SYNTHESIS OF AN ARGININE MIMIC FOR AN ANTIFUNGAL OCTAPEPTIDE LIBRARY

This thesis is presented in partial fulfilment of the requirements for the degree of Master of Science in Chemistry at Massey University, Palmerston North, New Zealand

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ABSTRACT

A family of bioactive antifungal octapeptides has been established[1-3]. The template for this library is based on peptides composed of two predefined amino acids, three arginines, and three unknown components. The arginine contains a guanidino group (pKa =12.48) which is readily protonated under physiological conditions, and hence can bind to fungal cell walls which possess a negatively charged surface. Other components of this peptide are used to kill the fungi by inhibition of their sodium transport system[1-5].

To date this story has survived on the fact that D-amino acids work best. However both D and L arginine derivatives are quite expensive. In collaboration with the research group in the University of Otago, it was planned to develop an effective and economic method to produce a series of compounds to replace arginine. These mimics were designed to be artificial amino acids as NH_2~~N*(R')~~COOR where the side chain R' carries a guanidino group. Thus the chiral carbon is now replaced by an amide group hence no stereocenter, no enantiomers but the guanidino group will retain a high pKa similar to that of the natural arginine. For the synthetic strategy, the target molecule was divided into two parts, one the amino acid backbone, and the other the guanidine containing side chain. Each segment was built up separately and finally combined together.

The backbone of the arginine mimic must possess two amino groups. One is at the N-terminal for the next step in peptide synthesis, the other one (N*) is used to connect to the guanidino containing side chain. This was reacted with selected acetate derivatives (*t*-butylchloroacetate, benzylchloroacetate, methylbromoacetate) that had potential to produce the desired backbone in good yield.

Construction of the side chain (R') to be composed of a di-protected guanidine and a carboxylic group was the most challenging and difficult part of this project. To achieve this, two different approaches were studied. One was to use a primary amino acid NH_2-$(CH_2)_x$-COOH (X=1,2,5) to react with a guanylating reagent to make the unprotected side chain, then two protecting groups were added to the two nitrogen containing groups of the guanidine. The other method was protection of a guanylating reagent (usually carboxamidine compounds) then reaction with a primary amino acid to make a di-protected guanidine containing side chain.

In amino acid and peptide chemistry, to avoid self condensation and by-product formation, selectivity of amino and carboxylic groups is very crucial hence application of different types of protecting groups are the basis of peptide construction. Therefore in this project, employment of suitable protecting groups at both N- and C-terminals was incorporated in this study.

Finally, one arginine mimic *N*-[*N'*-((9-fluorenyl)methoxycarbonyl)-2-aminoethyl]-*N*-*t*-butyloxycarbonylmethyl-3-*N',N''*-bis(*t*-butyloxycarbonyl)carbamidinopropanamide was successfully built up. It will be used for the construction of octapeptides for the current antifungal programme. The final product will be sent to University of Otago for bio-activity tests.

ACKNOWLEDGEMENTS

Firstly I would like to record my appreciation to Associate Professor David R. K. Harding as my research supervisor for his guidance and assistance in helping me complete my Masterate and opening the door to peptide chemistry.

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LIST OF ABBREVIATIONS

Arg	arginine
Boc	*t*-butyloxycarbonyl
$(Boc)_2O$	di-*t*-butyl dicarbonate
Cbz	benzyloxycarbonyl
$(Cbz)_2O$	dibenzyl dicarbonate
Cbz-OSu	benzyloxycarbonylsuccinimide
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DEAD	diethyl azodicarboxylate
DIEA	diisopropylethylamine
Fmoc	9-fluorenylmethyloxycarbonyl
Fmoc-OSu	*N*-(9-fluorenylmethoxycarbonyloxy)succinimide
HBTU	O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate
ortho-Br-Cbz	ortho-bromobenzyloxycarbonyl
ortho-Cl-Cbz	ortho-chlorobenzyloxycarbonyl
PNA	peptide nucleic acid
PPh_3	triphenylphosphine
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid

CHAPTER ONE
INTRODUCTION

1.1 A D-Octapeptide Combinatorial Library

The rapid development of resistance to current anti-infective, antimicrobial drug therapies requires innovation in the pharmaceutical and chemical research community. A perfect antimicrobial drug must contain two components. One is the drug delivery system which can locate the microbial cell targets and not the human host cells. The second component is destroying target cells by inhibiting metabolic pathways or by other routes. Peptides, due to their bioactivity and potential to overcome drug resistance of microbials have provided an increasingly important research focus in this regard[1,2].

Unlike the normal cells in the human host, fungal cell walls contain an abundance of negatively charged species[2]. This feature provides biochemists and chemists with a good basis for drug design that will lead to pathways to new drug delivery systems. If the drug molecule possesses a positive charge, it will easily bind to the surface of negatively charged cell walls of fungi and bacteria. To achieve this, a D-octapeptide combinatorial library was built[6]. It was synthesized manually using solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. This combinatorial library is composed of 324 peptide pools (each theoretically containing 5,832 separate peptides) with all peptides in theory being present in approximately equimolar amounts. Each peptide can be represented by the formula D-NH_2-$ABX_3X_2X_1RRR$-$CONH_2$ where, A and B are known and X represents any of 18 amino acids. Cysteine was excluded because a cysteine-targeting sublibrary had been constructed and glycine was excluded because it lacks a side chain (Figure 1). The three R groups are D-arginines which carry positive charges that can locate the entire peptide on the negatively charged fungal cell wall. The most active peptide library pools were identified by bioassay. The most suitable pools were deconvoluted by cycles

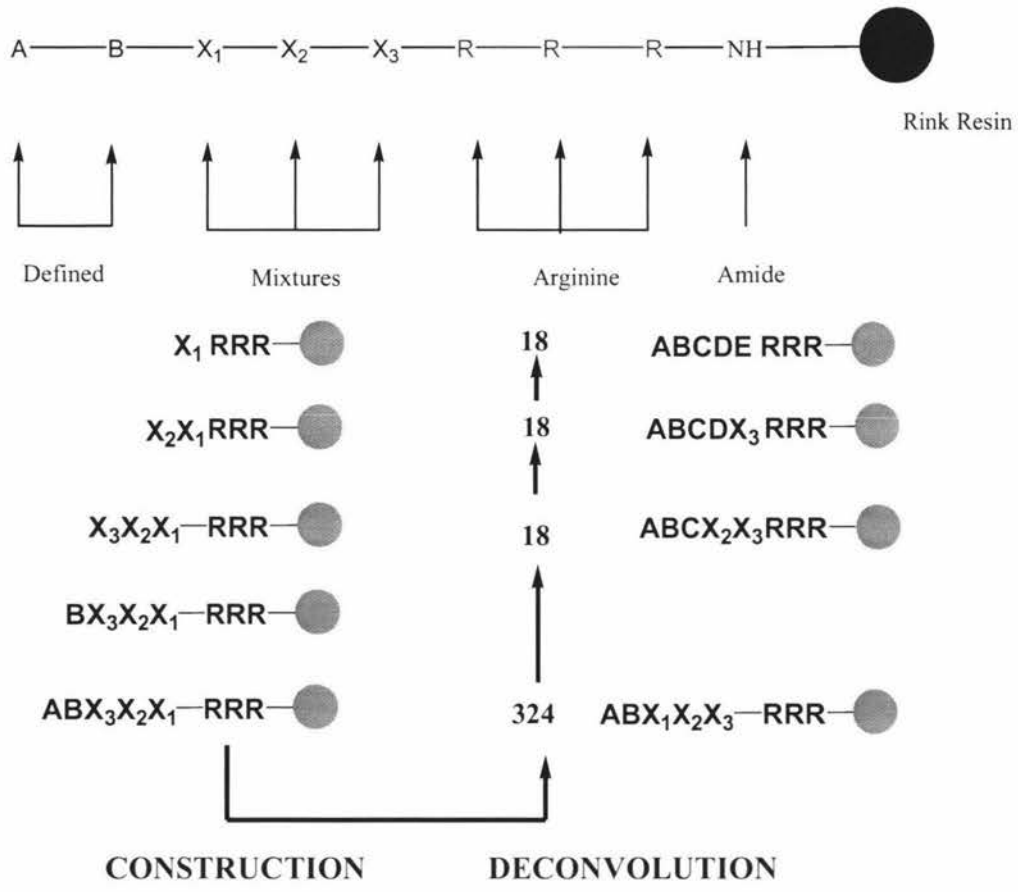
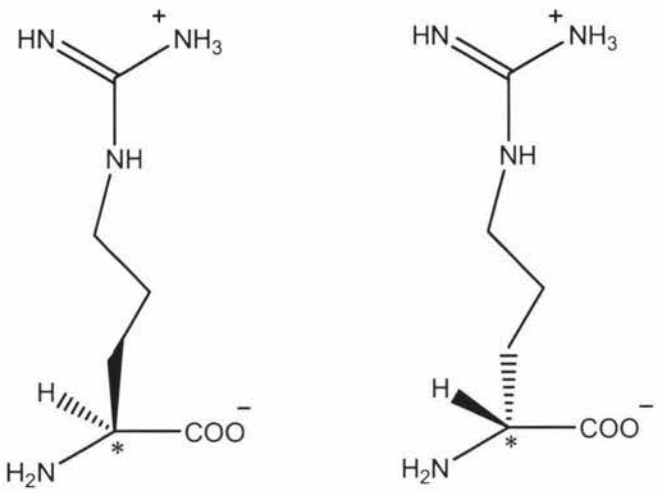


Figure 1. Designed D-octapeptide and the combinatorial library.

of resynthesis and bioassay to sequentially determine the optimal amino acids present at position X_3, X_2 and X_1. Finally the most potent peptides identified were manually resynthesized and purified for biotesting.

1.2 Natural Arginine and the Guanidino Group



L D

Figure 2. Structure of natural L and D arginine.

Arginine (symbol Arg or R) is an α-amino acid (Figure 2). It contains a chiral carbon (C* in Figure 2) and hence has a pair of enantiomers. The L form is one of the 20 common natural amino acids in mammals. It has numerous functions in the mammalian bodies. It aids disposal of ammonia, is used to make compounds in the body such as nitric oxide, creatine, L-glutamate, L-proline and can be converted to glucose and glycogen if needed. Arginine is a basic amino acid with a guanidino group which plays a significant role in arginine's chemical properties.

A typical guanidino group is composed of three nitrogen containing groups (two amines and one imine). Because of the conjugation between the double bond and the nitrogen lone pairs, the positive charge can be delocalized to form a stable resonance structure with a pK_a of 12.48. Thus the guanidino group can be positively charged in neutral, acidic and even strong basic environments to bind a single, monoacidic cation to form a guanidinium ion (Figure 3). The basicity of the guanidino group (pKa 12.48 for the guanidino group of arginine) is extremely high compared to the pKa's of other ammonium ions (pKa: from 8.80 of asparagine to 10.60 of proline). This creates the basis for specific intermolecular interactions that comprise key steps of many

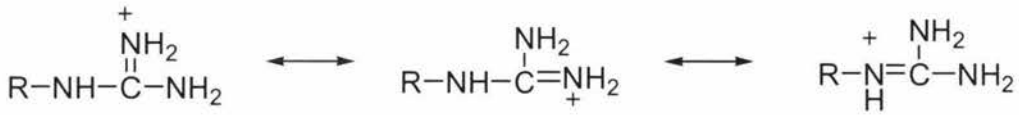
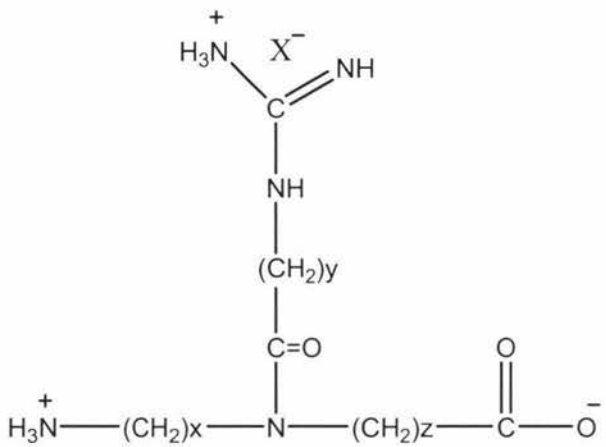


Figure 3. Resonance structures of guanidine.

biological reactions including enzyme-mediated processes and interaction of hormones with their receptors. Due to this unusual property, the guanidino group has attracted considerable attention recently[7-13]. For example, apart from applications in the pharmaceutical arena, scientists found that the guanidino group of arginine can act as a general base and play a significant role in enzyme-catalyzed proton abstractions[13].

Although the importance of arginine has been extensively reported and now it is widely used in chemical, biological and medical research, the high price limits its application. Compared with L-arginine which can be made by aerobic fermentation, D-arginine used in the combinatorial library system can not be obtained directly from natural sources. So the price is even more expensive. This prompted Associate Professor David Harding and his group at Massey University to look for other ways to synthesize an arginine mimic at reduced cost but that would retain the basicity and bioactivity of arginine. In addition, this project sought to look at an achiral mimic.

1.3 Structure of a Designed Arginine Mimic and the Synthetic Strategy



X^- = an anion

Figure 4. Structure of the target arginine mimic in this project.

The designed compound in Figure 4 contains N- and C-terminals as for normal amino acids. The side chain contains a guanidino group that should give similar chemical properties to those of natural arginine, hence an arginine mimic. The main difference between this designed compound and arginine is the achiral connection point of the backbone to the guanidino side chain. In this mimic, the backbone and guanidino side chain combination is composed of an amide bond and hence there is no enantiomer.

The basic strategy for the synthesis of these arginine mimics was to separate this molecule into two parts. The backbone to contain the N and C termini desired for peptide synthesis. A secondary amine in the backbone of this molecule was selected as the connection point with the side chain (Figure 5). The backbone was to be constructed by reaction of ethylenediamine with an α-haloacetate[14]. The side chain comprised a guanidino group designed to make the entire molecule carry out similar biological functions as those of natural arginine. A carboxylic acid group on the

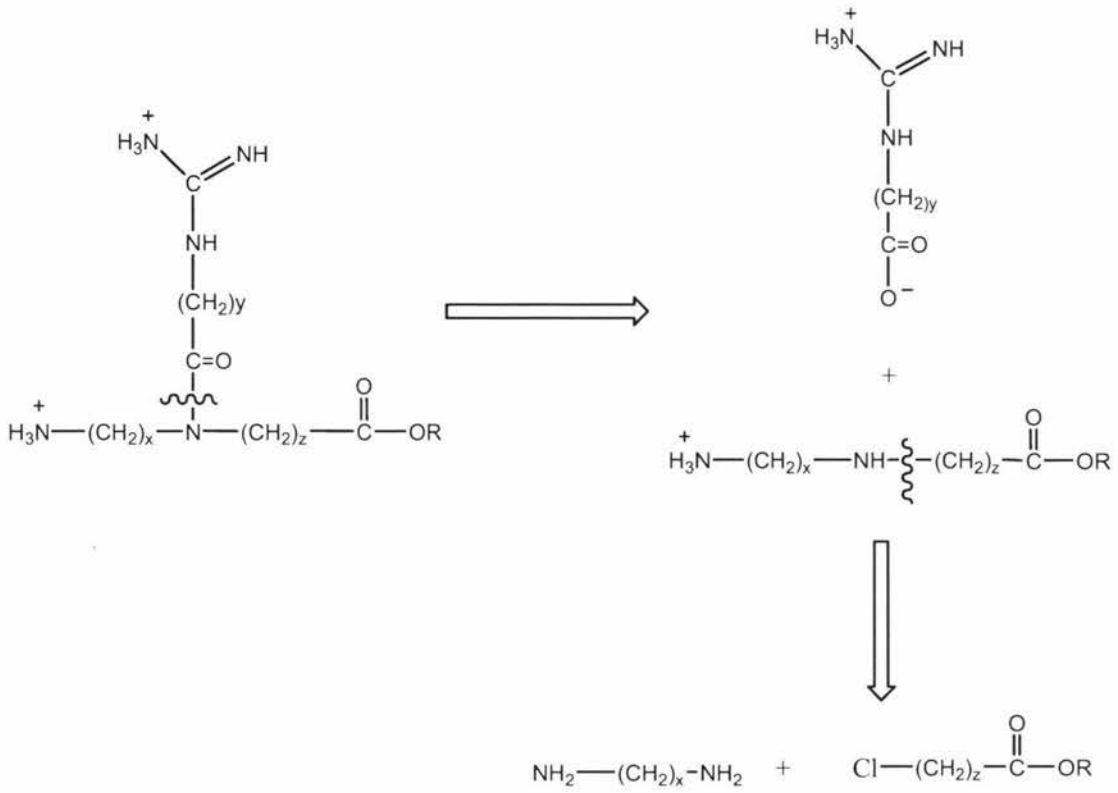


Figure 5. Synthetic strategy of arginine mimics.

chosen guanidino side chain was selected to form an amide bond with the secondary amine on the backbone. This carboxylic acid containing guanidino compound was to be built up by reaction of carboxamidine compounds with a primary non chiral amino acid. In this project, three amino acids were chosen: glycine, ß-alanine and 6-aminohexanoic acid (Figure 6).

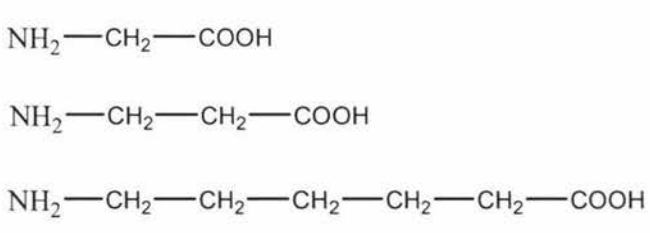
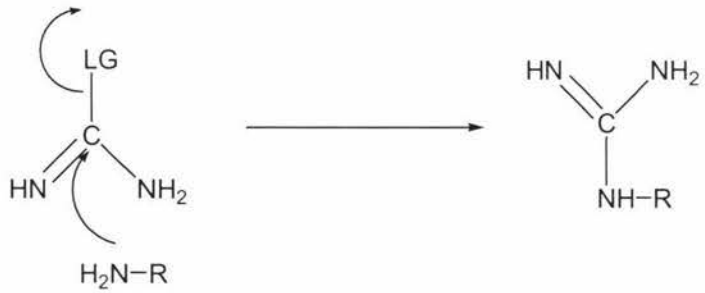


Figure 6. Structure and molecular weight of glycine, ß-alanine and 6-aminohexanoic acid.

After these two parts were produced separately, the carboxylic acid group of the side chain was to be activated with a coupling reagent (HBTU or DCC). Then it was to undergo a nucleophilic substitution with the secondary amine group in the middle of the backbone to form a new amide bond.

1.4 Strategy for the Synthesis of a Protected Guanidino Group

There are a number of different reagents that have been discovered and used to produce guanidino groups[15]. Their common characteristics are two free or protected amine groups and a leaving group (LG) connected to an amidino carbon (Figure 7). The normal process for formation of a new guanidino group is a nucleophilic attack by an amino group to the central carbon atom of the guanylating reagent.



LG= Leaving Group (SCH_3, SO_3H, pyrazole etc)

Figure 7. Structure of a typical guanylation reagent and the normal process of formation of a guandino group.

An ideal guanylating reagent must possess the following two features:

A. It should be stable in a pH range that will produce desired guanidine targets.
B. The leaving group must leave rapidly.

In order to avoid self condensation or side reactions in the coupling steps used in peptide synthesis, suitable protection of the guanidino groups is necessary.

Considering coupling reaction conditions, selectivity of protecting groups is crucial. In other words, a protecting reagent must be found that is easily attached to the guanidino group, yet is resistant to the strict reaction conditions in the following steps of peptide synthesis. Finally it must be easily removed. To make the di-protected guanidine compounds, there are two available routes: A. formation of guanidino group first then addition of protecting groups; B. protection of the starting reagent then reaction with primary amino acid to form the protected guanidino group.

Compounds containing carboxamidine functionality have now been widely utilized in guanidine synthesis. The general formula for the carboxamidine compounds is $RC(=NR_1)NR_2$ (R = Leaving Group, R_1 and R_2 =H or alkyl groups). Besides this group of compounds, thiourea and its derivatives meet the requirements of this project hence were used as well:

a. Group 1, carboxamidine compounds including pyrazoles[16] and sulfonic acid derivatives[17]. For this kind of reagent, the leaving group is adequate for efficiently conversion to the desired guanidines within a short period of time and under mild conditions. Their application will be introduced and discussed in Chapter Three.

b. Group 2, thiourea and its derivatives[18-23]. Their reactivity with primary amines or amino acids is low because of the poor leaving group or the strong carbon-sulphur double bond. Therefore Group 2 is not widely used in the preparation of unprotected guanidine compounds. However, some researchers mentioned that due to the stable leaving group (S and SCH_3) they can be easily protected before the guanylation reaction (with leaving groups that are not destroyed in the basic environment needed for the addition of protecting groups)[18,20]. When these compounds are protected, the electron withdrawing effect of the protecting group can make the central amidino carbon more electrophilic hence increase the reactivity[18-20]. However, even in this case, catalysts or other reagents (e.g. $HgCl_2$ and 2-chloro-*N*-methylpyridinium iodide) are still needed to aid the nucleophilic attack

of sterically hindered amines and amino acids. The details of the application of thiourea and its derivatives in guanylation will be introduced and discussed in Chapter Four.

Apart from these reagents, substituted guanidines can also be synthesized by using guanidine reacted with an amino acid ester through a Mitsunobu reaction. This will also be introduced in Chapter Four.

1.5 Protection of the N-Terminus in Peptide Chemistry

In peptide chemistry, it is customary that functional groups be blocked. For example, if we want to couple the C-terminal of an amino acid A: NH_2-CHR_1-COOH to the N-terminal of another amino acid B: NH_2-CH_2-CHR_2-COOH to produce a dipeptide, then in order to selectively acylate the amino group of B, we have to activate the carboxyl group of A. The activated carboxyl component, NH_2-CHR_1-CO-X, can acylate not only B to give A-B but also some unreacted A to yield the derivative AA. Also, by-products BB or BA can be obtained (Figure 8). To avoid this problem, blocking both the amino group of A and the carboxyl group of B is necessary. This will be discussed in Chapter Three and Five. Here the selection and application of blocking reagents to the amino groups is introduced.

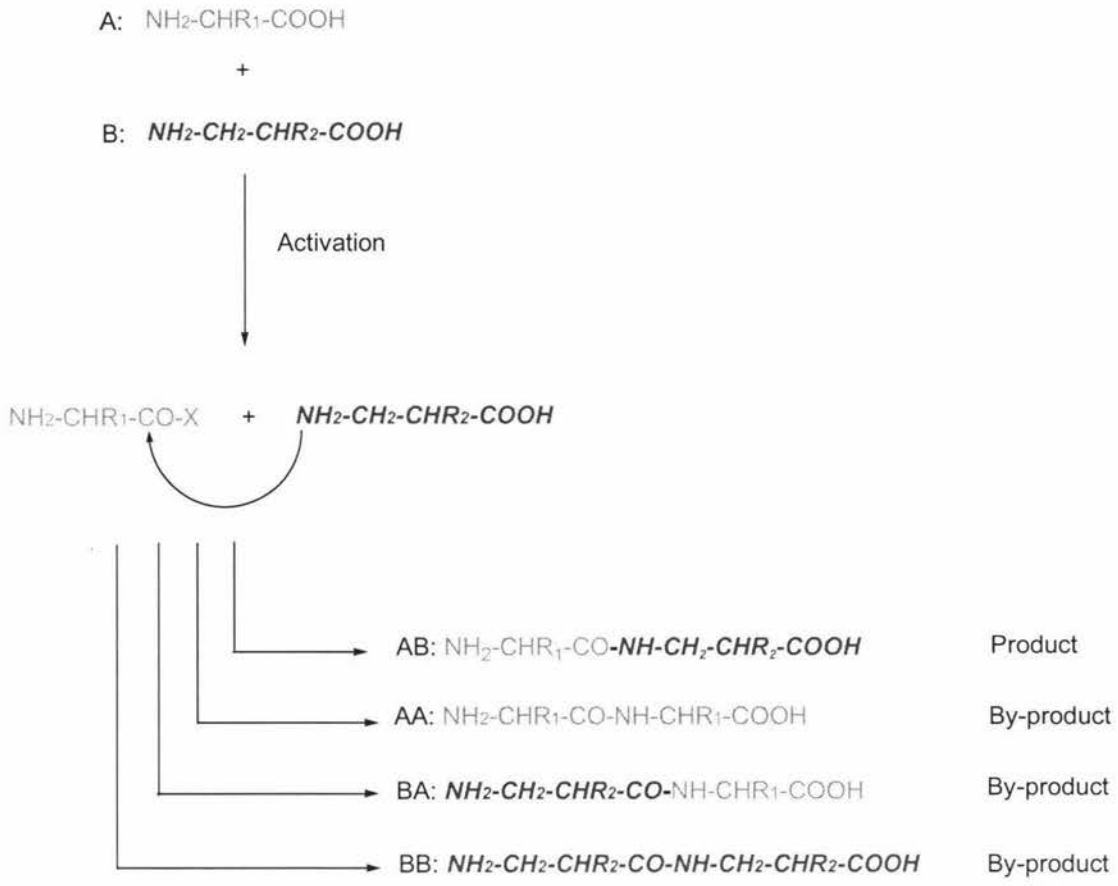


Figure 8. Four possibilities of di-peptide formation using unprotected amino acids.

There are three basic nitrogen species that need to be protected on the target arginine mimic (Figure 9). Two "amino" groups on the guanidine side chain (X), and one α-primary amine on the backbone (Y). Removal of the protecting groups is desirable ideally at different times. After the entire molecule of this mimic is constructed, the R group will be released at first then the arginine mimic can be used for solid phase peptide synthesis. The Y group is then removed for subsequent addition of amino acids. After the entire octapeptide is assembled, protecting group X can be cleaved from the peptide, often at the same time the peptide is cleaved from the resin.

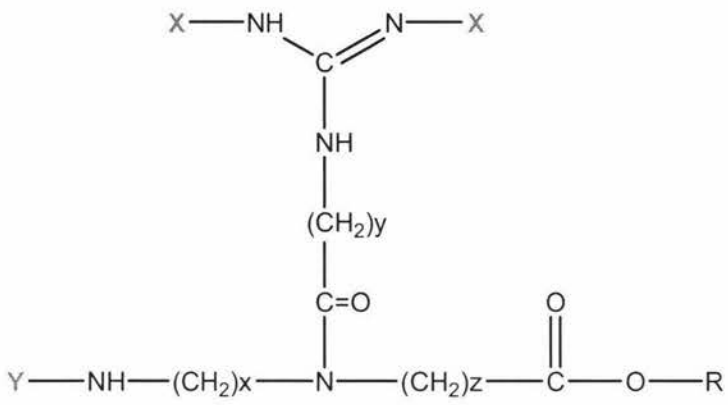


Figure 9. Designed protected arginine mimic

The protecting groups selected for blocking those two (X, Y) positions must of course be different. The X group must survive longer than Y. It should be stable under the conditions for cleavage of Y. For example, if Y can be cleaved in strong acid, X must be inactive in acidic solution. If Y needs a reducing reagent like H_2 to remove it, X should be resistant to this kind of environment.

In the last thirty years, organic chemists have developed many protecting reagents for different natural and artificial amino acids[15,24,25]. Most of them can be classified into four groups:

1. alkyl and alkylidenes,
2. sulphur and phosphorus derivatives,
3. acyl derivatives,
4. urethanes.

Addition and cleavage of alkyl and alkylidenes needs drastic conditions. Only bulky substituents can provide sufficient protection because the product is a secondary amine which also has nucleophilicity hence may interfere with the coupling reaction. Difficulty for formation of alkane imine bonds limits their application. For sulphur and phosphorus derivatives like tosyl group, most of the protection or removal reactions must be carried out in acidic environment, which might destroy other protecting groups like Boc (*t*-butyloxycarbonyl) situated on the N-terminal of

the peptide. Acyl protection, although the amide bond is weaker than alkane amine bonds, still needs strong cleavage conditions. Compared with the above three methods, conversion of free amine group into urethanes is now very popular in peptide chemistry. In this research project, the N-terminal protection focused on formation of urethane derivatives.

The application of urethanes was a very successful innovation in peptide chemistry in the last half of the 20th century[15,24]. Currently most of the popular commercial protecting reagents belong to this class. Compared with other reagents, urethane derivatives possess many advantages, like minimum amounts of by-products, simplicity of preparation, minimal racemization problems and fast cleavages. The protection process is usually a simple nucleophilic addition-elimination reaction. Urethane type reagents in peptide chemistry can be normally classified into three groups. One is removed by base-induced β-elimination and is typically for the Fmoc (9-fluorenylmethyloxy- carbonyl) group. Another is cleavage via acidolysis for Boc (*t*-butyloxycarbonyl) (Figure 10). The third one is the Cbz (benzyloxycarbonyl) group, which can be removed by both hydrogenolysis and acidolysis. The sequence for removing different protecting groups from the

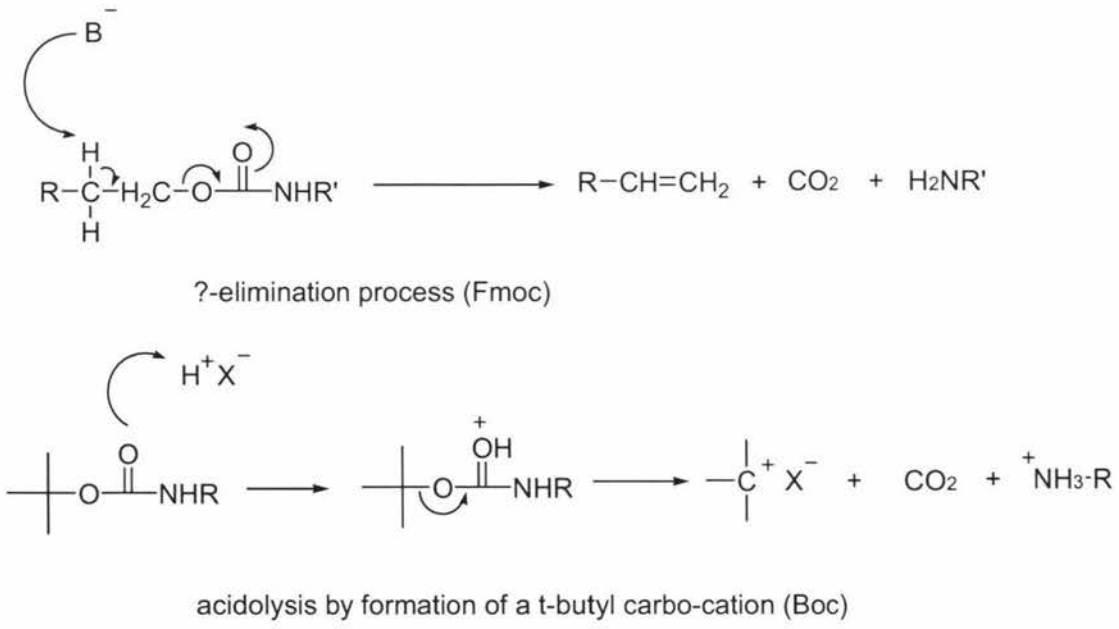


Figure 10. Mechanisms of cleavage of Fmoc and Boc groups.

arginine mimic determines what kind of protecting groups are situated on the guanidine. Because the protecting groups on the side chain guandine should survive until the solid phase peptide synthesis is completed, more stable protecting groups must be selected to make sure it is not destroyed under the conditions of cleavage of the other protected amino groups during the synthesis.

In peptide chemistry, the three most popular urethane type protecting groups[24] Boc, Cbz and Fmoc arise from the di-*t*-butyl dicarbonate $(Boc)_2O$; benzyloxycarbonyl succinimide (Cbz-OSu), *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) respectively. In this project, Fmoc was selected to protect the position Y (Figure 9) on the backbone and will be discussed in Chapter Five. The application of Boc and Cbz will be discussed in Chapter Four. Besides these three protecting reagents, other protecting groups were also explored. They will be introduced in Chapter Three and Seven.

1.6 Protection of the C-Terminus in Peptide Chemistry

Compared to N-terminal protection, C-terminal blocking is reactively facile[24]. Alkyl, aryl and hydrazide groups are the most popular C-terminal blocking groups in peptide chemistry. Hydrazide protection is limited to syntheses in which only moderately active acylating agents are used and not in large excess. In this project, on the basis of their high stability, ease of introduction and wide commercial availability, *t*-butyl and benzyl ester are preferred.

In this project, the main problem with C-terminal protecting groups does not come from their formation and removal. As was discussed early, to remove protection groups on N or C-terminals, one must make sure that the conditions applied should not destroy any other protecting groups. There are generally two methods for cleavage of normal *t*-butyl or benzyl ester, one is hydrolysis (basic or acidic), the other is hydrogenolysis in the case of benzyl esters. However, the main three N-terminal protecting groups: Boc, Fmoc, Cbz, are cleaved in acid, base and reducing

environment (H_2/Pd) respectively. Designed choice of the C-terminal protecting groups in this project was achieved after suitable N-terminal protecting groups were carefully selected.

1.7 Coupling Reactions

After preparation of the di-protected guanidine containing side chain and the protected backbone component, the next crucial step was to form a new amide bond between these two segments: the coupling reaction. Theoretically, a carboxylic acid should react with a primary amine. However, this is an endothermic reaction, and elevated temperatures are necessary which may be not suitable for peptide chemistry. To achieve the safe and fast formation the amide bond, the simplest way is to activate the N- or C-terminus.

The reason N-terminal activation is not widely applied[24] is that because to make it more reactive, one must increase the nucleophilicity of the amino group, by adding an electron-releasing substituent to it, for example *t*-butyl group. But this method also decreases the rate of acylation because of the bulkiness of the substituents.

Compared with the amino group, the carboxylic group is much easier to activate[24]. Chemists have developed a number of reactions to achieve it in the past half a century[15,24,25]. The basic mechanism of activation for the C-terminal is to employ an electron-withdrawing substituent which renders the carbon atom of the carboxyl sufficiently electrophilic to facilitate nucleophilic attack by amino groups. Acid halides, acid anhydrides, active esters, and many other reagents can be used to achieve it. Also when chemists select activation reagents, they not only focus on the activity of the C-terminal but also try to suppress racemization. Racemization is not a problem in this project which was deliberately designed to avoid chirality.

There are two common methods for the activation of the carboxylic group in peptide chemistry[15,25]. The first one is to convert it to a more active group. Application of this method is often related to the use of strong acid or base which may destroy the protecting groups on the target arginine mimic. Another method is to

employ a coupling reagent that can be used under mild conditions. The principle of this method is to convert the OH group of the carboxylic component into an active ester, such that a better leaving group is formed that can be easily attacked by the amino group. In this project, two popular reagents were selected, dicyclohexylcarbodiimide (DCC)[24] and O-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) [25](Figure 11). Their function and results will be discussed in Chapter Six.

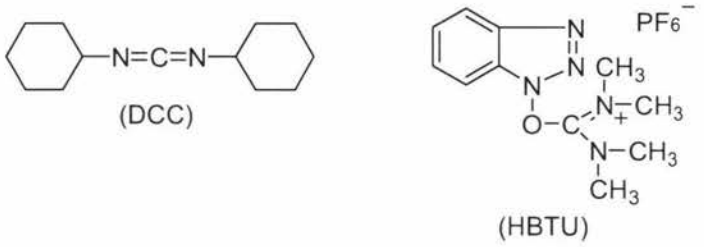


Figure 11. The structures of DCC and HBTU.

CHAPTER TWO
EQUIPMENT AND MATERIALS

2.1 Equipment

NMR data was obtained from a Bruker Advance, 400MHz and 500MHz apparatus (Germany). HPLC plus EI (electrospray ionization) /MS was obtained from a Waters (Model F00SHC 167M, Milford Massachusetts, USA) HPLC, and Micromass ZMD Mass Spectrometer. UV system used was from Hewlett Packard (Model 8452A Diode Array Spectrophotometer). A Cole-Parmer, Model 8891 sonicator was also used. Melting point/hot plate apparatus 1178VW 31-75-06 was from Cambridge Instrument Inc., (UK).

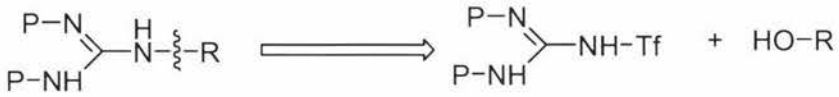
2.2 Materials

Solvents and common reagents were obtained from normal sources, generally AR grade was used unless otherwise stated. H_2O used was of Milli Q grade. Glycine, 6-aminohexanoic acid, thiourea were from Rhone-Poulenc, Ltd., Manchester, England; ß-alanine, pyrazole, cyanamide, benzyloxycarbonyl succinimide, di-*t*-butyl dicarbonate, ethylenediamine, diisopropylethylamine, *N*-(9-fluorenylmethoxycarbonyloxy)succinimide were from Sigma-Aldrich Company, Inc., New Zealand; S-methyl isothiourea, guanidine, *t*-butyl chloroacetate, methyl chloroacetate were from Acros Organics, Geel, Belgium.

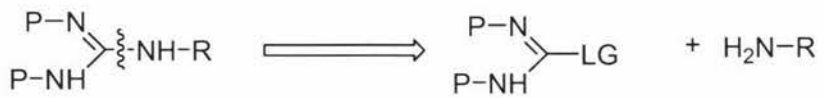
CHAPTER THREE
FORMATION OF THE PROTECTED GUANIDINO GROUP

3.1 Introduction

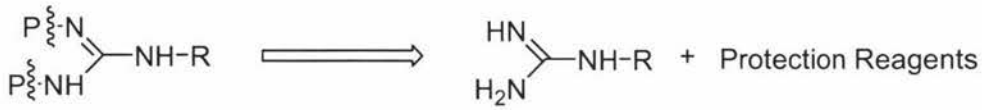
The preparation of the di-protected guanidino group was the most challenging job in this project. There are generally three popular routes to achieve this aim. One is to use guanidine as the starting material, protect it first then react it with an amine, alcohol, or carboxylic acid through a Mitsunobu reaction[26]. The second method is protection of the guanylating reagents followed by nucleophilic attack by a primary amino acid to construct the di-protected substituted guanidines. The third way is formation of the guanidino group then addition of the two protecting groups (Figure 12). In this Chapter, the A and C will be introduced and discussed.



Route A : synthetic route of using guanidine then Mitsunobu reaction.



Route B: Protection of carboxamidine reagent first then guanylation



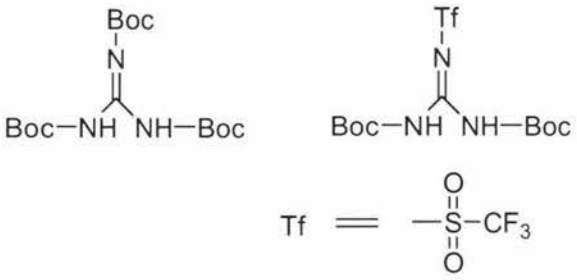
Route C: Formation of substituted guanidine using carboxamidine compounds, then protection

Tf=Triflic group, P=Protecting group (Boc or Cbz), LG=Leaving groups

Figure 12. The three routes for the formation of substituted guanidine.

3.2 Preparation of Guanidino Acids

3.2.1 *N,N',N''*-tri-Boc-guanidine and *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine



The application of *N,N',N''*-tri-Boc-guanidine and *N,N'*-di-Boc-*N''*-trifluoromethanesulfonylguanidine to peptide chemistry was first introduced by Murray Goodman et al. al. in 1998[27,28]. The general process was to protect guanidino group first, then use it to convert alcohols and amines to substituted guanidines. This invention is quite different from the method using carboxamidine compounds. In this method, the guanidino group comes from the guanidine itself not from a guanylating reagent.

In this method, there is a question. Why must the tri-protected guanidine (two Boc or Cbz, and one Tfa) be used to install the amine or alcohol into the substituted guanidine? Why can the di-protected guanidine not be applied? The answer is that the mechanism of this reaction is unlike guanylation that uses a carboxamidine compound which undergoes a nucleophilic substitution. Here the key conversion step is a Mitsunobu reaction[26,29]. Its basic concept is that triphenylphosphine (PPh_3) combines with DEAD (diethyl azodicarboxylate) to generate a phosphonium intermediate that binds to the alcoholic oxygen thus activating it as a leaving group. Substitution by a carboxylate, amine, or other nucleophile completes the process (Figure 13). In this case, the guanidine needs to be slightly acidic to protonate the DEAD intermediate (step 2). The negatively charged DEAD intermediate can react with the R-O-$^{+}PPh_3$ which is formed after step 2, to give the side product. This is why the di-protected

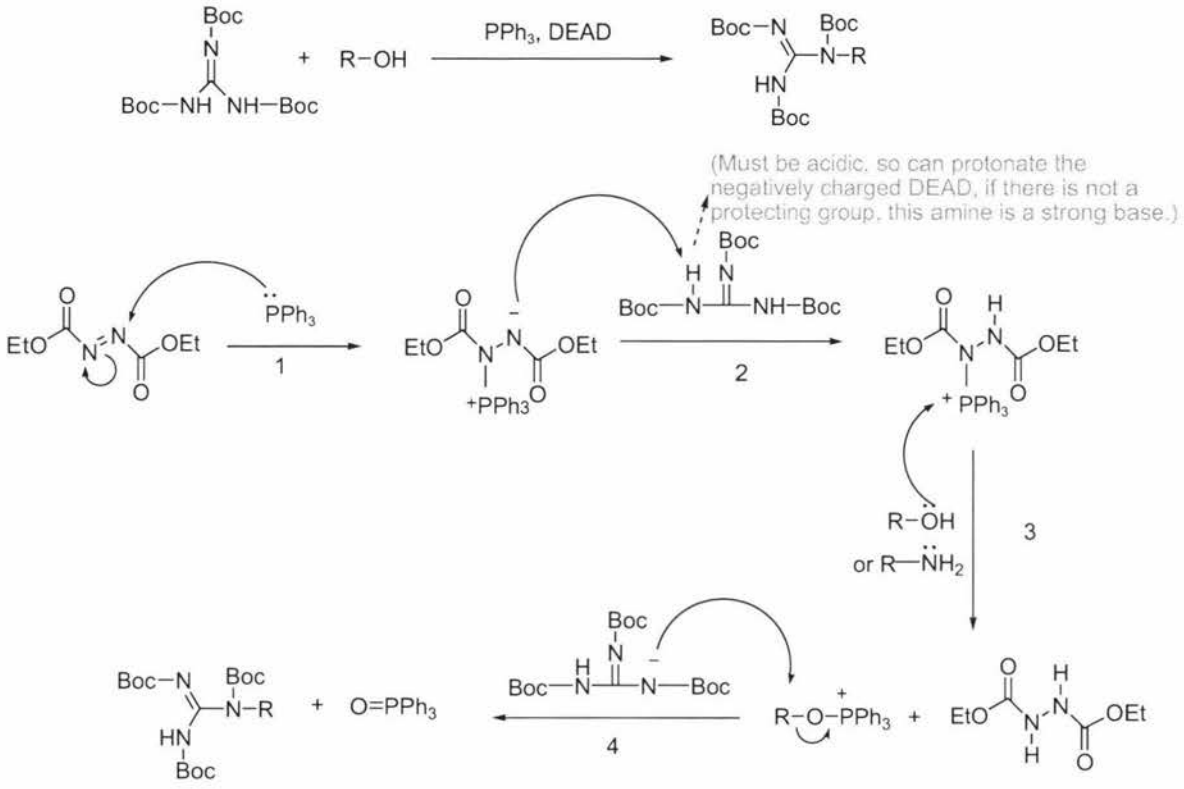


Figure 13. The mechanism of Mitsunobu reaction to achieve substituted guanidine formation. It must be noted that in step 3 there are many nucleophiles that can be used to trap the triphenyl phosphine.

guanidine is not suitable because the free amine group is a relatively strong base. When the secondary amine group is protected by the Boc, Cbz or Tfa groups, the electron withdrawing effect will decrease the nucleophilicity and basicity of this amine group. The acidity of the tri-protected guanidine is crucial for substituted guanidine formation. It determines the reactivity of the Boc, Cbz, Tfa protected guanidines, the selectivity of solvents and the yield with different amine or alcohols. Generally speaking, *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine is more acidic, and therefore more reactive than *N,N',N''*-tri-Boc-guanidine, and thus the guanylation reactions are preferably carried out in less polar reagents. If the starting reagents can not be effectively dissolved in non-polar solvents like benzene or dichloromethane, THF can be used.

Feichtinger et. al.[28] established a new route which could be used in the construction of a guanidino group containing side chain for the arginine mimics (Figure 14). Here the reagent selected in this project was *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl, not *N,N',N''*-tri-Boc-guanidine or the Cbz protected guanidine. The reason the author confirmed was due to the acidity of *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl. The trifluoromethanesufonyl group is a better leaving group than the other reagents. Another advantage is that when it reacts with an amine, the trifluoromethanesulfonyl group will form a by-product, triflic amide, which can be easily removed during a simple aqueous wash procedure. Thus the product is the desired di-Boc guanidine substitute for this project. But for *N,N',N''*-tri-Boc-guanidine after it has reacted with an amine, the product was still a tri-protected guanidine substitute. Further work[28] to rearrange the Boc group of the secondary amine on the guanidine substitute is needed.

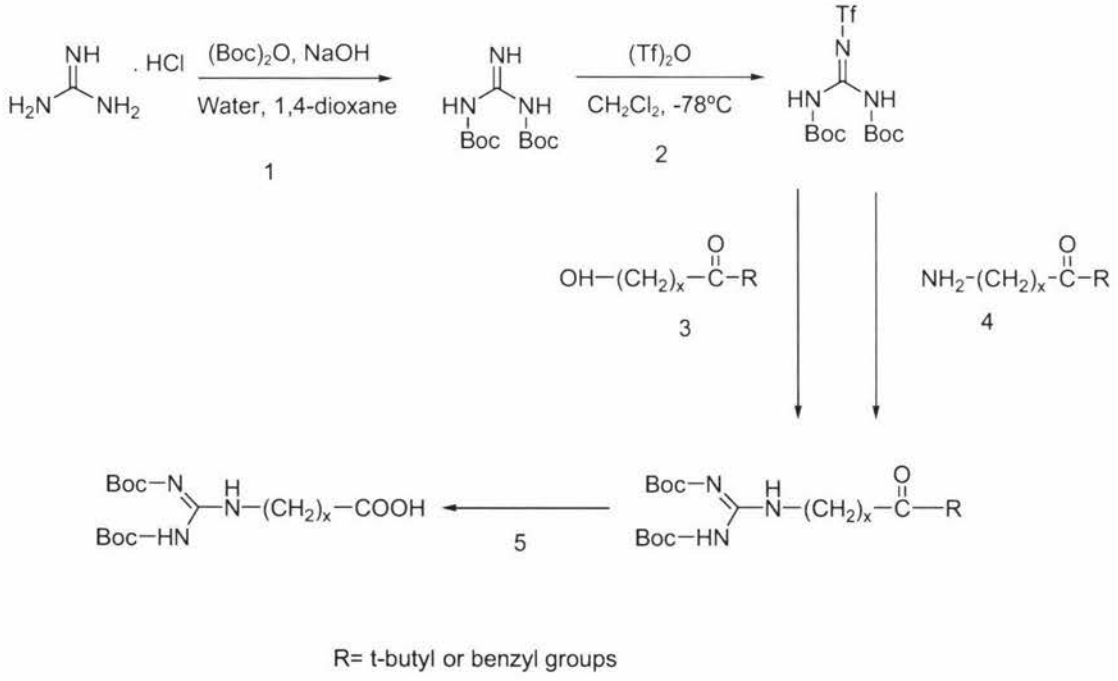


Figure 14. Synthetic strategy for making the di-Boc guanidino acid from the guanidine hydrochloride[28].

The *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine was synthesized from guanidine hydrochloride[28]. It was reacted with 2.5 equivalents of di-*t*-butyl dicarbonate to produce the di-Boc-guanidine. Then this product was stirred with triflic anhydride at -78 °C to give the starting material *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine which was used to react with suitable reagents to give the di-Boc-guanidino ester side chain. It must be noted that a lot of nucleophiles can be used for the Mitsunobu reaction to couple to the tri-protected guanidine. In this study there are two reagents which can be used to prepare the tri-Boc-guanidino ester side chain (step 3 and 4 in Figure 14), one an alcohol (3) and the other one is a primary amine (4). Another point that needs to be mentioned is that in this project, the desired side chain product should be a carboxylic acid that can be used to couple to the backbone segment of the arginine mimic. Because the carboxylic acid group is more reactive than the amine or alcohol in the Mitsunobu reaction[26,28], it must be protected by an alkyl or phenyl group. Two esters were prepared. After preparation of the tri-protected guanidino ester, cleavage of the alkyl or benzyl ester followed.

After *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine was used in this project, its reactivity with the t-butyl and benzyl esters of primary amino acids was investigated. Although this reagent could effectively convert the tri-protected guanidine to the desired substituted guanidines, there were still a lot of problems that made it less suitable for this project:

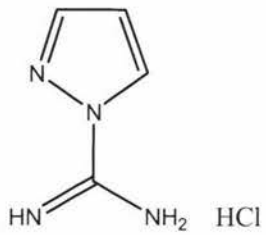
1. The preparation of *N,N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine is complex[28]. Other guanylating reagents like *N, N'*-bis(*t*-butoxycarbonyl)-S-methylisothiourea, *N,N'*-bis-*t*-butoxycarbonylthiourea, and 1H-pyrazole-1-carboxamidine hydrochloride are introduced in this Chapter and Chapter Four, could be produced in one simple step with high yield. The products could be easily separated by extraction or precipitation with satisfactory purity. However to synthesize *N, N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine, it needs synthesis of *N, N'*-bis (*t*-butoxycarbonyl) guanidine first followed by purification using flash column chromatography to separate it from the mono and tri-protected products. Then the di-Boc product needed a very cold environment to produce the *N, N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine because the reagent triflic anhydride is highly reactive. The

temperature must be strictly controlled in a cold (-65 °C, in a dry ice / methanol bath) anhydrous environment. High temperatures and water will cause a vigorous reaction which significantly decreases the final yield.

2. The other complexity is the selectivity for removal of protecting group. As the carboxylic acid group is more reactive than amine and alcohol in the Mitsunobu reaction[26,28], it must be blocked by a benzyl or alkyl group. Acid hydrolysis of these ester groups might destroy the N terminus protecting group (Boc). Hechinger ET. al.[28] did not investigate this problem. Their final products were a series of esters. However in this project, the acidic hydrolysis of the alkyl ester was avoided because the Boc group is not stable in the acid. Basic hydrolysis using Noah or KOH did not look very attractive for methyl or ethyl esters which have been confirmed in this study. Thus the carbonyl protecting group which was employed in this work was the benzyl group which can be removed with H_2/Pd in Mesh.

3. For other reagents to be introduced later, only two steps were needed to achieve the formation of the desired di-protected guanidino acids. The first step was to make the guanylating reagent, and the second step was guanylation of the primary amino acid. For *N, N'*-di-Boc-*N''*-trifluoromethanesulfonyl guanidine in Figure 14, it can be seen that there are four steps to get the desired product. The overall yield in this project was less than 30%. This is to be compared with *N,N'*-bis(*t*-butoxycarbonyl)-S-methylisothiourea which was subsequently selected as the suitable reagent to make the di-protected guanidino acid to make the same product with a yield of 75-80%

3.2.2 1H-Pyrazole-1-Carboxamidine Hydrochloride[16]



Although a number of guanylating reagents have been found and applied in peptide synthesis[15,24,25], there are only a few reagents that are effective in converting amino groups of amino acids to guanidine compounds. In this work for selection of relevant guanylating reagents, consideration was given to the following two aspects:

A) The problem of employing amino acids in a guanylation reaction is that some guanylating and protecting reagents are only soluble in special organic solvents such as chloroform and dichloromethane, but for most of the natural amino acids, their solubility in these solvents are very poor. A desirable guanylating reagents should be at least soluble in common solvents (e.g. mixture of water and 1, 4-dioxane, DMF, THF) which can effectively dissolve most primary amino acids;

B) For the challenge of producing an economic method to synthesize arginine mimics, the reaction conditions for guanylation should be as mild as possible.

Half a century ago, scientists treated amines with cyanamide, or 3,5-dimethyl-1-guanylpyrazole to produce guanidino compounds[30]. But these two reagents require a stung basic medium to give sufficient reactivity. Michael S. Bernatowicz et. al. published a paper on the application of 1H-pyrazole-1-carboxamidine hydrochloride in peptide synthesis[16,31]. In their work, they reacted cyanamide with pyrazole to make the guanylating reagent, and then converted amines to guanidino compounds in DMF. They also mentioned that this new reagent 1H-pyrazole-1-carboxamidine hydrochloride is quite soluble in water. Thus new guanidino acids can be produced by stirring amino acids (e.g. glycine, ß-alanine) with 1H-pyrazole-1-carboxamidine hydrochloride in aqueous Na_2CO_3 solution with satisfactory yields. Due to this simple procedure and satisfactory yields, this new guanylating reagent is now widely used in peptide chemistry to produce substituted guanidines [32-34].

Formation of 1H-pyrazole-1-carboxamidine hydrochloride can be simply achieved by refluxing pyrazole and cyanamide in 1,4-dioxane with HCl gas. Considering that cyanamide is not stable in air, the reaction is carried out under argon. During the course of the reaction the product precipitates and can be washed by anhydrous diethyl ether (Figure 15). Then it can be used for the guanylation of amino acids without further purification.

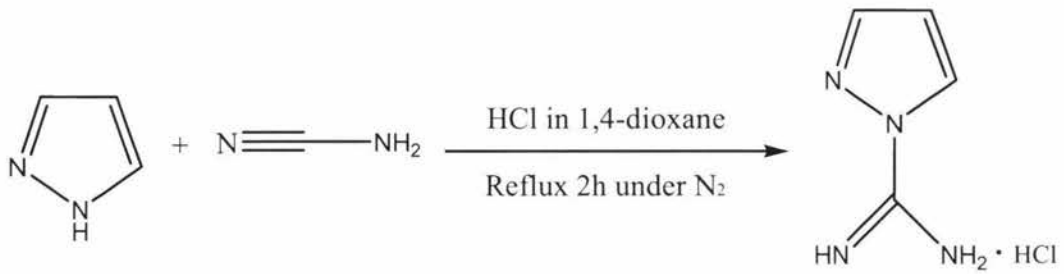


Figure 15. Formation of 1H-pyrazole-1-carboxamidine hydrochloride.

In this study, three primary amino acids, glycine, ß-alanine and 6-amino hexanoic acid were selected to react with 1H-pyrazole-1-carboxamidine hydrochloride, following the guide lines produced by Bernatiowicz et. al. (Figure 16). All of the reactions were fast, producing the desired product with satisfactory yield (Table 1).

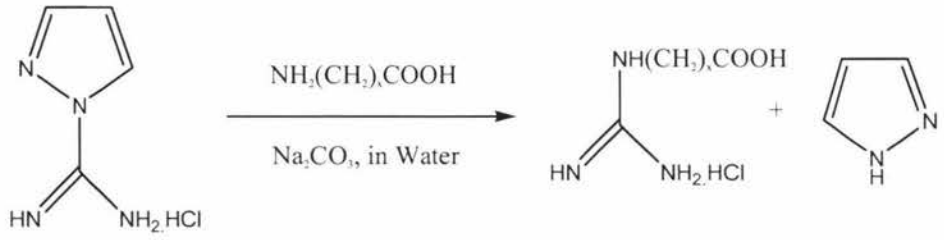


Figure 16. Reaction of 1H-pyrazole-1-carboxamidine hydrochloride with a primary amino acid

starting amino acid	reaction time	yield	melting point
glycine	3h	75%	271-274 °C
ß-alanine	3h	71%	213-214 °C
6-amino hexanoic acid	3h	70%	294-296 °C

Table 1. Experimental results for the reaction of 1H-Pyrazole-1-carboxamidine hydrochloride with three primary amino acids.

When glycine was reacted with 1H-pyrazole-1-carboxamidine hydrochloride, a white precipitate was formed during the process. Because the main by-products are pyrazole and NaCl and both of them are quite soluble in water, the precipitate can be easily purified by washing with a small portion of MeOH/H_2O (1:1). But when glycine was replaced by ß-alanine and 6-amino hexanoic acid in this reaction, the story was different. No solid precipitated even when stirring was continued overnight. But TLC and NMR results revealed that the conversion of the amino acids to guanidino acids had completed in three hours. The products were soluble in basic aqueous solution. Another way to purify them became essential.

In this case, to separate the products from unreacted ß-alanine and 6-amino hexanoic acid respectively after reaction completion (monitored by TLC), the solvent (water) was removed under reduced pressure. Then the residue was washed by MeOH to remove pyrazole. Finally the products were recrystallized from MeOH/H_2O. TLC showed the products were pure.

Also in their paper, Michael S. Bernatowicz et. al. discussed the possibility and effect of formation of N-amidinopyrazole-1-carboxamidine hydrochloride (Figure 17). This is a by-product which is formed by self-condensation of 1H-pyrazole-1-carboximidine (step 1). Theoretically, this compound could also be gained by nucleophilic attack by a primary amino acid to form *N*-alkyldiguanidines (step 2). In this study, under refluxing temperature, TLC and MS spectrum suggested that the formation of diguanidine derivatives was insignificant. It is believed that this

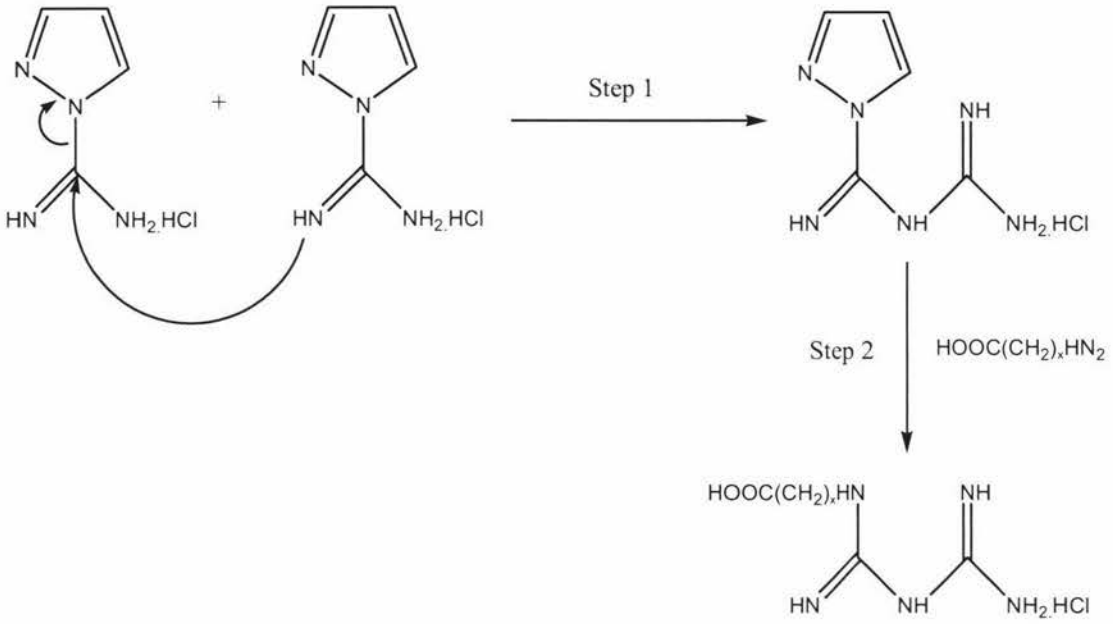
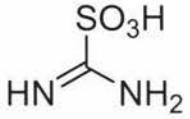


Figure 17. Self-condensation of 1H-pyrazole-1-carboxamidine (step 1) and formation of N-alkyldiguanidine (step 2)

self-condensation reaction takes a long time and needs high temperature because of the sterically hindered pyrazole group. Also, the product, 1H-pyrazole-1-carboxamidine, precipitates immediately after its formation, so its concentration in the reaction solution should be low compared with cyanamide and pyrazole, hence the opportunity of the reaction of two carboxamidine molecules is reduced. Even after refluxing the mixture of cyanamide and pyrazole under nitrogen overnight, there was no significant evidence that quantitative diguanidine derivative was produced. Preparation of 1H-pyrazole-1-carboximidine hydrochloride only needed refluxing for 2 h. When reacting primary amino acids with 1H-pyrazole-1-carboximidine hydrochloride, the aqueous basic solution is merely stirred at room temperature for 2-3 h. Thus the potential for formation of this by-product is not significant and will not have a serious effect on this reaction.

3.2.3 Aminoiminomethanesulfonic Acid



Unlike other basic guanylation reagents used in this project, aminoiminomethanesulfonic acid is an acidic compound[35]. Considering the SO_3H group might protonate amino group of primary amino acids hence decrease its nucleophilicity, a suitable base must be selected for the reaction. Audrey E. Miller et. al. (1986) investigated the reactivity of this reagent and introduced a good method for its preparation[17].

In this paper, one equivalent of potassium carbonate or sodium carbonate was used for completion of the reaction. CO_3^{2-} is a divalent ion so it was assumed that half equivalent was consumed to neutralize the SO_3H group, and the other half was applied to maintain the nucleophilicity of the amino group of the primary amino acid (Figure 18). Addition of extra base did not increase the yield.

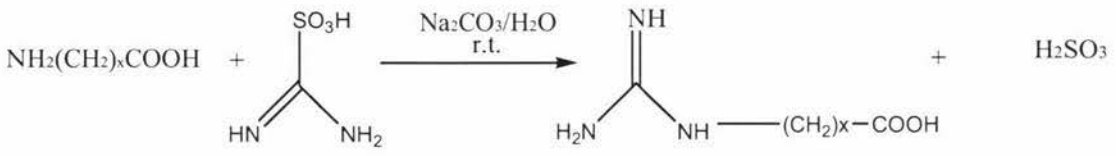


Figure 18. Formation of guanidino acids from reaction of aminoiminomethanesulfonic acid with primary amino acids.

The reaction yields with aminoiminomethanesulfonic acid with the three selected primary amino acids were satisfactory. The experimental results are shown in Table 2.

starting amino acid	reaction time	yield	melting point
glycine	24h	79%	273-275°C
ß-alanine	24h	75%	218-220 °C
6-amino hexanoic acid	24h	48%	294-296 °C

Table 2. Experimental results for the reaction of aminoiminomethanesulfonic acid with three primary amino acids.

In contrast to other papers[18,21,22] using guanylating reagents which focus on the conversion of amines to guanidino groups, Audrey E. Miller used the reagent mainly to convert different amino acids to form a guanidine containing amino acid. So this was very valuable for this study and it is widely used in formation of unprotected guanidino group containing amino acids[36].

3.2.4 Conclusion

Preparation of substituted guanidines using *N*, *N'*-di-Boc-*N''*-trifluoromethanesulfonylguanidine was successful. The strict reaction conditions, low yield and final complex steps to remove the carbonyl protecting group limited its application to this study. In comparison, 1H-pyrazole-1-carboximidine hydrochloride and aminoiminomethanesulfonic acid, as discussed in the literature, are very useful reagents to convert amino acids to guanidino acids. They can be easily prepared in excellent yield from readily available starting materials and have stability, reactivity and solubility properties desirable for a versatile reagent for efficient guanylation of primary amino acids. For both two methods, the reaction can be carried out under mild conditions (normal base, room temperature, in air). The reaction is usually completed within a relatively short time and gives satisfactory yields. In some cases, the product precipitates (glycine) during the course of the reaction so no further purification is needed.

3.2.5 Experimental

a. *N,N'*-bis (t-butoxycarbonyl) guanidine[28]

1, 4-Dioxane (50mL) was added to a solution of guanidine hydrochloride (2.39g, 25mmol) and sodium hydroxide (4.0g, 0.1mol) in water (25mL), and the resulting mixture was cooled to 0°C. Di-*t*-butyl dicarbonate (12g, 55mmol) was added in one portion, and the reaction mixture was allowed to warm to room temperature within 2h. After being stirred for 20h, the mixture was concentrated in vacuo to one-third of its

original volume. The resulting suspension was diluted with water (50mL) and extracted with ethyl acetate (3×50mL). The combined organic phases were washed with 10% citric acid, water and brine and dried with $MgSO_4$. After filtration and removal of the solvent under reduced pressure, the crude product was purified by flash column chromatography on silica gel (eluent, 100% CH_2Cl_2 to CH_2Cl_2/MeOH 97:3). N, N'-bis (*t*-butoxycarbonyl) guanidine (3.22g, 50%) was obtained as a colorless solid. ^{1}H NMR (DMSO-d6) δ 10.43 (s, 1H), 8.47 (s, 2H), 1.37 (s, 18H).

b. *N,N'*-di-Boc-*N''*-trifluoromethanesulfonylguanidine[28]

A solution of *N,N'*-bis (*t*-butoxycarbonyl) guanidine (0.52g, 2mmol) and triethylamine (0.29mL, 2.1mmol) in anhydrous CH_2Cl_2 (10mL) was cooled to -78 °C in a dry ice / methanol bath under nitrogen. Triflic anhydride (0.35mL, 2.1mmol) was added dropwise at a rate such that the reaction temperature did not exceed -65 °C. After the addition was complete, the mixture was allowed to warm to room temperature within 4h. The solution was washed with 2M aq. sodium bisulphate solution and water and then dried with $MgSO_4$. After filtration and removal of the solvent under reduced pressure, the crude product was purified by flash column chromatography on silica gel (eluent, CH_2Cl_2). The product was recrystallized from *n*-hexane to give 0.62g pure product (79%). ^{1}H NMR (DMSO-d6) δ 11.43 (s, 2H), 1.45 (s, 18H).

c. *N,N'*-di-Boc- *N''*-trifluoromethanesulfonylguanidine reacted with esters (benzyl and *t*-butyl) of ß-alanine[28]

To a solution of ß-alanine ester (0.33mmol) in THF (10mL) was added *N, N'*-di-Boc-*N'* trifluoromethanesulfonylguanidine (0.98mmol) and PPh_3 (0.49mmol) under nitrogen. The mixture was cooled to 0°C, and DEAD (0.10mL, 0.49mmol) was added dropwise at a rate such that the reaction mixture was completely colourless before addition of the next drop. After the addition was complete, the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with water and the solvent was evaporated under reduced pressure. The

crude product was purified by flash column chromatography on silica gel (eluent, CH_2Cl_2/ethyl ether 95:5), and finally a colourless solid was obtained (yield 36~45%).

d. 1H-Pyrazole-1-carboxamidine hydrochloride[16]

8.17g of pyrazole (0.12mol) and 5.05g of cyanamide (0.12mol) were dissolved in 120mL of 1,4-dioxane. Then 31mL of 4N HCl in 1,4-dioxane were added. The mixture was refluxed under nitrogen for 2h. During the course of the reaction the product crystallized. After cooling to room temperature, 30mL of anhydrous ether was added and the mixture was allowed to stand for half an hour. The white precipitate was collected by filtration and washed with anhydrous ether. Finally it was dried under vacuum to give 16.0g (91%). ^{1}H NMR (DMSO-d6) δ 9.75 (s, 4), 9.03 (d, 1), 8.13 (s, 1), 6.84 (t, 1).

e. *N*-(aminoiminomethyl)glycine[16]

1.5g of glycine (20mmol) and 2.93g of 1H-pyrazole-1-carboxamidine hydrochloride was dissolved in 20mL of 1 M aqueous Na_2CO_3 solution. Then the mixture was stirred at room temperature for 3h. The white precipitate formed was collected by filtration and washed with several small portions of MeOH/H_2O (1:1). The product was dried under vacuum to give 1.70g product as white solid. ^{1}H NMR ($CDCl_3$) δ 2.52 (s, 2H). (ß-alanine and 6 aminohexanoic acid were used to replace the glycine to make the relevant products).

f. Aminoiminomethanesulfonic Acid[17]

50mL of acetic anhydride was added slowly to 45g of 30% hydrogen peroxide cooled in an ice bath. Then two drops of concentrated sulfuric acid was carefully added. After the vigorous reaction had subsided, 110mL of acetic anhydride was slowly added to the cooled mixture. The mixture was allowed to warm to room temperature and left to stand for one day. Then 160mL of methanol was added and the solution was cooled to 10°C in an ice bath. A solution of thiourea (10g) in methanol

(500mL) was also cooled and then was added to the oxidizing mixture in portions at such a rate as to maintain the temperature at 10-20°C. After the addition was complete, the mixture was allowed to stand at room temperature for one day. Any crystals that formed were removed by filtration and discarded. After the filtrate was concentrated to about 100mL, the solid was filtered and washed with several portions of methanol, then dried under vacuum. Percentage yields varied from 55% to 75%. ^{1}H NMR ($CDCl_3$) δ 10.6 (s, 1H).

g. *N*-(aminoiminomethyl)glycine[17]

1.39g of potassium carbonate (10mmol) and 0.75g of glycine (10mmol) were mixed and dissolved in 20mL of water. Then 1.24g of aminoiminomethanesulfonic acid (10mmol) was added in small portions with good stirring. Then the mixture was stirred for 24h at room temperature. The white precipitate that formed was collected by filtration, and washed by small portions of water. Finally it was dried under vacuum to give 0.88g white solid product (75%). ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 2H). (ß-alanine and 6 aminohexanoic acid were used to replace the glycine to make the relevant products).

3.3 Protection of Guanidino Groups

3.3.1 Introduction to the Protection of Guanidino Group

There are three nitrogen containing groups in the guanidino group. How many and which ones should be protected is a serious problem. One, two or all three?

In Figure 19, it can be seen that the secondary amine C, compared with the other two groups A and B, is more difficult to react with another functional group due to the steric hindrance. The existence of this unprotected group should not interfere with future peptide synthetic steps, so its protection is not deemed necessary.

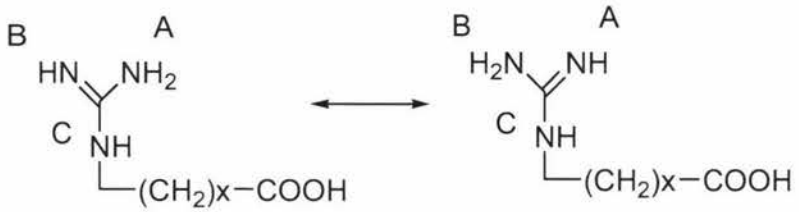


Figure 19. Structure of unprotected guanidino acids.

In the above figure, the group A and B can form a resonance structure and their reactivity should be the same. It is easy to put a protecting group on one of them. Some papers mentioned the preparation and application of 1-Boc-L-Arg and it is now commercially available[15,37,38]. To make it, the ratio of guanidino acid and $(Boc)_2O$ is normally 1:1.5. Percentage yield are reported to be more than 80%.

But after the first protecting group is added (suppose on A), the question arises as to whether group B needs to be protected or not and how easy is it to achieve this. The nucleophilicity of a functional group is determined not only by its basicity but also by structure. For example triethylamine has a pKa of 11.01, but the three ethyl groups prevent facile nucleophilic attack. In this case, the group B is a good base even after group A is protected due to the resonance structure. But the substituents on A and C make addition of another protecting group on B less facile than that of A.

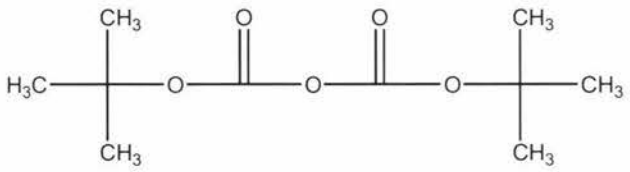
However, does this mean it is not necessary to protect B for the aims of this project? M. Bodanszky and A. Bodanszky[15,24] introduced the concept that large protecting groups (e.g. 1-adamantyl or mesitylene-2-sulfonyl group) on amine A can inhibit the group B from subsequent facile nucleophilic attack, hence no more protection is needed. But in their papers, they had to admit that after the reaction finished, there was still some di-protected product and further column work was needed for purification of the mono-protected product. So the activity of group B can not be ignored.

In this project, a downstream synthesis was envisaged to couple the guanidine containing side chain to the secondary amine containing backbone. The nucleophilic

capability of this secondary amine is thought to be hindered by the bulk of the backbone constituents. Thus if B of the guanidine is unprotected, it may contain enough nucleophilicity to produce undesired by-products.

Thus it was concluded that for the guanidino group applied in this project, it is necessary to protect both A and B. Mono-protection can be simply achieved by a number of methods[15]. Di-protection, due to the decreased activity of B required more stringent reaction conditions to achieve this aim.

3.3.2 Protection with Di-*t*-butyl Dicarbonate $(Boc)_2O$



t-Butyloxycarbonyl (Boc) is currently one of the favourite N-terminal protecting groups in peptide chemistry. Its main attraction to peptide chemists comes from two characteristics: excellent stability in strong base, and simplicity of cleavage with trifluoroacetic acid (TFA)[24,39,40].

It is known that in both solid phase and solution phase peptide synthesis, to achieve nucleophilicity for free amine groups, a basic environment is necessary. Some amino protecting groups like Fmoc are cleaved in base and undergo a β-elimination and hence are not very stable in basic environments. Compared with Fmoc, Boc groups can survive not only in aqueous inorganic and organic base solution but also in organic solvent with strong inorganic base (for example in THF, with LiH or NaH). The procedure for the cleavage of Boc group is very simple. Addition of a small amount of TFA to the peptide solution can remove it in minutes. By-products of this acidolysis reaction are carbon dioxide and *t*-butanol which are easy to remove.

Di-*t*-butyl dicarbonate $(Boc)_2O$ is amongst the most popular reagents for the addition of Boc group on the amine group. It has been used in peptide chemistry for many years[24]. This commercially available symmetrical anhydride reacts well with most of amino acids and aromatic amines (Figure 20). The general method of amine

protection using this reagent is to mix it with the target amino acid or amine in a cold mixture of 1,4-dioxane and aqueous NaOH (or $NaHCO_3$) solution (1:1) and then stir at room temperature from 1h to overnight. After the reaction is finished, the unreacted $(Boc)_2O$ can be destroyed by addition of aqueous HCl. The protected product can be extracted with a suitable organic solvent like chloroform or ethyl acetate. Further purification by flash column chromatography may be needed.

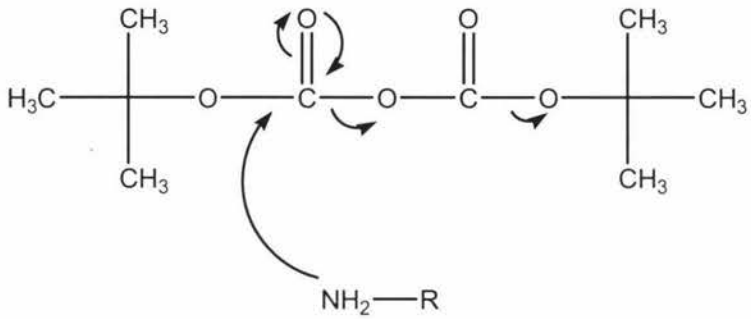


Figure 20. Structure of di-*t*-butyl dicarbonate and the mechanism of its amine protecting reaction.

Apart from the preparation of mono-Boc arginine, two papers reported the reaction and application of di-Boc arginine [41,42]. This compound is also commercially available. In this project, the original plan for the production of the di-Boc-guanidino acid side chain was to make the guandine substitutes at first then protect them as discussed earlier in this Chapter. In this case, although the above procedure looked unattractive and expensive, the process was improved in this study:

a. The ratio between guanidino acid and $(Boc)_2O$ used was 1:3 (the published procedure normally uses 1: 1.5).
b. 4N NaOH solution was used (1~2N for mono-protection). It was assumed that more base was needed to maintain the nucleophilicity of the guanidine nitrogens.
c. Stirring at 40°C overnight (for mono-protection 2~3h at room temperature) (Figure 21)

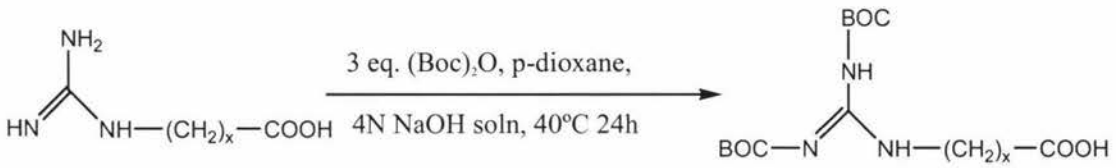
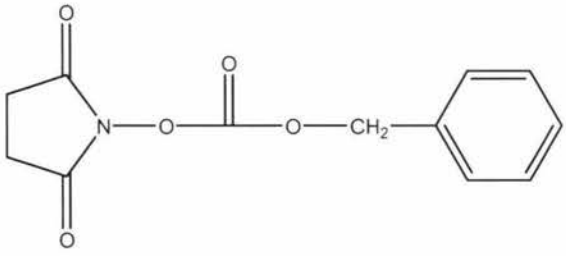


Figure 21. Preparation of di-Boc-protected guanidino acids

However, the result was disappointing. The final product was a mixture of mono-, di- and tri-protected compounds. After purification by flash column chromatography (dichloromethane: methanol, 95:5), the percentage yield of the desired di-protected guanidino acid was lower than 8%. More than 40% of the product was still the mono protected guanidine. Attempts to dissolve the mono-protected product in a mixture of dioxane and aqueous NaOH solution and then reacting it with extra $(Boc)_2O$ for 24h still were unsuccessful. Only 3~5% of the mono-protected product was converted to the di-protected guanidino acid. Production of di-Boc protected guanidino acid in decent yields was thus unsuccessful.

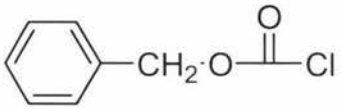
3.3.3 *N*-(Benzyloxycarbonyloxy)succinimide (Cbz-OSu)



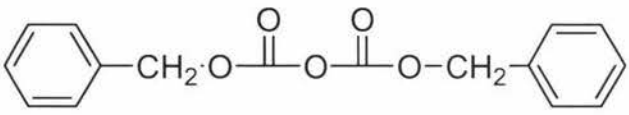
Failure to prepare di-Boc guanidino acids in suitable yields led to extension of this study to other protecting groups. One group that was applied to peptide chemistry earlier than Boc is the benzyloxycarbonyl group (abbreviated Cbz or Z). This group was discovered by Bergmann and Zervas in 1932[24]. The main advantage of this group is its stability in both normal acid and base. This characteristic of the Cbz group should be an advantage. A possible reason for the difficulty in putting two Boc groups on the guandino acids at the same time might be due to the steric effects of the Boc group on the amino group A preventing the addition of another Boc group on imine B. Compared to the *t*-butyl group, the benzyl group is less hindered. Therefore the Cbz group on amine A should be less likely to prevent another Cbz group addition. The

most popular methods for Cbz removal are hydrogenolysis using Pd/H_2 and acidolysis with HBr or anhydrous liquid HF. The by-products of those reactions are toluene and carbon dioxide which are easily separated from the product.

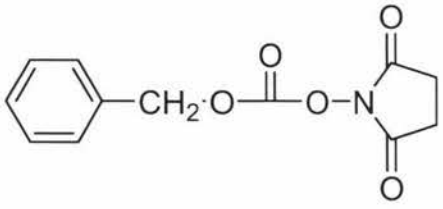
Figure 22 shows three of the most popular reagents used to generate Cbz groups. Benzyl chlorocarbonate, due to the active acyl halide bond, is the most reactive one. The benefit compared with the other two reagents is that its reaction with amine groups is rapid. However this compound decomposes on storage and has now been largely replaced by the other two reagents. Dibenzyl dicarbonate $(Cbz)_2O$, like *t*-butyl dicarbonate $(Boc)_2O$ is a symmetrical anhydride. Its reactivity is greater than benzyloxycarbonylsuccinimide (Cbz-OSu) but lower than benzyl chlorocarbonate. Both dibenzyldicarbonate and benzyloxycarbonylsuccinimide are reasonably stable at low temperature, and are widely used reagents for addition of Cbz group to amino groups. The succinimide compound was used in this study.



benzyl chlorocarbonate



dibenzyl dicarbonate



benzyloxy carbonylsuccinimide

Figure 22. Three reagents for the introduction of the Cbz group.

In Figure 23, the procedure for preparing di-Cbz guanidino acid is shown and is similar to the preparation of di-Boc guanidino acid[24]. Unfortunately, the reaction

yield was also low (about 10%). Most of the final product was still the mono-protected compound. Addition of extra sodium hydroxide and Cbz-OSu did not improve the yield.

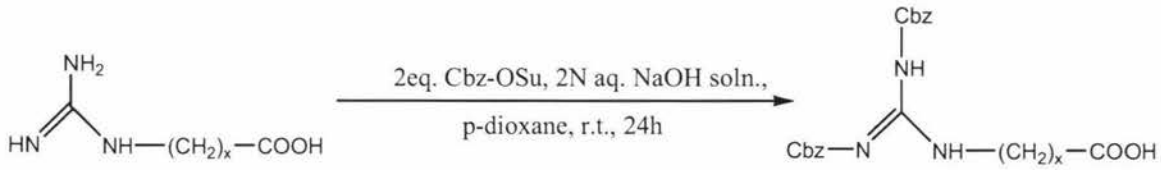
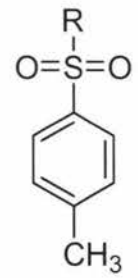


Figure 23. Preparation of di-Cbz protected guanidino acids using benzyloxycarbonylsuccinimide.

Considering the solubility of Cbz-OSu in a mixture of 1,4-dioxane and water is not very high (a suspension formed after addition of Cbz-OSu), THF was then used and both the guanidino acid and Cbz-OSu were dissolved. NaOH was replaced by two equivalents of diisopropylethylamine which works better in organic solvents. The yield was below 10% hence still not satisfactory. It looked that solubility and base is not the problem for this protection process. It would appear that the protecting group on amine A still hinders the addition of a second Cbz group on the guanidino group.

3.3.4 Tosyl Group for Guanidine Protection[43-45]



R= Amino acid

Compared with other urethane-type protecting groups, the tosyl group used for N-terminal protection is not very popular now. One reason is that the best method to remove it is treatment with sodium in liquid ammonia[44]. However its outstanding

stability was thought to be a possibility in this study. The main objective in this project was to achieve four protecting groups on the arginine mimic. One C-terminal and one N-terminal blocking group are on the backbone and there are two blocking groups on the guanidino group of the side chain. The problem is always the selective removal of any one group without destroying the other groups. If two tosyl groups are put on the guanidino group on the side chain (Figure 24), the C-terminal blocking group R might be easily removed by acidic hydrolysis because tosyl groups are quite stable under these conditions. As Boc and Fmoc can be separately destroyed under acidic and basic environments, the tosyl group had attraction in this project.

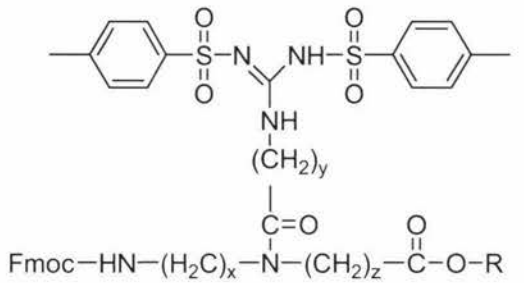


Figure 24. Di-tosyl protected arginine mimic.

The use of the tosyl group for masking the guanidino function of arginine was first conceived by Schwyzer and Li[43] who prepared N^{α}-p-(p'-methoxyphenylazo)-benzyloxycarbonyl-N^{G}-tosyl-L-arginine. Later, Ramachandran et. al. [45] improved the recipe and produced crystalline tosyl-L-arginine derivatives. However, in their recipe, they put only one tosyl protecting group on the guanidino group and made the mono protected product. It was noted that the activity of the amine B of the guanidino group could not be ignored. In this project, considerable effort was focused on the preparation of the di-protected guanidino group.

The reagent used for addition of the tosyl group on guanidine was *p*-toluenesulfonyl chloride (Figure 25).

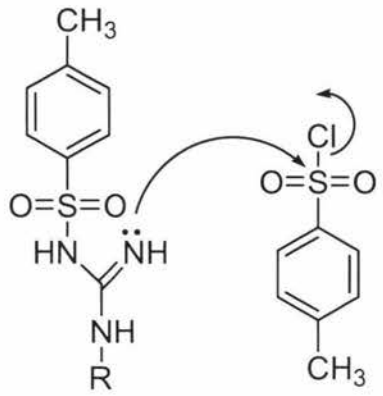


Figure 25. Attempted di-protection of a guanidino group with *p*-toluenesulfonyl chloride.

In this study, the method developed by Ramachandran et. al.[45] was attempted to make the di-tosyl-guanidino acid. The main product was still the mono-protected guanidino acid, and the yield of di-protected product was lower than 2%. Increasing the equivalents of the p-toluenesulfonyl chloride used did not improve the yield.

The benefit of the tosyl group for selective removal of different protecting groups on the arginine mimic remains attractive. However, considering the low yield, and drastic removal conditions (sodium in liquid ammonia), this route for guanidino protection was abandoned.

3.3.5 Experimental

a. 3-[*N,N'*-bis(tert-butoxycarbonyl)guanidino]propionic acid

N-(aminoiminomethyl)-ß-alanine (1.31g, 10mmol) was dissolved in 4N NaOH solution (20mL). Then the mixture of di-*t*-butyl dicarbonate (6.54g, 30mmol) in dioxane (20mL) was added dropwise over half an hour. After stirring for 2h, another portion of di-*t*-butyl dicarbonate (2.18g, 10mmol) was added. Then it was stirred at room temperature overnight. The mixture was extracted by ethyl acetate (4×20mL)

and the combined organic layers were washed by 2M aq. HCl solution until the pH became 6-7. Then it was dried under reduced pressure. The crude product was purified by flash column chromatography to give 0.27g product as a white solid (8%). ^{1}H NMR ($CDCl_3$) δ 10.02 (s, 1H), 3.71 (t, 2H), 2.76 (t, 2H), 1.46 (s, 18H).

b. 3-[*N,N'*-bis(benzyloxycarbonyl)guanidino]propionic acid

N-(aminoiminomethyl)-ß-alanine (1.31g, 10mmol) was dissolved in 4N NaOH solution (20mL). Then a mixture of benzyloxy-carbonylsuccinimide (7.47g, 30mmol) in dioxane (20mL) was added slowly. The mixture was stirred at room temperature overnight. The mixture was extracted with ethyl acetate (4×20mL) and the combined organic layers were washed by 2M aq. HCl solution until the pH became 3~4. After solvent was removed under vacuum, the crude product was purified by flash column chromatography (eluent, CH_2Cl_2/Methanol, 9:1) to give 0.39g of a white solid (9%). ^{1}H NMR ($CDCl_3$) δ 7.37~7.31, (m, 10H), 5.16 (s, 4H), 3.67 (t, 2H), 2.79 (t, 2H).

c. 3-[*N,N'*-bis(toluenesulfonyl)guanidino]propionic acid

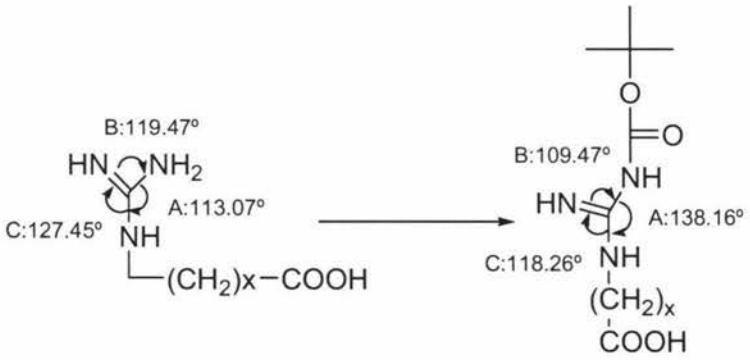
N-(aminoiminomethyl)-ß-alanine (1.31g, 10mmol) was dissolved in a mixture of water (15mL) and acetone (60mL), cooled to 0°C and stirred vigorously. Sufficient cold 4 N NaOH solution was added to maintain the pH at about 11 through out the reaction. Then *p*-toluenesulfonyl chloride (9.5g, 50mmol) dissolved in 15mL of acetone was added dropwise during a period of half an hour. Stirring was continued and the pH was maintained at about 11 by the addition of cold 4 N NaOH solution. The clear solution was stirred at 0°C for another 3 hours. The pH then was adjusted to 7 with 1 N HCl solution. Acetone was removed under reduced pressure at room temperature. Water (25mL) was added to the residual solution and the resulting aqueous phase was extracted with ether (3×40mL). The aqueous layer was cooled to 0°C and acidified to pH 3 with 6 N HCl solution. Then the aqueous phase was removed and saturated with NaCl, extracted with ethyl acetate (3×40mL). The residual oil was dissolved in the combined ethyl acetate extracts and washed repeatedly with a cold 0.1 N HCl solution. The organic phase was then washed with water until neutral, dried over $MgSO_4$ and evaporated to dryness under vacuum at

room temperature. The crude product was purified by flash column chromatography (eluent, CH_2Cl_2/n-hexane, 7:3) give a white solid. Percentage yield: 2%. ^{1}H NMR ($CDCl_3$) δ 7.80~7.85 (m, 8H), 3.19 (t, 2H), 2.83 (t, 2H), 2.30 (s, 6H).

3.4 Conclusion

In this Chapter, guanidine was investigated as a suitable starting material to make the di-protected guanidino acid side chain. After several attempts it was deemed unsuitable for this project. Both 1H-pyrazole-1-carboxamidine hydrochloride and aminoiminomethanesulfonic acid proved to be efficient guanylation reagents. In this study, all three selected primary amino acids reacted with both two guanylation reagents to produce guanidino acids within short period and gave high yields. Although two of the products did not precipitate from the reaction mixture, they could be easily purified by recrystallization.

In contrast to the ease of producing guanidino acids, attempts to make di-protected guanidino acids from addition of two Boc, Cbz or tosyl groups proved difficult. Why in this project, even with an increase in the basicity of the reaction solution and increasing the amount of the urethane reagent did not improve the yield? One could be the structure of guanidine (Figure 26). For a free guanidine or a guanidino acid in this project, the angles between three amines groups are somewhat



1 2

Figure 26. Angles of the guanidino group for the unprotected and mono protected guanidino acids.

similar and close to 120° (molecule 1 in Figure 26). In this case, there is enough space for addition of a protecting group. However, after the first addition (molecule 2 in Figure 26), the electrostatic force between the urethane substituent and the first protecting group distorts the structure. In Figure 21 it can clearly be seen that the angle A was changed from 113.07° to 138.16° when one Boc group was added. So, when one protecting group was added, the space for the lone pair electrons on the second amine group to attack another protecting reagent molecule is limited. The two bulky substituent and protecting group blocks these protecting reagent molecules from approaching the second amine. Thus this problem required another approach.

CHAPTER FOUR
CONVERSION OF PROTECTED CARBOXAMIDINE COMPOUNDS TO GUANIDINO ACIDS

4.1 Introduction

In Chapter Three, the application of several guanylating reagents toward guanidino group formation was discussed. Because di-protection of the guanidino group seemed to be quite difficult, another route had to be sought to solve this problem. The new idea was to put two protecting groups on the guanylation reagent first, then a primary amino acid would nucleophilic attack it to form a di-protected guanidino acid. Ureas which are very popular for guanidino group formation compared with substituted guanidines, have a simple structure. The leaving group is always small (like S-methyl or pyrazole) or even an atom (sulfur). In Figure 27 it can be seen that because the

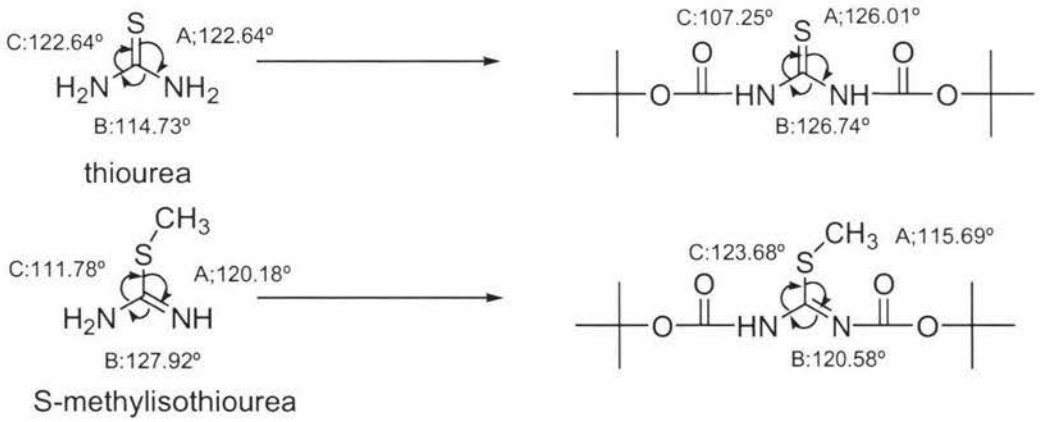


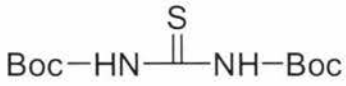
Figure 27. Angles of the guanidino group for thiourea and S-methylisothiourea and their di-Boc products.

sulfur and S-methyl groups are smaller than the carboxylic group of the guanidino acid in Chapter Three, there should be enough space for the second protecting group. When the di-protected reagents formed, it is supposed that the two bulky protected groups would repel each other hence provide enough space for the primary amino acid to nucleophilic attack the centre carbon atom in the next step.

Another benefit for the application of a pre-protected ureas is that some chemists found that for some guanylating reagents, addition of electronegative protecting groups can further increase the electrophilicity of the amidino carbon, hence making it more reactive to nucleophilic attack[20,46]. The ideal reagents are thiourea and its derivatives which are more stable than the guanylating reagents introduced in Chapter Three. Their stability minimizes the possibility of decomposition in the highly basic environment used in the protection process. Also, it was speculated that the activating effect of the protecting group would improve reactivity in our guanidine synthesis.

4.2 Protection of Carboxamidine Compounds and Conversion to Guanidino Acids

4.2.1 *N,N'*-bis-*t*-Butoxycarbonylthiourea



Thiourea and its di-protected derivatives are now widely applied to peptide chemistry, due to their solubility in most organic solvents (DMF, THF, 1, 4-Dixoane) and their stability under extreme conditions. Rather than using thiourea, di-protected derivatives for example *N,N'*-bis-*t*-butoxycarbonylthiourea are more popular in solution phase peptide synthesis[18,21]. Due to its high electronegativity, the sulphur atom of the thiourea is not a good leaving group. Thus when the thiourea reacts with an amine or amino group it is quite hard to form an unprotected guanidino group. The poor reactivity of free thiourea needs an extra electron withdrawing effect from protecting groups like Boc to activate the amidino carbon. Also the application of proper metal salts of mercury or copper are employed to facilitate the reaction[19,22]. Mercuric or cupric ions react with the sulphur to form insoluble sulphide precipitates, and hence weaken the double bond between the sulphur and amidino carbon (Figure 28). Until now, the exact intermediate and mechanism of bis-Boc-thiourea is unknown. Kim K.S. claimed that an in-situ generated bis-Boc-urea might be the reactive species which undergoes the guanylation for deactivated amines[22]. When bis-

Boc thiourea is subjected to the similar reaction conditions in the absence of an amine it is rapidly transformed into a less polar compound within a few minutes and then a complex is formed with the metal[22].

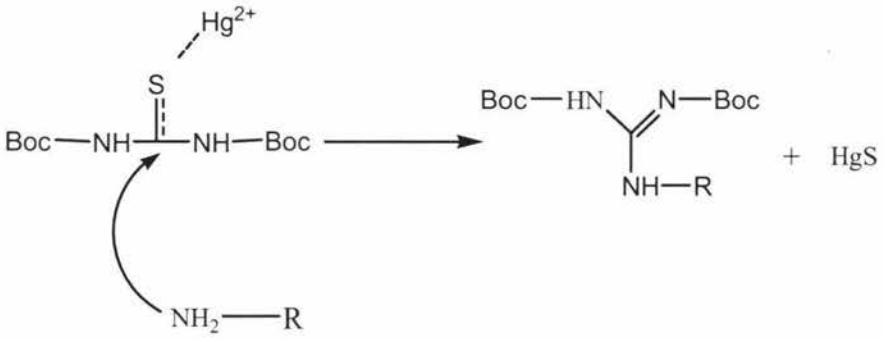


Figure 28. Basic mechanism for the reaction of *N,N'*-bis-*t*-Butoxycarbonylthiourea react with amines catalyzed by $HgCl_2$.

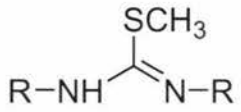
The precipitated HgS limits the application of this approach for solid phase peptide synthesis[19] (method to overcome this problem will be introduce below). Also, although this method solved the problem of di-protected thiourea with amines, its reactivity with amino acids was still quite low[19,22]. Until now, no report has stated that this is a good reagent for guanylation of amino acids. The reactions carried out in this study confirmed this conclusion. With the assistance of metal salt, the selected three primary amino acids treated with this reagent did not give any positive results.

In 1997, according to Kim's assumption of the intermediate of di-Boc-thiourea guanylation, Lipton et. al. published a paper that mentioned their application of Mukaiyama's reagent (2-chloro-*N*-methylpyridinium iodide) which has a similar function as the above metal salts for guanylation using di-Boc-thiourea[19]. Although this reagent solved the problem of formation of the insoluble by-product and can be applied in solid phase peptide synthesis, it is still not a good candidate for the nucleophilic amino acids due to poor yields.

The work in this study with di-Boc-thiourea failed to meet expectations. An alternative di-protected guanylating reagent was needed. After considering the

advantages and disadvantages of thiourea, one of its derivatives looked more promising and finally led to successful di-protected guanidine acid formation.

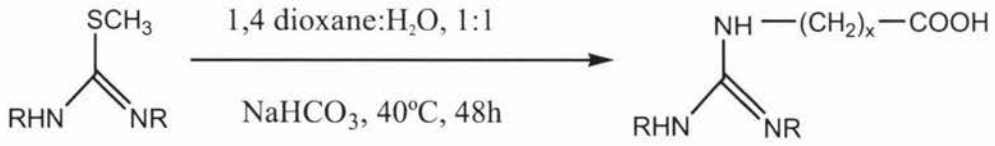
4.2.2 *N,N'*-bis(*t*-butoxycarbonyl)-S-Methylisothiourea



R=Boc, or Cbz

Attempts to apply thiourea to guanidine acid syntheses were not very successful due to low reactivity, even after addition of activating protection groups. Thus finding another thiourea derivative which possessed this advantage but was more reactive became paramount. Also, although some chemists mentioned the application of $HgCl_2$ to activate the sulphur group of the *N, N'*-bis-*t*-Butoxycarbonylthiourea, most peptide researchers believed that activation by suitable protection groups was sufficiently effective for conversion of less sterically hindered amines and amino acids to the desired guanidines[19,20,22,23].

In 2003, Jan Izdebski et. al. introduced the functionalization of S-methyl isothiourea with different types of protecting groups applied to guanidine acid synthesis[20,23] (Figure 29). In this study, the focus was placed on protection of isothiourea with the Boc and Cbz groups. The main reason for this was the simplicity of its production. The di-Boc isothiourea can be precipitated during the reaction, so it



1: R=BOC
2: R=Cbz

Figure 29. Formation of di-protected guanidine acids from S-methylisothiourea.

can be easily isolated and used without further purification. Isothiourea or its protected product is suitable for most of guanylating reactions and unreacted amines or amino acids can be washed out from the organic layer with aqueous acid. By-product formation was insignificant in this process hence no flash column chromatography was needed.

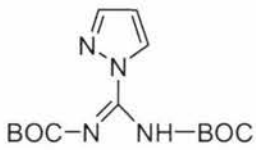
Table 3 lists the results of the formation of di-protected guanidine acids using *N,N'*-bis(*t*-Butyloxycarbonyl)-S-methylisothiourea in this study. Although the yields were lower than the published results (average 80% using N^{α}-alkoxycarbonylornithine), they were still satisfactory to this project. They were of course much better than the other reagents studied so far.

starting amino acid	reaction time	yield	melting point
glycine	48h	54%	117-121 °C
ß-alanine	48h	61%	104-105 °C
6-aminohexanoic acid	48h	48%	100-102°C

Table 3. Experimental results for the formation of di-Boc guanidine acids from the reaction of the *N,N'*-bis(t-butyoxycarbonyl)-S-methylisothiourea with three primary amino acids.

Also, in 2003 Jan Izdebski et. al. put two new protecting groups on S-methylisothiourea: ortho-Cl-Cbz and ortho-Br-Cbz[20,23]. They expected these two groups would increase the reactivity of the reagent due to the extra electron-withdrawing effects of bromine and chlorine atoms. Their results confirmed their assumption. Another benefit of these two reagents is their stability in trifluoroacetic acid (TFA). This was extremely important for this project because the blocking group at the C terminus of the backbone was to be an alkyl ester (details see Chapter Five), which would need an acidic environment to remove it. However, it is known that Boc is sensitive to acid hence the guanidine protection would be destroyed in this type of process. The application of ortho-Cl (or Br)-Cbz can avoid this problem. They were deemed worthy of further investigation.

4.2.3 1H-Pyrazole-1-[*N,N''*-bis(*t*-Butoxycarbonyl)]carboxamidine



The above two guanylating reagents used were thiourea and it's a derivative. These two compounds were readily protected with the butyloxycarbonyl (Boc) or benzyloxycarbonyl (Cbz) groups and production of the desired guanidino acids using S-methyl isothiourea was successful. After this study, an attempt was made to investigate another more reactive di-protected carboxamidine compound. To ensure such a reagent can undergo both protection and nucleophilic attack reactions smoothly, it must possess the following two characteristics:

a) In this study, most protecting groups are urethanes. Formation of the protected amine is via a nucleophilic substitution reaction (amine group of the carboxamidine attacks the protecting reagent), therefore the new carboxamidine compound should be a good base, otherwise it can not be react to the protecting reagent effectively. For example, aminoiminomethanesulfonic acid, as mentioned in Chapter Three, is a good guanylating reagent but can not be pre-protected by Boc or Cbz groups because of its acidity.

b) It must possess a good leaving group, so that when the nucleophilic substitution reaction takes place, this leaving group can be cleaved rapidly.

In Chapter Three, the usefulness of the free 1H-pyrazole-1-carboxamidine hydrochloride in guanidine acids formation was discussed. All three amino acids reacted with it successfully to give satisfactory yields. It is an organic base so there was no problem for reaction with a popular protecting reagent. The leaving group pyrazole has the pKa value of 11.25 so it can be deprotonated at about pH 12. If this reaction is carried out in a basic environment like LiH (Figure 30), the cleaved pyrazole cation can be stabilized thus making it a good leaving group.

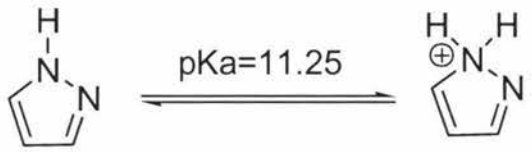


Figure 30. Protonation of pyrazole.

The other advantage of 1H-pyrazole-1-carboxamidine hydrochloride is that after the addition of the two protecting groups (Boc), the electron withdrawing effect of the t-butyloxycarbonyl groups will increase the electrophilicity of the amidino carbon, hence accelerate the following reaction (Figure 31). Also it is assumed that the protected compound 1H-pyrazole-1-[*N,N''*-bis(t-Butoxycarbonyl)]carboxamidine should react similarly to the analogous 1H-pyrazole-1-carboxamidine[18].

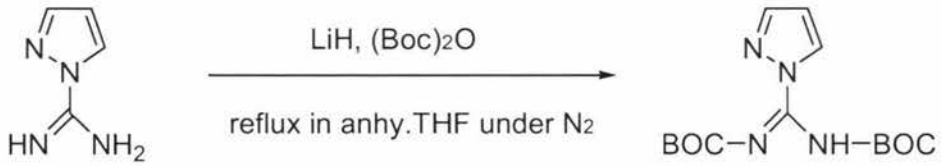


Figure 31. Formation of 1H-pyrazole-1-[*N,N''*-bis(*t*-Butoxycarbonyl)]carboxamidine

In 1994, Michal Lebl et. al. published a paper, introducing a new synthesis for the production of 1H-pyrazole-1-[*N,N''*-bis(*t*-butoxycarbonyl)] carboxamidine[46] (Figure 31). They reacted this reagent with a series of amines and amino acids to investigate their reactivity. Compared with other methods in which the reaction was carried out in a mixture of dioxane and water, Michal Lebl et. al. used anhydrous THF as the solvent in this reaction where LiH was used. Other methods for the preparation of di-protected carboxamidines mainly used aqueous Na_2CO_3, NaOH or $NaHCO_3$ as the base. The main reason for this was that the starting material 1H-pyrazole-1-

carboxamidine hydrochloride, although stable in the fridge, can be destroyed by water or oxygen at reflux temperature.

In section 3.2.2, the possibility of self-condensation of 1H-1-pyrazole carboxamidine to form N-amidinopyrazole-1-carboxamidine was discussed. It was confirmed that this reaction can proceed when heated for a long time.

However, when this recipe was used in this project (Figure 32), the following limitations were found:

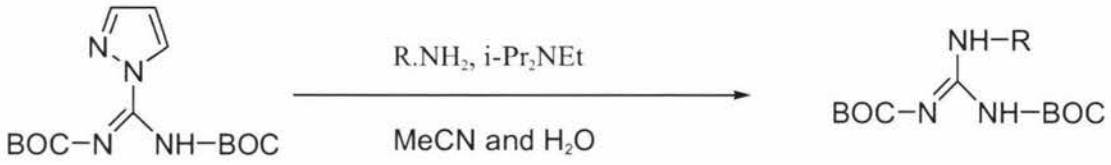


Figure 32. Production of guanidines from 1H-Pyrazole-1-[*N,N''*-bis(*t*-butoxycarbonyl)]carboxamidine.

1. Production of 1H-pyrazole-1-[*N,N''*-bis(*t*-butoxycarbonyl)] carboxamidine needed strict conditions. Firstly, LiH is essential in this reaction although Michal Lebl et. al. mentioned that n-hexane washed NaH can also be used with lower yield. In this project, the product of the reaction between 1H-Pyrazole-1-[*N, N''*-bis(*t*-Butoxycarbonyl)] carboxamidine and ß–alanine was an oil, and attempted recrystallization from hexane did not give a pure solid product. Secondly, as mentioned above, a small amount of water can destroy the whole reaction. High quality anhydrous THF must be used and the entire environment must be dry.

2. Michal Lebl et. al. (1994) mentioned that if LiH was used, the first portion of product can be recrystallized from n-hexane giving a yield 69%. Purification of the mother liquor by flash column chromatography would afford an additional portion of 26%. However, in this study, the pure solid product could not be obtained by recrystallization. The final product was a crude oil, needed to be pure by flash column chromatography.

3. Although results for the guanylation of some amino acids were given in the paper, it was reported that a number of amino acids (e.g. ß-alanine) did not work well with this reagent due to solubility problems. The ideal solvent like MeCN to dissolve this guanylation reagent did not always work well with some common amino acids. The solvent the authors suggested for guanidine preparation is a mixture of MeCN and H_2O with the ratio varying with different amino acids. In this study, only glycine could be completely dissolved with the guanylation reagent in MeCN/H_2O (80/20, 1g in 30 mL) gave a reasonable yield (55%). For ß-alanine and 6-amino hexanoic acid, the starting mixtures had to be very dilute and did not give satisfactory yields. Michal Lebl et. al. suggested that use of the alkyl ester could significantly improve the yield. However, cleavage of this kind of ester (e.g. acidic or basic hydrolysis, or reduction) can bring a new problem to this project: destruction of the Boc groups. This will be clearly discussed in Chapter Six.

Also, when preparing 1H-pyrazole-1-[*N,N''*-bis(*t*-butoxycarbonyl)] carboxamidine, a greater consumption of di-t-butyl dicarbonate (1:3) occured than with *N,N'*-bis-*t*-butoxycarbonylthiourea (1:2.2) and *N,N'*-bis(*t*-butoxycarbonyl)-S-methylisothiourea (1:2.6). Although a lot of problems occurred in the attempts to apply 1H-pyrazole-1-[*N, N''*-bis(*t*-butoxycarbonyl)] carboxamidine in the guanidine acids formation, this new reagent showed promise if the insolubility limitations could be improved.

4.3 Conclusion

In this chapter, three kinds of carboxamidine derivatives were examined. Thiourea and isothiourea due to its relatively stable electronegative leaving group, reacts with di-t-butyl dicarbonate smoothly without by-products. The di-Boc-product can be easily purified by extraction with suitable solvents to give a high yield. However, for di-Boc thiourea the stable structure also limits its usefulness for guanylation of amino acids. To increase the yield, elimination of the sulphur and SCH_3 groups need extra reagents ($HgCl_2$ or 2-chloro-N-methylpyridinium iodide). Due to the possibility of

formation of mercuric conjugates, it is still not the best reagent for synthesis of guanidine acid. 1H-pyrazole-1-[*N, N''*-bis(*t*-Butoxycarbonyl)] carboxamidine was more reactive than di-Boc thiourea, however its insolubility in common solvents in which some amino acids can dissolve limited its application in this project. Compared with them, di-Boc-S-methyl isothiourea is relatively active, with reaction times with primary amino acids of about 48h at high temperature. The yields of this reaction with the three amino acids used were satisfactory, hence here di-protected (Boc or Cbz) S-methyl isothiourea was finally selected in this project to synthesize the desired guanidine acids for peptide synthesis.

4.4 Experimental

a. *N,N'*-Bis-*t*-Butoxycarbonylthiourea[21]

0.571g of thiourea (7.5mmol) was dissolved in THF with stirring. The mixture was then cooled to 0 °C in an ice bath under N_2. 1.35g of hexane washed NaH (33.8mmol) was added. The ice bath was removed after 5 min and then the reaction mixture was allowed to stir at room temperature for 10 min. The reaction mixture was cooled again to 0 °C and 3.6g of di-t-butyl dicarbonate (16.5mmol) was added. After half an hour, the ice bath was removed and a slurry formed within 10 min. The reaction mixture was stirred for another 2 h at room temperature and was then quenched with an aqueous solution of saturated $NaHCO_3$ (10mL) solution. The mixture was poured into 250mL of water and extracted with ethyl acetate (3×70mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under vacuum to give 1.96 g (95%) solid product which can be used without further purification. ^{1}H NMR ($CDCl_3$) δ 1.61 (s, 18H)[21].

b. General guanylation reaction procedure using *N,N'*-bis-*t*-Butoxycarbonylthiourea[22]

To a mixture of 2mmol of amino acid, 0.35g of *N,N'*-bis-*t*-Butoxycarbonylthiourea (2mmol) and 0.67g of triethylamine (6.6mmol) in 4mL of DMF at 0 °C was added

0.59g of $HgCl_2$ (2.2mmol) with stirring. The resulting mixture was stirred at 0 °C for half an hour, diluted with 40mL of ethyl acetate, and filtered. The filtrate was washed with 20mL of water and 20mL of brine, dried over $MgSO_4$ and concentrated in vacuum. The residue was purified by flash column chromatography (hexane/ethyl acetate, 3:1). Yield: 5-12%.

c. *N,N'*-bis(t-Butoxycarbonyl)-S-methylisothiourea[20]

1.39g of S-methylisothiourea semisulfate (10mmol) was dissolved in a mixture of H_2O (30mL) and dioxane (30mL) followed by addition of 10mL of 1M aqueous NaOH solution (10mmol) and 5.81g of di-t-butyl dicarbonate (25mmol, 1.25equiv.). The reaction mixture was vigorously stirred overnight at room temperature. The precipitate formed was filtered and washed with a small amount of water. The filtrate was concentrated on a rotary evaporator to approximately half the volume and the solid that separated was isolated by filtration. The solids were combined and suspended in 100mL of H_2O at about 50 °C, shaken and filtered. Finally the product was dried under vacuum to give 2.3g white solid (97%). ^{1}H NMR ($CDCl_3$) δ 11.62 (s, 1H), 2.42 (s, 3H), 1.53 (s, 9H), 1.51 (s, 9H).

d. General guanylation reaction procedure using *N,N'*-bis(*t*-butoxycarbonyl)-S-methylisothiourea[23]

To a solution of $NaHCO_3$ (0.525g, 6.25mmol) in 30mL of dioxane:water (1:1), 5mmol of amino acid (glycine, ß-alanine, 6 aminohexanoic acid) was added followed by *N, N''*-bis(*t*-butoxycarbonyl)-S-methylisothiourea (3.75mmol). The mixture was stirred for 48 h at 40 °C. The solvent was removed under vacuum and the crystalline residue was dissolved in 400mL of ethyl acetate. The solution was washed with aqueous HCl (26mL of 0.3M) and water (6×20mL), dried over $MgSO_4$ and the solvent was evaporated to give an oil which solidified. Recrystallization from methanol gave yields of 48-61%.

CHAPTER FIVE
BACKBONE SYNTHESIS

5.1 Introduction

In Chapter One, the structure of natural arginine was discussed. Its chirality comes from the chiral carbon connecting the amino acid backbone and the guanidine side chain. In this project, to avoid chiral restrictions a new backbone was designed (Figure 33). It has the following features:

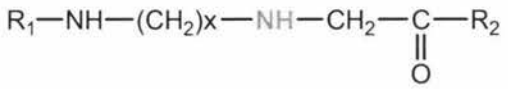


Figure 33. Designed backbone structure.

1. The secondary amine is essential as a branching point for attachment of the guanidino acids.
2. Both N and C-terminals were protected to avoid self-condensation and other side reactions in the next combination step and the following peptide synthesis.

The first feature requires the use of di-amines reagents (like ethylenediamine) for the backbone synthesis. The second feature involves the selection of protecting groups. In Chapter Four the reason for Cbz and Boc application to guanidine protection was discussed. Figure 34 outlines the basic principle of solid phase peptide synthesis (SPPS) and the requirements for the protection of the various functional groups.

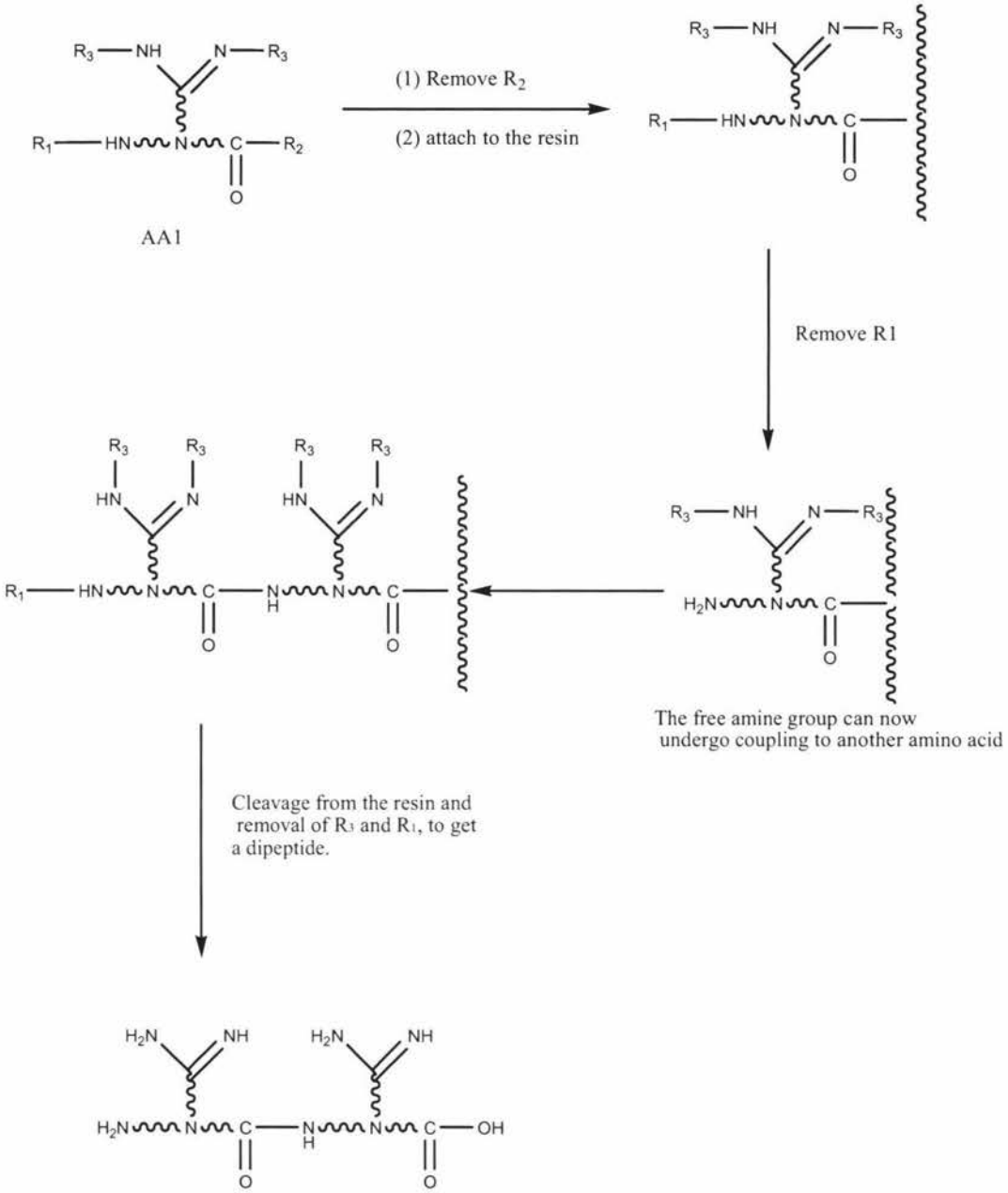


Figure 34. Basic process of solid phase peptide synthesis.

In this process, the protecting group R_3 must be conserved until the entire peptide is constructed. Its chemical properties must be dissimilar to R_1 and R_2 to make sure that it is stable in the environment of repeated removal of those two groups (R_1, R_2). The Fmoc group can be removed in a basic environment in which the Boc groups on the guanidine are stable (Figure 35). Fmoc technology in solid phase peptide synthesis has been used extensively; hence it was selected to protect the N terminus of the arginine mimics.

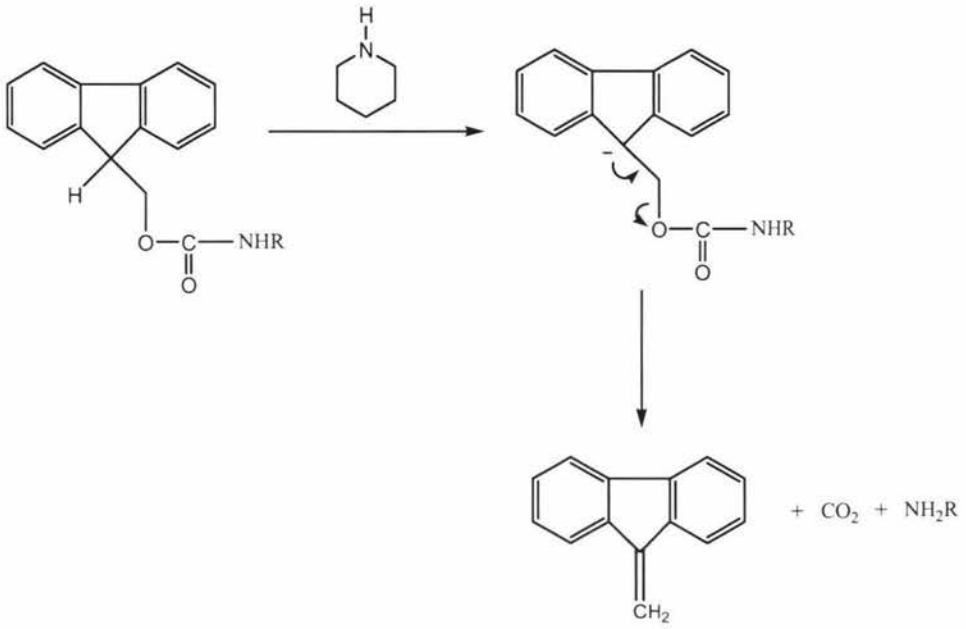


Figure 35. Mechanism for removal of the 9-fluoromethyloxycarbonyl group (Fmoc).

In addition to the protection of the amine group (N-terminal), the carboxylic acid group also needed to be protected (R_2 in Figure 34). M. Bodanszky introduced a series of methods for carboxyl protection including formation of alkyl ester and hydrazides[15,24]. In this study, the synthesis of the alkyl esters was chosen.

5.2 Synthesis and Discussion

Josey J.A. et. al. (1995) introduced a method for the synthesis of a series of PNA (peptide nucleic acid) Fmoc monomers using ethylenediamine[14]. Their method for formation of the backbone is shown in Figure 36. In this study, t-butyl chloroacetate was used instead of the t-butyl bromoacetate.

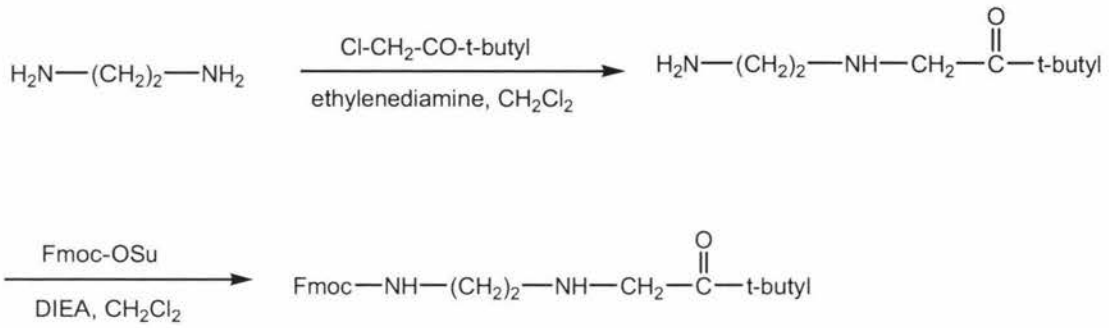


Figure 36. Reaction scheme for the construction of the protected backbone amino acid.

It is known that the electronegative carbonyl group can affect the nucleophilicity of the secondary amine, and the number of the methyl groups between them may determine the success and yield of the next combination step. Josey et. al. reported that secondary amines can effectively react with the carboxylic group of the guanidine acids when there is only one methyl end group between them[14]. Thus t-butyl chloroacetate was chosen as a desirable starting material for the construction of the amino acid backbone.

Another challenge arises in the step where ethylenediamine reacts with t-butyl chloroacetate. In Josy J.A. et. al. studies, a large excess of diamine was used (8 : 1)[14]. After the reaction finished, the excessive ethylenediamine was washed out with water. They claimed the yield of this reaction was 87% and no significant di-substituted product existed. In this study, HPLC and MS results confirmed this claim.

The paper[14] reported that in the final step the hydrochloride salt of product can be crystallized from a concentrated solution in a freezer. This crystallization process was hard to control. In some syntheses seeding did not yield a solid as the product. Finally, this procedure was improved by taking the concentrated solution and evaporation it to dryness under reduced pressure. The solid product was then recrystallized from warm acetone and n-hexane.

Other alkyl groups like benzyl and methyl esters were also investigated. Replacement of t-butyl chloroacetate by methyl chloroacetate which is also

commercially available gave the backbone with a methyl terminal. Condensation of chloroacetic acid with benzyl alcohol produced the benzyl chloroacetate which was applied in the same procedure to make the final product.

5.3 Conclusion

The methods of Josy J.A. et. al.[14] were successfully applied to the protected backbone synthesis required for this project. Use of different alkyl esters did not change the result significantly. Reactions proceeded rapidly and no extreme conditions were needed. HPLC/MS and NMR results supported acceptable purity for final products.

Nevertheless application of alkyl esters into the arginine mimic molecule results presents a selectivity problem. Most of the popular N-terminal protecting groups are unstable in either basic or acidic hydrolysis environment. Enzyme catalysed hydrolysis of alkyl ester and application of other more stable N-terminal protecting groups were thought to be an alternative solution in development of this new series of artificial amino acids.

5.4 Experimental

a. *t*-butyl *N*-(2-aminoethyl) glycinate[14]

To a vigorously stirred solution of ethylenediamine (30mL, 0.45mol) in CH_2Cl_2 (200mL) at 0 °C was added *t*-butyl chloroacetate (7.68g, 7.30mL, 0.051mol) in CH_2Cl_2 (40mL) over 1h. The resulting mixture was allowed to warm to room temperature within 1h and then stirred overnight. The reaction mixture was washed with water (3×50mL) and the combined aqueous washes was back-extracted with CH_2Cl_2 (50mL). The combined organic phases were dried over $MgSO_4$ and filtered. (This solution was used in the next step without further purification). The solution was then concentrated under reduced vacuum to give 7.53g colourless oil. Yield: 83%: ^{1}H NMR ($CDCl_3$) δ 3.27(s, 2H), 2.75(t, 2H), 2.63(t, 2H), 1.51(s, 3H), 1.43(s, 9H).

b. *t*-butyl *N*-(2-(*N*-9-fluorenylmethoxycarbonyl)aminoethyl)glycinate hydrochloride[14]

To a solution of *t*-butyl *N*-(2-aminoethyl) glycinate (7.53g, 0.043mol) in CH_2Cl_2 (300mL) was added diisopropylethylamine (7.35mL, 0.043mol) with vigorous stirring. A solution of *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (14.5g, 0.043mol) in CH_2Cl_2 (80mL) was added dropwise over 1h. The resulting solution was stirred overnight at room temperature and then washed with 1 N aq. HCl solution (5×50mL) and brine (50mL). The organic phase was dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give a yellow oil. This oil was dissolved in a minimum of warm acetone and then crystallized with n-hexane. The white solid was collected by filtration and dried in vacuo to give 8.65g of white solid. Yield: 51%: ^{1}H NMR (d_6-DMSO) δ 9.22(s, 2H), 7.86(d, 2H), 7.68(d, 2H), 7.54(t, 1H), 7.40(t,2H), 7.32(t, 2H), 4.34(d, 2H), 4.20(t, 1H), 3.83(s, 2H), 3.31(m, 2H), 2.95(t, 2H), 1.43(s, 9H).

CHAPTER SIX
COUPLING OF THE GUANIDINE CONTAINING SIDE CHAIN TO THE AMINO ACID BACKBONE

6.1 Introduction

After the two parts of the arginine mimic, the di-protected guanidino acid and the amino acid backbone had been prepared, an effective method to combine them together was required. It is known that one purpose of this project was to avoid the chirality of the natural arginine. In this study this was to be achieved by acylation of the backbone to the side chain.

The usual two methods of activation of C-terminal are to use preformed active esters or direct coupling reagents. The second method is basically an improvement on the first method. The purpose of the coupling reagent is to convert the carboxylic acid to an ester or anhydride for rapid reaction with an amino group.

In this study, the carboxylic group of the guanidine containing side chain must be combined with a secondary amine in the middle of the backbone. However, the two reactive centres connected to this secondary amine were both protected with a large protecting group (Fmoc on N-terminal and alkyl ester on C-terminal). A suitable coupling reagent must therefore be carefully selected.

Name	Structure	pKa of ammonium ion
ammonia	NH_3	9.26
ethylamine	$CH_3CH_2NH_2$	10.81
diethylamine	$(CH_3CH_2)_2NH$	10.49

Table 4. Basicity of common amines.

In this project, two popular reagents were selected, dicyclohexylcarbodiimide (DCC)[24] and O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU)[25]. Each of them was used for the coupling reaction and their effectiveness and reactivity were compared.

6.2 Discussion

The reaction mechanism for coupling with dicyclohexylcarbodiimide (DCC) and O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) is shown in Figure 37. Both of them readily react with various amines and hydroxyl groups and are widely used in peptide bond formation (acylation). DCC uusually

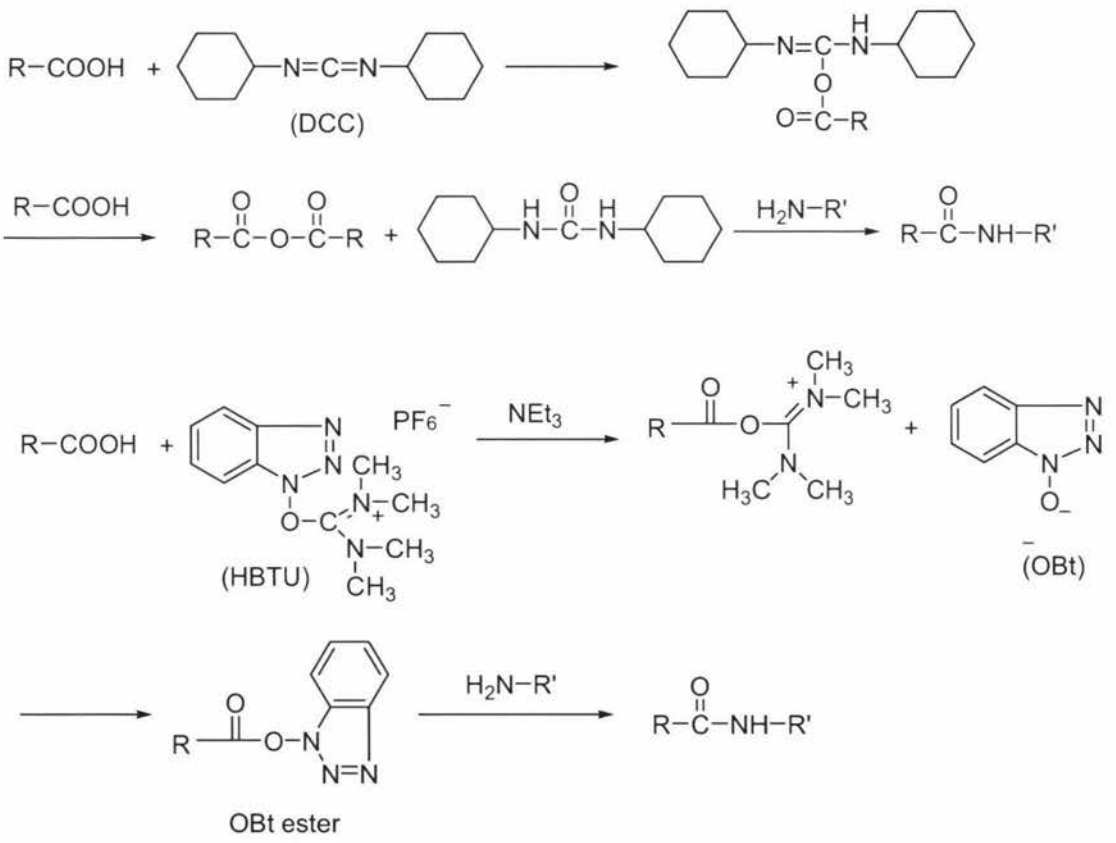


Figure 37. Structure and reaction scheme for the coupling reagents dicyclohexylcarbodiimide (DCC) and O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU)

converts a carboxylic acid to an iso-urea then an anhydride[15,24]. Then this anhydride is attacked by an amino group to form a new amide bond. HBTU has now become the preferred tool in Fmoc-peptide synthesis. Its main function is to convert amino acids in the presence of a tertiary base, to their corresponding OBt esters (Figure 37). Because the OBt component is an electron withdrawing group, it makes the adjacent carbonyl carbon electrophilic. This activated ester then is easily attacked by an amino group to form a new amide bond.

One advantage of using these two coupling reagents is the ease of product purification. For DCC, its by-product *N, N'*-dicyclohexylurea precipitates from the reaction mixture so it can be readily filtered off. Unreacted HBTU and its by-product are soluble in water and also can be destroyed by acid. After the reaction has gone to completion, they can be removed by simply washing with acid solution (e.g. 1M aq. HCl).

6.3 Conclusion

The final step in this synthetic strategy worked well. In both coupling reactions, the guanidine containing side chain and the backbone amino acids were successfully combined within a short time under mild conditions. As expected, the secondary amine on the backbone amino acid was a good nucleophile and effectively coupled with the guanidine containing side chain. The bulkiness of the two protecting group on the backbone amino acid did not hinder the nucleophilic reaction. It is assumed that the distance between the secondary amine and those two protecting groups minimized their steric hinderance. The yield using HBTU was a little higher than that of DCC; therefore it was selected as the coupling reagent of choice in this project.

6.4 Experimental

a. Coupling reaction to produce target arginine mimic using DCC: *N*-[*N'*-((9-fluorenyl)methoxycarbonyl)-2-aminoethyl]-*N*-*t*-

butyloxycarbonylmethyl-3-*N′,N″*-bis(*t*-butyloxycarbonyl)carbamidinopropanamide

To a solution of the 3-[*N,N′*-Bis(*t*-butoxycarbonyl)guanidino]propionic acid (0.343g, 1mmol) and *t*-butyl *N*-(2-(*N*-9-fluorenylmethoxycarbonyl)aminoethyl) glycinate hydrochloride (0.432g, 1mmol) in dichloromethane (20mL), dicyclohexylcarbodiimide (DCC) (0.22g, 1.1mmol) was added and the mixture was stirred at room temperature overnight. Then the reaction mixture was filtered. The filtrate was washed with saturated aq. $NaHCO_3$ solution (2×10mL) and water (10mL). The organic layer was dried over $MgSO_4$ and evaporated to dryness. Purification by flash column chromatography (CH_2Cl_2:MeOH, 95:5) yielded 0.31g product as a white solid (43%). ^{1}H NMR (DMSO-d6) δ 7.85 (d, 2H), 7.52 (d, 2H), 7.44 (t, 2H), 7.06 (t, 2H), 4.45 (d, 2H), 4.09 (t, 1H), 3.91 (s, 2H), 3.69 (m, 2H), 3.35 (t, 2H), 2.92 (t, 2H), 2.48 (t, 2H), 1.48 (s, 9H), 1.47 (s, 18H).

b. Coupling reaction to produce target arginine mimic using HBTU: *N*-[*N′*-((9-fluorenyl)methoxycarbonyl)-2-aminoethyl]-*N*-*t*-butyloxycarbonylmethyl-3-*N′,N″*-bis(*t*-butyloxycarbonyl)carbamidinopropanamide

3-[*N,N′*-Bis(*t*-butoxycarbonyl)guanidino]propionic acid (0.343g, 1mmol) was dissolved in DMF (5mL), then HBTU (0.379g, 1mmol) and (0.101g, 1mmol) was added. After standing for 1min, the orange solution was poured into a solution of *t*-butyl *N*-[2-(*N*-9-fluorenylmethoxycarbonyl) aminoethyl] glycinate hydrochloride (0.432g, 1mmol) and DIEA (2mL) in DMF (10mL). The reaction mixture was stirred at room temperature for 1h. Then the reaction mixture was poured into 25mL of dichloromethane, washed with 1M aq. HCl solution (10mL) and water (2×25mL). The organic phase was dried over $MgSO_4$ and evaporated to dryness. Purification by flash column chromatography (CH_2Cl_2:MeOH, 95:5) gave 0.39g product as a white solid (54%). ^{1}H NMR (DMSO-d6) δ 7.85 (d, 2H), 7.52 (d, 2H), 7.44 (t, 2H), 7.06 (t, 2H), 4.45 (d, 2H), 4.09 (t, 1H), 3.91 (s, 2H), 3.69 (m, 2H), 3.35 (t, 2H), 2.92 (t, 2H), 2.48 (t, 2H), 1.48 (s, 9H), 1.47 (s, 18H).

CHAPTER SEVEN

SUMMARY

One target arginine mimic was produced in this study. It contains a di-protected guanidino group attached to the backbone amino acid through an amide bond (Figure 38). The main purpose of this project was to produce an artificial amino acid which carries a guanidino group without racemization problems. There are still a number of problems to be solved in the future.

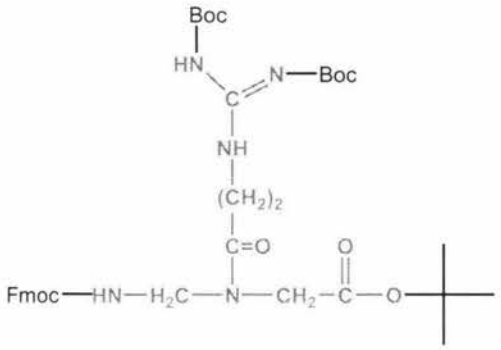


Figure 38. The arginine mimic: *N*-[*N'*-((9-fluorenyl)methoxycarbonyl)-2-aminoethyl]-*N*-*t*-butyloxycarbonylmethyl-3-*N',N''*-bis(*t*-butyloxycarbonyl)carbamidinopropanamide produced in this project.

Earlier in this study, the selectivity of the three protecting groups (guanidino group, N- and C-terminals of the backbone amino acid) was discussed as being crucial to the success of this project. These three protecting groups must possess different chemical properties so that cleavage of any one would not destroy the other two, otherwise self-condensation and/or by-product formation may happen in subsequent steps. For the arginine mimic produced in this study (Figure 38), a *t*-butyl ester was used as the blocking group on the C-terminal of the backbone amino acid but it caused a problem after the entire molecule was assembled. For construction of the octapeptides, the C blocking *t*-butyl ester of the arginine mimic must first be hydrolyzed, and the free carboxylic group will bind to the resin. This is then followed by deprotection of its backbone amino group and acylation of the carboxylic acid group by the second amino acid. Because for the designed octapeptides in this project,

there are totally need three arginines or its mimics. So, the *t*-butyl group must be selectively cleaved from the peptide in each cycle without affecting the semi-permanent protecting groups on the functional side chains. However, currently the most popular methods for the decomposition of esters involve the application of acid or base hydrolysis. The two important N protecting groups in this project: Boc and Fmoc are unstable in acid and base respectively.

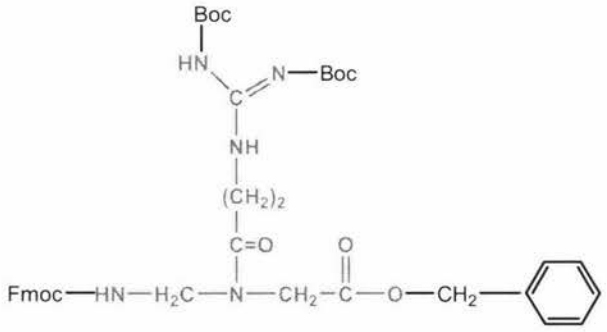


Figure 39. Designed arginine mimic in which benzyl ester is used as C-terminal block group.

To overcome this problem in the future, a new C-terminal protecting group or a new method for the hydrolysis of the alkyl ester should be employed. Esterification of the backbone amino acid with benzyl alcohol might be employed (Figure 39). Because it can be cleaved over H_2/Pd (Figure 40), so it allows for facile deprotection of the carboxyl group while maintaining the Fmoc and Boc groups in their respective places.

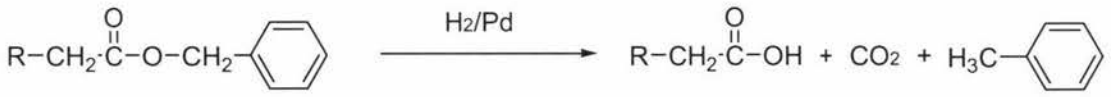


Figure 40. Mechanism for the removal of the benzyl group on the C-terminal by hydrogenolysis.

Another method to consider is enzyme catalyzed hydrolysis of alkyl esters. Due to high chemo-, regio- and stereoselectivity, enzymatic methods have gained interest

in peptide synthesis recently. Enzymes usually operate under very mild conditions, at neutral pH values and at temperatures of 20-50° C. Thus, under such conditions, acid or base-catalysed side reactions can be circumvented. Eggen I.F. et. al., developed a new process for the selective enzymatic hydrolysis of C-terminal *t*-butyl esters of peptide substrate using protease subtilisin[47]. In their research, the effectiveness of subtilisin was confirmed that high (up to quantitative) yields of the hydrolysed product of a number of amino acids and peptides esters could be obtained. So it looks very promising for this project. One thing must be noted the application of the enzyme is restricted by pH and solvents so carefully buffer selection is hence necessary.

At the start of this project, it was proposed to use the arginine mimic produced to make octapeptides using the solid phase peptide synthesis. The preparation of the di-protected substituted guanidine consumed considerable time and thus this objective was not achieved. For the future the hydrolysis of the C-terminal blocking groups has to be achieved to produce the target octapeptides.

CHAPTER EIGHT
CONCLUSION

At the starting point of this project, there were three main challenges identified for the synthesis of arginine mimics,

1. Guanidino group formation.
2. Protection of the N and C termini.
3. The coupling reaction between the backbone and the side chain.

A number of guanylating reagents for the formation of the guanidino group were identified at the start of this study. Some of them like aminoiminomethanesulfonic acid, 1H-pyrazole-1-carboximidine hydrochloride worked well in this project. Most of them might be suitable for formation of unprotected guanidino groups from amines but they did not seem useful for the amino acids used in this study. One conclusion made from this study is that the general method for addition of Boc or Cbz protecting groups to amino acids did not work very well for the preparation of the di-substituted guanidino groups targeted in this project. To overcome this problem, another idea proposed by peptide chemists in the last ten years is to protect the guanylating reagents (carboxamidine compounds) first then synthesize the di-protected substituted guanidines. The protecting groups are not only stable under the reaction conditions of formation of guanidino groups but their electron withdrawing effect also increases the reactivity of these reagents. *N,N'*-bis(alkoxycarbonyl)-S-methylisothiourea was a typical example and finally was selected as the starting material to make all di-protected substituted guanidines in this project. The coupling reaction, compared to the above guanidine effort, was relatively straight forward. With the assistance of the coupling reagent DCC and HBTU, a new amide bond between the amino acid backbone and the guanidine containing side chain was achieved with a satisfactory yield.

The main challenge for this study came from the selectivity required for the addition and removal of different types N and C-terminal blocking groups. In amino acid and peptide synthesis studies, to protect the functionality of different groups, the principle of maintaining blocking group selectivity is paramount. Although the

arginine mimic was successfully synthesized, the hydrolysis of the C-terminal blocking group for the ensuing solid phase peptide synthesis needs future study.

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