and a challenge.

In the area of organic synthesis, retrosynthetic analysis has been widely adopted, especially for the synthesis of complex molecules.It is also known as the most fundamental, essential and simplest method. [2][3] Its evident gist is achieved by converting a desirable target molecule to simpler precuersor without the consideration of starting materials. However, the procedure is commonly repeated until the uncomplicated or commercially available structures are obtained. Various of synthetic routes can be designed and compared, but only the most appropriate and logical one will be followed through. [4] This concept was formulated by E.J. Corey in his book *The Logic of Chemical Synthesis* and won the 1990 Nobel Prize in Chemistry accordingly. [5]

When starting from the complex molecules to work out the optimal starting materials and synthesis route, we need to consider the following three factors:

1) The reaction mechanism must guarantee to be reasonable and appropriate
2) Materials are easily accessible
3) Synthetic route is simple and the yield rate high as possible

1.2 Relevance and Significance of Phos-tag™ Technology and Phos-tag™ Acrylamide Molecule

1.2.1 Significance of Phos-tag™ Technology

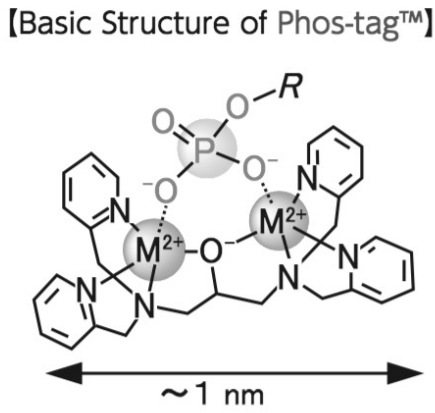


Figure 1: Basic structure of Phos-tag™ proposed by Dr. Eiji from *Waco*. [1]

As the general structure shown in figure 1, Phos-tag™,is a functional molecule discovered several years ago that can bind specifically with phosphorylated ions. It is a novel phosphate-binding tag in a physiological neutral pH (7.2) environment and a ligand which bind with an anionic substituent and particularly inclined to bind with phosphatemonoester group. This Phos-tag™ technology has been proved to be a powerful tool that could be applied in the separation of phosphorylated proteins (Phos-tag™ Acrylamide), purification of Phos-tag™ Agarose, and detection of Phos-tag™ Biotin through Western-Blotting (Figure 2).

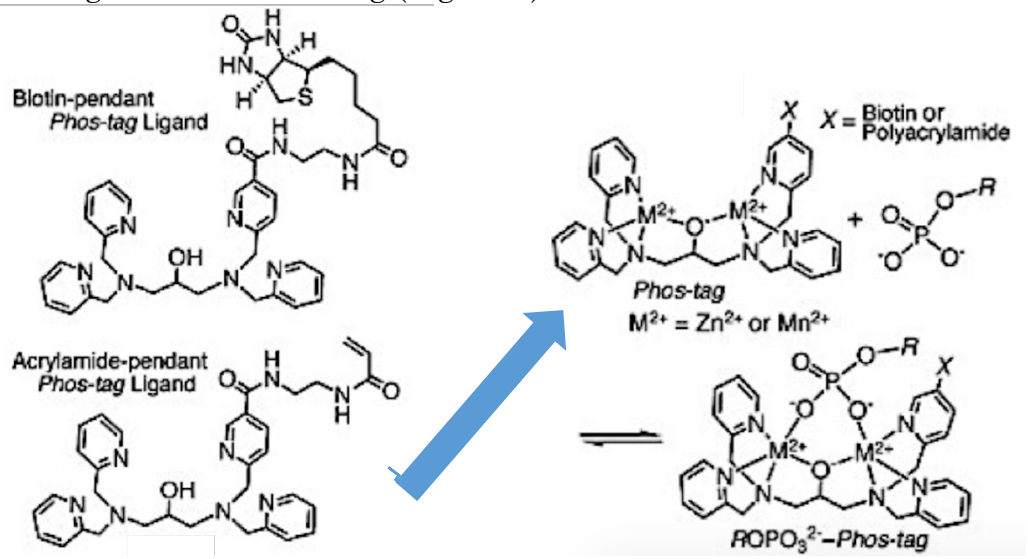


Figure 2: Structures of biotin-pendant and acrylamide-pendant Phos-tag™ ligands. [1][6]

Attributed to the high binding affinity and the specificity of the phosphorylated proteins, Phos-tag™ technology can be applied widely in some innovative methods for separation, purification, detection and analysis for protein phosphorylation to aid the investigation of regulatory pathways. It behaves as a metal chelate with space in the middle of its structure that can accommodate a phosphate group, making the Phos-tag™ bind tightly with the phosphate group. [6]

To be noted, the traditional method has some disadvantages. For example, as one of the conventional methods, radioactive isotope approach involves using radioisotope ^{32}P, which needs radiation equipments and has hazardous waste. In addition, the method has limitation in that specimens must be amenable to labeling. [10]

To test on the phosphorylation, phosphorylated antibody is typically employed. However, some new technologies such as Phos-tag™ have been developed and utilized instead of the traditional ones in recent years. Conventional methods include enzyme immunoassay (ELISA) and radioactive isotope methods. ELISA relies on antibodies that have amino specificity and its utility depends on timing factor in using immunoreaction of animals. [7] As for radioactive isotope methods, it is used as an agent for capturing substance that has a phosphatemonoester group in phosphorylation analysis techniques. Discovered by Dr. Eiji Kinoshita from Kinoshita University, Phos-tag™ labels anions, synthetic or naturally found, are especially effective for compounds that have phosphate group (preferably a phosphomonoester dianion). [8]

Phos-tag™ technology has greatly improved the instability of previous testing methods, and became, by so far, the most stable technique to test for phosphorylated compounds.

1.2.2 Relevance of Phos-tag™ Acrylamide Molecule

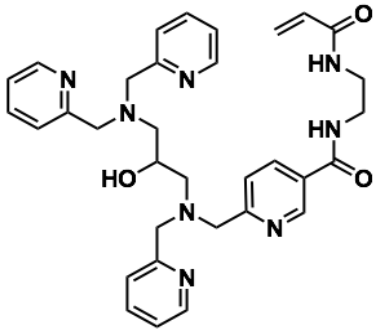


Figure 3: Phos-tag™ Acrylamide molecular structure[1]

MW: 594.72

Phos-tag™ Acrylamide, as illustrated in figure 3, is an acrylamide-pendant Phos-tag™ molecule. In making SDS-PAGE gels[2], addition of Phos-tag™ Acrylamide and $MnCl_2$ in Resolving Gel makes phosphorylated proteins bind to divalent metal ions in Phos-tag™ during the migration process. This process lowers the speed of migration and separates phosphorylated proteins from non-phosphorylated ones. [6] The considerable separation effect is illustrated below.

[2] Acrylamide-pendant Phos-tag™ ligand was an oily product with high viscosity; it should be completely dissolved by intensive pipetting.

The detection became possible because of mobility shift of phosphorylated proteins, a result of reversible phosphate trapping by the immobilized Phos-tag™ molecules in SDS-PAGE gel prepared. As shown in figure 4, phosphorylated proteins have different extents of electrophoretic migration compared with their non-phosphorylated counterparts. [10] Based on the degree of phosphorylation, there will be visible and distinct bands.

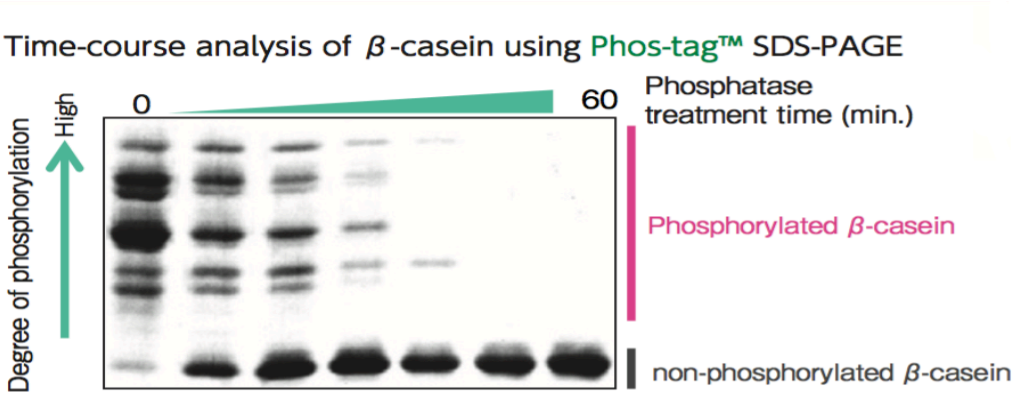


Figure 4: Phos-tag™ SDS-PAGE separation of phosphorylated protein from *Waco*.[1]

Mechanism is as following:

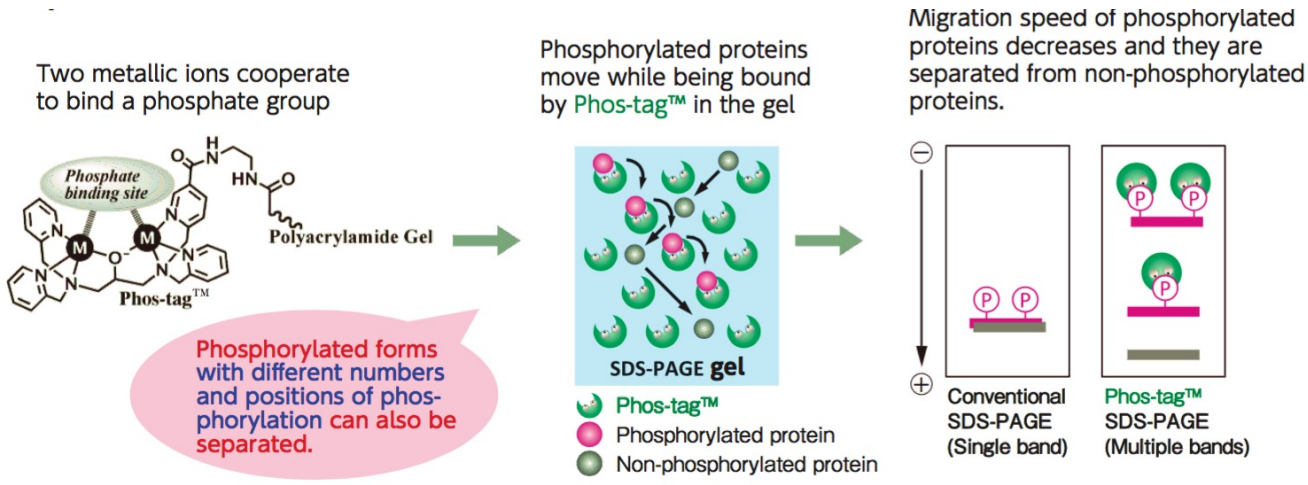


Figure 5: Phos-tag™ binding mechanism illustration from *Waco* [1]

The amino acid residue is phosphorylated by a protein kinase through adding a covalently bonded phosphate group, thus altering the structural configuration of the protein. As a result, activation, deactivation or the modification of its function occurs. This process is thus called protein phosphorylation. [10]

"Isolation and identification of phosphorylated macromolecules are essential for the deconvolution of most biological regulatory networks." [21] Protein phosphorylation is a type of modification that is a flexible mechanism for cells to respond to external

signals and environmental conditions. 30% of cellular proteins are estimated to be phosphorylated at any moment. [11] During many signal transduction pathways and myriad cellular process, phosphor-regulation is indispensable since the process includes, for example, cytoskeletal arrangement, metabolism, gene transcription, protein stability and apoptosis. [1] It enables us to acquire the information transmitted with living bodies via the phosphorylated proteins. Thus, phosphorylated protein plays an essential role in the modification of gene and cell functioning. [12] [13] [14]

1.3 Reductive Amination Reaction

Reductive amination reaction, also known as Borch reductive amination, is a branch of amination reaction that converts the carbonyl group (most often an aldehyde or a ketone) into an amine through an intermediate, imine, with ammonia equivalents or primary or secondary amines. Hydride reducing agents are used to make alkylated amines. Also, it is believed to be one of the most important ways to prepare amines, since the majority of amines in the pharmaceutical industry by following this reaction. [15]

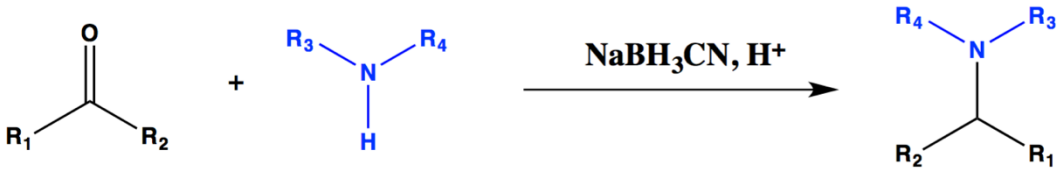
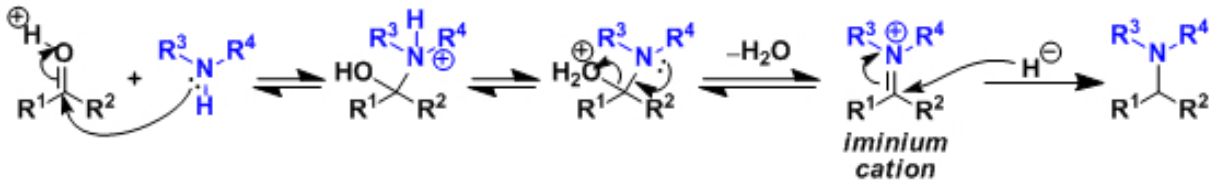


Figure 6: Borch reductive amination reaction

The reaction has high generality and sodium cyanoborohydride is used as the reducing agent. The benefit of this reducing agent is that it is a comparatively weak reductant which works under mildly acidic conditions. [16]

Monitoring pH is crucial to the clean reductive amination. Iminium cation needs pH of 6~7 when it undergoes reduction, while ketones and aldehydes usually need more acidic environment: pH of 3~4. $NaBH_3CN$ reacts well in this range of pH values without causing the occurrence of any unnecessary side reactions. [17]



Mechanism 1: Borch reductive amination

1.3.1 Mechanism 1: Borch Reductive Amination

Carbonyl group reacts with an amine to form a secondary amine, which is also called Schiff base. Then use sodium borohydride or sodium cyanoborohydride to reduce it to an amine. The reaction is carried out in a mildly acidic environment since it amplifies the electrophilicity of the protonated carbonyl group to stimulate the reaction and simultaneously avoid the decrease of nucleophilicity caused by over protonation of amine. Sodium cyanoborohydride is a better agent than sodium borohydride since cyanide has a positive inductive effect which diminishes the reactivity of the boron-hydrogen bond. As a result, sodium cyanoborohydride can only selectively reduce Schiff base, but not the carbonyl group of aldehyde and ketone. Thus, the side reactions won't take place. [16] [17]

1.4 Spectra Analysis and Research Planning

1.4.1 Spectra Analysis

^{1}H NMR, and UP-LC are used to identify the structure of products. [3]The fundamental principle of ^{1}H NMR is that spinning charge generates a magnetic field, and later chemical shift allows to identify the number of H-1 atoms. [18] [19] Thus, the functional groups presented could be deduced. These three structure identification tools are significant in this research. Also, when the target products are obtained, its binding effect (Phos-tag™ Acrylamide) with phosphorylated protein can be tested on organisms.

1.4.2 Research Plans

To investigate on this project, I planned on following steps:

1) Find textbooks on topics of introduction of organic chemistry and know basic reactions as well as their mechanisms. Search the organic synthesis procedures and useful handouts online. (I found the novice guide from blog *Not Voodoo, University of Rochester* and the PowerPoint from SIOC particularly useful.) Then propose a first draft of research topic and find its viability. My first research proposal on nicotine was forced to halt for now due to the restriction of SM. Continue this process and ask the advice from experienced researchers at lab and mentors from school. Finalize a topic and hypothesis to be implemented.

2) Search the literature for each keyword and read their synthesis methodology and conditions. Study the traditional synthesis route of Phos-tag™ series and evaluate its defects and potential ways to circumvent the difficulty in reaction. Then analyze appropriate retrosynthetic routes of the compound suitable for this specific molecule using the database and design an initial synthetic route.

3) Find relevant literatures regarding the specific name reaction mechanisms such as Borch reductive amination reaction. Study on its application in

[3] $CDCl_3$ with silver foil was used as an internal reference for ^{1}H NMR measurement.

synthesizing different molecules. Review and modify the first draft of reaction routes with a mentor, and formulate a more efficient synthetic route.

4) Conduct experiments in the laboratory, synthesize and optimize Phos-tag™ acrylamide molecule route as the experiments success and fail. Evaluate the differences between the previous synthesis approach and propose a mechanism of a novel synthetic route in this research.

5) Learn how to use various spectra analysis techniques and practice analyzing skills to confirm the structure of products. Purify the TM in order to test for its biological activity on proteins in biology labs. (not actualized due to time limit)

1.5 The Purpose of This Research

Phos-tag™ technology is by far one of the most stable ways to separate the phosphorylated proteins and non-phosphorylated proteins in SDS-PAGE gel. Its high demand and wide application make the price of it crucial be affordable. From an economics point of view, lower price and higher efficiency save resources. Thus, this research project aims to lower the cost of it from its commercial price found on Waco chemistry, that is, 2 mg of 336 USD, and the price proposed by literature as 60 USD/ g, by successfully synthesizing the acrylamide-pendant Phos-tag™ ligand with a higher yield rate than previously proposed. In the present investigation, it is synthesized by reacting product **2** with ethylenediamine. (Initial researchers/ Dr. Eiji group didn't propose a complete/ total synthesis of acrylamide- pendant Phos-tag™ ligand from cheapest and most available products; they bought the substrate, product **3**, from NARD Institute) [6]

The Phos-tag™ derivatives, which can be further synthesized by this method in an efficient way, have important uses as well. For example, the Phos-tag™ Biotin functions as detecting the substitute for the antibody of anti-phosphor used in Western blot. Phos-tag™ Agarose purifies the phosphorylated proteins via column chromatography.

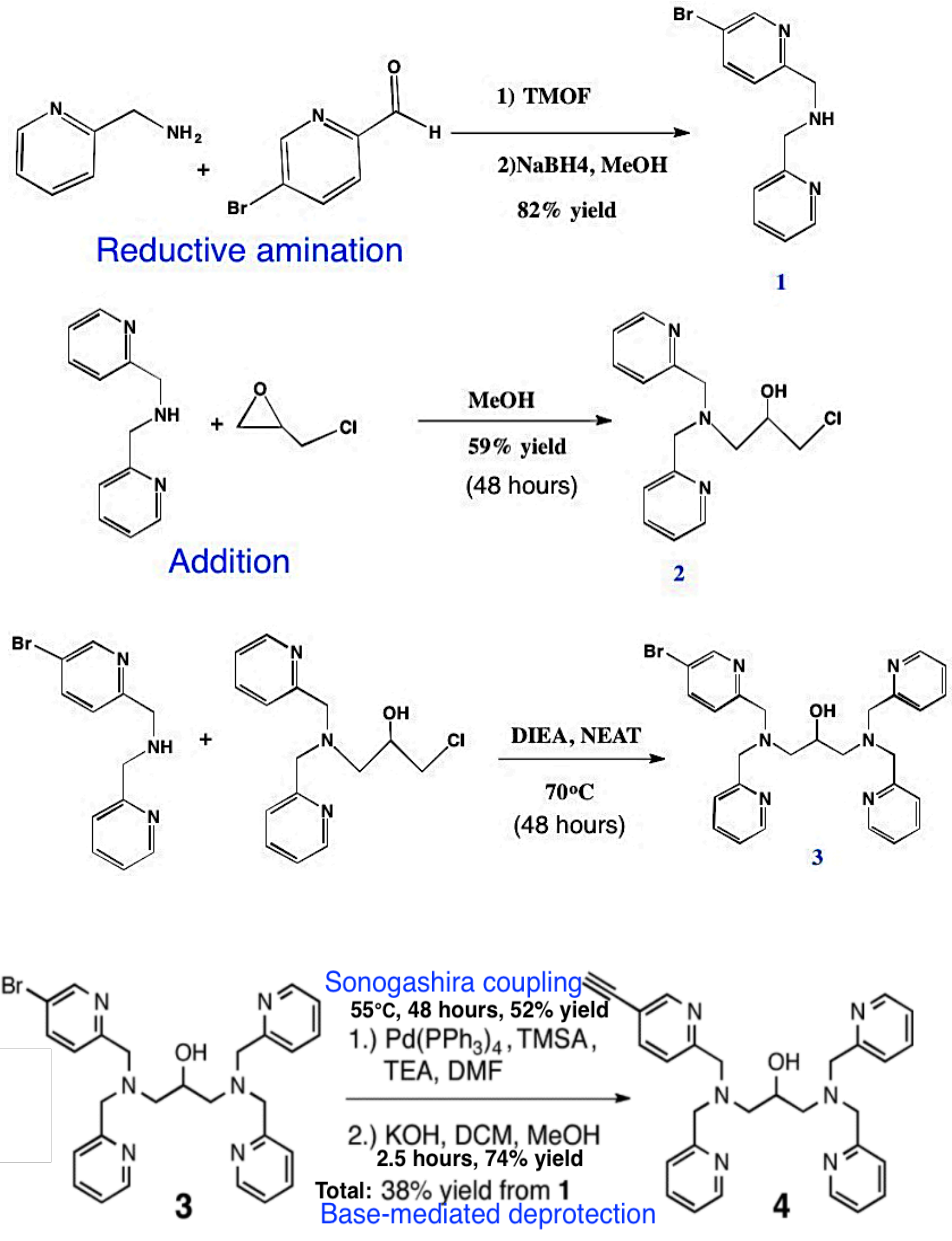
It has numerous application: NMR could identify the unknown donors bound with Phos-tag™; Chromatography could detect the mass changes of bound molecules; A specific number of groups could be identified. [20]

In addition, the laboratory environment played an role in determining this research topic. This research was performed during the summer program at National Institute of Biological Science (NIBS) Chemistry center, an organic synthesis auxiliary center that prepares demanded organic molecules for biology-related labs. The demand of costly Phos-tag™ from the bio-labs promoted me to investigate this topic with the aim of developing economic synthesis route.

2 RETROSYNTHETIC ANALYSIS

2.1 Previous Synthesis Route towards Phos-tag™ Acrylamide and Its Limitations

In this research, the previous synthetic route was proposed by Nicolas P. Tilmans at el. from Harbury lab, Stanford University. This is a modified route based on the 2004 literature of Koike, Tohru at el. and their U.S. patent. [36]



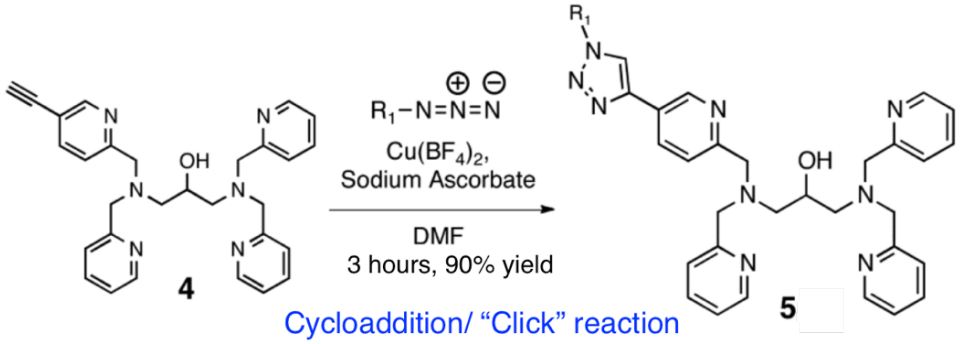


Figure 7: The previous synthesis route of Phos-tag™ [20] [21]

Product **3** could be obtained first, then after coupling/deprotection reactions the Phos-tag™ derivative **5** could be produced. The final product **5** obtained is a triazole-pendant Phos-tag™, which, I proposed has the same binding effect as the acrylamide-pendant Phos-tag™ does. However, this method is complicated in the synthetic steps, gives the target compound **3** in a low yield, and generates a mixture of products that are hard to separate. Over 3 steps of reaction, the yield of compound **3** is 31%, which means the third step of reaction has 64.1% of yield. As shown, the reaction is very time-consuming: multiple steps are 48 hours long, and has great difficulty in the post-modification of halogen and deviate from the topic of this research, that is, acrylamide-pendant Phos-tag™ ligand. [21]

However, the research from previous step did estimate a cost of $60/g for triazole-pendant Phos-tag™ ligand, which is significantly lower compared with the commercial price from Waco Chemistry Industry. [1] I aim to achieve a similar cost on synthesizing acrylamide-pendant Phos-tag™ ligand. Thus, I did an alternate retrosynthetic analysis on target.

2.2 Retrosynthetic Analysis

I found the '*Introduction to Organic Chemistry*' by Qiyi Xing very helpful in finding pertinent knowledge, especially chapter 24: basics of organic synthesis. The retrosynthetic analysis for this target molecule is not very challenging since the starting materials and functional groups involved are regularly repeated and uncomplicated.

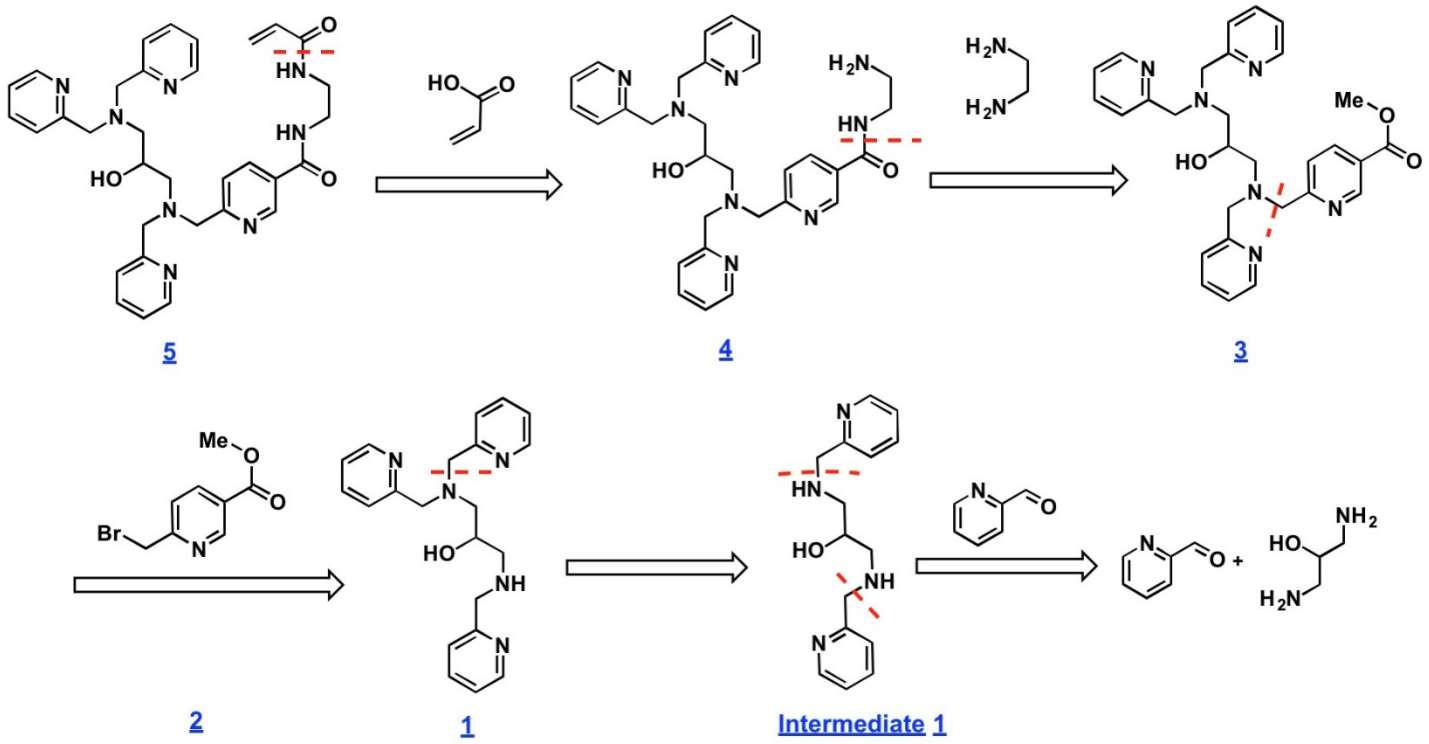


Figure 8: Retrosynthetic analysis scheme

The initial molecule that the synthesis starts with is the biotin-pendant Phos-tag™ ligand.

1) The product **5** can be formed by nitrogen atom undergoing amide formation reaction. Thus, the acrylic acid was chosen, and the essence is dehydration reaction and acrylamide condensation. The coupling reagents should be carefully chosen and experimented on later. In this step, I adopted the same substrates as the Dr. Eiji group did, but proceeded via different coupling reagents to obtain maximal yield. I worries about the yield in this step since hydroxyl group and carboxylic acid reacts under esterification reaction, producing by-products. However, yield won't be lowered as much from esterification reaction since it requires heat under reflux and acid catalyst. I chose a mild reaction condition is in this step, so few esters should be formed as by-product.

2) Then I decided to use aminolysis reaction to break down a smaller molecule first and then obtain the product from the last step. The monoester group is transformed into an amide group by the stronger nucleophile: primary or secondary amine. Thus, the substrate used and precursor molecule could both be identified.

3) Use the nucleophilic substitution to substitute the tertiary amine group by halogen, the pyridine and its derivative could attach to it via the substitution

reaction. The reflux and heat should be used. However, the substrate must be made in the laboratory.

4) The structure with three pyridine groups (product **1**) is identified from the last step of retrosynthesis. Therefore, Nitrogen atom attached to two pyridine groups can be reduced from nitrile intermediate to form this structure.

5) Then, use pyridine to react with the 'main part' that is, 1,3-diaminopropan-2-ol, to construct the desirable structure with three pyridine groups. This is made possible by the reduction of Schiff base to form structure from the last step of the analysis. The reducing agent could be NaBH4 or other weaker ones. Thus, the simplest starting materials are obtained. (The identification of final starting materials was inspired by Reaxys) [22]

Nonetheless, this retrosynthetic analysis has three potential challenges:

1) Whether reductive amination can produce three-pyridine derivatives is uncertain.
2) Can the aminolysis happen as expected?
3) The self-polymerization of acrylic acid is unpredictable.

These questions above will be illustrated during the experiment procedure.

3 PHOS-TAG™ SUBSTRATE PREPARATION

3.1 Apparatus and Experimental Setup

3.1.1 Apparatus Used

NMR (Varian) 400MHz
Rotary evaporator (BUCHI: Rotavapor R-3)
Magnetic mixer (IKA)
Ultra-Performance- Liquid Chromatography (Waters)
Electric balance to 0.1 mg (Daojin)
Glassware (Synthware)[4]
LC-MS (Agilent)
Vacuum pump (Leybold)
Ultrasound cleaner (Yuhua)

The following spectroscopic techniques were used to identify reactants and products:

NMR (Varian)
LC-MS (Agilent)
Ultra-Performance- Liquid Chromatography (Waters)

3.1.2 Experimental Setup

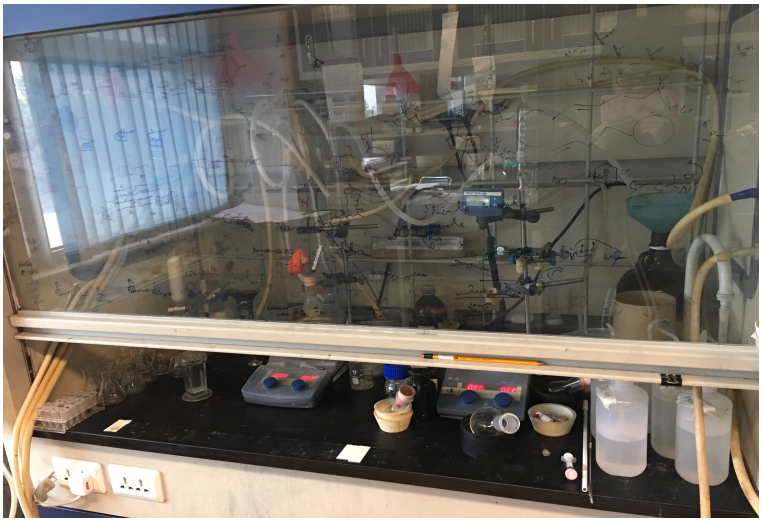


Figure 9: Fume hood in the lab

[4] All glassware used are emerged in the base tank for 1 hour and then cleaned with deionized and distilled water and dried in the oven at 120℃ overnight.

All experiments were conducted in the well-ventilated laboratory environment.

Figure 10: Rotary evaporator setup in lab

The rotavapor used differ in their pumps. There is diaphragm pump, water pump, and oil pump available in the laboratory. Based on the boiling point of different solvents, pumps with different vacuum pressure are used.

3.2 Starting Materials[5]

Agents	Molecular formula	Purity	Company of purchase
2-Pyridine carboxaldehyde	C_6H_5NO	98%	Energy Chemical
1,3-diaminopropan-2-ol	$C_3H_{10}N_2O$	98%	Energy Chemical
Sodium borohydride	$NaBH_4$	98%	Energy Chemical
Sodium cyanoborohydride	$NaBH_3CN$	95%	Shao-Yuan
Methyl 6-methylnicotinate	$C_8H_9NO_2$	98%	Energy Chemical
Ethanoic acid	CH_3COOH	AR	Beijing Tong Guang Chemicals
N,N-diisopropylethylamine	$[(CH_3)_2CH]_2NC_2H_5$	99%	Energy Chemical
N-bromosuccinimide	$C_4H_4BrNO_2$	98%	Energy Chemical
Azobisisobutyronitrile	$[(CH_3)_2C(CN)]_2N_2$	98%	Adamas
Deionized water	H_2O	N.A	N.A

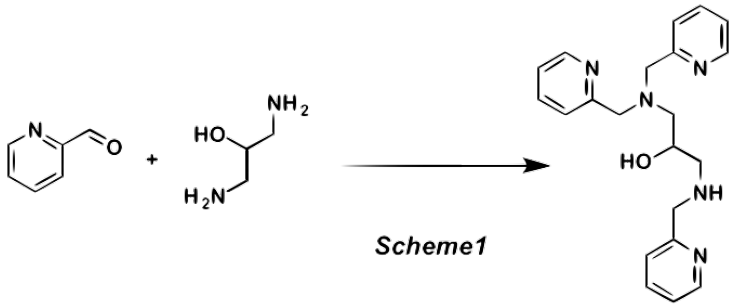
[5] All reagents, unless specifically stated, were used in the experiment as they were purchased from manufacturers without further purification and were of the best commercial quality.

Ammonia water	NH_4OH	25%	Beijing Tong Guang Chemicals
Argon gas	Ar	N.A	N.A
Ethanol	C_2H_5OH	AR	Beijing Tong Guang Chemicals
1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluoro phosphate	$C_{10}H_{15}F_6N_6OP$	99%	Energy Chemical
4-dimethylaminopyridine	$C_7H_{10}N_2$	98%	Energy Chemical
1-(3-dimethylaminopropyl)ethyl-carbodiimid monohydrochloride	$C_8H_{17}N_3 \cdot HCl$	99%	Energy Chemical
Dichloromethane	CH_2Cl_2	AR	Beijing Tong Guang Chemicals
Anahydrous sodium sulfate	Na_2SO_4	N.A	N.A
Saturated ammonium chloride	NH_4Cl	N.A	N.A
Ethylenediamine	$NH_2CH_2CH_2NH_2$	99%	Aladdin
Silica gel	N.A	N.A	N.A
1-Hydroxybenzotriazole	$C_6H_5N_3O$	99%	Energy Chemical
Methanol	CH_3OH	AR	Beijing Tong Guang Chemicals
Triethylamine	$(C_2H_5)_3N$	99.50%	J & K Chemicals
Tetrachloromethane	CCl_4	AR	Beijing Tong Guang Chemicals
Petroleum ether	C_6H_{14}	AR	Beijing Tong Guang Chemicals
Ethyl acetate	$C_4H_8O_2$	AR	Beijing Tong Guang Chemicals
Acrylic acid	$C_3H_4O_2$	99%	Aladdin

Table 2: List of reagents and chemicals used

3.3 Synthesis of Product 1

3.3.1 First Experiment on Product 1



Scheme1

1

Analysis: According to the properties of pyridines, organic solvent, specifically, methanol, is chosen to dissolve the pyridine. This is an aldehyde/ ketone condensation reaction with amine to get the imines. Then, in order to get amine from Schiff base, reducing agents are used. To start with, there is an important assumption made: Product **1** could be made by controlling the number of reductive amination reaction, obtaining only the product which undergoes three times of reductive amination. Thus, this primary assumption led to the start of this experiment. The reaction is shown in Scheme 1.

Synthesis of 1-(bis(pyridin-2-ylmethyl)amino)-3-((pyridin-2-ylmethyl) amino) propan-2-ol (1)

To a solution of methanol (10 mL) in a 25 mL three-necked bottle was added 2-pyridine carboxaldehyde (320 mg, 3.0 eq) and 1,3-diaminopropan-2-ol (100 mg, 1.0 eq). The mixture was stirred for 1 hour at room temperature. Thin-layer chromatography (TLC) revealed the formation of the Schiff base. Then sodium triacetoxyborohydride (550 mg, 9.0 eq) was added dropwise into the mixture in the ice bath under argon protection[6]. Then the ice bath was removed and the reaction mixture was kept stirring for 5 h at room temperature. Unfortunately, no desired product formed according to analysis of LC-MS and NMR spectra. UPLC showed showed no peak with a molecular weight of product **1** and ^{1}H NMR integration gave the wrong ratio.

Sodium triacetoxyborohydride was chosen as the boronizing reductive reagent because it is a relatively weaker one: A strong one might lead to four reductive amination reactions. NMR showed the majority of products were formed from a single reductive amination only. Thus the strength of the boronizing reductive reagent was adjusted, and another tryout was carried on.

3.3.2 Second Experiment on Product 1

[6] All glassware used to air sensitive experiments were oven-dried at least one hour before use.

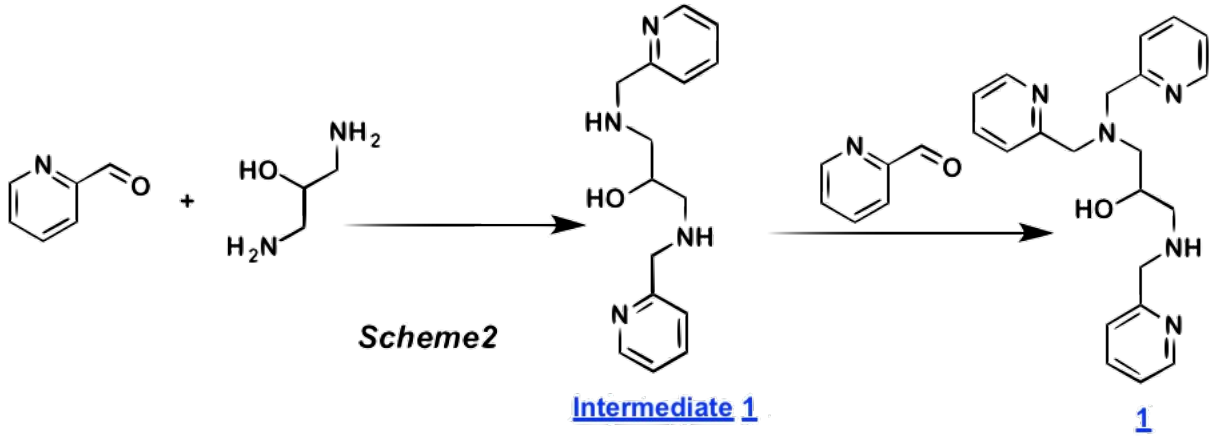
Synthesis of 1-(bis(pyridin-2-ylmethyl)amino)-3-((pyridin-2-ylmethyl) amino propan-2-ol (1)

To a solution of methanol (10 mL) in a 25 mL three-necked bottle was added the little magnet, 2-pyridine carboxaldehyde (320 mg, 3.0 eq) and 1,3-diaminopropan-2-ol (100 mg, 1.0 eq). The mixture was stirred for 1 hour at room temperature. TLC showed the formation of Schiff base, and then sodium borohydride (350 mg, 9.0 eq) was added into the mixture in the ice bath and under an argon atmosphere. The mixture was stirred at room temperature for five hours. LC-MS showed no trait of product **1**.

However, the majority of the intermediates underwent two reductive amination reaction. By comparing the results of these two experiments, I decided to modify the reaction conditions by using a stronger boronizing agent. [23]

3.3.3 Third Experiment on Product 1 and Modification of Reaction Scheme

Analysis: Since two times of experiments failed to obtain the target material, the phenomenon demonstrated the lack of reactivity of secondary amine could be the reason why the third reductive amination reaction didn't occur. The steric interference of pyridine made it imperative to obtain intermediate **1** before product **1**, and further let it undergo amination reaction. As the literature suggested, sodium cyanoborohydride is more effective in reducing secondary amine to form Schiff base. [16] [17] Thus, another experiment was designed to investigate whether product **1** would form.



Scheme2

Synthesis of 1,3-bis((pyridin-2-ylmethyl)amino)propan-2-ol (intermediate1)

The reaction was as shown in Scheme 2. To a solution of methanol (50 mL) in a 250 mL three-necked bottle was added 2-pyridine carboxaldehyde (3.1 g, 2.0 eq) followed by 1,3-diaminopropan-2-ol (1.37 g, 1.0 eq). Methanol must be added before the addition of all substrates since this was a NEAT reaction. The mixture was stirred at room temperature until TLC showed the formation of Schiff base. The eluent used

was DCM: MeOH=10:1 with one drop of ammonia water. After 1 hour, more 2-pyridine carboxaldehyde (775 mg, 0.5 eq) was added to ensure the complete reaction.

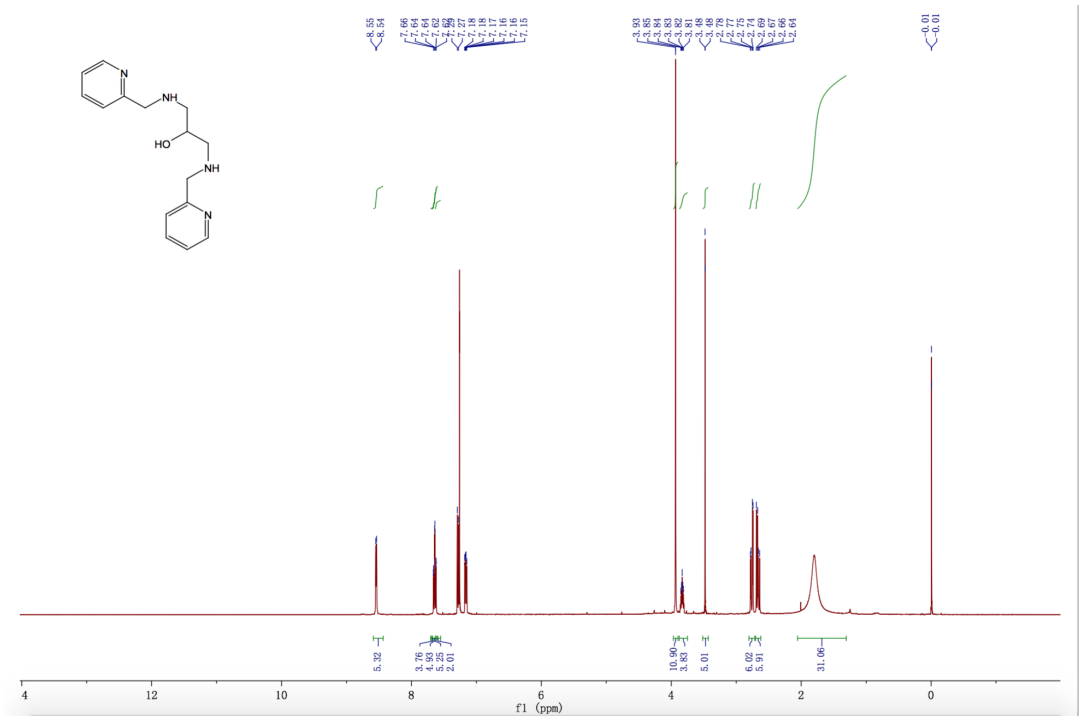
Then sodium borohydride (1.74 g, 3.0 eq) was added dropwise to the mixture in the ice bath and under an argon atmosphere. The mixture was stirred for 2 hours at room temperature. TLC showed positive results of the product, but let it react for another half hour. UP-LC showed peaks of *m/z:* 273 and 272.

Then sodium borohydride (580 mg, 1.0 eq) was added to the reaction mixture. Deionized water was added to quench the reaction in the ice bath. The resulting solution was extracted with DCM, and separated organic layer was dried over anhydrous sodium sulfate. After removal of the solvent in vacuum, the crude product was further purified by column chromatography (eluent used is DCM: MeOH= 5:1) to afford intermediate **1** of light yellow color. (3.90 g, 94 %).

^{1}H NMR ($CDCl_3$, 298 K): δ= 8.55 (d, 2H, ArH), 7.66 (m, 2H, ArH), 7.29 (d, 2H, ArH), 7.18 (m, 2H, ArH), 3.93 (s, 4H, $ArCH_2$), 3.85 (m, 1H, CH), 3.48 (s, 1H, OH), 2.78-2.64 (m, 4H, CH_2)

Figure 11: FUCHI Rotavapor with diaphragm pump

^{1}H NMR gave an affirmative result on the structure of target product made.



^{1}H NMR spectrum of compound intermediate 1

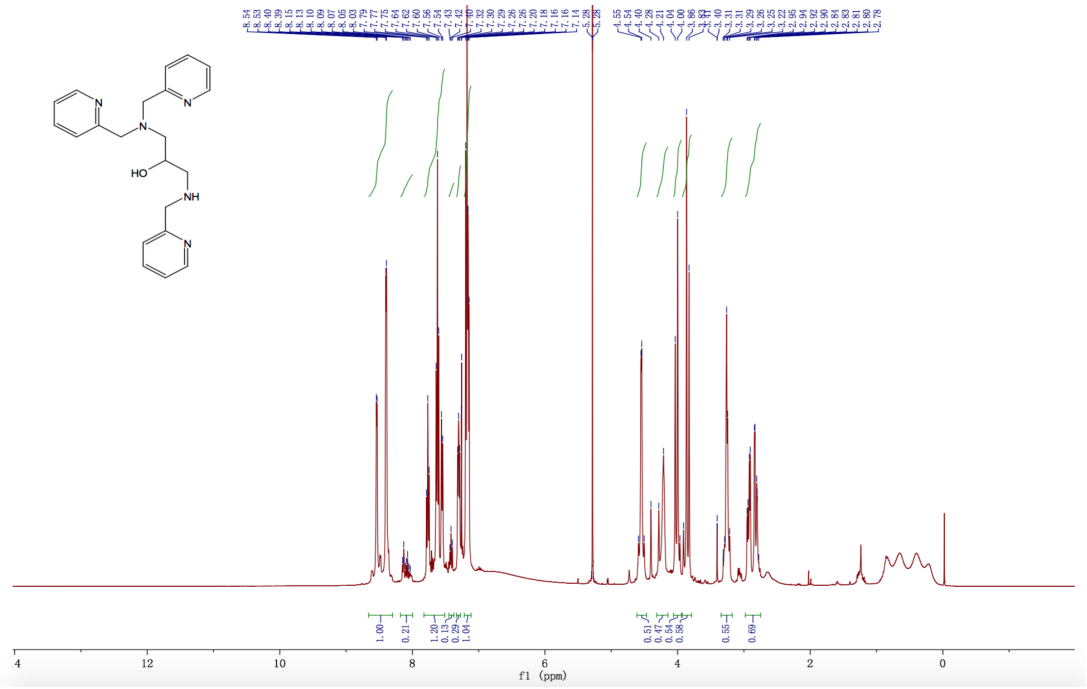
Synthesis From intermediate 1 to Product 1

To a solution of methanol (20 mL) in the bottle containing **1** (intermediate made from previous reaction scheme), 2-pyridine carboxaldehyde (900 mg, 1.1 eq) were added. Then the mixture was stirred at room temperature under argon protection overnight. [23] After 14 hours, sodium cyanoborohydride (1.44 g, 3.0 eq) was added into the reaction mixture while the flask was placed in the ice bath and under argon protection. Removed the ice bath once the addition of reductant was complete. Reacting mixture was stirred for 7 hours at room temperature and TLC with DCM: MeOH: NH_4OH=5:1:0.1 as eluent showed the target product **1**, the polarity of which was slightly smaller than that of the starting materials.

Methanol was used to quench the unreacted sodium cyanoborohydride. The resulting solution was partitioned between DCM and water, and the organic layer was dried over anhydrous sodium sulfate. After removal of the solvent in vacuum, the crude product was further purified by column chromatography, the eluent used was DCM: MeOH: NH_4OH=20:1:0.1 (2 mL of 1.00 mol/L ammonia water). Product **1** was obtained as pale yellow solid. (1.89 g, 72%)

It is worth noticing that TLC showed few of the central nitrogen atoms were attached to four pyridines. However, column chromatography separated the target product 1, which had three pyridines pendants. Also, since the intermediate corrupted rather easily, some impurity was disposed and yield sacrificed.

NMR showed the formation of the desirable product.



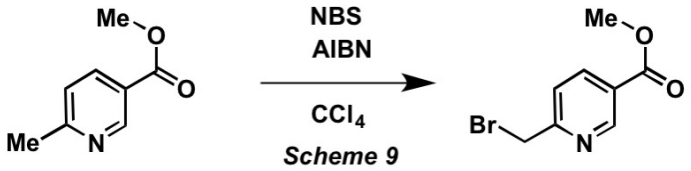
^{1}H NMR spectrum of product 1

The total yield for this product is 67.7%.

3.4 Synthesis of Product 3

According to the retrosynthetic analysis, the nucleophilic substitution was used to actualize the connection of 4th pyridine group. Product **2** must be prepared firstly from the free radical substitution of methyl 6-methylnicotinate as shown in Scheme 9.

3.4.1 Synthesis of Product 2



Scheme 9

2

Analysis: This is a typical bromination reaction, thus use the traditional agents: NBS, AIBN and CCl_4. The iniitation of free radical substitution requires heat or the UV light. Since NBS is very reactive, to avoid the double substitution on methyl group, the aluminum foil was used to control the amount of light. This is synthesized in a very similar method as the literature suggested. NBS was used with BPO as

catalyst, which gives a faster reaction rate, milder reaction conditions, and higher yield (60%). [12] However, I decided to use AIBN and CCl_4 as the reactants to see its effects on the reaction.

Synthesis of methyl 6-(bromomethyl)nicotinate (2)

According to Scheme 9, the experiment procedures were as follows. To a solution of ethanoic acid (2 mL) in a 100 mL round flask, methyl 6-methylnicotinate (5.00 g, 1.0 eq.) and carbon tetrachloride (50 mL) were added under argon atmosphere. (Replaced the air in the reacting system with argon gas using double row tube.) The reflux equipment was set up as shown in figure 11. The reaction vial was covered with foil. (The figure 11 only shows the reaction before I covered the reaction vial with foil.) The reaction mixture was heated to 60℃.

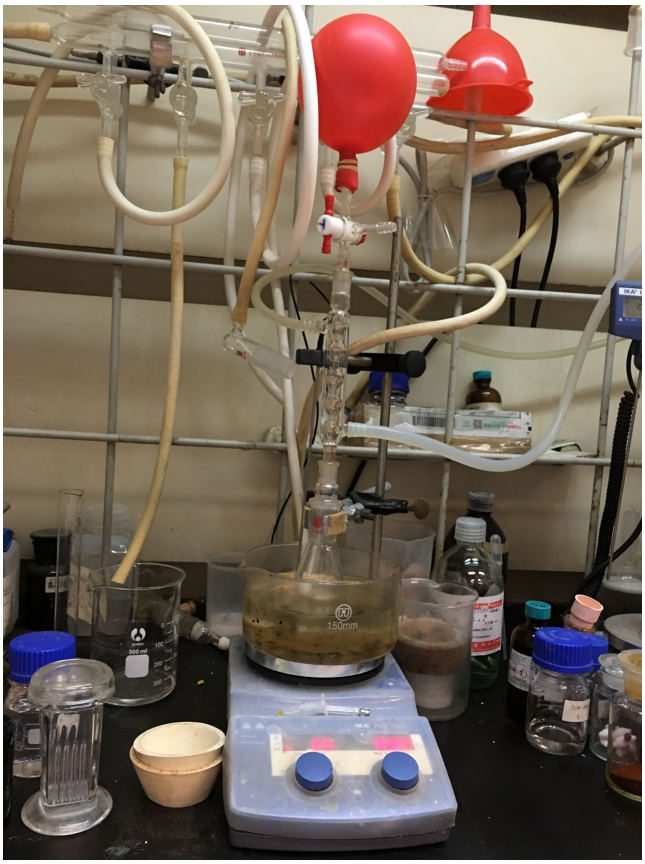


Figure 12: Reflux setup

N-bromosuccinimide (NBS) (6.0 g, 1.2 eq) was added, followed by Azobisisobutyronitrile (AIBN) (5.0 g, 0.1 eq) into the reaction mixture at room temperature. And then the mixture was refluxed for 3 hours. After cooling down to room temperature, additional AIBN (3.5 g, 0.07 eq) and NBS (1.2 g, 0.24 eq) were added, and was refluxed for 1 more hour in an oil bath and then was quenched with deionized water. The resulting solution was filtrated through a bed of silica gel to remove the precipitate formed during the reaction. (Later, I found out this was caused

by a miscalculation which led to insufficient addition of ethanoic acid.) After removing the solvent using rotavapor, the crude product was further purified by column chromatography with an eluent of PE: EA=10:1, as shown in figure 13, to give the target product **2** as a white solid[7]. (3.02 g[8], 46.7 %).

The relative low yield of this product was resulted from its difficult purification process. This reaction was governed by free radial substitution reaction, which produces many byproducts. Bromine free radical may attack the methyl group or carboxyl group. There are also possibilities that more than one bromine free radicals attach to one carbon. In order to minimize this situation, the amount of reagents added was limited. [24]

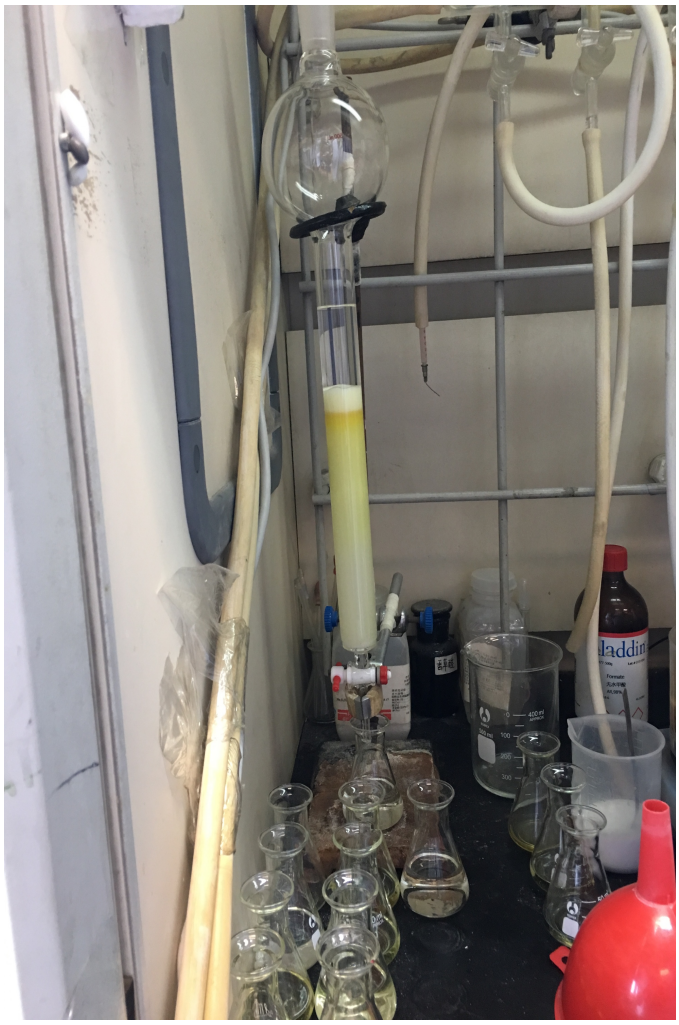
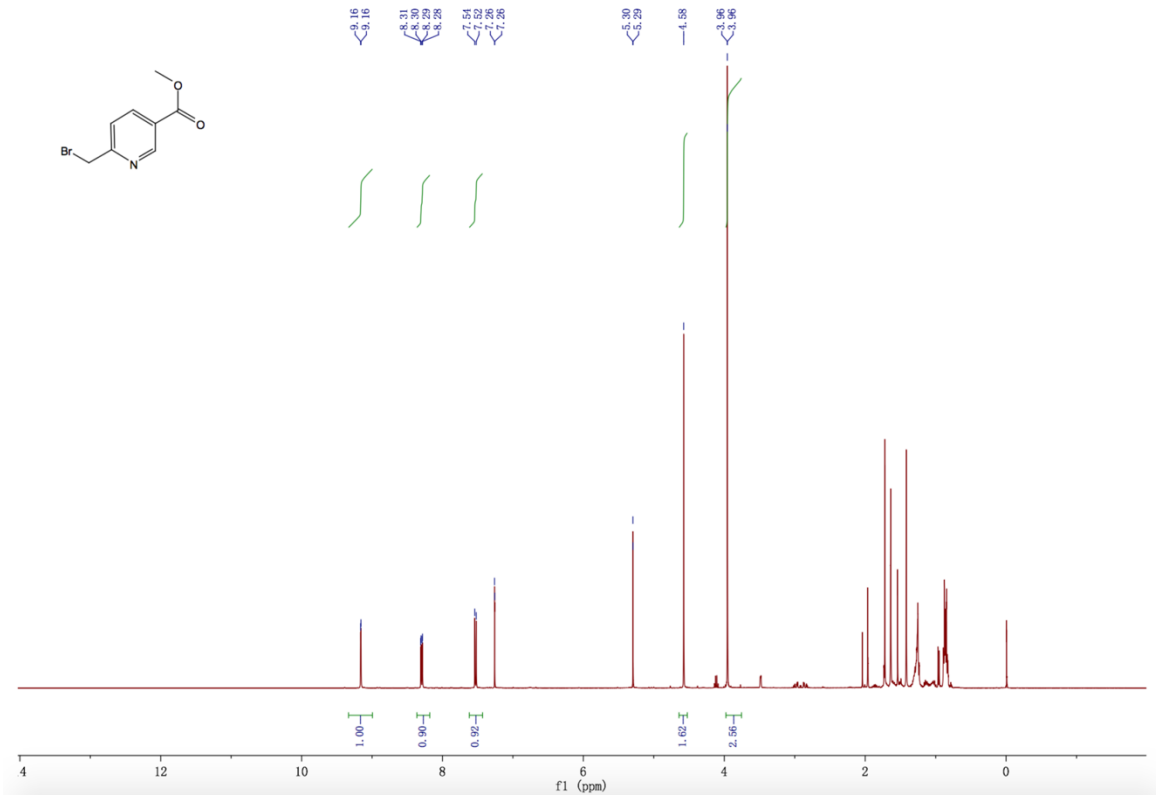


Figure 13: Column chromatography setup

[7] Product 2 gone corrupted quickly even in argon protection and refrigerator condition. Thus, the next scheme of reaction was carried immediately. (If the product is corrupted, the color changes to light red as shown in figure 14)

[8] Excess product 2 that was not used in next step pf reaction (1.07 g) was stored and given to other researchers in lab.



^{1}H NMR spectrum of product 2

NMR gave affirmative result on structure. [25]
^{1}H NMR ($CDCl_3$, 298 K): δ= 9.16 (s, 1H, ArH), 8.31-8.28 (m, 1H, ArH), 7.54 (d, 1H, ArH), 4.58 (s, 2H, $BrCH_2$), 3.96 (s, 3H, OCH_3).

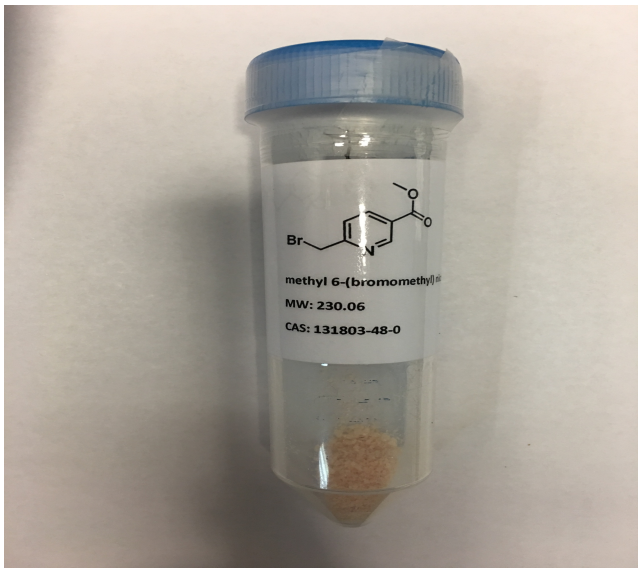
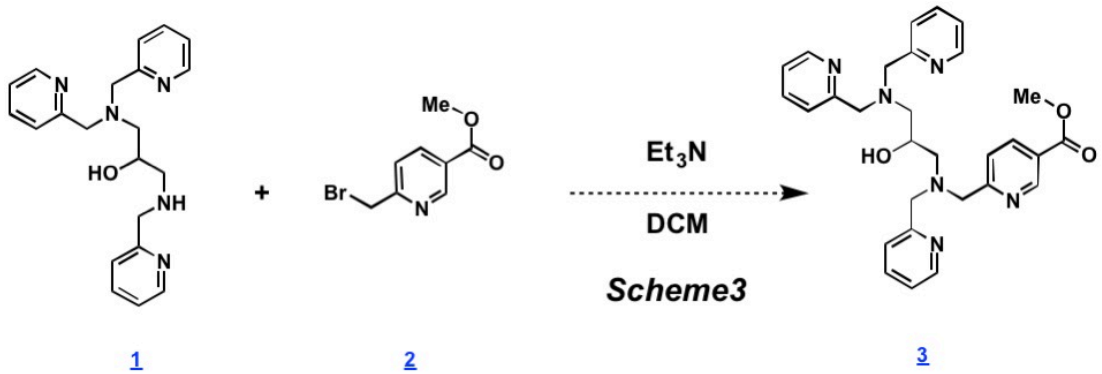


Figure 14: Product 2 obtained gone corrupted

3.4.2 Synthesis of Product 3[9]



Analysis: Et_3N was used as the common reagent in the dehalogenation reaction, which is analogous to Hofmann elimination. [26]

Synthesis of methyl 6-(((3-(bis(pyridin-2-ylmethyl)amino)-2-hydroxypropyl) (pyridin-2-ylmethyl)amino)methyl)nicotinate (3)

According to Scheme 3, the experiment procedures were as follows: To a solution of dichloromethane (DCM)[10] in a 100 mL flask product **1** (1.50 g, 1.0 eq) was added at -78℃. (Placed the reaction vial in acetone with the addition of dry ice to obtain -78℃). After stirring for 5 min, triethylamine (Et_3N) (1.7 mL, 3.0 eq) was added to the reacting mixture.

Figure 15: Adding dry ice in acetone

Figure 16: Substituting atmosphere with argon

[9] Dr.Eiji group purchased the product 3 from NARD institute, which leads to higher cost of Phos-tag™ acrylamide.
[10] All DCM used as solvent is solvent-free (no water).

Fresh product **2** (1.3 g, 1.4 eq) was added to the reaction mixture under argon atmosphere. The vial was removed from the acetone bath. Then the mixture was stirred at room temperature overnight.

TLC showed the product as well as a range of by-products, so extra product **2** (650 mg, 0.7 eq) was added to the reaction mixture and was stirred for four additional hours. Saturated ammonium chloride was used to quench the reaction and the resulting solution was partitioned between DCM and water, the organic layer was dried over anhydrous sodium sulfate.

After removal of the solvent in vacuum, the crude product was further purified by column chromatography with eluent DCM: MeOH=15:1, giving the target product **3** (1.384 g pure product and 116 mg impure mixture). The impure mixture was further purified by HPTLC to give the desired product.

I realized this step was similar to step 3 in original reaction route since the type of reaction undergoing was the same. However, the uses of bromine halogen made reaction harder. (During the free radical substitution in making product **2**, it was more difficult to gain electron through homolytic fission since it had smaller electronegativity than chlorine did.) In other aspects, the nucleophilic substitution reaction in this scheme was easier by bromine, which has larger C-X bond strength.

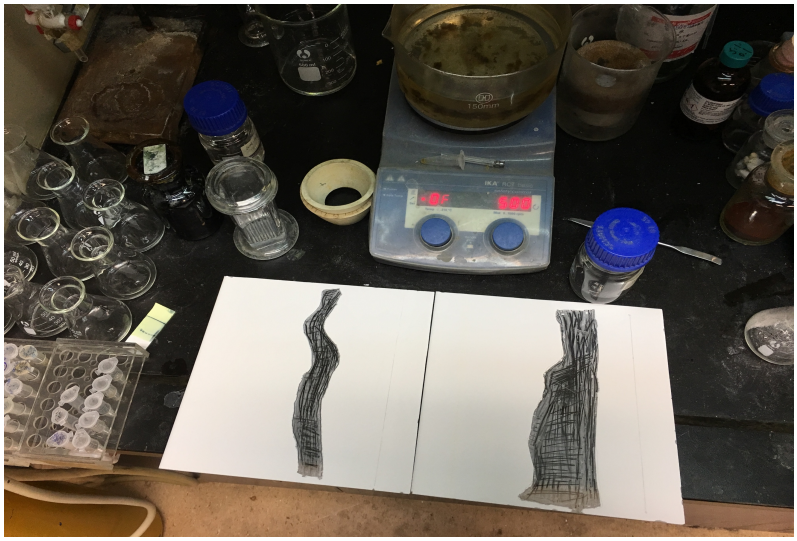
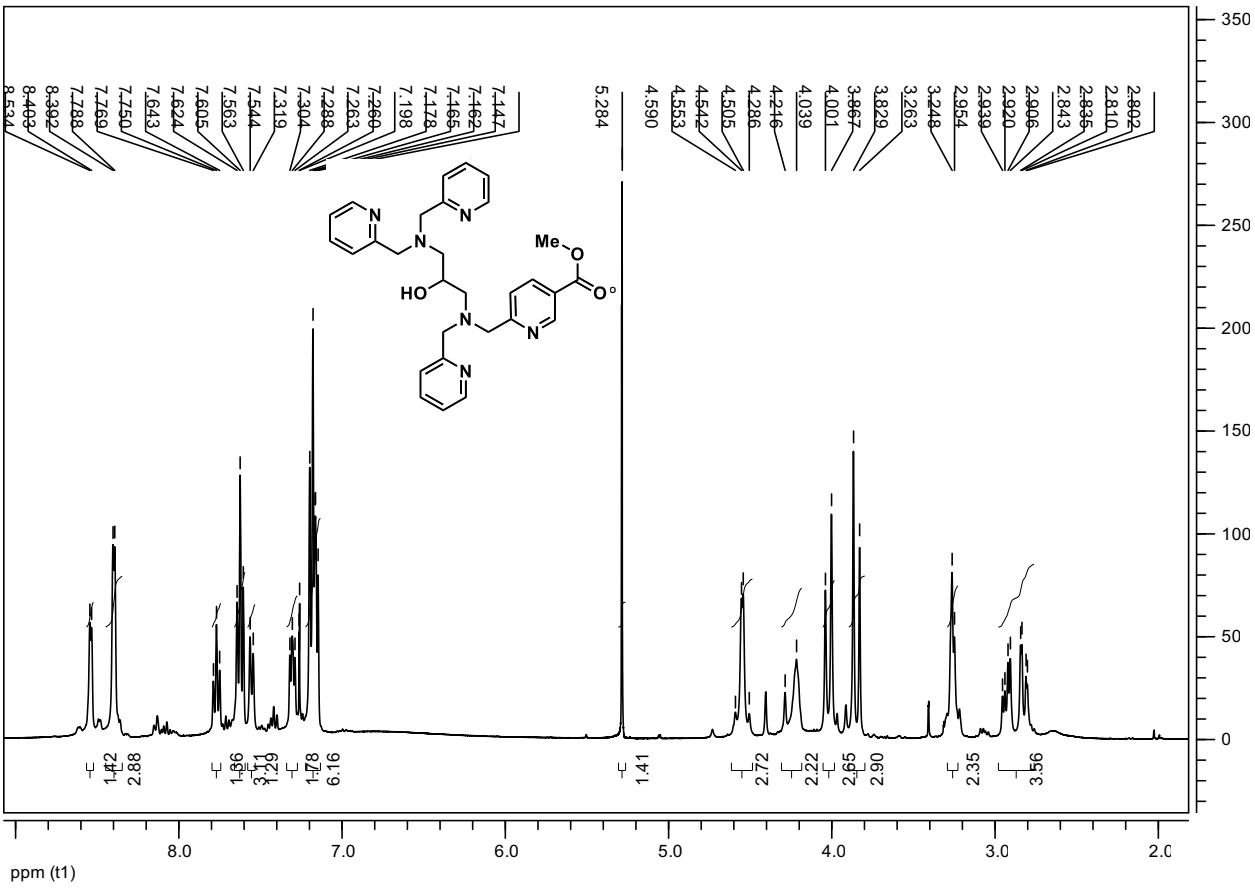


Figure 17: HPTLC collected target product

From HPTLC, additional 44.7 mg of target product was obtained. Total product **3** was light yellow oily solid. (1.43 g, 72%)

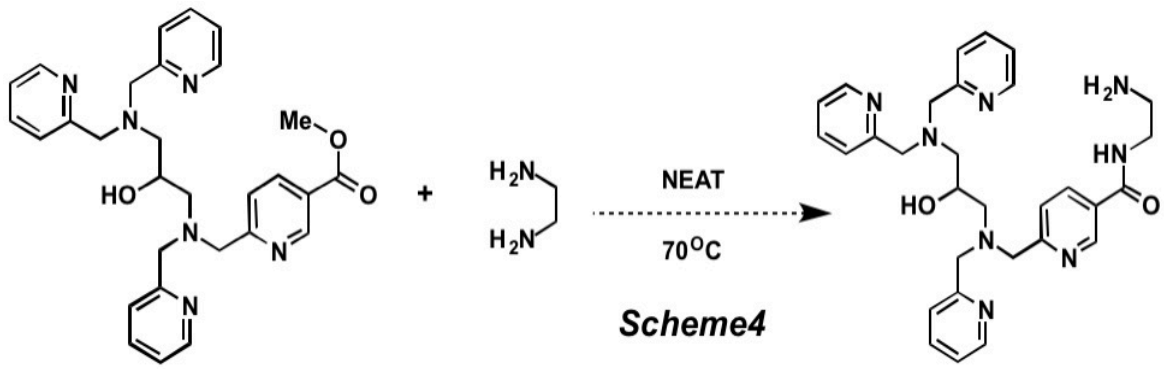
NMR gave an affirmative result on the structure of the product.



^{1}H NMR spectrum of product 3

3.5 Synthesis of Product 4

Analysis: From the obtained product from last scheme of reaction, methyl ester was designed to directly undergo aminolysis to get the single-side acrylamide structure. However, the ratio of two starting materials should be controlled to prevent the double aminolysis. This reaction procedure generally follows the literature of Dr.Eiji et al. [27]



Scheme4

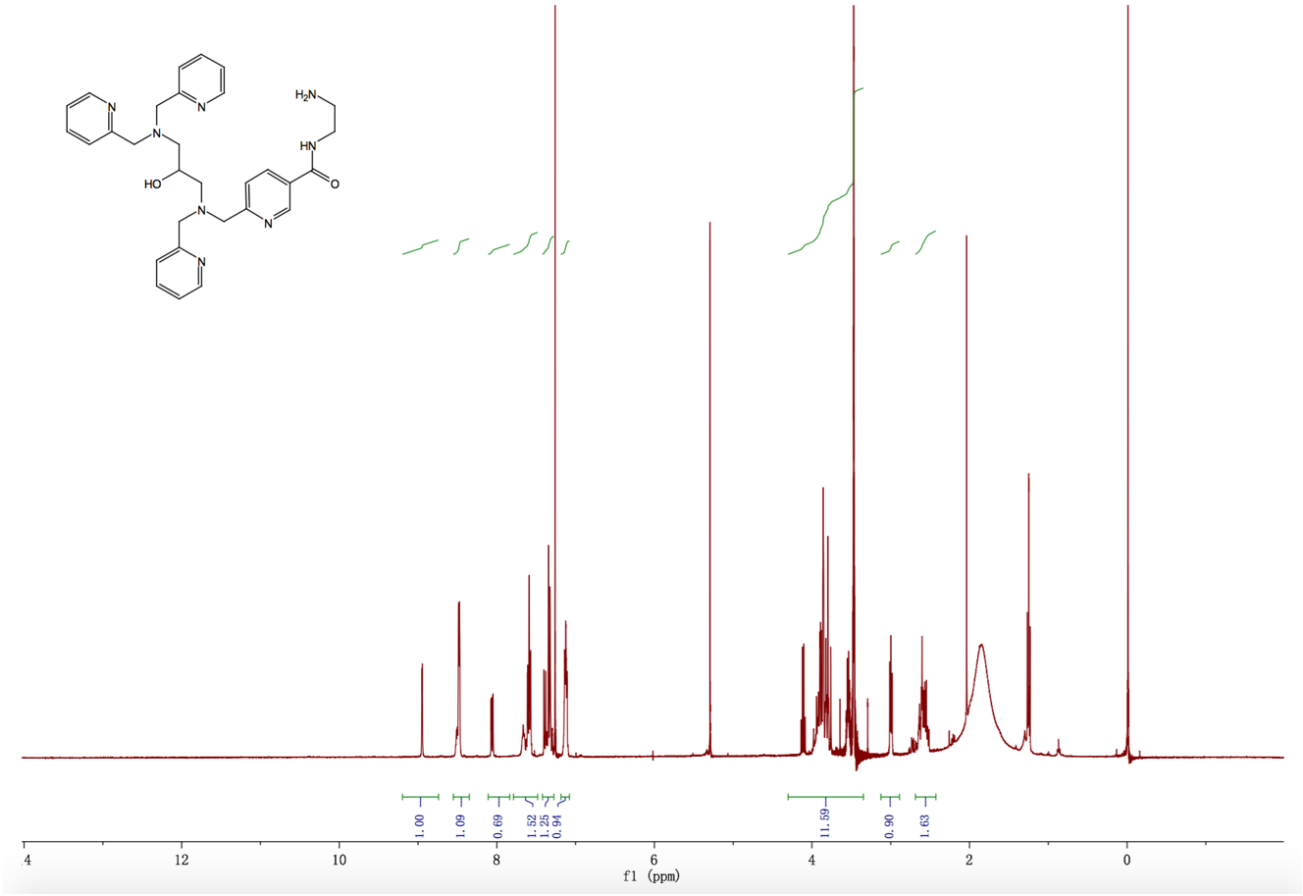
Synthesis of N-(2-aminoethyl)-6-(((3-(bis(pyridin-2-ylmethyl)amino)-2-hydroxypropyl)(pyridin-2-ylmethyl)amino)methyl)nicotinamide (4)

The reaction was as shown in the Scheme 4. To a solution of ethane-1,2-diamine (35mL) in a 100 mL three-neck flask with the Allihn condenser, a magnet, and product **3** (1.4 g, 1.0 eq) was added. The reaction mixture was heated at reflux for overnight.

After 17 hours of reaction, TLC showed the reaction had gone to completion. Saturated ammonium chloride was used to adjust the pH value to weakly alkaline. The product was isolated from the aqueous phase by successive extractions with DCM and water until no product remained in the water phase as judged by TLC. Dried the collected organic layer with anhydrous sodium sulfate.

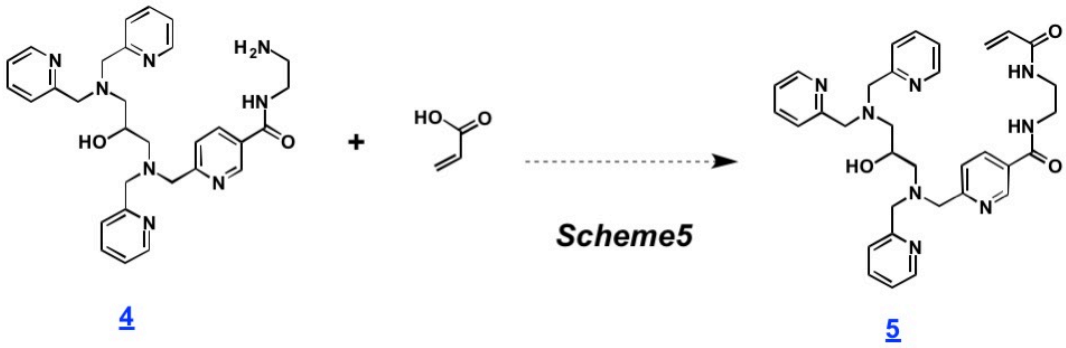
After solvent removal under reduced pressure, the crude residue was purified by column chromatography with eluent of DCM: MeOH: NH_4OH= 4:1:0.2. to give the target product **4** as a light yellow oily liquid. (1.40 g, 95%) [27]

NMR gave the affirmative result.



^{1}H NMR spectrum of product 4

3.6 Synthesis of Phos-tag™ Acrylamide/Product 5

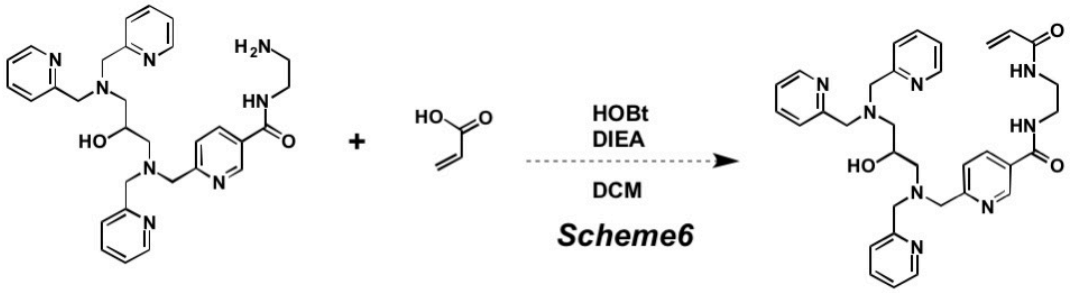


Analysis: The general reaction scheme is shown above. Since the acrylic acid has low tolerance and stability: it polymerizes with itself easily, especially when the temperature increases. There are many methods to prepare amides. Thus, the addition of acyl chloride dropwise to form acrylamide was not considered, since this involves thionyl chloride and Oxalyl chloride, which are strong reacting reagents, and

also the glorification of the carboxyl group is very toxic and time-consuming. Therefore, the experiment was continued by using ester or condensation agent.

The original synthesis route adopted this scheme as the last step of Phos-tag acrylamide formation. In that scheme, the coupling reagents and solvents chosen were not efficient. Therefore, the yield rate for it was 53%. [6] However, author approached different combinations of coupling reagents and tried to determine the most efficient ones for this critical step.

3.6.1 First Experiment on Phos-tag™ Acrylamide



4 5

Analysis: The active ester method has lower reactivity than either of acyl chloride and anhydries. Therefore, the products' racemization is less significant and the reaction can be conducted under higher temperature. At first HOBt and DIEA were chose as the coupling agents since DIEA is also known as Hünig's base, which is widely used as a selective reagent in the alkylation of secondary amines to tertiary amines. [28] Also, HOBt is not a very strong agent. This could avoid condensation reaction of secondary amine.

Synthesis of N-(2-acrylamidoethyl)-6-(((3-(bis(pyridin-2-ylmethyl)amino)-2-hydroxypropyl)(pyridin-2-ylmethyl)amino)methyl)nicotinamide (5)

As shown in the Scheme 6, ester 1-Hydroxybenzotriazole (HOBt) was chosen to perform acrylamide condensation. To a solution of DCM (1 mL) in a 10 mL specimen bottle, acrylic acid (8.64 mg, 1.5 eq), magnet, and HOBt (20 mg, 1.7 eq) were added. The reaction mixture was stirred at room temperature for 5 minutes. Then, to a solution of DCM (1 mL) in another 10 mL specimen bottle, product **4** (42.7 mg, 1.0 eq) and N,N-diisopropylethylamine (DIEA) (40.8 mg, 4.0 eq) were added dropwise under an argon atmosphere. The reacting mixture was slowly transferred from bottle #2 into bottle #1 via the 2 mL-syringe in ice bath condition. Then 4-dimethylaminopyridine (DMAP) (0.97 mg, 0.1 eq) was added, and reaction mixture was stirred at room temperature for 6 hours. The results weren't clear: TLC gave a vague indication of whether the reaction took place or not, and UP-LC showed cue of very few target products.

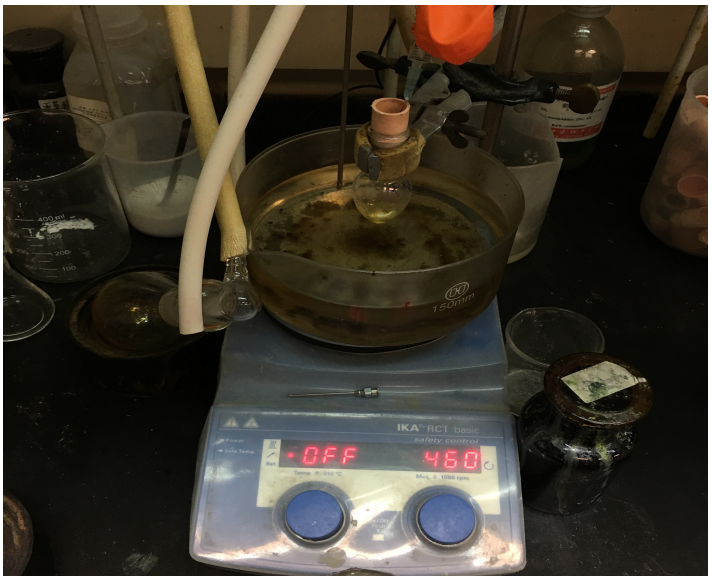
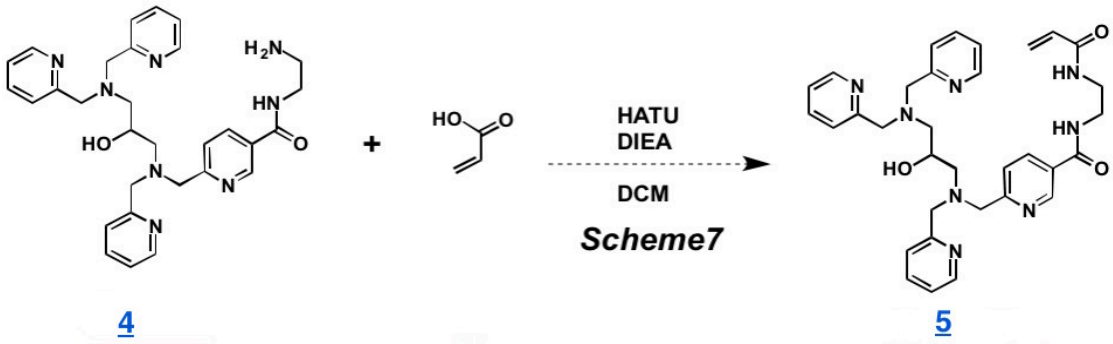


Figure 18: Magnetic mixture stirring at room temperature

I suspects that the DIEA attacked secondary amine, instead of the primary amine group, and meanwhile HOBt decomposed during the reaction. Therefore, the UP-LC showed no signal of correct weight of Phos-tag™. Another method was designed. [29]

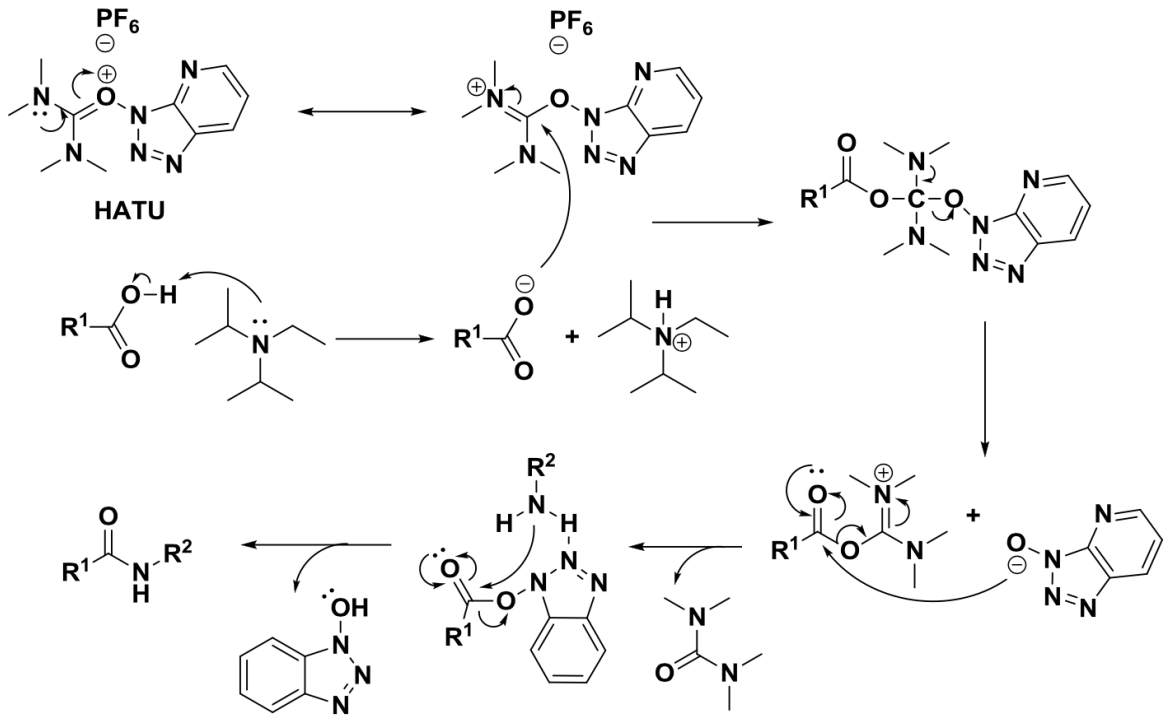
3.6.2 Second Experiment on Phos-tag™ Acrylamide



4 5

Analysis: This time, I chose condensation method, uronium salts type to be exact, to form amide. This method is the most commonly used way to synthesis amide. It doesn't need to separate the intermediates to form amide, which doesn't sacrifice yield and saves time. This time, another coupling reagent was chosen, which performed well in other acrylamide structure condensation: HATU. HATU has higher reactivity than HBTU. Therefore, when the reactivity of amine is not high enough, HATU is usually chosen. [30] The combination of the coupling agents is similar to referenced method, but DCM is used as an alternative solvent. This is due to higher boiling point of DMF, which makes the workup more difficult. Neighboring group effect, a result of the nitrogen atom on pyridine, stabilizes the incoming amine through a H-bonded cyclic transition state. This leads to the high coupling efficiency of HATU and its fast reaction rates. [31] However, its price is twice as much as that of HBTU.

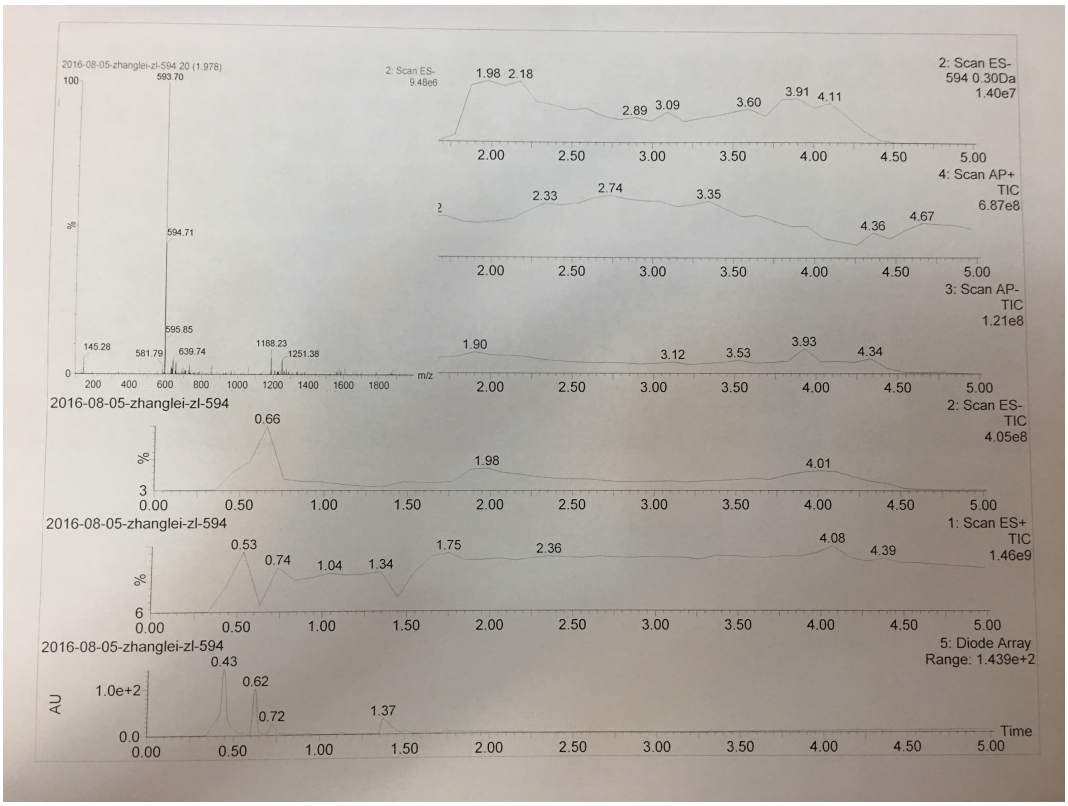
The reaction mechanism is as following:



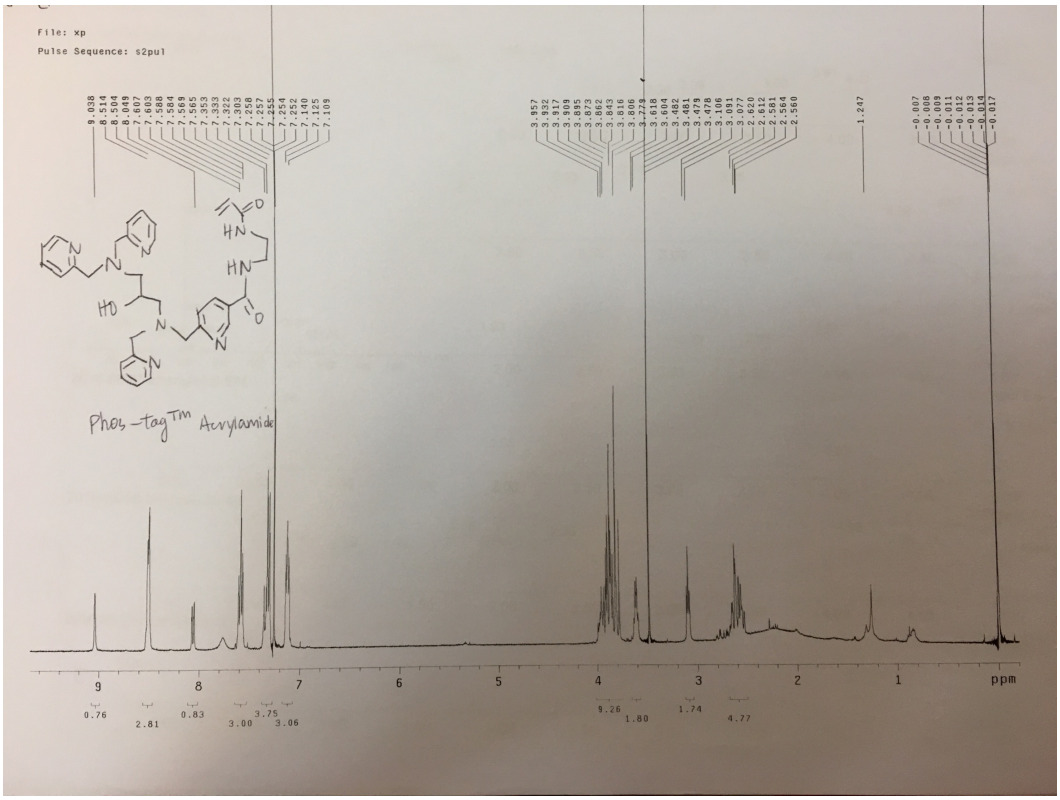
Mechanism 2: Carbodiimide condensation reaction

Synthesis of N-(2-acrylamidoethyl)-6-(((3-(bis(pyridin-2-ylmethyl)amino)-2-hydroxypropyl)(pyridin-2-ylmethyl)amino)methyl)nicotinamide (5)

The reaction scheme is as listed above. To a solution of DCM (2 mL) in a 10 mL specimen bottle, added a magnet, acrylic acid (8.64 mg, 1.5 eq), and 1-[bis(dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluoro phosphate (HATU) (76.0 mg, 2.5 eq) were added, followed by the addition of DIEA (40.8 mg, 4.0 eq) dropwise and product **4** (42.7 mg, 1.0 eq). The reaction mixture was stirred at room temperature for 3 hours. TLC showed the completion of reaction. Water was used to quench the reaction followed by DCM to extract the products.



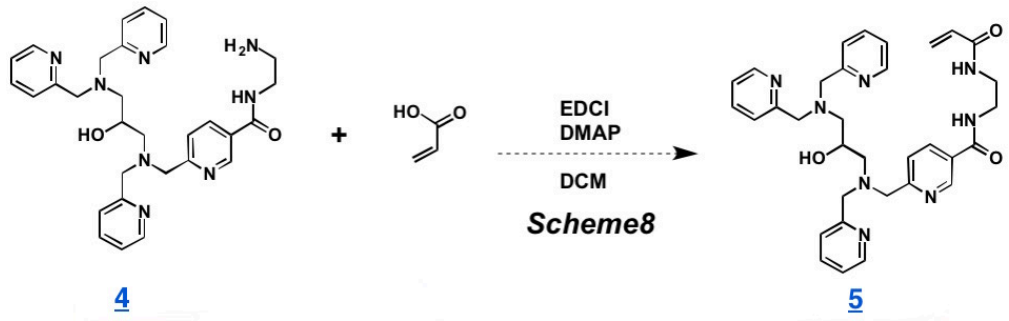
UP-LC figure 1: Affirmatory result of the Phos-tag™ Acrylamide



^{1}H NMR spectrum of product 5

UP-LC and NMR spectra both showed formation of the product. After purification by column chromatography (eluent: DCM: MeOH: NH4OH= 5:1:0.1), a light yellow product was obtained (41.2 mg, 89%).

3.6.3 Third Experiment on Phos-tag™ Acrylamide

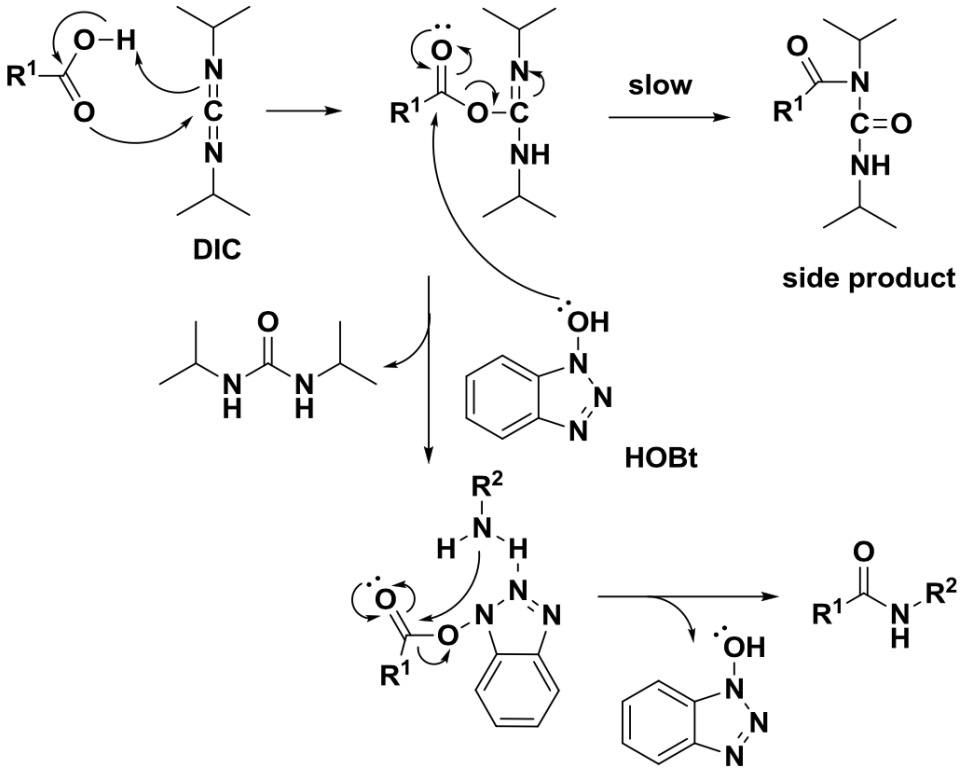


4 5

Scheme8

Analysis: Similarly, condensation reaction was selected but a different type was considered. Carbodiimide condensation reaction was used and after searching for appropriate coupling reagents, Scheme 8 was designed. I decided to try EDCI combination lastly, since the original reaction conditions from literature adopted EDCI and 4-methoxyphenol. [6] DMAP was used as a catalyst. It is especially useful for the arylamine which has great steric hindrance and low reactivity, so the reaction is able to be conducted at a milder condition with higher yield in a relatively similar time period.

Its reaction mechanism is as following:



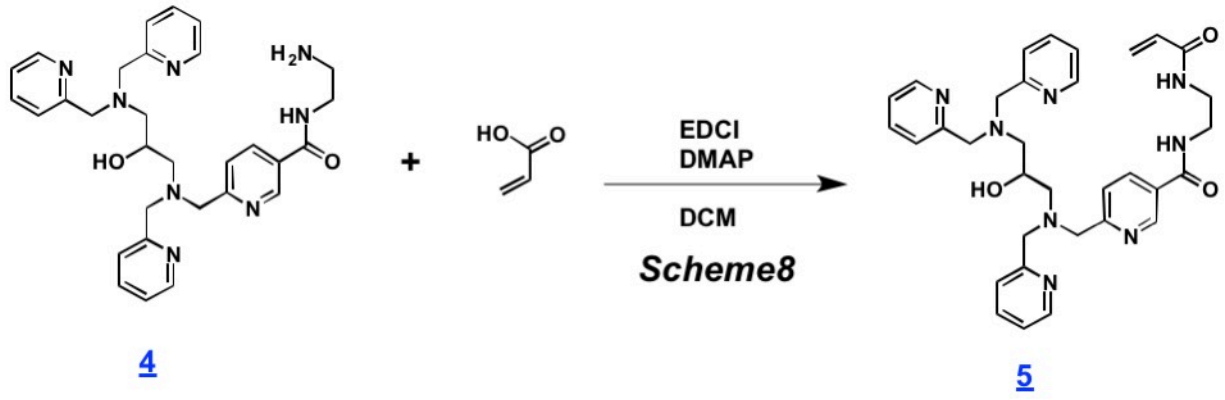
Mechanism 3: Uronium salts condensation reaction

Synthesis of N-(2-acrylamidoethyl)-6-(((3-(bis(pyridin-2-ylmethyl)amino)-2-hydroxypropyl)(pyridin-2-ylmethyl)amino)methyl)nicotinamide (5)

To a solution of DCM (2mL) in a 10 mL specimen bottle, added (3-dimethylamino propyl)ethyl-carbodiimid monohydrochloride (EDCI) (29.0 mg, 2.5 eq) and DMAP (3.0 mg, 0.5 eq). In the ice bath, acrylic acid (6 mg, 1.5 eq) was added dropwise. The reaction mixture was then stirred at the room temperature for 6 hours.

UP-LC showed target product **5**. Continued with scaling-up experiment. [33]

3.6.4 Fourth Experiment on Phos-tag™ Acrylamide



Synthesis of N-(2-acrylamidoethyl)-6-(((3-(bis(pyridin-2-ylmethyl)amino)-2-hydroxypropyl)(pyridin-2-ylmethyl)amino)methyl)nicotinamide (5)

To a solution of DCM (20 mL) in a 100mL three-necked flask, product **4** (1.3g,1.0eq), DMAP (130 mg, 0.5 eq) and EDCI (1.3 g, 2.5 eq) were added. Acrylic acid (260 mg, 1.5 eq) was added dropwise in the ice bath. And then the reaction mixture was stirred for 6 hours at room temperature. The flask was covered with aluminum foil whole time during the reaction since the reacting mixture was very light and heat sensitive.

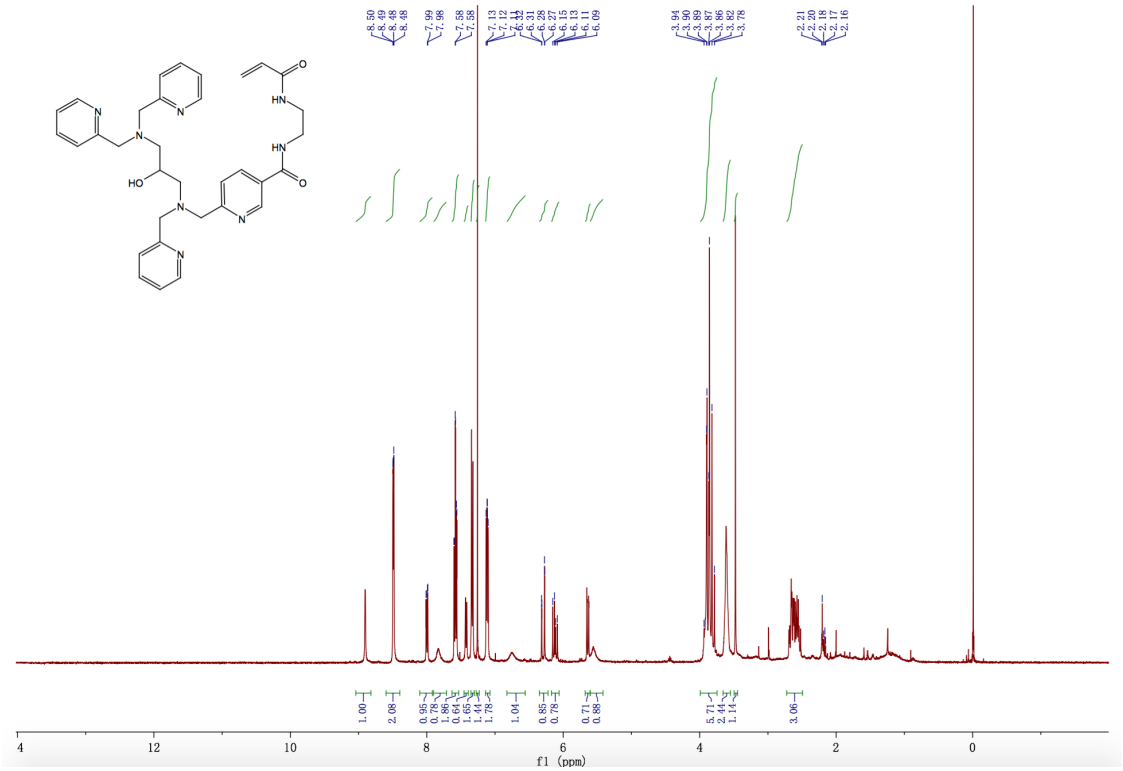
After 3.5h, TLC revealed the formation of products. Deionized water was added to a fraction of products to see which whether the products dissolve in the organic phase or water phase. TLC showed product **5** dissolved in the organic layer. Then reaction was quenched with deionized water and products extracted three times with DCM. The organic layer of the partitioned mixture was dried over anhydrous sodium sulfate. Excess solvents were removed via rotavapor with silica gel and wool. Finally, column chromatography with an eluent of DCM: MeOH: NH_4OH= 5:1:0.1 was used to separate the target material.

Product **5: Phos-tag™ acrylamide** is of pale yellow and oily texture. (1.3 g, 91%) In figure 19, target product was successfully synthesized and stored.[11] [12]

NMR gave affirmative result.

[11] The final product was given to other labs in NIBS for them to research on biological field.

[12] Phos-tag™ acrylamide must be stored in distilled water, at room temperature (28℃) with no direct contact with light, and used within three months of preparation. [10]



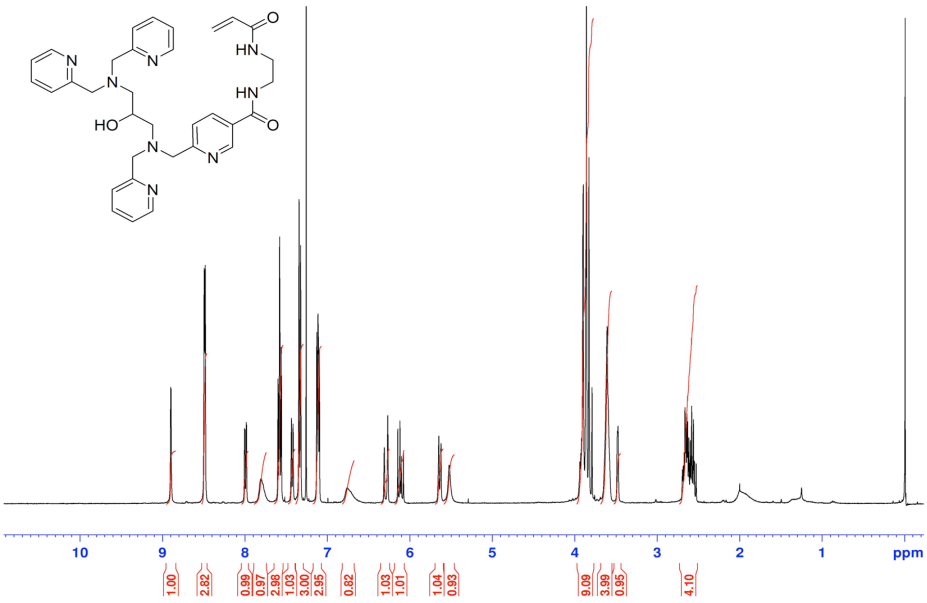
^{1}H NMR spectrum of Phos-tag™ acrylamide

Figure 19: Phos-tag™ Acrylamide prepared

4 CHARACTERIZATION AND ANALYSIS

4.1 ^{1}H NMR Data of Phos-tag™ Acrylamide

The analysis of data gave an affirmative result of the identity of the product.



^{1}H NMR spectrum of acrylamide-pendant Phos-tag™ ligand

^{1}H NMR data of acrylamide-pendant Phos-tag™ ligand:
^{1}H NMR ($CDCl_3$, 298K, 400 MHz): δ= 2.53 –2.69 (4H, m, NCCCHN), 3.57–3.66 (4H, m, CONCCH andCONCHC), 3.80 –3.94 (9H, m, NCCHCN and PyCHN), 5.65 (1H, d,J 10.3 Hz, COC=CH), 6.11 (1H, dd,J 17.0 and 10.3 Hz, COCH=C),6.29 (1H, d,J 17.0 Hz, COC=CH), 6.60 (1H, bs, NHCOC=C), 7.13(3H, t,J 6.2 Hz, PyH), 7.34 (3H, d,J 7.8 Hz, PyH), 7.44 (2H, d,J 8.0 Hz, PyH), 7.59 (3H, td,J 7.7 and 1.8 Hz, PyH), 7.73 (1H, bs,PyCONH), 7.99 (1H, dd,J 8.0 and 2.3 Hz, PyH), 8.50 (3H, d,J 4.3Hz, PyH), 8.90 (1H, d,J 1.8 Hz, PyH).

4.2 UP-LC Data of Phos-tag™ Acrylamide

The Phos-tag™ acrylamide product on UP-LC, as shown in UP- LC figure 1, gave: m/z: 595.3 for ES^+. The molecular weight of the product is 594, cation signal is 595. Therefore, it is a match.

4.3 Analysis of Potential Limitations in the Research

This is the first organic synthesis research project undertaken by this team. Thus, the unfamiliarity with the experimental procedures, safety protocol and synthesis

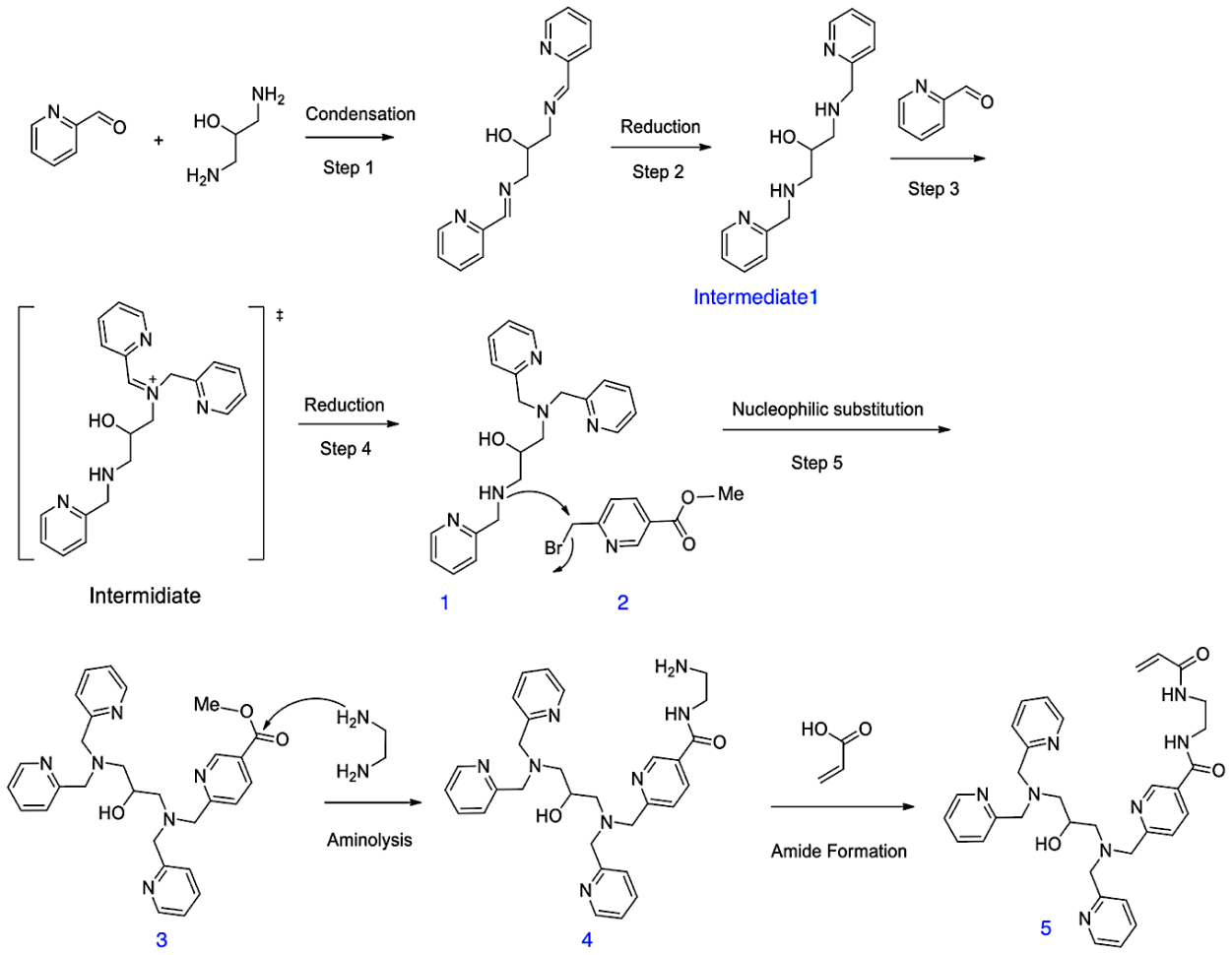
route modification led to time-consumption and inaccuracy. For instance, weighting the small amount of flammable liquid by syringe had larger percentage uncertainty due to the small-scaling experiments.

Another striking phenomenon is that intermediates went corrupted quickly. Since the intermediate was unstable, the experiment Scheme 9 was repeated multiple times to prepare the freshly made product **2** for the preparation of product **3** in following reactions.

In addition, during the experiments, the major obstacles came from the difficulty in purifying the products and the sensitivity of substrates. The majority of intermediates obtained have large polarity, thus during the process of column chromatography, products were partially stuck on the silica gel and showed tails on TLC. Basic substances such as Et_3N, pyridine, and ammonia water are commonly used to neutralize the slightly acidic property of silica gel. After consideration, even though Et_3N has a better effect of developing solvent, ammonia water is easier to remove from the product. Due to aforementioned character, yield rate will be lower when a small scale experiment is performed in the laboratory.

4.4 Mechanism 2: Proposed Possible Reaction Mechanism

Combined the experiment results with the reported literature, I proposed a possible reaction mechanism as follows:



Mechanism 2: Proposed overall reaction mechanism

5 BENEFITS OF THIS RESEARCH OUTCOME& CONCLUSION

Acrylamide-pendant Phos-tag™ ligand can be synthesized by this method in an efficient and convenient manner with the circumvention of the traditional route and trouble in bromination. Also, this can be applied to industrial mass production, lowering the difficulty in reaction, decreasing the need to monitoring the process, and increasing the yield. From this methodology, Phos-tag™ biotin can also be efficiently synthesized, and so do the series of Phos-tag™ derivatives.

Entry	Substrate	Product (proposed)	Scheme	Method	% Yield
1		1	1	$(CH_3COO)_3BHNa$	-
2		1	1	$NaBH_4$	-
3		1	2	$NaBH_4$, $NaBH_3CN$	67.7
4		2	9	NBS, AIBN, CCl_4	46.7
5	1,2	3	3	Et_3N, ice bath	72.0
6	3	4	4	75℃	95.0
7	4	5	6	HOBt, DIEA	-
8	4	5	7	HATU, DIEA	89.0
9	4	5	8	EDCI, DMAP	-
10	4	5	8	EDCI, DMAP	91.0
Total	-	-	-	-	42.1

Table 3: List of entries and percentage yield

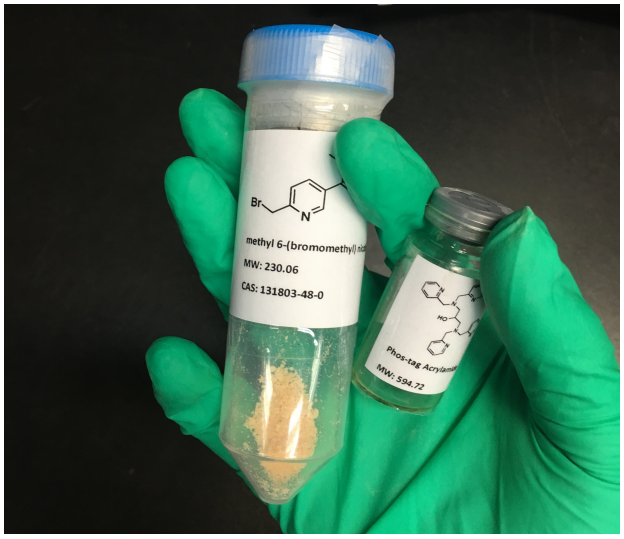


Figure 20: All products that are stored for future research

By crude calculation and rough cross-pricing on the starting materials, I found the cost of commercially made Phos-tag™ acrylamide can be lowered by multiple times.[13] Therefore, promising view on this method should be expected. The table below gave the demonstration of the rough pricing calculation.

Name of reagents used	Price available[14]/ CNY g^{-1}	Unit price/ CNYg^{-1}	Amount used/ g	Total cost/ CNY	Notes
2-Pyridine carboxaldehyde	510.12 CNY/100 g	5.1 CNY/g	4.775 g	24.4	
1,3-diaminopropan-2-ol	180 CNY/ 25 g	7.2 CNY/g	1.37 g	9.9	
Sodium borohydride	370.89 CNY/25 g	14.84 CNY/g	2.32 g	34.4	
Sodium cyanoborohydride	628.29 CNY/10 g	62.8 CNY/g	1.44 g	90.4	
Methyl 6-methylnicotinate[15]	1411 CNY/10 g	141.1 CNY/g	5 g	705.5	
Ethanoic acid	303 CNY/100 ml	3.03 CNY/ml	2 g	6.1	
N,N-Diisopropylethylamine	487 CNY/100 ml	4.87 CNY/ml	40.8 mg	0.2	DIEA
N-Bromosuccinimide	1342 CNY/1000 g	1.34 CNY/g	7.2 g	9.6	
Azobisisobutyronitrile	590 CNY/100 g	5.9 CNY/g	8.5 g	50.2	
1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate	6347 CNY/25 g	253.88 CNY/g	76 mg	19.3	HATU
4-Dimethylaminopyridine	372 CNY/25 g	14.88 CNY/g	133.97 mg	2.0	DMAP
1-(3-dimethylaminopropyl) ethyl-carbodiimidmonohydrochloride	1058 CNY/5 g	211.6 CNY/g	1329 mg	281.2	EDCI
PE	770 CNY/1000 ml	0.77 CNY/ml	N/A	N/A	
EA	750 CNY/1000 ml	0.75 CNY/ml	N/A	N/A	
Tetrachloromethane	25 CNY/500 ml	0.05 CNY/ml	N/A	N/A	
Triethylamine	206 CNY/100 ml	2.06 CNY/ml	1.7 g	3.5	
1-Hydroxybenzotriazole	2882 CNY/250 g	11.53 CNY/ml	N/A	N/A	
Ethylenediamine	593 CNY/250 ml	2.37 CNY/ml	35 g	83.0	
Acrylic acid	495 CNY/100 g	4.95 CNY/g	274.64 mg	1.4	

[13] Costs of solvents, recycling solvents, disposal of laboratory wastes, gases, other equipments, electricity and the Research& Development are ignored in this calculation. These expense should not be very considerable when converted into unit cost.

[14] Pricing information was found on SIGMA-ALDRICH website, using the pricing analysis. Information of relatively cheap and pure starting materials and all other reagents used were obtained. [34]

[15] Total product 2 made in the laboratory weighted 3.02 g. During the scheme 3 to prepare product 3, only 1.95 g product 2 was used. The remaining 1.07 g product 2 was stored. However, in the pricing calculation, I assumed that the product 2 was all used up. Commercial price for product 2 was 450 USD/g, therefore giving the value of 481.5 USD (roughly 3211 CNY) to remaining product 2. [35]

Phos-tag™ Synthesized (Result of this research)	**Commercial price:** 6900 CNY/10 mg	**Commercial price:** **690 CNY/mg**	**Made:** 1341.2 mg	1320.96 CNY	**New price[16]:** **0.98 CNY/mg**

Table 4: Costs and final price comparison of new synthetic approach to mass production

[16] The costs of starting materials in failed experiments were not considered, the percentage yield, however, was.

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SHIYUAN SUN 孙士元

Sun Shiyuan was born in 1986, and received his Bachelor's degree majored in materials science and engineering, minored in English from East China University of Science and Technology (ECUST) in 2009. Then he was admitted to the Shanghai Key Laboratory of Advanced Polymeric Materials in ECUST without qualification examination to pursue his Ph.D. degree under the supervision of Professor Aiguo Hu in 2009, and received his Ph.D. degree in 2014. His research focuses on synthetic polymer chemistry, and I had published many papers on the top journal in polymer materials field such as Polymer Chemistry, J. Polym. Sci.: Polym. Chem. , J. Mater. Chem. C and so on.

After he received his Ph.D. degree, he jointed Beijing National Day School in 2014, teaching AL/AP/IB chemistry in international department. In addition to conventional teaching, he also played the professional advantage of what he learned during Ph.D. study, and actively participated in the preparatory work of high-end laboratory, he has been undertaking organic synthesis and analysis of high-end experimental course since Sep, 2014. He also directed the students to participate in the first Royal Chemical Society Chemical Star Challenge, won the third prize in North China area. He was hired as part-time instructors and researchers by Haidian District Board of Educationfor the current school year.

Resume of teachers

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WAN
万永红

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Beijing National Day School, Chemistry teacher.	2005-2016