Synthetic Studies of Thymosin Alpha-1 Fragments

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Summary

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Master of Philosophy

Background: According to F&S (Frost & Sullivan)'s annual report, there have been more than 40 marketed peptides worldwide till 2005. In the past three years, three new peptide drugs have been approved by the US Food and Drug Administration (Knut, 2005), which suggests that a new era of treatment by peptide drugs is coming. Today, the benefits of peptide drugs have been accepted by increasing numbers of people, and hundreds of peptides are in development.

Aim: This research programme was designed to explore a suitable method for synthesis of the specific fragments of thymosin alpha-1 which were reported to have a potent anticancer activity. We were also interested in investigating new coupling reagents which would be more efficient, easily-operated, and economic in the liquid-phase.

Methods: To achieve accurate results, a series of analytical techniques was used in this study. This included TLC, LC, NMR, IR, Melting point, MS, as well as evaporation, extraction, desiccation, filtration and distillation.

Results: A new coupling reagent BEPB has been investigated that gives a better reaction yield in a much shorter time. It also has been proved by the synthesis of thymosin alpha-1 fragments in the liquid phase. No partial racemisation was observed.

Keywords: Peptide synthesis, Coupling reagents, 2-Bromine-1-ethyl-pyridinium bromide (BEPB), Protecting group.

Dedication

For Mum and Dad, with thanks

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Finally, I would like to give my love to my parents for their financial support and continuous encouragement.

Abbreviations

AIDS	Acquired Immure Deficiency Syndrome
APT	Attached proton test
BNP	Brain natriuretic peptide
BOMI	N-(1H-Benzotriazol-1-ylmethylene)-N-methylmethanaminium hexachloroantimonate Noxide
BOP	(1H-Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
Cbz	Benzyloxycarbonyl
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	N, N′-Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMF	Dimethylformamide
BEPB	2-Bromine-1-ethyl-pyridinium bromide
Fmoc	9-Fluorenylmethyloxycarbonyl
F&S	Frost & Sullivan consulting firm
HATU	O-(7-azabenzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate
HBTU	N-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]- -N-methylmethanaminium hexafluorophosphate N-oxide

HF	Hydrofluoric acid
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HOCt	Ethyl-1-hydroxy-1H-1,2,3-triazole-4-carboxylate
HODhbt	3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HOSu	N-Hydroxysuccinimide
IR	Infrared
L	Laevorotary
LC	Liquid chromatography
MeOH	Methanol
MS	Mass spectrum
NMR	Nuclear magnetic resonance
Nsc	N-2-(4-Nitrophenylsulfonyl) ethoxycarbonyl
OtBu	*Tert*-butyl ester
Pd	Palladium
PE	Petroleum
PTF	Benzyltriphenylphosphonium dihydrogen trifluoride
PTH	Parathyroid hormone

PyAOP	(1H-7-Azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	(1-H-Benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate
PyBrop	Bromotrpyrrolidinophosphonium hexafluorophosphate
PyCloP	Chlorotripyrrolidinophosphonium hexafluorophosphate
PyClU	1,1,3,3-Bis(tetramethylene)chlorouronium hexafluorophosphate
Ref.	Reference
SPPS	Solid Phase Peptide Synthesis
t-Boc	*Tert*-butyloxycarbonyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFFH	Tetramethylfluoromamidinium hexafluorophosphate
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Z	Benzyloxycarbonyl

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Chapter One: Introduction

1.1 Amino acids and peptides

1.1.1 Background of peptide drugs

By 2005 there were more than 40 marketed peptides explored worldwide. Around 270 peptides were in clinical phase testing, and about 400 in advanced preclinical phases. Natural peptides such as insulin, vancomycin, oxytocin, and cyclosporine and synthetically produced ones such as Fuzeon (enfuvirtide) and Integrilin (eptifibatide) are among the approved peptide-based drugs (Business, 2005). Over the last ten years, the attitude to research into peptide drugs has moved from reluctance to a widely accepted position. Increasing number of people has begun to do research on these kinds of drugs. In Europe, nearly 60% of the market comprises peptide-based therapeutics for the oncology area followed by cardiovascular, infection and metabolic therapeutics, and 90% of these peptides are devoted to developing the formulation and clinical trial of peptides (Anonymous, 2005).

'The rising need for new therapeutic approaches combined with the potential of peptides as active pharmaceutical ingredients (APIs) for effective drug formulation is contributing to rapid market development,' says the analyst of F & S (Frost & Sullivan consulting firm). This suggests that a new era of treatment through peptide drugs is coming.

The primary structures of peptides include amino acids. As these different amino acids are connected via amide bond to produce a peptide or a protein, then many different sequences are possible depending on the number of different building blocks and the length of the peptide. As all peptides display a high degree of conformational diversity, it follows that many diverse and highly specific structures can be observed (Sewald et al., 2002).

However, why did peptide drugs become so popular? First the common advantages and disadvantages of peptide drugs are listed in the **Table1-1**.

Despite the significant progress that has been made, technological challenges relating to the delivery of peptides, their instability *in vivo* and short half-life remain critical challenges. Moreover, costly and inefficient large-scale manufacturing and purification processes are also hampering market growth; for instance, 'Fuzeon' has a massive 106 separate steps in its production, which has contributed to its high price.

Meanwhile, cost-competitive, bulk manufacturing strategies are the key to sustained market expansion. Thus, an efficient and cost-effective scale up process, cutting-edge purification and separation methods with low cost and high quality are the commercial purposes. However, Knut suggested that it is a hard task to investigate the peptides not only because of instability, easy racemisation and hard purification but also due to the limited development of coupling reagents (Knut, 2005).

Advantages	Disadvantages
High activity	Low oral bioavailiability
High specificity	Injection required
Little unspecific binding to molecular structures other than desired target	Less stable
Minimization of drug-drug interactions	Difficult delivery; challenge to transport across membranes
Less accumulation in tissues	Challenging & costly synthesis
Lower toxicity	Solubility challenges
Often very potent	Risk of immunogenic effects
Biological & chemical diversity	Cleared from body quickly

Table 1-1 Advantages and disadvantages of peptide drugs

Sources: Frost & Sullivan, Drug & Market Development Publications, Roche, 5AM Ventures, and Richard DiMarchi

Perhaps one of the most representative examples of the convergence of those processes is 'Fuzeon' (enfuvirtide, T-20), an HIV fusion inhibitor approved by the US Food and Drug Administration (FDA) in 2004. Fuzeon is made up of a 36 amino acid peptide chain containing 14 different amino acids (**Figure 1-1**). It requires 106 chemical steps to make up the peptide chain compared to an average of eight to 12 steps to produce small molecules.

The structure of Fuzeon is as follows.

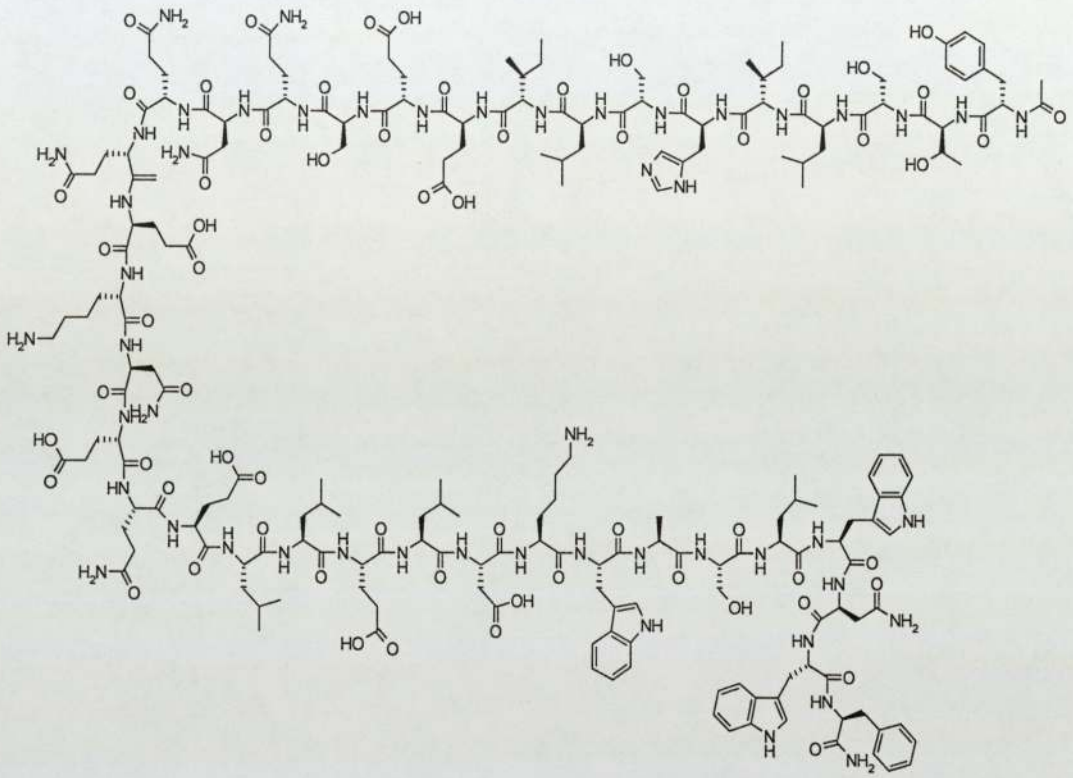
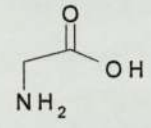
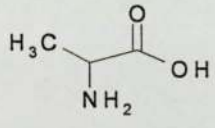
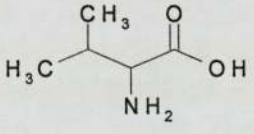
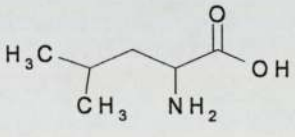
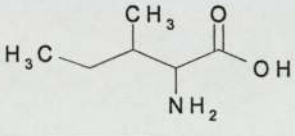
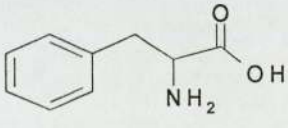
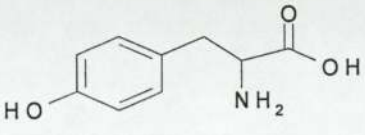


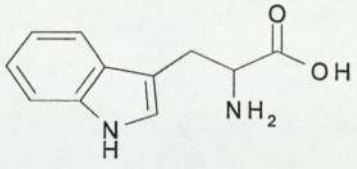
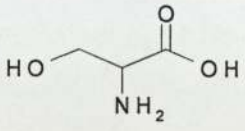
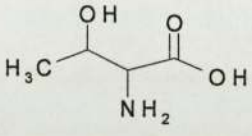
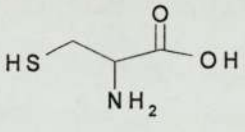
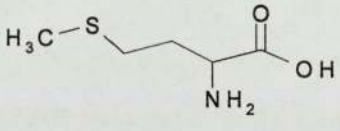
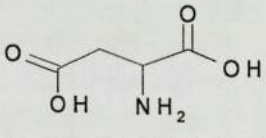
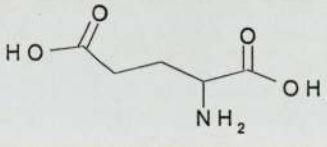
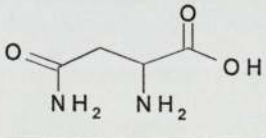
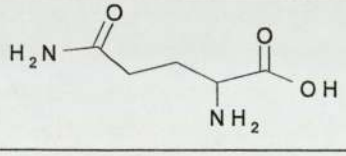
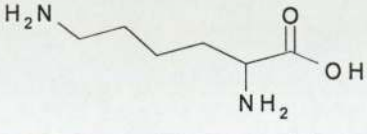
Figure 1-1 Structure of Fuzeon

From the figure above, we can imagine the difficulty in synthesizing such a complicated compound. Thus in the next several sections, I will introduce some background knowledge of peptides: the definition, building bricks, synthetic method and common knowledge in peptides synthesis.

1.1.2 Amino acids

Amino acids are the structural units of peptides. There are 20 essential amino acids in human bodies. The abbreviated names and general structures of those amino acids are listed in **Table 1-2** below.

Name	Abbreviated name	One-letter abbreviation	Structure
Glycine	Gly	G	
Alanine	Ala	A	
Valine	Val	V	
Leucine	Leu	L	
Isoleucine	Ile	I	
Phenylalanine	Phe	F	
Tyrosine	Tyr	Y	

Tryptophan	Trp	W	
Serine	Ser	S	
Threonine	Thr	T	
Cysteine	Cys	C	
Methionine	Met	M	
Aspartic acid	Asp	D	
Glutamic acid	Glu	E	
Asparagine	Asn	N	
Glutamine	Gln	Q	
Lysine	Lys	K	

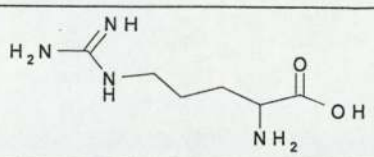
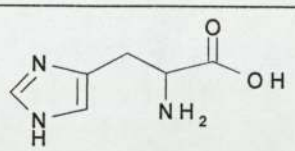
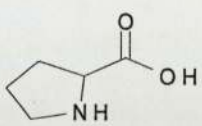
Arginine	Arg	R	
Histidine	His	H	
Proline	Pro	P	

Table 1-2 The 20 essential amino acids occurring in the human body

The structure of Fuzeon can be written as CH_3CO-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile--Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-NH_2 according to the abbreviations of amino acids in **Table 1-2**.

Table1-2 demonstrates one of the obvious features of amino acids (with the exception of Glycine) is that they all contain at least one stereogenic carbon atom; that is, the molecules are chiral. The structures below show the two forms of an amino acid (**Figure 1-2**) (Doonan, 2002).

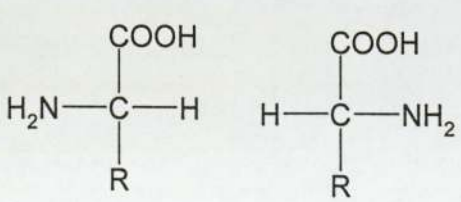


Figure 1-2 Fischer projections of two forms of an amino acid

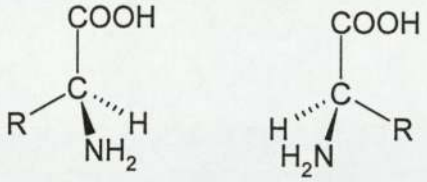


Figure 1-3 Stereo diagrams of two forms of an amino acid

The Fischer projection (**Figure 1-2**) and stereo diagram (**Figure 1-3**) on the left represents an amino acid with the L relative configurations, and the structure on the right in both figures are D relative configurations. Basically, the structure of amino acids in peptide can be represented as follows (**Figure 1-4**).

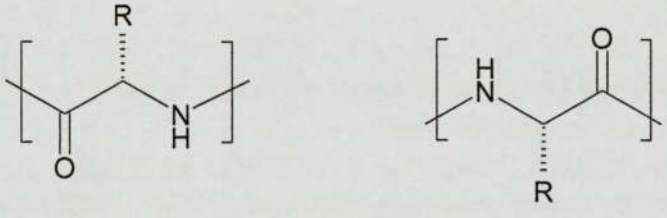


Figure 1-4 The representation of amino acids in peptides

Modern practise is to describe the configurations of chiral molecules using the Cahn-Ingold-Prelog R/S converntion (Cahn, 1966). For example, consider L-alanine, because priority of the R which is Me in **Figure 1-2** is lower than COOH. Viewing the molecule with the H at the back, the decreasing order of priority of the others is anticlockwise. Hence the configuration is S. However, for L-cysteine, priority of the R which is CH_2SH in **Figure 1-2** is greater than COOH because of the sulfur atoms.

Viewing the molecule with the H at the back, the decreasing order of priority of the others is clockwise. Hence the configuration is R. In spite of acceptance of the R/S convertion in nearly all areas of chemistry, the L/D convertion is still almost universally used to describe amino acids and carbohydrates. This topic has been dealt with in detail by Morris (Morris, 2001).

More interesting thing is that the amino acid structures in human living system are dominated by L configuration, which seems that L configuration is inherent. There is not an easy way to explain how it happened that the amino acids selected were entirely of one configuration. The same holds true of virtually all biomolecules that are chiral: one enantiomer is biologically active; the other is not (Doonan, 2002). Therefore, the configurations of peptides in synthetic procedure are very important. Racemization in peptide synthesis will be referred in detail in section 1.2.3.

1.1.3 Peptides

Two or more amino acids joined by amide bonds are called peptides. In each peptide, there is a free carboxyl called C-terminal and a free amino group called N-terminal. One peptide containing two amino acids is called dipeptide, one containing three amino acids is a tripeptide. Those containing more than ten amino acids are called polypeptides (Miller, 1980).

Peptides have a wide range of biological activities, for example, the level of glucose in the blood is regulated by the main peptide hormone: insulin; Insulin promotes the anabolism of glucose by enhancing glycolysis, glycogenesis and lipogenesis and preventing the breakdown of glycogen and fat to ensure that metabolism is normal. If insulin is absent or deficient, nutrient homeostasis becomes chaotic and diabetes mellitus follows.

1.2 Chemical synthesis of peptides

Amino acids are the basic units of peptides and proteins, but how to link these units together to form a peptide? The same question is how to synthesize a desired peptide? In this section we will introduce the method to synthesize a peptide in solution phase.

1.2.1 Introduction of protecting groups

It is different to synthesize a peptide in a manner like other amides being synthesized because amino acids contain both amino and carboxyl groups. For example, if Glycine is converted to its acid chloride, two molecules of the acid chloride would condense to form a cyclic diamide (Miller, 1980) (**Figure 1-5**):

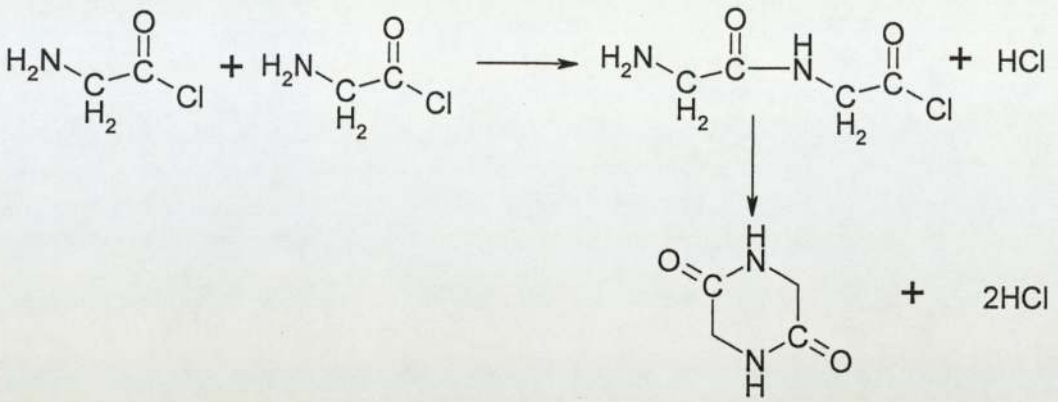


Figure 1-5 Formation of cyclic diamide

For the purpose of obtaining the expected peptide without by-product, it is first necessary to prohibit the functional groups of amino acids from the reacting with each other. A protecting group must be introduced to convert either the amino group or the carboxyl group to an unreactive functional group (Miller, 1980).

Over the last 70 years, great efforts have been devoted to develop protecting groups for peptide synthesis (Jakubke, 1977). Herein we will pay attention to some of the most widely used protecting groups in modern peptide synthesis.

1.2.1.1 Protection of amino groups

The most important development in peptide synthesis was made in 1932 by Bergmann and Zervas (Bergmann, 1932), who firstly introduced the benzyloxycarbonyl protecting group. The essential point about benzyloxycarbonyl amino acids (Cbz amino acids) is

that, as esters of carbamic acid, the nitrogen atom does not have nucleophilic properties and will not influence peptide bond formation. Benzyloxycarbonyl amino acids are easily prepared by reaction with benzyloxycarbonyl chloride, as shown in **Figure 1-6.**

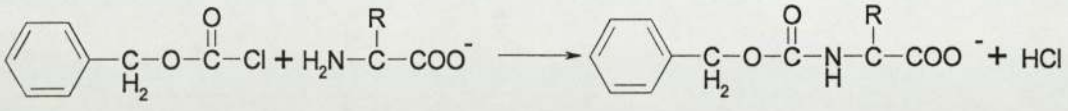


Figure 1-6 Amino protection by benzyloxycarbonyl

Equally important, benzyloxycarbonyl protecting group can be removed under a variety of conditions including treatment with liquid HF which do not affect peptide bonds (Doonan, 2002).

Subsequently another improved amino protecting group: *tert*-butyloxycarbonyl (*t*-Boc), was developed. The structure of a *t*-Boc amino acid is shown in **Figure 1-7.** This group is removable by treatment with aqueous TFA. Availability of these two protecting groups removable under different conditions provides a strategy for synthesis of peptides containing lysine. The α-amino group can be protected with *t*-Boc and the side chain amino group with Cbz. During synthesis the α-amino group may then be deprotected with TFA for chain elongation under conditions where the side-chain amino protecting group is unaffected (Doonan, 2002).

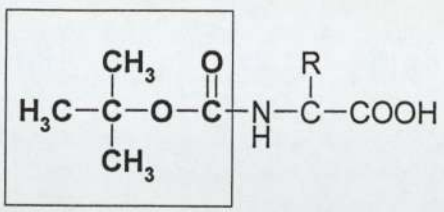


Figure 1-7 Protected amino acid by *t*-Boc

A further important advance was made by Carpino and his colleague (Carpino,1970), who introduced the 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group (**Figure 1-8**). The protecting group is removable under very mild conditions by treatment with an organic base. This is because of the acidity of the proton at position 9 of the fluorenyl group (Doonan, 2002). The deprotection reaction is shown in **Figure 1-9**.

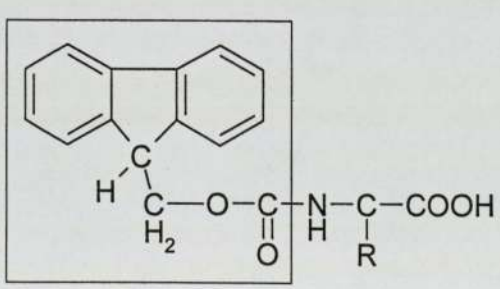


Figure 1-8 Protected amino acid by Fmoc

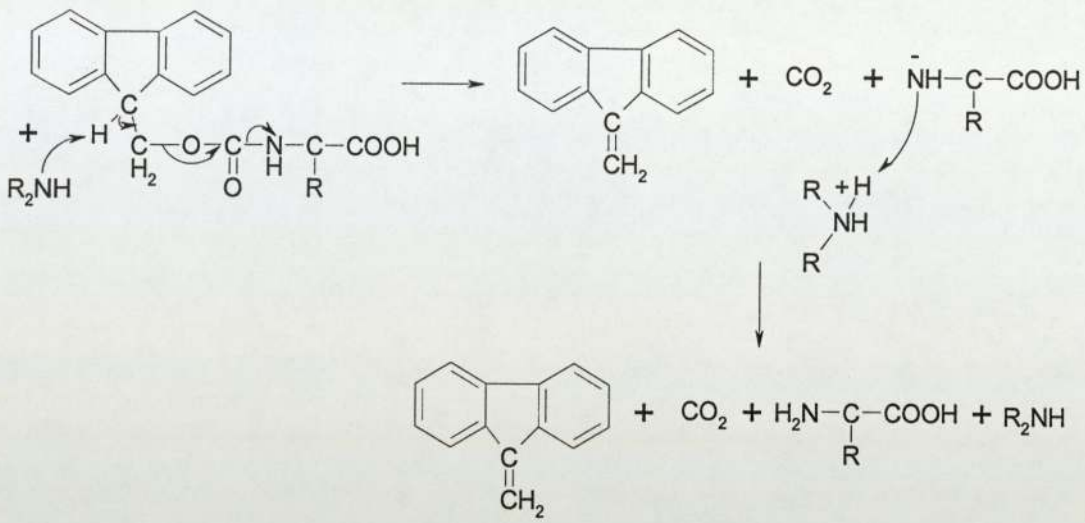


Figure 1-9 Deprotective reaction of Fmoc amino acid

1.2.1.2 Protection of carboxylic acid groups

Carboxyl group protection is usually carried out by conversion to esters. They must be unreactive in the process of peptide bond formation but easily removable during chain elongation or formation of the final deptotected product. Two widely used derivatives are benzyl esters (Wang, 1973) (**Figure 1-10**) and *t*-butyl esters (Sheehan, 2000) (**Figure 1-11**). The benzyl group can be removed by HF and the *t*-butyl group can be removed by treatment with TFA. It is important to have two protecting groups removable under different conditions because of the need for differential protection of amino acids with carboxylic acid side chains (Aspartic acid and Glutamic acid; see **Table 1-2**) (Doonan, 2002).

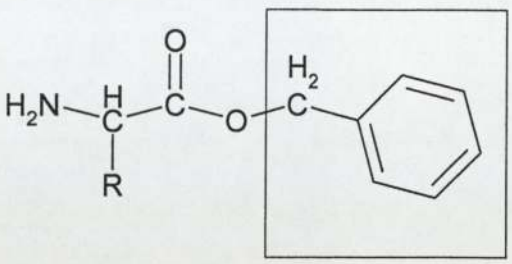


Figure 1-10 Benzyl ester protection

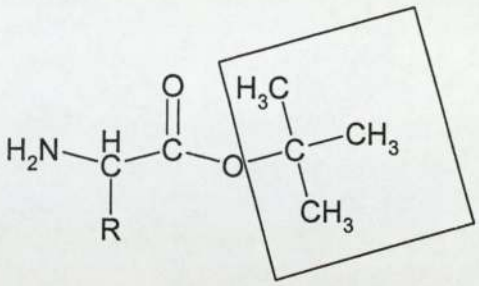


Figure 1-11 *t*-Butyl ester protection

1.2.1.3 Protection of the side-chains

Except for those residues already mentionded (Aspartic and Glutamic acids, Lysine), the side chains of Cysteine, Serine, Threonine, Tyrosine, Histidine and Arginine always need to be protected. Whether or not Tryptophan, Methionine and the amino acids with amide side chains (Asparagine and Glutamine) are protected depends on the approach to peptide synthesis being used (Doonan, 2002).

The choice of side chain protecting agents will depend on the protecting group used for the α-amino function, because it is essential that the side chain remain protected when the N-terminal amino acid is deprotected for chain elongation. The two main approaches for SPPS (Solid Phase Peptide Synthesis) are summarized in **Table 1-3.** When the *t*-Boc group is used for N-protection, then side chains are protected with groups based on the benzyl (phenylmethyl) function (e.g. the benzyl ether for threonine). Selective removal of the *t*-Boc group is achieved using TFA, which does not affect the

side-chain protecting groups. The latter are removed with liquid HF. When Fmoc is used for N-protection, the side chains are protected with groups based on the *t*-butyl function (e.g. the *t*-butyl thioether of cysteine). Fmoc is removed by treatment with an organic base such as piperidine and the side chains are deprotected with HF (Doonan, 2002).

N-protection	**N-deprotection**	**Side-chain protection**	**Side-chain deprotection**
t-Boc	TFA	Bz based	HF
Fmoc	Piperidine	t-Bu based	TFA

Table 1-3 Summaries of the two main approaches to side-chain protection

The *t*-Boc/benzyl strategy was the first to be developed and is very widely used. However, it has the disadvantage that the N-deprotection and side-chain deprotection are both carried out under acidic conditions, and there is the danger that the deprotection process may not entirely specific. With the Fmoc/*t*-butyl strategy the two phases of deprotection are carried out under completely different conditions, so specificity is more certain (Doonan, 2002).

1.2.2 Introduction of coupling reactions

Carboxyl components can be activated as acyl halides, acyl azides, acylimidazoles, anhydrides, esters etc. There are different ways of coupling reactive carboxyl derivatives with an amine:

- An intermediate acylating agent is formed and isolated then subjected to aminolysis;
- A reactive acylating agent is formed from the acid in a separate step, followed by immediate treatment with the amine;
- The acylating agent is generated *in situ* from the acid in the presence of the amine, by the addition of an activating or coupling agent (Christian, 2005).

The basis of peptide bond formation is the conversion of the carboxylic acid function of one amino acid to a reactive acyl derivative that is susceptible to nucleophilic attack by the amino group of the second amino acid. Most of methods currently in use are based on activation of the amino acid using dicyclohexylcarbodiimide (DCC) (**Figure 1-12**) (Doonan, 2002).

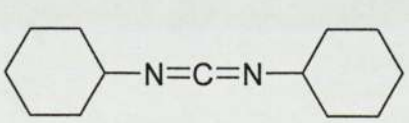


Figure 1-12 Structure of dicyclohexylcarbodiimide(DCC)

In the most straightforward application of DCC coupling, the protected amino acids are mixed in the presence of the coupling agent.

The carboxylic acid function of the N-protected amino acid reacts with the DCC to form an O-acylisourea intermediate; this is an active ester of the amino acid. In the second reaction the amino group of the C-protected amino acid (or of the growing peptide) attacks the carbonyl function of the intermediate to form a peptide bond and liberate dicyclohexylurea. The product is the protected peptide. Removal of the protecting group R_2 allows the peptide chain to be extended at the N-terminal end (**Figure 1-13**).

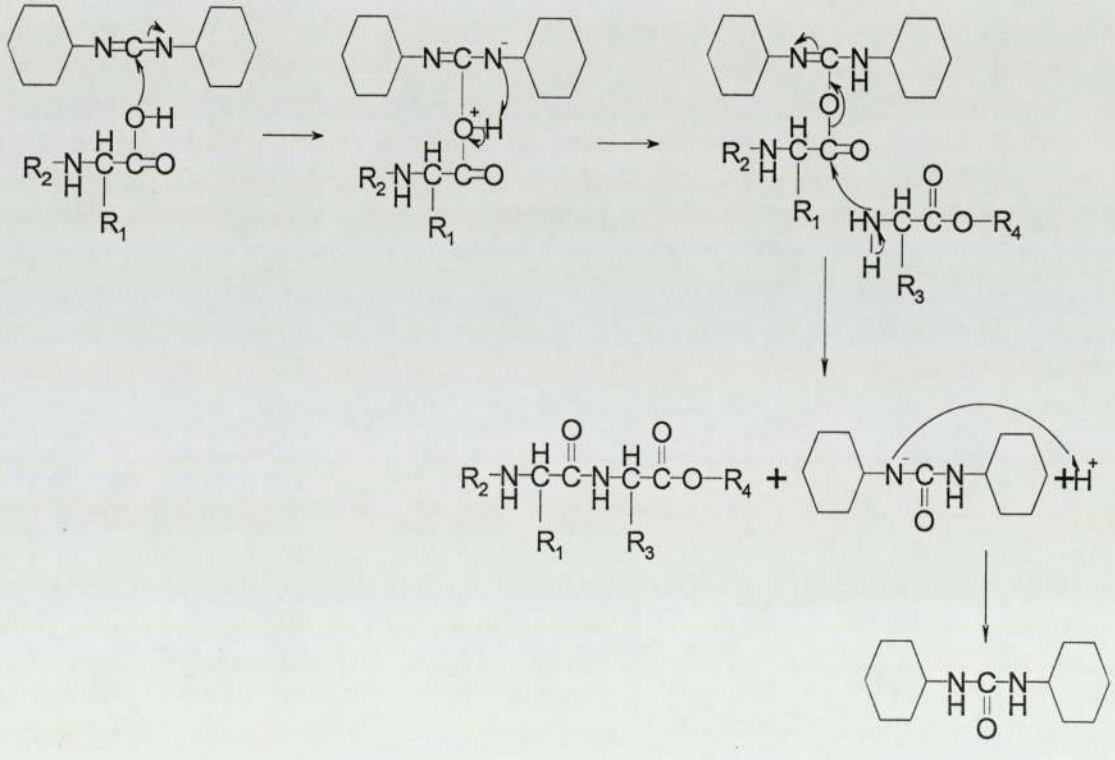


Figure 1-13 Procedure of coupling by DCC

In recent years, peptide coupling reactions have been significantly advanced in accord with the development of new peptide coupling reagents in organic synthesis. Development of new peptide coupling reagents has been steadily accelerated in the past few years. So-Yeop Han and her colleague Young-Ah Kim classify coupling reagents into eight types including phosphonium, uranium, immonium, carbodiimide, imidazolium, organophosphorous, acid halogenating and other coupling reagents according to the structure similarity in solution phase peptide synthesis (Han, 2004).

The figures below give us some examples of each type of coupling reagents.

Phosphonium Reagents:

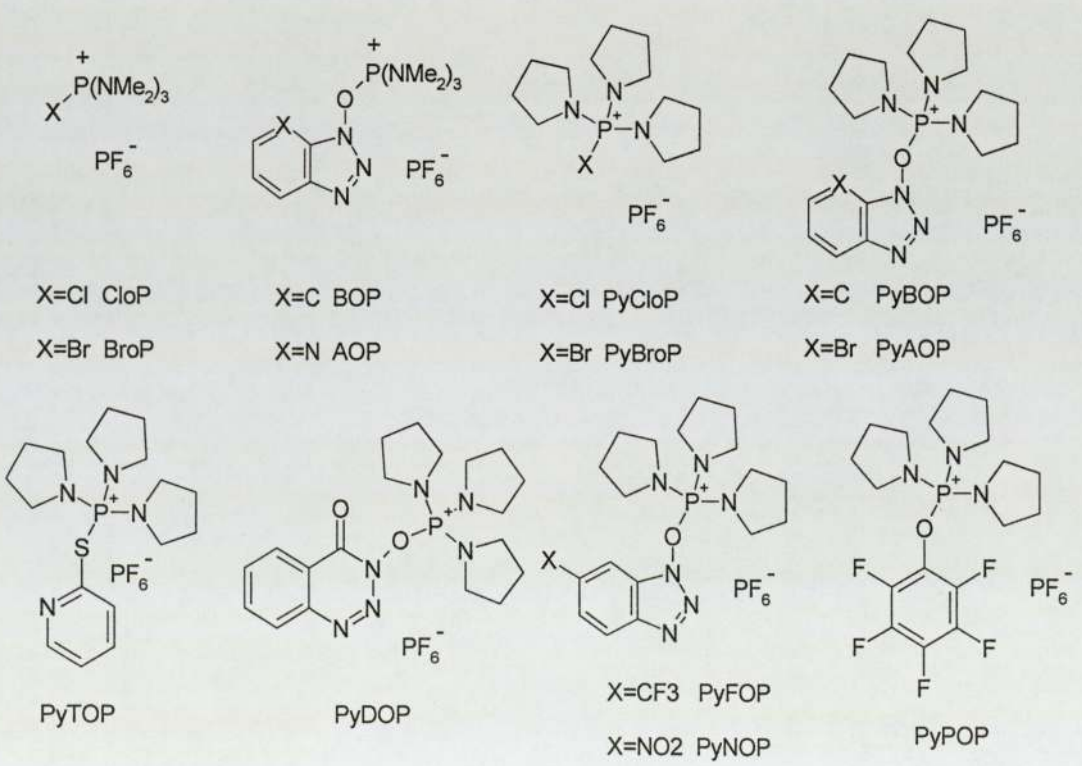


Figure 1-14 Examples of phosphonium typal coupling reagents (Coste, 1990)

Uranium Reagents:

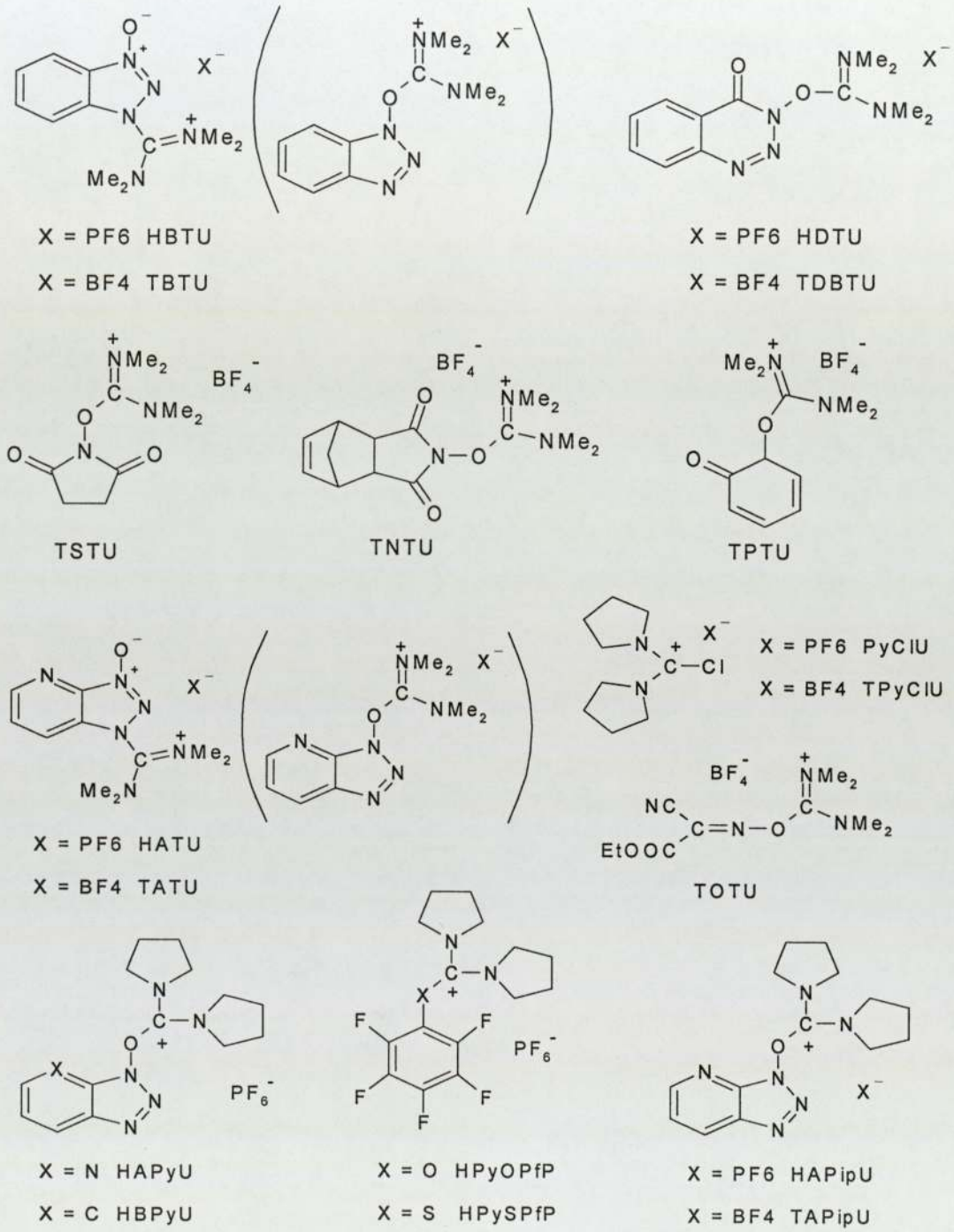


Figure 1-15 Examples of uranium typal coupling reagents (Dourtoglou, 1978)

Immonium Reagents:

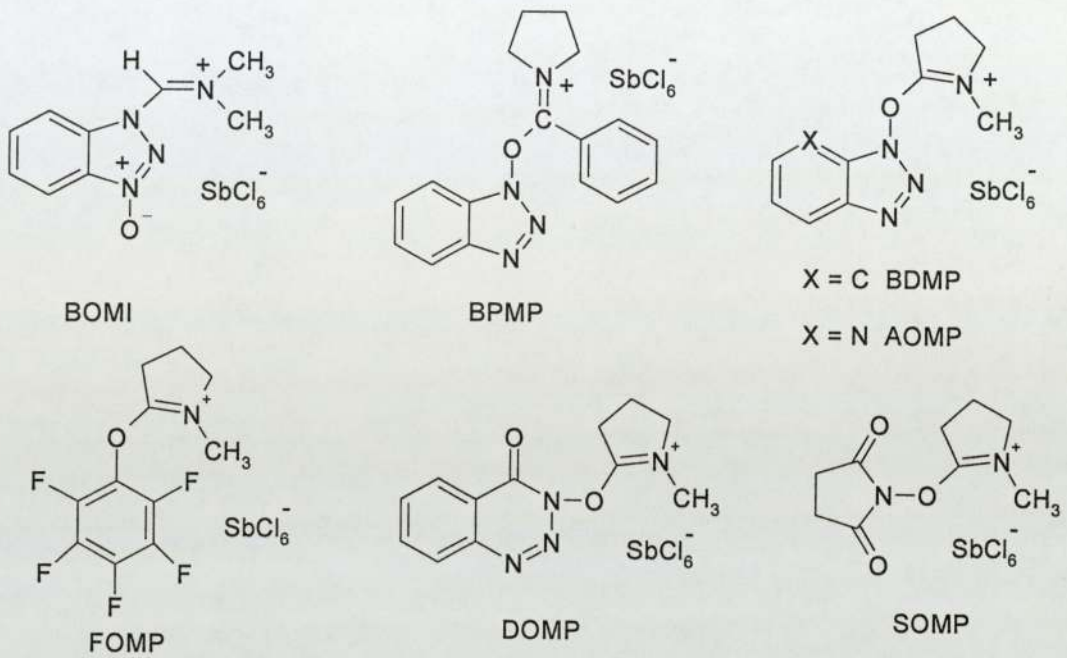
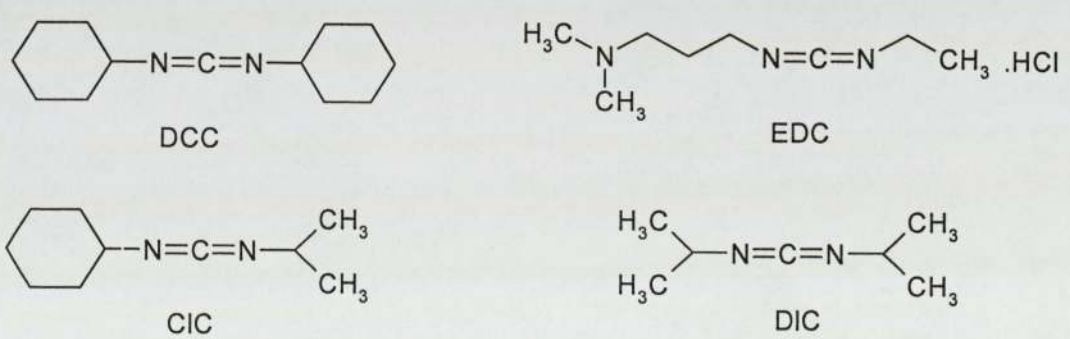


Figure 1-16 Examples of immonium typal coupling reagents (Li, 2000)

Carbodiimide Reagents:



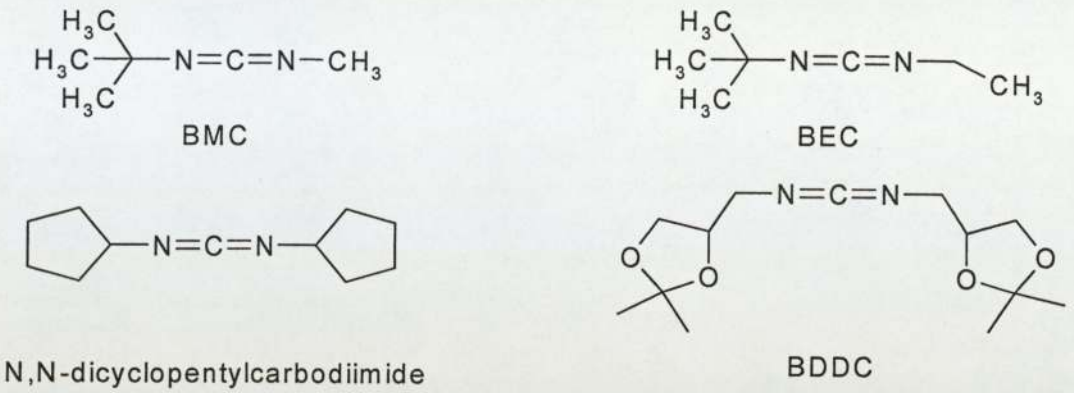


Figure 1-17 Examples of carbodiimide typal coupling reagents (Izdebski, 1997)

Imidazolium Reagents:

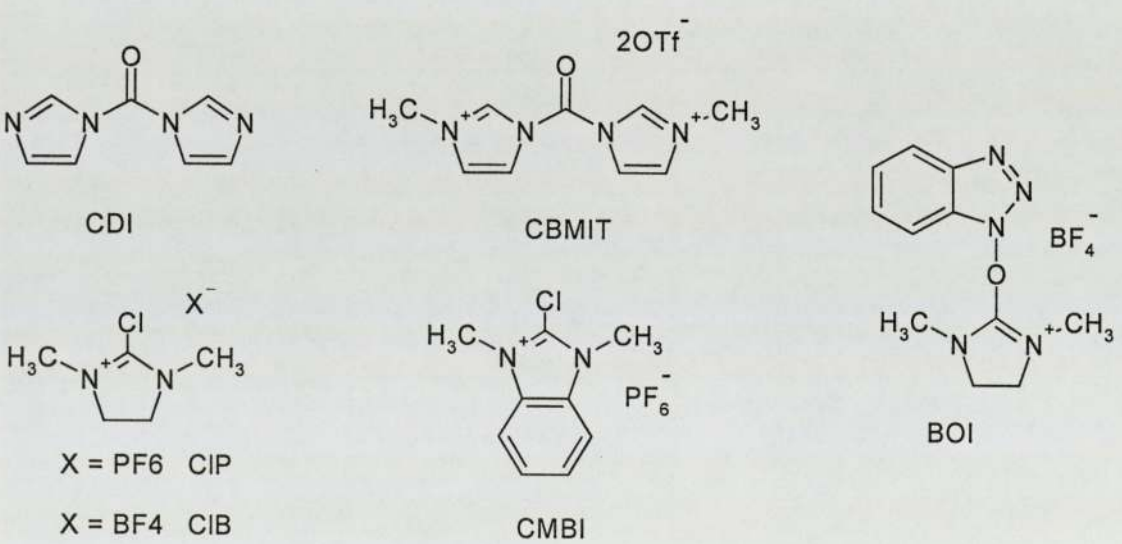
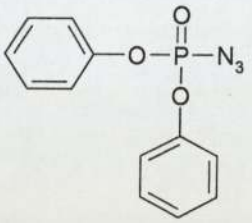
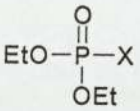


Figure 1-18 Examples of imidazolium typal coupling reagents (Kato, 1995; Li, 2000; Li, 1999)

Organophosphorous Reagents:



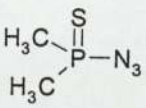
DPPA



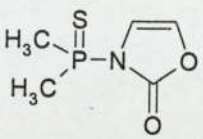
X = CN DECP

X = Br DEPB

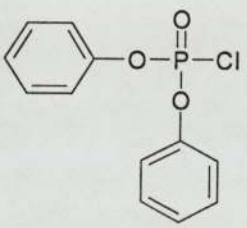
X = Cl DEPC



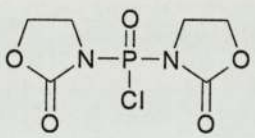
MPTA



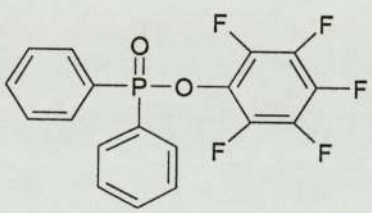
MPTO



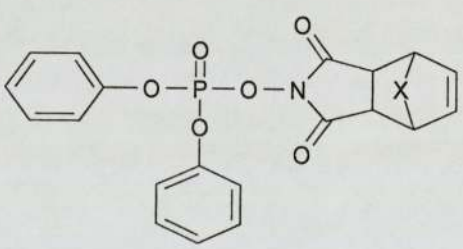
DPP-Cl



BOP-Cl

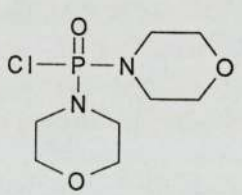


FDPP

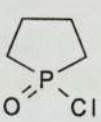


X = O ENDPP

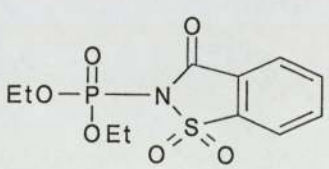
X = C NDPP



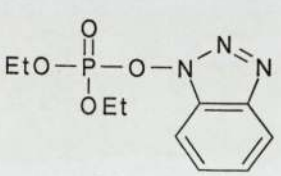
BMP-Cl



Cpt-Cl



DEBP



BDP

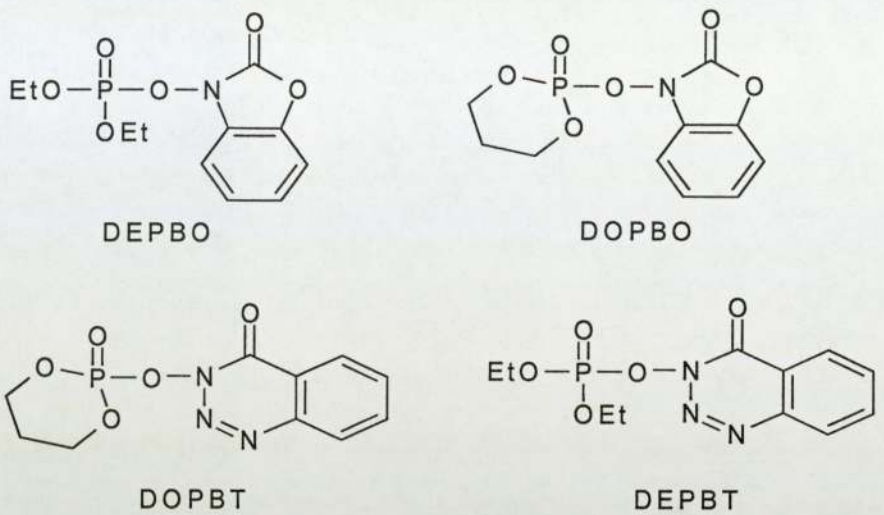


Figure 1-19 Examples of organophosphorous typal coupling reagents (Shioiri, 1972)

Acid halogenating Reagents:

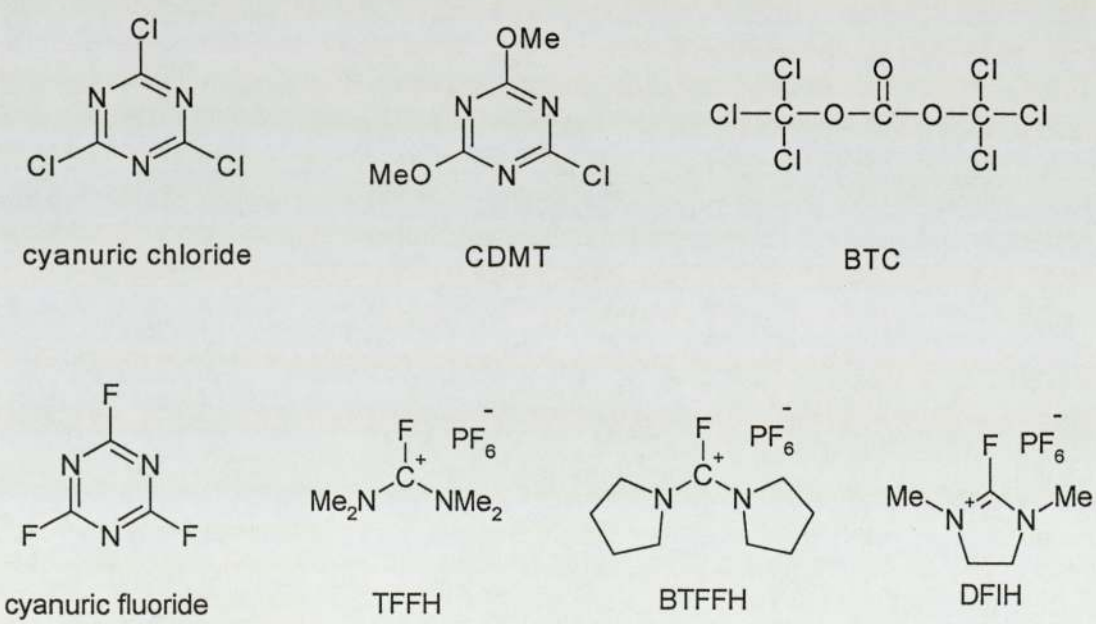


Figure 1-20 Examples of acid halogenating typal coupling reagents (Kaminski, 1987; Falb, 1999)

1.2.3 Racemization during peptide synthesis

1.2.3.1 Mechanism of Racemization

Partial racemization in coupling reactions of amino acids was a problem encountered frequently in peptide synthesis, what is more, D-amino acids in a peptide is hard to separate from all-L molecules. So the minimization of partial racemization is also an important task in peptide synthesis.

Racemization can occur at the C-terminal amino acid residue in the course of a coupling reaction due to the ionization of the α-hydrogen and the formation of an oxazolone intermediate.

The mechanism of racemization in peptide synthesis is shown in **Figure 1-21**.

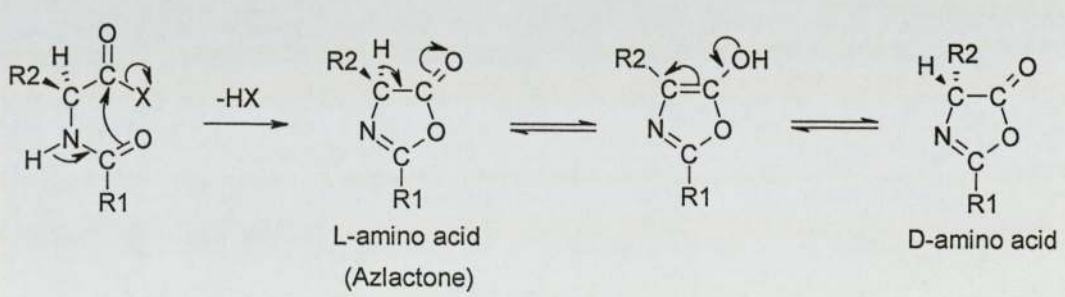


Figure 1-21 Mechanism of racemization in peptide synthesis

Activation of carboxyl function of *N*-acyl (acetyl, benzoyl, peptidyl and so on) amino acids easily forms azlactones, which give rise to chirality unstable intermediates via tautomerization of the azlactone.

1.2.3.2 Racemization Suppressant Additives

A peptide coupling reagent with an appropriate racemization suppressing agent assures suppression of the undesired racemization and other side reactions, and thus minimizes the loss of the optical integrity at the chiral centre.

In 1970, König and Geiger first reported the use of HOBt as a racemization suppressant additive in peptide coupling reactions with carbodiimide coupling reagents (König,1970). With this technique, additives such as HOBt, HOAt, HODhbt, *N*-hydroxytetrazole, HOCt, and PTF (**Figure 1-22**) have roles in not only suppressing racemization, but also enhancing the reactivity.

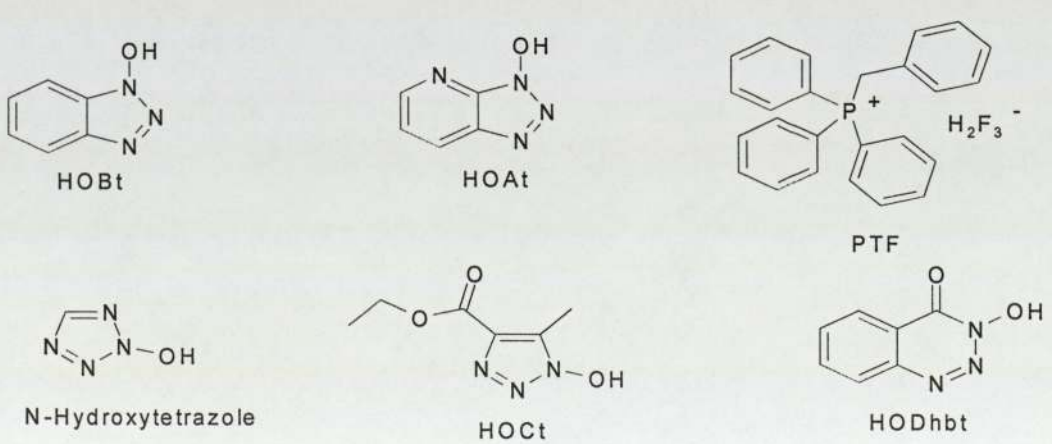


Figure 1-22 Structure of several racemization suppressant additives

HODhbt has been limited in its widespread adoption due to the side reaction of ring opening. HOAt has been reported to be more efficient than HOBt because of an anchimeric assistance effect caused by the pyridine ring (Carpino, 1993; Carpino, 1995; Carpino, 2001). Later, *N*-hydroxytriazoles and *N*-hydroxytetrazoles were examined for their coupling efficiency (Spetzler, 1998; Robertson, 1999; Jiang, 1998). Ramage reported the coupling reaction of dipeptides with DIC and the newly designed HOCt for a racemization study. Racemization with DIC/HOCt activation was negligible for all amino acids except Histidine (Spetzler, 1998; Robertson, 1999; Jiang, 1998). More recently, Carpino and Henklein reported polyhydrogen fluoride additives, $Py(HF)_n$ (Carpino, 2003). For example, the efficiency of the coupling reaction for HBTU combinated with PTF was as good as HATU. Unfortunately, PTF was unsuitable for phosphonium or organophosphorous reagents due to the high strength of the P–F linkage. For inorganic additives, the lowest level of racemization was occasionally found in the presence of $CuCl_2$ combined with various coupling reagents (Gibson, 1995; Nishiyama, 1999; Nishiyama, 2001). However, the improvement in yield was not sufficient by addition of $CuCl_2$. In addition, the Cu(II)-based complexes, $Cu(OBt)_2$ and $Cu(OAt)_2$ also showed the ability to function as racemization suppressants (Van Den Nest, 2001).

Figure 1-23 shows mechanism of racemization suppressant additive in peptide coupling procedure through carbodiimide activation. The mechanism of the carbodiimide activation, which is complex and depending on the solvent, starts by a proton transfer,

followed by addition of the carboxylate to form the O-acylisourea **(1)**. This is the most reactive species that can attack the amino component to give the corresponding amide. However, the O-acylisourea **(1)** can undergo a rearrangement to give the N-acylurea **(2)**, which is not reactive, or sustain an intramolecular cyclization to give a 5(4H)-oxazolone **(3a)**, which is less reactive than **(1)** and can tautomarize with the corresponding change of chirality **(3b)**. If activation is carried out in a solvent of low dielectric constant such as $CHCl_3$ or CH_2Cl_2, the formation of **(1)** occurs instantaneously in the absence of a nucleophile or base and can be stable for many hours. However, if the activation is carried out in a more polar solvent such as DMF, no immediate reaction can be detected, and a complex mixture of starting amino acid, symmetrical anhydride **(4)**, and **(2)** is formed. If the activation is carried out in the presence of an extra equivalent of acid, **(4)** is formed, which is also very reactive. At the beginning of the 70's, 1-hydroxybenzotriazole (HOBt: X represents C , Y represents H in **Figure 1-23**) was proposed as an additive to DCC to reduce racemization and from then on other benzotriazole derivatives such as 1-hydroxy-5-chlorobenzotriazole (Cl-HOBt: X represents C , Y represents Cl in **Figure 1-23**) or 1-hydroxy-7-azabenzotriazole (HOAt: X represents N , Y represents H in **Figure 1-23**) have also been used. The OBt active esters are less reactive than **(1)**, but are more stable and less prone to racemize. All these factors make the addition of benzotriazole derivatives almost mandatory to preserve the peptide bond formation by carbodiimide activation of low yields and undesired side reactions (Marder, 2003).

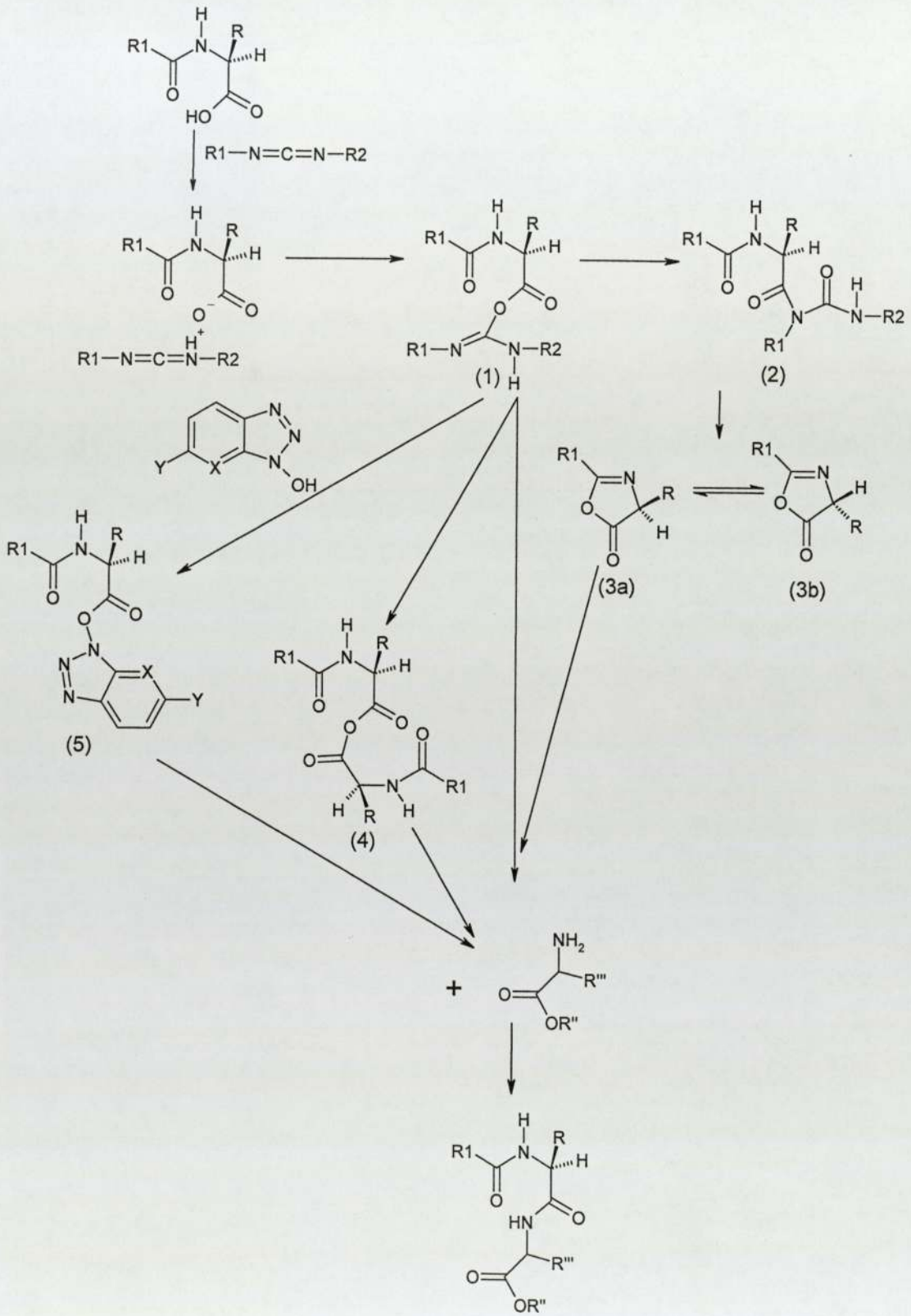


Figure 1-23 Mechanism of peptide bond formation through carbodiimide activation

The racemization suppressant additives can adjust the activity of acid component by forming HOBt kind active esters, as O-acylisourea **(1)** is among the most reactive species; the less reactive active esters are more stable and less prone to racemize. If the HOBt active ester is left alone in solution, racemization still happens slowly.

In the last decade onium (phosphonium and aminium/uronium) salts of hydroxybenzotriazole derivatives have been introduced. Although, they have been rapidly adapted for research purposes, only a few of them have been found compatible with current industrial requirements and synthetic strategies and therefore adopted by the industry. The specie that reacts with onium salts is the carboxylate (**Figure 1-24**) and therefore the presence of at least one equivalent base is essential. The intermediate species acyloxy-phosphonium or amidinium salts have not been detected and react immediately with the benzotriazole derivative (an extra equivalent of it is added in some synthetic protocols) to give **(5)**, which react with the amino component to give the corresponding amide (Marder, 2003).

For reagents based on HOBt analogues (HBTU, HTCU), the intermediate species acyloxy phosphonium or amidinium salts have not been detected and react immediately with the benzotriazole derivative (Marder, 2003).

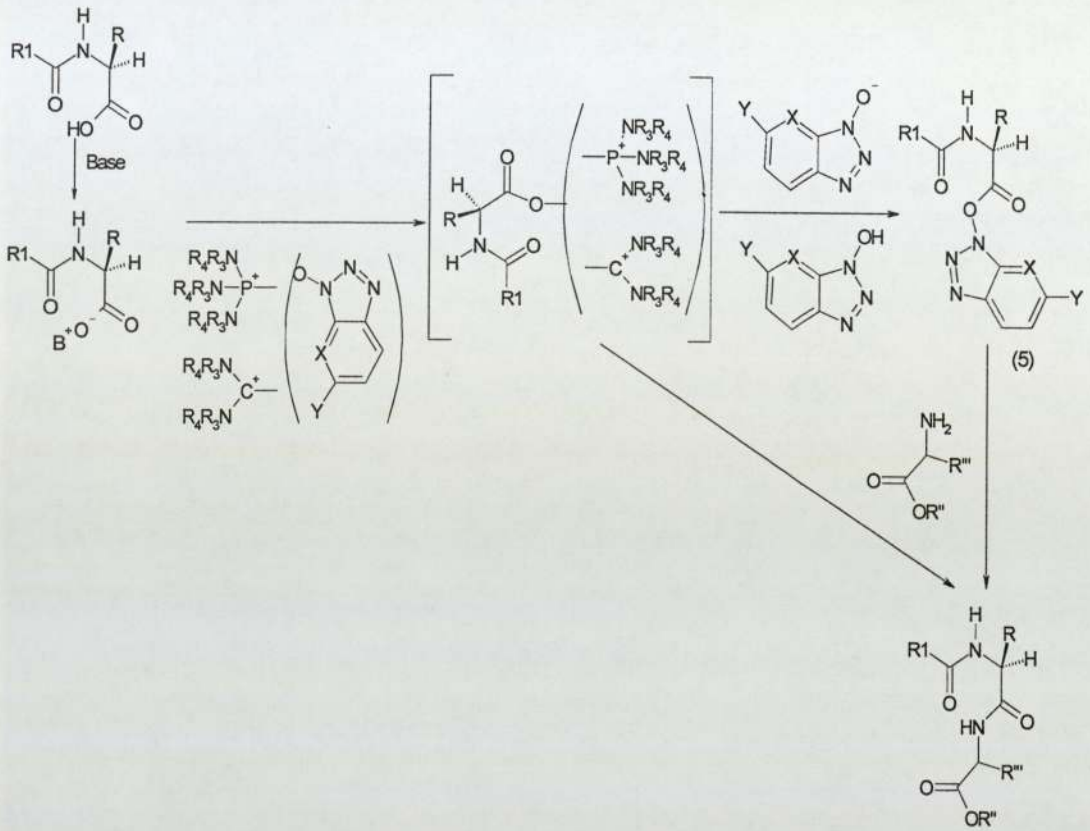


Figure 1-24 Mechanism of peptide bond formation through onium salts activation

N-α protected histidine when activated is especially prone to racemization due to the reactivity of the imidazole nucleus. This topic has been dealt with in detail by Robertson and Jones (Robertson, 1999) (Jones, 1980). Standard tests were done by König (König, 1970). Racemization detection about cysteine was investigated by Siedler (Siedler, 1996).

Chapter Two: Results and Discussion

2.1 Method development

2.1.1 Studies of N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl (Nsc) groups

For the protection of amino and carboxyl functions in peptide synthesis, a wide variety of base-labile protecting groups cleaved by β-elimination had been proposed, among them there were several groups on the basis of substituted 2-sulfonylethanols (Hardy, 1968). Cleavage of the groups of this type usually involved a short-term treatment with a strong inorganic base in an aqueous or mixed solution (Samukov, 1994).

The structure of N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl-amino acids (Nsc-amino acids) have the general formula like **Figure 2-1**:

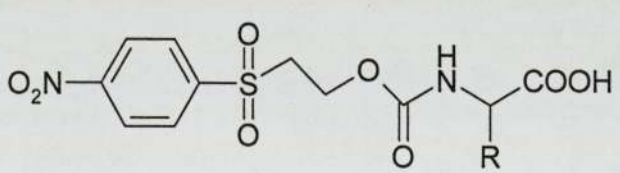


Figure 2-1 General formula of N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl-amino acids

N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl-amino acids (Nsc-amino acids) represent a class of protected amino acid derivatives which are used in the chemical synthesis of peptides. In these derivatives, N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl (Nsc) group serves as a temporary N-protection which can be selectively removed after each step of

the peptide chain elongation. Nsc-Group is fairly resistant to acidic reagents and can be cleaved according to the β-elimination mechanism by organic bases in aprotic solvents. Mild conditions of the cleavage allow for use of the temporary N-Nsc-protection in the peptide synthesis together with the acid-sensitive side chain protection of widely used *t*-butyl or benzyl type, thus providing the so-called 'orthogonality' of the synthetic strategy (Samukov, 2001).

Recently N-Nsc-amino acids, methods for their preparation, and their employment for the solid phase peptides synthesis have been disclosed in US Patent 6,265,590 (Samukov, 2001). However, Solubility problems which were still encountered during procedure of deprotection and the risk of side reactions appeared to limit the wide application of these protecting groups in peptide chemistry. In addition, all the amino acids which were described in the patent were protected by N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl. No derivatives relating to Nsc group were introduced.

The aim of our programme was to choose a better amino protecting group with good solubility and simple operation based on Nsc-group. Learning from 2-(4-nitrophenylsulfonyl) ethanol and its 7 analogues, we synthesized their chloroformates which were used for protecting amino groups because their chloroformates has good reactive activity with amino groups and the synthetic procedure was efficient and economic.

An example of this procedure was the preparation of N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl Asparagine *tert*-butyl ester (Nsc-Asn-OtBu) which is shown below. 2-(4-nitrophenylsulfonyl) ethylchloroformate was first synthesized from 2-(4-nitrophenylsulfonyl) ethanol and diphosgene (trichloromethyl chloroformate) (**Figure 2-2**).

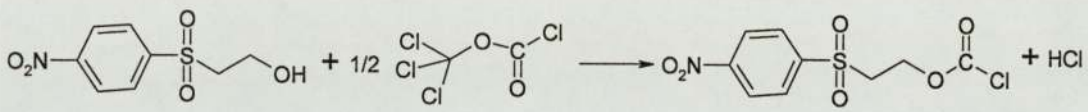


Figure 2-2 Preparation of 2-(4-nitrophenylsulfonyl) ethylchloroformate

Then, Nsc-Asn-OtBu can be prepared by the treatment of H-Asn-OtBu (Preparation method will be referred in section 2.2.2.1) with 2-(4-nitrophenylsulfonyl) ethylchloroformate in the presence of a base (Triethylamine) and at a temperature of 0 °C (**Figure 2-3**).

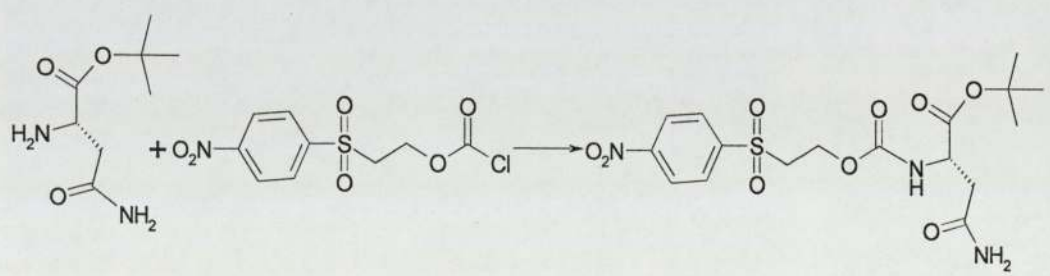
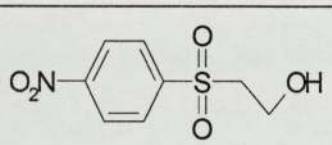
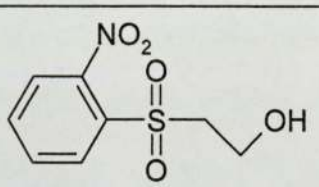
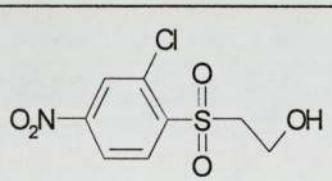
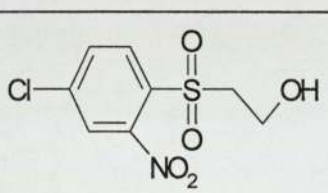


Figure 2-3 Preparation of Nsc-Asn-OtBu

Figure 2-2 and **2-3** indicated a general process of preparation of Nsc-amino acids, but the conditions of individual reaction are various; herein we list a series of reactions in **Table 2-1** which presents the structure of the 8 Nsc-ethanols and the results.

Name	Structure	-Chloroformate		-Asn-OtBu	
		Reaction time	yield	Reaction time	yield
2-(4-nitrophenylsulfonyl) ethanol		1 hour	87%	22h	73%
2-(2-nitrophenylsulfonyl) ethanol		1 hour	91%	--	No reaction occurs
2-(2-chloro-4-nitrophenylsulfonyl) ethanol		1 hour	82%	33h	64%
2-(4-chloro-2-nitrophenylsulfonyl) ethanol		1 hour	86%	--	No reaction occurs

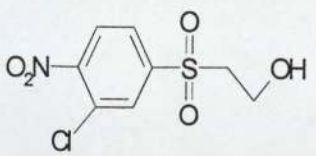
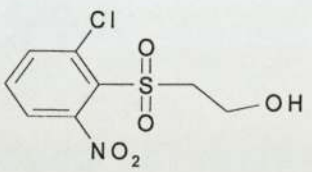
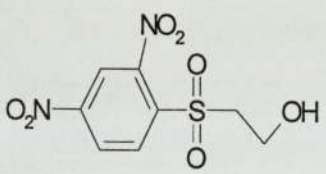
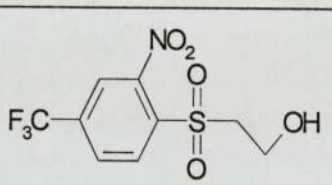
2-(3-chloro-4-nitrophenylsulfonyl) ethanol		1 hour	88%	--	No reaction occurs
2-(2-chloro-6-nitrophenylsulfonyl) ethanol		1 hour	81%	26h	67%
2-(2,4-dinitrophenylsulfonyl) ethanol		1 hour	78%	--	No reaction occurs
2-(2-nitro-4-trifluoromethylphenylsulfonyl) ethanol		1 hour	92%	16h	78%

Table 2-1 Results of preparation of Nsc-Asn-OtBu-series product

Table 2-1 indicated that 2-(4-nitrophenylsulfonyl) ethylchlroformate, 2-(2-chloro-4-nitrophenylsulfonyl) ethylchloroformate, 2-(2-chloro-6-nitrophenylsulfonyl) ethylchloroformate and 2-(4-trifluoromethyl-2-nitrophenylsulfonyl) ethylchloroformate can be attacked by amino group of H-Asn-OtBu. The others did not seem to be reactive within 36 hours.

We then measured the reaction time and yield of the deprotective procedure of Nsc-derivative protecting groups with piperidine and diethtylamine. According to the patent (Samukov, 2001), we knew that the Nsc-group could be cleaved due to the β-elimination mechanism by organic bases in aprotic solvents. The possible mechanism of this reaction is shown below (**Figure 2-4**).

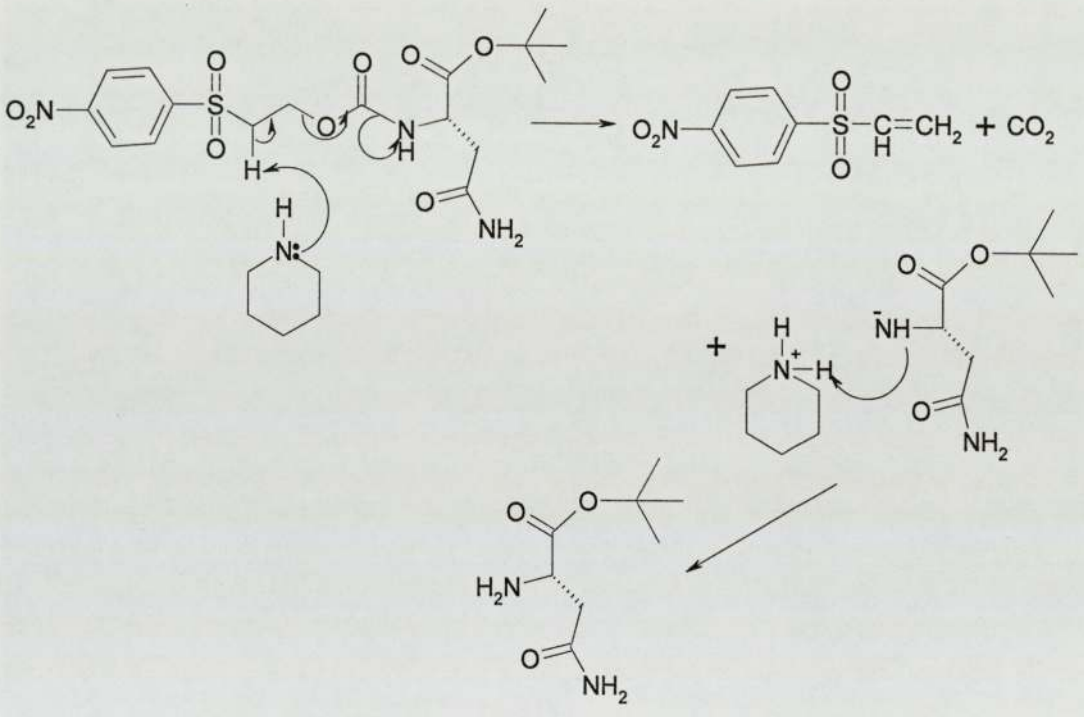


Figure 2-4 Mechanism of deprotection of Nsc-Asn-OtBu by piperidine

By detecting the four products obtained from the previous which were mentioned in **Table 2-1**, the results of deprotective procedure were collected in **Table 2-2**.

Reactant	Deprotected by piperidine		Deprotected by diethylamine	
	Reaction time	Yield of product	Reaction time	Yield of product
N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl Asparagine *tert*-butyl ester	41h	67%	26h	74%
N-2-(2-chloro-4-nitrophenylsulfonyl) ethoxycarbonyl Asparagine *tert*-butyl ester	53h	62%	41h	54%
N-2-(2-chloro-6-nitrophenylsulfonyl) ethoxycarbonyl Asparagine *tert*-butyl ester	48h	58%	35h	57%
N-2-(2-nitro-4-trifluoromethyl phenylsulfonyl) ethoxycarbonyl Asparagine *tert*-butyl ester	29h	72%	17h	75%

Table 2-2 Results of deprotection of Nsc-Asn-OtBu series product

The **Table 2-2** demonstrates that Nsc-series amino protecting groups displayed low activity when deprotected by piperidine and diethylamine. Compared with the rest, the protecting group 2-(2-nitro-4-trifluoromethylphenylsulfonyl) ethanol has achieved a

better results in protecting and deprotecting reaction with a shorter reaction time and a higher yield. Although it is not as good as the traditional amino protecting groups such as Fmoc, it is still feasible.

2.1.2 Studies of coupling reagents

As we mentioned in section 1.2.2, many new peptide coupling reagents were being designed, synthesized and commonly used in peptide synthesis. Among these reagents, HOBt- and HOAt-based uronium, phosphonium and immonium salts, such as BOP (Castro, 1975), HBTU (Dourtoglou, 1978), and BOMI (Li, 1999; Li, 2000) have been proven to be very efficient. The predominance of carbodiimide (Sheehan, 1955; Sarantakis, 1976; Shechan, 1961) and active ester techniques (Wieland, 1951; Bodanszky, 1955) have been gradually replaced with onium salts. These reagents can efficiently promote the formation of unhindered amide bonds, while the chain assembly of sterically hindered peptides containing N-methyl or Cα, Cα–dialkyl amino acid residues is inefficient using the above reagents except the HOAt-derived onium salts (Humphrey, 1997). However, these HOAt-based reagents are expensive and unsuitable for large scale peptide synthesis, and may react with amino components to form the corresponding guanidinium derivatives, especially in peptide segment condensation and cyclization (Albericio, 1998; Story, 1994). As an alternative approach, halogenated coupling reagents, such as PyBroP (Frerot, 1991), BroP (Coste, 1990), CIP (Akaji, 1994), TFFH (Carpino, 1995; Vojkovsky, 1997), BTFFH (El-Faham, 1998), PyCIU

(Coste, 1991), CDTP (Przybylski, 1996), CMMM, BOP-Cl (Van der Auwera, 1987; Van der Auwera, 1986), and BEMT (Li, 1999), were also found to be efficient for the synthesis of hindered peptides, especially for the scale-up of the preparation of peptides since they are inexpensive. Unfortunately, these halogenated reagents, except BOP-Cl and BEMT, usually result in high racemization during coupling reactions, especially for segment condensation. Therefore, it is necessary to develop more efficient and inexpensive coupling reagents to meet the needs of the synthesis of increasingly challenging peptides and peptidomimetics, as well as the establishment of peptide libraries (Li, 2000).

Mukaiyama did lots of research in 2-halo- and 2-bromopyridinium iodides, and used them to synthesize esters (Mukaiyama, 1975), lactones (Mukaiyama, 1976) and carboxamides (Bald, 1975). Furthermore, 1-ethyl-2-halo-pyridinium salts, was used as highly efficient coupling reagents for hindered peptide synthesis both in liquid-phase and the solid-phase. The general structure of 1-ethyl-2-halo-pyridinium salts were shown in **Figure 2-5** (Li, 2000).

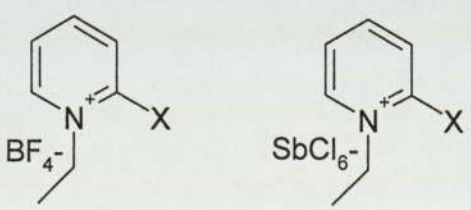


Figure 2-5 Structure of 1-ethyl, 2-halo-pyridinium salts

The tetrafluoroborate and hexachloroantimonate counterions were adopted to improve the solubility of these pyridinium compounds (Li, 2000). Herein, bromide salt was adopted as the coupling reagent instead of tetrafluoroborate and hexachloroantimonate and X represents bromine element (**Figure 2-6**).

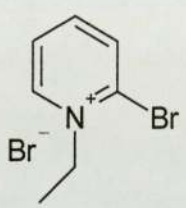


Figure 2-6 Coupling reagent 2-bromine-1-ethyl-pyridinium bromide (BEPB)

To test the reaction time and yield between DCC method and 2-bromine-1-ethyl-pyridinium bromide method, Z-Ala-Glu(OtBu)-Asn-OtBu (Thymosin alpha-1 26-28 derivative) was synthesized from H-Glu(OtBu)-Asn-OtBu and Z-Ala by using these two coupling reagents. HOBt was added as a racemization suppressant additive. The procedures of these two reactions are shown in **Figure 2-7**.

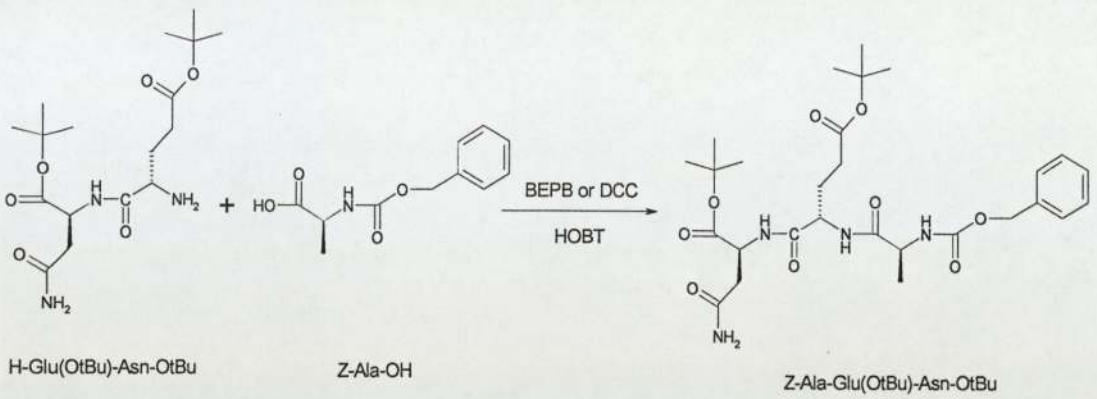


Figure 2-7 The coupling reactions using two different coupling reagents

Mechanism of BEPB-mediated coupling reactions is interpreted in section 2.2.2.1 and the operation of the reactions is described in section 3.2.2 in detail.

The TLC method shows that BEPB (2-bromine-1-ethyl-pyridinium bromide)-mediated coupling reaction completed within 2 minutes. On the other hand, the DCC-mediated reaction completed around 1 hour. The yield of tripeptide obtained by BEPB-mediated method was 82.7% and the yield of DCC-mediated method was 80.4%. According to polarimetric analysis the $[\alpha]_D^{20}$ (C=2.0, in THF) of the products of these two methods were both within -34.25° to -34.00°.

From these test results we assume that 2-bromine-1-ethyl-pyridinium bromide (BEPB) is a potential ideal coupling reagent because it is more efficient, economic, and easily

prepared. Thus, it is applied in the synthesis of thymosin alpha-1 fragments in our research.

2.1.3 Coupling method for synthesizing thymosin alpha-1 fragments

To optimize the method for synthesizing thymosin alpha-1 fragments, a series of tests were set up here:

- Comparison between HOBt and CF_3-HOBt as racemization suppressant additive;
- Comparison between TEA and DIPEA as base;
- Comparison between acetonitrile and DCM as solvent.

All the tests were developed in the synthetic procedure of protected tripeptide Z-Ala-Glu(OtBu)-Asn-OtBu. Results of the tests are shown in **Table 2-3** and operations are referred to in section 3.2.3.

Coupling Reagent: **Racemization suppressant additive:** **Base:** **Solvent:**	**Time**	**Yield**	**$[\alpha]_D^{20}$ (C=2.0 in MeOH)**
BEPB HOBt TEA Acetonitrile	<2 min	82.2%	-34.00 °
BEPB CF_3-HOBt TEA Acetonitrile	10-15 min	81.4%	-35.15 °
BEPB HOBt DIPEA Acetonitrile	<3 min	80.6%	-36.50 °
BEPB CF_3-HOBt DIPEA Acetonitrile	10-15 min	78.8%	-33.60 °
BEPB HOBt TEA DCM	<5 min	70.2%	-35.20 °

Table 2-3 Results of the tests for coupling method

According to these data, it was concluded that the first entry which took the shortest time and highest yield is the best combination for BEPB coupling. Therefore, in the synthesis of thymosin alpha-1 fragments, we choose BEPB as the coupling reagent, HOBt as a racemization suppressant additive, TEA as base and acetonitrile as solvent.

2.2 Thymosin alpha-1 fragments design and synthesis

2.2.1 Introduction of thymosin alpha-1

The importance of the thymus gland in the development and senescence of immunological competence in animals and man is now generally accepted. Although there is little knowledge of the molecular events by which the thymus gland exerts control over T cell development, it appears that a vital part of the process occurs via a hormonal mechanism. The thymus produces a family of polypeptides termed thymosin and perhaps several other thymic hormones and/or factors which play an important role in the maturation, differentiation and function of T cells. Thymosin has been found to induce T cell differentiation and enhance immunological functions in genetically athymic mice, in adult thymectomized mice, in tumor bearing mice and in mice with casein-induced amyloidosis (Wang, 1979).

Several polypeptide factors present in the thymus gland have been implicated to play important roles in the development and maintenance of immunological competence in

man and animals. The importance of the immune system in the defence against cancer and tumor cells is now widely recognized. In recent years, a few polypeptides shown to be able to stimulate maturation, differentiation and function of T cells have been isolated from bovine thymus. Among them, the acidic peptide thymosin .alpha-1, has been intensively studied. Its structure and activity have been described in U.S. Pat. No. 4,079,127 (Wang, 1979).

The structure of thymosin alpha-1 is as follows:

Ac-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH (Wang, 1979).

2.2.2 Synthesis of thymosin alpha-1 fragments 25-28 tetrapeptide

The methods to synthesize thymosin alpha-1 fragments are various. The traditional method to synthesize thymosin alpha-1 was referred in United States Patent 4,148,788 (Wang, 1979). Boc was used as the amino protecting group and OBzl as the carboxylic protecting group. We use Z protecting group and OtBu protecting group as the amino protecting group and carboxylic protecting group respectively. To vary the synthetic method of the patent, we attempted to synthesize thymosin alpha-1 25-28 tetrapeptide through two different routes:

Route 1 was to synthesize the tetrapeptide from the 28th amino acid Asn to the 25th amino acid Glu by means of elongating the amino acids chain one by one.

Route 2 was to synthesize two dipeptides which were Glu-Asn and Glu-Ala first. Then, tetrapeptide was synthesized by means of connecting the two dipeptides together.

The procedure of the tetrapeptide synthesis is described as follows and the operation in detail will be described in section 3.2.3.

Route 1:

2.2.2.1 Synthesis of Z-Glu(OtBu)-Asn-OtBu

Z-Asn-OtBu was first synthesized as the carboxyl terminal from Z-Asn-OH and 2,2,2-trichoro-acetimidic acid *tert*-butyl ester (Armstrong, 1988) in the presence of $BF_3 \cdot Et_2O$.

The procedure was shown in **Figure 2-8**.

Trichloromethylamide was hard to remove by simple solvent extraction, so the crude product was purified by passing through a silica column with dichloromethane-acetone (8/1) as elution sovent. The final yield of Z-Asn-OtBu was 81.7%.

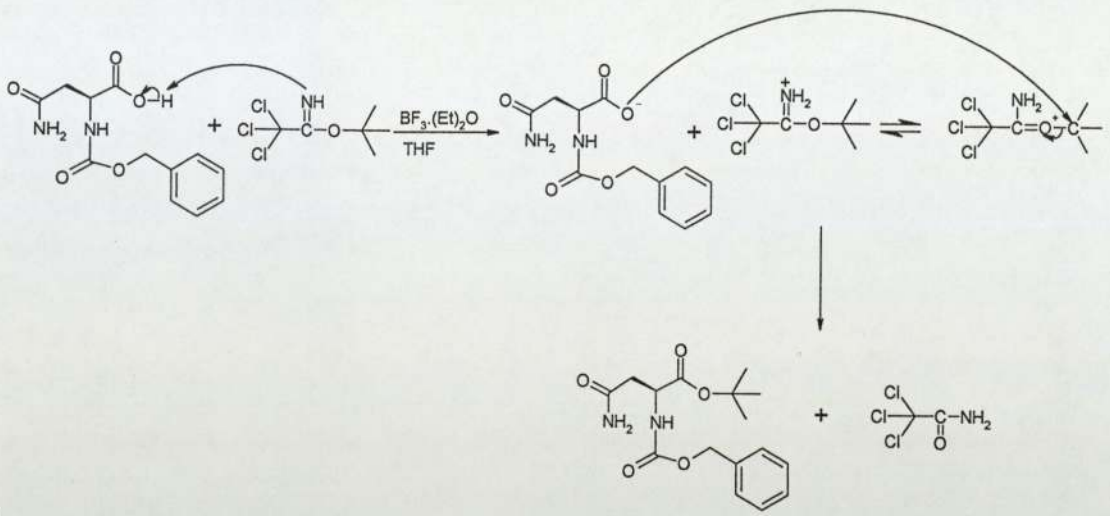


Figure 2-8 Preparation of Z-Asn-OtBu

Before coupling the Z-Asn-OtBu was first hydrogenated with hydrogen in the presence of palladium 5% on carbon (**Figure 2-9**) (Bergman, 1932), and the yield of this reaction was 96.5%.

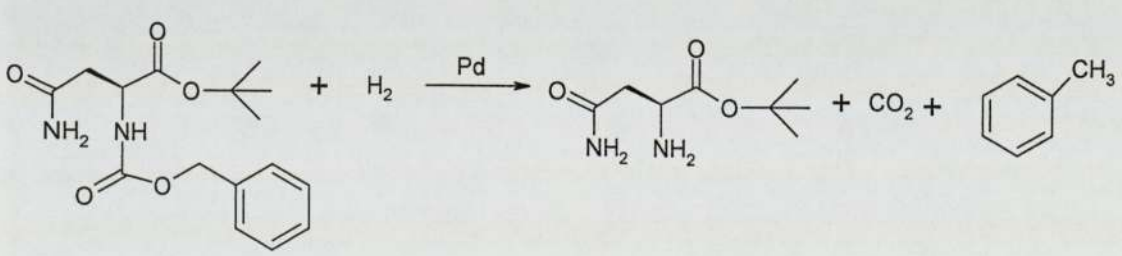


Figure 2-9 Hydrogenation of Z-Asn-OtBu

For the preparation of Z-Glu(OtBu)-OH, Z-Glu-OH was first treated with paraformaldehyde in the presence of 4-methylbenzenesulfonic acid to form 5-oxazolidinone structure. The Glutamic acid 5-oxazolidinone was then protected with *tert*-butyl ester by 2,2,2-trichoro-acetimidic acid *tert*-butyl ester (Armstrong, 1988) in the presence of $BF_3 \cdot Et_2O$. After purifying using a silica column with chloromethane as elution solvent, the glutamic acid 5-oxazolidinones *tert*-butyl ester was hydrolyzed by LiOH and give the product Z-Glu(OtBu)-OH. The procedure is shown in **Figure 2-10** to **Figure 2-12**.

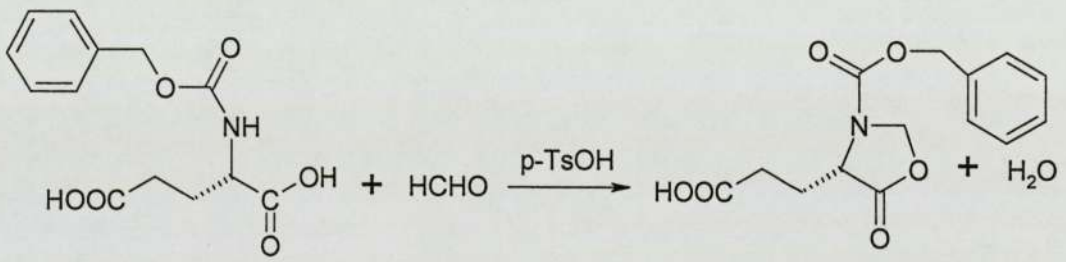


Figure 2-10 Formation of glutamic acid 5-oxazolidinones

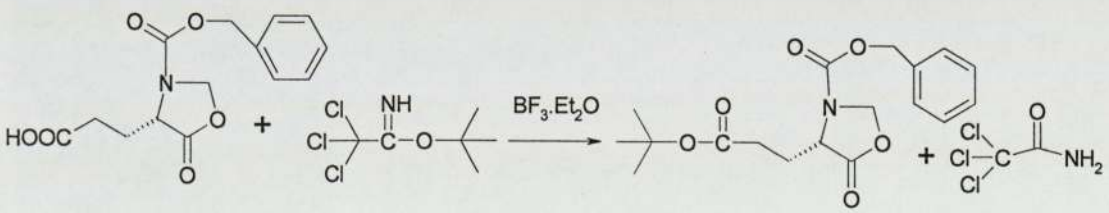


Figure 2-11 Add *tert*-butyl by *tert*-butyl trichloroacetimidate

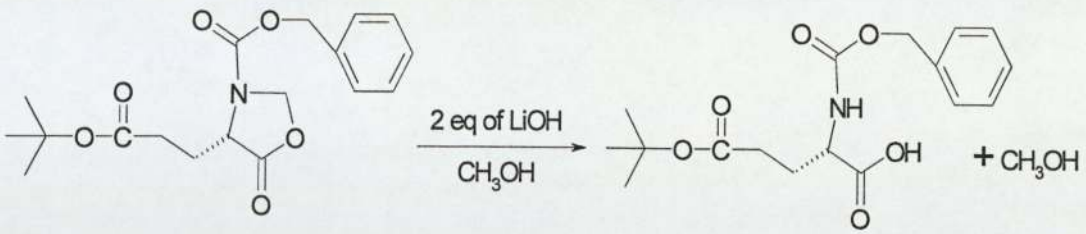


Figure 2-12 Hydrolysis of glutamic acid 5-oxazolidinones *tert*-butyl ester by LiOH.

The final yield of Z-Glu(OtBu)-OH was 71.5%. Z-Glu(OtBu)-OH directly coupled with H-Asn-OtBu in the presence of BEPB as coupling reagent (**Figure 2-13**).

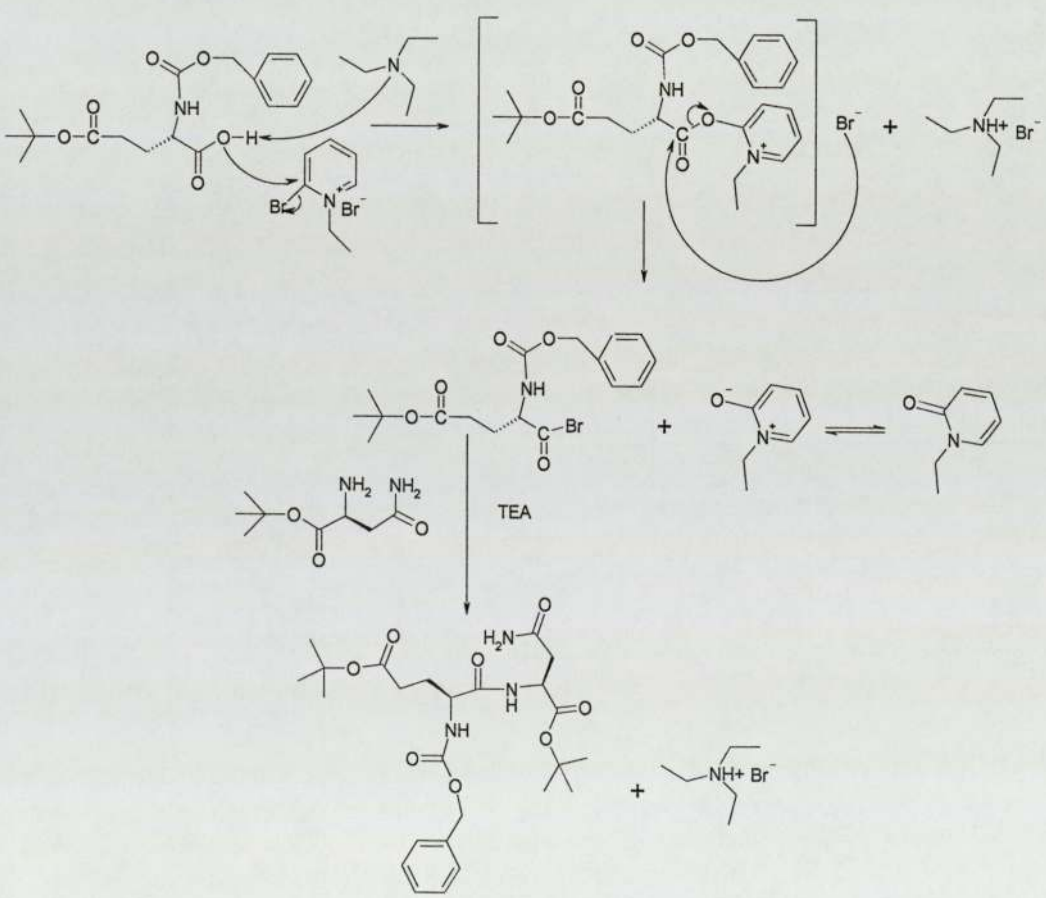


Figure 2-13 The possible mechanism of preparation of Z-Glu(OtBu)-Asn-OtBu in the presence of BEPB (Li, 2000).

2.2.2.2 Synthesis of Z-Ala-Glu(OtBu)-Asn-OtBu

Z-Glu(OtBu)-Asn-OtBu obtained from the procedure above was first hydrogenerated by hydrogen in the presence of palladium 5% on carbon (**Figure 2-14**).

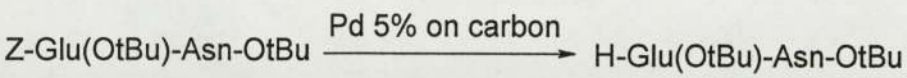


Figure 2-14 Hydrogenation of Z-Glu(OtBu)-Asn-OtBu

The resulting product H-Glu(OtBu)-Asn-OtBu was coupled with Z-Ala-OH (**Figure 2-15**). The coupling reaction was treated with BEPB/HOBt method.

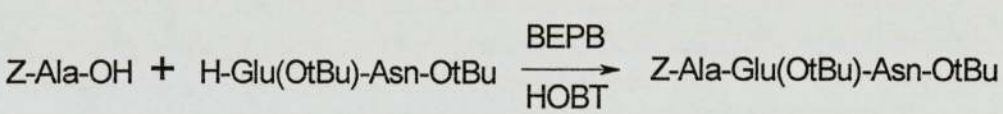


Figure 2-15 Synthesis of Z-Ala-Glu(OtBu)-Asn-OtBu

2.2.2.3 Synthesis of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

Z-Ala-Glu(OtBu)-Asn-OtBu obtained from the procedure above was first hydrogenated by hydrogen in the presence of palladium 5% on carbon (**Figure 2-16**).

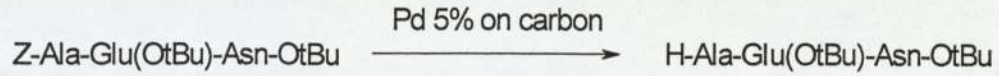


Figure 2-16 Hydrogenation of Z-Ala-Glu(OtBu)-Asn-OtBu

The resulting product H-Ala-Glu(OtBu)-Asn-OtBu was coupled with Z-Glu(OtBu)-OH (Figure 2-17). The coupling reaction was treated with BEPB/HOBt method.

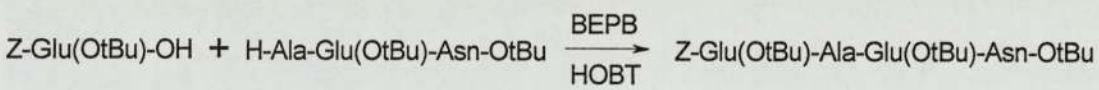


Figure 2-17 Synthesis of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

Route 2:

2.2.2.4 Synthesis of Z-Glu(OtBu)-Asn-OtBu

See section 2.2.2.1.

2.2.2.5 Synthesis of Z-Glu(OtBu)-Ala-OH

The preparation of Z-Glu(OtBu)-OH was refered in section 2.2.2.1. Z-Glu(OtBu)-OH was first treated with N-hydroxysuccinimide(HOSu) and DCC to form N-hydroxysuccinimide active ester (**Figure 2-18**) (Anderson, 1963).

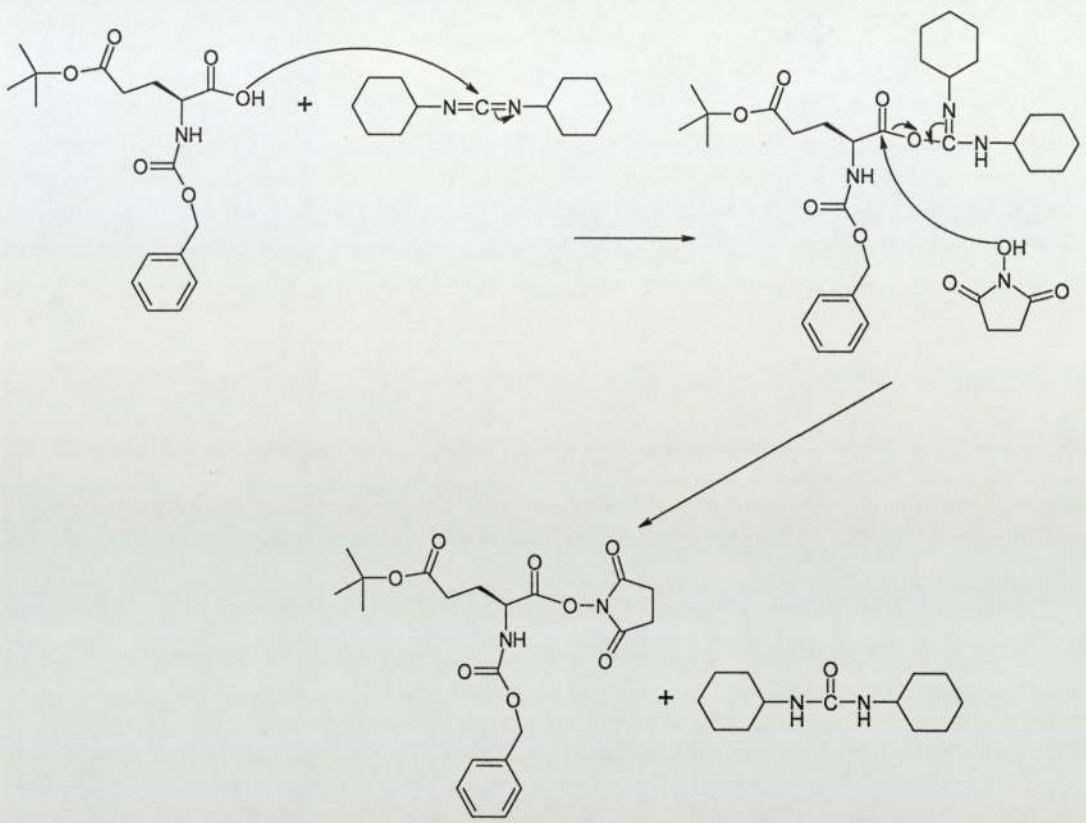


Figure 2-18 Mechanism of preparation Z-Glu(OtBu)-HOSu active ester

The solution of crude Z-Glu(OtBu)-HOSu was directly poured into the L-Alanine Sodium salt solution to synthesize Z-Glu(OtBu)-Ala-O$^-$Na$^+$ (**Figure 2-19**).

The final yield of dipeptide obtained by HOSu active ester method was 73% and the $[\alpha]_D^{20}$ (C=2.0, in MeOH) of dipeptide was -6.9°.

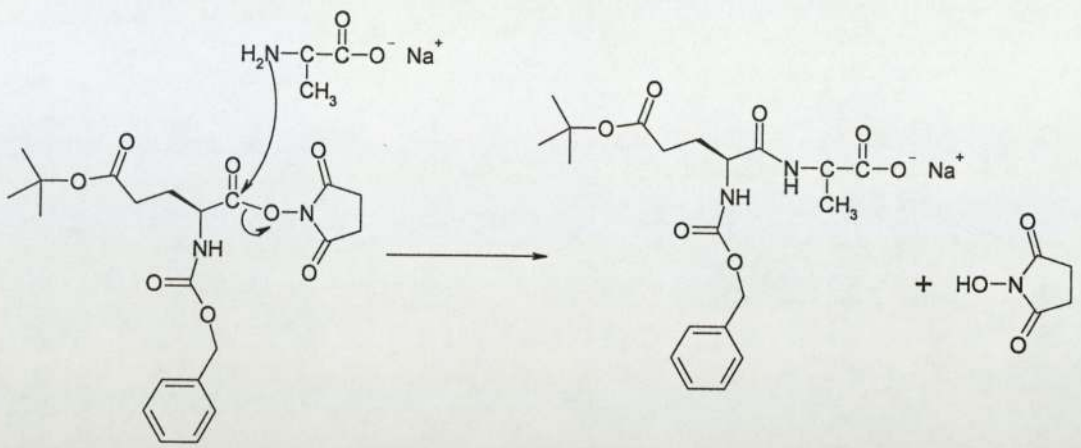


Figure 2-19 Preparation of Z-Glu(OtBu)-Ala-O$^-$Na$^+$ by active ester method

2.2.2.6 Synthesis of tetrapeptide Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

Z-Glu(OtBu)-Asn-OtBu obtained from the procedure mentioned in section 2.2.2.1 was first hydrogenerated by hydrogen in the presence of palladium 5% on carbon (**Figure 2-20**).

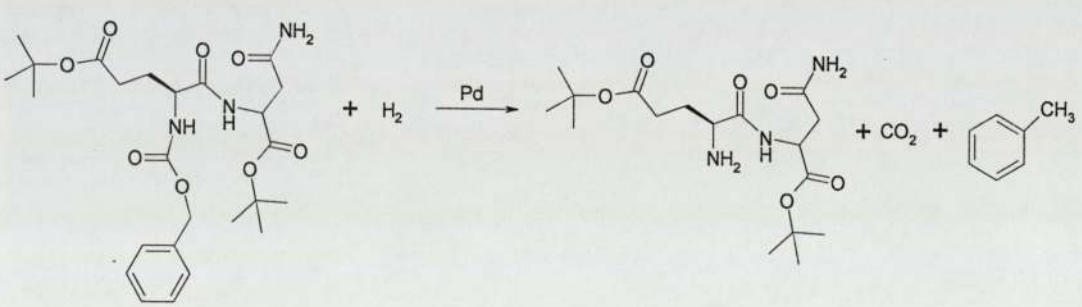


Figure 2-20 Hydrogenation of Z-Glu(OtBu)-Asn-OtBu

The yield of the reaction above was 96.5% and the the product H-Glu(OtBu)-Asn-OtBu was coupled with Z-Glu(OtBu)-Ala-OH (**Figure 2-21**). The coupling reaction was subjected to the BEPB/HOBt method.

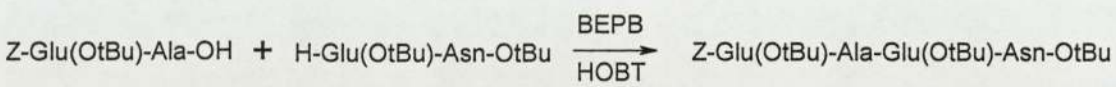


Figure 2-21 Preparation of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

The results for tests of the two routes are shown in the **Table 2-4** below. From the table below, the final yield of the product Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu through route 1 was slightly higher than that through route 2. In addition, the procedure of route 1 was not as complicated as that of route 2. Therefore, it is better to synthesize the peptide fragment analogues of thymosin alpha-1 by means of elongating the amino acids chain one by one as in route 1.

Route 1		**Route 2**	
Product	Yield	Product	Yield
Z-Glu(OtBu)-Asn-OtBu	85.6%	Z-Glu(OtBu)-Asn-OtBu	85.6%
H-Glu(OtBu)-Asn-OtBu	96.5%	H-Glu(OtBu)-Asn-OtBu	96.5%
Z-Ala-Glu(OtBu)-Asn-OtBu	82.7%	Z-Glu(OtBu)-Ala-OH	73.6%
H-Ala-Glu(OtBu)-Asn-OtBu	94.3%	Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu	70.6%
Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu	70.3%		

Table 2-4 Results of the tests

2.2.3 New peptide designed from thymosin alpha-1 fragments

Method 1: Alanine substitute:

According to the structure of Thymosin alpha-1 25-28, there are two Glutamic acids, their side chains are more complicated than that of the other two amino aicds. To test the contribution of the glutamic acids to the biological activity of the thymosin alpha-1 25-28, herein, we designed two tetrapeptides which were different from the original peptide (Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu) by replacing with a different amino acid. We chose Alanine as the substitution for Glutamic acid because that side chain of Alanine was methyl which could be made a different comparison with the complex side chain of Glutamic acid. If the replaced tetrapeptide had no biological activity, the Glutamic acid group would be proved to be necessary. Otherwise, there could be other groups account for biological activity. The synthesis procedures for two designed tetrapeptide are described as follows and the experimental details are described in section 3.2.4.

2.2.3.1 Synthesis of designed tetrapeptide Z-Glu(OtBu)-Ala-Ala-Asn-OtBu

The synthesis procedure of Z-Glu(OtBu)-Ala-Ala-Asn-OtBu (**Figure 2-22**) is similar to that of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu which is described in section 2.2.3 in **Route 1**.

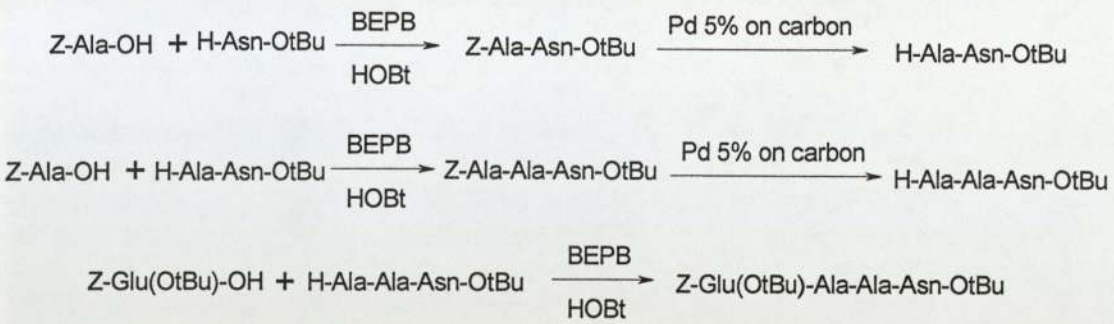


Figure 2-22 Preparation of Z-Glu(OtBu)-Ala-Ala-Asn-OtBu

The reaction time and yield for each step is listed below in **Table 2-5.**

Product	**Reaction Time**	**Yield**	**$[\alpha]_D^{20}$ (l = 20cm)**
Z-Ala-Asn-OtBu	2min	84.9%	-16.3 ° (C=2.0, in MeOH)
H-Ala-Asn-OtBu	60min	97.1%	-14.4 ° (C=2.0, in MeOH)
Z-Ala-Ala-Asn-OtBu	4min	83.5%	-29.7 ° (C=2.0, in MeOH)
H-Ala-Ala-Asn-OtBu	100min	91.0%	-25.4 ° (C=2.0, in MeOH)
Z-Glu(OtBu)-Ala-Ala-Asn-O tBu	10min	76.1%	-13.2 ° (C=1.0, in MeOH)

Table 2-5 Results of synthetic procedure of Z-Glu(OtBu)-Ala-Ala-Asn-OtBu

2.2.3.2 Synthesis of designed tetrapeptide Z-Ala-Ala-Glu(OtBu)-Asn-OtBu

The synthetic procedure of Z-Ala-Ala-Glu(OtBu)-Asn-OtBu (**Figure 2-23**) is similar to that of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu which has been described in section 2.2.2.3. The reaction procedure is shown in **Figure 2-23**, reaction time and yield is listed in **Table 2-6**.

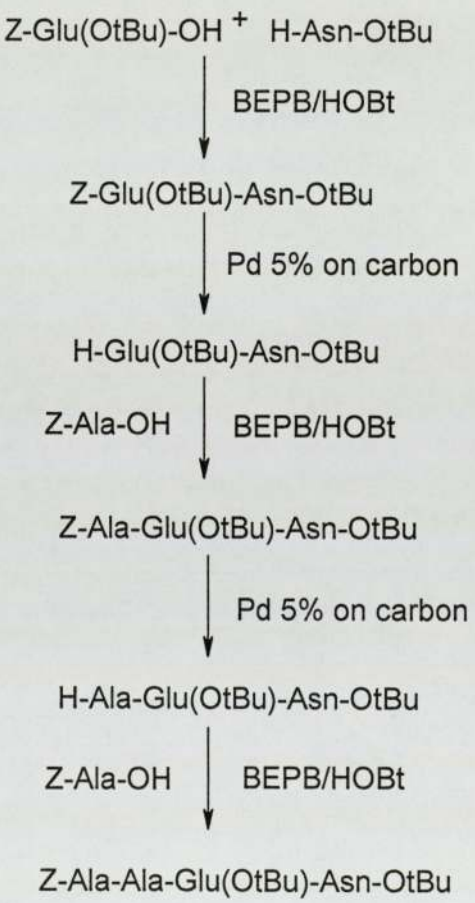


Figure 2-23 Preparation of Z-Ala-Ala-Glu(OtBu)-Asn-OtBu

Product	Reaction Time	Yield	$[\alpha]_D^{20}$ (l = 20cm)
Z-Glu(OtBu)-Asn-OtBu	2 min	85.6%	-18.2 ° (C=2.0, in MeOH)
H-Glu(OtBu)-Asn-OtBu	60 min	96.5%	-16.8 ° (C=2.0, in MeOH)
Z-Ala-Glu(OtBu)-Asn-OtBu	2 min	82.7%	-34.2 ° (C=2.0, in THF)
H-Ala-Glu(OtBu)-Asn-OtBu	110 min	94.3%	- 30.1° (C=2.0, in MeOH)
Z-Ala-Ala-Glu(OtBu)-Asn-OtBu	10 min	70.8%	-18.6 ° (C=1.0, in MeOH)

Table2-6 Results of synthetic procedure of Z-Ala-Ala-Glu(OtBu)-Asn-OtBu

The two modified tetrapeptides Z-Glu(OtBu)-Ala-Ala-Asn-OtBu and Z-Ala-Ala-Glu(OtBu) -Asn-OtBu will be used directly into the pharmacological experiments to test the biological activity compared with Thymosin alpha-1 25-28 tetrapeptide.

Method 2: D-Amino acid substitution:

According to the structure of Thymosin alpha-1, all the amino acids are L configuration. To test the influence of optical isomer on the biological activity of the thymosin alpha-1 25-28, herein, we designed two tetrapeptides which were optically different from the original peptide (Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu) by replacing with a D-amino acid. The synthetic procedures of the designed peptide are described as follows and their experimental details are described in section 3.2.5.

2.2.3.3 Synthesis of dipeptide Z-D-Glu(OtBu)-Asn-OtBu

The synthetic procedure of Z-D-Glu(OtBu)-Asn-OtBu (**Figure 2-24**) is similar to that of Z-Glu(OtBu)-Asn-OtBu which is described in section 2.2.3.1.

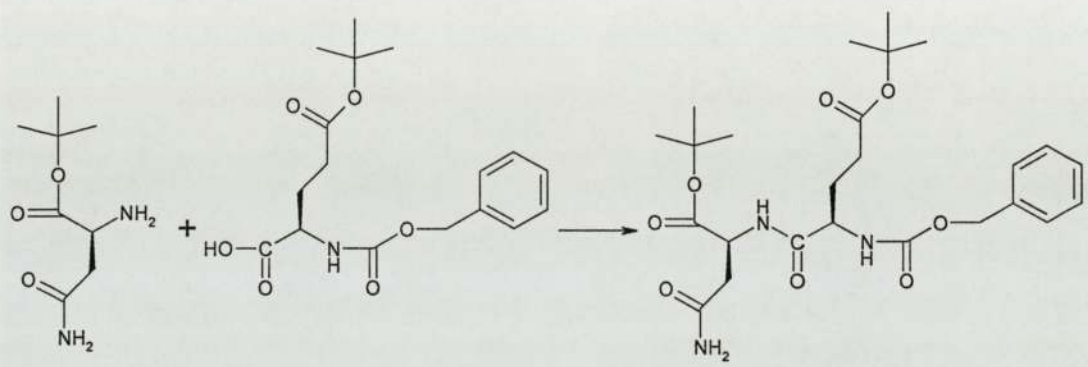


Figure 2-24 Preparation of Z-D-Glu(OtBu)-Asn-OtBu

The yield of the product Z-D-Glu(OtBu)-Asn-OtBu was 85.3% and the $[\alpha]_D^{20}$ (C=2.0, in MeOH) of the product was 13.1°.

2.2.3.4 Synthesis of tripeptide Z-D-Ala-Glu(OtBu)-Asn-OtBu

The synthetic procedure of Z-D-Ala-Glu(OtBu)-Asn-OtBu (**Figure 2-25**) is similar to that of Z-Ala-Glu(OtBu)-Asn-OtBu which is described in section 2.1.2.

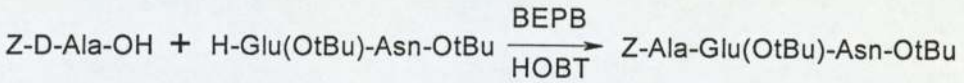


Figure 2-25 Preparation of Z-D-Ala-Glu(OtBu)-Asn-OtBu

The yield of the product Z-D-Ala-Glu(OtBu)-Asn-OtBu was 82.1% and the $[\alpha]_D^{20}$ (C=2.0, in THF) of the product was +23.4°.

2.2.3.5 Synthesis of tetrapeptide Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

The synthetic procedure of Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu (**Figure 2-26**) is similar to that of Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu which is described in section 2.2.3.3.

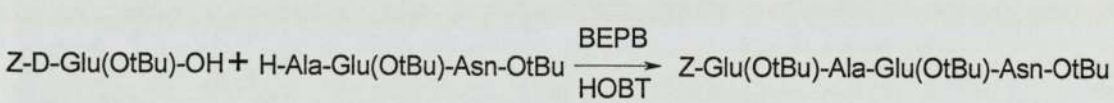


Figure 2-26 Preparation of Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

The yield of the product Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu was 70.7% and the $[\alpha]_D^{20}$ (C=1.0, in MeOH) of the product was +14.9°.

The three replaced tetrapeptides Z-Glu(OtBu)-Ala-D-Glu(OtBu)-Asn-OtBu, Z-Glu(OtBu)-D-Ala-Glu(OtBu)-Asn-OtBu and Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-

-OtBu could be used directly in the pharmacological experiments to test the biological activity compared with Thymosin alpha-1.

2.2.3.6 Conclusion

From the results of the synthesis of Thymosin alpha-1 fragments, it can be concluded that the new coupling reagent BEPB is effective because it can give yields as high as DCC-mediated reactions. Another advantage of EBPB-mediated reactions is that the reaction time is much shorter than DCC-mediated reactions. In addition, there is no partial racemization in the EBPB-mediated reactions.

Compared with the traditional coupling reagents DCC, EDC, and HBTU which have been widely used, more research on BEPB is required such as the study of its use for coupling other amino acids. The advantages of BEPB; its effectiveness, ease of use and low cost, make it an attractive reagent for peptide synthesis.

Chapter Three:

Experimental

3.1 Instrumentation and Materials

3.1.1 Instrumentation

All melting points were measured on a Reichert-Jung ThermoGalen hot stage microscope and were corrected. Proton NMR spectra were obtained on a Bruker AC 250 instrument operating at 250 MHz as solutions in d_6-DMSO (referenced from δDMSO=2.50ppm) and $CDCl_3$ (referenced from $\delta CDCl_3$=7.26ppm) unless otherwise stated. Infrared spectra were recorded as KBr discs on a Mattson 3000 FTIR spectrophotometer. Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) was carried out on a Hewlett-Packard 5989B quadrupole instrument connected to an electrospray 59987A unit with an APCI accessory and automatic injection using a Hewlett-Packard 1100 series autosampler. Structures were drawn, manipulated and optimized using ChemDraw Ultra 8.0.3 (CambridgeSoft Corporation) and MDL ISIS/Draw 2.5 (MDL Information Systems, Inc.).

3.1.2 Materials

All solvents used in this thesis were purchased from Fisher Scientific and were used without further purification. $CDCl_3$ and d_6-DMSO for proton NMR were purchased from Apollo Scientific Limitied. Amino acids were supplied by Avocado Organics and general chemicals were obtained from Fisher Chemicals and Acros Organic.

Thin Layer Chromatography (TLC) was carried out using aluminium backed Merck Silica Gel 60 F_{254} plates and 50% sulphuric acid water solution was used to develop TLC plates. All column chromatographic purifications were accomplished on silica gel 60 (200-400 mesh) with the appropriate solvent gradients.

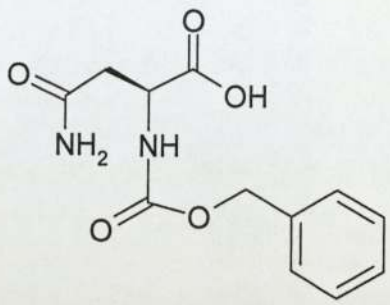
3.2 Chemical synthesis methods

3.2.1 Methods for N-2-(4-nitrophenylsulfonyl) ethoxycarbonyl (Nsc) series amino protecting groups research

3.2.1.1 Preparation of Z-Asn-OH

To a solution of L-Asparagine monohydrate 20.0 g (133 mmol) in 318ml 10% Na_2CO_3 solution (300 mmol) was added THF (56 ml) and benzyl chloroformate (158 mmol, 22.7 ml) in THF (56 ml) at 0 °C. The reaction mixture was stirred overnight and was poured into water (300 ml). The mixture was carefully acidified with 2.5 N HCl in aqua and a white precipitate formed. The precipitate was filtered out and washed extensively with diethyl ether and water to give 31.0 g (91.03%) product as white solid.

Product: Z-Asn-OH

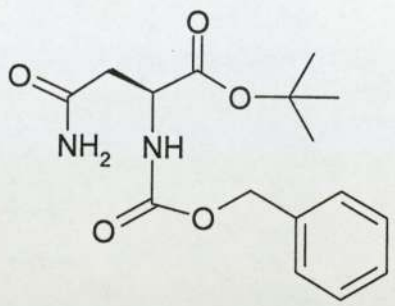


Mp 163-165 °C (Merck, 2004/2005); $[\alpha]_D^{20}$=+6 ° (c=1.6 g/100 ml in CH_3COOH); ^1H NMR (DMSO-d6): δ_H 2.56-2.76 (overlapping with DMSO, m, J=4.5 Hz, 2H, CH_2-CO) δ_H 4.30-4.38 (q, J=4Hz, 1H, CH-CO) δ_H 5.03 (s, 2H, CH_2-O) δ_H 7.35 (s, 5H, Aromatic) δ_H 7.60 (d, 2H, NH_2)

3.2.1.2 Preparation of Z-Asn-OtBu

Z-Asn-OH (26.65 g, 100 mmol) was suspended in THF (300 ml) with stirring at 0 °C. *Tert*-butyl trichloroacetimidate (35.5 g, 162.5 mmol) was added followed by $BF_3{\cdot}Et_2O$ (2.5 ml, 20 mmol). This mixture was stirred for 5 minutes at 0 °C, then allowed to be warmed up to room temperature with stirring. The resulting mixture was stirred for 120 minutes further and became a clear solution. Most of the THF was removed by evaporation at room temperature by reduced pressure. The residue was diluted with water (100 ml) and extracted with DCM (2×50 ml). The DCM layer was washed with water (10 ml). After dried with anhydrous $MgSO_4$, the solvent was evaporated out and give the crude product as white solid. Then, the crude product was purified by passing through a silica column with DCM-acetone (8/1) as elution solvent.

Product: Z-Asn-OtBu

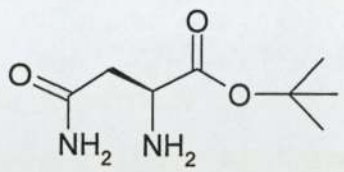


Mp 106.3-108.2 °C (Ref. 107-108.5 °C Vasella, 1983); IR: ν_{max} 3388 (ν^{as}_{NH}), 3325 (ν^{s}_{NH2}), 3188 (ν_{NH}), 2980 (ν_{CH3}), 2929 (ν_{CH2}), 1741 ($\nu_{C=O}$ Ester), 1698 ($\nu_{C=O}$ Amide), 1655 (β_{NH}), 1537 ($\nu_{C=C}$ Aromatic), 1365 (δ_{CH3}), 1224 (ν_{C-O-C}), 1153 (ν_{C-O-C}), 844 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 1.43 (s, 9H, $(CH_3)_3$-C) δ_H 2.69-2.93 (dddd, J=4.5 Hz, 2H, CH_2-CO) δ_H 4.44 (t, J=4Hz, 1H, CH-CO) δ_H 5.09 (s, 2H, CH_2-O) δ_H 5.93-5.96 (d, J=8Hz, 1H, NH) δ_H 7.33 (s, 5H, Aromatic)

3.2.1.3 Hydrogenation of Z-Asn-OtBu

Pallladium on carbon 5% (3.70 g) was added to a solution of Z-Asn-OtBu (37.366 g, 157.85 mmol) in MeOH (380 ml). The mixture was hydrogenated with vigorous stirring for 1hr. Pd/C was removed by filtration and the filtrate was evaporated. The residue was worked up by acetonitrile to give the product.

Product: H-Asn-OtBu



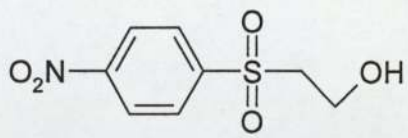
Mp 102.7-104.5 °C (Ref. 101-102 °C Callahan, 1963); IR: ν_{max} 3407 (ν^{as}_{NH}), 3337 (ν^{s}_{NH2}), 3223 (ν_{NH}), 2945 (ν_{CH3}), 1698 ($\nu_{C=O}$ Ester), 1639 ($\nu_{C=O}$ Amide), 1533 ($\nu_{C=C}$ Aromatic), 1318 (δ_{CH3}), 1267 (ν_{C-O-C}), 1192 (ν_{C-O-C}), 738 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR (DMSO-d6): δ_H 1.41 (s, 9H, $(CH_3)_3$-C) δ_H 2.69-2.93 (overlapping, m, 2H, CH_2-CO) δ_H 3.73 (t, J=6Hz, 1H, CH-CO)

3.2.1.4 Preparation of 2-(4-nitrophenylsulfonyl) ethylchloroformate

To a solution of 2-(4-nitrophenylsulfonyl) ethanol (2.31 g, 10.0 mmol) in THF (30 ml) was added TEA (4 ml, 28.5 mmol). The pH value of the mixture was around 9. Diphosgene (1.98 g, 10.0 mmol) in dichloromethane (5 ml) was added dropwise to the stirred solution over 30 minutes at 0 °C. The mixture was stirred for another one hour. The precipitate was removed by filtration and the filtrate was evaporated to give the product as yellowy solid.

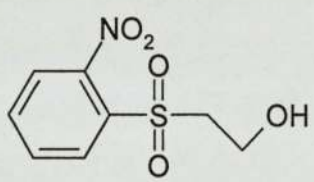
The preparation of the other seven analogues chloroformate was as the same operation as described above.

Reactant: 2-(4-nitrophenylsulfonyl) ethanol (Verhart, 1988)



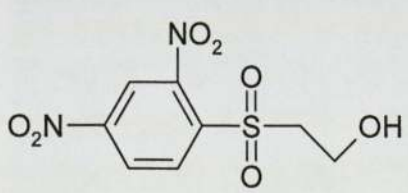
Mp 125-126 °C; ^{1}H NMR ($CDCl_3$): δ_H 1.57 (s, 1H, OH) δ_H 3.41 (t, J=6.5Hz, 2H, CH_2-SO_2) δ_H 4.07 (t, 2H, CH_2-OH) δ_H 8.13-8.44 (dd, J=9Hz, 4H, Aromatic)

Reactant: 2-(2-nitrophenylsulfonyl) ethanol (Carpino, 1972)



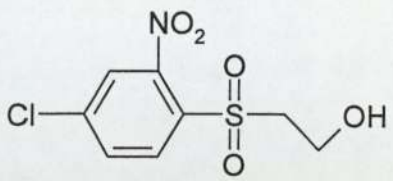
Mp 88-89 °C; ^{1}H NMR ($CDCl_3$): δ_H 2.25 (s, 1H, OH) δ_H 3.85 (t, J=6.5Hz, 2H, CH_2-SO_2) δ_H 4.14 (t, 2H, CH_2-OH) δ_H 7.80-7.84 (m, 3H, Aromatic) δ_H 8.17-8.20 (m, 1H, Aromatic)

Reactant: 2-(2,4-dinitrophenylsulfonyl) ethanol (Samukov, 1988)



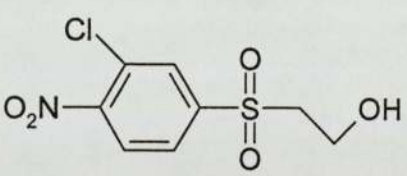
Mp 117-119 °C; ^{1}H NMR ($CDCl_3$): δ_H 3.01-3.67 (2×sep, J=4Hz, 2H, CH_2-SO_2) δ_H 4.16-4.23 (m, 2H, CH_2-OH) δ_H 8.57 (d, J=9Hz, 1H, H-6 in Phenyl) δ_H 8.74-8.79 (dd, J=2Hz, 1H, H-5 in Phenyl) δ_H 9.13 (d, J=2Hz, 1H, H-3 in Phenyl)

Reactant: 2-(4-chloro-2-nitrophenylsulfonyl) ethanol



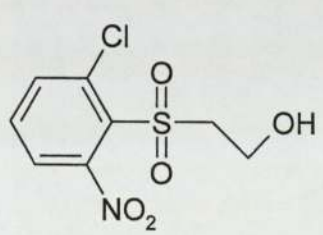
^{1}H NMR ($CDCl_3$): δ_H 2.91-3.67 (2×sep, J=4Hz, 2H, CH_2-SO_2) δ_H 4.16 (m, 2H, CH_2-OH) δ_H 7.94 (d, 1H, H-5 in Phenyl) δ_H 8.27 (s, 1H, H-6 in Phenyl) δ_H 8.31 (t, 1H, H-3 in Phenyl)

Reactant: 2-(3-chloro-4-nitrophenylsulfonyl) ethanol



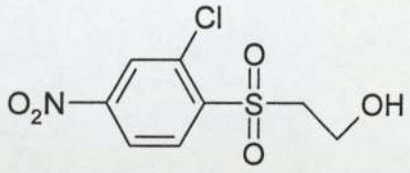
^{1}H NMR ($CDCl_3$): δ_H 3.88 (t, J=6 Hz, 2H, CH_2-SO_2) δ_H 4.16 (t, J=6Hz, 2H, CH_2-OH) δ_H 7.77 (dd, 1H, H-6 in Phenyl) δ_H 7.85 (d, 1H, H-2 in Phenyl) δ_H 8.16 (d, 1H, H-4 in Phenyl)

Reactant: 2-(2-chloro-6-nitrophenylsulfonyl) ethanol



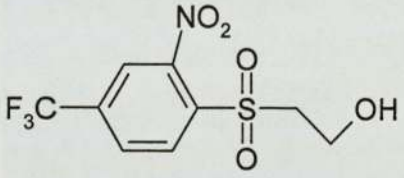
^{1}H NMR ($CDCl_3$): δ_H 3.76 (t, J=6Hz, 2H, CH_2-SO_2) δ_H 4.17 (t, J=6Hz, 2H, CH_2-OH) δ_H 7.49 (dd, 1H, H-4 in Phenyl) δ_H 7.65-7.76 (m, 2H, H-3, 5 in Phenyl)

Reactant: 2-(2-chloro-4-nitrophenylsulfonyl) ethanol



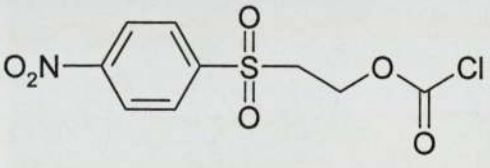
^{1}H NMR ($CDCl_3$): δ_H 3.70 (t, J=6Hz, 2H, CH_2-SO_2) δ_H 4.08 (t, J=6Hz, 2H, CH_2-OH) δ_H 8.34 (overlapping, m, 3H, Aromatic)

Reactant: 2-(2-nitro-4-trifluoromethylphenylsulfonyl) ethanol



^{1}H NMR ($CDCl_3$): δ_H 2.95-3.67 (2×sep, J=4Hz, 2H, CH_2-SO_2) δ_H 4.18 (m, 2H, CH_2-OH) δ_H 8.21 (d, 1H, H-6 in Phenyl) δ_H 8.49-8.58 (t, 2H, H-3, 5 in Phenyl)

Product: 2-(4-nitrophenylsulfonyl) ethylchloroformate (Verhart, 1988)

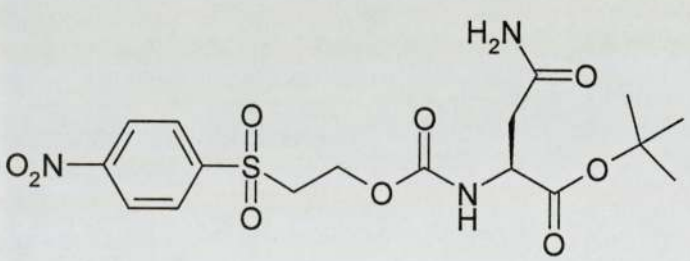


Mp 115.0-116.9 °C (Ref. 119-122°C Verhart, 1988); ^{1}H NMR ($CDCl_3$) δ_H 6.20 (m, 2H, CH_2-SO_2) δ_H 6.56-6.75 (sex, 2H, CH_2-OH) δ_H 8.09-8.42 (dd, J=9Hz, 4H, Aromatic)

3.2.1.5 Preparation of 2-(4-nitrophenylsulfonyl) ethyloxycarbonyl Asparagine *tert*-butyl ester (Nsc-Asn-OtBu)

H-Asn-OtBu (1.882 g, 10 mmol) was added to DCM (20 ml). TEA (2.0 ml, 14.2 mmol) was also added to make the pH around 9, followed by the addition of 2-(4-nitrophenylsulfonyl) ethylchloroformate (3.23 g, 11 mmol) in several portions. The mixture was stirred for 20 hour at room temperature. The resulting mixture was filtered and the filtrate was evaporated in vacuo. The residue was recrystallized with ether (20 ml) and gave yellow solid as crude product. The crude product was purified by silica column with DCM-Acetone (3.5/1) as elution solvent.

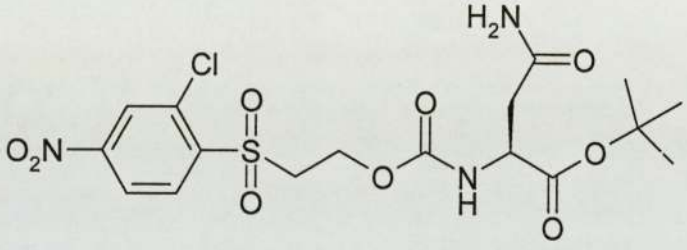
Product: 2-(4-nitrophenylsulfonyl) ethyloxycarbonyl Asparagine *tert*-butyl ester (Nsc-Asn-OtBu)



Mp 105.0-160.9 °C; IR: ν_{max} 3415 (ν^{as}_{NH}), 3332 (ν^{s}_{NH2}), 3188 (ν_{NH}), 1713 ($\nu_{C=O}$ Esters), 1660 ($\nu_{C=O}$ Amides), 1523 (ν^{as}_{NO2}), 1303 (ν^{s}_{NO2}), 852 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^{1}H NMR (DMSO-d6) δ_H 1.34 (s, 9H, $(CH_3)_3$-C) δ_H 2.09-2.27 (m, 2H, CH_2-CO) δ_H 2.65-2.91 (qq, J=6Hz, 2H, CH_2-SO_2) δ_H 3.25 (t, 1H, CH-CO) δ_H 3.57 (t, 2H, CH_2-O) δ_H 8.15-8.45 (dd, J=9Hz, 4H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 28.7; (CH_2) 36.4, 54.0,

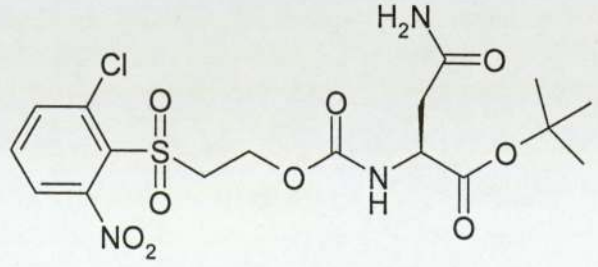
60.2; (CH) 37.2, 128.9, 129.2; (C) 82.1, 145.0, 152.9, 155.9, 169.8, 172.3; m/z 468 ($M+Na^+$), 913 ($2M+Na^+$).

Product: 2-(2-chloro-4-nitrophenylsulfonyl) ethyloxycarbonyl Asparagine *tert*-butyl ester



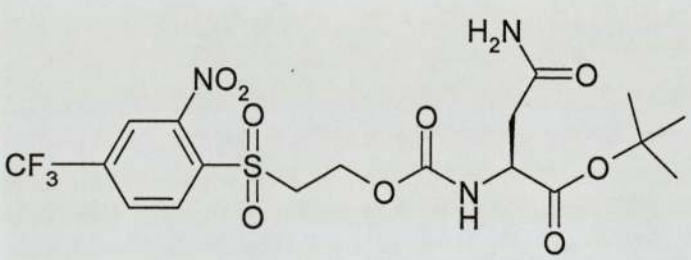
Mp 116.9-120.0 °C; IR: ν_{max} 3483 (ν^{as}_{NH}), 3369 (ν^{s}_{NH2}), 3090 (ν_{NH}), 1732 ($\nu_{C=O}$ Esters), 1679 ($\nu_{C=O}$ Amides), 1522 (ν^{as}_{NO2}), 1347 (ν^{s}_{NO2}), 885 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR (DMSO-d6) δ_H 1.35 (s, 9H, $3CH_3$-C) δ_H 2.11-2.18 (m, 2H, CH_2-CO) δ_H 2.77-2.99 (qq, J=6Hz, 2H, CH_2-SO_2) δ_H 3.24 (t, 1H, CH-CO) δ_H 3.73 (t, 2H, CH_2-O) δ_H 8.24-8.53 (m, 3H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 28.7; (CH_2) 36.4, 53.5, 60.2; (CH) 37.2, 123.0, 125.2, 130.6; (C) 82.1, 133.4, 148.6, 154.3, 155.9, 169.8, 172.3; m/z 502 ($M+Na^+$), 981 ($2M+Na^+$).

Product: 2-(2-chloro-6-nitrophenylsulfonyl) ethyloxycarbonyl Asparagine *tert*-butyl ester



Mp 112.6-114.3 °C; IR: ν_{max} 3430 (ν^{as}_{NH}), 3320 (ν^{s}_{NH2}), 3190 (ν_{NH}), 1710 ($\nu_{C=O}$ Esters), 1660 ($\nu_{C=O}$ Amides), 1537 (ν^{as}_{NO2}), 1364 (ν^{s}_{NO2}), 797 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^{1}H NMR (DMSO-d6) δ_H 1.37 (s, 9H, $(CH_3)_3$-C) δ_H 2.18-2.23 (m, 2H, CH_2-CO) δ_H 2.79-3.11 (qq, J=6Hz, 2H, CH_2-SO_2) δ_H 3.22 (overlapping, m, 1H, CH-CO) δ_H 3.75 (t, 2H, CH_2-O) δ_H 7.96-8.02 (m, 3H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 28.7; (CH_2) 36.4, 52.5, 60.2; (CH) 37.2, 123.0, 136.0, 137.0; (C) 82.1, 133.4, 134.0, 149.6, 155.9, 169.8, 172.3; m/z 502 ($M+Na^+$), 981 ($2M+Na^+$).

Product: 2-(2-nitro-4-trifluoromethylphenylsulfonyl) ethyloxycarbonyl Asparagine *tert*-butyl ester



Mp 108.0-109.2 °C; IR: ν_{max} 3435 (ν^{as}_{NH}), 3327 (ν^{s}_{NH2}), 3170 (ν_{NH}), 1727 ($\nu_{C=O}$ Esters), 1673 ($\nu_{C=O}$ Amides), 1545 (ν^{as}_{NO2}), 1375 (ν^{s}_{NO2}), 796 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^{1}H NMR (DMSO-d6) δ_H 1.36 (s, 9H, $(CH_3)_3$-C) δ_H 2.17-2.27 (m, 2H, CH_2-CO) δ_H 2.77-3.08 (qq, J=6Hz, 2H, CH_2-SO_2) δ_H 3.35 (overlapping, m, 1H, CH-CO) δ_H 3.78 (t, 2H, CH_2-O) δ_H 8.32-8.63 (m, 3H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 28.7; (CH_2) 36.4, 53.0, 60.2; (CH) 37.2, 122.5, 129.5, 133.9; (C) 82.1, 123.1, 136.9, 137.1, 148.5, 155.9, 169.8, 172.3; m/z 536 ($M+Na^+$), 1049 ($2M+Na^+$).

3.2.1.6 Deprotection of 2-(4-nitrophenylsulfonyl) ethyloxycarbonyl Asparagine *tert*-butyl ester (Nsc-Asn-OtBu)

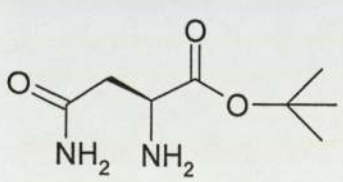
Method 1: Deprotected by piperidine

Nsc-Asn-OtBu (500 mg) was dissolved in DMF (30 ml). Piperidine (10 ml) was added to the solution. The solution was stirred for 26 hours. Then, deionized water (50 ml) was added followed by 10% HCl (10 ml). After separating the solid from the mixture, 10% NaOH solution was dropped into the solution until no precipitate separating out of the solution. The precipitate was collected and dried in vacuum overnight.

Method 2: Deprotected by diethylamine

Nsc-Asn-OtBu (500 mg) was dissolved in DMF (30 ml). Diethylamine (10 ml) was added to the solution. The solution was stirred for 41 hours. Then, deionized water (50 ml) was added followed by 10% HCl (10 ml). After separating the solid in the mixture, 10% NaOH solution was added dropwise into the solution until no more precipitate came out. The precipitate was collected and dried in vacuum overnight.

Product: H-Asn-OtBu



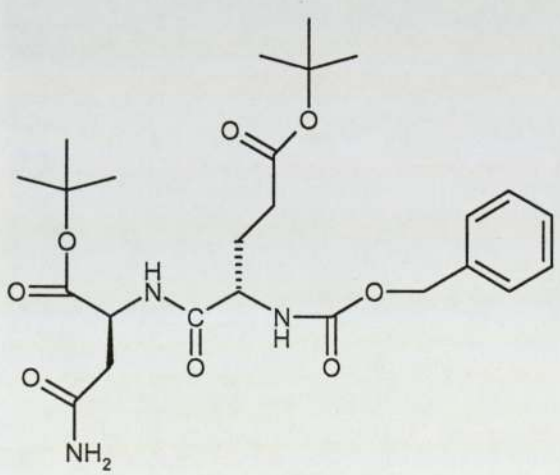
Mp 102.7-104.5 °C (Ref. 101-102°C Callahan, 1963); ^{1}H NMR (DMSO-d6): δ_H 1.41 (s, 9H, $(CH_3)_3$-C) δ_H 2.69-2.93 (overlapping, m, 2H, CH_2-CO) δ_H 3.73 (t, J=6Hz, 1H, CH-CO)

3.2.2 Methods for coupling reagents research

3.2.2.1 Hydrogenation of Z-Glu(OtBu)-Asn-OtBu

Palladium on carbon 5% (0.20 g) was added to a solution of Z-Glu(OtBu)-Asn-OtBu (2.00 g, 3.94 mmol) in MeOH (20 ml). The mixture was hydrogenated with vigorous stirring for one hour. Pd/C was removed by filtration. The filtrate was divided into two groups and evaporated for the next two tests.

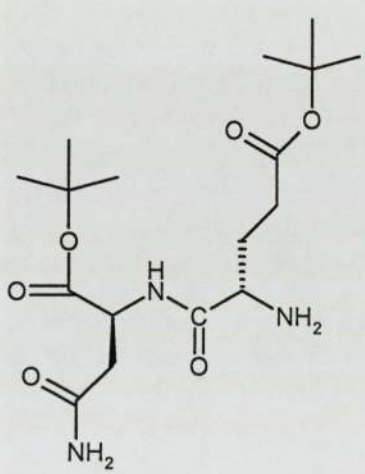
Reactant: Z-Glu(OtBu)-Asn-OtBu



Mp 150.6-152.8 °C; $[\alpha]_D^{20}$= -18.2° (c=2 g/100 ml in THF); IR: ν_{max} 3431 (ν^{as}_{NH}), 3309 ($\nu^{s}_{NH_2}$), 3218 (ν_{NH}), 2977 (ν_{CH_3}), 2923 (ν_{CH_2}), 1723 ($\nu_{C=O}$ Esters), 1673 ($\nu_{C=O}$ Amides),

1636 (β_{NH}), 1527 ($\nu_{C=C}$ Aromatic), 1364 (δ_{CH3}), 1250 (ν_{C-O-C}), 1168 (ν_{C-O-C}), 845 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.42-1.45 (ss, 18H, $2(CH_3)_3$-C) δ_H 1.95-2.14(m, 2H, CH_2-CO of Glu) δ_H 2.36 (m, 2H, CH_2-CH of Glu) δ_H 2.77-2.92 (m, 2H, CH_2-CO of Asn) δ_H 4.27 (t, H, CH-CO of Glu) δ_H 4.70 (m, H, CH-CO of Asn) δ_H 5.08 (s, 2H, CH_2-O of Glu) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 27.5, 27.7; (CH_2) 27.5, 31.3, 36.6, 65.4; (CH) 49.4, 53.6, 127.7, 127.8, 128.3; (C) 79.6, 80.5, 137.0, 155.9, 170.2, 171.1, 171.2, 171.7; m/z 530 ($M+Na^+$), 1037 ($2M+Na^+$).

Product: H-Glu(OtBu)-Asn-OtBu



Mp 125.4-127.6 °C; $[\alpha]_D^{20}$= -16.8° (c=2 g/100 ml in MeOH) IR: ν_{max} 3431 (ν^{as}_{NH}), 3309 (ν^{s}_{NH2}), 3218 (ν_{NH}), 2977 (ν_{CH3}), 2923 (ν_{CH2}), 1723 ($\nu_{C=O}$ Esters), 1673 ($\nu_{C=O}$ Amides), 1636 (β_{NH}), 1527 ($\nu_{C=C}$ Aromatic), 1364 (δ_{CH3}), 1250 (ν_{C-O-C}), 1168 (ν_{C-O-C}), 845 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.44-1.47 (ss, 18H, $2(CH_3)_3$-C) δ_H 1.85-2.13(dddd, J=7.5Hz, 2H, CH_2-CO of Glu) δ_H 2.40 (t, 2H, CH_2-CH of Glu) δ_H 2.82 (m, 2H, CH_2-CO of Asn) δ_H 3.61 (t, H, CH-CO of Glu) δ_H 4.70 (m, H, CH-CO of Asn) δ_H 5.97-6.51 (ss,

2H, NH_2-CO of Asn) δ_H 8.22 (d, 1H, NH-CO); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 27.5, 27.7; (CH_2) 27.5, 31.3, 36.6; (CH) 49.4, 53.6; (C) 79.6, 80.5, 155.9, 170.2, 171.1, 171.2, 171.7.

3.2.2.2 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu by BEPB/HOBt

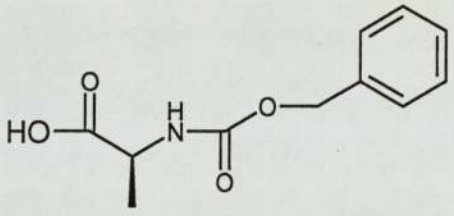
H-Glu(OtBu)-Asn-OtBu (from 1.00 g Z-Glu(OtBu)-Asn-OtBu, 1.97 mmol) and Z-Ala-OH (420 mmg, 1.88 mmol) were dissolved in acetonitrile (10 ml). HOBt (277 mg, 2.05 mmol) was added and the mixture was stirred at -10 °C. TEA (0.55 ml, 3.88 mmol) was added followed by 2-bromo-1-ethyl-pyridinium bromide (534 mg, 2.00 mmol). The reaction mixture was stirred for 5 minutes. To the resulting mixture was added water (20 ml). After extracted with CH_2Cl_2 (2×20 ml), the CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and dried over anhydrous $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with diethyl ether to give the product.

3.2.2.3 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu by DCC/HOBt

H-Glu(OtBu)-Asn-OtBu (from 1.00 g Z-Glu(OtBu)-Asn-OtBu, 1.97 mmol) and Z-Ala-OH (420 mmg, 1.88 mmol) were dissolved in acetonitrile (10 ml). HOBt (277 mg, 2.05 mmol) was added and the mixture was stirred at -10 °C. DCC (423 mg, 2.05 mmol) in acetonitrile (3 ml) was added slowly through a syringe over 2 minutes. After

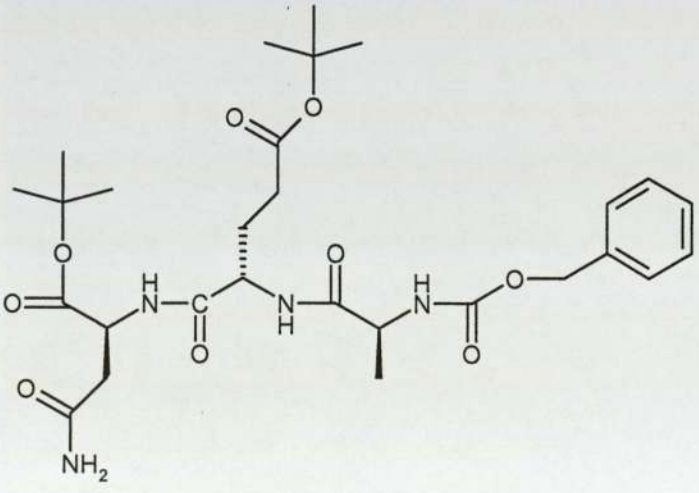
filtering out the precipitate, added water (20 ml) to the resulting mixture. After extracted with CH_2Cl_2 (2×20 ml), the CH_2Cl_2 layer was then washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and dried over $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with ether to give the product.

Reactant: Z-Ala-OH



Mp 84.6-86.2 °C (Merck, 2004/2005); $[\alpha]_D^{20}$=-14.2 ° (c=2 g/100 ml in CH_3COOH); ^1H NMR (DMSO-d6): δ_H 1.48 (d, J=6.2 Hz, 3H, $-CH_3$) δ_H 4.43 (q, J=4Hz, 1H, CH-CO) δ_H 5.65 (s, 2H, CH_2-O) δ_H 7.35 (s, 5H, Aromatic) δ_H 7.80 (d, 2H, NH_2)

Product: Z-Ala-Glu(OtBu)-Asn-OtBu



Mp 158.7-160.8 °C; $[\alpha]_D^{20}$= -34.2° (c=2 g/100 ml in THF); IR: ν_{max} 3404 (ν^{as}_{NH}), 3312 (ν^{s}_{NH2}), 2979 (ν_{CH3}), 2929 (ν_{CH2}), 1726 ($\nu_{C=O}$ Esters), 1661 ($\nu_{C=O}$ Amides), 1632 (β_{NH}), 1535 ($\nu_{C=C}$ Aromatic), 1366 (δ_{CH3}), 1259 (ν_{C-O-C}), 1159 (ν_{C-O-C}), 841 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.36-1.39 (ss, 3H, CH_3-C of Ala) 1.42-1.45 (ss, 18H, $2(CH_3)_3$-C) δ_H 1.97-2.11(m, 2H, CH_2-CO of Glu) δ_H 2.36 (m, 2H, CH_2-CH of Glu) δ_H 2.84 (m, 2H, CH_2-CO of Asn) δ_H 4.25 (m, H, CH-CO of Glu) δ_H 4.52 (m, H, CH-CO of Ala) δ_H 4.70 (m, H, CH-CO of Asn) δ_H 5.09 (s, 2H, CH_2-O of Z) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 18.1, 27.5, 27.7; (CH_2) 27.9, 31.1, 36.6, 65.4; (CH) 49.4, 50.0, 51.2, 127.7, 127.8, 128.4; (C) 79.7, 80.5, 137.0, 155.7, 170.3, 170.8, 171.1, 171.8, 172.4; m/z 601 ($M+Na^+$), 1179 ($2M+Na^+$).

3.2.3 Methods for coupling of synthesizing thymosin alpha-1 fragments

3.2.3.1 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu by BEPB/HOBt with TEA in acetonitrile

H-Glu(OtBu)-Asn-OtBu (from 1.00 g Z-Glu(OtBu)-Asn-OtBu, 1.97 mmol) and Z-Ala-OH (420 mmg, 1.88 mmol) were dissolved in acetonitrile (10 ml). HOBt (277 mg, 2.05 mmol) was added and the mixture was stirred at -10°C. TEA (0.55 ml, 3.88 mmol) was added followed by 2-bromo-1-ethyl-pyridinium bromide (534 mg, 2.00 mmol). The reaction mixture was stirred for 2 minutes. Then, water (20 ml) was added and the resulting mixture was extracted with CH_2Cl_2 (2×20 ml), the CH_2Cl_2 layer was

washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and dried over anhydrous $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with diethyl ether to give the product.

3.2.3.2 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu by BEPB/HOBt with DIPEA in acetonitrile

H-Glu(OtBu)-Asn-OtBu (from 2.00 g Z-Glu(OtBu)-Asn-OtBu, 3.94 mmol) and Z-Ala-OH (850 mg, 3.80 mmol) were dissolved in acetonitrile (15 ml). HOBt (554 mg, 4.10 mmol) was added and the mixture was stirred at -10°C. DIPEA (0.98 ml, 8.00 mmol) was added followed by 2-bromo-1-ethyl-pyridinium bromide (1.086 g, 4.00 mmol). The reaction mixture was stirred for 3 minutes. Then, water (20 ml) was added and the resulting mixture was extracted with CH_2Cl_2 (2×20 ml), the CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and dried over anhydrous $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with diethyl ether to give the product.

3.2.3.3 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu by BEPB/CF_3-HOBt with TEA in acetonitrile

H-Glu(OtBu)-Asn-OtBu (from 2.00 g Z-Glu(OtBu)-Asn-OtBu, 3.94 mmol) and Z-Ala-OH (850 mg, 3.80 mmol) were dissolved in acetonitrile (15 ml). CF_3-HOBt (833

mg, 4.10 mmol) was added and the mixture was stirred at -10°C. TEA (1.12 ml, 8.00 mmol) was added followed by 2-bromo-1-ethyl-pyridinium bromide (1.086 g, 4.00 mmol). The reaction mixture was stirred for 15 minutes. Then, water (20 ml) was added and the resulting mixture was extracted with CH_2Cl_2 (2×20 ml), the CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and dried over anhydrous $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with diethyl ether to give the product.

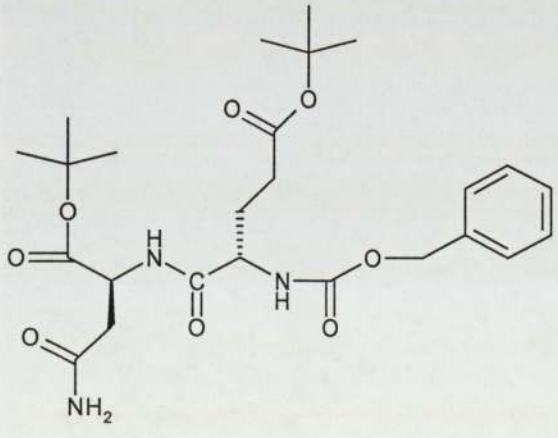
3.2.3.4 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu by BEPB/CF_3-HOBt with DIPEA in acetonitrile

H-Glu(OtBu)-Asn-OtBu (from 2.00 g Z-Glu(OtBu)-Asn-OtBu, 3.94 mmol) and Z-Ala-OH (850 mg, 3.80 mmol) were dissolved in acetonitrile (15 ml). CF_3-HOBt (833 mg, 4.10 mmol) was added and the mixture was stirred at -10°C. DIPEA (0.98 ml, 8.00 mmol) was added followed by 2-bromo-1-ethyl-pyridinium bromide (1.086 g, 4.00 mmol). The reaction mixture was stirred for 15 minutes. Then, water (20 ml) was added and the resulting mixture was extracted with CH_2Cl_2 (2×20 ml), the CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and dried over anhydrous $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with diethyl ether to give the product.

3.2.3.5 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu by BEPB/CF_3-HOBt with DIPEA in acetonitrile

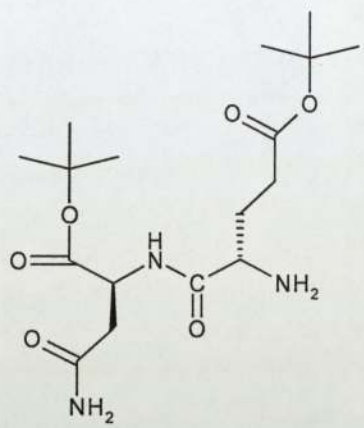
H-Glu(OtBu)-Asn-OtBu (from 2.00 g Z-Glu(OtBu)-Asn-OtBu, 3.94 mmol) and Z-Ala-OH (850 mg, 3.80 mmol) were dissolved in dichloromethane (10 ml). HOBt (554 mg, 4.10 mmol) was added and the mixture was stirred at -10°C. DIPEA (1.12 ml, 8.00 mmol) was added followed by 2-bromo-1-ethyl-pyridinium bromide (1.086 g, 4.00 mmol). The reaction mixture was stirred for 5 minutes. Then, water (20 ml) was added and the resulting mixture was extracted with CH_2Cl_2 (2×20 ml), the CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and dried over anhydrous $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with diethyl ether to give the product.

Reactant: Z-Glu(OtBu)-Asn-OtBu



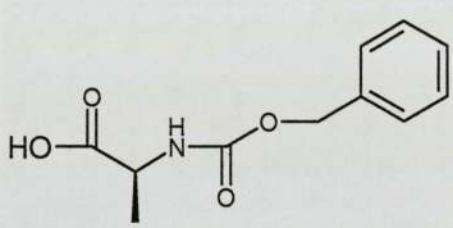
See Section 3.2.2.1

Product: H-Glu(OtBu)-Asn-OtBu



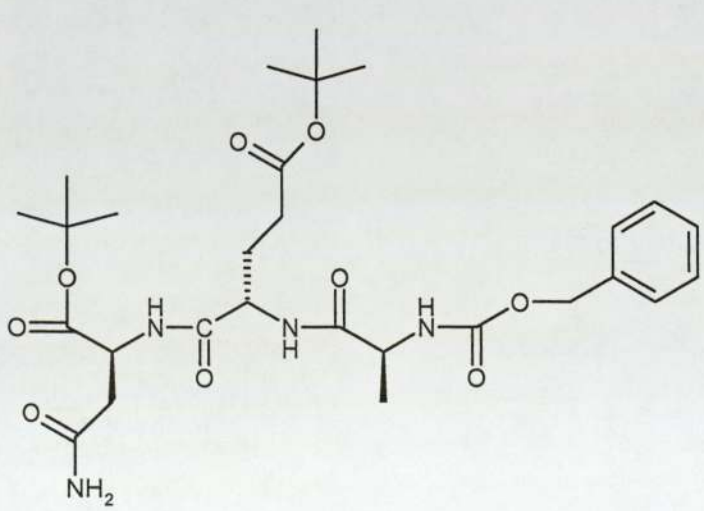
See Section 3.2.2.1

Reactant: Z-Ala-OH



See Section 3.2.2.2

Product: Z-Ala-Glu(OtBu)-Asn-OtBu



Mp 158.7-160.8°C; $[\alpha]_D^{20}$= -34.00 °, -34.25 °, -35.15 °, -36.50 °, -33.60 °, -35.20 ° respectively (c=2 g/100 ml in THF); IR: ν_{max} 3404 (ν^{as}_{NH}), 3312 (ν^{s}_{NH2}), 2979 (ν_{CH3}), 2929 (ν_{CH2}), 1726 ($\nu_{C=O}$ Esters), 1661 ($\nu_{C=O}$ Amides), 1632 (β_{NH}), 1535 ($\nu_{C=C}$ Aromatic), 1366 (δ_{CH3}), 1259 (ν_{C-O-C}), 1159 (ν_{C-O-C}), 841 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.36-1.39 (ss, 3H, CH_3-C of Ala) 1.42-1.45 (ss, 18H, $2(CH_3)_3$-C) δ_H 1.97-2.11(m, 2H, CH_2-CO of Glu) δ_H 2.36 (m, 2H, CH_2-CH of Glu) δ_H 2.84 (m, 2H, CH_2-CO of Asn) δ_H 4.25 (m, H, CH-CO of Glu) δ_H 4.52 (m, H, CH-CO of Ala) δ_H 4.70 (m, H, CH-CO of Asn) δ_H 5.09 (s, 2H, CH_2-O of Z) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 18.1, 27.5, 27.7; (CH_2) 27.9, 31.1, 36.6, 65.4; (CH) 49.4, 50.0, 51.2, 127.7, 127.8, 128.4; (C) 79.7, 80.5, 137.0, 155.7, 170.3, 170.8, 171.1, 171.8, 172.4; m/z 601 ($M+Na^+$), 1179 ($2M+Na^+$).

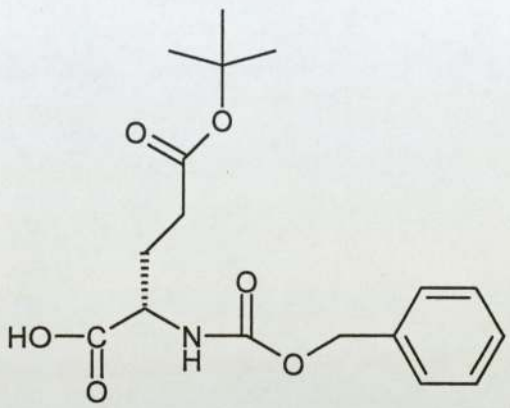
3.2.4 Methods for synthesis of thymosin alpha-1 fragments of the 25-28 tetrapeptide

Route 1:

3.2.4.1 Preparation of Z-Glu(OtBu)-OH

Z-Glu(OtBu)-OH was prepared by Dr. Kejun Zhao and was used directly for the my experiment.

Product: Z-Glu(OtBu)-OH



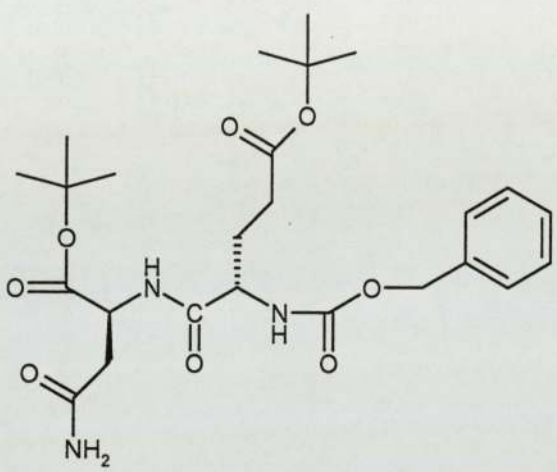
Mp 163-165°C (Merck, 2004/2005); $[\alpha]_D^{20}$= -13.5° (c=2 g/100 ml in MeOH); ^{1}H NMR ($CDCl_3$): δ_H 1.41 (s, 9H, $(CH_3)_3$-C) δ_H 1.93-2.22(m, 2H, CH_2-CO of Glu) δ_H 2.36 (m, 2H, CH_2-CH of Glu) δ_H 4.37 (m, 1H, CH-CO) δ_H 5.10 (s, 2H, CH_2-O) δ_H 7.33 (s, 5H, Aromatic)

3.2.4.2 Preparation of Z-Glu(OtBu)-Asn-OtBu

H-Asn-OtBu (19.76 g, 105 mmol) and Z-Glu(OtBu)-OH (33.74 g, 100 mmol) were dissolved in acetonitrile (400 ml). HOBt (27.68 g, 205 mmol) was added and the mixture was stirred at -10°C. TEA (55 ml, 388 mmol) was added followed by 2-Bromo-1-ethyl-pyridinium bromide (53.40 g, 200 mmol). The reaction mixture was stirred for 5 minutes. Then, water (400 ml) was added and the resulting mixture was extracted with CH_2Cl_2 (2×300 ml), the CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (300 ml), 5% HCl (300 ml) and dried over $MgSO_4$. The solution was evaporated to give a white solid. The solid was washed with ether to give the crude

product. The crude product was purified by silica column with DCM-Acetone (5/1) as elution solvent.

Product: Z-Glu(OtBu)-Asn-OtBu

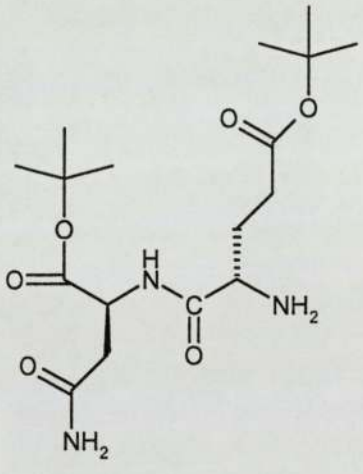


Mp 150.6-152.8°C; $[\alpha]_D^{20}$= -18.2° (c=2 g/100 ml in THF); IR: ν_{max} 3431 (ν^{as}_{NH}), 3309 (ν^{s}_{NH2}), 3218 (ν_{NH}), 2977 (ν_{CH3}), 2923 (ν_{CH2}), 1723 ($\nu_{C=O}$ Esters), 1673 ($\nu_{C=O}$ Amides), 1636 (β_{NH}), 1527 ($\nu_{C=C}$ Aromatic), 1364 (δ_{CH3}), 1250 (ν_{C-O-C}), 1168 (ν_{C-O-C}), 845 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.42-1.45 (ss, 18H, $2(CH_3)_3$-C) δ_H 1.95-2.14(m, 2H, CH_2-CO of Glu) δ_H 2.36 (m, 2H, CH_2-CH of Glu) δ_H 2.77-2.92 (m, 2H, CH_2-CO of Asn) δ_H 4.27 (t, H, CH-CO of Glu) δ_H 4.70 (m, H, CH-CO of Asn) δ_H 5.08 (s, 2H, CH_2-O of Glu) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 27.5, 27.7; (CH_2) 27.5, 31.3, 36.6, 65.4; (CH) 49.4, 53.6, 127.7, 127.8, 128.3; (C) 79.6, 80.5, 137.0, 155.9, 170.2, 171.1, 171.2, 171.7; m/z 530 ($M+Na^+$), 1037 ($2M+Na^+$).

3.2.4.3 Preparation of Z-Ala-Glu(OtBu)-Asn-OtBu

The Z-Glu(OtBu)-Asn-OtBu (25.380 g, 50 mmol) was hydrogenated in the present of Palladium 5% on carbon (2.5 g) to give the product H-Glu(OtBu)-Asn-OtBu for the preparation of Z-Ala-Glu(OtBu)-Asn-OtBu.

Product: H-Glu(OtBu)-Asn-OtBu

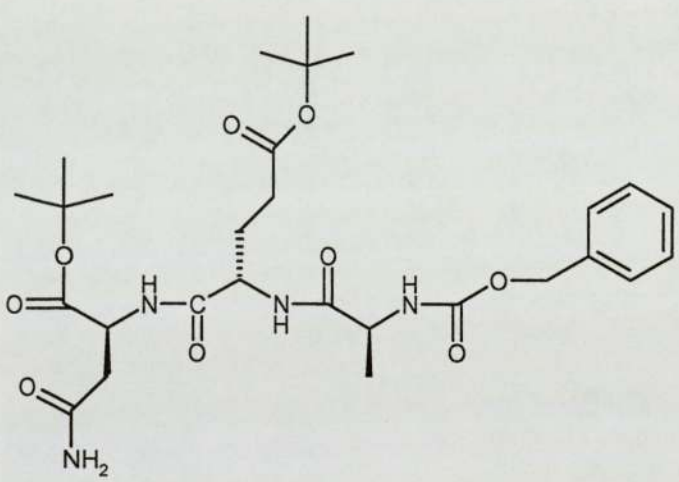


See 3.2.2.1.

H-Glu(OtBu)-Asn-OtBu (from 25.380 g Z-Glu(OtBu)-Asn-OtBu, 50 mmol) and Z-Ala-OH (12.287 g, 55 mmol) were dissolved in acetonitrile (300 ml). HOBt (6.75 g, 50 mmol) was added and the mixture was stirred at -10°C. TEA (14.2 ml, 100 mmol) was added followed by 2-Bromo-1-ethyl-pyridinium bromide (13.35 g, 50 mmol). The reaction mixture was stirred for 10 minutes. Then, water (400 ml) was added and the resulting mixture was evaporated under reduced pressure. The precipitate was separated

by filtration and was washed with 10% HCl (50 ml), saturated NaHCO3 (60 ml) with 10% Na_2CO_3 (10/1 v/v) and deionizing water (50 ml) to give the crude product. The crude product was purified by silica column with DCM-Acetone (3/1) as elution solvent.

Product: Z-Ala-Glu(OtBu)-Asn-OtBu



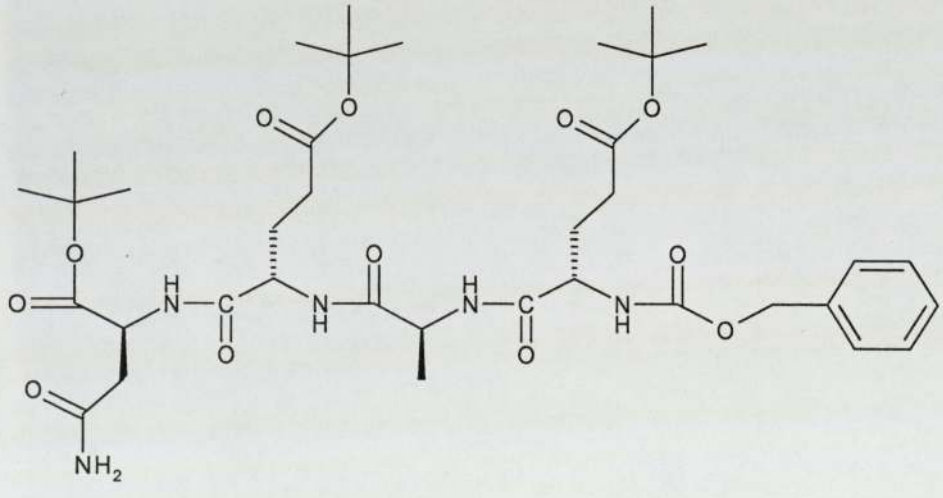
See section 3.2.2.3

3.2.4.4 Preparation of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

The Z-Ala-Glu(OtBu)-Asn-OtBu (5.787 g, 10 mmol) was hydrogenated in the present of Palladium 5% on carbon (0.58 g) to give the product H-Ala-Glu(OtBu)-Asn-OtBu for the preparation of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu.

H-Ala-Glu(OtBu)-Asn-OtBu (from 5.787 g Z-Ala-Glu(OtBu)-Asn-OtBu, 10 mmol) and Z-Glu(OtBu)-OH (3.711 g, 11 mmol) were dissolved in acetonitrile (60 ml). HOBt (1.351 g, 10 mmol) was added and the mixture was stirred at -10°C. TEA (3.09 ml, 22 mmol) was added followed by 2-Bromo-1-ethyl-pyridinium bromide (2.670 g, 10 mmol). The reaction mixture was stirred for 15 minutes. Then, water (100 ml) was added and the resulting mixture was evaporated under reduced pressure. The precipitate was separated by filtration and was washed with 10% HCl (50 ml), saturated NaHCO3 (60 ml) with 10% Na_2CO_3 (10/1 v/v) and deionizing water (50 ml) to give the crude product. The crude product was purified by silica column chromatography with DCM-Acetone (3/1) as elution solvent.

Product: Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu



Mp 205.1-207.3°C; $[\alpha]_D^{20}$= -7.7° (c=1 g/100 ml in THF); IR: ν_{max} 3428 (ν^{as}_{NH}), 3315 (ν^{s}_{NH2}), 3205 (ν_{NH}), 2972 (ν_{CH3}), 2925 (ν_{CH2}), 1726 ($\nu_{C=O}$ Esters), 1677 ($\nu_{C=O}$ Amides), 1526 ($\nu_{C=C}$ Aromatic), 1365 (δ_{CH3}), 1255 (ν_{C-O-C}), 1161 (ν_{C-O-C}), 847 ($\gamma_{C=H}$ Aromatic)

cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.41 (s, 30H, $10CH_3$) 2.04 (s, 4H, $2CH_2$-C of Glu) δ_H 2.35(s, 4H, $2CH_2$-CO of Glu) δ_H 2.84 (m, 2H, CH_2-CO of Asn) δ_H 4.45-4.69 (ss, 4H, 4CH-CO) δ_H 5.09 (s, 2H, CH_2-O of Z) δ_H 7.31 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 18.1, 27.5, 27.7, 27.9; (CH_2) 27.9, 28.1, 31.1, 31.3, 36.6, 65.4; (CH) 49.4, 50.0, 51.2, 51.5, 127.7, 127.8, 128.4; (C) 79.7, 80.5, 80.7, 137.0, 155.7, 170.3, 170.8, 171.1, 171.3, 171.8, 172.4, 172.6; m/z 786 ($M+Na^+$), 1549 ($2M+Na^+$).

Route 2:

3.2.4.5 Preparation of H-Glu(OtBu)-Asn-OtBu

See 3.2.2.1.

3.2.4.6 Preparation of Z-Glu(OtBu)-Ala-OH

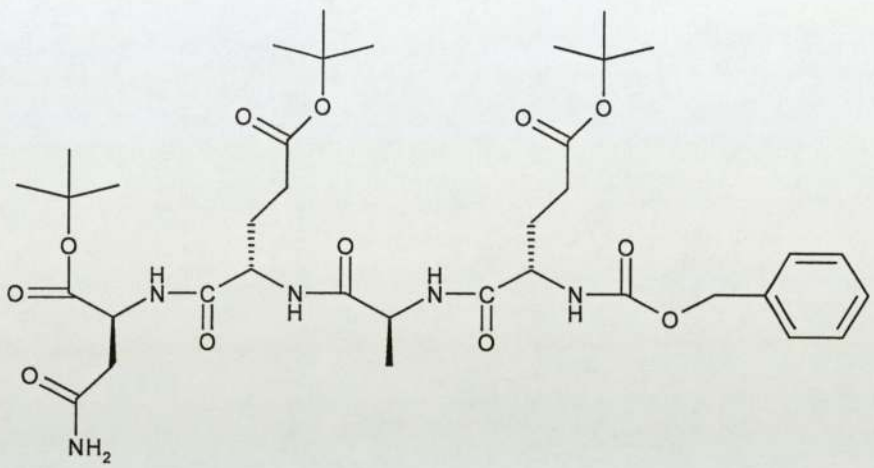
A solution of Z-Glu(OtBu)-OH (26.99 g, 80 mmol) and N-hydroxysuccinimide (10.13 g, 88 mmol) in anhydrous THF (300 ml) was cooled in an ice-water bath and DCC (18.16 g, 88 mmol) in THF(100 ml) was added in five portions with stirring. The mixture was kept in fridge for 48 hours. After filtering out the precipitate, the filtrate was poured into a solution of L-Alanine sodium salt which was prepared by adding sodium hydroxide (3.2 g, 80 mmol) dropwise to L-Alanine (7.127 g, 80 mmol) at 0 °C. The mixture was stirred for 8h and washed with diethyl ether (50 ml). The aqua layer was then acidified by 10% HCl (50 ml) and the mixture was extracted with DCM (2×100 ml). The DCM

layer was evaporated to give the crude product which was purified by silica column with DCM-Acetone (5/1) as elution solvent.

3.2.4.7 Preparation of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

H-Glu(OtBu)-Asn-OtBu (4.29 g, 11.5 mmol) and Z-Glu(OtBu)-Ala-OH (4.70 g, 11.5 mmol) were dissolved in acetonitrile (50 ml). HOBt (1.71 g, 12.65 mmol) was added and the mixture was stirred at -10°C. TEA (3.59 ml, 25.3 mmol) was added followed by 2-Bromo-1-ethyl-pyridinium bromide (3.38 g, 12.65 mmol). The reaction mixture was stirred for 5 minutes. Then, water (50 ml) was added and the resulting mixture was evaporated under reduced pressure. The precipitate was separated by filtration and was washed with saturated $NaHCO_3$ (20 ml), 5% HCl(20 ml) and deionizing water(20 ml) to give the crude product. The crude product was purified by silica column with DCM-Acetone (5/1) as elution solvent.

Product: Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu



Mp 205.1-207.3°C; $[\alpha]_D^{20}$= -7.7° (c=1 g/100 ml in THF); IR: ν_{max} 3428 (ν^{as}_{NH}), 3315 (ν^{s}_{NH2}), 3205 (ν_{NH}), 2972 (ν_{CH3}), 2925 (ν_{CH2}), 1726 ($\nu_{C=O}$ Esters), 1677 ($\nu_{C=O}$ Amides), 1526 ($\nu_{C=C}$ Aromatic), 1365 (δ_{CH3}), 1255 (ν_{C-O-C}), 1161 (ν_{C-O-C}), 847 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.41 (s, 30H, $10CH_3$) 2.04 (s, 4H, $2CH_2$-C of Glu) δ_H 2.35(s, 4H, $2CH_2$-CO of Glu) δ_H 2.84 (m, 2H, CH_2-CO of Asn) δ_H 4.45-4.69 (ss, 4H, 4CH-CO) δ_H 5.09 (s, 2H, CH_2-O of Z) δ_H 7.31 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 18.1, 27.5, 27.7, 27.9; (CH_2) 27.9, 28.1, 31.1, 31.3, 36.6, 65.4; (CH) 49.4, 50.0, 51.2, 51.5, 127.7, 127.8, 128.4; (C) 79.7, 80.5, 80.7, 137.0, 155.7, 170.3, 170.8, 171.1, 171.3, 171.8, 172.4, 172.6; m/z 786 ($M+Na^+$), 1549 ($2M+Na^+$).

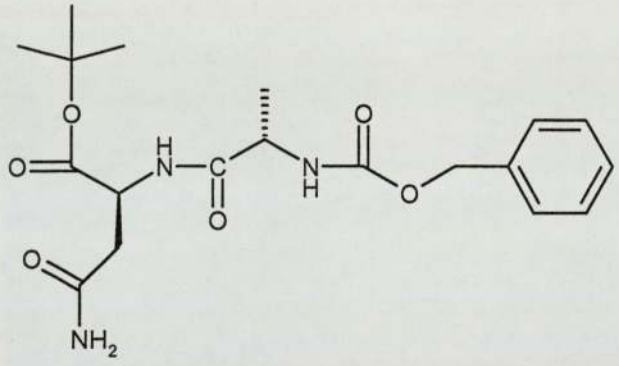
3.2.5 Methods for new peptide designed from thymosin alpha-1 fragments

Method 1: Alanine substitute:

3.2.5.1 Preparation of Z-Glu(OtBu)-Ala-Ala-Asn-OtBu

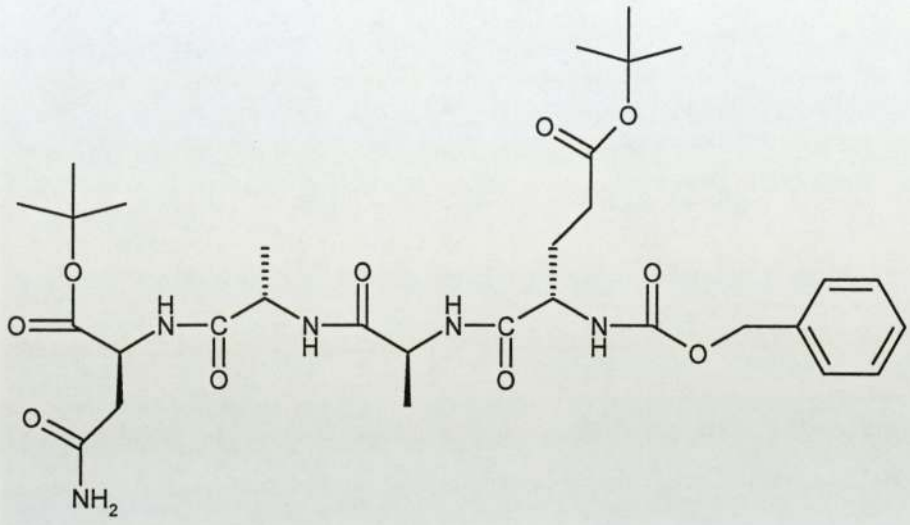
Preparation of H-Ala-Asn-OtBu was similar to preparation of H-Glu(OtBu)-Asn-OtBu which was mentioned in section 3.2.3.2 and 3.2.2.1.

Product: Z-Ala-Asn-OtBu



Mp 150.7-152.8 °C; $[\alpha]_D^{20}$= -16.3 ° (c=2.0 g/100 ml in MeOH); IR: ν_{max} 3427 (ν^{as}_{NH}), 3309 (ν^{s}_{NH2}), 2976 (ν_{CH3}), 1733 ($\nu_{C=O}$ Esters), 1655 (β_{NH}), 1545 ($\nu_{C=C}$ Aromatic), 1365 (δ_{CH3}), 1239 (ν_{C-O-C}), 1161 (ν_{C-O-C}), 847 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.39 (ss, 3H, CH_3-CH of Ala) δ_H 1.45 (s, 9H, $(CH_3)_3$-C) δ_H 2.72-2.92(m, 2H, CH_2-CO of Asn) δ_H 4.31 (s, H, CH-CO of Asn) δ_H 4.27 (m, H, CH-CO of Ala) δ_H 5.09 (s, 2H, CH_2-O of Ala) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 18.9, 27.9; (CH_2) 37.3, 66.9; (CH) 49.6, 50.5, 128.0, 128.1, 128.5; (C) 82.4, 136.3, 156.0, 170.0, 172.6, 172.8; m/z 416 ($M+Na^+$), 809 ($2M+Na^+$).

Product: Z-Glu(OtBu)-Ala-Ala-Asn-OtBu

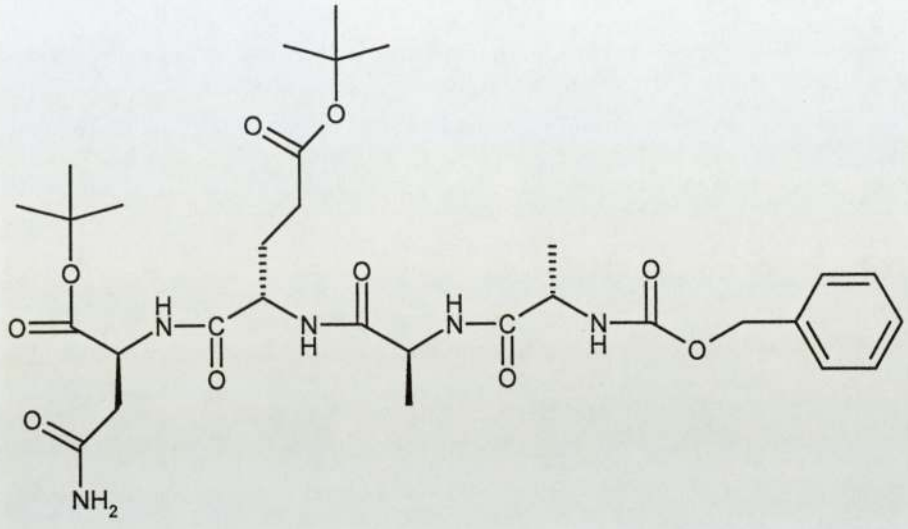


Mp 115.5-117.2 °C; $[\alpha]_D^{20}$= -13.2° (c=1.0 g/100 ml in MeOH); IR: ν_{max} 3305 (ν^s_{NH2}), 2973 (ν_{CH3}), 2928 (ν_{CH2}), 1725 ($\nu_{C=O}$ Esters), 1661 ($\nu_{C=O}$ Amides), 1522 ($\nu_{C=C}$ Aromatic), 1363 (δ_{CH3}), 1261 (ν_{C-O-C}), 1152 (ν_{C-O-C}), 850 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.39 (ss, 3H, CH_3-CH of Ala) δ_H 1.45 (s, 9H, $(CH_3)_3$-C) δ_H 2.72-2.92(m, 2H, CH_2-CO of Asn) δ_H 4.31 (s, H, CH-CO of Asn) δ_H 4.27 (m, H, CH-CO of Ala) δ_H 5.09 (s, 2H, CH_2-O of Ala) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 18.1, 18.3, 27.7, 27.9; (CH_2) 27.9, 31.3, 36.5, 66.8; (CH) 49.4, 50.1, 50.3, 53.5, 127.1, 127.6, 128.9; (C) 82.1, 82.3, 136.1, 155.9, 169.8, 171.1, 171.3, 172.4, 172.6, 173.2; m/z 672 ($M+Na^+$), 1321 ($2M+Na^+$).

3.2.5.2 Preparation of Z-Ala-Ala-Glu(OtBu)-Asn-OtBu

Z-Ala-Ala-Glu(OtBu)-Asn-OtBu was prepared following the similar operation as section 3.2.4 described.

Product: Z-Ala-Ala-Glu(OtBu)-Asn-OtBu



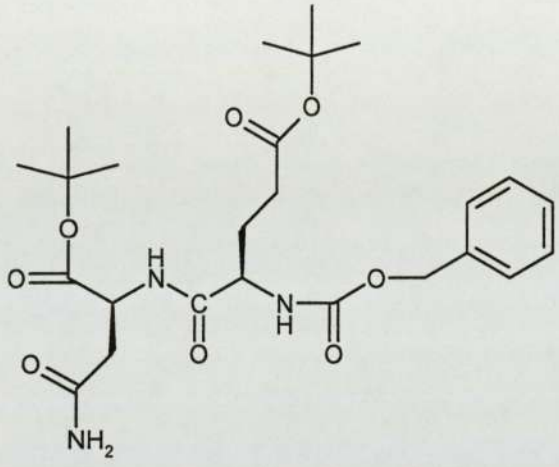
Mp 188.5-190.3 °C; $[\alpha]_D^{20}$= -18.6° (c=1.0 g/100 ml in MeOH); IR: ν_{max} 3305 (ν^{s}_{NH2}), 2973 (ν_{CH3}), 2928 (ν_{CH2}), 1725 ($\nu_{C=O}$ Esters), 1661 ($\nu_{C=O}$ Amides), 1522 ($\nu_{C=C}$ Aromatic), 1363 (δ_{CH3}), 1261 (ν_{C-O-C}), 1152 (ν_{C-O-C}), 850 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.39 (ss, 3H, CH_3-CH of Ala) δ_H 1.45 (s, 9H, $(CH_3)_3$-C) δ_H 2.72-2.92(m, 2H, CH_2-CO of Asn) δ_H 4.31 (s, H, CH-CO of Asn) δ_H 4.27 (m, H, CH-CO of Ala) δ_H 5.09 (s, 2H, CH_2-O of Ala) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 17.8, 17.9, 28.7, 28.9; (CH_2) 27.9, 30.7, 36.5, 66.8; (CH) 49.8, 50.4, 50.6, 53.9, 127.4, 127.7, 129.0; (C) 82.4, 82.6, 136.4, 156.2, 169.9, 171.3, 171.5, 172.6, 172.8, 173.4; m/z 672 ($M+Na^+$), 1321 ($2M+Na^+$).

Method 2: D-amino acid substitute:

3.2.5.3 Preparation of designed dipeptide Z-D-Glu(OtBu)-Asn-OtBu

Preparation of Z-D-Glu(OtBu)-Asn-OtBu was similar to preparation of Z-Glu(OtBu)-Asn-OtBu which was mentioned in section 3.2.4.2.

Product: Z-D-Glu(OtBu)-Asn-OtBu

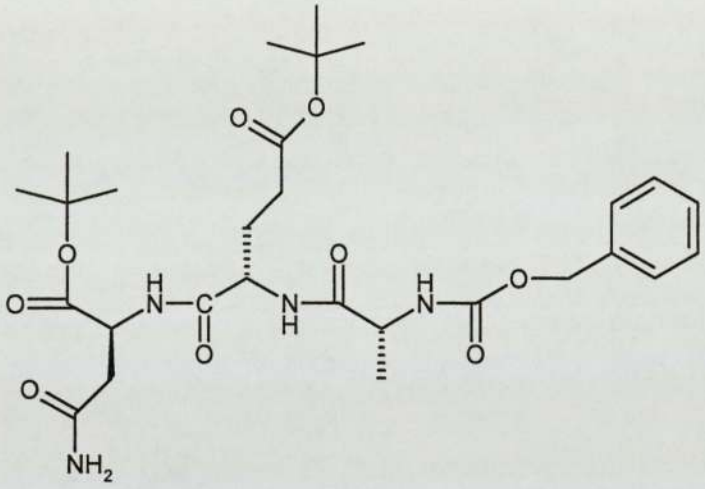


Mp 150.6-152.8°C; $[\alpha]_D^{20}$= +13.1° (c=2.0 g/100 ml in MeOH); IR: ν_{max} 3415 (ν^{as}_{NH}), 3335 (ν^{s}_{NH2}), 2981 (ν_{CH3}), 2927 (ν_{CH2}), 1721 ($\nu_{C=O}$ Esters), 1668 ($\nu_{C=O}$ Amides), 1522 ($\nu_{C=C}$ Aromatic), 1363 (δ_{CH3}), 1252 (ν_{C-O-C}), 1154 (ν_{C-O-C}), 843 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.42-1.45 (ss, 18H, $2(CH_3)_3$-C) δ_H 1.95-2.14(m, 2H, CH_2-CO of Glu) δ_H 2.36 (m, 2H, CH_2-CH of Glu) δ_H 2.77-2.92 (m, 2H, CH_2-CO of Asn) δ_H 4.27 (t, H, CH-CO of Glu) δ_H 4.70 (m, H, CH-CO of Asn) δ_H 5.08 (s, 2H, CH_2-O of Glu) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 27.5, 27.7; (CH_2) 27.4, 31.3, 36.6, 65.4; (CH) 49.5, 53.8, 127.6, 127.8, 128.3; (C) 79.7, 80.5, 137.0, 155.9, 170.1, 171.1, 171.3, 171.6; m/z 530 ($M+Na^+$), 1037 ($2M+Na^+$).

3.2.5.4 Preparation of designed dipeptide Z-D-Ala-Glu(OtBu)-Asn-OtBu

Preparation of Z-D-Ala-Glu(OtBu)-Asn-OtBu was similar to preparation of Z-Ala-Glu(OtBu)-Asn-OtBu which was mentioned in section 3.2.2.2.

Product: Z-D-Ala-Glu(OtBu)-Asn-OtBu



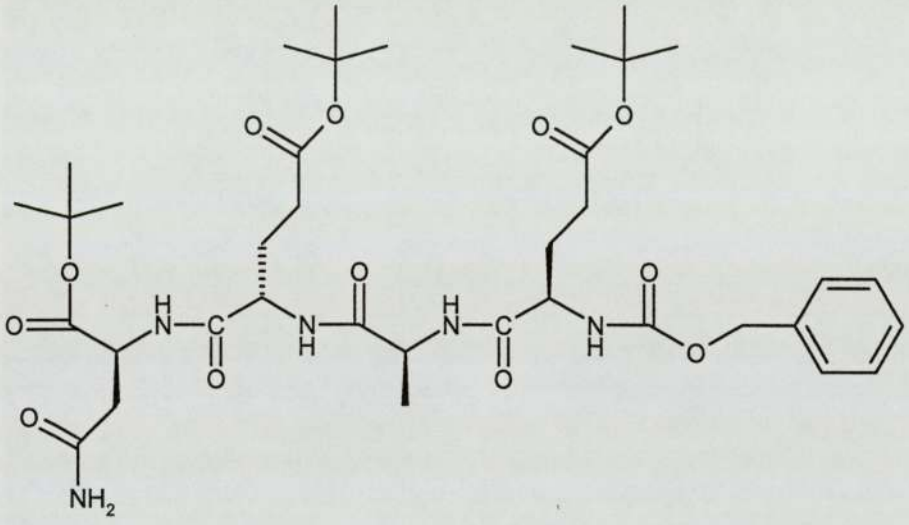
Mp 158.7-160.8°C; $[\alpha]_D^{20}$= +23.4° (c=2.0 g/100 ml in THF); IR: ν_{max} 3404 (ν^{as}_{NH}), 3312 (ν^{s}_{NH2}), 2979 (ν_{CH3}), 2929 (ν_{CH2}), 1726 ($\nu_{C=O}$ Esters), 1661 ($\nu_{C=O}$ Amides), 1632 (β_{NH}), 1535 ($\nu_{C=C}$ Aromatic), 1366 (δ_{CH3}), 1259 (ν_{C-O-C}), 1159 (ν_{C-O-C}), 841 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.36-1.39 (ss, 3H, CH_3-C of Ala) 1.42-1.45 (ss, 18H, 2$(CH_3)_3$-C) δ_H 1.97-2.11(m, 2H, CH_2-CO of Glu) δ_H 2.36 (m, 2H, CH_2-CH of Glu) δ_H 2.84 (m, 2H, CH_2-CO of Asn) δ_H 4.25 (m, H, CH-CO of Glu) δ_H 4.52 (m, H, CH-CO of Ala) δ_H 4.70 (m, H, CH-CO of Asn) δ_H 5.09 (s, 2H, CH_2-O of Z) δ_H 7.33 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 17.9, 27.5, 27.7; (CH_2) 27.9, 31.1, 36.6, 65.4; (CH) 49.4, 50.2, 51.2, 127.7, 127.8, 128.4; (C) 79.7, 80.5, 137.0, 155.7, 170.3, 170.8, 171.1, 171.8, 172.4; m/z 601 ($M+Na^+$), 1179 ($2M+Na^+$).

3.2.5.5 Preparation of designed dipeptide

Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu

Preparation of Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu was similar to preparation of Z-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu which was mentioned in section 3.2.4.4.

Product: Z-D-Glu(OtBu)-Ala-Glu(OtBu)-Asn-OtBu



Mp 202.2-203.9°C; $[\alpha]_D^{20}$= +14.9° (c=1.0 g/100 ml in MeOH); IR: ν_{max} 3428 (ν^{as}_{NH}), 3315 (ν^{s}_{NH2}), 3205 (ν_{NH}), 2972 (ν_{CH3}), 2925 (ν_{CH2}), 1726 ($\nu_{C=O}$ Esters), 1677 ($\nu_{C=O}$ Amides), 1526 ($\nu_{C=C}$ Aromatic), 1365 (δ_{CH3}), 1255 (ν_{C-O-C}), 1161 (ν_{C-O-C}), 847 ($\gamma_{C=H}$ Aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.41 (s, 30H, $10CH_3$) 2.04 (s, 4H, $2CH_2$-C of Glu) δ_H 2.35(s, 4H, $2CH_2$-CO of Glu) δ_H 2.84 (m, 2H, CH_2-CO of Asn) δ_H 4.45-4.69 (ss, 4H, 4CH-CO) δ_H 5.09 (s, 2H, CH_2-O of Z) δ_H 7.31 (s, 5H, Aromatic); ^{13}C NMR APT (DMSO-d_6): δ_C (CH_3) 18.1, 27.5, 27.7, 27.9; (CH_2) 27.7, 28.1, 31.1, 31.3, 36.6, 65.4;

(CH) 49.4, 50.0, 51.2, 51.7, 127.7, 127.8, 128.4; (C) 79.7, 80.5, 80.7, 137.0, 155.7, 170.3, 170.8, 171.1, 171.3, 171.8, 172.4, 172.6; m/z 786 ($M+Na^+$), 1549 ($2M+Na^+$).

References

Akaji, K., Kuriyama, N. and Kiso, Y. (1994) *Tetrahedron Lett.* **35**, 3315.

Albericio, F., Bofill, J. M., El-Faham, A. and Kates, S. A. (1998) *J. Org. Chem.* **63**, 9678.

Anderson, G. W., Zimmerman J. E. and Callahan F. M. (1963) *J. Amer. Chem. Soc.* **85**, 3039.

Anonymous. (2005) http://www.drugresearcher.com/news/ng.asp?n=57290-peptide-market-finally

Armstrong, A., Brackenridge, I., Richard, F., Jackson, W. and Kirk, J. M. (1988) *Tetrahedron Lett.* **29**, 2483.

Bald, E., Saigo, K. and Mukaiyama, T. (1975) *Chem. Lett.*, 1163.

Bergmann, M. and Zervas, L. (1932) *Ber. Dtsch. Chem. Ges.* **65**, 1192.

Bodanszky, M. (1955) *Nature* **175**, 685.

Business. Watching Peptide Drugs Grow Up (2005) *Chem. & Eng. News* March **83**, 17.

Cahn R. S., Ingold C. K., and Prelog V. (1966) *Angew. Chem. Int. Ed. Engl.* **5**, 385.

Callahan, F.M. et al. (1963) *J. Am. Chem. Soc.* **85**, 201.

Carpino L. A. (1993) *J. Am. Chem. Soc.* **115,** 4397.

Carpino, L. A. and El-Faham, A. (1995) *J. Am. Chem. Soc.* **117**, 5401.

Carpino L. A. and El-Faham A. (1995) *J. Org. Chem.* **60,** 3561.

Carpino L. A. and Ferrer F.J. (2001) *Org. Lett.* **3**, 2793.

Carpino, L. A. and Han, G. Y. (1970) *J. Am. Chem. Soc.* **92**, 5748.

Carpino, L. A. and Han, G. Y. (1972) *J. Org. Chem.* **37**, 22, 3404.

Carpino L. A., Ionescu D., El-Faham A., Beyermann M., Henklein P., Hanay C., Wenschuh H. and Bienert M. (2003) *Org. Lett.* **5,** 975.

Castro, B., Dromoy, J. R., Evin, G. and Selve, C. (1975) *Tetrahedron Lett.*, 1219.

Christian, A. G., Montalbetti, N. and Falque V. (2005) *Tetrahedron.* **46**, 10827.

Coste, J., Dufour, M.-N., Pantaloni, A. and Castro, B. (1990) *Tetrahedron Lett.* **31**, 669.

Coste, J., Frerot, E. and Jouin, P. (1991) *Tetrahedron Lett.* **32**, 1967.

Coste, J., Le-Nguyen, D. and Castro, B. (1990) *Tetrahedron Lett.* **31,** 205.

Doonan, S. (2002) *Peptides and Proteins.* The Royal Society of Chemistry, Cambridge, p. 4.

Dourtoglou, V., Ziegler, J. C. and Gross, B. (1978) *Tetrahedron Lett.* **15**, 1269.

Dourtoglou, V., Ziegler, J. C. and Gross, B. (1984) *Synthesis* 572.

El-Faham, A. (1998) *Chem. Lett.*, 671.

Falb, E., Yechezkel, T., Salitra, Y. and Gilon, C. (1999) *J. Pept. Res.* **53,** 507.

Frerot, E., Coste, J., Pantaloni, A., Dufour, M.-H. and Jouin, P. (1991) *Tetrahedron* **47,** 259.

Gibson F. S. and Rapoport H. (1995) *J. Org. Chem.* **60**, 2615.

Nishiyama Y., Tanaka M., Saito S., Ishizuka S., Mori T. and Kurita K. (1999) *Chem. Pharm. Bull.* **47,** 576.

Nishiyama Y., Ishizuka S. and Kurita K. (2001) *Tetrahedron Lett.* **42,** 8789.

Han, S. Y. and Kim Y. A. (2004) *Tetrahedron* **60,** 2447.

Hardy, P. M., Rydon, H. N. and Thompson, R. C. (1968) *Tetrahedron Lett.*, 2525.

Hoeg-Jensen, T., Jakobsen, M. H. and Holm, A. (1991) *Tetrahedron Lett.* **32,** 6387.

Humphrey, J. M. and Chamberlin, A. R. (1997) *Chem. Rev.* **97**, 2243.

Izdebski, J. and Kunce, D. J. (1997) *Pept. Sci.* **3,** 141.

Jakubke, H.-D. and Jeschkeit, H. (1977) *Amino acids, peptide and proteins.* Macmillan London, p. 37.

Jiang L., Davision A., Tennant G. and Ramage R. (1998) *Tetrahedron* **54**, 14233.

Jones J. H., Ramage W. I. and Witty M. (1980) *J. Int. J. Pept. Prot. Res.* **15,** 301.

Kaminski, Z. J. (1987) *Synthesis* 917.

Kato, S., Morie, T. and Yoshida, N. (1995) *Chem. Pharm. Bull.* **43,** 699.

Knut, A. (2005) *Current Protein and Peptide Science* **3**, 205.

König, W. and Geiger R. (1970) *Chem. Ber.* **103,** 788.

Li, P. and Xu, J. C. (1999) *Tetrahedron Lett.* **40**, 8301.

Li, P. and Xu, J. C. (1999) *Chem. Lett.* 1163.

Li, P. and Xu, J. C. (2000) *J. Pept. Res.* **55**, 110.

Li, P. and Xu, J. C. (1999) *Tetrahedron Lett.* **40**, 3605.

Li, P. and Xu, J. C. (2000) *Tetrahedron Lett.* **41,** 721.

Li, P. and Xu, J. C. (2000) *Tetrahedron.* **56**, 8119.

Li, P. and Xu, J. C. (2000) *Tetrahedron* **56,** 9949.

Marder O. and Albericio F. (2003) *Chimica Oggi-Chemistry Today* **June**, 6.

Miller, B. (1980) *Organic Chemistry: The Basis of Life.* The Benjamin/Cummings Publishing Company, Inc.

Morris D. G. (2001) *Stereochemistry* The Royal Society of Chemistry, Cambridge, 23.

Mukaiyama, T., Usui, M. and Shimada, E. (1975) *Chem. Lett.*,1045.

Mukaiyama, T., Usui, M. and Saigo, K. (1976) *Chem. Lett.*, 49.

Orlowska, A. J. (1994) *Pol. J. Chem.* **68,** 713.

Przybylski, J., Wasilewski, K. and Legowska, A. *Pol. PL* 170331 B1, filed 10/30/92, published 11/29/96, assigned to Uniwersytet Gdanski, Pol., 6 pp (Poland).

Robertson N., Jiang L. and Ramage R. (1999) *Tetrahedron* **55,** 2713.

Samukov, V. V., Sabirov, A. N. and Troshkov, M. L. (1988) *J.Gen.Chem.USSR (Engl.Transl.)* **58**, 1277.

Samukov, V. V., Sabirov, A. N. and Pozdnyakov, P. I. (2001) *United Stated Patent:* US 6,265,590 B1.

Samukov, V. V., Sabirov, A. N. and Pozdnyakov, P. I. (1994) *Tetrahedron Lett.* **35**, 7821.

Sarantakis, D., Teichman, J., Lien, E. L. and Fenichel, R. (1976) *Biochem. Biophys. Res. Commun.* **73**, 336.

Sewald, N. and Jakubke H.-D. (2002) *Peptides: chemistry and biology.* WILEY-VCH, Weinheim, Germany.

Shechan, J. C., Cruickshank, P. A., Boshart, G. L. (1961) *J. Org. Chem.* **26**, 2525.

Sheehan, D. (2000) *Physical Biochemistry*, Wiley, Chichester, p. 46.

Sheehan, J. C. and Hess, G. P. (1955) *J. Am. Chem. Soc.* **77**, 1067.

Shioiri, T., Ninomiya, K. and Yamada, S. (1972) *J. Am. Chem. Soc.* **94,** 6203.

Shioiri, T. and Yamada, S. I. (1974) *Chem. Pharm. Bull.* **22,** 849.

Siedler, F., Weyher, E., and Moroder, L. (1996) *J. Pept. Sci.* **2** (4) 271.

Spetzler J. C., Meldal M., Felding J., Vedsø P. and Begtrup M. (1998) *J. Chem. Soc. Perkin Trans.* **1,** 1727.

Story, S. C. and Aldrich, J. V. (1994) *Int. J. Pept. Protein Res.* **43**, 292.

Thern, B., Rudolph, J. and Jung, G. (2002) *Tetrahedron Lett.* **43,** 5013.

Vasella, A., Voeffray, R., Pless, J. and Huguenin, R. (1983) *Helv. Chim. Acta.* **66**, 4, 1241.

Van der Auwera, C. and Anteunis, M. O. (1986) *Bull. Soc. Chim. Belg.* **95**, 203.

Van der Auwera, C. and Anteunis, M. J.O. (1987) *Int. J. Pept. Protein Res.* **29**, 574.

Van Den Nest W., Yuval S. and Albericio F. (2001) *J. Pept. Sci.* **7,** 115.

Verhart, C. G. J. and Tesser, G. I. (1988) *Recl. Trav. Chim. Pays-Bas.* **107**, 621.

Vojkovsky, T. and Drake, B. (1997) *Org. Prep. Proced. Int.* **29**, 497.

Wang, S.-S. (1979) *United States Patent.* 4,148,788.

Wang, S.-S. (1973) *J. Am. Chem. Soc.* **95**, 1328

Wieland, T. and Schaefer, W. (1951) *Angew. Chem.* **63**, 146.

Zouikri, M., Vicherat, A., Aubry, A., Marraud, M. and Boussard, G. (1998) *J. Pept. Res.* **52,** 19.