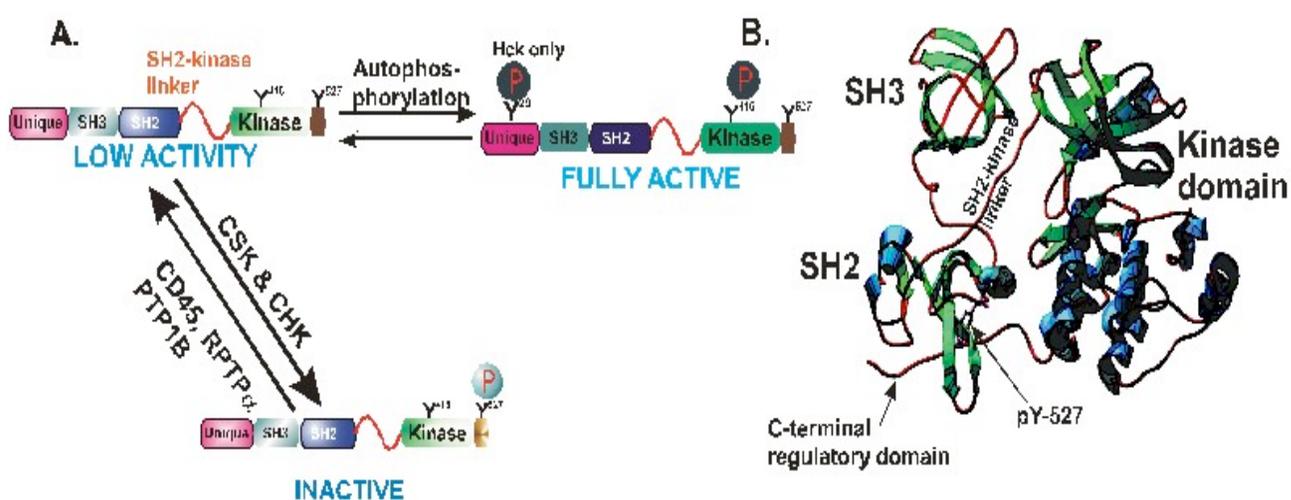


Structural Optimization of a Bivalent Tyrosine Kinase Inhibitor



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Abbreviations

List abbreviation of chemicals that I used in this thesis paper and its complete names are as follows:

- 1) **DAP = 2, 3 Diamino propionic acid**
- 2) **ADPOC = 1-(1'-Adamantyl)-1-methyl-ethoxycarbonyl)**
- 3) **ELISA = Enzyme Linked Immunosorbent Assay**
- 4) **Fmoc = 9-FluorenylmethylOxycarbonyl**
- 5) **HBTU = 2-(1H Benzotriazole-1, yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate.**
- 6) **HMBA = 4-Hydroxy methyl benzoic acid**
- 7) **HOBT = 1-Hydroxy benzotriazole hydrate**
- 8) **HTRF = homogenous time-resolved fluorescence**
- 9) **Nmm = N-methyl morpholine**

Preface

The Src families of tyrosine kinases are SH2 and SH3 domain containing proteins involved in the transmission of extra cellular signals from the plasma membrane to distant locations in the cytoplasm and nucleus. Elevated levels of kinase activity have been directly associated with a variety of cancers including human breast and colon cancer. Inhibitors that can be precisely targeted to specific members of a kinase family could be of great therapeutic value.

We have synthesized a peptide-based inhibitor that is designed to simultaneously bind to the SH2 and the active site region of the Src kinase. We are going to detect the protein tyrosine kinase activity and screening of potent inhibitor using non-radioactive method due to simple, rapid, efficient, safe, and no need to expose to radiation. ELISA and Htrf methods are non-radioactive methods which help to characterize and purify protein tyrosine kinases.

The goal of this research is to make a potent inhibitor that work at a 3000 fold higher affinity for binding the active site of mutated Src.

Chapter 1: Tyrosine Kinase

Introduction:

Tyrosine kinase is an enzyme that transfers the gamma phosphate group of ATP. In other words, tyrosine kinases catalyze the phosphorylation of tyrosine residues. Signal transduction is a process by which our cell converts one kind of stimulus or signal into another. Furthermore, Kinase plays a role in signal transduction and it is involved in the transmission of growth factor signals from the plasma membrane to the nucleus. Tyrosine kinases can be divided into two categories including receptor tyrosine kinases and non-receptor tyrosine kinases. Receptor tyrosine kinases contain an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain. The transmembrane domain anchors the receptor in the plasma membrane, while the extracellular domains bind to the growth factors. On the other hand, non-receptor tyrosine kinases are located in the cytoplasm and nucleus and they exhibit unique kinase regulation and substrate phosphorylation. Deregulation of these kinases has also been linked to several cancers including colon cancer and breast cancer and therefore, selective receptor and non-receptor Protein tyrosine kinase inhibitors represent a hopeful class of anti-tumor agents.

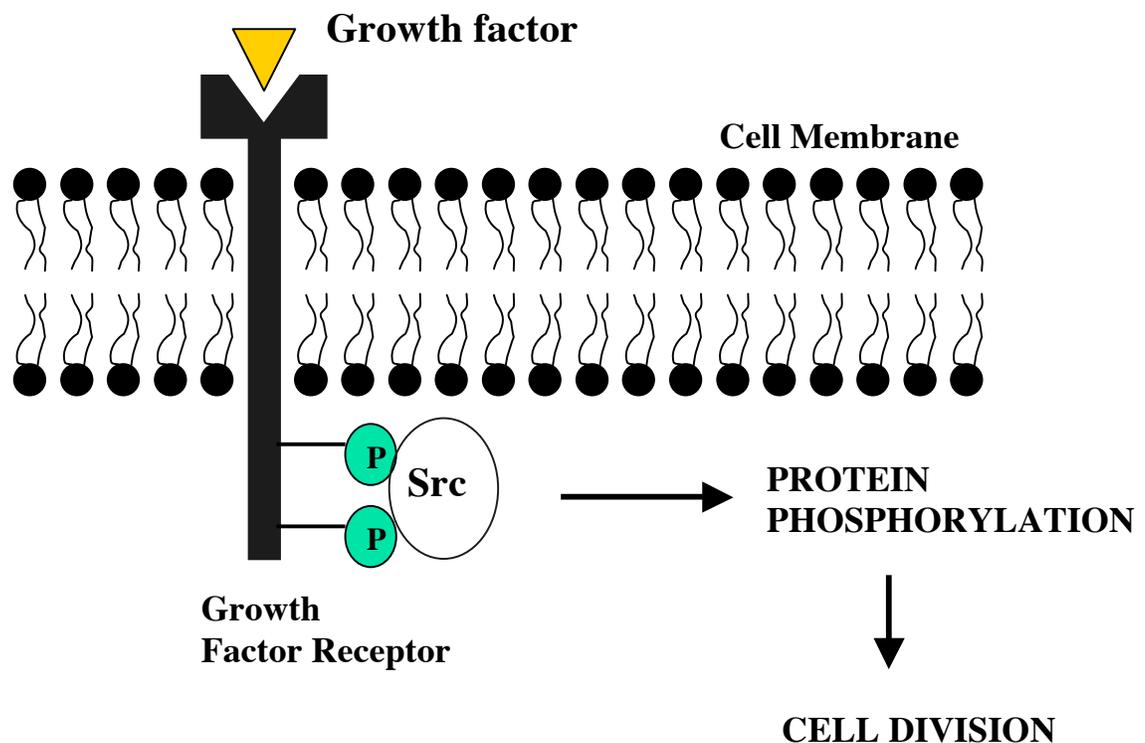
Src is a non-receptor protein tyrosine kinase that plays various roles in cell signalling. It is one of proteins that involved in a system that is an interconnected system of control and communication. It involves in several functions, including cell growth, differentiation, adhesion, and movement. Src is an enzyme that belongs to a family of proteins called tyrosine kinases. Src belongs to the family of non-receptor tyrosine

kinase. Other Src family kinase members are Fyn, Yes, Blk, Fgr, Hck, Lck, Lyn, and Yrk. Kinase members including Blk, Fgr, Hck, Lck, Lyn, Yrk are expressed in definite tissues.

Src can also transduce signals from other receptors to internal signaling pathways, which transfer these signals to the nucleus, cytoskeleton and other cellular components. Therefore, Src can perform through the growth factor receptors in order to affect proliferation and cell growth. Src also plays different roles since it can be also found in the cytoplasm, and between cells.

Figure #1 illustrates the concept of how Src involves in signal transduction and how it leads to cell division. Growth factor binds to a cell surface receptor. This promotes the association of Src with the receptor, which leads to protein phosphorylation and cell division.

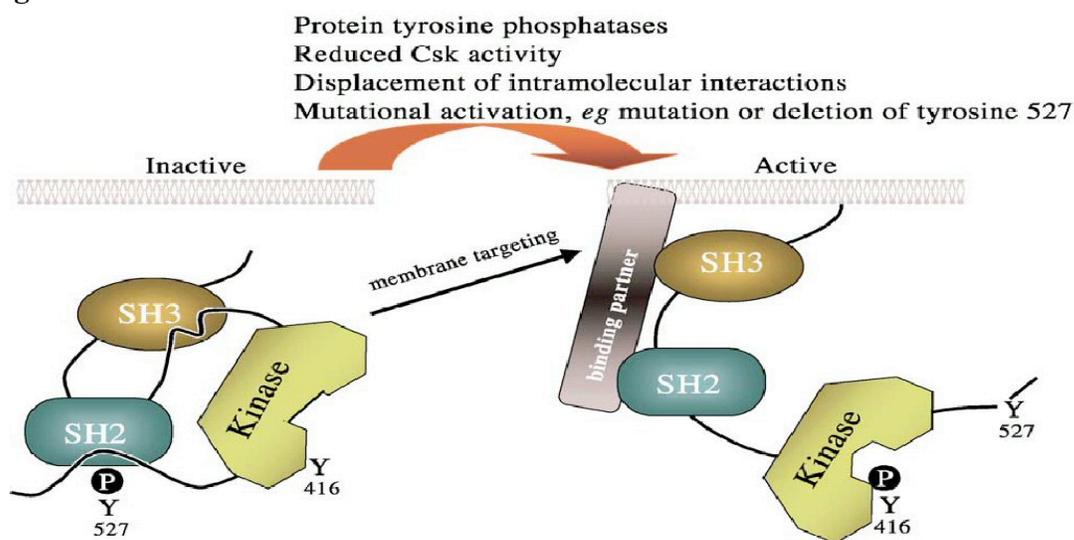
Figure #1:



Chapter 2: Regulation of Src

Src has three binding sites called SH3, SH2 and the kinase or SH1 catalytic domain that contains the kinase active site. SH2 and SH3 stand for Src homology 2 and 3 respectively. SH2 and SH3 are called protein- protein interaction domains since these domains play a role in protein-protein interaction. Figure #2 shows how these two domains play key roles in directing the activity of the enzyme and its interaction with other proteins. SH2 interacts with phosphotyrosine, and SH3 interacts with poly proline type II helices. When the Src is in the inactive form, SH2 domain is coordinated to the Tyr527 that is phosphorylated and SH3 domain correlate with a protein-rich linker, between catalytic and SH2 domains. During the active form of Src, the SH2 and SH3 domains regulate the activity of protein tyrosine kinase by forming an interaction with proteins and other substrate proteins.

Figure # 2:



Src regulation: conformational opening and activation

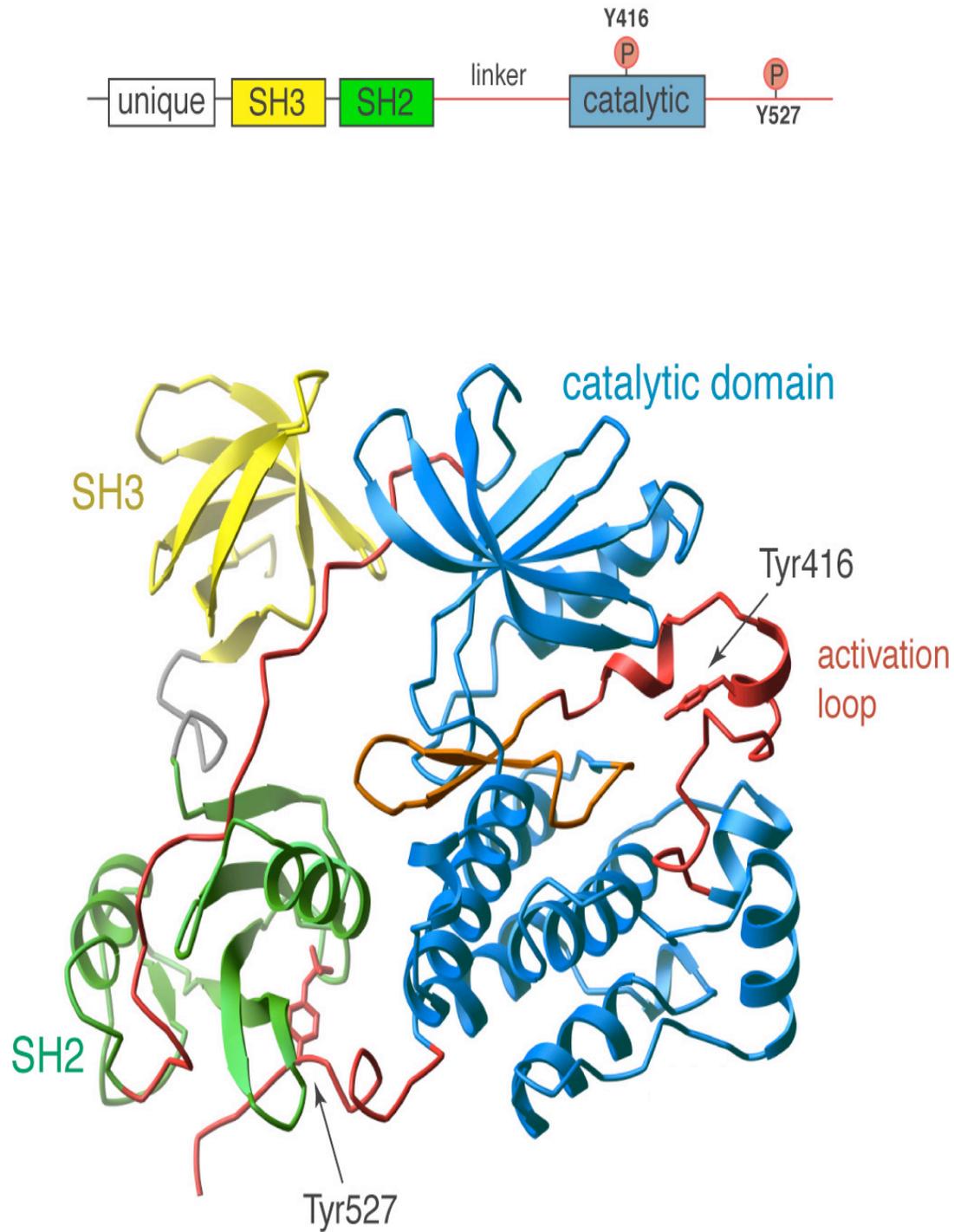
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² SRC, pro-oncogene tyrosine- protein kinase. Retrieved April 27, 2006 from www.ebi.ac.uk/interpro/potm/2003_2/page_2.htm .

According to the crystallographic studies (LaFevre, M., 1998), SH2 and SH3 domains regulate the family of Src kinase by forming intramolecular contacts other sites that are on the enzyme. Past studies reveal the orientation of catalytic domain in the inactive form of Src; it lies opposite to both SH2 and SH3. However, the orientation of the catalytic site in the active conformation of Src kinase has not been identified. Moreover these domains play a role in regulation of the cell cycle and cell division by interacting with other proteins.

These SH2 and SH3 domains play an important role in substrate recognition. SH2 and SH3 domains play two important roles in substrate recognition including activation of Src-family tyrosine kinases, assisting tyrosine kinases in recognizing cellular substrates. Binding leads the catalytic domain to phosphorylate and thus the kinase activation would be coupled to the substrate recognition. Figure #3 illustrates all three domains including catalytic domain, which is in blue, SH3 domain, which is in yellow, and SH2 domain, which is in green. These SH3 and SH2 domains arbitrate both intramolecular and intermolecular interactions that are vital in signal transduction. Figure #3 is a three dimensional structure of Src kinase Hck, which was founded by Dr. John Kuriyan.

Figure # 3:



3

³ Dual roles of the SH3 and SH2 domains of SRC family kinases. Retrieved February 4, 2006 from <http://www.pnb.sunysb.edu/faculty/miller/dualrole.htm>

Src play an important role in cell signaling but organisms that are deficient in Src can continue to exist since Src is one of the nine members, which can balance for one another.

Src is inactive under normal conditions and it can become continuously active when point mutations or over expression of Src occur or through mutations in proteins that regulates it. In other words, Src can become active from inactive throughout protein interactions and control of its phosphorylation state. Especially Src becomes activated when Tyr416 is auto-phosphorylated and therefore P-Tyr416 is being displaced from its binding pocket and eventually substrate is allowed to bind to the active site.

Src becomes inactivated via phosphorylation of Tyr527, which inactivate the Src throughout the interaction of P-Tyr527 with SH2 domain since these interactions lead the Src to fold in such a way that it's active site is not explicitly shown, and therefore the substrate cannot be able to bind to its active site. This Src regulation can be seen in figure #2. On the other hand, dephosphorylation of Tyr527 activates the Src by allowing the substrates to interact with the active site since the active site would not be hidden within the fold. Moreover, growth factor including platelet-derived and adhesion kinase are bind to SH2 domain and thus causing the Src to be in active form.

Chapter 3: Mutation in Src

Src should be switched on only at particular times and it should be inactive rest of the time. Alteration in Src activity arises when there is an imbalance between dephosphorylation and phosphorylation. This resulted disruption in Src activity leads to several cancers including colon and breast cancer. First, Src was separated as an oncogene, which is called v-Src that is a virus, Rous Sarcoma Virus. c-Src, which is a

cellular protein was found to have Tyr527, which leads the Src to be active continuously and resulted mutations in c-Src or mutation in proteins that regulate it since mutation is a permanent change in a DNA sequence. Increase in proteins including PTPalpha, SHP-1 and PTP 1 B can be observed at cancer cells. These proteins activate the Src by dephosphorylating Tyr 527. Furthermore, single point mutation in a SH2 region meaning alteration in one single amino acid can lead to activation.

Chapter 4: Goal of the research

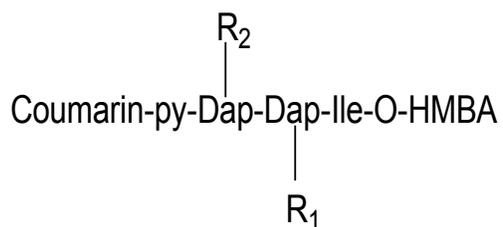
There are several studies have been done previously on Src kinase to determine a potent inhibitor. For instance, Alexander Silberman Institute of Life Sciences, and Hebrew University of Jerusalem, Israel reported that PP1 (4-Amino-5-(4-methylphenyl)-7-(*t*-butyl) pyrazolo [3, 4-*d*] - pyrimidine) was behaved as non-competitive with ATP for the inhibition of Src (Karni. R. 2003). Peptide based inhibitors are synthesized based on combinatorial library methods and these strategies has now been developed to transform modest binding consensus sequences into high-affinity ligands (D.S Lawrence, 2001).

Therefore, the goal of this research is to synthesize a more potent bivalent inhibitor, directed to both SH2 and active domain of mutated Src kinase so that it could be a best therapeutic drug for breast cancer, colon cancer and other types of cancers. In other words, the objective of this research is to make effective inhibitors for kinase (Src) in order to prevent from cancers since mutagens in kinase or elevated level of kinase activity leads to cancers.

Bivalent inhibitor is a peptide- based inhibitor, which is designed to simultaneously bind to SH2 and active site regions of the Src kinase. Bivalent inhibitor is a more potent inhibitor than the monovalent inhibitor due to entropy. Sequence that is

directed to the SH2 region is Coumarin-pTyr-Glu-Glu-Ile-Glu and the active site directed peptide is coumarin-Glu-Glu-Leu-Leu- (F₅) Phe-NH₂. GABA is a flexible linker, which connects both the SH2 directed and active site directed peptides. GABA stands for Gamma Amino Buteric Acid. This peptide-based bivalent inhibitor binds at 230 fold higher affinity than the simple active site directed inhibitor. The reason for this is that Glu, Tyr and Ile were determined to participate in high affinity interactions with residues on SH2 domain (Yeh, R., Lee, T., and Lawrence, D.S. 2001). Furthermore, this sequence is a derivative of SH2 and active site domains.

However, the goal of this research is to make an inhibitor that work at a 3000 fold higher affinity for binding the active site of mutated Src. Therefore, SH2 directed peptide sequence is going to be alternated so that the resulted bivalent peptide would have a higher affinity than previously synthesized bivalent inhibitor. Now the SH2 directed peptide sequence is going to be as follows:



Now, we have to test the newly synthesized inhibitor on Src Kinase (protein tyrosine kinase) to determine if it is indeed more potent inhibitor than those inhibitors that has been synthesized previously. As we all know that protein tyrosine kinase plays an important role where it activates several pathways that lead to differentiation, metabolism, and proliferation. Moreover, this protein tyrosine kinase helps to transfer γ

phosphate of ATP to tyrosine residues that are on protein substrates. This resulted phosphorylation of residues leads to enzymatic activity.

Chapter 5: Methods to detect PTK

There are several methods that can be useful in order to detect the PTK (protein tyrosine kinase) activity. These methods are radioactive and non-radioactive where, ELISA, HTRF are non-radioactive methods, and Scintillation proximity assay and Filter paper radioactive methods are radioactive methods. Moreover, these methods are useful in various ways such as help to characterize and purify the protein tyrosine kinases. In addition to purification, these methods are helpful in developing and screening of potent protein tyrosine inhibitors. These methods are also very useful to learn about PTK reaction mechanism and kinetics.

ELISA stands for Enzyme- Linked Immunosorbent Assay. Advantages of ELISA method involves simple, inexpensive, rapid, efficient, convenient, safe, and requires washing, which is a drawback. HTRF stands for Homogeneous Time-Resolved Fluorescence. Advantages of using this HTRF method involves no washing or liquid transferring steps, simple, high sensitivity, easy to handle and dispose. Scintillation proximity assay (SPA method) is useful to detect the tyrosine kinase activity and it is similar to HTRF. Comparison between SPA and HTRF methods is that SPA requires fewer reagents and faster compare to HTRF. In addition, SPA uses radioactivity and not as sensitive as HTRF. However, HTRF uses non-radioactivity, which leads to be safer.

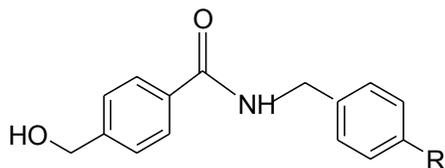
Advantage of filter paper radioactive method is high sensitivity and it is called gold standard or traditional assay. Disadvantage: we will be exposed to radiation. Not good for high throughput screening. We have used ELISA and HTRF methods although

radioactive methods can be useful too. We avoided of using radioactive method since we did not want to expose ourselves to radioactive rays.

Chapter 6: Results and Discussion

Results and Discussion:

HMBA (15) is a type of resin, which is a supporter for SH₂, directed peptide inhibitor and its structure can be seen below. Moreover, this resin is a base labile and stable to TFA and peptide release can be affected with a variety of nucleophiles to generate peptides with various C-terminal carboxy modifications.



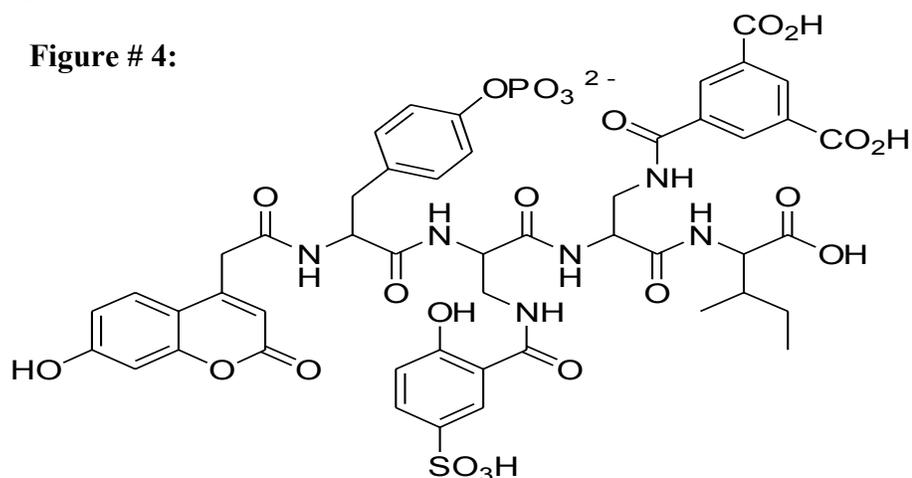
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HMBA resin

The side chain of DAP called ADPOC was removed by treating with acid, TFA (Tri Fluoro Acetic acid) in di chloro methane (1a-2a). 1, 3, 5-Benzenetricarboxylic acid was added with the activating reagents including HBTU and NMM after removal of ADPOC (3a). Then the side chain of DRP was removed using 50% Tri Fluoro Acetic acid in di chloro methane (4a-5a). 5-Sulfosalicylic acid dihydrate was added with the activating reagents such as HBTU, HOBT, and NMM (6a).

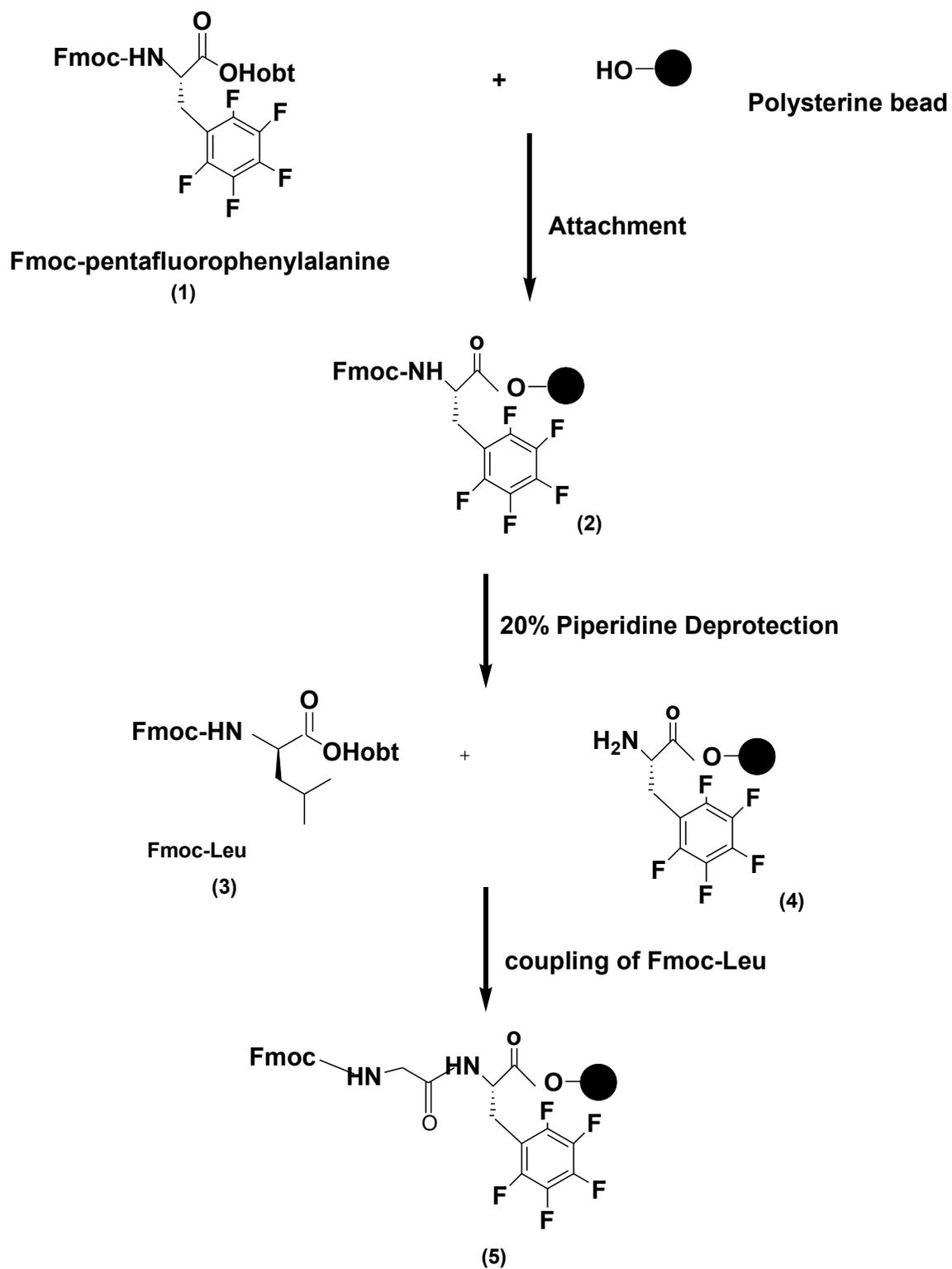
The purpose of adding the activating reagents is that these reagents activate the carboxyl group so that nucleophile can attack since carboxyl group is not that reactive by it self. After adding the sulfuric acid, 3-Bromopropylamine hydro bromide (8a) was added in order to complete the SH₂ directed peptide inhibitor (9a). The resulted SH₂ directed peptide inhibitor (9a) was purified in HPLC.

5-Sulfosalicylic acid dihydrate was added to the active site sequence such as H₂N-(GABA)₆-EELL-(F₅) Phe-RINK (10a-11a) and this sequence was synthesized in a linear fashion using solid phase peptide synthesis. Then the sulfur, which is more electronegative and therefore good nucleophile that attacks the SH₂, directed peptide inhibitor. More specifically the nucleophile attacks carbon that is been attached with the bromine and the bromine leaves since it is a better leaving group. Now, the desired and more potential inhibitor has been obtained after cleaving the peptide from the resin and purified by HPLC (13a). Following is the sequence of the higher affinity inhibitor, which has been synthesized based on library scan strategy. Library scan strategy replaces specific residues in a consensus peptide sequence. Residues of coumarin, Dipropionic acid and Drp, the following new SH₂ directed peptide binds to a site with a 3300 fold (Yeh, R., Lee, T., and Lawrence, D.S. 2001) whereas the previous SH₂ sequence binds to a site with only a 250 fold.

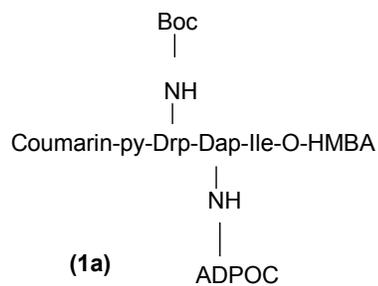
Following residues of new SH₂ directed inhibitor participate in high interactions with residues on SH₂ domain. Coumarin alone displays 37 fold higher affinity to the site. The reason for choosing DAP rather than some other residues containing diamine was that the side chain of the DAP is short and therefore it can limits the conformational mobility of substituents.



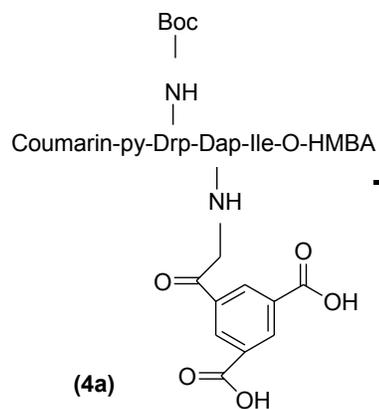
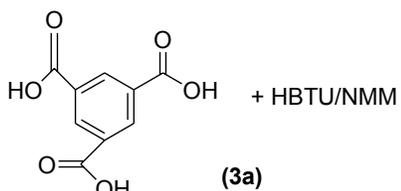
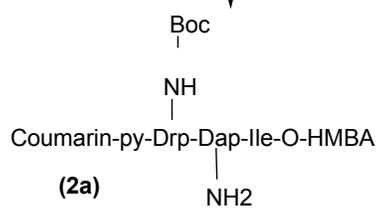
Scheme# 1



Scheme # 2:

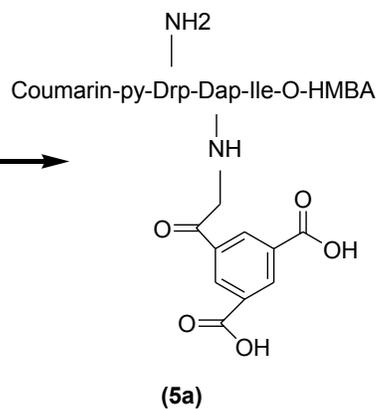
Boc
|

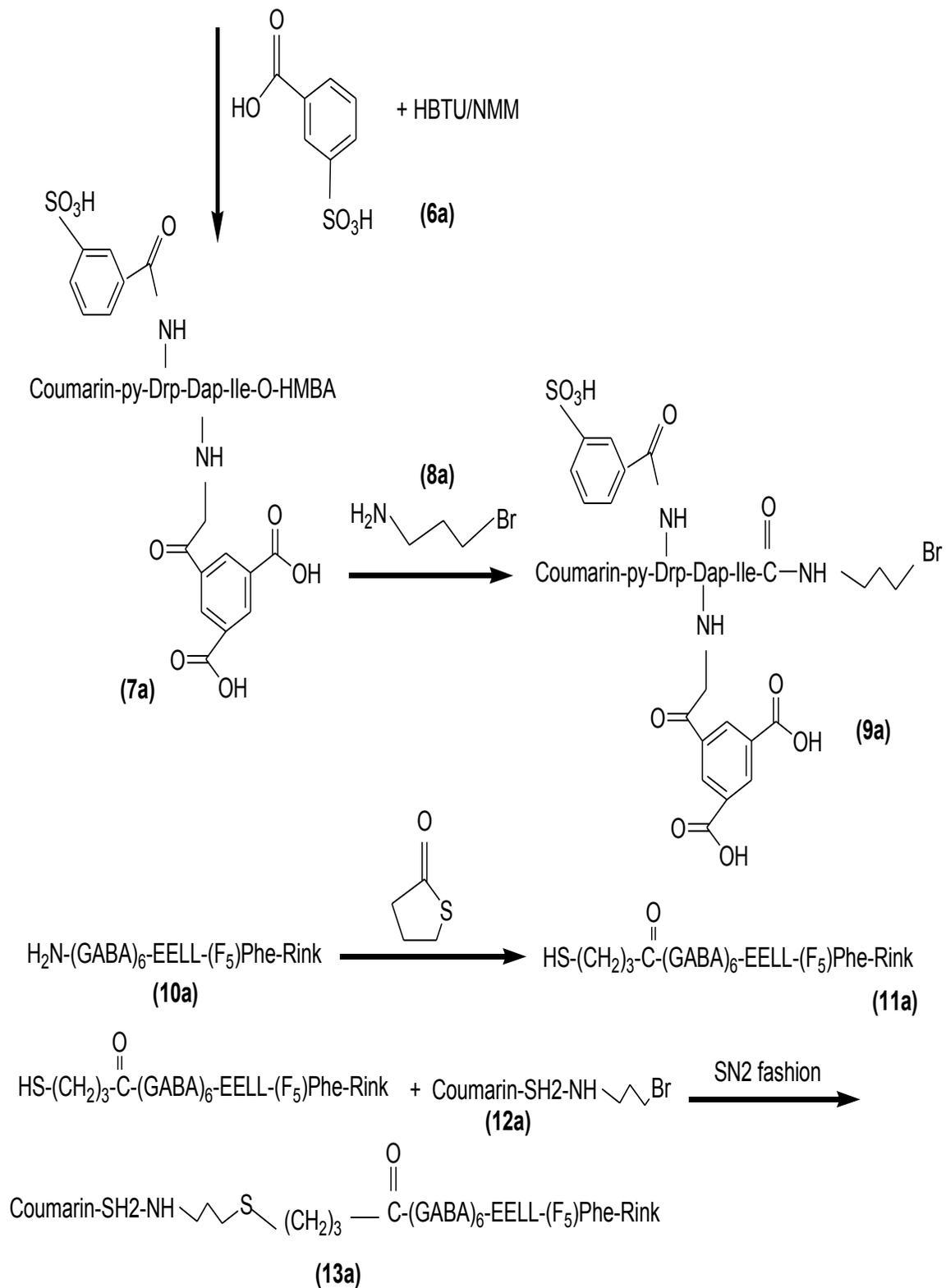
3% TFA in DCM



50% TFA

DCM





Peptide that is synthesized via older approach was determined to work at 250 fold (1-5). Active site directed peptide was cleaved from the resin by coupling Hydrazine monohydrate to the peptide instead of 3-Bromopropylamine hydro bromide (8a) since we did not observe any absorbance peak of coumarin on HPLC. This may be due to bulkier group of 3-Bromopropylamine hydro bromide (8a) relative to hydrazine. We made sure the cleavage of peptide from the resin in HPLC where we observed a peak at absorbance of 330, which refer to coumarin. However, due to insufficient time, we did not able to test the peptide (scheme # 2) on enzyme.

Chapter 7: Conclusion

Conclusion:

Under normal conditions, Src is only active at certain times. However, mutated Src kinases that are missing tyrosine 527 are active all the time. This leads to several cancers and diseases including Colon, Breast Cancers, Arthrosclerosis, Psoriasis, and Restnoises. Bivalent inhibitors are inhibitors that bind to two different sites such as SH2 and the active site (protein binding site). Again, SH2 domain interacts with phosphor tyrosine. The peptide that is synthesized via new approach (scheme # 2) has a higher affinity for both SH2 and active site domain although we do not have enough time to test it on enzyme. In other words, we had insufficient time to perform an enzyme assay. One of the reasons for high affinity is because the side chain, Dap is a short side chain, which limits the conformational mobility of substituents, attached the side amine moiety (S.D Lawrence, 2001). Furthermore, this peptide is promising to be the tightest binding peptide based ligands described for any protein-protein interaction site.

Chapter 8: Procedure

Experimental:

The following is the sequence of a bivalent inhibitor that I made: coumarin-pTyr-Glu-Glu-Ile-Glu-(GABA) 8-Glu-Glu-Leu-Leu-(F5) Phe (scheme # 1, older approach). I made the substrate such as tryrosine-glu-glu-glu-ala in order to do the enzyme assay. I cleaved it from the resin using 95% TFA since the peptide is base labile meaning stable to acid and can be removed with mild base and then purified on HPLC. The inhibitor was tested on Src kinase to determine if it is more potent than those inhibitors previously reported. IC_{50} represents the inhabitation of enzyme by 50%. According to the experiment, the active site control peptide is a poor inhibitor. More over, inhibitory potency increases as a function of tether length and reaches a maximum at eight GABA units. We also found out that the inhibitor worked 230 fold better with coumarin.

The assay that has been performed is ELISA method (Figure #5) to detect the tyrosine kinase activity. This ELISA method includes obtaining a 100% activity of the substrate using ATP (10 μ l of 50mM in Tris), substrate (10 μ l), enzyme (10 μ l), and cations (Mg^{2+} & Mn^{2+}). Moreover, 0% activity of the substrate all mentioned above except the substrate also obtained. Obtain IC_{50} using fixed concentration of ATP, substrate, enzyme, and VARYING concentration of inhibitor and therefore diluted inhibitor by 2 fold. For instance, 2.5mM inhibitor solution was prepared by taking 41.7 μ l from the stock solution (6.03mM of inhibitor) and dilute it with Tris (58.3 μ l). Then 25 μ l was taken from 2.5mM inhibitor and transferred to another tube, which was labeled as tube #2. This is how 2 fold dilutions were performed until tube #9. Therefore, the trend among the varying

concentration of inhibitor and IC_{50} was able to observe. Potent of inhibitor increases as IC_{50} decreases.

Phosphorylated substrate binds to the plate. Observation of blue color once the antibody that links to HRP binds to the substrate and later yellow color (Figure # 5) was observed once 1M H_2SO_4 was added. Pale yellow color and low absorbance will be observed in the presence of inhibitor. In HTRF method, Kinase phosphorylates the substrate. HTRF uses europium labeled anti-phosphotyrosine antibody to detect phosphorylation. The energy donor and the energy acceptor interact with the phosphorylated substrate. This leads to energy transfer between each other when the energy donor is excited at 337nm. Signal is detected when the energy donor (Europium) and the energy acceptor (allophycocyanin) are in contact. Signal at 665nm energy transfer corresponds to the amount of phosphorylated product.

Illustration of ELISA method can be seen in Figure # 5:

Figure # 5:

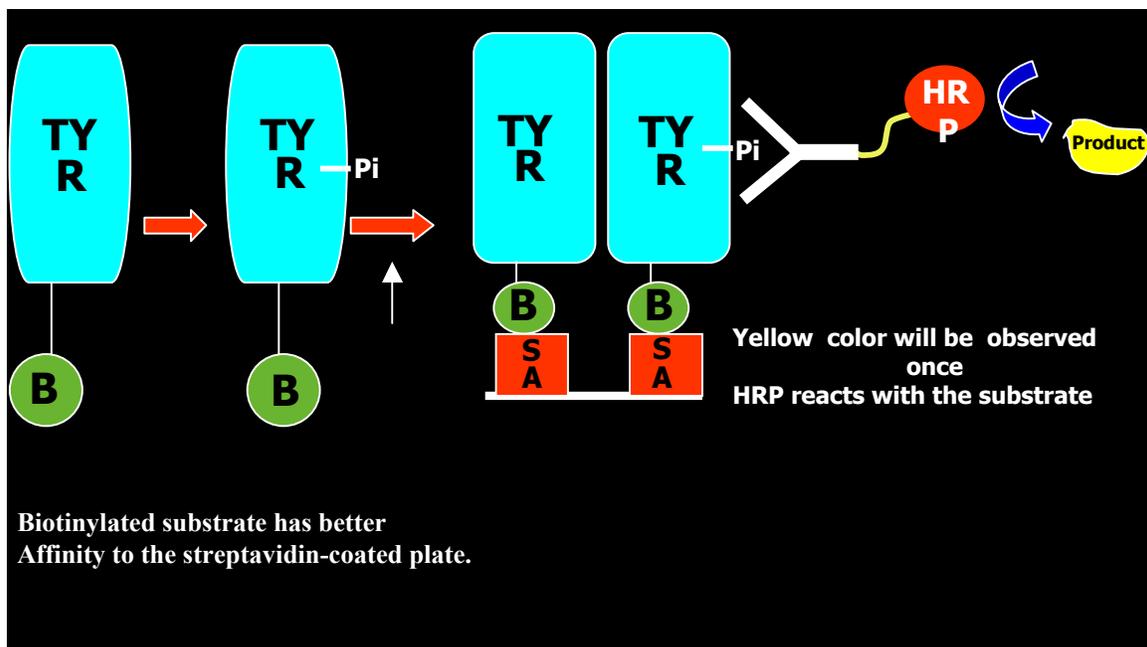
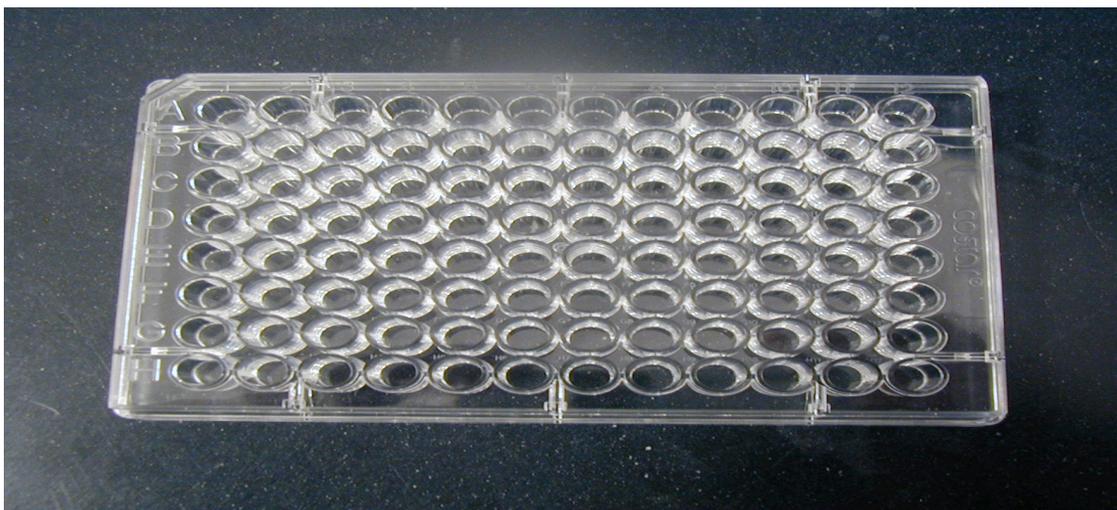


Figure # 6 is the picture of a micro titer plate, which contains 96 well where each can hold one compound. Assay involves ELISA method is very sensitive since only $20\mu\text{L}$ of sample is enough to run the assay. Certain assays require only $3\mu\text{L}$. There is even micro plate titer that contains 1536 wells, which can detect 1536 compounds at once. Therefore, this micro titer plate can detect many compounds and thus save time.

Figure #6



Closer view of the micro titer wells.



Figure # 6 & 7 are the pictures of a micro titer in a victor plate reader.

Figure # 6:

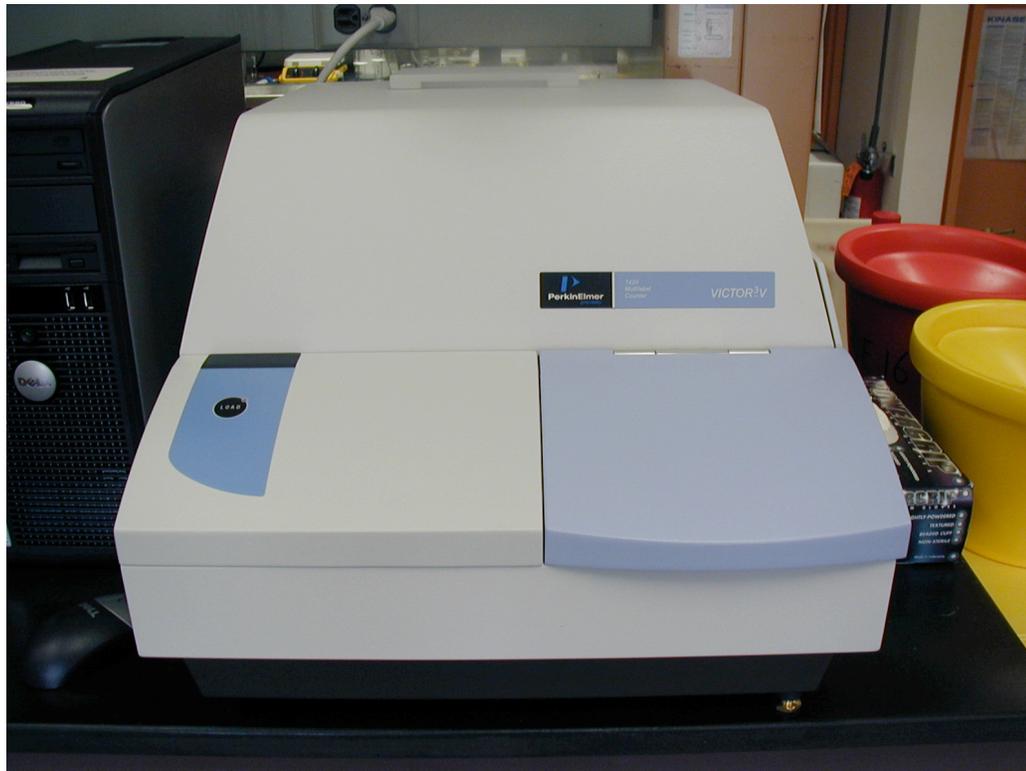


Figure # 7:



Chapter 9: Places where chemicals are obtained

A. General:

All the chemicals were purchased from Aldrich, Sigma and Advanced Chemtech.

Chapter 10: Principles of Peptide synthesis

B.Synthesis:

The new high affinity inhibitor has been made using solid phase synthesis (Figure# 4, Scheme # 2). The solid phase synthesis has several advantages including simplicity, speed, efficiency, and convenience. More over, it avoids excess purification during each coupling and deprotection steps. In addition, in solid phase synthesis, the by products that form during each coupling and deprotection process are washed using the solvents (DCM & DMF). In addition to washing the by products, it's easy to separate peptide products from other reagents and the resin.

During the coupling reactions, high solubility of intermediates forms. The basic procedure for this peptide synthesis is to attach the amino acid to an insoluble resin, which acts as a supporter. The resin protects the polypeptide chain during synthesis and keeps it insoluble. After adding the first amino acid to the resin (1), it is necessary to quantitate how much amino acid is on the resin. We can achieve this by monitoring the Fmoc cleavage at 301 nm.

Next, excess free amines are blocked using acetic anhydride and NMM. To make sure capping is complete, we do a Kaiser test. Clear beads indicate that all amines are modified (no primary amino acid). Blue beads indicate incomplete coupling (reveals the presence of primary amino acid). We made our desired peptide using FMOC chemistry, which is base labile and acid stable. FMOC uses T-Butyl ethers and esters for

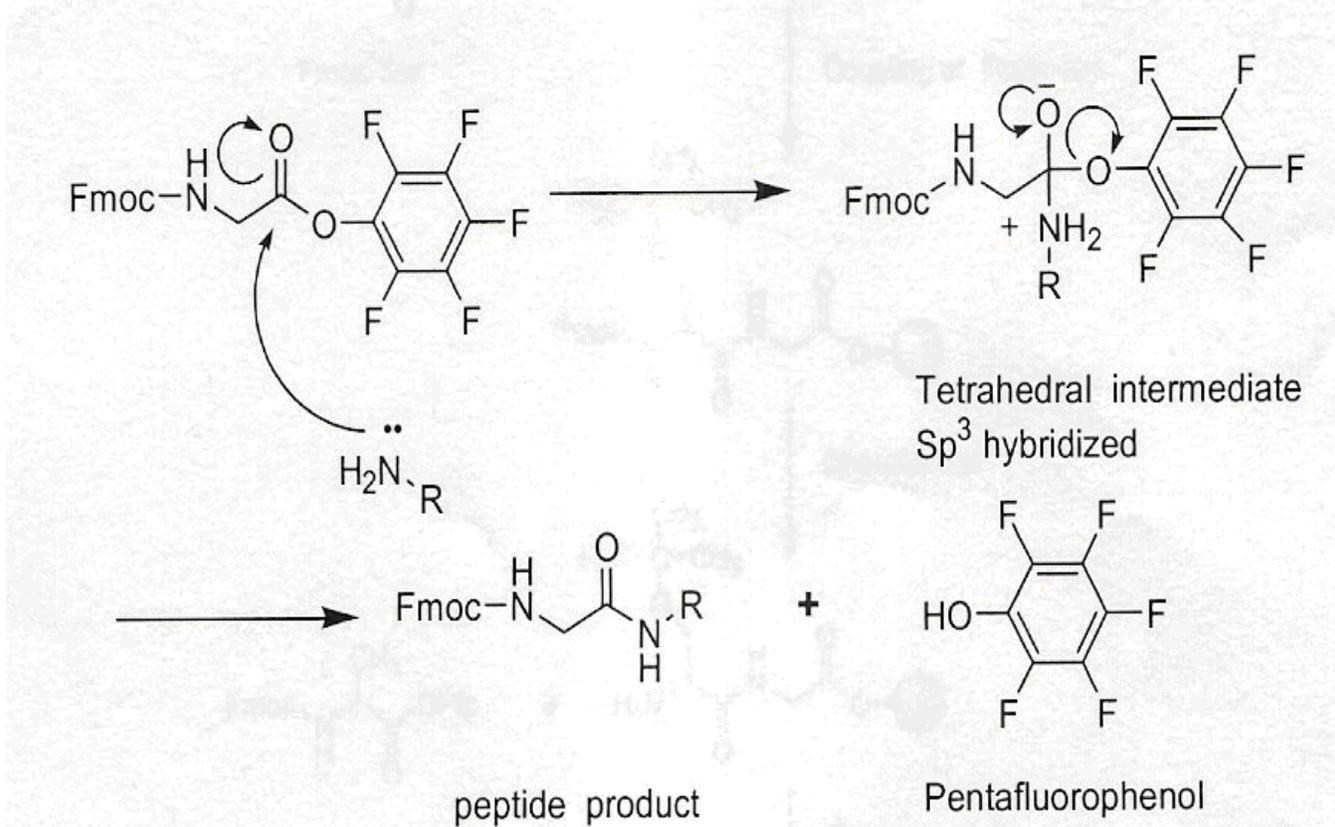
side chain. One cycle of peptide synthesis consists of removing the Fmoc group and coupling of the desired amino acid. We remove the Fmoc group with 20% piperidine (step 1-2, Scheme # 1) in DMF. A Kaiser test is then performed. The presence of dark blue beads indicates the presence of free amines. By adding this 20% piperidine solution (base), we only remove the Fmoc group and prevent other protecting groups and linkage from leaving the resin.

The desired amino acid is then activated with HBTU, HOBT and NMM and coupled to the resin. Amino acids were coupled by adding 3 equivalents HBTU, 3 equivalent HOBT, 3 equivalent amino acid, and 9 equivalents NMM with the solvent DMF and add to the resin. Then shake it for about 2hrs. Kaiser test indicated if coupling was complete. Finally the polypeptide is placed into a solution of TFA to cleave the linkage to the solid support and remove all side chain protecting groups.

As we can see the coupling mechanism # 1, the OH of the carboxylic group has been replaced with the electron-withdrawing group since these are better leaving group than the OH. In addition to better leaving group, these compounds react well toward the nucleophile.

Mechanism # 1:

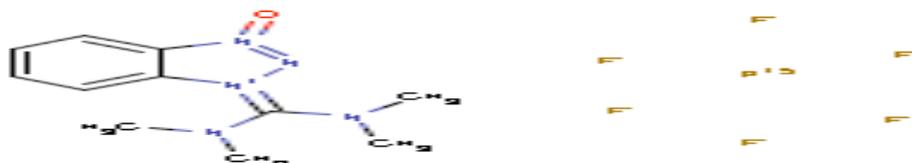
Coupling of an amine to the Pfp ester of glycine.



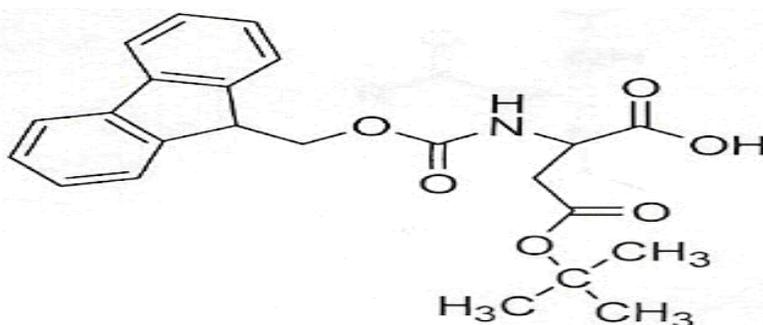
Following is the activated derivative of carboxylic acid that has been used in the peptide synthesis since the carboxylic group is unreactive toward amine which is a nucleophile. HOBT ester is an active ester that is one of the reagents used in the peptide synthesis. HOBT stands for 1-Hydroxybenzotriazole.



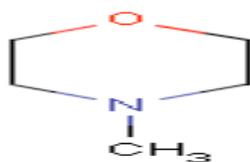
Following is an activated reagent called HOBT which stands for 2-(1H-Benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate. In other words, it is an activator for peptide synthesis.



The side chain of the amino acid is protected with t-butyl esters in Fmoc chemistry and it is illustrated below. This side chain protecting group can be cleaved with TFA.

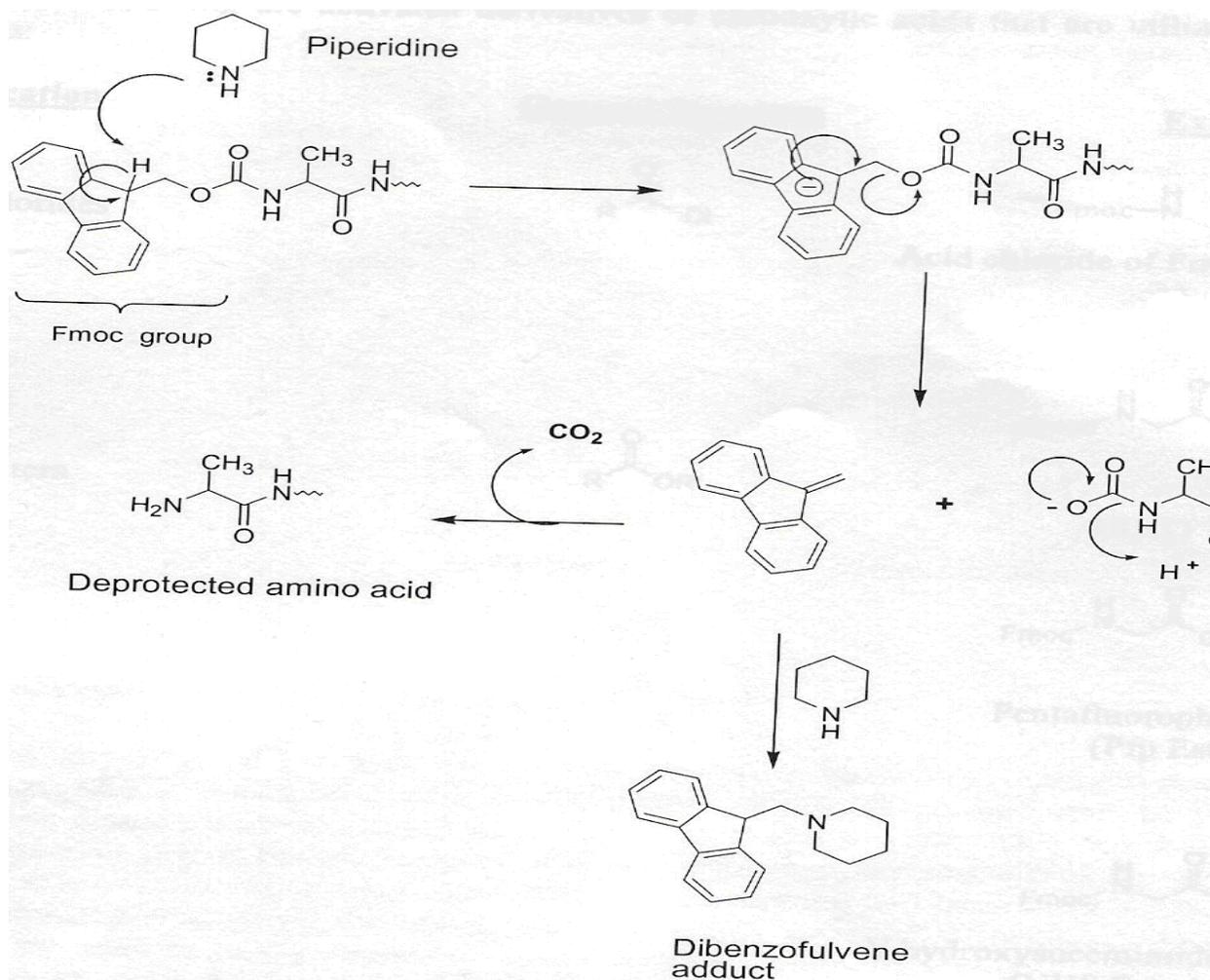


Below is the structure of N, Methyl Morpholine (NMM), which acts as a nucleophile (base).



Mechanism # 2 depicts the deprotection mechanism or removal of fmoc, which protects the primary amine and fmoc can be removed using 20% piperidine.

Mechanism # 2:



The overall approach to solid phase peptide synthesis is shown above where the first amino acid is attached to a bead called resin. Amine would be deprotected to give the free amine. Then another amino acid will be added to produce a dipeptide and then the second amino acid will be deprotected. Then tripeptide, tetrapeptide, and the peptide chain are extended in this manner until the target peptide is complete. The resin will be systematically washed and filtered after each coupling and deprotection in order to

eliminate the byproducts and impurities. Finally, the desired peptide can be obtained by simply cleaving it from the supporter, resin using 95% TFA.

Chapter 11: New approach

B. New approach in synthesizing a potent inhibitor:

Scheme #2 is a new approach to synthesize a potent inhibitor. This new schematic approach differs from the older approach (Scheme #1). For instance, the sequence of SH2 has synthesized separately, sequence of active site has synthesized separately, and finally both sequences are combined to form thioether product. However, according to the older approach, the all the amino acids are coupled in a linear fashion. Reasons for synthesizing the inhibitor via new approach was that we are treating with 50% of TFA to remove the Boc, which is a side chain of the DAP since Boc is an acid labile and stable to base. However, if we synthesize via older way, then when we treat with 50% TFA, then the peptide would be cleaved from the resin.

We can observe below, that 3% and 50% of TFA was used to remove both the side chains of Drp and DAP respectively. Then 1, 3, 5- Benzenetricarboxylic acid was coupled with HBTU and NMM. 5- Sulfosalicylic acid was added. Hydrazine monohydrate was coupled to the active site directed sequence. Ester was added to the active site directed sequence by stirring with the sequence so that the cyclo sulfur ester would attack the ester via Michael addition. Finally the SH2 sequence was coupled with the active site sequence, which was treated with cyclo sulfur ester.

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