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Synthesis of Model Tripeptides and Approaches to the Preparation of a Transition State Analogue of Phosphoramidon

Nadina Monberg
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

SYNTHESIS OF MODEL TRIPEPTIDES AND APPROACHES TO THE PREPARATION OF A TRANSITION STATE ANALOGUE OF PHOSPHORAMIDON

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NADINA MONBERG

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FOR SOTI

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LIST OF ABBREVIATIONS

Ac	Acetyl
ACE	Angiotensin Converting Enzyme
Ac_2O	Acetic Anhydride
Anal Calcd	Combustion Elemental Analysis Calculated
Bn	Benzyl
bp	Boiling Point
^0C	Degree in Celsius
Cbz-Cl	Benzyl chloroformate
d	Doublet
DBQ	2,6-Dibromoquinone 4-Chloroimide
DMF	Dimethylformamide
equiv	Equivalent (molar)
Et	Ethyl
g	Gram(s)
GC	Gas Chromatography
Glu	Glutamic Acid
h	Hour(s)
His	Histidine

Hz	Hertz
IR	Infrared
J	Coupling Constant (NMR) in Hertz
Leu	Leucine
m	Multiplet(s)
m	Meta
M	Moles Per Liter
Me	Methyl
MHz	Megahertz
min	Minute(s)
mmole	Millimole(s)
mg	Milligram(s)
mL	Milliliter(s)
mp	Melting Point
NMR	Nuclear Magnetic Resonance
o	Ortho
p	Para
Ph	Phenyl
PheGly	Phenylglycine
ppm	Parts Per Million

psi	Pound Per Square Inch
q	Quartet
R_f	Retention Factor
s	Singlet
t	Triplet
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TLN	Thermophylic Bacterium *Bacillus Thermoproteolyticus*
TMS-Br	Trimethylsilyl Bromide
t_r	Retention Time
TS	Transition State
UV	Ultraviolet

CHAPTER I

INTRODUCTION

A. Endogenous vasoactive peptides

Since their discovery, endogenous vasoactive peptides have attracted considerable interest because of their concerted actions on the heart, vascular smooth muscle and kidney, as well as their ability to alter the release of hormones and neurotransmitters. Although much information has been obtained regarding their vasoconstrictor and mitogenic actions, their involvement in modulating the activity of the cardiovascular system under normal conditions has not been fully elucidated. A better understanding of their role in the pathogenesis of a variety of diseases such as hypertension, artherosclerosis or acute renal failure is required (Nayler, 1990; Masaki et al., 1992). The endogenous vasoactive peptides presumably act through a variety of mechanisms to control vascular tone and peripheral blood flow (Said, 1983). Some of these peptides (such as angiotensin II, vasopressin and neuropeptide Y) are potent vasoconstrictors while others (such as atrial natriuretic peptide and bradykinin) are vasodilators acting in parallel to maintain homeostasis (Kramer, 1990). The peptides are known to be released mostly by endothelial cells to mediate vasoconstriction in

response to various chemical and physical stimuli (Doherty, 1992).

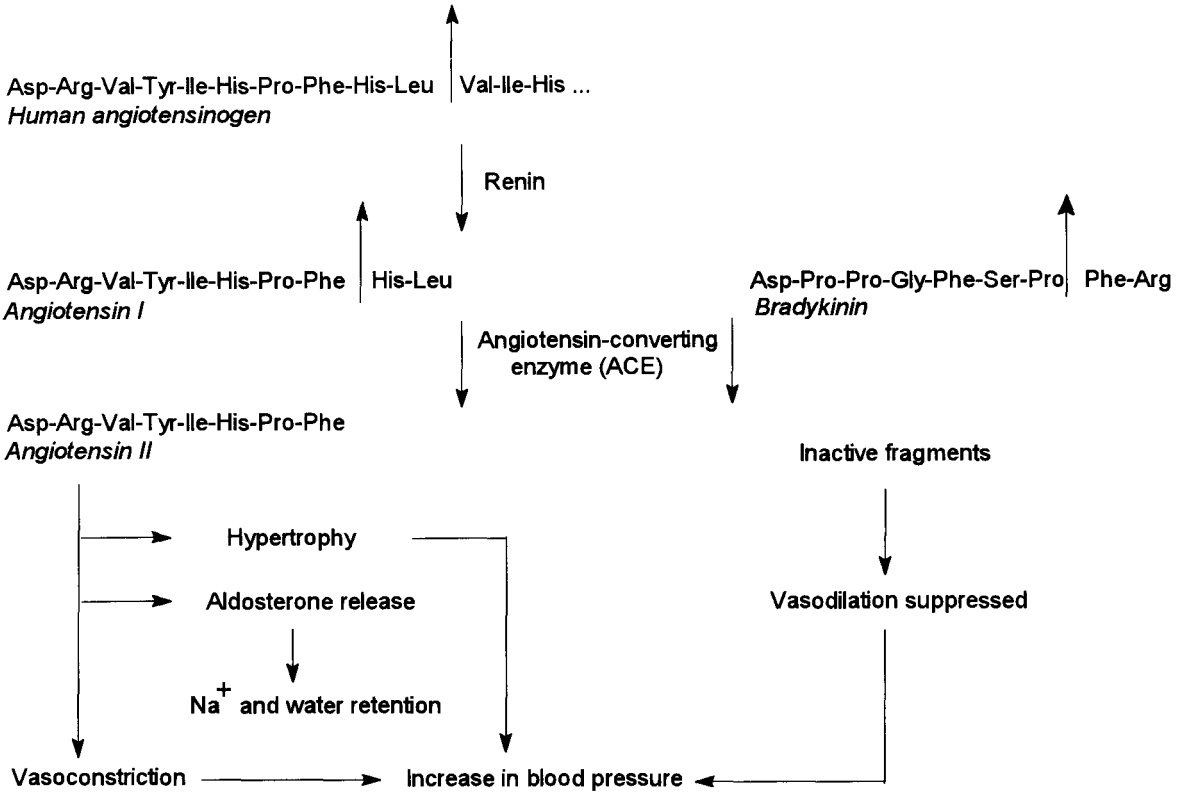


Figure 1 : The renin-angiotensin system (Redshaw, 1993)

Angiotensin II is a potent vasoconstrictor acting on smooth muscle and in the central nervous system. It is an octapeptide which in vascular smooth muscle acts directly via receptors to constrict the arteries and arterioles while in the adrenal cortex releases aldosterone which induces sodium and water retention, resulting in a further hypertensive effect through increased plasma volume (Redshaw, 1993). It is

responsible for the full pressor effect (raise in blood pressure involving vasoconstriction) of the renin-angiotensin system (Figure 1) and believed to participate in well known events in atherogenesis such as the control of smooth muscle cell growth and proliferation (Ambrosioni et al., 1992).

In the renin-angiotensin system, which correlates renal disease with an increase in high blood pressure, a Leu-Val bond connection of a circulating globular protein known as angiotensinogen, or renin substrate, is hydrolysed specifically by the aspartic acid proteinase, renin, produced in the kidney. This process releases the N-terminal decapeptide, angiotensin I, which has no known biological activity. Angiotensin-converting enzyme (ACE) then cleaves a two amino acid fragment from the C-terminus of this decapeptide to give the octapeptide, angiotensin II (Alhenc-Gelas et al., 1990).

The key factor in the production of angiotensin II (as seen from Figure 1) is the angiotensin-converting enzyme (ACE), which warrants further discussion. ACE, a glycoprotein widely distributed in mammals, is a membrane-bound enzyme localized mainly in endothelial cells (especially pulmonary) with an expression level higher in atria than in ventricles (Uratha et al., 1993). ACE is also a well known zinc-containing carboxypeptidase that has a central role in blood pressure homeostatis (Studdy et al., 1989), namely, inactivation of ACE can lower or prevent expected increases in blood pressure. As a result, understanding its mode of action and the discovery of enzyme specific inhibitors can lead to the development of useful pharmaceutical agents for high

blood pressure therapy (Ehlers et al., 1989).

Unfortunately, size (1.3-1.6 kDa) and heavy glycosylation have made structure elucidation of ACE a very difficult task (Redshaw, 1993). The amino acid sequence was not determined until 1988 (Soubrier et al., 1988) after the first inhibitors of ACE had been marketed. Even today, ACE structural information is not useful for inhibitor design, and the most relevant factors in inhibitor preparation remain a knowledge of the enzyme's substrate specificity together with an understanding of the catalytic mechanism (Redshaw, 1993). Despite these drawbacks, the fact that there is an essential zinc ion in its active site encourages comparison with other zinc metalloproteinases. Among them, thermolysin *(TLN)* (Matthews et al., 1974), is a well known zinc-dependent enzyme and its structure has been determined by X-ray crystallography. Although the amino acid sequence of thermolysin is not necessarily related to the sequence of other zinc-requiring peptidases such as ACE or CPA (carboxypeptidase A, another zinc-dependent enzyme) (Christianson et al., 1986), there is increasing evidence that the active sites of these zinc enzymes have common features (Kester et al., 1977; Cushman et al., 1977; Ondetti et al., 1977). As such, recent X-ray structures of thermolysin-inhibitor complexes have proven useful in elucidating the mode of binding of related zinc peptidases including ACE (Holland et al., 1994; Tronrud et al., 1992). Moreover, commercial availability and cost makes thermolysin easier to study. Therefore, understanding thermolysin's mode of action can aid better

understanding of ACE.

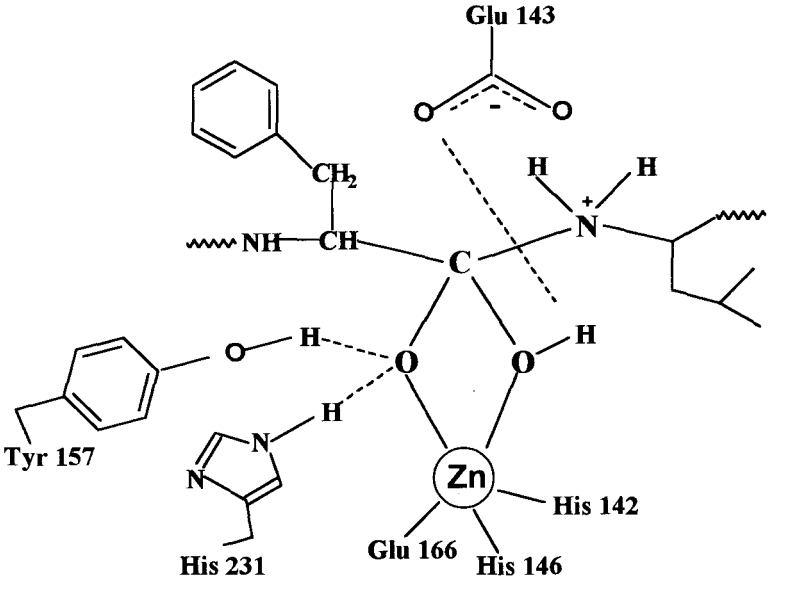
B. Thermolysin

Thermolysin is a thermostable, zinc-containing endopeptidase isolated from the thermophilic bacterium *Bacillus thermoproteolyticus* (Copie et al., 1990). It has a molecular weight of 34,600 and contains 316 residues in its single polypeptide chain (Holland et al., 1992). Thermolysin is related to other peptidases such as ACE, collagenase, enkephalinase and other neutral endopeptidases, and has been recognized to play important roles in cellular and hormonal metabolism (Copie et al., 1990). In addition, thermolysin has proven to be an advantageous system for the detailed evaluation of protein-ligand binding effects (Morgan et al., 1991).

The enzyme is particularly suited for studying the mechanism of zinc-promoted peptide hydrolysis because a variety of crystallographic, inhibitor, substrate, and chemical modification data are available. Importantly, the crystal and solution conformations appear to be very similar on the basis of available experimental comparisons (Hangauer et al., 1984) and the findings that the crystalline enzyme still hydrolyzes peptides. The relevance of the crystal structure (which has been determined to 1.60 angstrom resolution (Holmes et al., 1982)) is further enhanced by the fact that, in general, thermolysin changes very little upon binding numerous inhibitors; this feature makes thermolysin a good model enzyme for mechanistic studies of zinc

peptidases (Hangauer et al., 1984).

The crystal structure of thermolysin together with computer graphics analysis of the active site, and the x-ray structure of an *N*-carboxymethyl dipeptide inhibitor (Mozingo et al., 1984) have led to a proposal of its mechanism (Tronrud et al., 1986).



(Copie et al., 1990)

Figure 2: **Postulated tetrahedral intermediate formed during thermolysin's enzymatic action (Presumed hydrogen interactions are drawn as dashed lines. Thermolysin's zinc ion is shown coordinated to three protein amino acid residues and two substrate ligands).**

As shown in Figure 2 (Tronrud et al., 1986, Fersht, 1985), the protein consists mainly of two spherical domains with a deep cleft in the middle, which constitutes the active site. In its native state, thermolysin has a single water molecule bound to the zinc ion.

The aforementioned inhibitor, *N*-(1-carboxy-3-phenylpropyl)-l-leucyl-l-tryptophan, has been observed to bind to thermolysin with the carboxyl group close to leucine interacting with the zinc. In addition, the leucyl side chain was found to be required by the active site due to its nestled location in the hydrophobic specificity pocket.

Together with three ligands from the protein (Glu-166, His-146, and His-142), the zinc coordination is essentially tetrahedral. As a peptide substrate binds, the carbonyl oxygen (previously mentioned) forms a fifth zinc ligand and displaces the bound water molecule towards Glu-143. The water molecule, activated by the combined influence of Glu-143 and the metal, attacks the carbonyl carbon of the substrate forming a tetrahedral intermediate in which two of the oxygens become zinc ligands, thus creating a pentacoordinated zinc ion. A proton, accepted by Glu-143 from the attacking water molecule, is shuttled to the leaving nitrogen, leading to cleavage of the scissile bond and displacement of products. In such a mechanism, Glu-143 performs the dual role of assisting the attack of the water molecule on the carbonyl carbon of the peptide bond and also acting as the proton donor to the nitrogen (Fersht, 1985).

C. Transition state and enzyme catalysis

The term transition state (TS) refers to the configuration along the reaction coordinate that a specie must pass through on its way to product formation (Figure 3).

The transition state corresponds to the highest energy state in the course of a reaction; it is a dynamic, reactive and unstable state in which bonds are only partially formed or broken. According to transition state theory, the physical entities under consideration during a reaction are the reagents, or ground states, and the most unstable species in the reaction pathway, the transition state(s). The importance of transition state theory is that it relates the rate of a reaction to the difference in Gibbs Free Energy (G) between the transition state and the ground state. This is an important consideration when comparing the relative reactivities of pairs of substances or the rates of a given reaction under different sets of conditions.

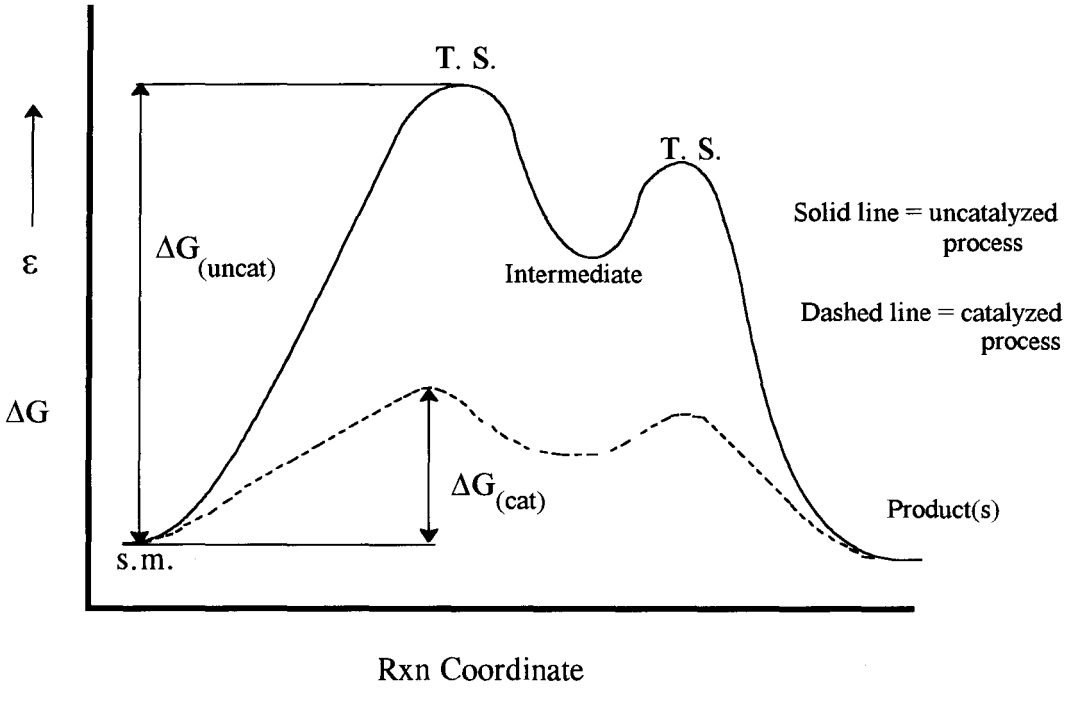


Figure 3 : Effect of catalyst on reaction pathway.

The difference in height on the energy surface between the starting materials

and the transition state is the reaction's activation energy (or ΔG^{TS}), and is the energy barrier that must be overcome before the reactants can convert to product. The higher a reaction's activation energy is, the more difficult the path to products. An enzyme, however, can speed up a reaction by lowering the activation energy thus providing a smaller energy "hill" in the reaction pathway (Bender et al., 1984). Thus, enzymes function as catalysts in reactions, meaning that they alter the rate of a reaction without themselves undergoing change. The enzyme is able to function as a catalyst by converting the substrate into a specific product through formation of a reactive TS. One of the enzymes primary functions in its role as the catalyst, however,

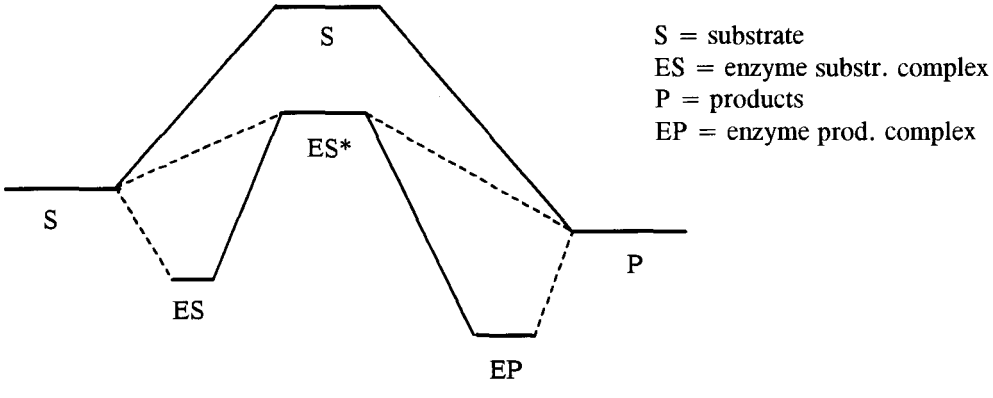


Figure 4 : Energy levels in catalysis vs. normal reaction

is not its ability to strongly bind the substrate or the product but rather its ability to discriminate between substrate, product and the transition state. In 1946 Linus Pauling suggested that an enzyme binds more strongly to the transition state than to the

reactants or products (Pauling, 1946). When the enzyme captures the transition state, it is stabilized and as a result, less energy is needed to access this structure. The energy barrier is lowered and the reaction is generally accelerated - often by factors of several orders of magnitude in rate (Figure 4). The enzyme's effect is catalytic because the products immediately diffuse away from the active site following formation, enabling the enzyme to bind and transform new molecules of substrate repeatedly (Lerner et al., 1988). To provide for the catalytic affinity, interactions between the enzyme and the substrate are extensive with a specificity reflected in the transition state (Wolfenden, 1969). The binding interactions are quite numerous and can include hydrophobic, ionic, π-stacking and hydrogen bonding.

D. Transition State Analogues

Transition state analogues are molecules designed to exploit and manifest the special interactions that distinguish the substrate in the transition state from the substrate in the ground state (Jencks, 1966), and can be used to probe differences in enzyme binding at various stages in the reaction coordinate rather than to establish absolute structures. Pauling predicted that it would be possible to utilize transition state analogues as enzyme inhibitors (Pauling, 1946). He predicted that, given a reaction for which an enzyme exists, a stable substance might mimic the native, reactive transition state in shape and charge. Such a transition state analogue should bind very tightly to

the enzyme, inhibiting its catalytic action by filling the binding site at its most complementary state and thereby preventing the active site from binding to its true substrate (Lerner et al., 1988; Bartlett, 1984). This theory has been validated over the last 20 years, as a significant number of compounds have been synthesized that behave as TS analogues in just the manner Pauling predicted. An example of the depiction of enzyme action is supported by studies that show that stable mimics of transition states (such as phosphonates) are held tightly by enzymes that employ the putative mechanism (Wolfenden, 1969, 1972, 1991) (Figure 5). In this mechanism, a phosphorus atom has been substituted for a reacting carbonyl in the transition state's tetrahedral ensemble, yielding a stable compound known as a phosphonate ester. The distribution of charge on the phosphonate oxygen atoms of the molecule resembles that of the transition state. In addition, the phosphorus-oxygen bonds are about 20 percent longer than ordinary carbon-oxygen bonds, which enables the analogue to mimic the elongated bonds of the transition state (Lerner et al., 1988). Importantly, phosphorus esters and amides by virtue of their sp^3 hybridized center, closely resemble the transition state achieved during the hydrolysis of certain carbonyl compounds (Hanson et al., 1989). As a result, many of these phosphorus-based analogues have been used as enzyme inhibitors (Dreyer et al., 1989; McLeod et al., 1991) and as haptens for the production of catalytic antibodies (Lerner et al., 1987).

The arrival of stable TS analogues, conceived to mimic the structure of an

intermediate in the path of a substrate's transformation to product, have made it

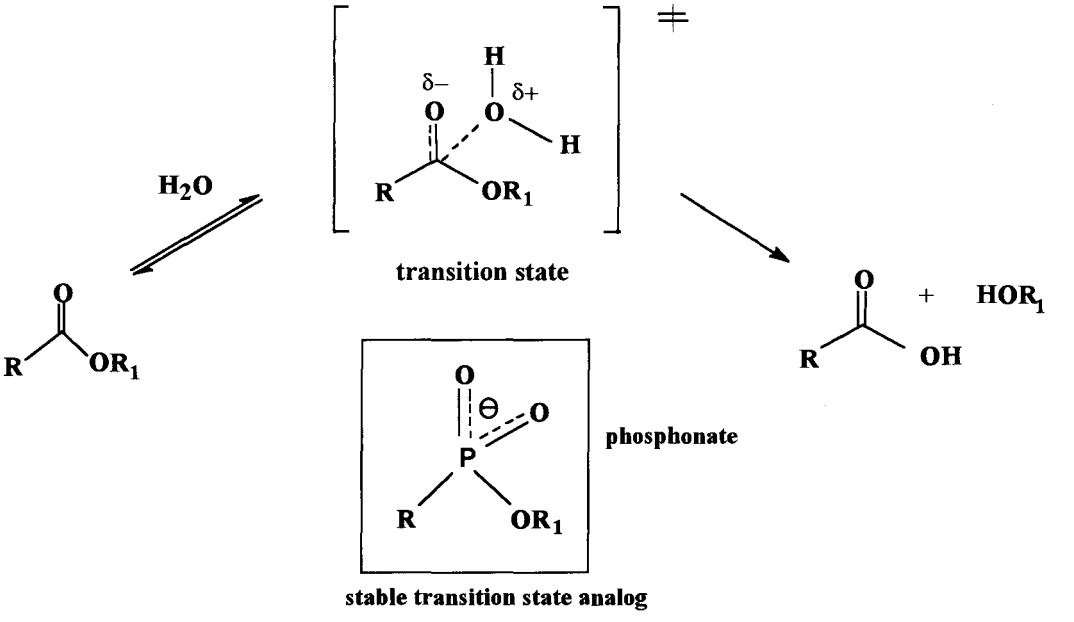
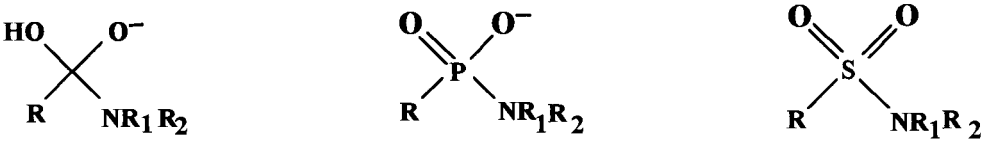


Figure 5: Hydrolysis of ester and phosphonate analog

possible to examine the degree of enzyme binding/distortion energy, and to probe the specificity and selectivity of this interaction. Most TS analogues behave as potent inhibitors because, unlike the native substrate, they are not transformed into product and remain tightly held in the catalytic domain, thereby inactivating the enzyme. Some enzyme inhibitors have been used to study the role of individual enzymes, to understand enzyme mechanisms and to aid in the development of pharmacological and agricultural agents (Bartlett et al., 1987). Among them, peptides containing a transition state-analogue in place of a hydrolyzing amide bond have received

considerable attention. Typically, peptide TS analogues contain a phosphonamidate or a sulfonamide moiety (Moree et al., 1993) which show the best resemblance to the transition state during the hydrolysis of the amide bond from both a steric and electronic point of view (Figure 6). These TS analogues are used in the development of protease inhibitors such as thermolysin, renin and pepsin.



a **b** **c**

(R = alkyl, R_1 = alkyl, R_2 = H, alkyl)

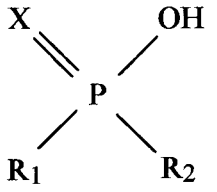
Figure 6 : TS analogues of amide bond hydrolysis.

E. Phosphorus-Containing TS Analogues as Thermolysin Inhibitors

Some of the most potent inhibitors of thermolysin are transition state analogues which incorporate phosphorus-containing tetrahedral groups capable of binding to the active site zinc in a bidentate fashion (Holmquist, 1977; Galardy, 1982).

Phosphonate, phosphinate, phosphonamidate and phosphoramidate analogues (Figure 7) designed to the same transition state show different binding propensity to thermolysin (Bartlett et al., 1990). The enhanced binding and inhibitory potency of the

phosphonamidate analog has been attributed to specific hydrogen-bonding by the



Phosphonate: X = O, R_1 = OR, R_2 = alkyl, aryl
Phosphinate: X = O, R_1 = R_2 = alkyl, aryl
Phosphonamidate: X = O, R_1 = NHR, R_2 = alkyl, aryl
Phosphoramidate: X = O, R_1 = NHR, R_2 = OR'

Figure 7: **Phosphorous containing monoacids as TS analogues.**

amidate (Bartlett et al., 1987). Grobelny and co-workers noted that the binding energy difference were due to increased basicity of the phosphonamidate anion (better ligates the active site zinc atom), whereas solvation effects were negligible (Grobelny et al., 1989).

A well known phosphorus-containing inhibitor used to probe the mechanism of thermolysin is phosphoramidon [N-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-

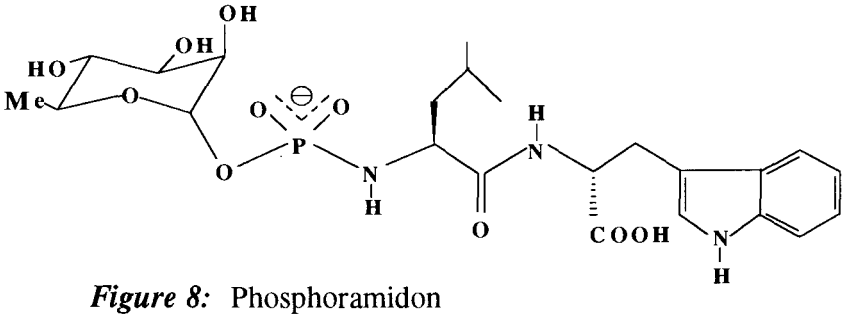
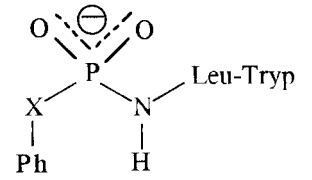


Figure 8: Phosphoramidon

leucyl-L-tryptophan] (Figure 8). This naturally occuring potent inhibitor (K_3 = 2.8 x 10^{-8} M) (Suda et al., 1973), consists of a rhamnose sugar group attached to a leu-tryp

moiety through a phosphate P=O (Umezawa et al., 1972). The phosphoramidon-thermolysin complex was crystallized, and showed monodentate Zn-ligation to one of the phosphate oxygens, 2.0 angstroms from the zinc atom, resulting in a tetrahedral complex which closely resembles the transition state during the catalytic process (Tronrud et al., 1986). Additionally, the crystal structure reaffirmed that the *N*-phosphoryl group mimics a hydrated peptide since it was seen that the P-O bonds were only slightly longer than the corresponding C-O bonds, and both the phosphoryl group and a hydrated peptide are tetrahedral (Hangauer et al., 1984). Interestingly, removal of the rhamnose moiety from phosphoramidon resulted in a slightly tighter binding inhibition (Komiyama et al., 1975) indicating that the sugar is not essential for thermolysin recognition.

Based on the effectiveness of phosphoramidon as an inhibitor of thermolysin, a series of related phosphoramidates and phosphonamidates have been synthesized and proved to be potent inhibitors not only of thermolysin, but also of other zinc peptidases including carboxypeptidase A, endothelial converting enzyme (ECE) and angiotensin converting enzyme (ACE) (Jacobsen et al., 1981; Bigaud et al., 1994). As an example, Bertenshaw truncated the phosphoramidon structure in an attempt to inhibit ECE, and the study found that the sugar moiety was not essential for anti-ECE activity (Bertenshaw et al., 1993). Previous studies (Jacobsen et al., 1981; Bigaud et al., 1994) also reaffirmed that the rhamnose moiety was of very little importance for the



$X = CH_2, O$ Bertenshaw et al., 1993.

Figure 9 : Bertenshaw's Truncated Version of Phosphoramidon

inhibition of ACE or ECE, whereas the phosphoryl group of phosphoramidon was an absolute requirement. Furthermore, the tryptophan residue of phosphoramidon appeared to be important for the ECE inhibition while thermolysin inhibition seemed to depend greatly on the leucine residue. It was concluded that *in vivo* ECE and themolysin differ only in the way they recognize phosphoramidon.

F. Statement of the Problem

Phosphorus-containing analogues have made significant contributions to our understanding of TS structure; however, several drawbacks to their application (resulting in deficits in current understanding) do remain. Some of these drawbacks include; (A) a lack of an essential hydrogen-bonding or solvation interaction in some phosphonates and phosphinates leaves them ineffectual, (B) instability to acid for many phosphonamidates, and (C) when used as haptens, fail to result in antibodies with substantial catalytic activity suggesting that key elements in binding amidases may be absent in phosphonamidates (McLeod et al., 1991).

Despite these drawbacks, phosphorus-containing analogues do offer useful advantages. Certain phosphorus esters and amides, by virtue of their sp^3 hybridized center, closely resemble the transition state achieved during the hydrolysis of certain carbonyl compounds. Another important advantage is the use of ^{31}P NMR, a sensitive nucleus, capable of revealing highly diagnostic chemical and biochemical information. In addition, the use of x-ray, as in the case of the x-ray crystal structure of a thermolysin-phosphoramidate transition state analog, has given tremendous insight into the TS-protein interaction (Tronrud et al., 1986). Doubtless, the systematic design and use of phosphorus-containing analogues will remain a promising path toward more specific and potent inhibitors (or probes) of protein structure and function.

In this study, our aim is to enhance our understanding of the utility of phosphorus-containing transition state analogues by attempting to synthesize a tripeptide-based TS analogue that resembles phosphoramidon. The chosen transition state analogue could be used to suppress the enzyme thermolysin, thus furthering our understanding of the requirements of zinc-containing enzymes.

The project's concept is based on Bertenshaw's truncated version of phosphoramidon (Figure 9), and the discovery that the sugar moiety has very little importance in the inhibition of the enzyme. Therefore, the main focus of the project is centered on synthesizing a transition state analogue of phosphoramidon without the sugar group. First, the tripeptide acetyl-*l*/*d*-phenylglycine-*l*-leucine-*l*-tryptophan,

methyl ester was selected to serve as a natural substrate for this transition state analogue, and was synthesized as the "S" and "R" isomeric forms **9a** and **9b**. This tripeptide closely resembles phosphoramidon's peptide sequence with the sole exception of the substitution by an *N*-acetyl phenylglycine group. The acetyl group was elected as an end cap for the amino group to better stimulate the carboxamide junction of an extended peptide chain. Phenylglycine was chosen because the enantiomers of the 2-phenyl phosphonate amino acid analogue are easily prepared, and the recent report by Bertenshaw revealed that the benzyl and phenoxy phosphonate analogues of this tripeptide showed parallel biological activity to phosphoramidon (Bertenshaw et al., 1993). In our case, we synthesized both "S" and "R" isomers of the tripeptide to serve as the natural and unnatural models for the TS analogue - which when synthesized, are racemic at the α-phosphorus center. [Note: the "R" configuration for a phosphonate α center corresponds to the amino acid "S" configuration]. We selected the *l*-leu-*l*-tryp dipeptide to maintain consistency with phosphoramidon's structure. More importantly however, the leucyl moiety was used due to its importance in known binding affinity to zinc-containing enzymes (as previously mentioned, the active site of thermolysin requires leucine in its hydrophobic pocket).

Once we obtained these native tripeptides, we will attempted the synthesis of the phosphono-substituted tripeptide. In the past, phosphorus-containing peptides have been prepared by conversion of a phosphono-derivative of an amino acid to a methyl

phosphonochloridate, and condensed with either the amine moiety of a flanking amino acid (Khatri et al., 1980; Bartlett et al., 1986; Bertenshaw et al.; 1993), or an alcohol (Bartlett et al., 1990; Sampson et al., 1991). Subsequent hydrolysis of the phosphorus methyl ester affords the phosphonic acid. Yuan and Chen (1990, 1992) reported a three component, one pot Pudovik synthesis of α-substituted, dimethylphosphonamidates from simple starting materials. Phosphorylation of dipeptide amines under basic conditions or with phase transfer catalysts can be conducted (Ma et al., 1992; Li et al., 1992).

In our case, we will first attempt the preparation of optically active phosphonamidate diastereomers **13** and **14** by an imine Pudovik reaction (Arbusov et al., 1978; Zon, 1981). Although more inventive approaches exist, this strategy permits for: a) rapid acquisition of the materials, b) variation in the amino acid R group, and c) inexpensive starting materials. Following acquisition of **13** and **14**, the next steps attempts to modify these intermediates into compounds suitable for coupling to the dipeptide *l*-leu-*l*-tryp, methyl ester **8** to obtain the desired phosphono-substituted tripeptide. The attempted modifications included thionation, acetylation, hydrolysis, dealkylation and chlorination. These transformations resulted in the formation of a racemic salt representing a phosphorus analogue of acetyl-phenylglycine. The salt was the subject of further investigations in an effort to couple it to the deprotected dipeptide to yield the expected phosphono-substituted peptide.

This thesis will describe (a) the preparation of the native tripeptide acetyl-phenylglycine-*l*-leucine-*l*-tryptophan, methyl ester synthesized as the "S" and "R" isomers (Figure 10); (b) the preparation of the phosphorus analogue of acetyl-phenylglycine (Figure 11), and (c) attempts for the preparation of the phosphorus-containing tripeptide (Figure 12).

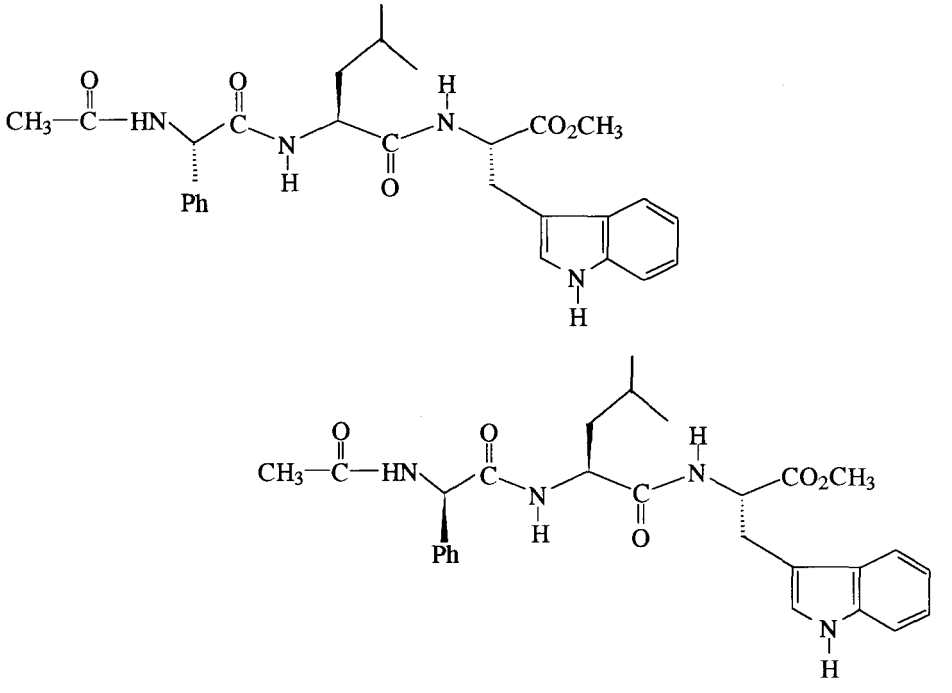


Figure 10 : Model tripeptides

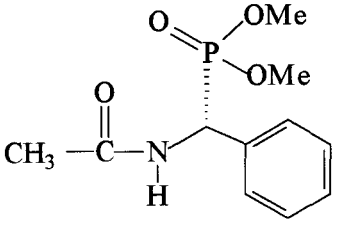


Figure 11 : Phosphorus analogue of acetyl-phenylglycine

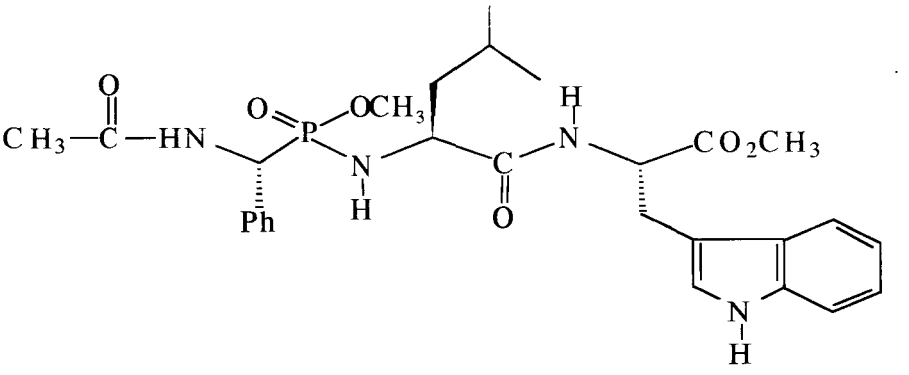


Figure 12 : Phosphorus substitute tripeptide (TS analogue)

CHAPTER II

RESULTS AND DISCUSSION

The specific aim of the project is to synthesize a phosphoramidon-like transition state analogue capable of strongly binding thermolysin. This analogue could also improve our understanding of the effect that a sp^3-hybridized phosphorus center substituting for a peptide carbonyl has on the conformation and dynamics of a peptide backbone (this can be achieved through the use of NMR or molecular modeling). Additionally, if active against the enzyme thermolysin, it could be used to more precisely probe the requirements of zinc protease inhibition.

The first goal toward the preparation of the TS analogue started with the synthesis of native tripeptides acetyl-*d/l*-phenylglycine-*l*-leucine-*l*-tryptophan, methyl ester **9a** and **9b** (Part A). We selected this tripeptide because it resembles phosphoramidon's peptide sequence and connectivity except for the sugar group rhamnose, which was shown to have a minor role in the inhibition of the enzyme. The native tripeptide is intended to serve both as a model for comparison of enzyme interaction and as a synthetic entry for the phosphonate transition state analogue. The amino acids used were *l*-phenylglycine, *l*-leucine and *l*-tryptophan, methyl ester and

were appropriately protected prior to coupling. Both tripeptides were successfully synthesized by conventional solution methods using DCC as the amide-forming coupling reagent. Following their successful acquisition, we also attempted to synthesize the native tripeptides by solid phase peptide synthesis techniques, however, without success.

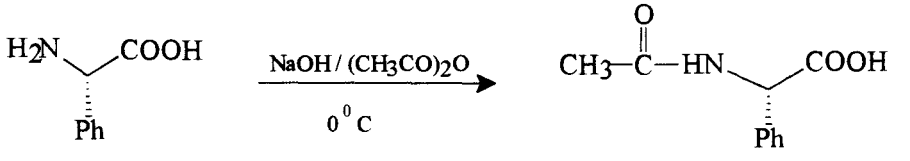
The second goal of the project was the preparation of a phosphorus analogue of acetyl-phenylglycine (Part B). This analogue will be reacted with the dipeptide *l*-leu-*l*-tryp, methyl ester **8** to obtain the phosphono-substituted tripeptide **24**. The synthesis for the analogue started with preparation of optically active phosphonamidate diastereomers **13** and **14** which could be later separated into their corresponding isomers. Following their acquisition, we tried to separate each diastereomer pair by column chromatography and GC but to date, both techniques have been unsuccessful.

Final steps attempted to modify **13** and **14** into compounds suitable for coupling with the dipeptide *l*-leu-*l*-tryp, methyl ester **8** to obtain the phosphorus-containing TS analogue **24** (Part C). Attempted modifications included thionation, acetylation, hydrolysis, dealkylation and chlorination.

Part A: Synthesis of acetyl-l-phenylglycine-l-leucine-l-tryptophan, methyl ester; the ***native tripeptide.***

The first goal of the project was the synthesis of the native tripeptide **9a** as both a model and a synthetic entry toward the preparation of the phosphonate precursor. The three amino acids used to construct the tripeptide were *l*-phenylglycine, *l*-leucine and *l*-tryptophan methyl ester and were end protected at the amine terminus prior to coupling when necessary.

Commercially available *l*-phenylglycine **1** was successfully blocked as the acetamide using 1M NaOH and $(CH_3CO)_2O$ at 0 ^{0}C, to provide *N*-acetyl-*l*-phenylglycine **2** in a 74% yield (Eqn. 1) (Bodanszky et al., 1984). These reaction conditions were selected after several attempts to N-acetylate **1** with acetic anhydride



(1)

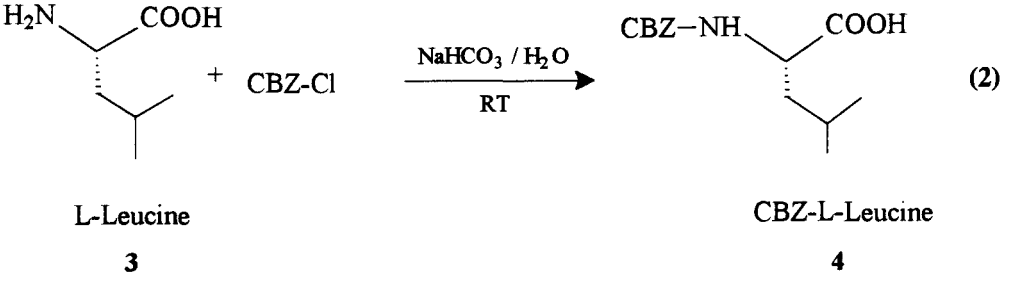
L-Phenylglycine
1

N - Acetyl-L-Phenylglycine
2

under neutral or acidic conditions failed. Under basic conditions, the reaction proceeded rapidly, had the highest yield, and the product was easily recrystallized from water. The acetyl group was selected as an end cap due to its resemblance to a native peptide amide bond and the ease of identification by NMR. Evidence of acetyl-*l*-

phenylglycine **2** formation is supported by the presence of two carbonyl peaks in ^{13}C NMR (at δ 169.2 and 172.3 ppm) and a singlet near 2.0 ppm in the ^{1}H NMR indicative of the $CH_3C(O)$ moiety. Additionally, the 192-194 ^{0}C melting point was close to the literature value (190-192 ^{0}C) (Hongo et al., 1981).

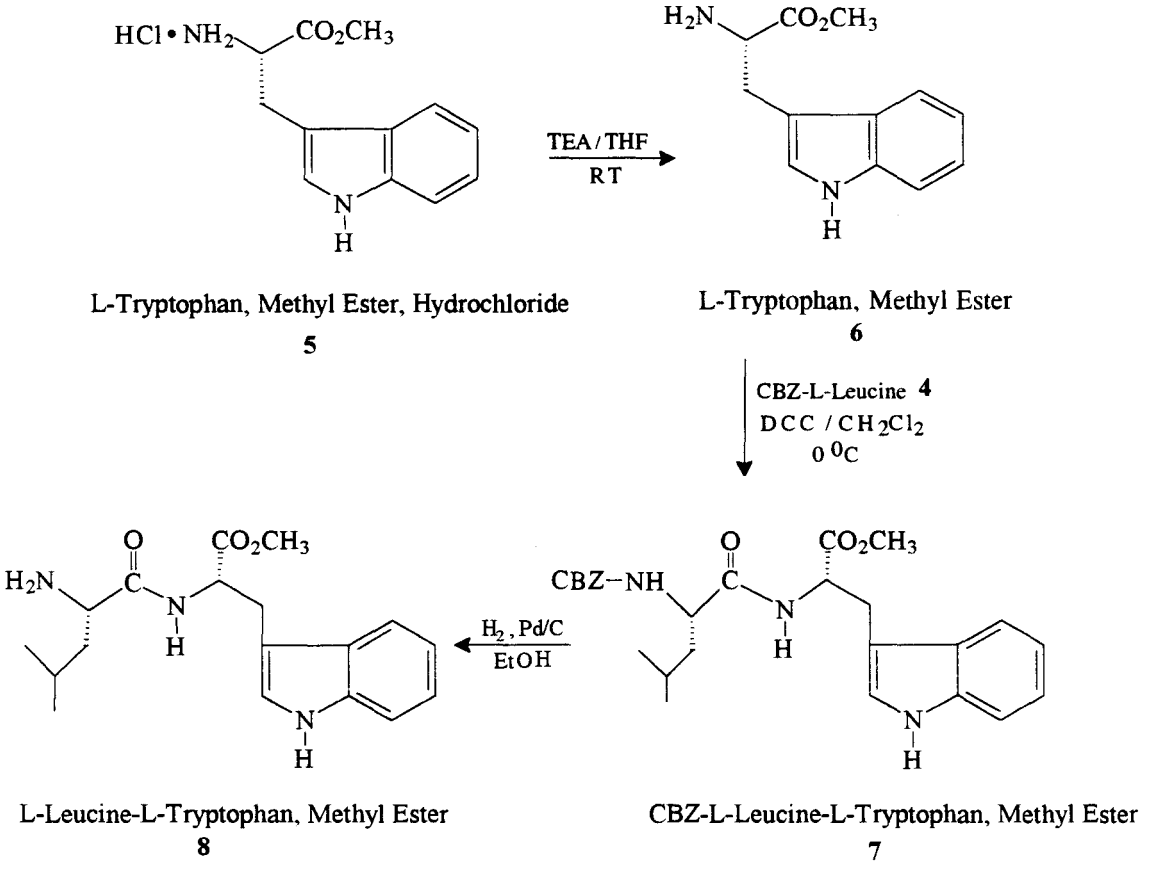
The amine terminus of *l*-leucine was blocked next in preparation for coupling to tryptophan. *l*-Leucine **3** was reacted with benzyl chloroformate (Cbz-Cl) using Schotten-Bauman conditions (CH_2Cl_2, aqueous $NaHCO_3$, RT), to yield N-Cbz-*l*-leucine **4** in a 75% yield (Eqn. 2). A thick, light-yellow oil was formed after Kugelrohr distillation. However, prior literature listed Cbz-leu as a solid (Choudry et al., 1980).



Compound **4** did not solidify even after prolonged storage in the freezer. The ^{13}C NMR spectrum shows the requisite Cbz-carbonyl and the ^{1}H NMR indicates the addition of a benzyl methylene group (δ 5.24 ppm, 2H) and five aromatic protons in the correct integration. These data were sufficient evidence to support that the correct product was formed despite the difference in physical composition.

Having these two amino acids securely end blocked, preparation of the dipeptide

was conducted next. First, *l*-tryptophan, methyl ester hydrochloride **5** was converted to the primary amine using triethylamine (TEA) in THF at room temperature to give *l*-tryptophan, methyl ester **6**. Cbz-*l*-leucine **4** was reacted with dicyclohexyl carbodi-



Scheme 1: Synthesis of l-leucyl-l-tryptophan, methyl ester **8**

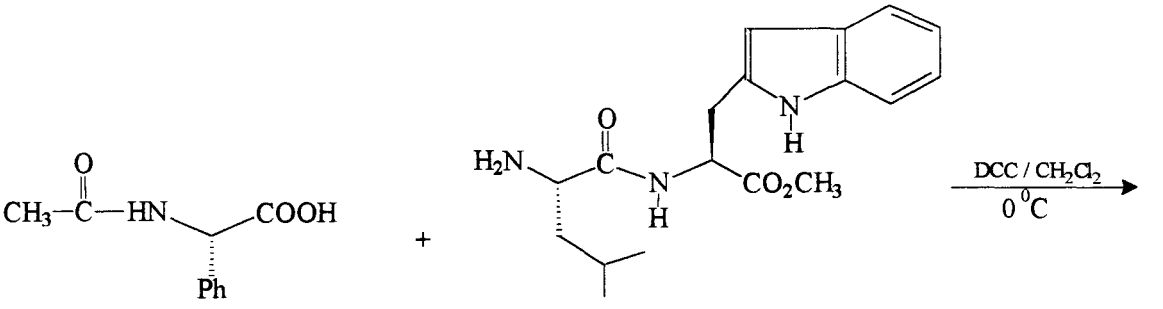
imide (DCC) (anhydrous CH_2Cl_2, 0 ^{0}C), to form the activated complex which undergoes coupling with **6** to form the Cbz dipeptide **7** in 77% yield. The crude dipeptide was obtained initially in the form of a viscous oil but after purification by

flash chromatography, a white solid was obtained. The ^{1}H NMR spectrum shows the correct integration of protons including a 6 H containing doublet at 0.87 ppm from the gem dimethyl of Cbz-*l*-leucine, the methyl ester singlet (3 H), benzyl-Cbz methylene (as a doublet), and all the aromatic protons. The ^{13}C NMR is also consistent with the structure as evidenced by the two carbonyl groups, as are other physical data including rotation and melting point (Shiba et al., 1974).

The next stage in the synthesis was to append a phenylglycine to the leu-tryp dipeptide. To accomplish this, deprotection via hydrogenolysis of the Cbz group was first conducted using 10% Pd/C in ethanol under balloon hydrogen pressure to give *l*-leucyl-*l*-tryptophan, methyl ester **8**. However, this compound was short lived and had to be immediately reacted in the next step within 24 h due to [potential] cyclization. NMR spectra were therefore taken on the protected dipeptide **7** and compared to that of Cbz-*l*-leucine and *l*-tryptophan, methyl ester hydrochloride. All the correct signals are present in the ^{1}H NMR and the amine N-H peaks appear as expected: doublets for the two amine groups in the backbone owing to amide rotomers and a singlet for the tryptophan indole amine. Additionally, ^{13}C NMR shows three carbonyl peaks, the aromatic ring carbon atoms, and also accounts for the remaining carbon atoms.

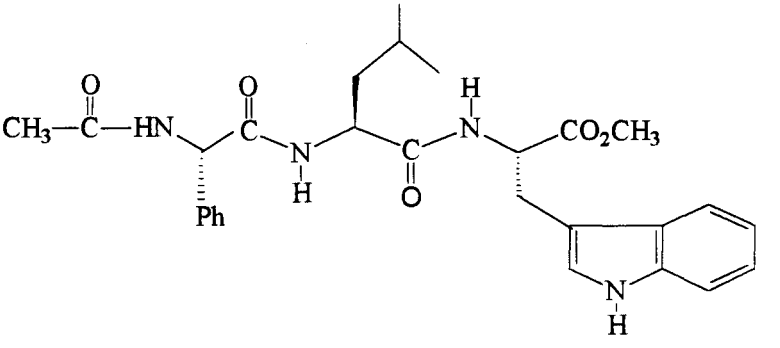
The last step in this synthetic sequence involved coupling of acetyl-*l*-phenylglycine **2a** or acetyl-*d*-phenylglycine **2b** to the deprotected dipeptide **8** to produce the native tripeptides **9a** and **9b** (Scheme 2; shown for the *l* isomer). The

synthesis was achieved by simultaneously combining equimolar amounts of **2a** or **2b** and DCC at 0 ^{0}C for 30-40 minutes to form the corresponding activated complex, followed by the addition of the deprotected dipeptide **8.**



Acetyl-L-Phenylglycine
2a

L-Leucine-L-Tryptophan, Methyl Ester
8

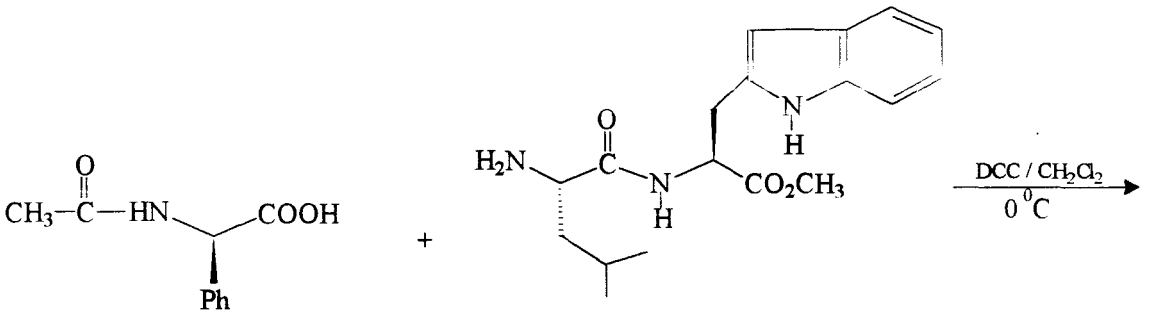


Acetyl-L-Phenylglycine-L-leucine-L-Tryptophan, Methyl Ester
9a

Scheme 2: Synthesis of acetyl-L-phenylglycine-L-leucine-L-tryptophan, methyl ester **9a**

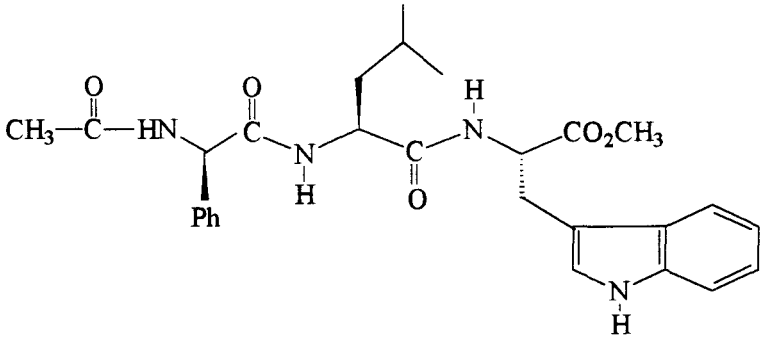
In this way, addition of the carboxylic acid to the diimide first allowed the formation of the intermediate activated O-acylisourea which undergoes nucleophilic substitution by

the amine from the deprotected dipeptide to yield the tripeptide product **9a** or **9b** (bearing a newly formed amide linkage) and dicyclohexylurea as side product (Streitweiser and Heathcock, 1985). The solvent for this reaction was anhydrous CH_2Cl_2 since dicyclohexylurea (DCU) - the coupling by-product of the reaction is insoluble and can precipitate out for easy removal by filtration. However, the ^1H NMR showed that a significant amount of DCU was still present in the crude mixture following filtration, and trituration with hot CH_2Cl_2 was necessary to remove the DCU. Repeated recrystallizations from CH_2Cl_2 and diethyl ether were also needed to obtain the pure tripeptide. The large number of purification procedures reduced the overall recovery but tripeptide **9a** (Scheme 2) was still obtained in a 52% yield. Similarly, tripeptide **9b** (Scheme 3) was obtained in a 49% yield starting with *d*-acetyl phenylglycine. Both compounds are in the form of white powders, with similar melting points. The ^1H NMR of both S (*l*) and R (*d*) isomers is consistent with the proposed structure. The ^{13}C NMR shows the requisite four carbonyl peaks, twelve unique aromatic peaks and ten aliphatics. When comparing the ^1H NMR and ^{13}C NMR spectra of both diastereomers, they appear nearly identical as would be expected. Our preliminary spectral examination did not reveal any distinct differences that could be attributed to the sole change in chirality at the phenylglycine α-center. The spectra show similar peaks with similar chemical shifts throughout, especially for the phenylglycine and acetyl groups. A basic COSY spectrum of the S isomer shows



Acetyl-D-Phenylglycine
2b

L-Leucine-L-Tryptophan, Methyl Ester
8



Acetyl-D-Phenylglycine-L-leucine-L-Tryptophan, Methyl Ester
9b

Scheme 3: Synthesis of Acetyl-D-phenylglycine-L-leucine-L-tryptophan methyl ester **9b**

strong peak intensities along the diagonal and the cross peaks that indicate coupling between the Trp-Trp ^{1}H's, OCH_3-Trp ^{1}H's and Leu-Leu ^{1}H's, consistent with the proposed structure.

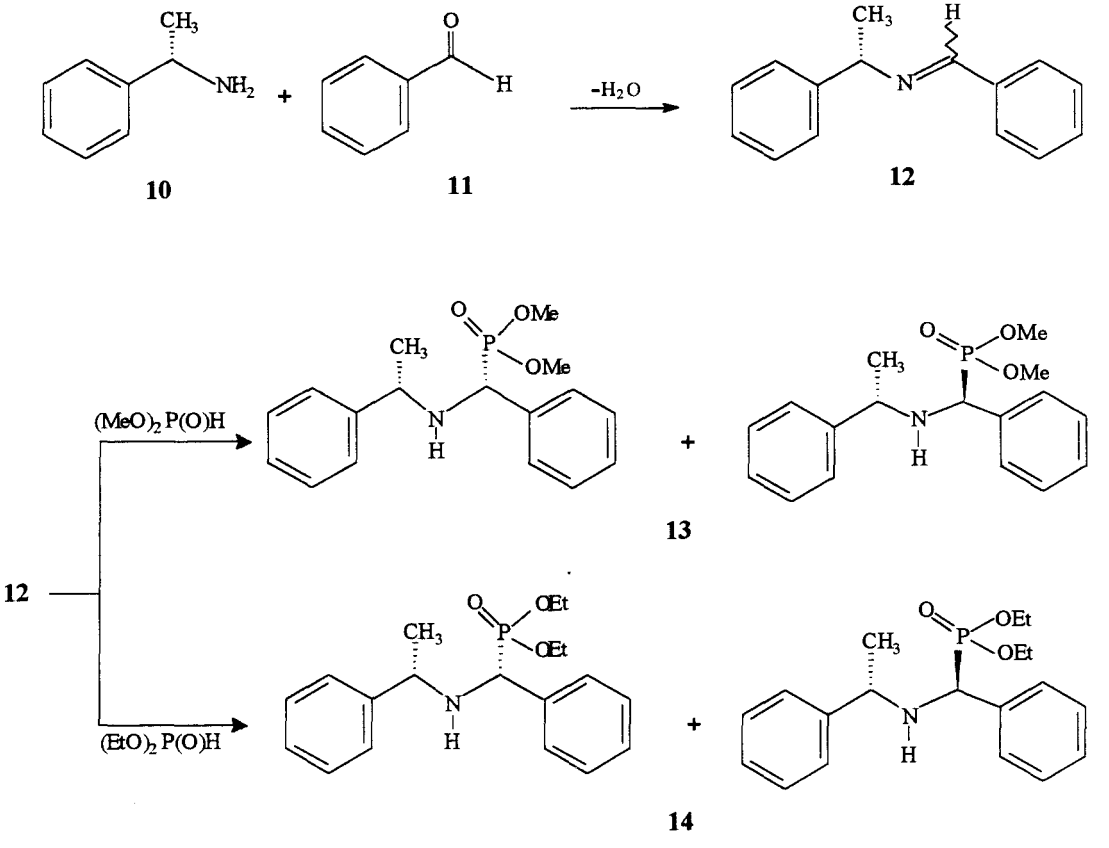
Having obtained the native tripeptides, the synthesis of a terminal phosphono-tripeptide isostere was next undertaken. However, since the insertion of the phosphonamidate moiety into a peptide backbone is rather difficult, a three-tier

approach was designed. The challenge was to first synthesize pairs of optically active phosphonamidate ester diastereomers, followed by their modification into suitable acid derivatives, and finally coupling with the unprotected dipeptide **8** to form the phosphorus-containing tripeptide.

Part B: Synthesis of phosphonamidate compounds.

Synthesis of the phosphonamidate analogues of phenylglycine diastereomers was first attempted by an asymmetric induction method, described initially by Gilmore and McBride, 1972. In this synthesis, the Schiff base between (S)-(-)-α-methylbenzylamine and benzaldehyde was prepared, followed by addition of diethylphosphite or dimethyl phosphite to the pre-formed imine to give diastereomeric mixtures of **13** (methyl esters) or **14** (ethyl esters) in 86.0% and 89.3%, respectively (Zon, 1981) (Scheme 4). Separation of each diastereomer pair was attempted by column chromatography and GC but to date, both techniques have been unable to resolve the stereoisomers. Each of the product sets (**13** or **14**) contains two centers of asymmetry with the α-methyl benzylic center established as the S configuration thereby leading to 2 stereoisomers. The ^{1}H and ^{13}C NMR indicate the correct products as a diastereomeric mixture although the NMR signals appear as duplicates throughout, making the spectra quite complex. In order to confirm the structures of diastereomers **13** and **14**, two alternate procedures

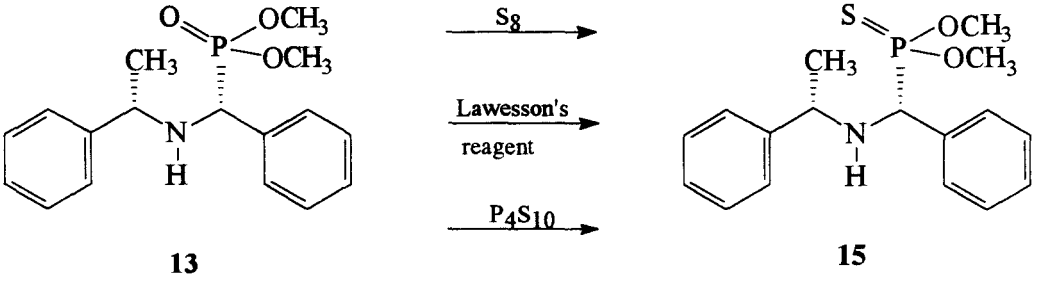
were used to synthesize them: one employing K_2CO_3 (Lekszko et al., 1977) and another using $MgSO_4$ (Suarez, 1994) as dessicant to promote formation of the Schiff bases. The ^1H, ^{13}C, ^{31}P spectral data along with melting points for the products from these procedures were compared to the spectra and melting points from the first procedure and were found to be identical. However, the yields from the last two procedures were much lower than the first: 50.2% and 29.3%, respectively. It was concluded that the



Scheme 4: Synthesis of (R,S)-dimethyl and diethyl-N-(S)-(methylbenzyl)--aminobenzyl phosphonate

the phosphonamidate diastereomers do form in each case, and the original procedure would be preferable (based on yield) for further study.

The next step attempted to transform the diastereomer mixture of phosphonamidate compounds **13** into phosphonamidothioates by replacing the phosphoryl (P=O) oxygen with a sulfur atom (Eqn. 3) (Table 1). It was hoped that the less polar nature of the P=S linkage would afford a more easily separable mixture of the diastereomers, and permit progress of the synthesis at the same time. Several procedures were attempted to exchange P=O for a P=S. First, S_8 with toluene and stirring for 48 hours was tried but thin layer chromatography did not indicate formation of any new compounds, and showed that almost all of the starting material remained. Lawesson's reagent (2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide), which has been shown to exchange a P=O for a P=S, was tried next (Cava et al., 1985). Starting material and Lawesson's reagent (0.5 equiv.) were dissolved in



13 **15** (3)

toluene and brought to reflux for 2-3 hours. TLC indicated numerous spots, and after purification by column chromatogaphy only starting material and Lawesson's by-

products were recovered. The majority of the by-products showed two SCH$_3$ signals between 2-4 ppm and a OCH_3 singlet at 3.9 ppm corresponding to the anisyl group of $(MeS)_2P(S)Ar$. The phenyl protons were split into a 2:2 quartet pattern, 1 ppm apart, and the ^{31}P NMR gave one signal at 84.2 ppm corresponding to the P=S linkage of these by-products. Additionally, by-products formed from the $(CH_3CH_2O)_2P(O)R$ phosphonamidate failed to show the ethoxy signals expected from the diethyl esters.

Table 1: Summary of Sulfurization Conditions

Starting material	**Reaction conditions**	**Expected products**	**Observations**
13 14	S_8, toluene, 48 hr	P=S	TLC: only sm
13 14	Lawesson, reflux, toluene, purif. by column	P=S	Lawesson's by-products isolated
13	Lawesson, reflux, toluene, purif. by extraction	P=S	sm + Lawesson's by-prod. isloated
14	P_4S_{10}, CH_2Cl_2, reflux	P=S	sm recovered

With the thought that phosphorus ester hydrolysis may have accompanied P=O/P=S exchange, an extraction procedure was attempted in place of the column. After reflux, the solution was diluted in diethyl ether, washed with 10% HCl, neutralized with

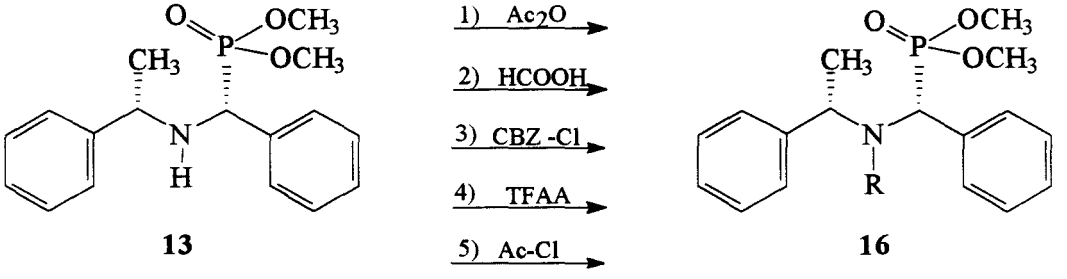
NaOH and washed with ether. However, Lawesson's by-products similar to those described before were again obtained.

In an effort to eliminate additional impurities, Lawesson's reagent was recrystallized and toluene was redistilled. All the reactions were repeated under the same conditions, but the results remained dissapointing; either starting material or Lawesson's by-products were collected. Combinations of reactions using recrystallized Lawesson's with regular toluene or purified toluene with regular Lawesson's were tried and were unsuccessful. A final trial to conduct a P-O/P=S exchange used P_4S_{10} in CH_2Cl_2 and overnight reflux. After recrystallization from absolute ethanol, ^{31}P NMR indicated starting material. It is not yet fully understood why all of these reactions failed to give the desired product. Perhaps combined steric factors from the phenyl and phosphorus ester methoxy or ethoxy groups are a problem or intramolecular hydrogen bonding between the amine proton and the oxygen weakens one of the P-O bonds, making the replacement slower.

While still searching for a good sulfurization reagent or different conditions, our alternative approach sought to acetylate the amine group of either compounds **13** and **14** (Eqn. 4) (Table 2). The acetyl group was chosen as a blocking group since this truncated amide linkage resembles the native peptide structure. The intent at this point was that after successfully blocking the amine group to form **16**, debenzylation would leave the desired acetyl-NH-phosphonamidothioate moiety. This fragment would be

coupled with the unprotected dipeptide **8** using DCC methodology to obtain the phosphorylated tripeptide.

Initial acetylation attempts used acetic anhydride, TEA, DMAP, pyridine conditions at room temperature overnight or at reflux for 1-2 hours. In each case, thin



13 **16** **(4)**

1), 5) R = $CH_3C(O)$
2) R = HC(O)
3) R = Cbz
4) R = $CF_3C(O)$

layer chromatography showed that a majority of the starting material was still present after stirring overnight or after refluxing for up to 3 hours. Workup consisted of several washings with 0.50 M cupric sulfate to remove the pyridine. For all trials, ^{1}HNMR of the crude mixture did not indicate the expected product. The CH_3 singlet from the acetyl group at 2.0 ppm was not seen. In some cases, extra peaks (not seen before) appeared between 5-6 ppm and indicated possible decomposition or elimination pathways. In other cases, all the peaks between 3-4 ppm (from the starting material) disappeared entirely. Workup was changed from washing with cupric sulfate solution

to removal of pyridine on the rotary evaporator followed by column chromatography. Mixtures of starting material with pyridine and different by-products similar to those described above were collected. Since acetylation conditions failed to give the desired product the reactivity of compound **13** was in question and new routes were considered (Eqn. 4). A mixture of formic acid (catalytic), acetic anhydride and overnight reflux were tried, but thin layer chromatography again indicated only the presence of starting material.

Table 2 : Summary of Acetylation Conditions

Starting material	**Reaction conditions**	**Expected products**	**Observations**
13 14	Ac_2O, TEA, RT CH_2Cl_2, DMAP	N-Ac	brown tar
13	HCOOH, Ac_2O reflux 24 hr	N-HC(O)	TLC: only sm; rxn didn't occur
13	TFA, $CHCl_3$, RT then reflux 3 hr	N-Ac	TLC: only sm; rxn didn't occur
13	Acetyl-Cl, RT	N-Ac	TLC: only sm

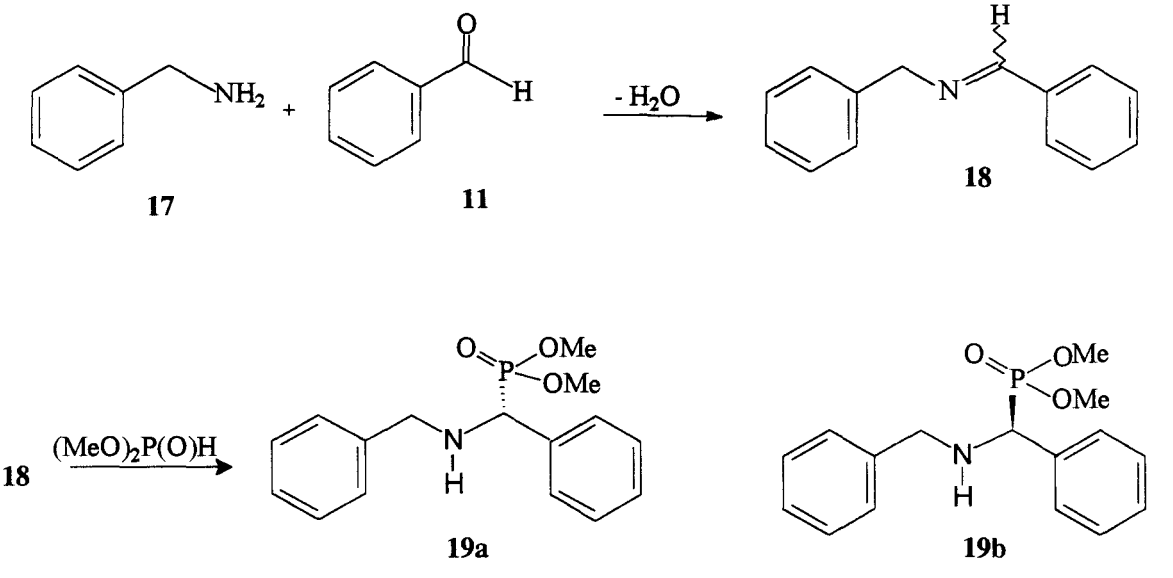
Protection of the amine moiety of **13** with a Cbz was tried next (CBZ-Cl, toluene/$NaHCO_3$) (Eqn. 4). After stirring for 2 hours at room temperature only

starting material and Cbz-Cl were present on thin layer chromatography plate. Since a biphasic system was used and the starting material was partially soluble in water, probably the starting material was in the aqueous layer, and did not react well with Cbz-Cl. A small amount of soap or tetrabutylammonium bromide were added as phase transfer catalysts, but after stirring overnight at room temperature, thin layer chromatography indicated that starting material, Cbz-Cl and benzyl alcohol remained (from the decomposition of Cbz-Cl). Trifluoroacetic anhydride in $CHCl_3$ at room temperature conditions was tried next in an attempt to increase the acetylation reactivity but this reaction also failed to produce the product as evidenced by thin layer chromatography. Even after reflux was applied for 3 hours, the amount of product did not increase. The last attempt at acylation employed acetyl chloride which also failed to produce the desired product.

For all the failed acetylation reactions it is believed that the main reason this is such a difficult reaction is due to the overall geometry of the molecule. The two phenyl groups along with the phosphonate moiety are large enough to sterically hinder the attack on the reagent by the amino NH group. The acetylating reagents probably have a difficult time channeling between these sterically congested groups in order to reach the amino group and react with it. With this in mind, the next approach was to change one end of the molecule and create a less crowded system.

Benzylamine was chosen to replace (S)-(-)-α-methylbenzylamine in the synthesis

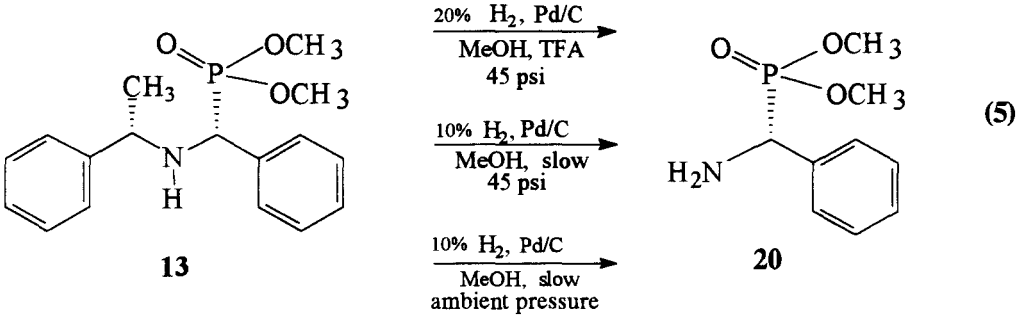
of phosphonamidate diastereomers **19a** and **19b** thus eliminating one chiral center and a methyl group. The failure of the chiral auxiliary sequence convinced us to alter our strategy. All the acetylation reactions were repeated under the conditions previously described and the results were still negative. Only starting material was seen on TLC and ^{1}H NMR. This proved that a) the α-methyl group in **13** or **14** did not sterically hinder the acetylation since the reaction failed even in the group's absence and, b) even in the absence of the methyl group the system was probably still crowded. In all the



Scheme 5: Synthesis of (R,S)-dimethyl-N-benzyl-aminobenzyl phosphonate.

prior amine protection sequences we hoped to achieve a resolvable diastereomer pair as a benefit. Our alternative approach then was to remove the *N*- benzyl group to obtain a

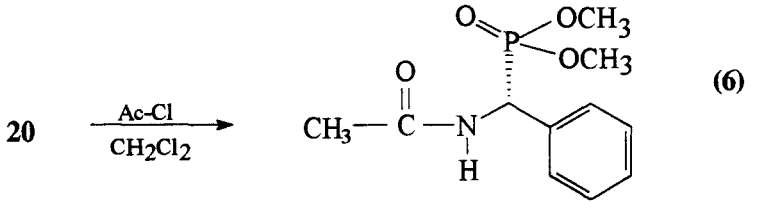
smaller molecule at the amine end which would have a greater chance at acetylation (Eqn. 5). The primary amine **20** was successfully obtained in a 91% yield by hydrogenolysis of **13** using 20% Pd/C and 1-2 eq. TFA in the Paar hydrogenator (40 psi). Initially, 10% Pd/C at balloon pressure followed by 10% Pd/C and 40 psi were tried, but the reaction showed a significant amount of starting material even after 16 h. Increasing the Pd catalyst concentration to 20% reduced the amount of unreacted starting material left after 16 h, but the reaction remained incomplete. When 1-1.5 or 2 eq of TFA was added however, the reaction finished in 2.5-3 h at 40 psi and in 4-5 h



13 **20** **(5)**

at ambient pressure. After purification by column chromatography, ^{1}H NMR shows the broad amine peak, 2 pairs of doublets from the 2 OCH_3 groups, a doublet from the chiral C-H and the phenyl protons with the correct integration. Additionally, ^{13}C NMR spectra confirmed the structure through chemical shifts and the correct number of carbon absorbencies.

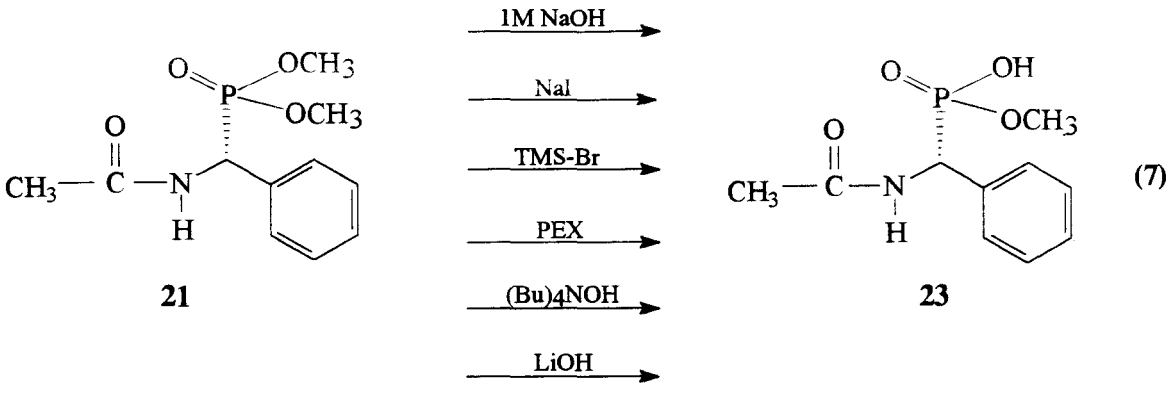
The primary amine of **20** was acetylated next using 2 eq of acetyl chloride in CH_2Cl_2 at room temperature for 2-3 h (Eqn. 6). Following workup and recrystallization from $CHCl_3$/pet ether, the *N*-acetyl derivative **21** was obtained in a 51.3% yield. The ^1H NMR shows the acetyl CH_3 at 1.93 ppm, the 2 pairs of doublets from the methoxy groups and the chiral H which now shifted to 5.5 ppm from 4.27 ppm (for **20**) and appears as a quartet.



(6)

21

Although the removal of the α-methylbenzyl group reduced our opportunities for a chiral synthesis, the scheme could be advanced. Having successfully synthesized **21**, the next step involved hydrolysis of one of its methyl esters to obtain the target **23**. Twelve different procedures were tried unsuccessfully (Table 3) including 1M NaOH (1, 1.2, 1.5, 2.0 eq) NaI (Morita et al., 1978), TMS-Br (Schmidt, 1981; McKenna et al., 1979), PEX, $(Bu)_4NOH$, LiOH, at room temperature or at reflux. Either no reaction, 'partial' (both methoxy peaks are seen but one is smaller than the other) or complete hydrolysis of both esters takes place after aqueous workup (in the case of 'partial' hydrolysis the reactions were started at room temperature then heated to reflux

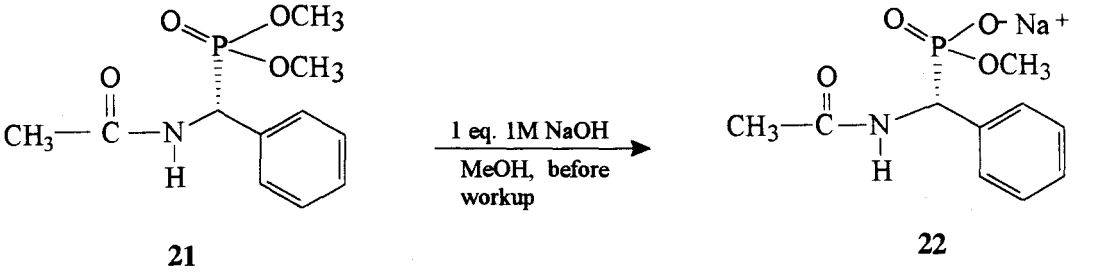


21 23 (7)

Table 3: Summary of Hydrolysis Conditions

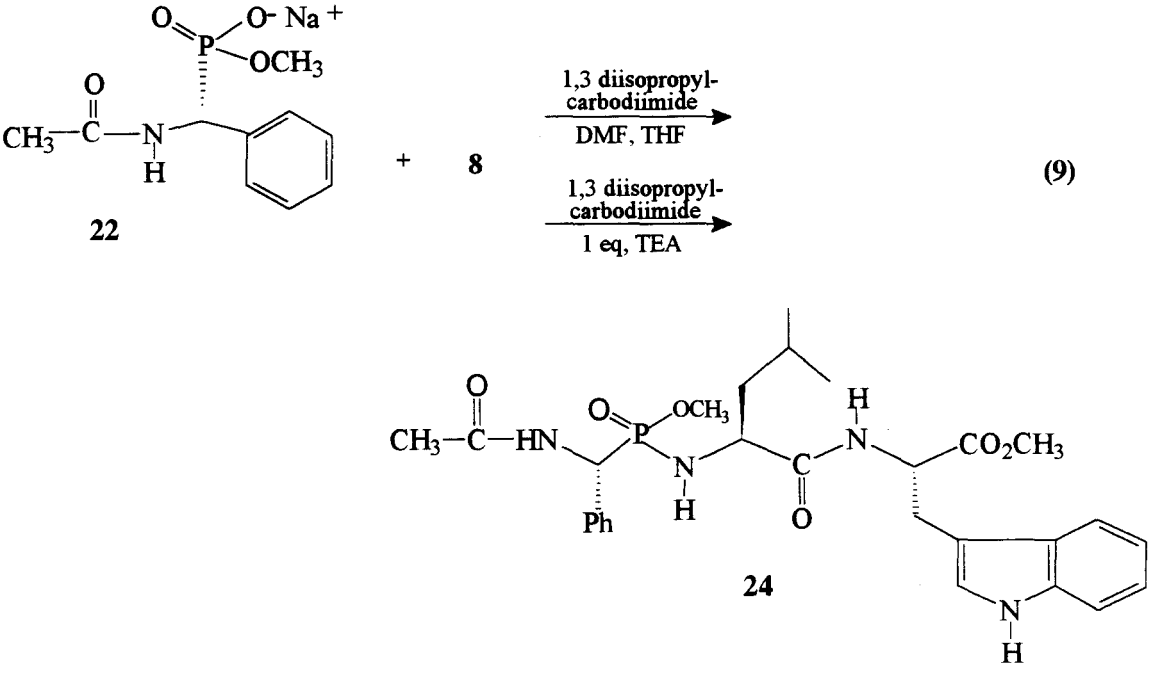
Starting material	Reaction conditions	Expected products	Observations
21	1M NaOH, 1eq, reflux	23	starting material
21	1M NaOH, 1.2eq, RT to reflux	23	"partial hydrolysis"
21	1M NaOH, 1.5 eq, reflux	23	diacid
21	1MNaOH, 1.5 eq, RT to reflux, 48hr	23	diacid
21	1MNaOH, 2eq RT(8h) to reflux (4h)	23	diacd
21	NaI, 1eq, RT	23	starting material
21	NaI, 1eq + 1xtal I_2 RT	23	starting material
21	NaI, 2eq, RT	23	starting material
21	TMS-Br, 1eg, RT	23	diacid
21	LiOH, 1eq, RT to reflux	23	starting material
21	2N NaOH, 0.5eq, RT	23	starting material
21	2N NaOH, 1 eq	23	diacid

and allowed to proceed for 12-48 h). When this was first observed. extra care was given in the extraction step, by reducing the strength of the acid (from 20% HCl to 15, 10 and 5%). It is important to mention that this reaction worked well on the sterically more demanding compound **13** (Eqn. 11). The hydrolysis of **13** was conducted with 1 eq of 1M NaOH in ethanol with reflux for 5 hours. After aqueous workup with 20%HCl and one recrystallization, the pure product was obtained in a 74% yield. This reaction worked so well in fact, that we were quite confident that it would work on the smaller compound **21**. However, after many attempts and careful extraction procedures the reaction failed to give the desired product **23** (Eqn. 7). It is not fully understood why the reaction leads to other products since it worked consistently on **13** under the same conditions. One reason for the poor isolation of **23** could be the aqueous workup where enough water pushes the reaction toward diacid formation. With this in mind, the next approach was to isolate the phosphonate salt which formes prior to workup (Eqn. 8). This suggests that the monoacid does form but it is in the form of a salt which is unstable to the aqueous workup. The phosphonate salt monoester **22** was isolated from the reaction between 1 eq of **21** and 1 eq of 1M NaOH at reflux after thin



21 **22** **(8)**

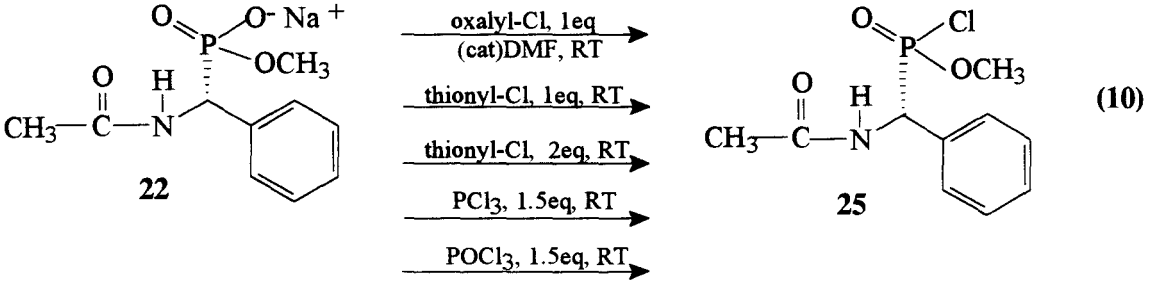
layer chromatography indicated consumption of the starting material (Eqn. 8). The ^1H NMR shows the acetyl singlet and one doublet at 3.7 ppm from the remaining methoxy group, a doublet from the chiral H and the phenyl protons with the correct integration. Most importantly, ^{31}P NMR shows a singlet at 17.9 ppm, - approximately a 7 ppm shift upfield from **21**. Coupling between the salt and the deprotected dipeptide **8** was attempted in an effort to obtain the phosphorus-containing tripeptide **24**. The reaction was tried several times, using equimolar amounts of salt, deprotected dipeptide and 1,3 diisopropylcarbodiimide as coupling reagent in absolute ethanol as solvent.



The reaction was run at 0 ^0C for 5 h and allowed to proceed to room temperature. After 30 h, ^1H NMR indicated only dipeptide and DCU. The reaction was repeated using

equimolar amounts of salt, 1,3 diisopropylcarbodiimide and trifluoroacetic acid (to restore the protonated form of the acid) with two equivalents of deprotected dipeptide and DMF/THF as a solvent. After 30 h however, ^{1}H NMR indicated decomposed dipeptide. This reaction was abandoned and efforts were shifted toward converting the phosphorus acid salt **22** to a phosphoryl chloride **25** (Eqn. 10) which would then be coupled to the amine of a deprotected dipeptide (Table 4).

Oxalyl chloride, thionyl chloride (Musiol et al, 1994; Bhongle et al., 1987; Camp et al., 1992), PCl_3 and $POCl_3$ were tried as chlorinating agents in 1, 1.5 and 2 eq. for the formation of phosphoryl chloride. For reactions with oxalyl chloride, 0.1 eq of DMF was used as recomended for the production of acid chlorides (Musiol et al, 1994). All reactions were unsuccessful and only starting material was isolated for reactions employing oxalyl-Cl, thionyl-Cl and $POCl_3$. Decomposition of starting material took place when PCl_3 was used.

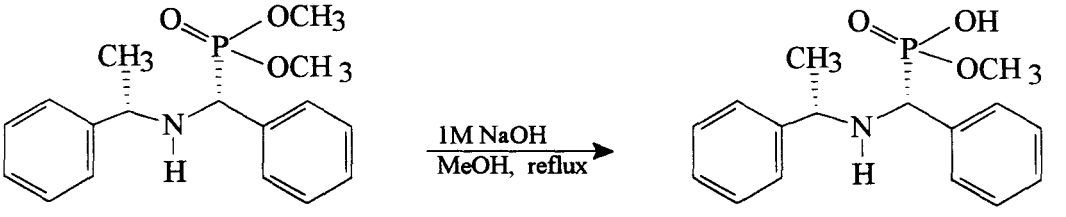


(10)

Table 4: Summary of Chlorination Conditions

Starting material	Reaction conditions	Expected products	Observations
22	oxalyl-Cl, 1 eq (cat)DMF, RT	P(O)-Cl **25**	starting material
22	thionyl-Cl, 1 eq RT	P(O)-Cl **25**	starting material
22	thionyl-Cl, 2 eq, RT	P(O)-Cl **25**	starting material
22	PCl_3, 1.5 eq, RT	P(O)-Cl **25**	starting material
22	$POCl_3$, 1.5 eq, RT	P(O)-Cl **25**	starting material

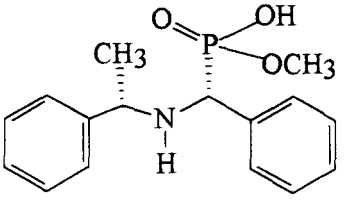
In addition to sulfuration and acetylation reactions, also conducted with **13** and **14**, included the hydrolysis of the phosphorus methyl ester of **13**. A solution of **13** with 1M NaOH in absolute ethanol at reflux followed by workup and recrystallization



13 **26** **(11)**

from water were used to obtain the mono methyl ester **26** in 74% yield. The ^1H NMR shows 3 pairs of doublets: one from the aliphatic CH_3, one from the P-OCH_3, and one

due to the α-CH phosphoryl. In addition, a quartet from the $CH(CH_3)$ on the second chiral center is also seen. The ^{13}C NMR shows the four aliphatic and twelve aromatic peaks as expected. The ^{31}P NMR has two peaks appearing at 12.36 and 12.57 ppm in a 1:3 ratio representing the diastereomer pair. Coupling between **26** and **8** (deprotected dipeptide Leu-Trp) was tried next as an attempt at obtaining a phosphorus-containing peptide **24** (Eqn. 12). 1,3 Diisopropylcarbodiimide or DCC were tried as coupling reagents. DMF had to be used as a solvent due to the poor solubility of **26** in any other organic solvent. The reactions were conducted at 0 °C for 5-7 h and continued at RT overnight. Only starting materials and DCU were seen on TLC and by ^1H NMR.

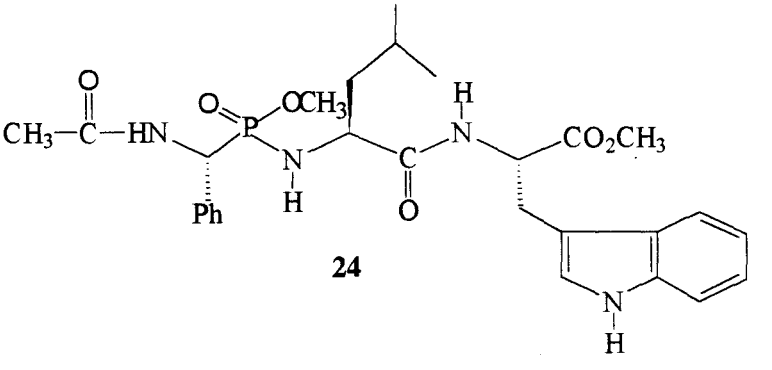


26

\+ **8**

DCC, DMF

(12)

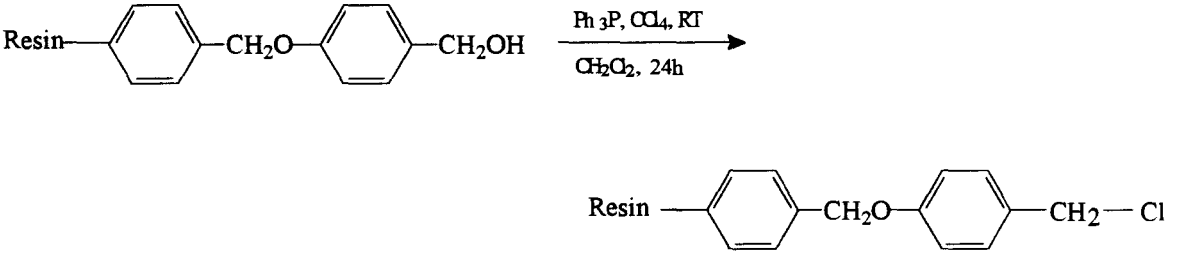


24

Part C: Solid phase peptide synthesis.

In parallel studies, we also attempted to synthesize the tripeptide **9a** by solid phase peptide synhesis. This is a faster method currently used mainly for the preparation of large peptides. Our approach involved the use of 9-fluorenylmethyl chloroformate (FMOC) blocked amino acids supported on a p-alkoxybenzylalcohol resin (Wang resin) or Cbz blocked amino acids supported on a Merrifield resin (1% crosslinked polystyrene - divinylbenzene). However, before the synthesis of **9a** was attempted, we first tried to synthesize the tripeptide Leu-Leu-Leu as a simple model.

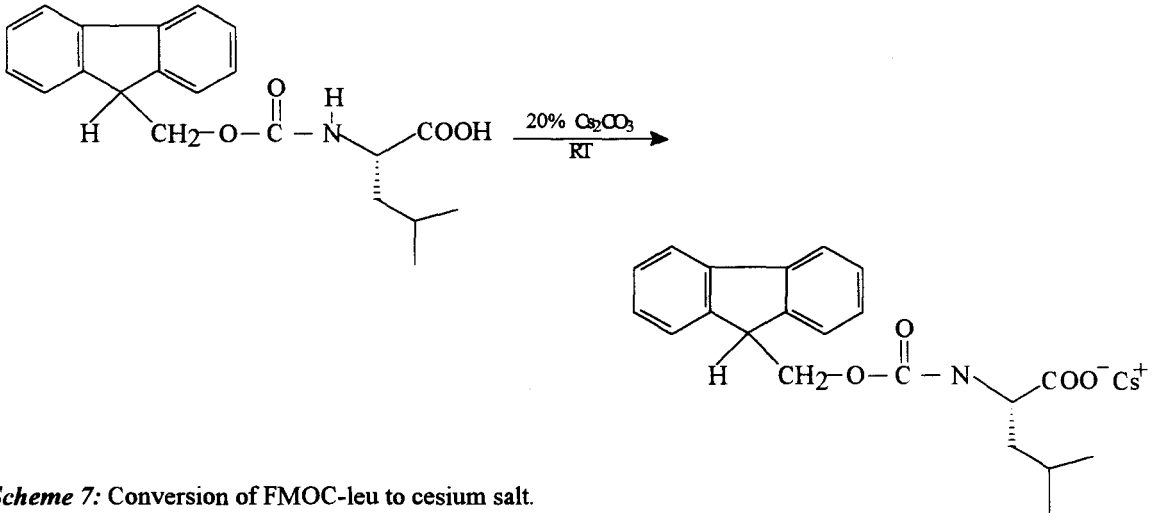
For the FMOC blocked amino acids with p-alkoxybenzylalcohol resin method



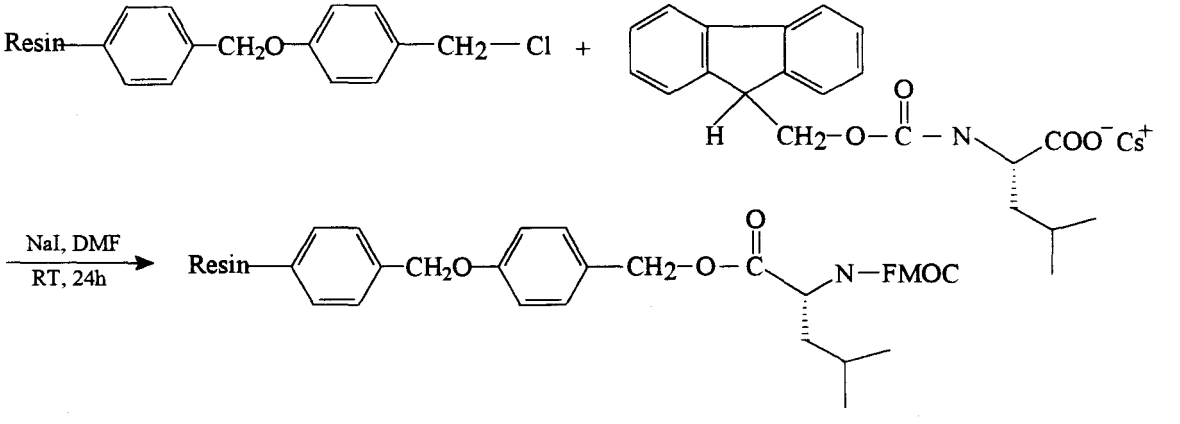
Scheme 6: Chlorination of the resin.

the following outline was used: a) chlorination of the resin with Ph_3P and CCl_4 (Scheme 6); b) conversion of FMOC-Leu to the cesium (Cs) salt; c) coupling of FMOC-Leu-Cs salt to the chlorinated resin via NaI; d) deprotection of FMOC group by cyclohexylamine/DMF, followed by coupling with FMOC-Leu via DCC; e) repetition of step d) followed by ninhydrin test.

Initially, we attempted to chlorinate the resin (p-alkoxybenzylalcohol) using 1 eq of Ph_3P and 2 eq of CCl_4 per gram of resin. The reaction was carried out in an Erlenmeyer flask placed in a shaker for 36 h at room temperature. Following reaction washing with methylene chloride and drying, the resin was stored in the freezer. FMOC-Leu was next converted to FMOC-Leu-cesium salt salt using a 20% Cs_2CO_3

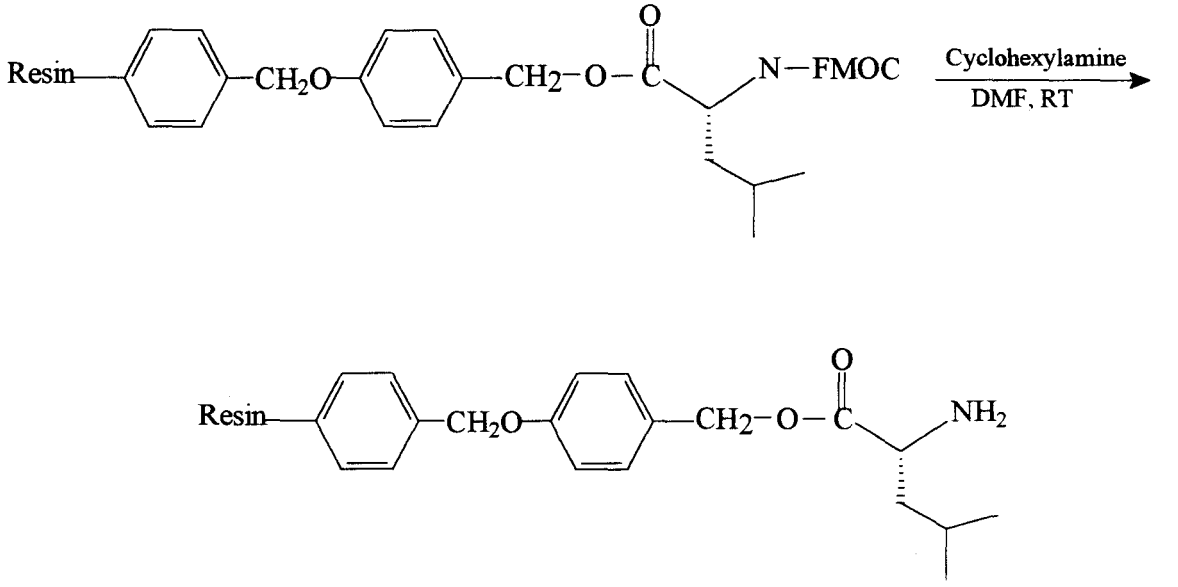


Scheme 7: Conversion of FMOC-leu to cesium salt.



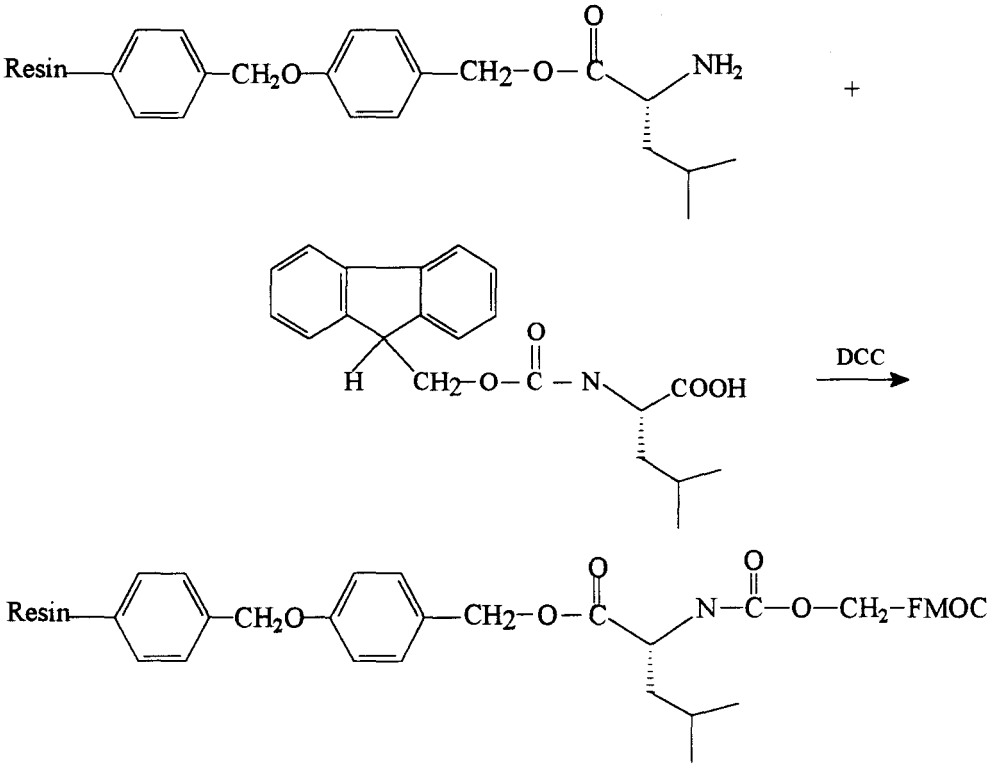
Scheme 8: Coupling of FMOC-leu-Cs salt to the chlorinated resin.

solution at RT (Scheme 7); the salt was then coupled to the chlorinated resin via NaI in DMF over 24 h (Scheme 8). The resin was then washed with DMF (2 x 50 mL), CH_2Cl_2 (2 x 50 mL), MeOH (2 x 50 mL), and dried. At this point it was important to find out how much of the amino acid coupled onto the resin. Usually the resin is sent for elemental analysis; in this case, it was assumed that approx 50% coupling took place and we proceeded to the next step. A 50:50 mixture of cyclohexylamine and DMF was used to soak the resin for 1 h to remove the FMOC group (Scheme 9). After several washings with DMF (50 mL), CH_2Cl_2 (50 mL), EtOH (50 mL), CH_2Cl_2 (50 mL), DMF (50 mL), the resin was reacted with FMOC-Leu and DCC to obtain



Scheme 9: Deprotection of FMOC group.

FMOC-Leu-Leu-resin. The last 2 steps (soaking in 50:50 mixture of cyclohexylamine/DMF and coupling with DCC) were repeated once more (Scheme 10) After washing with DMF (50 mL) and CH_2Cl_2 (50 mL) and overnight drying under vacuum, a ninhydrin test was performed on a small quantity of resin to determine the amount of coupling present.

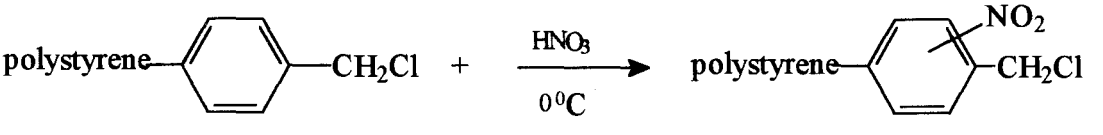


Scheme 10: Coupling of FMOC-leu via DCC.

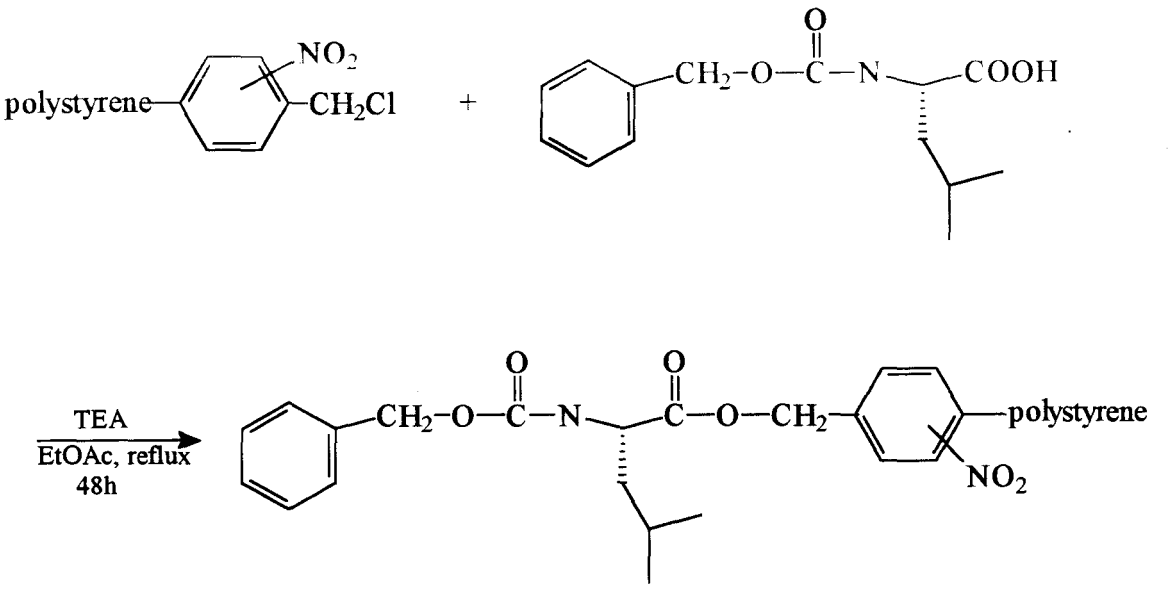
Three solutions were prepared for the ninhydrin test; 1) 2 mL of 0.01 KCN dil. to 100 mL with pyridine; 2) 500 mg ninhydrin in 10 mL of n-BuOH; 3) 80 g

phenol in 20 mL n-BuOH. A small amount of resin was added to 3 test tubes and 3-4 drops of each solution to each tube. The test tubes were heated in boiling water for 3-4 min. Upon heating, the beads turned bluish - corresponding to approximately 80% coupling (this was the first test performed on the resin; usually, elemental analysis is performed after each step to determine the amount of coupling). The final step involved deprotection from the resin via 19:1 TFA:anisole solution, followed by extractions with MeOH and ether. After drying, a dark brown residue was left. However, the ^{1}H NMR did not show the correct product; and only a singlet at 2.0 ppm and small impurities between 3.5 - 4.5 ppm were seen.

For the preparation of Cbz-blocked Leu with Merrifield resin the following outline was used: a) nitration of the resin; b) esterification of the resin with Cbz-Leu; c) removal of Cbz-group and coupling with a second Cbz-Leu; d) repeat of step c) and coupling with a third Cbz-Leu; e) cleavage of Cbz and peptide from the resin. The first step involved nitrating the resin by HNO_3 at 0 °C for 2 h (Scheme 11), followed by a series of washes: 3:1 dioxane/H_2O (100 mL), 3:1 dioxane/3N HCl (100 mL),

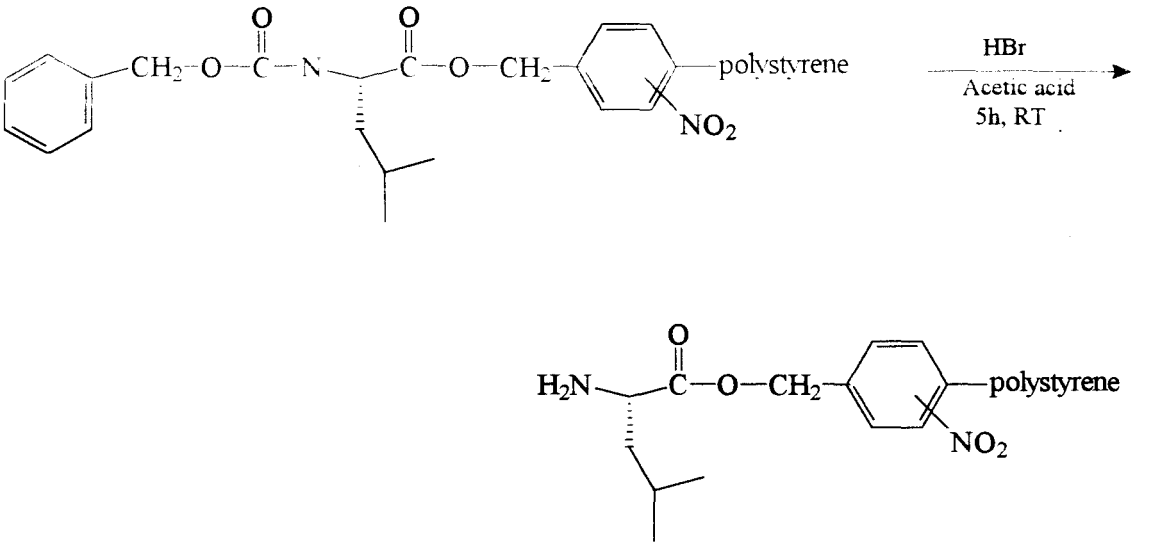


Scheme 11: Nitration of the resin.

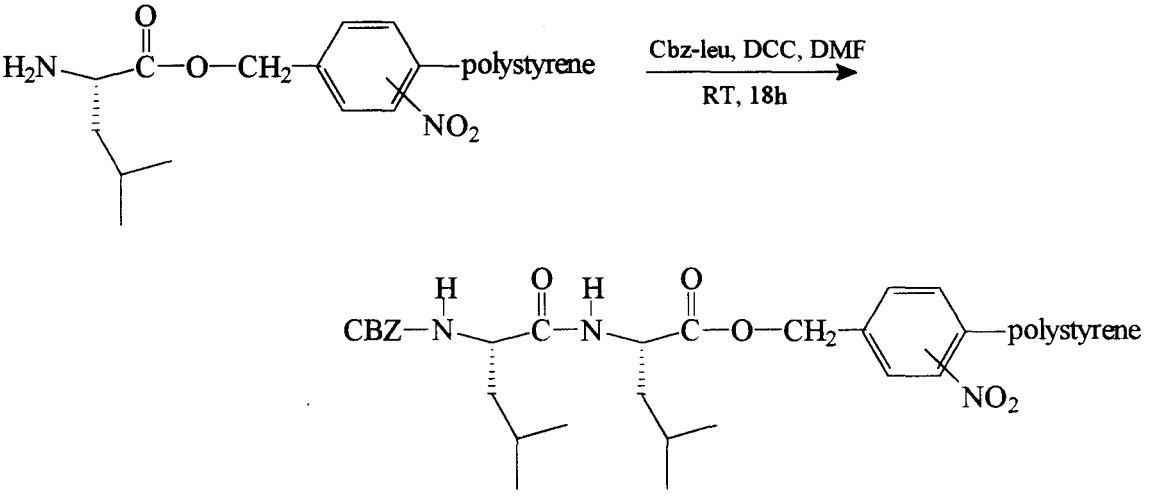


Scheme 12: Esterification of the resin.

H_2O (300 mL, soaking for 2 h), dioxane (2 x 50 mL)MeOH (2 x 100 mL) and drying. The next step was esterification with equimolar amounts of TEA and Cbz-Leu at reflux in EtOAc (Scheme 12) for 48 h, followed by the addition of a TEA/EtOAc/glacial acetic acid solution with continued reflux for another 4 h. After washings with ethyl acetate, ethanol, water, methanol (2 x 50 mL) and drying, deprotection of the Cbz group was conducted as follows. A solution of 30% HBr in glacial acetic acid (Scheme 13) was used to soak the resin for 5 h in the shaker, followed by washings with glacial acetic acid (2 x 50mL), ethanol (2 x 50 mL), DMF (2 x 50 mL) and neutralization by TEA in DMF. A coupling reaction between Cbz-Leu and DCC in DMF was tried



Scheme 13: Deprotection of the Cbz group.



Scheme 14: Coupling with Cbz-leu.

next (Scheme 14). It was left in the shaker at RT for 18 h, then washed with DMF (50 mL), EtOH (50 mL) and glacial acetic acid (50 mL) to remove DCU. The last two steps (deprotection of Cbz by 30% HBr solution and coupling with Cbz-Leu) were repeated once more followed by cleavage of the peptide from the resin. This step involved washing the resin in 30% HBr/EtOAc solution overnight, then with acetic acid, EtOH, DMF and 2N NaOH. The filtrate was treated with 20% HCl, extracted with EtOAc, and after drying a yellow liquid was obtained. Again, ^{1}H NMR did not show the expected product. Only a doublet at 2.2 ppm corresponding to 7H was seen. For both procedures, we believe that the coupling steps were successful as indicated by the ninhydrin test. The reagents used in the deprotection steps however, were probably too harsh and could have decomposed the desired products.

Since both solid phase procedures failed to give the expected Leu-Leu-Leu tripeptide, the synthesis of the native tripeptide was not attempted; instead, work was resumed on the synthetic pathways.

Conclusion

The native tripeptide acetyl-phenylglycine-*l*-leucine-*l*-tryptophan, methyl ester was synthesized as the "S" and "R" isomers **9a** and **9b**, to serve as the natural and unnatural model for the transition state analogue of phosphoramidon. The tripeptides were synthesized using solution methods and DCC as coupling reagent in a 52.3% and

49.2% yields, respectively. Several purification steps such as washings, tirturations and recrystallizations were nedeed to remove all of the by-product DCU and these manipulations account for the reduced yield. The tripeptides have similar features in the ^{1}H and ^{13}C NMR spectra: they show nearly identical chemical shifts with small changes in the ^{13}C NMR. A COSY spectrum of the S isomer was taken and showed strong peak intensities along the diagonal; moreover, the cross-peaks indicate coupling between tryptophan's protons, methoxy-tryptophan protons and leucine's protons.

A phosphorus analogue of acetyl-phenylglycine was also synthesized, and approaches toward the preparation of the phosphorus-substituted tripeptide were initiated. The phosphorus analogue of acetyl-phenylglycine was initially synthesized as a diastereomeric mixture (**13** and **14**) which could not be separated into its individual stereoisomers by conventional flash chromatography or GC. Efforts shifted toward modifying the diastereomeric mixture into compounds which could be easier separated as well as being suitable for coupling with the deprotected dipeptide to obtain the phosphorus-substituted tripeptide. Modifications included attempted thionation of the P=O, acetylation of the amino group, hydrolysis of one of the methoxy groups, dealkylation of a benzyl group and chlorination of a phosphonate ester. Only two steps, namely, dealkylation and hydrolysis were successful in converting the phosphonamidate diastereomer **13** into a stable phosphonate salt **22**. This salt represents a phosphorus analogue of acetyl-phenylglycine. Attempts to hydrolyze the

salt to a monoacid or to chlorinate it were not successful. It was hoped that in this way the salt could be coupled to the deprotected dipeptide **8** to obtain the desired phosphono TS analogue.

Future studies could explore alternative routes for insertion of the analogue **22** into the peptide backbone, to obtain the transition state analogue of phosphoramidon. When this will be achieved, we could enhance our understanding in two areas: a) on the effect that a phosphorus center substituting for a peptide carbonyl can have on the conformation and dynamics of a peptide backbone, and b) on the requirements of zinc-containing enzymes The native tripeptides, **9a** and **9b** will serve as essential models for study in both areas, especially for comparison of enzyme interaction.

CHAPTER III

EXPERIMENTAL SECTION

General methods. Commercially available reagents were purchased from Aldrich Chemical Co., (Milwaukee, WI), Chemical Dynamics Co., (South Plainfield, NJ), and Sigma Chemical Co., (St. Louis MO). All solvents and reagents were purified when necessary by standard literature methods (Perrin et al., 1988). Moisture sensitive reactions were conducted under an argon atmosphere utilizing standard techniques (Brown et al., 1975).

Melting points were determined on a Mel-Temp melting point apparatus and are uncorrected. Analytical thin layer chromatography (TLC) was conducted on E. Merck aluminum-backed, 0.2 mm silica gel TLC plates. Visualization was accomplished with an ultraviolet lamp and ninhydrin (5% ninhydrin in ethanol), ammonium molybdate (2% ammonium molybdate in 90:10:1 distilled water-concentrated sulfuric acid-ceric ammonium sulphate) and DBQ (0.5% 2,6-dibromoquinone chlorimide in ethyl ether) stains with heat. Flash chromatography was performed with Kieselgel 60, 230-400 mesh (Merck) (Still et al., 1978). Elemental analyses were performed by Midwest Microlab Ltd., Indianapolis, IN.

Proton (^{1}H), carbon (^{13}C) and phosphorus (^{31}P) NMR spectra were taken at 300 MHz, 75.7 MHz and 121.5 MHz using a Varian VXR 300 NMR instrument. The chemical shifts of the ^{1}H NMR and ^{13}C spectra were referenced to tertramethyl silane (TMS) (δ = 0.00 ppm). Chemical shifts of the ^{1}H peaks are relative to the deuterated chloroform singlet (δ = 7.24 ppm) and ^{13}C peaks are relative to the middle of the deuterated chloroform triplet (δ = 77.00 ppm). ^{31}P NMR spectra were taken in deuterated solvents with phosphoric acid as an external standard in chloroform-d (δ = 0.00 ppm). Infrared data (IR) were obtained on a Perkin-Elmer Model 1310 instrument in chloroform, methanol or ethyl acetate in a liquid cell. Rotation data were recorded on a Perkin-Elmer Model 241 Polarimeter (Na lamp) at room temperature in appropriate solvents (c = 100 x g/mL).

Part A: Synthesis of Phe-Leu-Trp Tripeptide

N-Acetyl-*l*-phenylglycine 2a. (Hongo et al., 1981). *l*-Phenylglycine **1** (5.0 g, 3.3 mmol) and 33.0 mL of 1M NaOH solution were stirred vigorously and chilled to 0 ^{0}C. Cold NaOH (6.66 mL, 1M) and 0.31 mL of acetic anhydride were added and the mixture was checked for basicity. The intermittent addition of NaOH and acetic anhydride (same amounts) was repeated 4 more times while testing for alkalinity at each step. Stirring continued for 30 min and the solution was then acidified to pH 1 with concentrated HCl and the solution allowed to crystallize at 0 ^{0}C overnight. White crystals were collected by filtration and washed with cold water. Recrystallization from water affords long, needle-like crystals. Yield: 74%. Melting point: 192-194 ^{0}C. $[\alpha]_{D^{24}} = +203.87^0$ (c 2.3, MeOH). ^{1}H NMR (D_2O): δ 1.99 (s, 3H), 5.43-5.46 (d, *J* = 7.6 Hz, 1H), 7.31-7.44 (m, 5H), 8.45-8.47 (d, *J* = 7.5 Hz, 1H). ^{13}C NMR: δ 22.9, 56.3, 127.1, 127.8, 128.4, 136.9, 169.2, 172.3.

Cbz-*l*-Leucine 4. (Choudry, et al., 1980). *l*-Leucine **3** (2.624 g, 20.0 mmol) and sodium bicarbonate (4.2 g, 50.0 mmol) were dissolved in 25 mL of water and 15 mL of a saturated $NaHCO_3$ solution at room temperature using a magnetic stirrer. Carbobenzyloxy chloride (Cbz-Cl) was added dropwise over a period of 30 minutes. The reaction was monitored by TLC for loss of CBZ-Cl, and was left stirring

overnight. At completion, the reaction mixture was extracted once with 20 mL of diethyl ether and the aqueous layer was acidified to pH 3 with 20% HCl while cooling and stirring. The resulting white precipitate was extracted into 45 mL of ethyl acetate (3 x 15 mL). The ethyl acetate layers were combined and dried over anhydrous magnesium sulfate for 1 h. The mixture was filtered and the solvent was removed *in vacuo* to afford Cbz-*l*-leucine which was in the form of a thick, pale-yellow oil. Purification by Kugelrohr distillation at 0.5 mm pressure yields 75.1%. R_f = 0.16 (MeOH:$CHCl_3$, 1:9). Boiling point: 122 ^{0}C. $[\alpha]_{D24}$ = -5.796^0 (c 2.45, EtOAc). ^{1}H NMR ($CDCl_3$): δ 0.87-0.95 (m, 6H), 1.53-1.70 (m, 3H), 4.39-4.40 (m, 1H), 5.09-5.13 (m, 2H), 5.23-5.26 (d, *J* = 8.5 Hz, 1H), 7.31-7.32 (m, 5H), 10.51 (br, 1H). ^{13}C NMR: δ 21.7, 22.9, 24.8, 41.4, 52.4, 67.1, 128.0, 128.1, 128.4, 135.9, 156.0, 177.7.

***l*-Tryptophan, methyl ester 6.** *l*-Tryptophan, methyl ester hydrochloride **5** (5.0 g, 19.6 mmol) was dissolved in 40 mL of distilled THF. Triethylamine (2.74 g, 19.3 mmol) was added, and the reaction was stirred for 20 minutes. The mixture was filtered through a frit with celite and the solvent was removed *in vacuo*. *l*-Tryptophan methyl ester was obtained in the form of light - yellow oil and was used directly in the next step without further purification.

Cbz-*l*-leucine-*l*-tryptophan, methyl ester 7. (Shiba et al., 1974). Cbz-*l*-leucine **4** (6.06 g, 22.9 mmol) and DCC (4.72 g, 22.9 mmol) were dissolved in 150 mL of distilled methylene chloride and chilled to 0 ^{0}C. *l*-Tryptophan methyl ester **6** from the prior step was added and the reaction mixture was stirred under argon atmosphere for 3 h at 0 ^{0}C. The reaction was monitored by TLC for loss of starting material **6**. At completion, the reaction mixture was filtered through a pad of Celite and the solvent was removed *in vacuo* to yield the crude product as a yellow, sticky oil. Purification by flash chromatography using diethyl ether resulted in a white solid. Yield: 49.9%. R_f = 0.52 (Et_2O). Melting point: 67-68 ^{0}C. $[\alpha]_{D^{24}}$ = +23.6 (c 1.0, Et_2O). H NMR ($CDCl_3$): δ 0.86-0.88 (d, 6H), 1.23-1.77 (m, 3H), 3.27-3.29 (d, 2H), 3.65 (s, 3H), 4.22-4.28 (m, 1H), 4.86-4.93 (m, 2H), 4.98-5.04 (m, 2H), 5.05-5.23 (d, *J* = 8.6 Hz, 1H), 6.67-6.70 (d, *J* = 7.6 Hz, 1H), 6.93 (s, 1H), 7.07-7.15 (m, 6H), 7.28-7.32 (m, 5H), 7.48-7.50 (d, 1H), 8.14 (br, 1H). ^{13}C NMR: δ 21.7, 22.8, 24.5, 27.4, 41.5, 52.3, 52.7, 53.7, 66.8, 109.2, 111.3, 118.3, 119.4, 122.0, 123.2, 127.9, 128.0, 128.4, 135.9, 136.2, 154.6, 156.1, 172.0, 172.2.

***l*-Leucine-*l*-tryptophan, methyl ester 8.** (Rambhav et al., 1976). Cbz-*l*-leucine-*l*-tryptophan methyl ester **7** (2.0 g, 4.3 mmol) was dissolved in 30 mL of absolute ethanol and approximately 10.0 mg of palladium on activated carbon (10% Pd/C) was added under a blanket of argon gas. The reaction mixture was evacuated

under vacuum aspirator and exchanged for a hydrogen atmosphere and allowed to proceed overnight at RT. The reaction was checked by TLC. The next morning, at completion, the mixture was filtered through celite and the solvent was evaporated *in vacuo*. The crude product was used directly in the next step without further purification.

(S)-N-acetyl-*l*-phenylglycine-*l*-leucine-*l*-tryptophan, methyl ester 9a. N-Acetyl-*l*-phenylglycine (0.08 g, 0.43 mmol) and DCC (0.09 g, 0.43 mmol) were dissolved in 10 mL of distilled CH_2Cl_2 and stirred for 30-45 minutes at 0 ^{0}C. *l*-Leucine-*l*-tryptophan, methyl ester **8** (0.20 g, 0.43 mmol) was added and the reaction mixture was stirred under an argon atmosphere for 4 hours at 0 ^{0}C, then at RT overnight. The solution was filtered through a pad of Celite and neutralized to pH 8-9 by the dropwise addition of 5% $NaHCO_3$. After the organic phase was dried over anhydrous magnesium sulfate, it was evaporated in vacuo to obtain a solid, yellow residue. The solid was washed 3-4 times with hot CH_2Cl_2 to remove DCU then air dried. The solid was redissolved in 20 mL EtOAc and washed with 20% HCl (2 x 10 mL), 10% NaOH (2 x 20 mL) and brine. The solution was dried over Na_2SO_4, filtered and concentrated to a solid. Recrystallization (3x) from CH_2Cl_2/Et_2O afforded white, powdery crystals. Yield: 52.3%. R_f = 0.20 (EtOAc:CH_2Cl_2 9:1). Melting point: 178-180 ^{0}C. $[\alpha]_{D^{24}}$ = + 44.4 (c 0.9, $CHCl_3$). ^{1}H NMR: δ 0.34-0.58 (d,1H), 0.68-

0.73 (d, 1H), 0.81-0.87 (m, 4H), 1.50-1.55 (m, 3H), 1.96 (s, 3H), 3.14-3.24 (2d, 2H), 3.67 (s, 3H), 4.71-4.80 (m, 1H), 4.80-4.87 (m, 1H), 5.0-5.1 (m, 1H), 5.88-5.90 (d, 1H), 6.78-6.79 (d, 1H), 7.02-7.63 (m, 10H), 7.98 (s, 1H). ^{13}C NMR: δ 22.1, 22.6, 22.9, 24.7, 27.4, 41.3, 52.0, 52.3, 52.8, 56.7, 109.5, 111.3, 118.2, 119.4, 122.0, 123.1, 126.9, 127.1, 127.3, 128.3, 128.8, 129.0, 135.9, 137.8, 169.9, 170.2, 171.1, 171.9. Anal. calcd. for $C_{28}H_{34}N_4O_5 \cdot 1.5\ H_2O$: C, 63.02; H, 6.61; N, 10.50. Found: C, 63.14; H, 6.57; N, 10.43.

(R)-N-acetyl-*d*-phenylglycine-*l*-leucine-*l*-tryptophan, methyl ester 9b. N-Acetyl-*d*-phenylglycine (0.08 g, 0.43 mmol) and DCC (0.09 g, 0.43 mmol) were dissolved in 10 mL of distilled CH_2Cl_2 and stirred for 30-45 minutes at 0 ^{0}C. *l*-Leucine-*l*-tryptophan, methyl ester **8** (0.20 g, 0.43 mmol) was added and the reaction mixture was stirred under an argon atmosphere for 4 hours at 0 ^{0}C, then at RT overnight. The solution was filtered through a pad of Celite and neutralized to pH 8-9 by the dropwise addition of 5% $NaHCO_3$. After the organic phase was dried over anhydrous magnesium sulfate, it was evaporated in vacuo to obtain a solid, yellow residue. The solid was washed 3-4 times with hot $CH_2\ Cl_2$ to remove DCU, then air dried. The solid was redissolved in 20 mL EtOAc and washed with 20% HCl (2 x 10 mL), 10% NaOH (2 x 20 mL) and brine. The solution was dried over Na_2SO_4, filtered and concentrated to a solid. Recrystallization (3x) from CH_2Cl_2/Et_2O afforded white,

powdery crystals. Yield: 49.6%. R_f = 0.22 (EtOAc:CH_2Cl_2 9:1). Melting point: 176-178 ^{0}C. ^{1}NMR: δ 0.51-0.58 (d, 1H), 0.62-0.69 (d, 1H), 0.79-0.81 (m, 4H), 1.52-1.68 (m, 3H), 1.94 (s, 3H), 3.10-3.21 (2d, 2H), 3.63 (s, 3H), 4.55-4.59 (m, 1H), 4.66-4.75 (m, 1H), 4.91-5.01 (m, 1H), 5.77-5.86 (d, 1H), 6.74-6.78 (d, 1H), 6.98-7.33 (m, 10H), 7.87 (s, 1H). ^{13}C NMR: δ 19.8, 20.1, 21.4, 22.3, 23.9, 40.4. 51.8, 52.2, 52.4. 56.3, 109.8, 111.7, 118.3, 119.8, 121.9, 122.7, 124.1, 127.1, 128.1, 128.5, 128.8, 136.1, 138.2, 170.1, 171.3, 171.6, 172.0.

Part B: Synthesis of Phosphonamidate Compounds

(R,S)-Dimethyl-N-(S)-(α-methylbenzyl)-α-aminobenzyl phosphonate 13. Method A. Benzaldehyde (2.15 mL, 24.75 mmol), (S)-(-)-α-methyl benzyl amine (3.19 mL, 24.75 mmol), toluene (50 mL) and p-toluene sulfonic acid (cat. amt.) were brought to gentle reflux using a Dean-Stark condenser and maintained for 3 h. When the water volume in the collector remained constant and the reaction solution turned clear, the reaction was stopped and cooled to RT. Dimethyl phosphite (2.29 mL, 0.025 mol) was added and the solution was refluxed for 4 hours, then stirred slowly, at RT overnight. The solvent was removed, and the remaining yellow oil was dissolved in diethyl ether (60 mL) and washed with 20% HCl solution (70 mL). The aqueous layer was neutralized to pH 7 by the cautious addition of NaOH pellets at 0 ^{0}C then

extracted with EtOAc (2 x 25 mL). A solid formed upon evaporation that was purified by flash chromatography (chloroform/diethyl ether/pet ether; 3:3:4). The product was collected as a mixture of diastereomers and to date could not be separated by chromatographic methods. Yield: 86%. R_f = 0.24 (diethyl ether). Melting point: 74-76 ^{0}C. $[\alpha]_{D^{24}}$ = -37.88 (c 2.5, MeOH). ^{1}H NMR: δ 1.30-1.32 (d, *J* = 6.35 Hz, 3H), 2.21 (br, 1H), 3.41-3.46 (dd, *J* = 10.3 Hz, 3H), 3.71-3.86 (q, *J* = 10.1 Hz, 1H), 4.08-4.14 (d, *J* = 20.17 Hz, 1H), 7.24-7.32 (m, 10H). ^{13}C NMR: δ 22.14, 53.26 and 53.35 (chiral C), 53.61 and 53.70 (chiral C), 56.63, 58.65, 126.45, 126.72, 126.86, 126.97, 127.59, 127.63, 128.0, 128.10, 128.20, 128.26, 128.29. ^{31}P NMR: δ 27.2, 27.5. Anal. calcd. for $C_{17}H_{22}NO_3P$: C, 63.94; H, 6.95; N, 4.39. Found: C, 63.87; H, 6.97; N, 4.39.

Method B. A mixture of benzaldehyde (2.03 mL, 0.02 mol), anhydrous potassium carbonate (4.0 g) and (S)-(-)-α-methyl benzyl amine toluene (60 mL) was heated on a steam bath for 10 min, then cooled to RT. Toluene was removed, potassium carbonate was filtered off and the residue was refluxed with diethyl phosphite (2.57 mL, 0.02 mol) or dimethyl phosphite (1.83 mL, 0.02 mol) at 120-140 ^{0}C for 30 min. After cooling to RT, the remaining oil was dissolved in diethyl ether (60 mL) and washed with 20% HCl solution (70 mL). The aqueous layer was neutralized with NaOH pellets then washed with EtOAc (2 x 25 mL). The organic

layers were combined and dried to afford a yellowish oil which was purified by flash column chromatography using ether as a solvent. Yield **13**: 30.7%; **14**: 50.2%. The ^{1}H NMR and melting point were identical to those obtained from method A.

Method C. Benzaldehyde (1.38 mL, 0.014 mol), toluene (20 mL), (S)-(-)-α-methyl benzylamine (1.76 mL, 0.014 mol) and magnesium sulfate (4.0 g) were stirred at RT for 3 hr under Ar gas. Dimethyl phosphite (1.25 mL, 0.014 mol) was added and the reaction was stirred at RT overnight. TLC did not indicate complete product formation, so the solution was refluxed for 4 h or until all starting material was consumed. Workup was the same as for the previous procedures. Yield:**13**: 29.3%. (reaction with **14** was not conducted). The ^{1}H NMR and melting point were identical to those obtained from method A.

(R,S)-Diethyl-N-(S)-(α-methylbenzyl)-α-aminobenzyl phosphonate 14. Method A. Benzaldehyde (2.15 mL, 24.75 mmol), (S)-(-)-α-methyl benzylamine (3.19 mL, 24.75 mmol), toluene (50 mL) and p-toluene sulfonic acid (cat. amt.) were brought to gentle reflux using a Dean-Stark condenser and maintained for 3 h. When the water volume in the collector remained constant and the reaction solution turned clear, the reaction was stopped and cooled to RT. Diethyl phosphite (3.22 mL, 0.025 mol) was added and the solution was refluxed for 4 hours, then stirred at RT overnight.

The solvent was removed, and the remaining yellow oil was dissolved in diethyl ether (60 mL) and washed with 20% HCl solution (70 mL). The aqueous layer was neutralized to pH 7 by NaOH pellets in an ice bath then extracted with EtOAc (2 x 25 mL). The combined organic layers were concentrated to a yellow oil, which was purified by flash chromatography (chloroform/diethyl ether/pet ether; 3:3:4). The product was collected as a mixture of diastereomers and so far could not be separated by chromatographic methods. Yield: 89.29%. R_f = 0.12 (3:3:4 $CHCl_3$:ether:pet. ether). Melting point: 38-40 ^{0}C. $[\alpha]_{D^{24}}$ = -35.01 (c 2.6, MeOH). ^{1}H NMR: δ 1.06-1.10 (m, 3H), 1.28-1.33 (m, 6H), 2.21 (br, 1H), 3.67-3,76 (m, 2H), 4.05-3.85 (m, 1H), 4.06-4.19 (m, 3H), 7.21-7.32 (m, 10H). ^{13}C: δ 16.46, 16.54, 22.32, 55.27, 57.13, 59.14, 62.88, 126.72, 127.0, 127.07, 128.32, 128.35, 128.38, 128.41, 128.53. ^{31}P NMR: δ 24.35, 24.76. Anal. calcd. for $C_{19}H_{26}NO_3P$: C, 65.69; H, 7.55; N, 4.03. Found: C, 65.64; H, 7.61; N, 4.10.

(R,S)-Dimethyl-N-benzyl-α-aminobenzyl phosphonate 19. The procedure used to synthesize **13** and **14** was used here except that benzylamine was substituted for (S)-(-)-α-methylbenzylamine. Purification by column chromatography (chloroform/diethyl ether/pet ether; 3:3:4) afforded a clear oil which solidified in the refrigerator. Yield: 76.6%. R_f = 0.15 (chloroform/diethyl ether/pet ether 3:3:4). Melting point: 30-32 ^{0}C. ^{1}H NMR: δ 2.09 (br, 1H), 3.50-3.54 (d, *J* = 11.39 Hz,

3H), 3.69-3.73 (d, J = 11.42 Hz, 3H), 3.99-4.06 (d, J = 20.02 Hz, 1H). ^{13}C NMR: δ 50.99, 53.67, 58.22, 60.26, 127.14, 128.01, 128.06, 128.31, 128.36, 128.52, 128.57, 128.61. ^{31}P NMR: δ 26.47. Anal. calcd. for $C_{16}H_{20}NO_3P$: C, 62.94; H, 6.60; N, 4.59. Found: C, 62.82; H, 6.64; N, 4.70.

(R,S)-Dimethyl-α-aminobenzyl phosphonate 20. Compound **13** (1.234 g, 3.87 mmol) was first dissolved in 14 mL MeOH and 0.25 g of palladium on activated carbon (10% Pd/C) and 0.4 mL CF_3COOH were added and the mixture was placed in a Paar hydrogenator (H_2, 45 psi) for 3 h. Upon completion, the solution was filtered through Celite, evaporated to a yellow oil, neutralized by $NaHCO_3$ and extracted into ethyl acetate (2 x 15 mL). The combined organic layers were concentrated to a yellow oil which was purified by flash chromatography ($CHCl_3$:MeOH:Hexanes 85:10:5). Yield: 90.6%. R_f = 0.18 ($CHCl_3$:MeOH:Hexanes 85:10:5). ^1H NMR: δ 1.96 (br, 2H), 3.57-3.60 (d, J = 11.34 Hz, 3H), 3.70-3.73 (d, J = 11.37 Hz, 3H), 4.27-4.31 (d, J = 17.34 Hz, 1H), 7.33-7.47 (m, 5H). ^{13}C NMR: δ 29.66, 52.86, 54.86, 127.57, 127.65, 127.93, 127.97, 128.53, 128.56, 136.61. ^{31}P NMR: δ 26.37.

Note: This reaction also takes place at ambient H_2 pressure but it requires 5 h for completion.

(R,S)-Dimethyl-α-acetylaminobenzyl phosphonate 21. Compound **20** (0.123

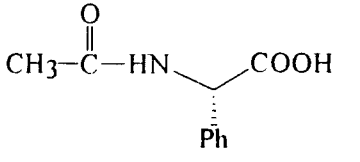
g, 0.75 mmol) was dissolved in methylene chloride (5 mL) at RT. Acetyl chloride (0.08 mL, 2 eq) was added slowly and the reaction was allowed to proceed for 1 h when TLC indicated consumption of starting material. (It was noted that shortly after the addition of acetyl chloride the solution turns milky). The solvent was evaporated and the mixture was neutralized by $NaHCO_3$ to pH 7 and extracted with ethyl acetate (3 x 10 mL). After drying over Na_2SO_4 and removal of solvent a pale yellow solid remains. Recrystallization from $CHCl_3$/pet. ether and a few drops of ether afords white crystals. Yield: 51.35% R_f = 0.25 (10% MeOH/EtOAc). Melting point: 136-138 ^{0}C. ^{1}H NMR: δ 1.92 (s, 3H), 3.34-3.38 (d, *J* = 10.55 Hz, 3H), 3.71-3.74 (d, *J* = 10.74 Hz, 3H), 5.45-5.60 (q, *J* = 9.47 Hz, 1H), 7.20-7.45 (m, 5H), 7.65-7.70 (m, *J* = 9.03 Hz, 1H). ^{13}C NMR: δ 22.86, 48.88, 50.50, 53.76, 128.20, 128.25, 128.28, 128.65, 128.68, 134.88, 170.0 ^{31}P NMR: δ 24.63. Anal. calcd. for $C_{11}H_{16}NO_4P$: C, 51.36; H, 6.27; N, 5.45. Found: C, 51.32; H, 6.24; N, 5.39.

(R,S)-Methyl-α-acetaminobenzyl phosphonate sodium salt 22. Compound **21** (0.187 g, 0.73 mmol) was dissolved in absolute ethanol (3 mL) at RT. One equivalent of 1M NaOH solution (0.73 mL) was added and the solution was stirred ar RT under Ar overnight. The reaction was checked by TLC and the following day, TLC indicated complete consumption of starting material. The solvent was removed under vacuum, and the residue was washed with absolute ethanol (3 x 2 mL). The

remaining white compound was dried under vacuum overnight. Yield: 90%. R_f = on the baseline (10% MeOH/$CHCl_3$). Melting point: ^{1}H NMR: δ 1.88 (s, 3H), 3.34-3.37 (d, 3H), 5.92-6.02 (d, 1H), 7.22-7.24 (m, 5H). ^{31}P NMR: δ 17.93 ppm.

Methyl hydrogen-N-(S)-α-methylbenzyl-α-aminobenzyl-(R,S) phosphonic acid 26. To compound **13** (0.200g, 0.63 mmol) were added to 1 mL of 1 M NaOH and 4 mL of absolute ethanol and the reaction was refluxed for 5 h. At completion, the solution was cooled to RT, acidified to pH 3 by 0.75 ul of 20% HCl, and extracted three times with 6 mL of isopropanol:chloroform (1:9). The organic layers were combined and dried over Na_2SO_4. The drying agent was filtered off, and removal of the solvent gave a white residue which was recrystallized from water. Yield: 74.2%. R_f = 0.29 (MeOH:$CHCl_3$ 1:9). Melting point: 189-191 ^{0}C. $[\alpha]_{D^{24}}$ = -17.26 (c 1.0, D_2O). ^{1}H NMR: δ 1.51-1.54 (d, J = 7.0 Hz, 3H), 3.31-3.35 (d, J = 9.9 Hz, 3H), 4.13-4.18 (d, J = 15.7 Hz, 1H), 4.50-4.51 (q, 1H), 7.22-7.35 (m, 10H). ^{13}C NMR: δ 22.4, 56.53, 56.62, 60.82, 131.93, 132.94, 133.02, 133.48, 133.61, 133.91, 134.02, 139.97. ^{31}P NMR: δ 12.34, 12.55. Anal. calcd. for $C_{16}H_{20}NO_3P$: C, 62.94; H, 6.60; N, 4.59. Found: C, 62.81; H, 6.64; N, 4.53.

^{1}H NMR Spectrum:



2a

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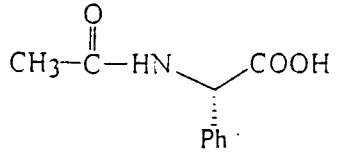
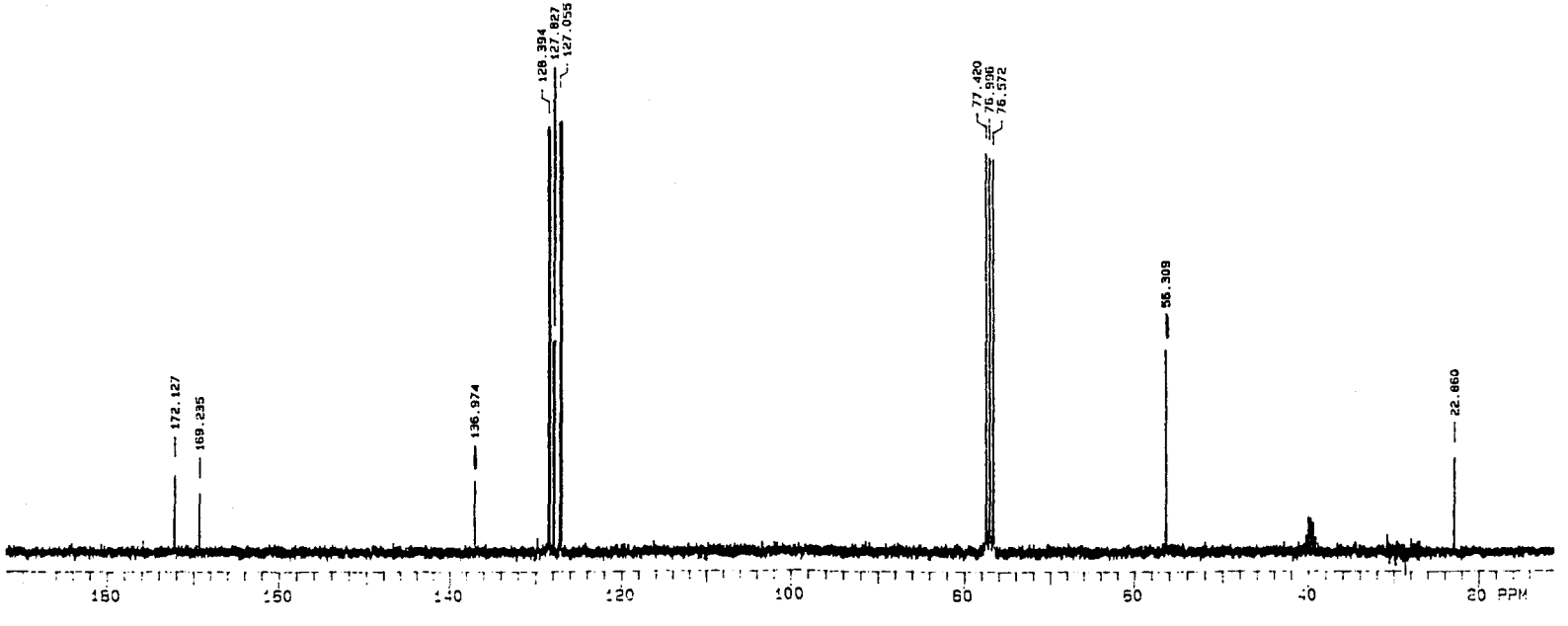


2a



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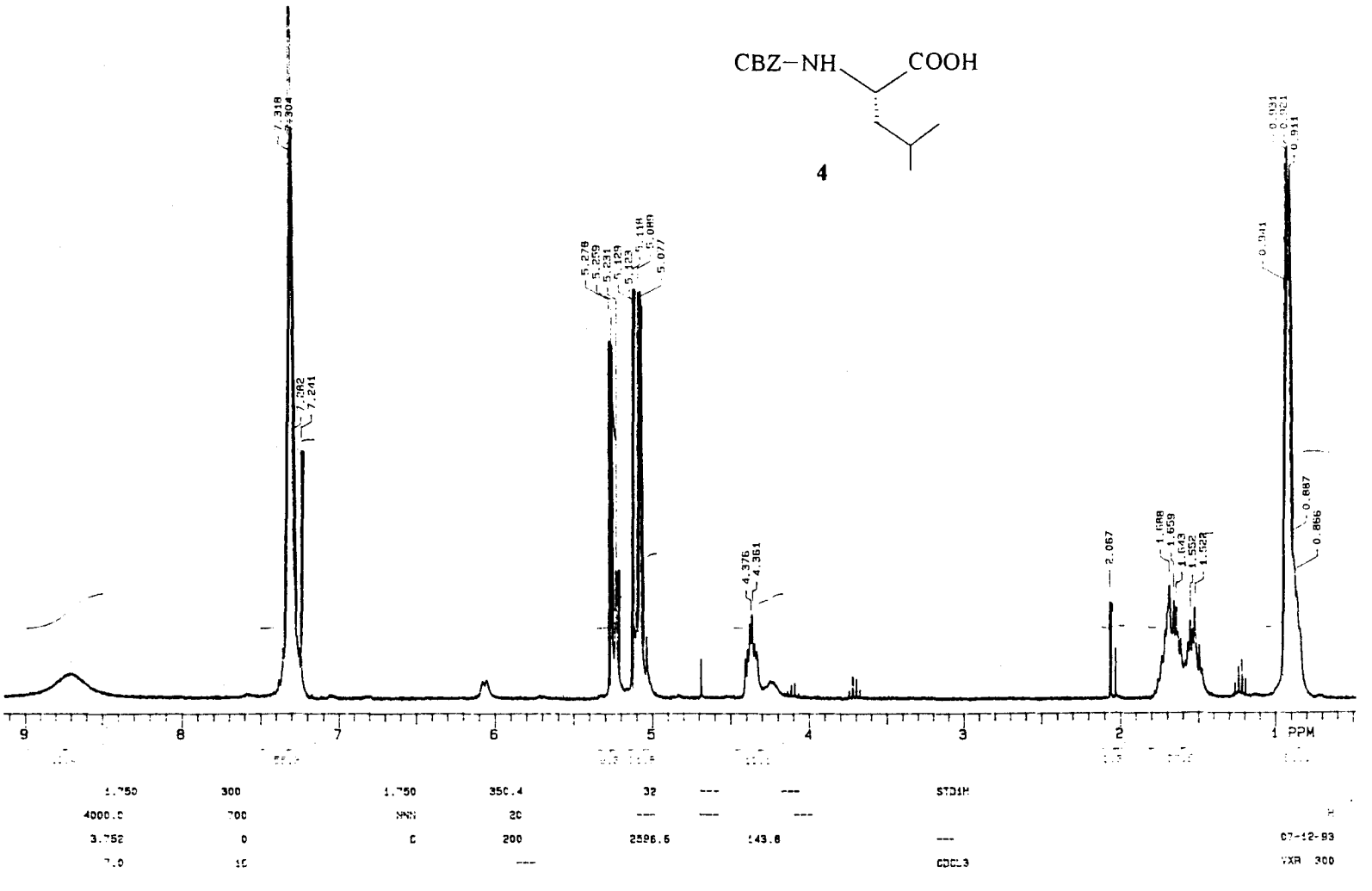
8.7	704	17.5	64.0				CDCL3	VXR 300

^{1}H NMR Spectrum:



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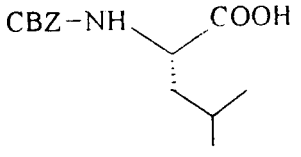
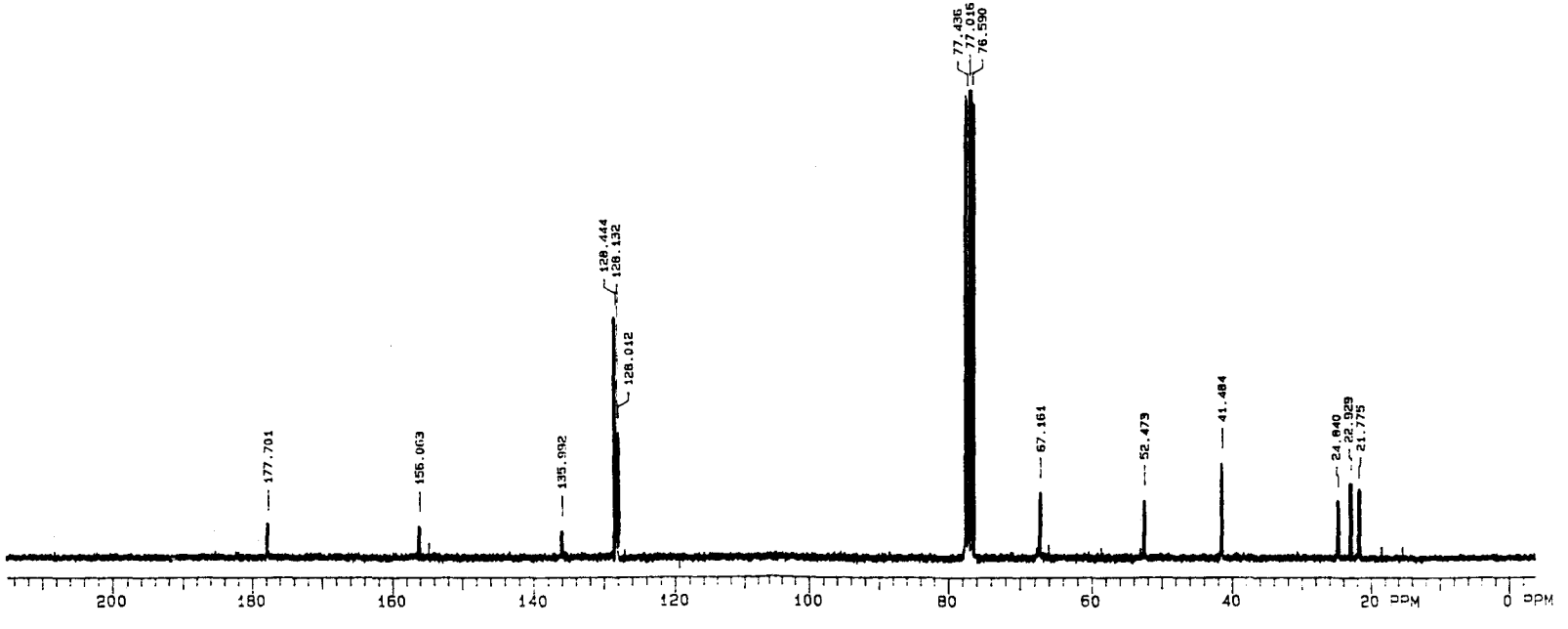


4



13.750	75	1.750	350.3	64	---	---	STD13C	
16501.7	700	YYY	5	1.000	---	---		C
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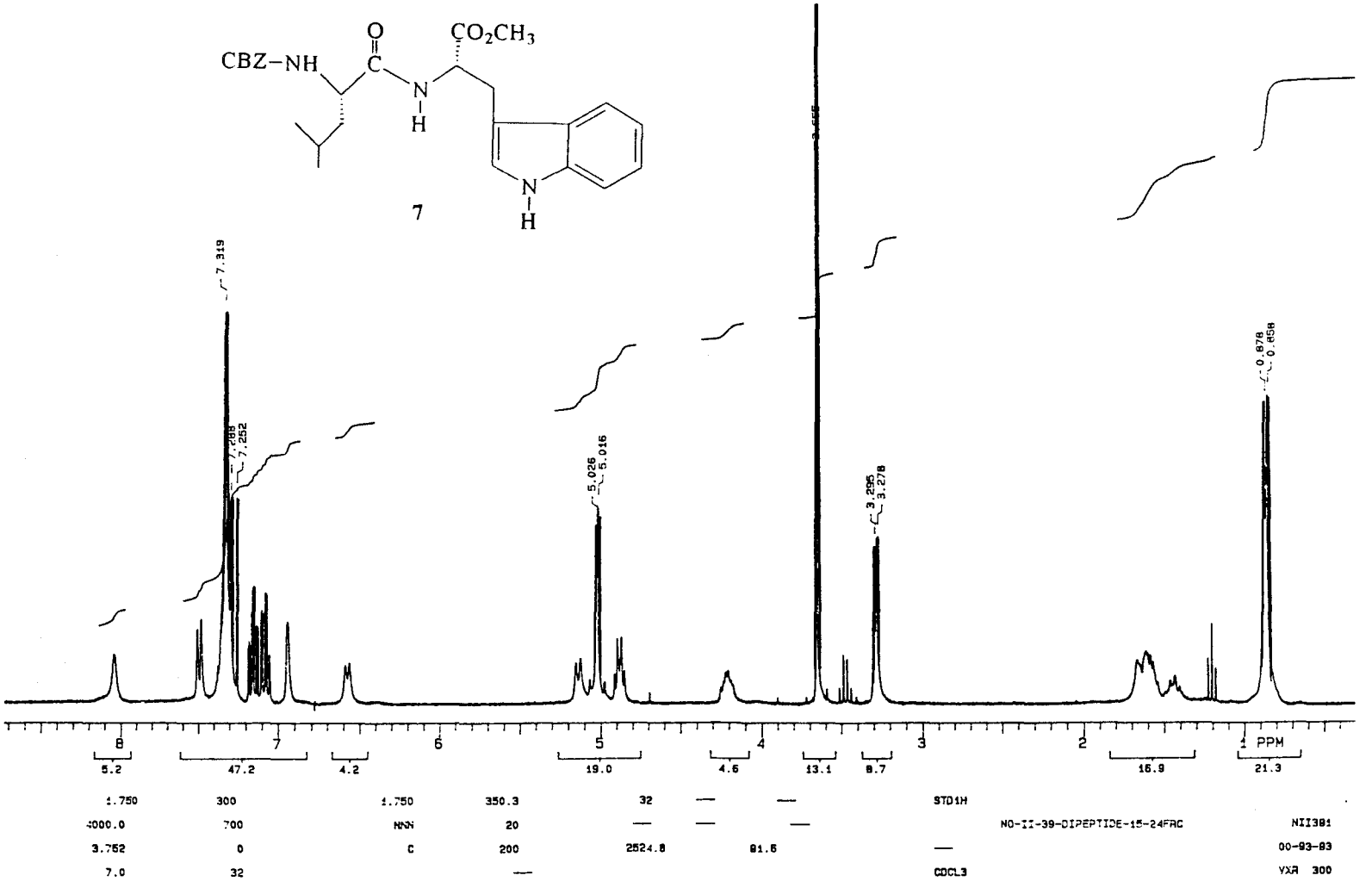
^{1}H NMR Spectrum:



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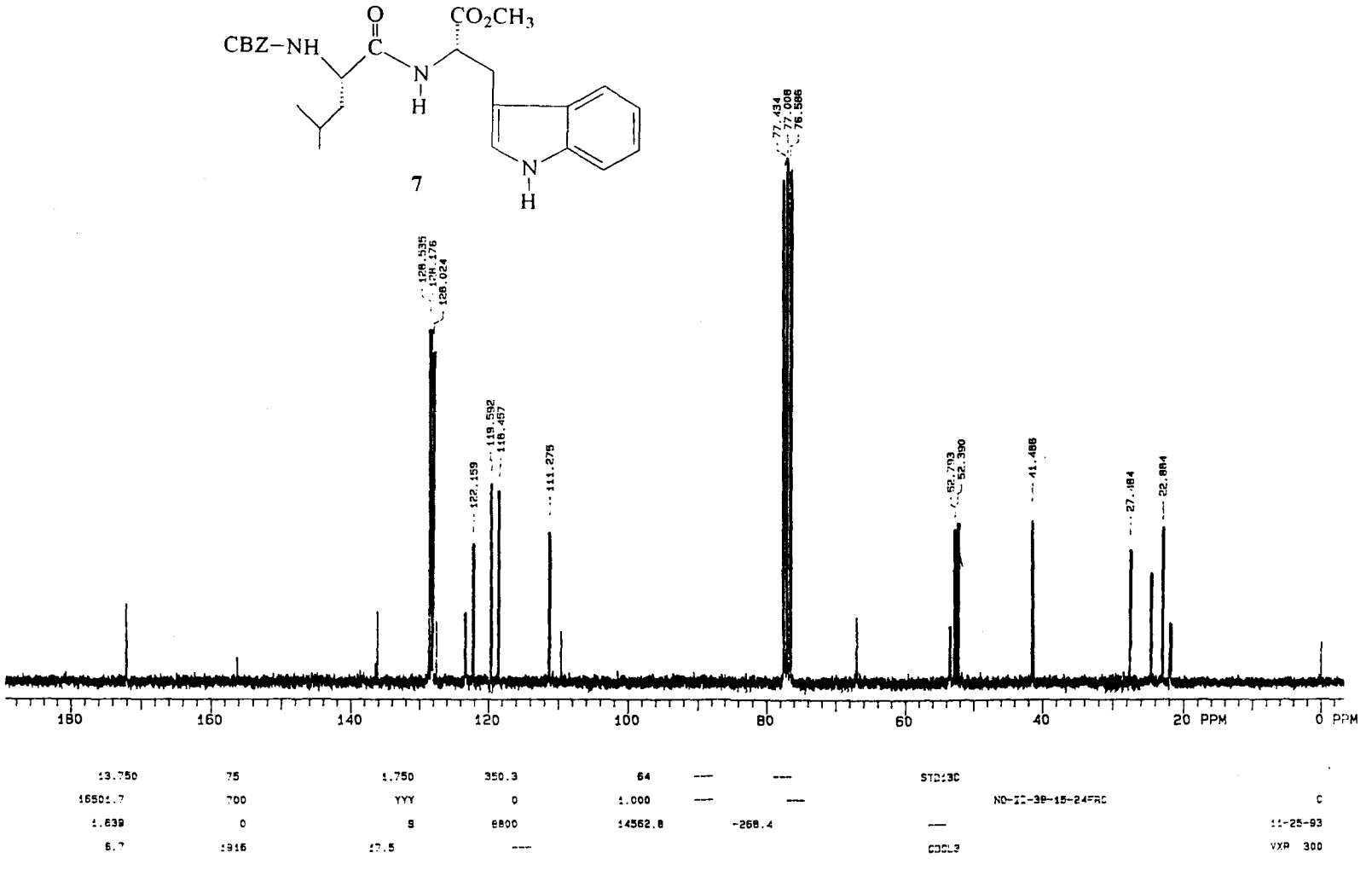

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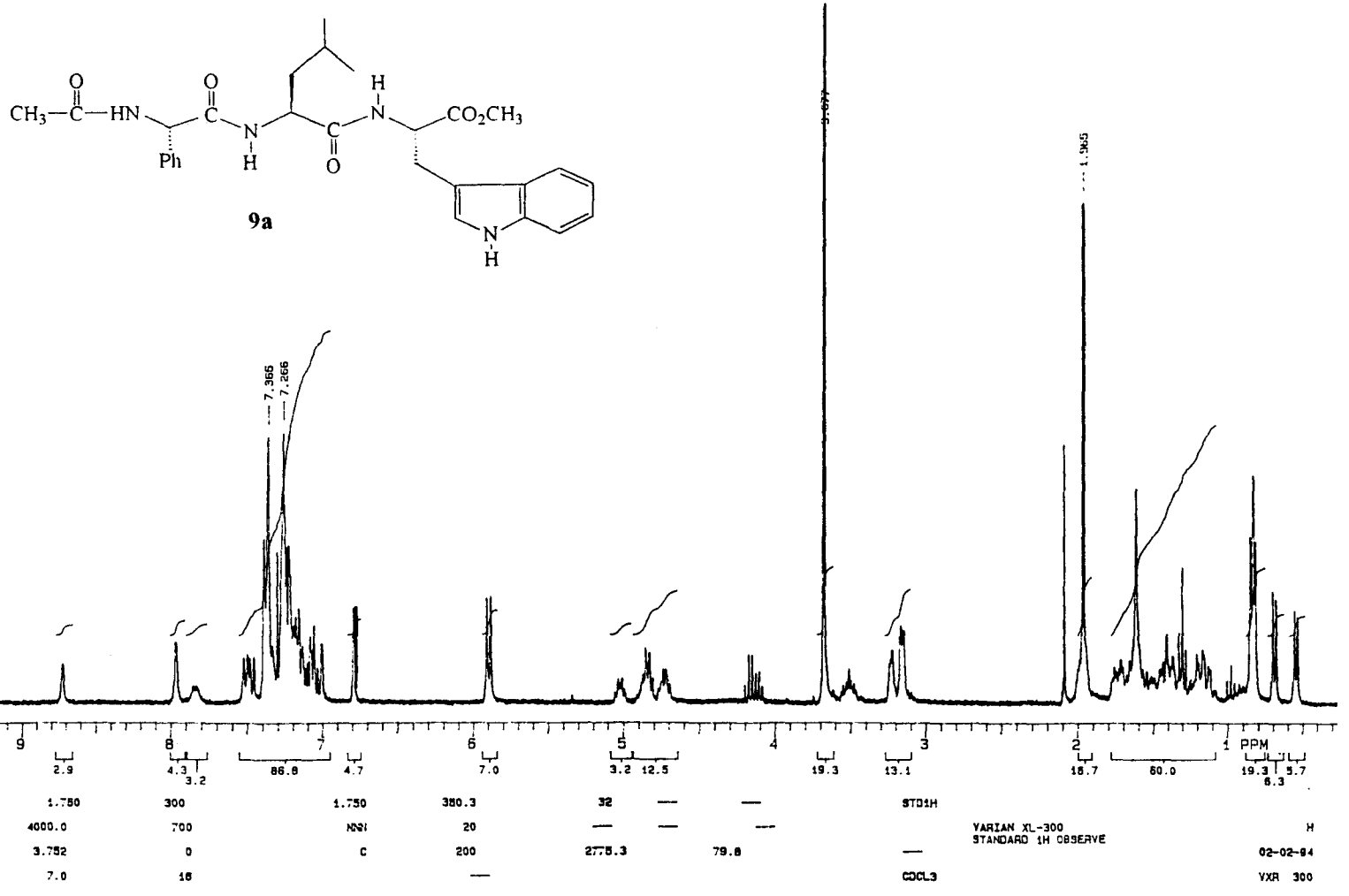

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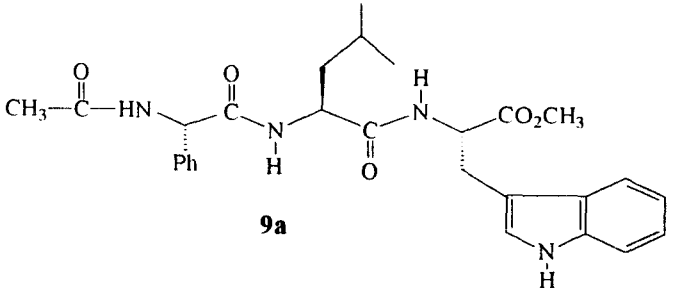

9a

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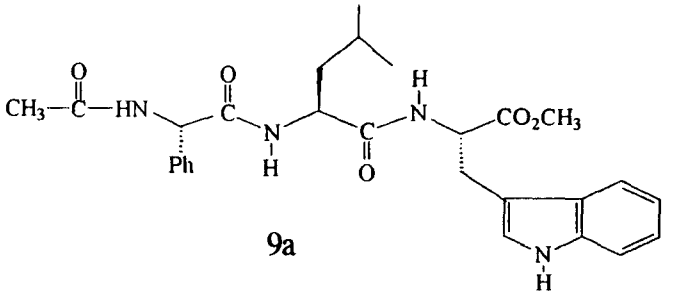

9a

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DATE 05-03-94
SOLVENT CDCL3
FILE COSY

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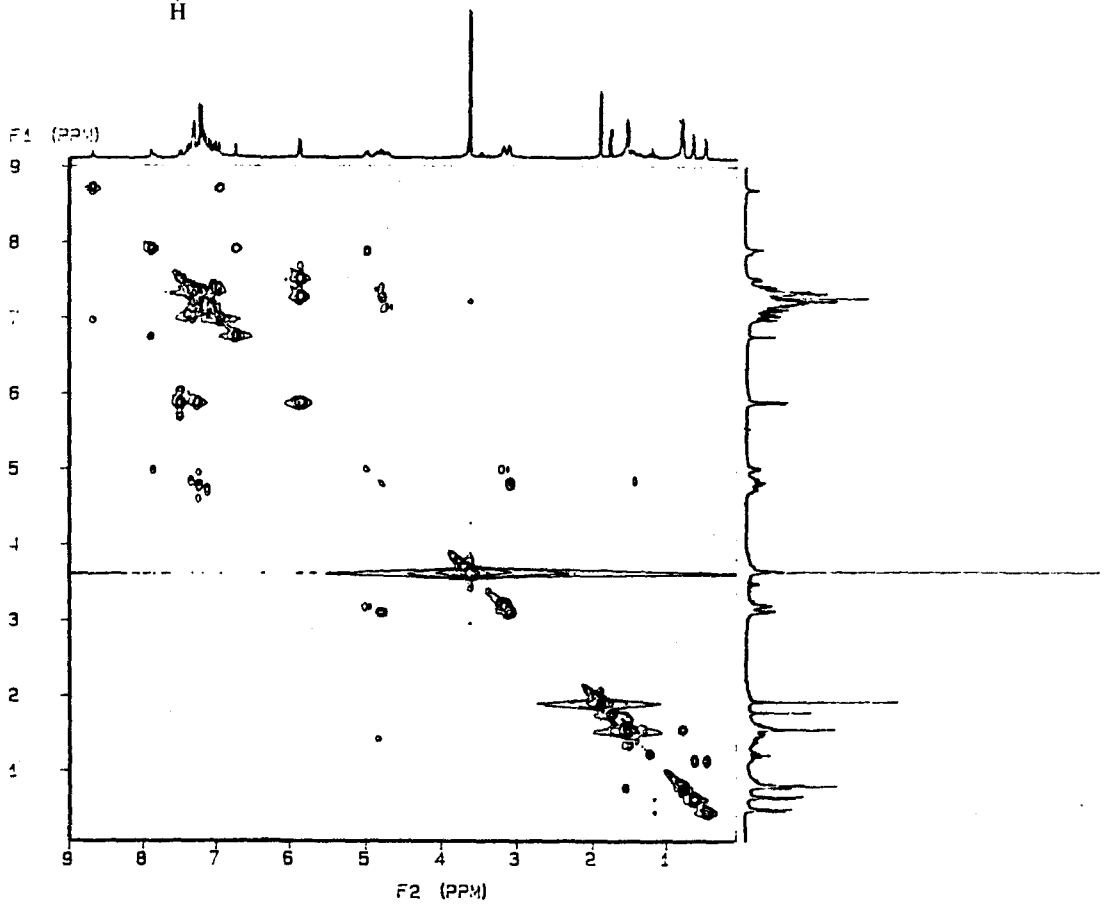

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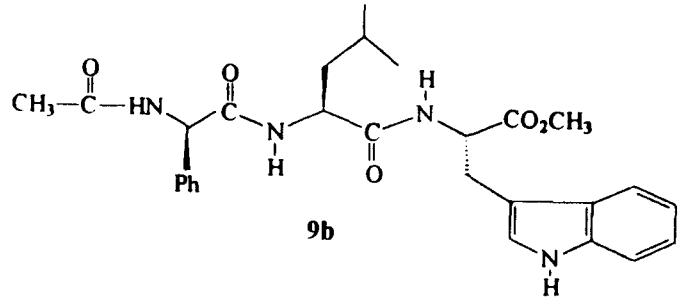

9b

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7.0	16			---					CDCL3	YXR 300

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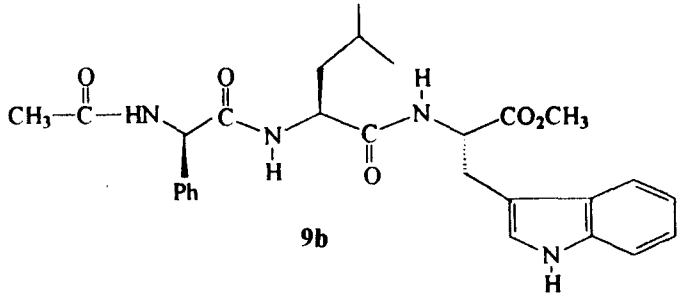

9b

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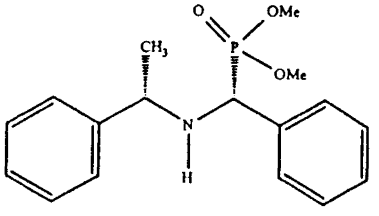
8.7	1992	17.8	—			CDCL3		VXR 300

^{1}H NMR Spectrum:



13

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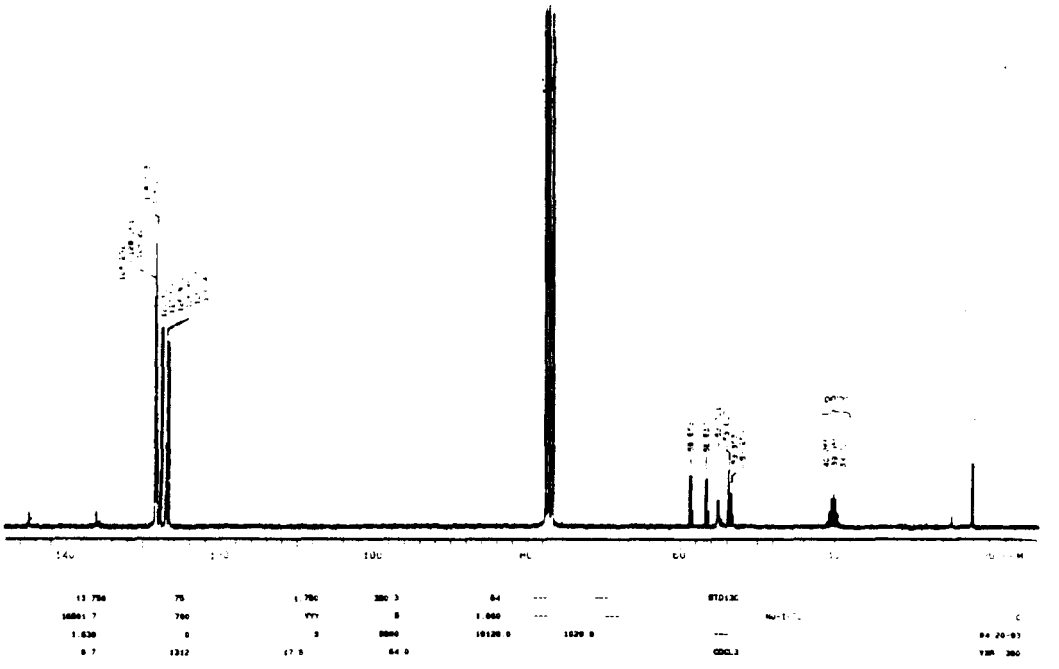

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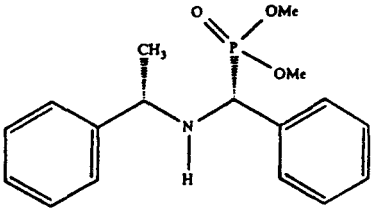
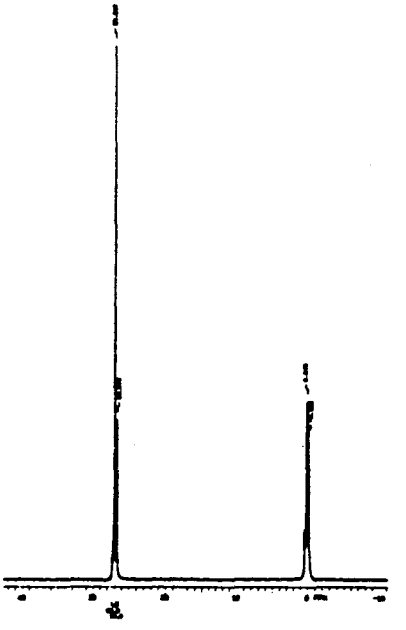


13



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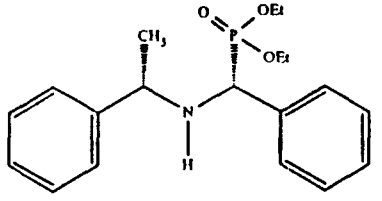

14

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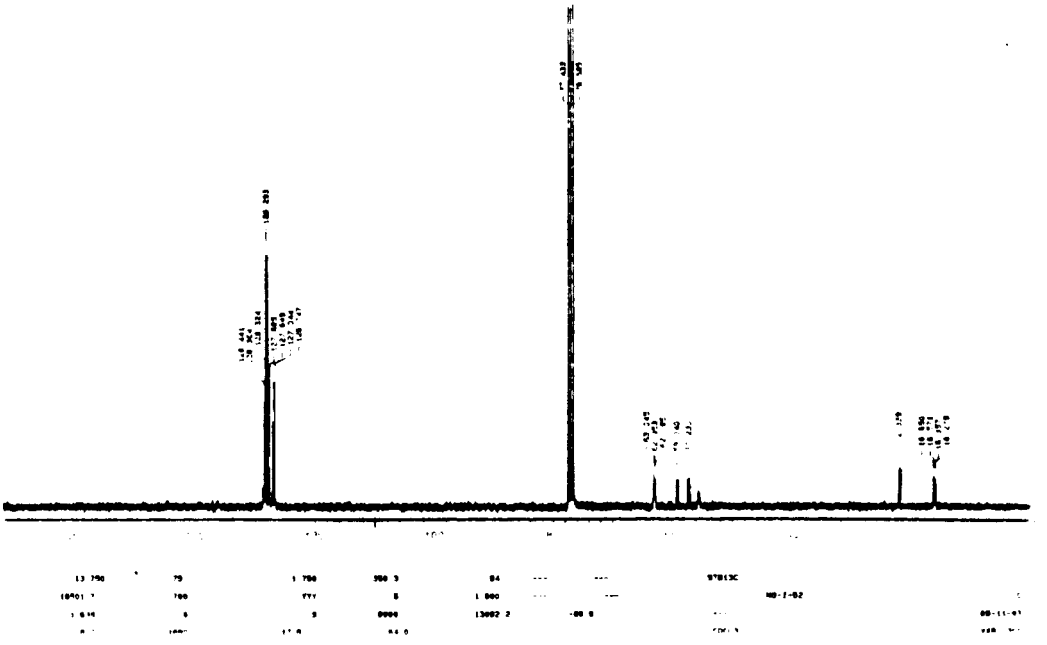

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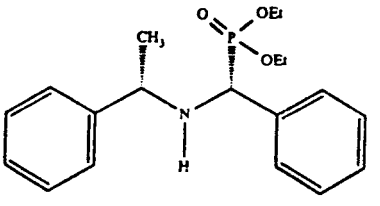
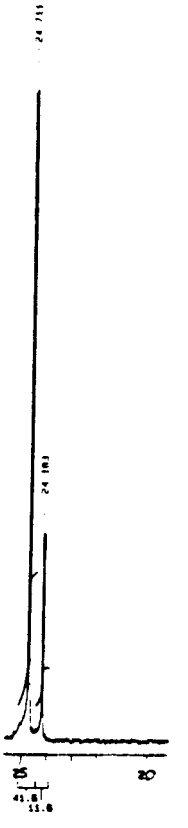


14



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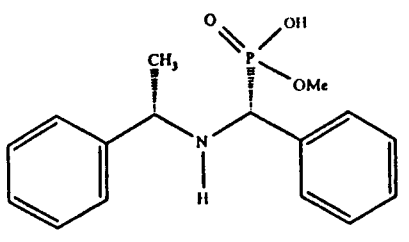

26

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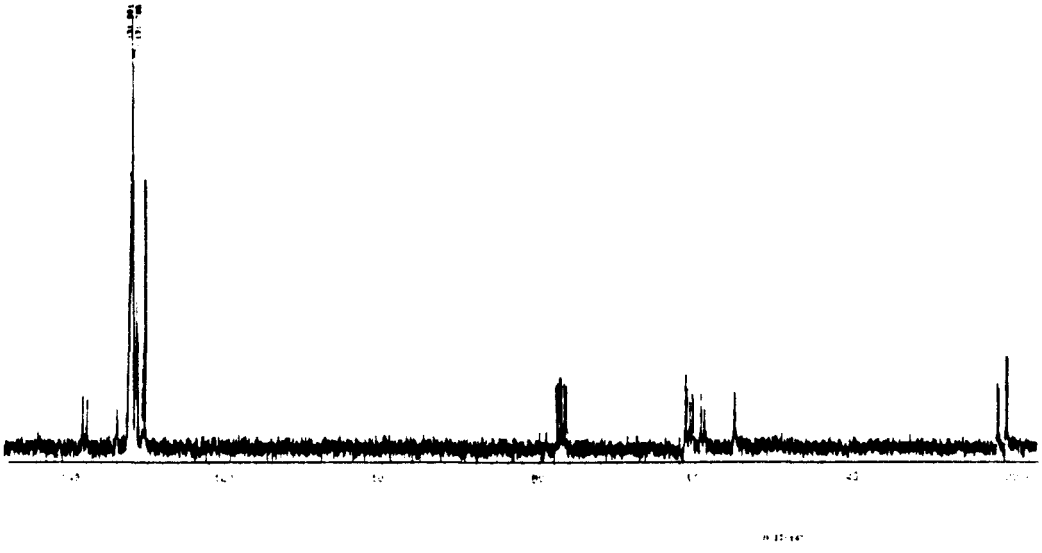

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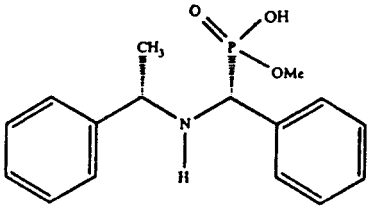
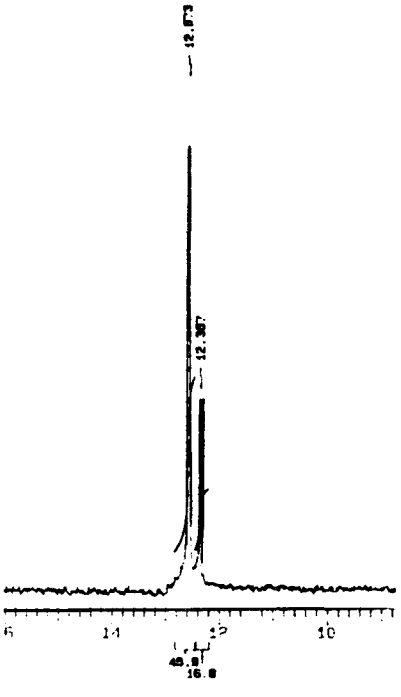


26



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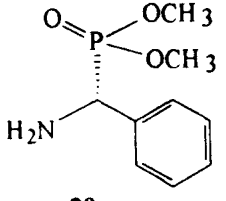

20

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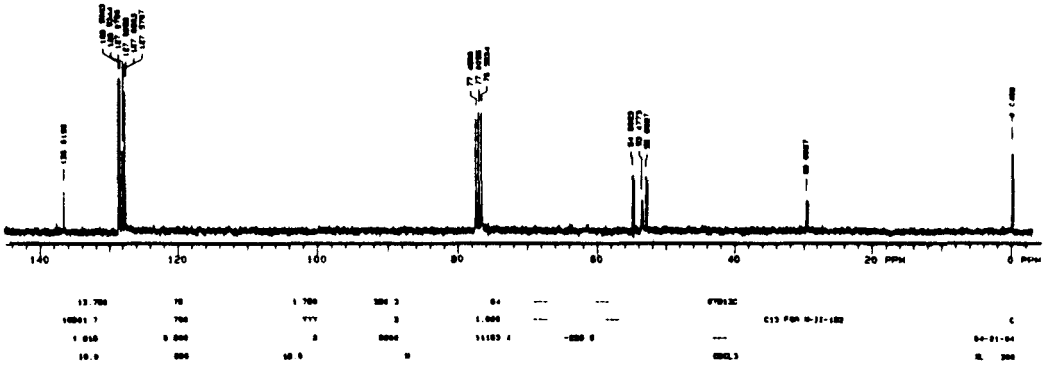

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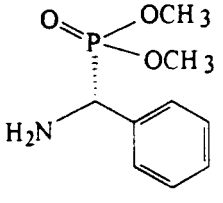

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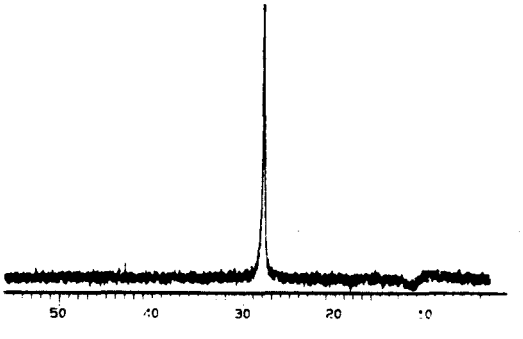

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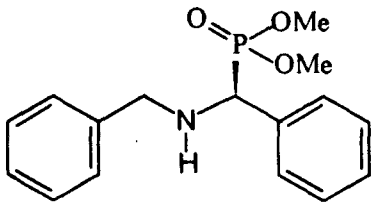

19a

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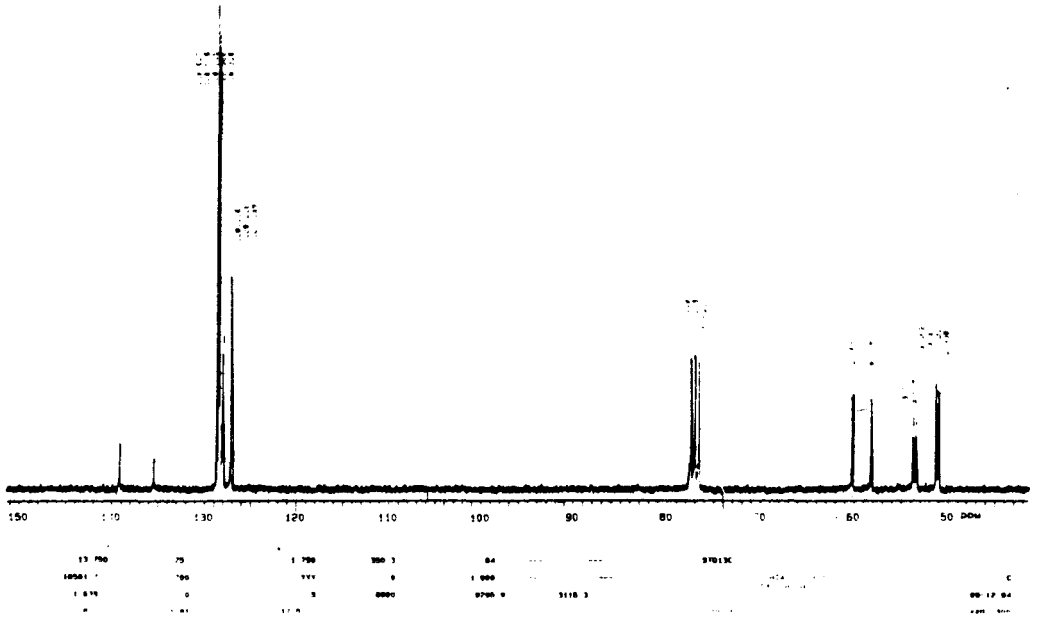

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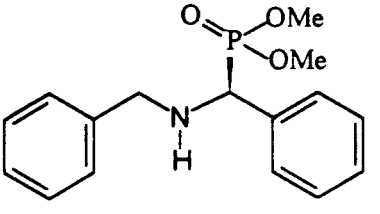

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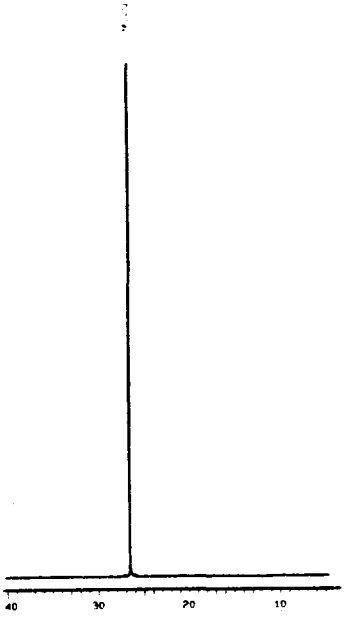

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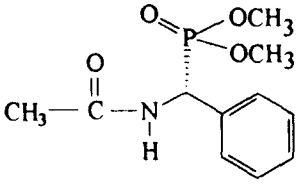

21

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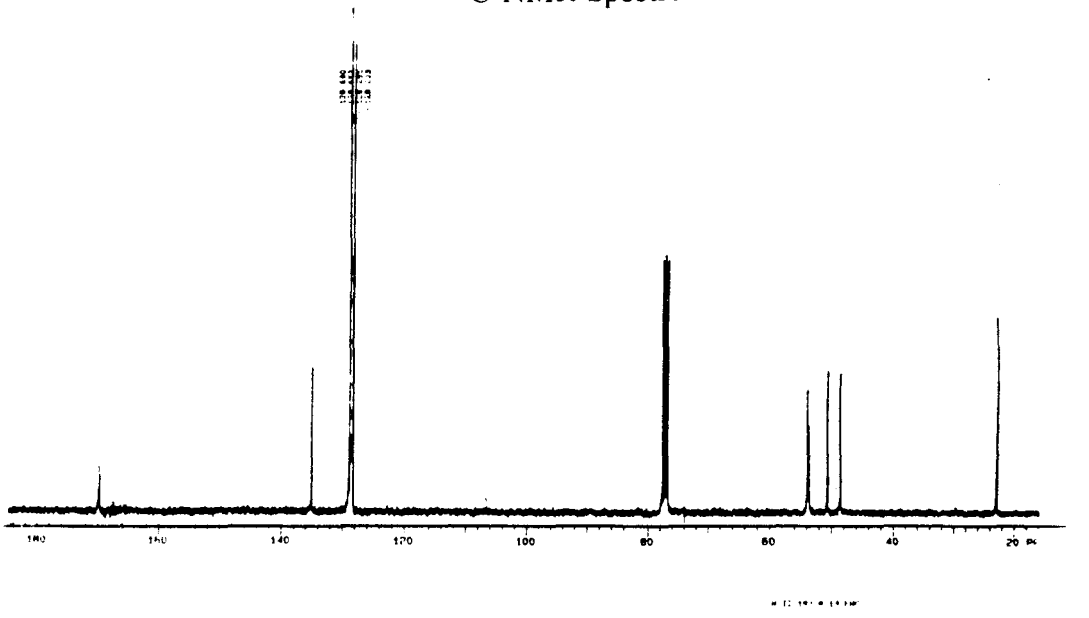

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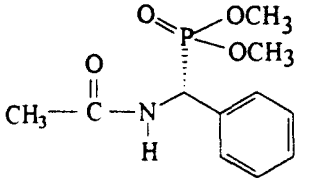

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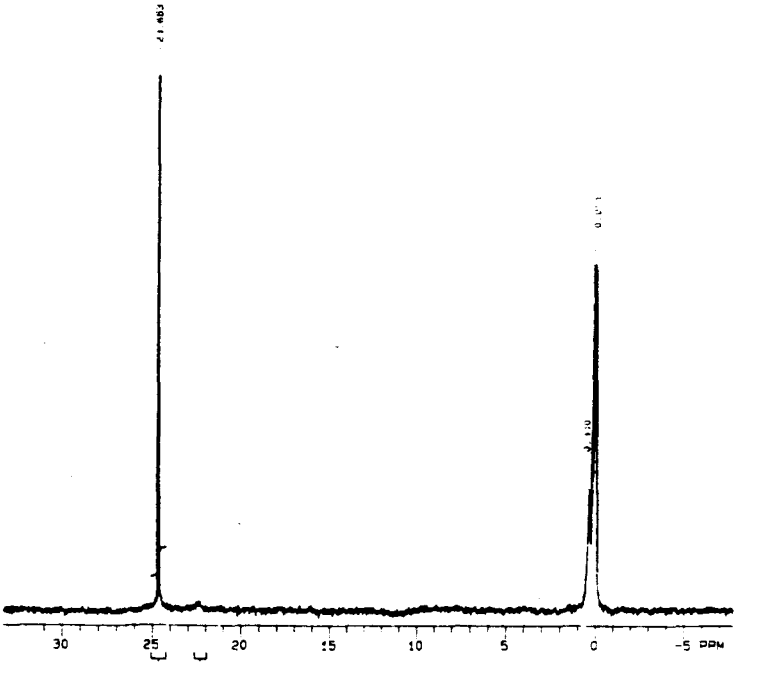

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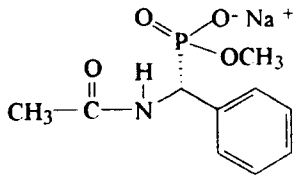

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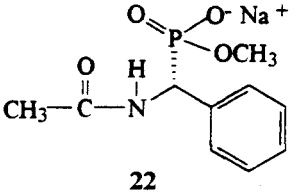

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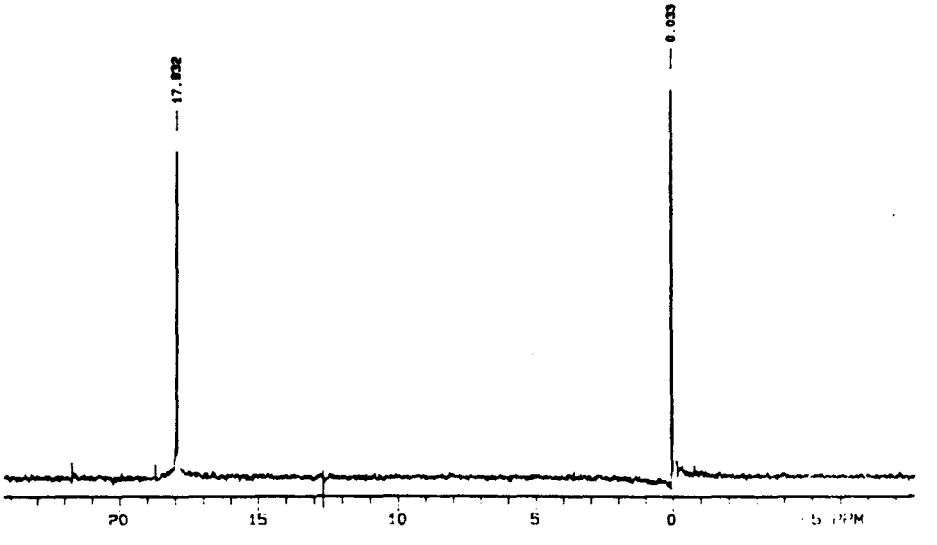

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VITA

Nadina Cezara Monberg

Nadina C. Monberg was born on April 4, 1968 in Bucharest, Romania. After completing her eighth grade, she moved with her family to United States to join the rest of her relatives which have been living in northwest Indiana since 1930's. In May, 1987 she graduated from Hanover Central high school and in the fall entered Purdue University Calumet. She participated in the university's co-op program for four semesters, working for the quality department at Inland Steel Co. in East Chicago, Indiana. In May 1992, she received a Bachelor of Science in Chemistry, and two weeks later started summer school and teaching assistant duties at Loyola University Chicago. In August, 1992 she became a full time graduate student at Loyola, where she was awarded a GAANN fellowship for 3 years. Research toward her master's thesis was guided by professor Charles M. Thompson, and was successfully defended on March 24, 1995. On December 12, 1994, she started emplyment with Abbott Laboratories in the neuroscience division.

References

Professor Charles M. Thompson
Deparment of Chemistry
University of Montana
Missoula, Montana 59812

Professor Duarte Freitas
Deparment of Chemistry
Loyola University Chicago
6525 N. Sheridan Rd., Chicago, Illinois 60626

Professor David Crumrine
Department of Chemistry
Loyola University Chicago
6525 N. Sheridan Rd., Chicago, Illinois 60660

THESIS APPROVAL SHEET

The thesis submitted by Nadina C. Monberg
has been read and approved by the following committee:

Charles M. Thompson, Ph.D., Director
Professor, Chemistry
Loyola University Chicago
(Current address: University of Montana)

Duarte Freitas, Ph.D.
Professor, Chemistry
Loyola University Chicago

David Crumrine, Ph.D.
Associate Professor, Chemistry
Loyola University Chicago

The final copies have been examined by the director of the committee
and the signature which appears below verifies the fact that any necessary changes have
been incorporated and that the thesis is now given final approval
by the committee with reference to the content and form.

The thesis is, therefore, accepted in partial
fulfillment of the requirements for the degree of M. S..

March 28th 1995
Date

Charles M. Thompson
Director's Signature