INVESTIGATIONS OF THE ACTIVE SITE OF MICROSOMAL LEUCINE AMINOPEPTIDASE BY PROBING WITH ETHYLENEDIAMINE DERIVATIVES

INVESTIGATIONS OF THE ACTIVE SITE OF MICROSOMAL LEUCINE AMINOPEPTIDASE BY PROBING WITH ETHYLENEDIAMINE DERIVATIVES

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ABSTRACT

The active site of porcine microsomal aminopeptidase was probed by studying the inhibition of the enzyme using derivatives of ethylenediamine and diaminopropionic acid. In addition, some amino acids, substituted hydroxamates and phosphates were also tested.

In order to synthesize diaminopropionic acid derivatives, CBZ-amino acid p-nitrophenyl esters were reduced to the corresponding aldehydes by lithium tri-t-butoxy-aluminohydride. Through the Strecker synthesis, the aldehyde intermediates were converted to diaminopropionitrile analogues which were then hydrolysed in acid to the desired products. Unfortunately, these compounds were not potent inhibitors for this enzyme.

α-Amino acids were found to be better inhibitors than their ß-amino counterparts and the K_i of α-leucine was about 7-fold lower than its ß-analogue. The amino group position of the amino acids is therefore important for enzyme recognition. On the other hand, N-alkylation of ethylenediamine was observed to abolish its inhibition potential. Furthermore, another unexpected finding in this work is that N- or O- methylation of the hydroxamate group hinders the ability of these inhibitors to act as a bidentate zinc ligand. Although some phosphate derivatives that we tested showed poor inhibitory potency, phosphonamidate, a

potential transition state analogue, might serve as a powerful inhibitor.

In summary, the relationship between the structure and inhibitory potency of some inhibitors was demonstrated.

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ABBREVIATIONS

CBZ or Z	Carbobenzoxy
C-13	Carbon-13
DAPA	2,3-Diaminopropionic acid
δ	NMR chemical shift, in ppm downfield from Tetramethylsilane
DAPN	2,3-Diaminopropionitrile
DECP	Diethylcyanophosphonate
DMSO	Dimethysulfoxide
EDTA	N,N,N',N'-Ethylenediaminetetraacetic acid
HPLC	High Pressure Liquid Chromatography
IR	Infra-red
kD	Kilodalton
LAH	Lithium aluminum hydride
LTBA	Lithium tri-t-butoxyaluminohydride
MAP	Microsomal Aminopeptidase
m.p.	Melting Point
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TPCK	Tosyl-L-Phenylalanine chloromethyl ketone
Tris	Tris-(hydroxymethyl)aminomethane

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1. INTRODUCTION

There is considerable interest for the development of highly potent specific enzyme inhibitors among enzymologists and synthetic chemists alike. Inhibitors for enzymes that play very crucial physiological roles can be potentially used in chemotherapy. For example, captopril (Cushman et al., 1977; Ondetti et al., 1977) is currently used as an antihypertensive drug because of its potency as an inhibitor for angiotensin converting enzyme, which plays a role in the regulation of blood pressure. Inhibitors can also be used for the purification and characterization of enzymes. Hydroxamic acid inhibitors for thermolysin (Nishino and Powers, 1979) and lisinopril for angiotensin converting enzyme (Bull et al., 1985) are now used to prepare affinity chromatographic columns for these enzymes.

Aminopeptidase is an N-terminal exopeptidase involved in the degradation of neurotransmitters including enkephalins which are endogenously produced painkillers. Inhibitors for this enzyme may be used in the future as analgesic drugs. Therefore, the aim of this work was to further probe the active site of aminopeptidase, develop a new series of powerful inhibitors and apply the compounds for clinical trials if appropriate.

The first section of this chapter involves the relevant theoretical basis of

the use of transition state theory and inhibition kinetics to study enzymes and design of inhibitors. The classification of enzymes and a more detailed illustration of zinc metalloenzymes will be examined in the following section. The focus, then, will be on one such zinc enzyme, aminopeptidase and its catalytic mechanisms, physiological roles, and its inhibitors in the background of clinical significance as drugs. Finally, the last section deals with the survey and design of research, and the different goals and tools employed in this work.

1.1 Enzyme Kinetics

Enzymes, as biological catalysts, are able to lower the chemical activation energy of a reaction, thereby enhancing the rate of reaction. In this section , a general picture of the strategies used by enzymes, in order to achieve rate accelerations, will be discussed.

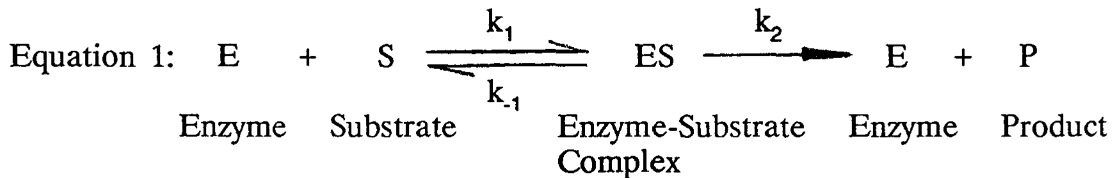
1.1.1. Transition State Theory

In this theory (Pelzer and Wigner, 1932), the only physical entities concerned are the ground state and the transition state. This theory is able to relate the rate of a reaction to the difference in Gibbs' free energy between the ground and transition states (Fersht, 1985). By assuming the two states are in thermodynamic equilibrium, the concentration of the transition state can be calculated from their differences in energy. Multiplying the concentration of the transition state by the rate constant for its decomposition will, therefore, derive

the overall rate of reaction.

1.1.2 Factors Affecting Rate Acceleration (Fersht, 1985)

Generally, non-enzymatically catalyzed reactions in solution are slow because the catalyst and the substrate must be brought together in the proper orientations for the reaction to occur. These intermolecular reactions are 100,000 times slower in rate than their intramolecular enzymatic counterparts. Enzymatic reactions can be said to be intra-molecular because the chemical change occurs in the confinement of an enzyme-substrate complex (ES, Equation 1).



The theoretical basis for rate acceleration can be explained by entropy or the degree of randomness or disorder of a system. The sum of translational, rotational and internal entropies contribute to the entropy of a given molecule. The union of two molecules in the case of bimolecular reactions in solutions leads to the loss of one set of rotational and translational entropies; in enzymatic catalysis, this entropy loss occurs upon formation of the E-S complex intermediate. This makes the catalytic groups part of the same molecule as the substrate, so there is no loss of entropy as the enzyme catalyses the formation of the transition state. The loss of 'disorder' is fully compensated for by the enzyme-substrate binding energy. This reduction of entropy loss enables the enzyme to speed up

its reaction by 10^8 - 10^9 fold.

Another factor contributing to the rate acceleration is enzyme-transition state complementarity. If the enzyme is complementary to the substrate, there will be a decrease in binding energy on the formation of the transition state because the substrate's three-dimensional status changes to give a poorer fit. Therefore, an increase in activation energy will result. On the other hand, enzyme-transition state complementarity is favoured because the increase in binding energy is fully realized as the reaction proceeds to the transition state. The reaction rate, k_{cat}, will increase.

The Michaelis-Menten equation can be expressed in the following way:

$$V = [E][S]\, k_{cat} / K_M$$

Enzymes are able to maximize k_{cat}/K_M by being complementary to the transition state of substrate. The maximum reaction rate for a particular substrate concentration also depends on the individual values of k_{cat} and K_M. While maintaining k_{cat}/K_M constant, the maximum rates are obtained by increasing k_{cat} and K_M together in equal magnitudes.

1.1.3 Enzyme Inhibition

An inhibitor is considered a substance that can reduce the rate of an enzyme-catalyzed reaction. Studies of inhibitors can provide information on (Segel, 1976): 1.) the specificity of an enzyme. 2.) the kinetic mechanism of the reaction. 3.) the physiochemical nature of the active site., and 4.) the

applications of these compounds for their desired pharmacological effects.

Reversible inhibitors can be classified as competitive, noncompetitive and uncompetitive inhibitors. A competitive inhibitor can combine with the free enzyme in a way that prevents substrate binding. This type of inhibitor is usually a substrate analogue, an alternate substrate of the enzyme, or the final product of the reaction. The substrate and inhibitor are mutually exclusive because in competing for the same site, binding of one provides steric hindrance for the other, or both can have distinct but overlapping binding sites (Segel, 1976). Noncompetitive inhibition occurs when substrate and inhibitor are not mutually exclusive and both compounds bind randomly, reversibly and independently of each other. An uncompetitive inhibitor is a substance that only binds reversibly to the enzyme-substrate complex but not to the free enzyme. The resulting ESI complex is catalytically inactive.

Irreversible inhibitors can be classified into two types: 1.) an affinity labelled or active-site-directed irreversible inhibitor (Baker et al., 1961)., and 2.) the so-called mechanism-based inactivator, enzyme-activated irreversible inhibitor, or suicide inhibitor (Abeles and Maycock, 1976; Walsh, 1983). The former type of inhibitor resembles a normal substrate but contains a chemically reactive group which can form covalent bonds with the active site residue after the initial formation of an enzyme-inhibitor complex (Fersht, 1985). For example, an inhibitor of chymotrypsin is tosyl-L-phenylalanine chloromethyl ketone (TPCK) which has a structure that is similar to the normal substrate, tosyl-L-phenylalanine

methyl ester. It can irreversibly attach to the histidine moiety of the chymotrypsin active site causing inactivation (Schoellmann and Shaw, 1963). A suicide inhibitor, which contains a latent reactive functional group, is a relatively unreactive compound in the absence of target enzyme. However, upon binding to the active site as a normal reversible inhibitor, certain catalytically induced transformations can occur to create a reactive specie which can then covalently bond to a neighboring functional group and inactivate the enzyme. As an example, propargylglycine is a suicide inhibitor for γ-cystathionase (Walsh and Abeles, 1973). Due to its specificity and potency, this kind of inhibitor is highly desirable for use as a pharmacological agent.

1.2 Proteases

1.2.1 Classification

Proteases can be classified according to their locations of actions as exopeptidases and endopeptidases. They can also be divided into four major groups according to their nature of the catalytic active site residues: the metallo-, serine-, carboxyl- and thiol-peptidases (Hartley, 1960). Zinc, copper, and iron are the three most common metals found in most metalloenzymes. The following subsection will deal with zinc enzymes because aminopeptidase is now known to belong to this category.

1.2.2 Zinc metalloenzymes

Zinc, although present in all living things in very minute amounts, is very

important in life. Zinc may assume one of the three roles in metalloenzymes: catalytic, structural, and regulatory (Vallee and Galdes, 1984).

Zinc may have direct involvement in catalysis, e.g. aminopeptidase (Himmelhoch, 1970; Lehky et al., 1973). Some proteins require this transition metal for their overall structural oligomeric quaternary stability, for example, the dimer form of B. subtilis α-amylase (Vallee et al., 1959). In addition to catalysis at the active site, bovine lens leucine aminopeptidase was found to be activated by zinc after the metal becomes bound to the regulatory site of the native enzyme (Carpenter and Vahl, 1973).

Zinc metalloenzymes can be divided into different categories which are listed in Table I. The important contributions of Keilin and Mann (1940) on zinc biochemistry were their discoveries of the first zinc enzyme, carbonic anhydrase from bovine red blood cells and they established for the first time the physiological role of this metal in organisms.

1.3 Aminopeptidases

First isolated by Linderstrom-Lang in 1929 from mammalian sources, aminopeptidase catalyzes the hydrolysis of the N-terminal residues of proteins, peptides and amino acid amides and is therefore classified as an exopeptidase.

TABLE I
Classification of Zinc Metalloenzymes and some Selected Examples (Vallee & Galdes, 1984)

Class [examples]	Source	Reference
1. **Oxidoreductases**		
Alcohol dehydrogenase	Vertebrates yeast, human	Vallee, 1955. Arslanian et al., 1971. Pares and Vallee, 1981.
2. **Transferases**		
Aspartate Trans-carbamylase,	E. coli	Rosenbusch and Weber, 1971.
DNA polymerase	E. coli	Slater et al., 1971.
3. **Hydrolases**		
Aminopeptidase	Mammals	Himmelhoch, 1970 Carpenter and Vahl, 1973. Van Wart and Lin, 1981.
Carboxypeptidase	Mammals	Bodwell and Meyer, 1981. Wintersberger et al., 1962.
4. **Lyases**		
Carbonic anhydrase, Fructose 1, 6-bisphosphate aldolase	Animals, yeast, bacteria	Keilin and Mann, 1940. Rutter and Ling, 1958. Sugimoto and Nosoh, 1971. Baldwin et al., 1978.
5. **Isomerases**		
Phosphomannose isomerase	Yeast	Gracy and Noltman, 1968.
6. **Ligases**		
pyuvate carboxylase	Yeast	Scrutton et al., 1970.

Although its carboxypeptidase counterpart was extensively studied, less attention was focussed on this enzyme. Subsequent isolation and studies of aminopeptidase were done from pig kidney (Spackman et al., 1954; Himmelhoch, 1970; Pfleiderer, 1970; Lehky et al., 1973; Van Wart and Lin,1981), bovine lens (Carpenter et al., 1971, 1972, 1973; Hanson and Frohne, 1976; Van Loon-Klaassen et al., 1979, 1980), hog intestinal brush border (Benajiba and Maroux, 1980) and human tissues (Little et al., 1976; Yokozeki and Sato, 1987, etc.). Since porcine kidney and bovine lens aminopeptidases are most extensively studied, these two will be discussed.

1.3.1 Porcine Kidney (Microsomal) Aminopeptidases

In mammalian cells, especially in liver and kidney, at least two different kinds of aminopeptidase exist, microsomal in origin and the other cytoplasmic, but they differ in their substrate specificities.

The aminopeptidase from porcine kidney, having a molecular weight of 280,000, appears to contain two catalytic active 140 kD subunits (Wacker et al., 1976). Two moles of firmly bound zinc per mole of enzyme were also found (Lehky et al., 1973). Approximately 400 carbohydrate residues are present in this glycoprotein and contribute to 20% of the molecular mass (Wacker et al., 1971).

This enzyme was classified as one of the zinc metalloenzymes and is supported by the following evidence. Firstly, the intrinsic zinc to protein ratio increases during enzyme purification whereas the extrinsic ratio decreases to zero.

The former becomes constant in a small integral number with complete purification (Lehky et al., 1973). The fact that this metalloenzyme can be isolated with maximum activity, without loss of the metal, does not support the possibility of being just a metal-protein complex. Secondly, metal-binding or chelating agents are able to inactivate the enzyme by removing the metal from the active site. Enzyme inhibitors were also seen to protect aminopeptidase from EDTA inactivation (Chan et al., 1982; Chan, 1983). Thirdly, the apoenzyme activity can be restored by adding zinc. In a similar manner, the holoenzyme can be inactivated by metal loss. Both activities are directly proportional to the amount of zinc added or lost, respectively (Lehky et al., 1973).

At a prolonged period of incubation, a peptide can be hydrolysed completely when sufficient enzyme is present. The rate of hydrolysis is directly influenced by the side chains' nature of the N-terminal residues. The rate decreases as the size of aliphatic side chain becomes smaller and the aromatic residues are less sensitive than the larger aliphatic ones (Smith and Spackman, 1954). Leucyl amides are among those hydrolysed most rapidly. More polar residues, e.g., lysine or aspartate are relatively resistant to the catalytic action of the enzyme. In addition to the necessary requirement of having a free N-terminal in the L-configuration, hydrolysis will be stopped or strongly hindered just before the encounter of the prolyl imide bond or the ß-peptide bond of the aspartyl residue (Light, 1967).

A regulatory site was also found in each subunit (Van Wart and Lin,

1981) and the enzymatic activity can be modulated by the binding of divalent metal ions to this site. The enzyme is activated by Mn(II) and inhibited by Ni(II), Cu(II), Hg(II), Zn(II) and Cd(II). These phenomena were also observed more than three decades ago (Spackman et al., 1954). Moreover, this exopeptidase is relatively insensitive to heat and extreme pH, but can be denatured by alcohols and other organic solvents. The most useful pH for peptide hydrolysis is between pH 7.0 to 7.3 (Wachsmuth et al., 1966) but the pH optimum is in the region of pH 7.8 - 8.0 (Himmelhoch, 1970). Tyrosyl and histidyl residues were shown to be part of the active site of aminopeptidase and were essential for its catalytic activity (Femfert and Pfleiderer, 1969; Pfleiderer and Femfert, 1969; Femfert et al., 1972).

1.3.2 Bovine Lens Leucine Aminopeptidase

Bovine lens leucine aminopeptidase, having a molecular weight of 327,000 (Kettman and Hanson, 1970; Melbye and Carpenter, 1971), is composed of six identical subunits arranged as a trimer of dimers (Carpenter and Harrington, 1972), which contrasts the dimeric form of the porcine kidney counterpart. The presence of two zinc atoms per subunit molecular weight of 54,000 was discovered (Carpenter and Vahl, 1973).

As in the case of the porcine enzyme, bovine aminopeptidase prefers hydrophobic substrates. Limited tryptic digest of the enzyme between Arg-137 and Lys-138 do not affect either the catalytic activity or the substrate binding (Van Loon-Klaassen et al., 1979) because digested active fragments still remain intact.

1.3.3 Other Aminopeptidases

Apart from mammalian and human sources, aminopeptidases are found extensively in micro-organisms, especially bacteria. Aminopeptidases from Aeromonas Proteolytic and Clostridial histolyticum are extracellular enzymes (Kessler and Yaron, 1976; Prescott and Wilkes,1976). Some thermophilic (either thermostable or thermolabile) aminopeptidases from bacterial origin are also well characterized (Roncari et al., 1976). These enzymes also preferentially hydrolyse peptides containing aliphatic or aromatic amino acid residues. Recent studies of Aeromonas aminopeptidase by Makinen et al. (1982) show the involvement of at least one tyrosyl and one carboxylic acid residues in the catalytic activity of the enzyme, and the participation of both tyrosine and histidine in zinc ligand formation.

1.3.4 Proposed Mechanism of Peptide Hydrolysis by Aminopeptidase

The catalytic mechanism of this metalloenzyme involves stepwise interactions between the metal ion of the enzyme and the substrate. Some early work by Smith and Spackman (1954) suggested a sequential mechanism involving the combined co-ordination between the N-terminal amino group and the amidic nitrogen with the metal ion to form a chelate complex (Figure 1a). The simultaneous electron withdrawal from the C-N bond, through the metal ion interaction with nitrogen, would enable the attack of hydroxyl ion at the carbon of the amide bond. A nucleophilic displacement of the amino group would result.

Another mechanism was recently proposed by Chan et al. (1982) in which a concerted proton-transfer process occurs after the generation of the pentacoordinated structure upon substrate binding (Figure 1b). The bound hydroxyl ion would then act as a general base leading to attack by another water molecule on the carbonyl carbon of the scissile peptide bond. A tetrahedral intermediate is formed and its breakdown is prompted by the donation of a proton by the zinc bound water molecule. The hydrolysed products are then diffused out of the active site to generate the free enzyme.

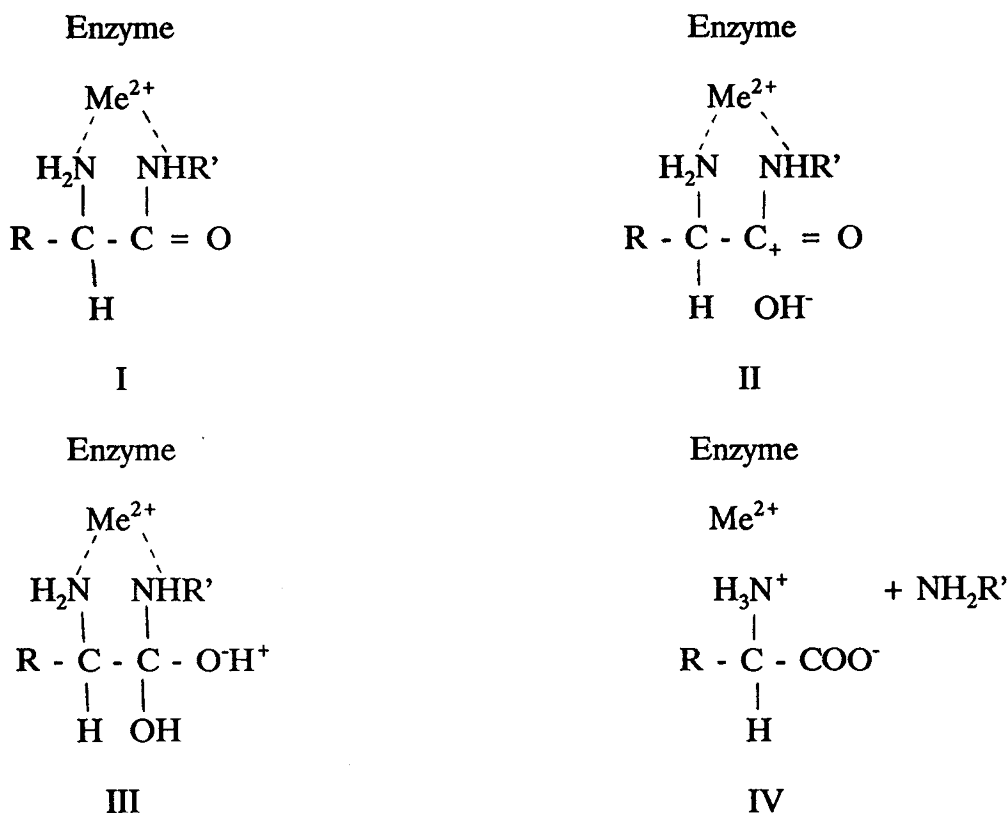


Figure 1a. **Proposed mechanism of the hydrolysis of polypeptides by leucine aminopeptidase (Smith and Spackman, 1954, Figure 8).**

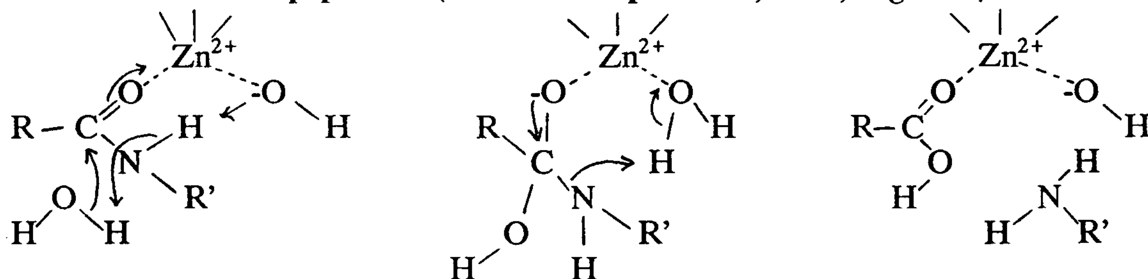


Figure 1b. **Postulated mechanism of the peptide hydrolysis catalyzed by leucine aminopeptidase (Chan et al., 1982, Figure 3).**

1.4 Physiological Roles of Aminopeptidase

1.4.1 Neurophysiological Role

The endogenous opioid pentapeptides, the enkephalins, are neurotransmitters which are rapidly inactivated after their release from cerebral neurons. Both the Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and Met-enkephalin (Tyr-Gly-Gly-Phe-Met) exhibited a weak and transient analgesic effect when injected into the rat brain and displayed only a short half-life (Belluzi et al., 1976). These features imply that they are degraded by peptidases present in the central nervous system whose role is to maintain homeostatic levels of these neurotransmitters.

Three kinds of hydrolytic enzymes involved are now known. The Tyr-Gly bond of enkephalins can be cleaved by the first type, the aminopeptidases, which is purified from rat, bovine, monkey and human brain (Hambrock et al., 1976; Vogel and Altstein, 1980; Gros et al., 1985). While the second type, the dipeptidyl aminopeptidases can hydrolyse the Gly-Gly bond (Graves et al., 1978). The third hydrolytic enzyme, enkephalinases, are dipeptidyl carboxypeptidases responsible for Gly^{3}-Phe^{4} bond cleavage and is present in the brain (Malfroy et al., 1978). These peptidases are metalloenzymes and possibly all contain zinc (Shimamura et al., 1983). In addition to the CNS aminopeptidases, these enzymes are also present in plasma (Venturelli et al., 1987) and the kidneys (Gros et al., 1985). Gros et al. (1985) isolated a puromycin insensitive but bestatin sensitive, enkephalin-hydrolyzing aminopeptidase in rat brain which resembled

aminopeptidase M purified from kidney brush border membranes. Also, Leu-enkephalin in human plasma (and other sources) is mainly degraded by aminopeptidase and two different aminopeptidase inhibitory polypeptides were found in the blood with molecular weights of 9000 and 2000 (Venturelli et al., 1987). The protecting mechanisms by these polypeptides maintain the physiological plasma levels of enkephalins.

1.4.2 Recent Designs of Enkephalin Degrading Enzyme Inhibitors as Potential New Analgesics

If the degradation of these morphine-like enkephalins involve certain peptidases, then inhibitors of these enzymes can prolong the natural pain-killing effects of these pentapeptides and they may be used as potential analgesics. In addition, owing to the probable role of enkephalins in emotional and behavioural controls, enkephalin degrading enzyme inhibitors can also be applied as new psychoactive agents (Roques and Fournie - Zaluski, 1986).

(a) Tools to study the protection of enkephalins from degrading enzymes by enzyme inhibitors

Firstly, the protecting ability of the inhibitor could be evaluated by the co-administrations of increasing concentrations of a given inhibitor with a fixed dose of [^{3}H]Leu-enkephalin in vivo (Roques and Fournie - Zaluski, 1986). In this assay, the animals (e.g., mice) were killed 5 minutes after dose administration and the intact [^{3}H]Leu-enkephalin was determined. By comparing with control

animals, the degree of protection could be ascertained.

Secondly, washed brain slices were used; this experiment resembled more the actual physiological situation (Roques and Fournie - Zaluski, 1986). In this test, the enkephalins were cleaved only by membrane-bound enzymes and the appearance of tritiated Tyr, Tyr-Gly and Tyr-Gly-Gly could be monitored. Addition of inhibitor decreased the formations of these metabolites. Lower radioactivity would correspond to higher inhibitory potency.

Thirdly, in vitro inhibition kinetic assays could be performed by using radioactive Leu- or Met-enkephalin as the substrate in the presence of different aminopeptidase inhibitors (Shimamura et al., 1983).

(b) Inhibitors of enkephalin-degrading enzymes

In the past 10 years, a lot of attention has been focussed on the development of inhibitors for aminopeptidase, particularly enkephalinase, in order to elucidate the modes of actions of these enzymes and to search for potential drugs.

Bestatin and amastatin, compounds of microbial origin, are good inhibitors for aminopeptidase (Umezawa et al., 1976; Rich et al., 1984) and recently they were also found to inhibit enkephalin metabolism (Chaillet et al., 1983; Cohen et al., 1983; Shimamura et al., 1983). These naturally occurring hydrophobic substances were proposed to be an analogue of the transition state or tetrahedral intermediate for amide bond hydrolysis formed via co-ordination with zinc. Angiotensin III and actinonin are two recent potent inhibitors of

enkephalin-degrading enzymes (Hachisu et al., 1987; Shimamura et al., 1987). The former was found to inhibit aminopeptidase and dipeptidyl aminopeptidase while the latter also inhibited enkephalinase. The K_i values of the two inhibitors were in the micromolar range and both showed powerful analgesic action. The hydroxylamido moiety of actinonin may be able to ligate with the metal in the active site.

In addition, synthetic inhibitors have been developed. Thiol and hydroxamate derivatives of amino acids and peptides were among the most potent ones (Roques et al., 1980; Blumberg et al., 1981; Coletti-Previero, 1982; Bouboutou et al., 1984). These inhibitors have high affinities for the enzymes by co-ordinating strongly with the zinc metal. Thiorphan and kelatorphan were two powerful inhibitors for enkephalinase (Roques et al., 1980; Bouboutou et al., 1984) and were proposed for possible clinical use as new analgesic and psychoactive drugs. The locations of the ligating groups were crucial and the proposed mode of binding at the active site of enkephalinase is illustrated in Figure 2.

ENKEPHALINASE

```
                          Zn2+     S1'            S2'          IC50(nM)
             ----------   O  ‾   CH2Ø‾‾‾‾‾‾‾‾ Ra ‾O-
                          ||      |            |  |
Enkephalins     Tyr-Gly-Gly-C-NH-CH-CO-NH-CH-C=O

                                  Ø
                                  |
                                 CH2 O        O-
                                  |  //        |
Thiorphan                  -S-CH2-CH-C-NH-CH2-C= O                4.7b

                                  Ø
                                  /
                         HO O    CH2 O    CH3 O-
                          | ||    |  ||    |  |
Kelatorphan             H-N-C-CH2-CH-C-NH-CH-C=O                  4.0

                                  Ø
                                  /
                           HO O CH2 O       O-
                            | || /  //        |
                           H-N-C-CH-C-NH-CH2-C=O                  10.0

                                  Ø
                                  /
                         HO O    CH2 O        O-
                          | ||    |  //        |
                      CH3-N-C-CH2-CH-C-NH-CH2-C=O                 70,000
```

a: Leucine or Methionine residue.
b: K_i value

Figure 2. Model of the active site of enkephalinase and the binding of enkephalins, thiorphan, kelatorphan, and derivatives (selected schematic representation of Fig. 1, Roques et al., 1980; and Table I, Bouboutou et al., 1984).

1.5 Survey and Design of Research

Basically, there are several reasons for us to study aminopeptidase. Unlike other zinc proteases, aminopeptidase has not been extensively studied. Apart from its significant physiological function, this N-terminal exopeptidase was used for protein and peptide sequencing in the past (Smith and Hill, 1960). Although the crystal structure of the enzyme-inhibitor is not known, a lot of structural and mechanistic insight can be gained through the study and design of its inhibitors, in addition to considerable background for future X-ray crystallographic studies. Furthermore, an abundant supply of the enzyme is available from commercial sources.

Strikingly within the last 15 years, a number of inhibitors bearing a variety of different functional groups and side chains were found and developed for aminopeptidase. Earlier work done by Birch et al. (1972) provided the preparation of amino acid chloromethylketone analogues and they were discovered to be strong reversible inhibitors of leucine aminopeptidase. Bacterially derived bestatin (Umezawa et al., 1976) and amastatin (Rich et al., 1984), the tertiary butyl derivative of H-Thr-Phe-Pro-OH (Jost et al., 1972) as well as the phenylthiophenylacetic acid (Miller and Lacefield, 1979) were later found to have strong inhibition potencies. The two oxygen atoms of the α-hydroxy-carbonyl functional groups of the former two natural inhibitors were proposed to act as a bidentate ligand for the active site zinc in a manner similar to that proposed for N-hydroxypeptides and hydroxamates (Nishino and Powers, 1979). Although, the

latter two inhibitors did not give much information to the structure of the catalytic pocket and did not resemble normal substrate analogues, they did provide strong evidence to confirm the favourable binding of hydrophobic compounds to aminopeptidase.

As with other zinc proteases (Cushman et al., 1977; Nishino and Powers, 1978, 1979; Ondetti et al., 1979; Fournie-Zaluski et al., 1984), hydroxamate and thiol derivatives of amino acids were also extremely strong inhibitors for aminopeptidase (Chan et al., 1982; Baker et al., 1983; Chan, 1983; Pickering et al., 1985; Ocain and Rich, 1987). Among these inhibitors, L-leucinthiol and L-lysinethiol (having Ki's of 22 and 0.91 nM, respectively) were the most potent reversible ones ever found. The thiol and the bidentate hydroxamate groups were proposed to act as highly effective zinc ligands. Recently, N-monobenzyl phosphonobenzyl derivatives of amino acids and N-2-halogeno-2, 2-diphenyl-acetyl mono- and dipeptides were also synthesized and tested to be good reversible inhibitors (Weiss et al., 1986, 1987). The large and hydrophobic nature of these compounds partly contribute to their tight binding.

In order to further probe the active site of aminopeptidase, inhibition studies in the presence of two ligands simultaneously for this porcine microsomal enzyme was conducted in our laboratory (DiGregorio et al., 1988). The results were then analyzed using Yonetani-Theorell kinetics (1964) to determine the degree of interaction between the two inhibitors by measuring the α-constant. Two inhibitors are said to be mutually exclusive when α is infinite. On the other

hand, binding of the first inhibitor decreases the affinity of the enzyme for the other inhibitor when α is between 1 and ∞. Synergistic (or positive co-operative) binding between the two occurs when α is between 0 and 1.0.

The combined effect of an α-amino binding site together with a hydrophobic pocket renders the active site of this N-terminal exopeptidase highly specific in its binding to substrates. In the study by DiGregorio et al. (1988), ammonia and isoamylamine were used to probe this amine binding site. These two inhibitors were found to be mutually exclusive competitors for the same site (Table IIa). The binding between ammonia and α-amino acids or mercaptoethylamines was also found to be mutually exclusive. Moreover, due to the proximity of the hydrophobic pocket to the amine binding site, tighter binding was achieved by introducing an extended side chain to the substrates (or inhibitors).

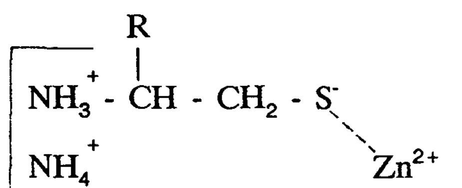
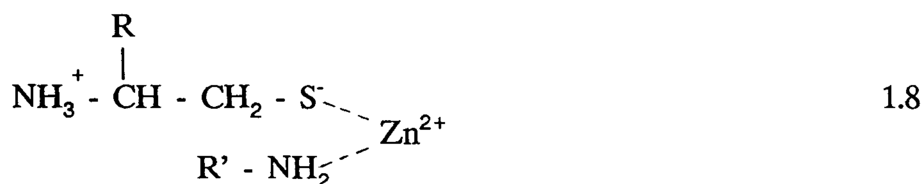
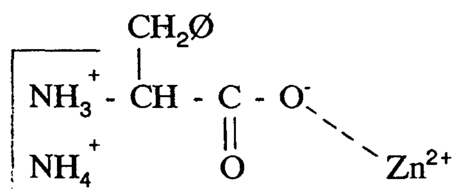
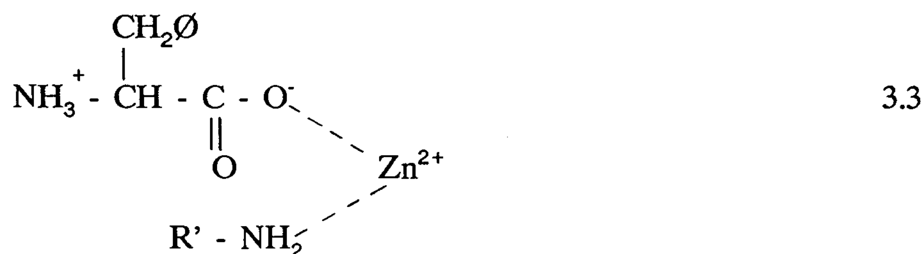
On the other hand, when ammonia was replaced by isoamylamine in similar experiments, the corresponding α constant was lowered drastically (Table IIa). To rationalize this, the active site was postulated to be able to accept the isoamyl alkyl moiety in a second hydrophobic site when the first one is occupied (eg., by an α-amino acid); the amino group of isoamylamine was proposed to ligate with the zinc atom. This second amine binding mode was further supported by the fact that ethylenediamine bound 13-fold tighter than ethylamine in analogy with ß-mercaptoamine (Table IIb). Furthermore, the introduction of a carboxylate group to ethylenediamine, as in DL-2, 3-diaminopropionic acid (DAPA), increased

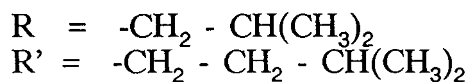
its affinity by 28-fold. This enormous improvement in binding suggests enhanced interactions between the second amino group and the zinc. Since two separate hydrophobic sites were proposed, introducing non-polar substituents to DAPA might provide promising high affinity inhibitors. With this background, the aim of this work is to synthesize these derivatives as a new series of inhibitors for aminopeptidase. Hopefully, some of these compounds would be of pharmacological importance and become future analgesic drugs.

In order to further study the zinc ligation power of hydroxamates and ethylenediamine, some substituted analogues were synthesized and tested. Also, α- and ß-amino acids, together with some phosphate derivatives, were also used to probe the active site of porcine kidney microsomal aminopeptidase.

TABLE II (DiGregorio et al., 1988, Table II, III)

a) Interactions of Ammonia or Isoamylamine in the Presence of Some Zinc Ligands

Proposed mode of binding[a]	Interaction constant (α)
	∞
	1.8
	∞
	3.3
	∞



R = $-CH_2 - CH(CH_3)_2$
R' = $-CH_2 - CH_2 - CH(CH_3)_2$

a: Areas of possible hindrance are enclosed by square brackets.

b) **Inhibition Constants of Some Amino Derivatives**

Inhibitors	Ki (mM)
NH_2 - CH_2 - CH_2 - NH_2	19
NH_2 - CH_2 - CH_3	250
NH_2 - CH_2 - CH_2 - CH_2 -NH_2	103
NH_2 - CH_2 - CH_2 - COOH	47
NH_2 - CH_2 - $CH(NH_2)COOH$ (D,L)	0.69
NH_2 - CH_2 - CH_2 - SH	0.0032

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzyme and Substrates

Microsomal aminopeptidase (MAP) from porcine kidney (EC 3.4.11.2) (Type IV-S) and substrate, L-leucine p-nitroanilide, were obtained from Sigma Chemical Co. The enzyme (containing 2.6 mg protein/ml in 3.5 M $(NH_4)_2SO_4$, pH 7.7, 10 mM $MgCl_2$) was supplied as a suspension and stored at 0-4°C. A substrate stock solution of 50 mM was prepared and stored at 0-4°C for several weeks.

2.1.2 Inhibitors

N-methylethylene diamine, N,N-dimethylethylene diamine, methyl phosphonic acid, dimethyl phosphite, diethyl phosphite, dimethyl phosphinic acid, diethyl methylphosphonate, diphenyl phosphate, phenyl phosphonic acid and phenyl phosphate, disodium salt di-hydrate were obtained from Aldrich Chemical Co. p-Phenylthio-phenyl acetic acid was a gift from Lilly Research Laboratories. L-Alanine, L-valine and acetohydroxamic acid were purchased from Sigma Chemical Co. Glycine was supplied by Fisher Scientific Co. ß-Leucine was generously provided by Dr. J. Michael Poston of the National Institutes of Health, Bethesda, MD.

2.1.3 Synthetic Reagents

CBZ-glycine p-nitrophenyl ester, its leucine analogue, isoamylamine, ß-phenylethylamine, lithium aluminum hydride, L-leucinamide HCl, N-methyl hydroxylamine HCl, ethyl formate and ethyl caproate were purchased from Sigma Chemical Co. Lithium tri-t-butoxyaluminohydride, diethyl cyano-phosphonate, ammonium acetate and O-methyl hydroxylamine HCl were obtained from Aldrich Chemical Co. Sodium cyanide and sodium metal were products of BDH Chemical Ltd. C^{13}-sodium cyanide was supplied by Cambridge Isotope Laboratories while hydroxylamine HCl and ethyl acetate were obtained from Fisher Scientific Co.

2.1.4 Other Chemicals

Tris base was supplied by Canadian Scientific Products Ltd. 2,4-Dinitrophenylhydrazine, ninhydrin, 2,4,6-collidine and tetramethylsilane were purchased from Sigma. Deuteriated solvents for NMR were products of MSD Isotopes.

2.2 Enzyme Kinetics

2.2.1 Enzyme Assays

All assays for aminopeptidase were performed in 0.1M Tris-HCl buffer pH 7.5 containing 0.3M NaCl at 25°C (Pickering et al., 1985). The enzyme was diluted one hundred times from stock into 0.1 M Tris-HCl containing 0.01 mM $ZnCl_2$ and 0.2mg bovine serum albumin per ml. After the addition of enzyme

(1.3 μg/ml), microsomal aminopeptidase activity was determined by measuring the rate of change in absorbance at 405 nm caused by the hydrolysis of the chromogenic substrate, L-leucine p-nitroanilide. The absorbance change was monitored for five minutes using a Gilford 2400 spectrophotometer. Five samples could be followed simultaneously.

2.2.2 Kinetic Experiments

In order to determine the inhibition constants, assays were performed in duplicate at four different substrate concentrations ranging from 0.5 to 2 mM at a fixed enzyme concentration. Preliminary inhibition assays were first done to estimate the range of inhibitor concentrations used. Five different inhibitor concentrations were then employed ranging from about zero to two K_i values. Lineweaver-Burk plots (Lineweaver and Burk, 1934) were constructed with initial velocity values to determine the mode of inhibition and Dixon plots (Dixon, 1953) were used to determine K_i values.

The original inhibitor stock solutions were adjusted to pH 7.5 when necessary before the assays were performed. In cases where the K_i values were too high to be determined, the lower limit of K_i was approximated by the following equation.

$$Ki = \frac{V'}{V-V'} \times \frac{Km[I]}{Km + [S]} \quad \text{for competitive inhibitors}$$

where V' = activity with inhibitor

V = activity without inhibitor

$[I]$ = inhibitor concentration

$[S]$ = substrate concentration

Km = Michaelis constant

2.3 Synthesis of Inhibitors

The progress of the synthesis and the purity of the synthesized compounds were checked by thin-layer chromatography on silica gel plates (Analtech, silical gel G) in different solvent systems. The synthesis of CBZ-leucinal was also followed with a Beckman HPLC with a reverse-phase Bondapak C8 column using gradient of 40 to 100% methanol as the mobile phase. The eluted peaks were monitored at 280 nm. The ^{1}H NMR were taken with a 90 MHz Varian EM390 in ($^{2}H_{6}$)-dimethyl sulfoxide solutions (30-60mg/ml) using tetramethylsilane as internal reference. Carbon-13 NMR spectra were obtained on a Bruker WM250 operating at 62.87 MHz and a Perkin Elmer 283 infra-red spectrophotometer was used to take IR spectra.

Aldehydes and ketones were detected on TLC plates with a spray which consisted of 0.4% 2,4-dinitrophenyl hydrazine in 2N hydrochloric acid (Stahl, 1969). All primary and secondary amines and amino acids were identified using ninhydrin spray reagent. Upon spraying and heating on hot plates, amines and amino acids appeared as purple spots. Ninhydrin spray was prepared by dissolving 0.2 g

ninhydrin in 100 ml of isopropyl alcohol and stirred it until a clear yellow-orange solution was formed. Then 0.1 ml of 2,4,6-collidine was added. The solution would be stable for weeks at room temperature. Hydroxamic acids were identified on TLC plates as reddish-brown spots by spraying with a five percent solution of ferric chloride in 0.5 N HCl (Stahl, 1969).

2.3.1 Synthesis of 3-Isobutyl-DAPA and N'-Substituted Derivatives

(a) Synthesis of CBZ-Leucinal

In a 50 ml three-necked flask equipped with dry argon inlet, magnetic stirring bar, and kept at 0^0 C by immersion in an ice bath, CBZ-L-leucine-p-nitrophenyl ester (5.2 mmoles) and 5.2 ml of THF were placed. Lithium tri-t-butoxyaluminohydride (LTBA) (13 mmoles), was added slowly over a period of 30 minutes to the stirring solution generating a yellow coloration immediately. The mixture was then stirred for one hour at 0^0 C. The resulting aldehyde was detected on TLC plates as a yellow spot by 2,4-dinitrophenylhydrazine spray with a running solvent of 1% methanol in methylene chloride. R_f = 0.32. A minor spot with a R_f of 0.1, CBZ-leucinol, was also detected by iodine vapour. After transferring to a 250 ml round bottom flask and washing with THF, dilute H_3PO_4 was added slowly. White precipitate was formed but it redissolved by adding more acid to the solution and then the THF was evaporated. The aqueous layer was extracted with 70 ml of methylene chloride and the combined organic extracts were washed with five 100 ml portions of 0.5M sodium bicarbonate buffer at the

pH of 10.5 to remove all the p-nitrophenolate. The organic layer was separated, dried with magnesium sulfate, and the solvent removed by rotary evaporation under vacuum. A colourless oil was obtained. The product was recrystallized from methylene chloride/hexane, chloroform/hexane or from hexane alone. m.p. 95-97^{0}C yield = 72%. The aldehyde could also be recrystallized from water/ethanol or water/methanol bisolvent systems but they were avoided because of the possibility of formation of the aldehyde hydrate in aqueous medium.

In order to further characterize the product, different analytical and spectroscopic methods were employed. Firstly, silver mirror was formed after warming for 5 minutes in Tollen's test and secondly, positive chromic acid test was confirmed with the formation of a green precipitate.

That reducing agent, LTBA, was unable to reduce the protecting CBZ group verified with IR spectroscopy. Two distinct peaks at 1700 cm^{-1} and 1530 cm^{-1} were observed which corresponded to the esteric and amido bifunctionalities of CBZ group (the aldehyde carbonyl peak was also overlapped in this region). NMR (DMSO): δ0.9 [6 H, d, $(CH_3)_2$ - C], 1.3 - 1.8 [3 H, m, CH_2 - CH of side chain], 3.6 [1 H, m, >CHNH], 5.0 [2 H, s, -CH_2 of benzoxyl group], 7.4 [5 H, s, aromatic] and 9.6 [1 H, s, CHO] HPLC: Retention time = 13.2 minutes in methanol gradient = 40/100%.

(b) **Synthesis of CBZ-3-Isobutyl-2,3-diaminopropionitrile and N-Substituted Derivatives**

(i) **Modified Strecker Synthesis - with DECP**

To a mixture of CBZ-leucinal (4 mmoles) and diethylcyanophosphonate (4.8 mmoles) dissolved in 20 ml of THF in a 100 ml round bottom flask, 1.1 ml of ß-phenethylamine (8.8 mmoles) or 0.87 ml of isoamylamine (8.8 mmole) in THF (20 ml)was added. The mixture was stirred at room temperature for 3 hours and the solvent was evaporated in vacuo. The resulting oil was redissolved in methylene chloride and washed with a calculated amount of dilute HCl to remove excess amine. The organic layer was dried and evaporated. R_f = 0.60 in 1% CH_3OH in CH_2Cl_2. The product was positive to ninhydrin spray and iodine vapour. No colour was observed in control experiment. Yield = 93%.

NMR (DMSO): **a). CBZ-N-ß-phenylethyl-3-isobutyl-DAPN** δ0.9 [two d, 6 H, gem dimethyl], 1.3 - 1.8 [m, 3 H, CH_2-CH of side chain], 2.7 - 3.1 [m, 2 H, HN-CH-CH<], 3.7 - 4.2 [m, 4 H, NH-CH_2CH_2-aromatic], 5.0 [s, 2 H, CH_2 of benzoxyl group], 7.2 [s, 5 H, aromatic group of the amine], 7.4 [s, 5 H, aromatic protecting group]. IR (KBr) = No 2260-2240 cm^{-1} nitrile stretching vibration signal (Bellamy, 1960). **b). CBZ-N-isoamyl-3-isobutyl-DAPN** δ0.9 - 1.0 [q, 12 H, gem dimethyl of leucine and isoamyl amine], 1.2 - 2.0 [m, 8 H, CH_2 -CH, CH_2CH_2CH of side chains], 2.7 - 3.2 [m, 2 H, HN-CH-CH], 5.0 [s, 2 H, $CH_2$of benzoxyl group]. 7.4 [s, 5 H, aromatic protecting group].

(ii) <u>Modified Strecker Synthesis - with amine free base</u>

To a mixture of CBZ-leucinal (4 mmoles) and ß-phenylethylamine (5 mmoles), dissolved in 7.7 ml of methanol, was added carbon-13 sodium cyanide (8 mmoles) slowly with stirring. The solution was stirred overnight. After evaporation, the product was extracted into methylene chloride, which was washed with two volumes of dilute basic sodium carbonate buffer and followed by dilute phosphoric acid. The substituted amino nitrile was obtained as a colourless oil after drying and evaporation. R_f = 0.60 with 1% methanol in methylene chloride as solvent by detecting with ninhydrin spray and iodine vapour. Yield = 50%.

The N-isoamyl derivative was synthesized in the same way with similar yield. The N-unsubstituted CBZ-3-isobutyl-DAPN was produced in the presence of 2 and 6 equivalents of NaCN and ammonium acetate, respectively. R_f = 0.45 in 2% CH_3OH in CH_2Cl_2. Yield = 70%. The ninhydrin positive spots of the products were not observed in control experiments and all confirmed by H^1 - NMR.

(iii) <u>Hydrolysis of Amino-Nitrile</u>

To CBZ-N-ß-phenylethyl-3-isobutyl-DAPN (0.4 g), dissolved in 0.6 ml of glacial acetic acid, was added 1.9 ml of concentrated HCl with stirring. The solution was heated up to 75^{0}C for 4 hours and the acids were removed by evaporation. The residue was dissolved in water and basified with NaOH until pH was around 12. The aqueous layer was then washed with cool ethyl acetate

to remove the byproducts and subsequently concentrated by evaporating under reduced pressure at 35^{0}C. Excess butanol was added and water evaporated continuously until no more precipitates were formed. After filtration, the butanol extract was dried and the solvent removed. A white solid was obtained. R_f = 0.66 in CH_3OH/CH_2Cl_2 (30:70). Yield = 12%. IR : 1610 cm^{-1} ($-COO^-$ signal).

The N-isoamyl-3-isobutyl-DAPA was synthesized in the same way. R_f = 0.6 in CH_3OH/CH_2Cl_2 (30:70) while the 3-isobutyl-DAPA had a R_f of 0.5 in $CHCl_3/CH_3OH/H_20$ (50:40:10).

2.3.2 Synthesis of N-ß-Phenylethyl-DAPA and N-Isoamyl DAPA

These compounds were synthesized in the same way as in section 2.3.1. but CBZ-glycine-p-nitrophenyl ester was used as the starting material. The resulting Z-glycinal had a R_f of 0.23 in CH_3OH/CH_2Cl_2 (2:98) and was positive to 2,4-dinitrophenyl hydrazine spray. Yield = 85%. NMR (DMSO): δ4.2 [d, 2 H, $-CH_2-$], 5.0 [s, 2H, benzyl CH_2], 7.4 [s, 5 H, aromatic], 9.6 [s, 1 H, CHO].

The substituted amino nitriles had R_f values of 0.51 and 0.50 in the above solvents with yields of 35% and 41% for the N-ß-phenylethyl and N-isoamyl derivatives, respectively. One interesting observation to note was that both nitriles had different colours after ninhydrin spray with intense purple for the former and reddish purple for the latter. After hydrolysis, both of the substituted DAPA derivatives had R_fs of around 0.47 and 0.45 in $CH_3OH/CHCl_3$/17% NH_4OH (40:40:20), respectively, and the yields were about 10-20%.

2.3.3 Synthesis of L-Isobutyl-ethylenediamine

After generating L-leucinamide free base from the hydrochloride salt, white crystals were formed: m.p. 96 °C. R_f = 0.78 in $CHCl_3/CH_3OH/17\%$ NH_4OH (50:45:5). Yield = 69%.

Leucinamide (2.1 mmoles), dissolved in 4.2 ml of dried tetrahydrofuran, was transferred to a 25 ml three-necked flask. Lithium aluminum hydride (LAH), 6.3 mmoles, was slowly added with stirring and the resulting mixture was refluxed for 3 hours. Upon cooling, ethylacetate was slowly added until no more bubbles were formed. The pH of the solution was adjusted to 2 by dilute HCl and the organic solvent was then evaporated under vacuum. Dilute NaOH was added to the cloudy mixture until pH was around 12. The desired product was then extracted into methylene chloride, dried, filtered and evaporated. The resulting oil had a R_f of 0.55 in $CHCl_3/CH_3OH/17\%$ NH_4OH (50:45:5).

The dihydrochloride salt of isobutyl-ethylenediamine was generated after the addition of 3M HCl with stirring for 15 minutes and evaporating any excess acid. Solidification occurred and the salt was further recrystallized with methanol and methylene chloride. m.p. 172-174 °C. Yield 25%.

2.3.4 Synthesis of (N^1-Isoamyl)-Isobutyl-ethylenediamine

To CBZ-L-leucine p-nitrophenyl ester (2.7 mmoles) in a 25 ml round bottom flask, 5.4 ml of isoamylamine was added and stirred for an hour. The excess amine was evaporated under reduced pressure at 30 °C and 25 ml of

methylene chloride was added. The p-nitrophenol was then extracted into 300 ml of 0.5 M $NaCO_3$ buffer at the pH of 10.5. The organic layer was washed with 20 ml of dilute HCl, dried and evaporated. Crystallization occurred and white crystals were formed. m.p. 90°C. R_f = 0.60 in CH_3OH/CH_2Cl_2 (2:98). Yield = 77%. Product was not ninhydrin positive because the protecting CBZ group was still intact.

CBZ-L-leucine isoamylamide (1.7 mmoles) was dissolved in 0.24 ml of glacial acetic acid and 2.0 ml of concentrated HCl was then slowly added with stirring. The resulting mixture was heated up to 60°C for 4 hours and the excess acid evaporated. White solids were formed. The deprotected amide was dissolved into dilute HCl, washed with CH_2Cl_2 and basified to pH 12 with dilute NaOH. Then the free base of amide was extracted into methylene chloride. A colourless oil was obtained upon solvent evaporation with a R_f = 0.7 in CH_3OH/CH_2Cl_2 (15:85) in ninhydrin and iodine sprays. Yield = 86%.

To L-leucine isoamylamide (1.45 mmoles), dissolved in 2.9 ml of THF, was added lithium aluminum hydride (4.35 mmoles) slowly with refluxing for 8 hours. Ethyl acetate was added until no more bubbles were formed and dilute HCl was then used to acidify the mixture. After evaporation and basifying to pH 12, the product was extracted into methylene chloride. R_f = 0.2 in CH_3OH/CH_2Cl_2 (15:85). Yield = 52%.

2.3.5 Synthesis of Aliphathic Hydroxamic Acid and Derivatives

The synthesis of formohydroxamic acid was performed by Richard Pfuetzner using the method described by Fishbein et al. (1969). The formohydroxamic acid was recrystallized from ethyl acetate. m.p. 77-78°C. O- and N-Substituted hydroxamic acids were synthesized by Mrs. Jutta Kaiser.

a) N-Methyl-acetohydroxamic acid

R_f [$CH_2Cl_2/CH_3OH/HCOOH$; 90/10/0.25] = 0.64. This compound was $FeCl_3$ spray positive.

b) O-Methylacetohydroxamic acid

It was crystallized at 0°C but melted at room temperature. It could be detected by iodine vapour but not with $FeCl_3$ spray. R_f = 0.57 in CH_2Cl_2/CH_3OH (90:10). NMR (DMSO) : δ1.7 [3H, S, CH_3], 3.6 [3H, s, -OCH_3].

c) O-Methyl caprohydroxamic acid

A colourless oil was obtained with a R_f = 0.52 in CH_3OH/CH_2Cl_2 (5:95) in iodine vapour but not with $FeCl_3$ spray.

NMR (DMSO) : δ0.8 - 1.0 [3H, t, CH_3.], 1.1 -1.7 [6H, m, $(CH_2)_3$], 2.2 - 2.4 [2H, t, -CH_2-CO-], 3.6 [3H, s, -OCH_3].

d) Hydroxamic Acid Assay

A stock solution of 10 mM acetohydroxamate and a reagent of 10% w/v $FeCl_3$ in 0.1 N HCl were prepared. 0, 25, 50, 75, 100 μls of the hydroxamate stock were pipetted into five clean test tubes and deionized H_2O was added to make up 100 μl in volume. To each of the test tubes, one ml of the reagent was

then added and the absorbance at 540 nm was measured after 30 minutes standing at room temperature. A standard curve was constructed by plotting O.D. against μmoles of acetohydroxamate after subtracting the blank.

In order to determine the unknown concentration of N-methylacetohydroxamate, the above procedures were repeated with appropriate dilutions and the amount of hydroxamate was determined by extrapolating from the standard curve. The solution was then assayed for inhibition with aminopeptidase.

2.4 Analytical Tests for Aldehyde

2.4.1 Tollen's Test (Pasto and Johnson, 1979)

Solution A was prepared by dissolving 3g of silver nitrate in 30ml of deionized water while solution B was a solution of 10% NaOH. These reagents were only prepared right before use.

To a mixture of solutions A and B (1 ml each) in a clean test tube, dilute ammonia was added dropwise until the silver oxide was just dissolved. To this solution was added a few drops of a dilute solution of the compound to be tested. A silver mirror was deposited on the walls of the test tube when warmed with running hot water and subsequent standing at room temperature for 45 minutes.

2.4.2 Chromic Acid Test (Pasto and Johnson, 1979)

To a solution of 1g of chromium trioxide dissolved in 1 ml concentrated sulfuric acid, 3 ml of deionized water was added. A few milligram of the compound to be tested was dissolved in 1 ml of acetone and then several drops of the above reagent were added. A green precipitate would be formed to indicate the presence of aldehyde in a positive test.

3 RESULT

3.1 Synthesis of Aminopeptidase Inhibitors

A schematic presentation of the strategies used in the synthesis of substituted diaminopropionic acid (DAPA) derivatives is shown in Figure 3.

Carbobenzoxy (CBZ)-leucine p-nitrophenyl activated ester (or its glycine analogue), was used as the starting material. It was first reduced to the corresponding aldehyde, CBZ-leucinal, by using lithium tri-t-butoxy-aluminohydride (Weissman and Brown, 1966), a much milder reducing agent than the lithium aluminum hydride (Brown and Mcfarlin, 1958). The same aldehyde intermediate could also be synthesized from CBZ-leucine through the reduction of the acid chloride intermediate with LTBA (Brown and Rao, 1958). Both methods were tried but the former was selected since it represents a shorter synthetic pathway and gives higher yields. Alternatively, pyridinium chlorochromate oxidation of CBZ-leucinol can also generate the aldehyde. Other than chemical methods, L-leucinal can also be made by enzymatic oxidation of L-leucinol using alcohol dehydrogenase and it was later found to be a strong competitive inhibitor for porcine kidney aminopeptidase (Anderson et al., 1982).

CBZ-leucinal was the key precursor for the synthesis of 3-isobutyl-diaminopropionitrile (DAPN) and its derivatives through the Strecker synthesis. It is a well-known classical method for the preparation of α-amino nitriles from

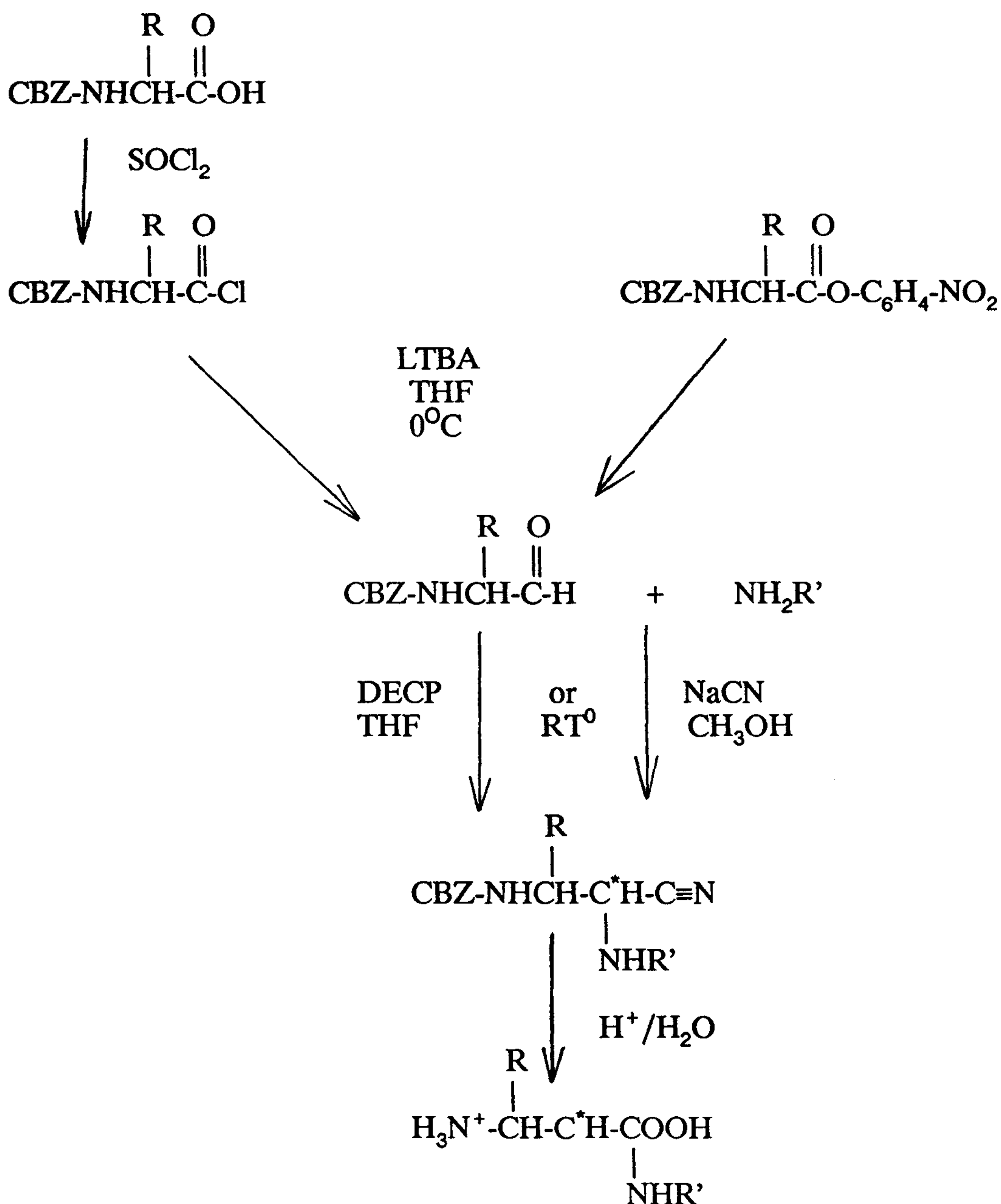
aldehydes or ketones in the presence of alkaline cyanides and salts of amines, and is well documented in literature (e.g. Steiger, 1942; Becker, 1973, etc.). Other modified ways are also known. One of them was to use diethylcyanophosphonate (DECP) as the cyanation reagent under mild reaction conditions (Harusawa et al., 1979). Inorganic cyanide and the free amine base in methanol were utilized in a second modified Strecker method. Both methods worked perfectly well and will be discussed.

When the nitrile product from the DECP method was first analyzed by IR spectroscopy, no nitrile stretching vibration peak at 2260-2240 cm^{-1} could be detected. It was known that "the introduction of an oxygenated group into a nitrile molecule resulted in quenching of the C≡N absorption intensity to a large extent and the absence of any strong absorption signal in the 2200-2300 cm^{-1} region could not be taken as evidence for the absence of C≡N groups unless oxygen was known to be absent" (Bellamy, 1960). The presence of two oxygen atoms in the CBZ group could be used to explain the failure in detecting the nitrile stretching frequency. Therefore, C-13 sodium cyanide was used in the second modified Strecker method as a solution to the above problem. The presence of the nitrile group in the product was then analyzed by carbon-13 NMR spectroscopy. The C-13 NMR spectrum obtained from CBZ-N-ß-phenylethyl-3-isobutyl-DAPN is illustrated in Figure 4. The chemical shifts for organonitrile carbons appear at 112 to 126 ppm (Levy and Neilson, 1972; Pretsch et al., 1983) and the spectrum showed two distinct peaks at around 118 ppm with similar

intensities. Since the cyanide could attack on either side of the planar aldehyde carbonyl moiety, a chiral carbon was generated and the two peaks should correspond to the C≡N groups of the two diastereoisomers formed. Proton NMR also confirmed the structure of the desired products.

The hydrolysis of the CBZ-DAPN derivatives to the ultimate products was carried out in concentrated HCl. The carbobenzoxy protecting group is cleavable with HCl under mild conditions (Ben Ishai and Berger, 1952) and the nitrile group could be hydrolysed to the corresponding carboxylic acid with heating (Steiger, 1942; Becker, 1973). Low yields were obtained (about 10-20%) probably because of incomplete hydrolysis.

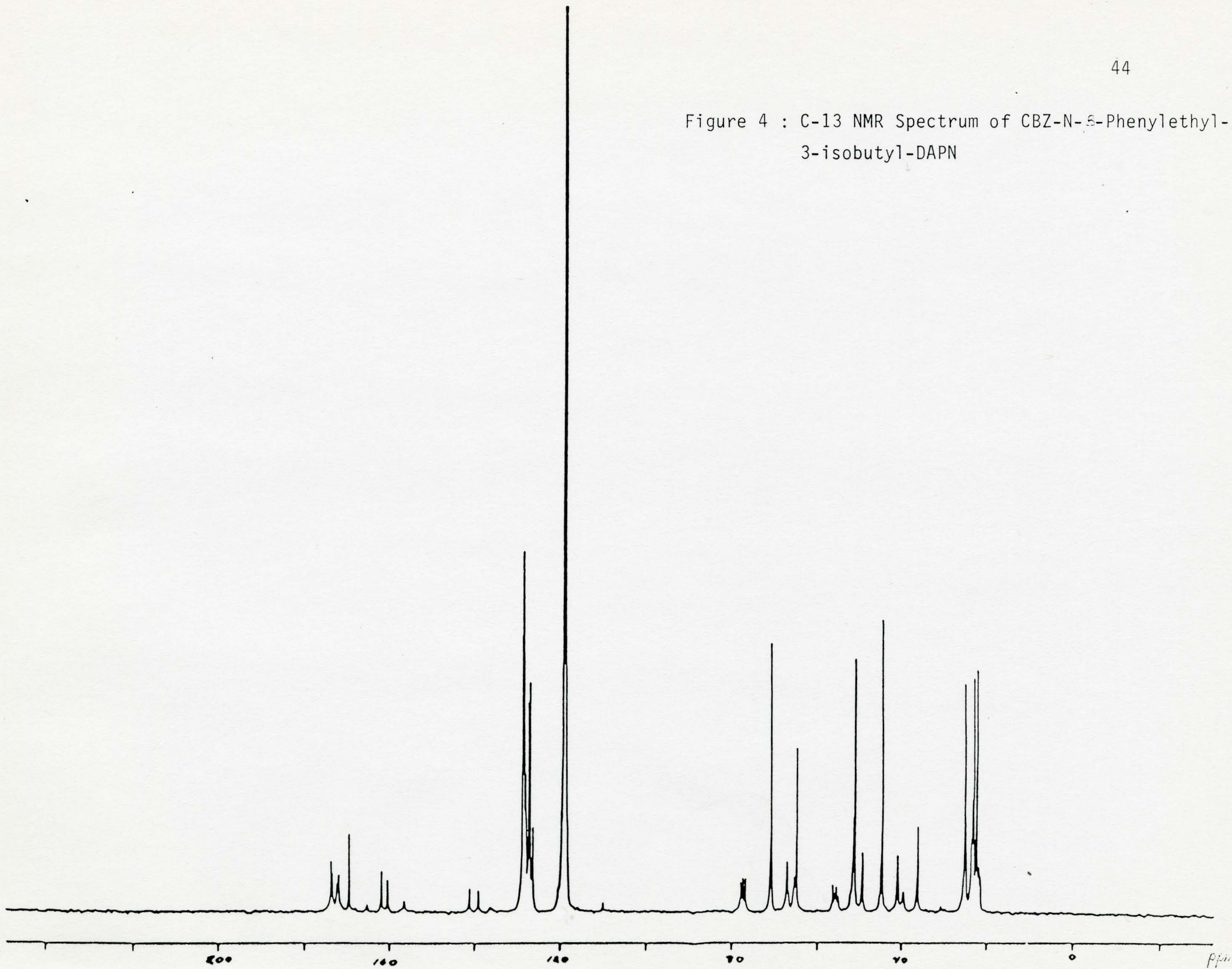
Synthesis of isobutyl-ethylenediamine involved the direct reduction of leucinamide to the corresponding amine by lithium aluminum hydride (Figure 5a). The synthetic scheme of N-isoamyl isobutyl-ethylenediamine is shown in Figure 5b. Synthesis of the amide, deprotection of the N-terminal and vigorous reduction of amide were the three strategies used. The labile CBZ group could be cleaved easily without hydrolysing the stable secondary amide bond.



* New Chiral Centre; LTBA = Lithium tri-(t)- butoxyalumino-hydride
CBZ = $C_6H_5CH_2OCO$-
R = -H or $-CH_2CH(CH_3)_2$
R' = -H , $-CH_2CH_2CH(CH_3)_2$, OR $-CH_2CH_2C_6H_5$

Figure 3 : Strategies for the Synthesis of Substituted DAPA Derivatives

Figure 4 : C-13 NMR Spectrum of CBZ-N-β-Phenylethyl-3-isobutyl-DAPN



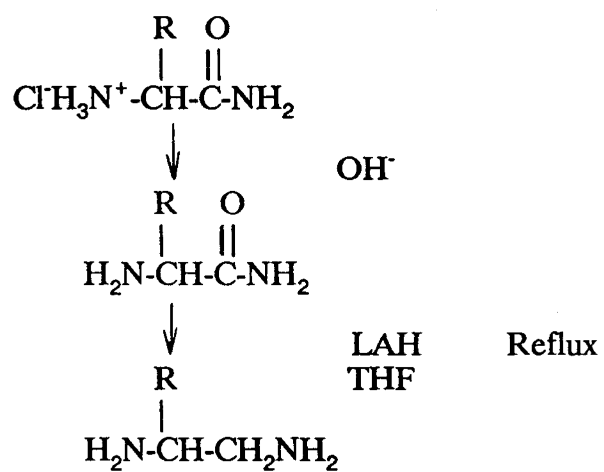
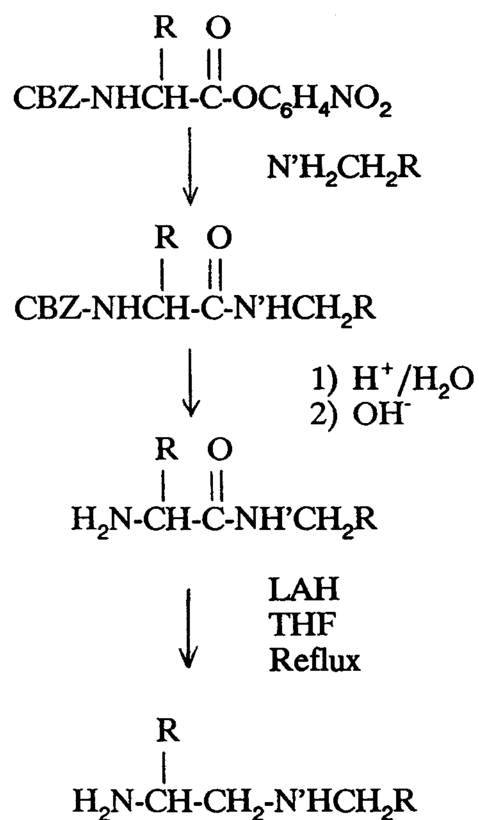


Figure 5a : **Synthesis of L-isobutyl-ethylenediamine**



R = $-CH_2CH(CH_3)_2$
LAH = Lithium Aluminum Hydride

Figure 5b : **<u>Synthesis of (N'-isoamyl)-L-isobutyl-ethylenediamine</u>**

3.2 Inhibitor Studies

3.2.1 A Comparison of α- and ß-Amino Acid Inhibitors for Aminopeptidase

Some α- and ß-amino acids were used as inhibitors for aminopeptidase in order to investigate the effects of changing the relative positions of the N- and C-termini on inhibition strength. Also, since DAPA-based derivatives are ß-amino acid analogues, the results obtained in this section would have direct contributions to explain the poor inhibition potencies of the DAPA compounds.

In general, for amino acid inhibitors, incorporation of a hydrophobic side chain at the α-carbon enhanced the inhibition, as illustrated in Table III. L-leucine bound 6- to 7- fold tighter than L-alanine and L-valine, but more than 100-fold than glycine.

Valine was found to bind 2.5-fold tighter than the racemic ß-leucine but the actual differences in affinity between the two compounds would be about half this amount if only the active enantiomer of ß-leucine was used. Therefore, valine and ß-leucine would have similar affinities for the enzyme by assuming the 'inactive' isomer of the latter to have no inhibition strength. Using a similar analysis, the enzyme showed a 7-fold binding affinity for leucine than its ß-amino counterpart. Although both compounds have the same molecular formula, placement of the amino group two carbons away from the carboxyl group, as in ß-leucine, will be rationalized later to retard correct binding of the inhibitor. Further evidence showed that the K_i value of alanine was nearly two-fold lower than its ß-analogue.

TABLE III

Carboxylic Acid Derivatives as Inhibitors for Aminopeptidase

Inhibitor	Ki (mM)	Inhibition Mode	Reference
$H_2N\text{-}CH_2\text{-}COOH$	>400	c	
CH_3 | $H_2N\text{-}CH\text{-}COOH$ (L)	29	c	
$H_2N\text{-}CH_2CH_2COOH$	47	mn[a]	(DiGregorio et al., 1988)
R | $H_2N\text{-}CH\text{-}CH_2\text{-}COOH$ (D,L)	58	c	
R | $H_2N\text{-}CH\text{-}COOH$ (L)	23	c	
R | CH_2 | $H_2N\text{-}CH\text{-}COOH$ (L)	4.0 1.2[b]	c	(Chan et al., 1982)
$R'\text{-}S\text{-}R'\text{-}CH_2COOH$	4.0	mn	

R = $-CH(CH_3)_2$
R' = $-C_6H_5$
[a]c = competitive, mn = mixed - noncompetitive
[b] The assay was done in the absence of 0.3 M NaCl.

3.2.2 Effect of N-Alkylation of Ethylenediamine on Inhibitory Strength

The second amino group of ethylenediamine was proposed to act as a zinc ligand, in an analogous manner to the thiol group of ß-mercaptoethylamine. It was also noted (DiGregorio et al., 1988) that two amino groups separated by two methylene units represented a more favorable binding situation than separation by three $-CH_2-$ units (as in 1,3-propanediamine). In order to obtain more information on the proposed mode of binding of ethylenediamine, substituted derivatives were tested as inhibitors for aminopeptidase, as shown in Table IV.

Mono- and di-methyl substitutions on one of the two amino groups increased the K_i's by about 5-fold. One reason for the weaker binding of the substituted diamine could be attributed either to steric hindrance and/or to the change in pKa upon substitution. N-Methyl inclusion of ethylenediamine possibly increases the pKa of the amino group and the potential for this group to act as a zinc ligand would be greatly reduced. The results therefore suggest that a free amino group in ethylenediamine is crucial for binding.

The inclusion of an isobutyl group in ethylenediamine improved binding by 38-fold because this non-polar side chain was able to fit inside the hydrophobic pocket of the enzyme. However, N-substitution of a hydrophobic isoamyl group decreased binding by more than 20-fold because of large steric interference.

TABLE IV

Substituted Ethylenediamines as Binding Probes

Inhibitor	Ki (mM)	Mode	Reference
$NH_2CH_2CH_2NH_2$	19	mn[a]	(DiGregorio et al., 1988)
CH_3-$NHCH_2CH_2NH_2$	90	nc	
$(CH_3)_2$-$NCH_2CH_2NH_2$	100	nc	
$NH_2CH(R)CH_2NH_2$	0.5	c	
$NH_2CH(R)CH_2NHR'$	10	c	

R = $-CH_2-CH(CH_3)_2$

R' = $-CH_2CH_2-CH(CH_3)_2$

[a]mn = mixed-noncompetitive

nc = noncompetitive

c = competitive

3.2.3 Inhibition by 2,3-Diaminopropionic Acid Derivatives

Compounds based on a 2,3-diaminopropionic acid backbone were synthesized and tested as inhibitors for this microsomal enzyme in order to probe the newly proposed second hydrophobic and amine sites.

As shown in Table V, attaching a hydrophobic isoamyl or phenylethyl group to the nitrogen at the second position of DAPA increased the K_i's by about 10- to 20-fold. The results can be explained partly by steric hindrance which in turn, affected the ligating power of the amino group. Since N-isoamyl- and N-ß-phenylethyl-DAPA were assayed as racemic mixtures, the amounts of the two enantiomers of each compound were expected to be approximately equal. Direct comparisions could therefore be made with DAPA and N-substitution was obviously not favoured for inhibitor binding.

On the other hand, 3-isobutyl-DAPA and the N-substituted derivatives were assayed as a mixture of diastereoisomers. Since these isomers were not mirror images and possessed two chiral centers, they might not be present in equal quantities, although the C-13 NMR spectrum at the nitrile stage before hydrolysis supported the equality of them. The results obtained from these compounds therefore were less representative than the two racemic N-substituted DAPA compounds described above. Although 3-isobutyl-DAPA and DAPA seemed to have similar affinity for the enzyme, non-polar N-substitutions on the former did not increase the inhibition constants in significant magnitude. Since these inhibitors did not show strong inhibitory potencies, the stereoisomers were not

separated.

A Dixon and Lineweaver-Burk plot of aminopeptidase inhibition by 3-isobutyl-DAPA are illustrated in Figures 6 and 7 to show the degree of accuracy by which the experiments were performed.

TABLE V

Binding of Diaminopropionic Acid Derivatives

Inhibitor [b]	**Ki (mM)**	**Mode**	**Reference**
NH_2 | NH_2CH_2CH-COOH (D,L)	0.7	c[a]	(DiGregorio et al., 1988)
$NHCH_2R$ | NH_2CH_2-CH-COOH (D,L)	6.6	c	
$NHCH_2R'$ | NH_2CH_2-CH-COOH (D,L)	13.5	c	
R NH_2 | | NH_2CH-CH-COOH	0.9	c	
R $NHCH_2R$ | | NH_2CH-CH-COOH	1.3	c	
R $NHCH_2R'$ | | NH_2CH-CH-COOH	2.7	c	

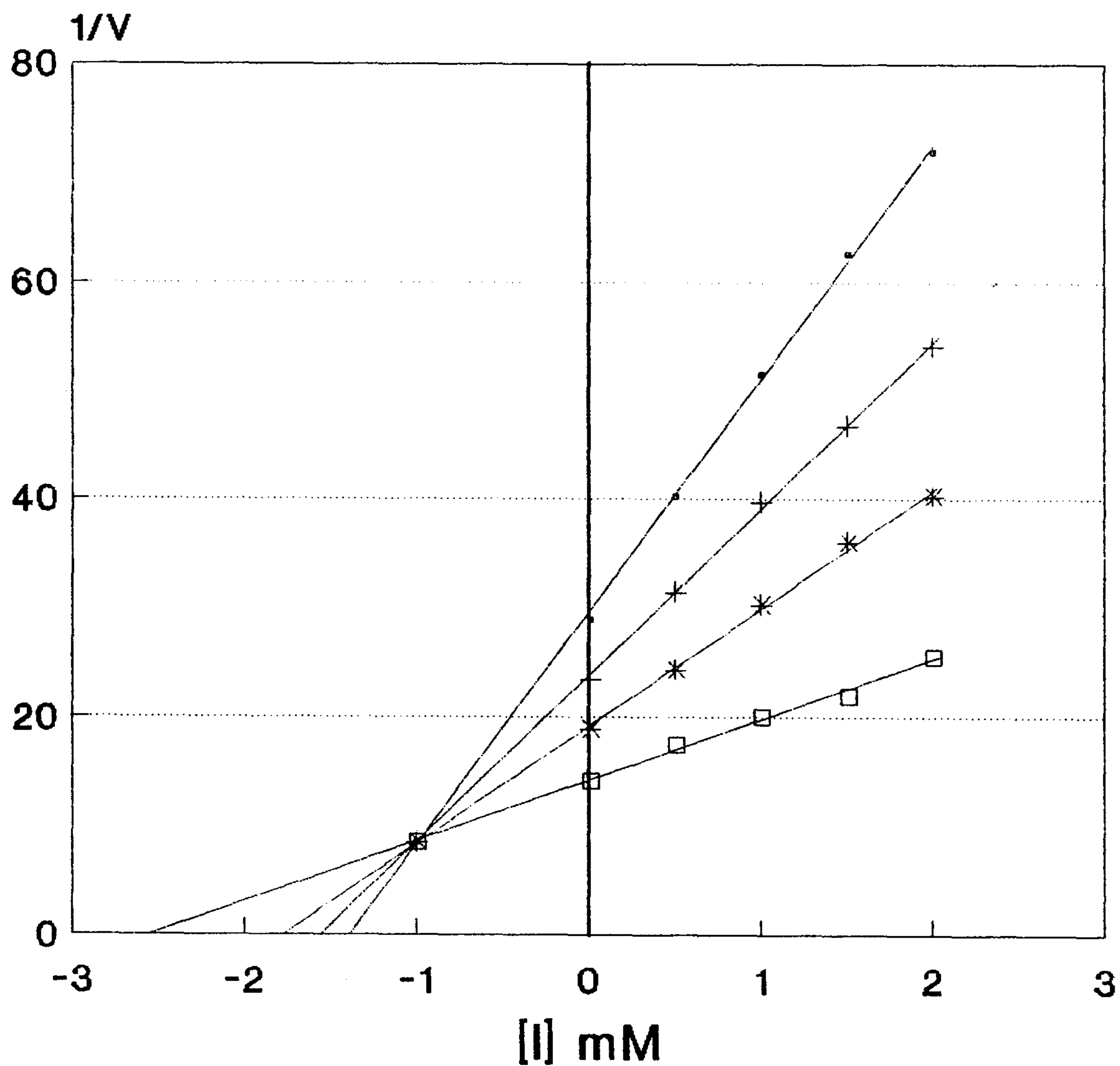
R = $-CH_2CH(CH_3)_2$

R' = $-CH_2-C_6H_5$

c[a] = competitive

[b] 3-Isobutyl-DAPA and N-substituted derivatives (i.e. the last three compounds) were tested as a mixture of diastereoisomers.

Figure 6: **Dixon Plot of Aminopeptidase Inhibition by 3-Isobutyl-DAPA**

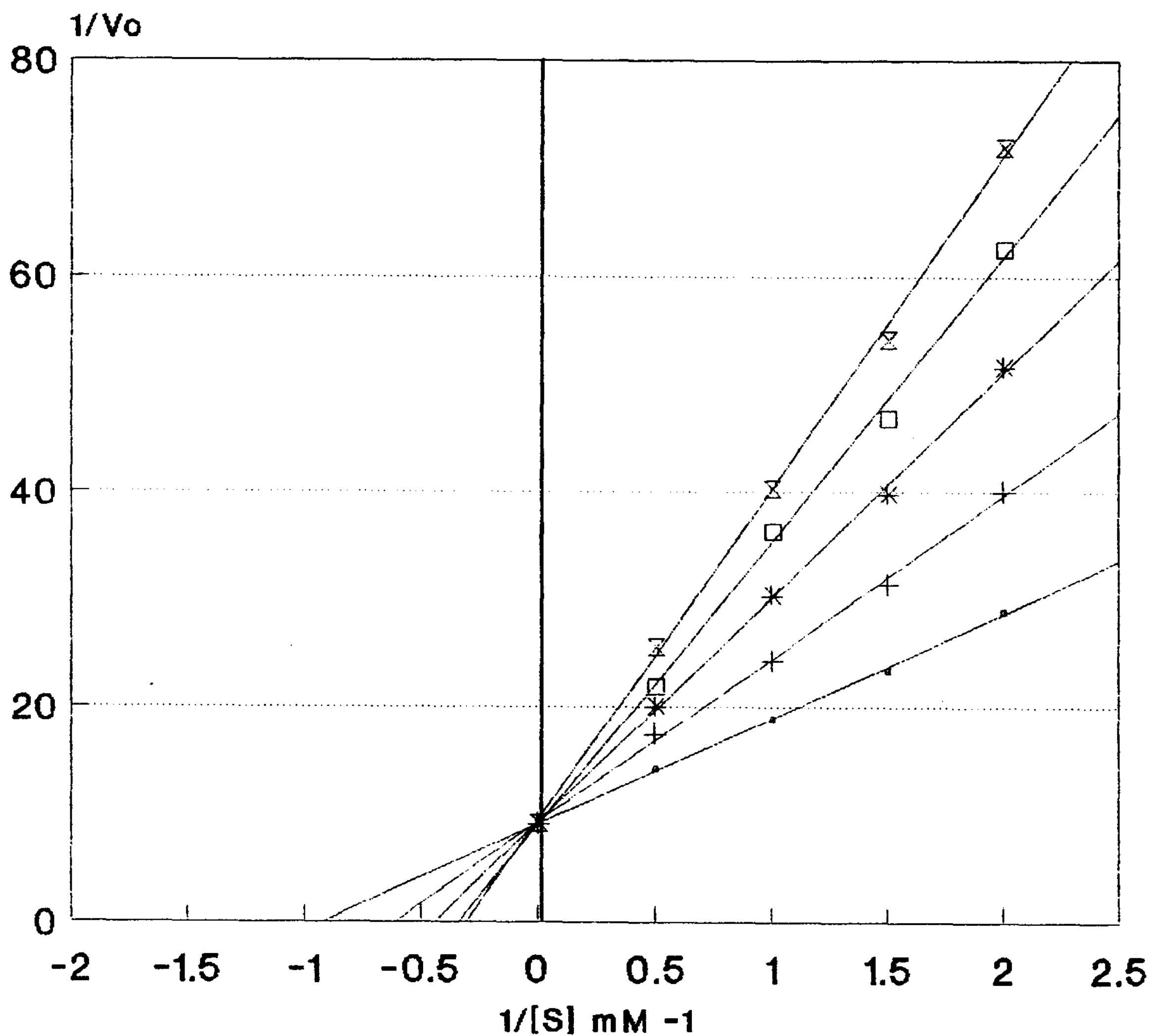


Substrate Conc.

—•— 0.5 mM —+— 0.67 mM —✳— 1.0 mM —□— 2.0 mM

A mixture of diastereoisomers

Figure 7 : **Lineweaver-Burk Plot of Aminopeptidase Inhibition by 3-Isobutyl-DAPA**



Inhibitor Conc.

[0] mM   [0.5] mM   [1.0] mM

[1.5] mM   [2.0] mM

Initial velocity in units of △A/min

3.2.4 Effects of O- and N-Methyl Substitutions on Hydroxamate Inhibition Power

Hydroxamates of amino acids and analogues were found to be good inhibitors for aminopeptidases (Chan et al., 1982; Baker et al., 1983), thermolysin (Nishino and Powers, 1978), enkephalinase (Bouboutou et al., 1984) and probably other zinc enzymes. The hydroxamate fragment is able to act as a bidentate zinc ligand. The effects of introducing substituents to the hydroxylamido group were investigated and changes, if any, of inhibitory strength were recorded.

As shown in Table VI, a more hydrophobic hydroxamate derivatives showed better binding but either N- or O- methyl substitutions on the hydroxylamido group impaired the inhibitory potency. N-methylation of acetohydroxamate increased the K_i by 18-fold. O-Methylated acetohydroxamate and caprohydroxamate could not inhibit the enzyme even at high concentrations. The results further suggested the crucial importance of the hydroxamate group in metal binding and the effects encountered by alkyl substitutions.

TABLE VI

Hydroxamate Inhibitors

Inhibitor	Ki (mM)	Mode	Reference
O OH || | H-C-NH	4.4	c[a]	
O OH || | CH_3-C-NH	0.81 2.0	 c	(DiGregorio et al., 1988)
O OH || | CH_3-C-NCH_3	35	c	
O OCH_3 || | CH_3-C-NH	>850	nd	
O OH || | $CH_3(CH_2)_4$C-NH	0.12	c	(Pickering, 1985)
O OCH_3 || | CH_3-$(CH_2)_4$C-NH	>180	nd	
O OH || | H_2N-CH_2C-NH	2.1	c	(Chan et al., 1983)

[a]c = competitive

nd = not determined

3.2.5 The Importance of the Amino Group of Phosphate Derivatives on Binding

When some phosphate derivatives were used for inhibition studies, they were found to be extremely poor inhibitors for the enzyme. Their inhibition constants were too high to be determined and therefore the lower limits of their K_i values were approximated by using the equation shown in section 2.2.2.

Attaching an amino group to methylphosphonate enhanced the binding since aminomethyl phosphonate had a low mM K_i while the unsubstituted one was unable to produce any inhibition at high concentration. The insertion of two ethyl groups in the methylphosphonate backbone did not improve the affinity, as illustrated by the diethyl methylphosphonate run. Unfortunately, no synergistic binding was observed between ammonia and methylphosphonate in preliminary assay. Since aminomethyl-phosphonate binds 360-times tighter than aminoethane (K_i = 250 mM), the phosphonate group must contribute to binding and might be able to coordinate with the zinc atom.

TABLE VII

Binding of Phosphate Derivatives

Ligand	Ki (mM)	Reference
$NH_2CH_2\text{-}P(=O)\text{-}(OH)_2$	0.70[a]	(Chan, 1983)
$CH_3\text{-}P(=O)\text{-}(OH)_2$	>950[b]	
$CH_3\text{-}P(=O)\text{-}(OC_2H_5)_2$	>950	
$H\text{-}P(=O)\text{-}(OCH_3)_2$	>950	
$H\text{-}P(=O)\text{-}(OC_2H_5)_2$	>950	
$HO\text{-}P(=O)\text{-}(CH_3)_2$	>950	
$C_6H_5\text{-}P(=O)\text{-}(OH)_2$	>200	
$C_6H_5O\text{-}P(=O)\text{-}(OH)_2$	>80	
$(C_6H_5O)_2\text{-}P(=O)\text{-}OH$	>60	

a: Actual K_i value; b: Lower limit of the K_i

4. DISCUSSION

4.1 Binding Modes of Inhibitors

The specificity of aminopeptidase as an N-terminal exopeptidase requires both the substrate and inhibitor to possess a free primary amino group and a specific side chain for the requirement of tight binding to the enzyme. In addition, compounds containing a powerful zinc ligand will lead to a dramatic improvement in inhibitory potency and probably without any concomitant loss of specificity (Ondetti et al., 1977). For a catalytic reaction to occur, the zinc ion has to be correctly located at the active site of the enzyme in order to polarize the carbonyl group of the scissile amide bond and make it more susceptible to hydrolytic cleavage. Therefore, both the choice and location of the zinc ligands on the inhibitor backbone are very crucial in the development of highly effective inhibitors. The following discussions will focus on the possible binding modes of the test inhibitors determined experiementally from Lineweaver-Burk and Dixon plots. From this information, a more detail picture of the active site can be drawn.

α-Amino acids were generally found to have a higher inhibitory potency than their ß-amino counterparts (Table III). These amino derivatives appear to bind in the same manner as natural substrates (DiGregorio et al., 1988). From

Dixon plots, the K_i's of α-leucine and α-alanine were about 7- and 2- folds lower than that of ß-leucine and ß-alanine respectively. This empirical observation would explicitly suggest the presence of a specific binding site in the catalytic pocket for α-amino substrates, i.e. a α-amino binding site is present. Another interesting observation has been introduced in the result section: in general, the larger the hydrophobic side chain of the amino acid inhibitor, the better the enhancement of inhibition. This can be explained by the presence of a hydrophobic binding site. Thus, in the leucine case, the stronger binding of the α-analogue can be attributed to its larger side chain. That is, α-leucine possesses an isobutyl side chain while ß-leucine has an isopropyl group. In addition, α-valine, which also has an isopropyl side chain, has a similar affinity for the enzyme as ß-leucine: the latter binds in such a way that its ß-amino group and the isopropyl side chain fits into the proposed primary amino and the hydrophobic binding sites. A third binding site, that involves the zinc atom binding with the carboxyl or carbamoyl functionalities of carboxylic acid or amide (peptide) substrates respectively, is also present. In consideration of this zinc binding site, the carboxyl group of ß-leucine will be one carbon removed from the normal substrate position of the scissile amide bond. However, Table III reviews that the location of the carbonyl moiety to be unimportant for binding. α-valine and ß-leucine possess nearly equal inhibitory potencies.

In order to obtain further conformation for the proposed binding mode of ethylenediamine (DiGregorio et al., 1988), in which the second amino group

of this compound was bound to the zinc, some N-substituted ethylenediamine derivatives were tested (Table IV). As expected, N-mono and dimethyl substitutions at the second amino group were shown to be unfavourable. If the postulated mode of binding of ethylenediamine holds, the N-methyl substitutions would impose steric interference on the second amino group to act as a zinc ligand.

Surprisingly, however, N-methyl and N, N-dimethylethylenediamine showed non-competitive inhibitions for the enzyme (Table IV). Therefore, it appears that binding of these inhibitors is independent, and not mutually exclusive with respect to the substrate. It follows then that these diamines might bind to some sites other than the active sites.

Interestingly enough, ethylenediamine was recently classified to be a metal chelating agent in analogy with EDTA (Vallee and Wacker, 1970). Furthermore, EDTA inactivation of aminopeptidase was enhanced by the presence of this diamine (Chan W.W.-C., personal communications). To explain this result, it can be proposed that the inhibition of the enzyme by ethylenediamine might not only be through a simple electrostatic interaction, but it may probably involve chelating the zinc out of the active site. Thus, the finding of DiGregorio et al. (1988) stating that the second amino group "interact" with the zinc atom might be an over-simplified picture. However, more evidence is indeed necessary to determine the validity of this hypothesis.

On the other hand, since isobutyl ethylenediamine is found to be a

competitive inhibitor (Table IV), the hydrophobic isobutyl group of this compound seems to have a direct contribution towards binding; that is, alkyl substitution improves the inhibitory potency of ethylenediamine relative to the unsubstituted diamine. By analogy with another competitive inhibitor, leucinthiol, isobutyl ethylenediamine should bind in a similar manner but the affinity of the thiol functionality was some 20,000-fold higher for the enzyme than the amino functionality (Chan, 1983). This observation is not surprising because the second amino group was previously found to be a much weaker zinc ligand than the thiol and hydroxamate for both aminopeptidase and thermolysin (Nishino and Powers, 1978; Chan, 1983; DiGregorio et al., 1988). Therefore, once the hydrophobic and primary amino sites are filled, the vast changes in the magnitudes of inhibitory potency are predominantly influenced by the nature of the zinc ligand. Again, N-alkylation of isobutylethylenediamine was proved to be unfavourable in zinc binding as shown by the higher K_i of N-isoamyl-isobutylethylenediamine. The N-substitution of a hydrophobic group, therefore, decreases the affinity of the amino group to act as a zinc ligand.

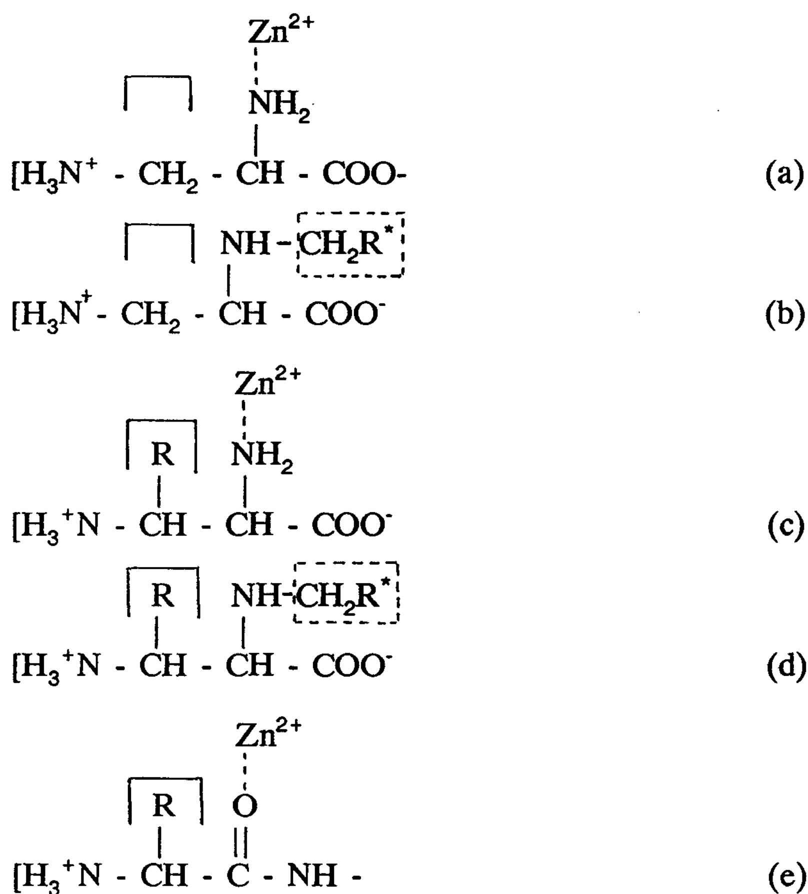
The above discussions concerning the binding modes of ß-amino acids and ethylenediamine will have direct relevance in the inhibition studies of 2,3-diaminopropionic acid and its derivatives since one can view the structure of DAPA is both a combination of a ß-amino acid and a diamine. The racemic mixture of DAPA was found to bind about 70- and 28-folds tighter than ß-alanine and ethylendiamine respectively (DiGregorio et al., 1988). The potential of the

amino group in the two position of DAPA to act as a zinc ligand would be enhanced by the carboxyl group because its electron withdrawing effect inductively decreases the pKa of the protonated amino group enabling stronger zinc coordination (DiGregorio et al., 1988). Our initial goals are to develop a new series of inhibitors using DAPA as the backbone structure and to employ these DAPA derivatives for further probing of the enzyme active site.

As discussed earlier in connection with the unfavourable N-alkyl substitutions, inclusion of a large hydrophobic group onto the second amino group of DAPA would be expected to generate steric hindrance effects. It also will increase the pKa of this group and thus decrease its potential to zinc ligate. However, N-alkylation of the amino group in the two position of 3-isobutyl-DAPA does not significantly impair binding relative to the unsubstituted isobutyl diamino acid (Table V). One can surmise that the enzyme's catalytic pocket can accept another large non-polar group with relative ease after the first hydrophobic site has already been occupied, in this case, by the isobutyl group. Implicitly, DAPA and 3-isobutyl-DAPA can be regarded as a mono-amino substituted ß-alanine and ß-homoleucine respectively. Therefore, in connection with previous discussions about the binding of ß-amino acids, the carboxyl groups of these compounds seem to be unable to interact with the zinc metal but the second amino group in DAPA derivatives now has this potential. In fact, since ß-leucine and α-valine have similar affinities for the enzyme, it would be reasonable to predict that ß-homoleucine might possess similar inhibitory potency as leucine, i.e., ca. Ki 4mM,

because they possess the same isobutyl side chain. If this prediction is correct, inclusion of an amino group at the 2-position of ß-homoleucine to form 3-isobutyl-DAPA should increase its binding affinity. Indeed, Table V shows this prediction to be experimentally viable: 3-isobutyl-DAPA is at least 4-fold more potent than the ß-homoleucine counterpart. The proposed binding modes of some of these inhibitors are shown in Figure 8. Again, our result is in agreement with the mode of binding of DAPA proposed by DiGregorio et al. (1988). Unfortunately, the derivatives of 2,3-diaminopropionic acid are not potent inhibitors of aminopeptidase as was previously suspected.

In order to obtain further information for the binding mode of hydroxamate derivatives, some N- and O-methyl substituted derivatives of the hydroxyamido group were synthesized. Recently, Wilkes and Prescott (1987) studied the hydroxamate-induced spectral perturbations of cobalt substituted aeromonas aminopeptidase and they confirmed that the mode of inhibition involved a co-ordination of the metal ion in the active site of the enzyme to the anionic form of the hydroxamate group of the inhibitors. The results of Wikes and Prescott's experiment confirmed the hydroxamate group to act as a bidentate ligand.



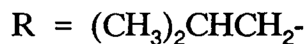
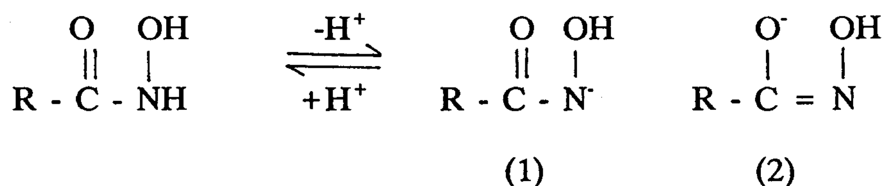


Figure 8 : Schematic diagram showing the proposed interaction between the active site of aminopeptidase and (a) diaminopropionic acid, (b) N-isoamyl-DAPA, (c) 3-isobutyl-DAPA, (d) N-isoamyl-3-isobutyl-DAPA and (e) substrate.

* Areas of possible steric hindrance are endosed by dotted lines.

The anionic form of the inhibitor, where the two oxygen atoms of the hydroxamate group are closer together than in the protonated form, possesses two resonance forms (Holmes and Mathews, 1981):



Note that the resonance form (2) contributes double bond character to the anion and decreases the C - N and eventually the O - O internuclear distances. If this model is correct, then N-methyl-acetohydroxamate will not be able to exist in the deprotonated form, it will not be resonance stabilized and the two oxygens will be further apart from each other. Thus, the results obtained seem to be consistant with this proposed mode of binding for hydroxamate (Table VI) . The reduced interaction between the N-methyl hydroxamic acid with the zinc can also be explained by the steric constraints imposed by the methyl group.

Morever, O-methyl hydroxamates were found to be extremely weak inhibitors. A possible rationale for this observed behavior might be that the strength of binding is directly proportional to the number of free oxygen atoms coordinating with the zinc atom. Similar results were also obtained with substitutions on peptide hydroxamate inhibitors for thermolysin (Nishino and Powers, 1978) and enkephalinase (Bouboutou et al., 1984).

In the search for other potential inhibitors, some phosphate derivatives

were tested and the results have been tabulated in Table VII. N-phosphorylated dipeptides were observed to be potent inhibitors for enkephalinase (Altstein et al., 1982 and 1983) but several N-terminal phosphate substituted hydrophobic amino acids were unable to inhibit rat brain and kidney aminopeptidases (Weiss et al., 1986 and 1987). These observations can be explained as follows: enkephalinase, an endopeptidase, should therefore have specific recognition at the substrates interior and relatively indiscriminate recognition at its N-terminal. Thus, incorporation of a phosphate group at the N-terminal of the dipeptide inhibitor should still enable binding with the zinc atom (see Figure 2). The situation becomes reversed when considering the exopeptidase, aminopeptidase. Weiss et al. have demonstrated that the externally discriminate aminopeptidase avoids binding a potentical inhibitor that has its N-terminal blocked by a phosphate group, probably because of steric hinderance offered at the α-amino binding site. Our results also show that the presence of an amino group is essential for the binding of these phosphate probes. This phenomenon can be illustrated by the higher inhibitory potency of aminomethyl-phosphonate when compared to other non-amino derivatives (Table VII). As stated earlier, this is most likely due to the enhanced binding which occurs with amino substituted derivatives at the α-amino binding site.

4.2 Binding Subsites in Aminopeptidase

The work presented thus far supports that there are three distinct binding

sites in microsomal aminopeptidase: namely, an α-amino binding site, a hydrophobic pocket and a zinc binding site. However, certain finding of DiGregorio et al. (1988) suggests the presence of other closely located amino and hydrophobic sites. Thus, further work is required to develop new inhibitors to probe these new binding sites.

The finding of the stronger inhibitory potency of α-amino acids when compared to their ß-analogues provides evidence that the α-amino and the hydrophobic binding sites of the substrate are located adjacent to each other. Our results also support the idea that the zinc atom is located in close proximity to its ligand since N-alkylations of DAPA derivatives and O-methylation of hydroxamates generate strong steric interferences and decreases binding affinity. The increase in steric bulk will push the ligand away from its natural zinc binding location. Incidently, the second amine binding site nomenclature adopted by DiGregorio et al.(1988) is likely to be the zinc atom binding site referenced in this work. In fact, it is very difficult to locate the second hydrophobic site although experimental results confirm its existence. Since extensive testing was not performed, conclusive results at this stage cannot be obtained. However, it seems plausible to state that this second hydrophobic pocket would be located at the position naturally occupied by the alkyl side chain of the second N-terminal amino acid residue of a peptide substrate. The postulated active site of microsomal aminopeptidase is summarized in Figure 9.

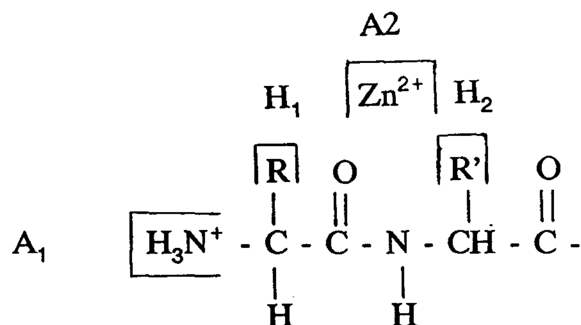
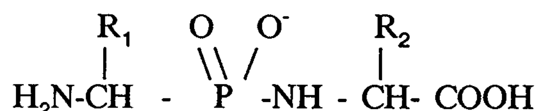


Figure 9 : Postulated active site of aminopeptidase.

H_1 and H_2 are the hydrophobic sites for the first and second amino acids' side chains binding sites; A_1 represents the binding site for the N-terminal amino group; A_2 illustrates the location of the zinc metal and it is also possibly the second amine site proposed by Digregorio et al., (1988).

4.3 Future Design of Inhibitor

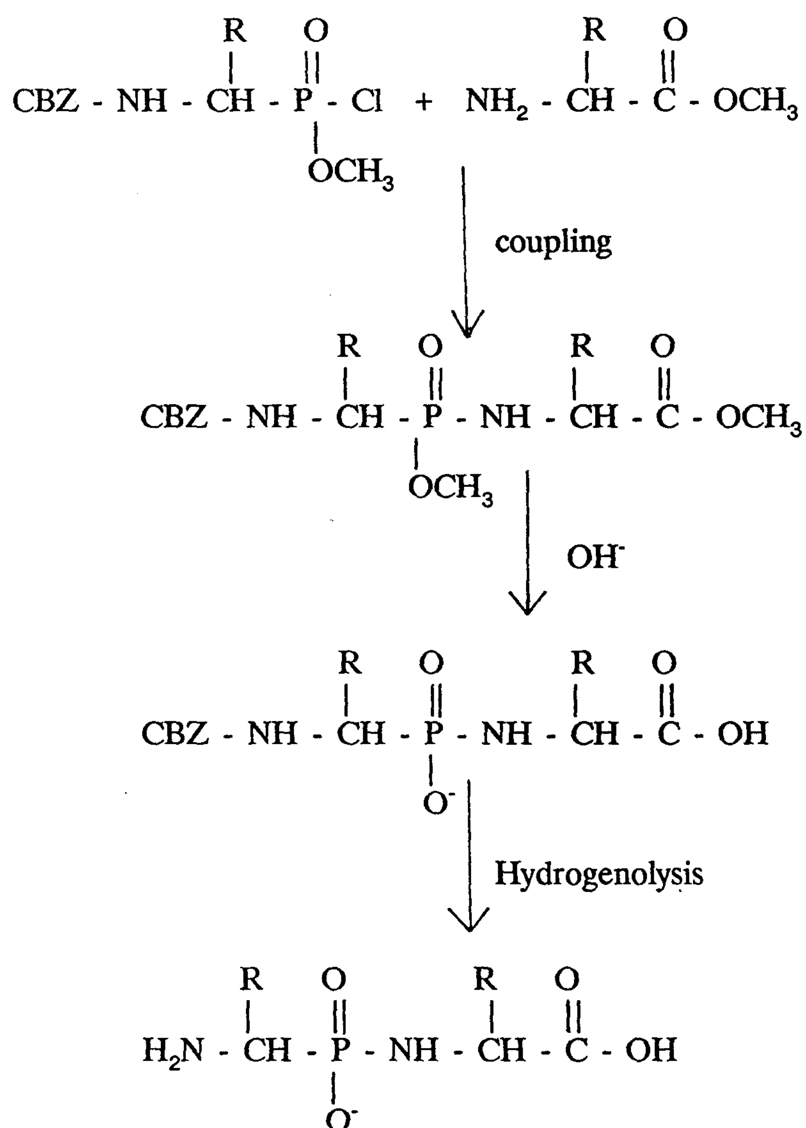
Although the inhibitors developed are not potent enough for clinical trials, the increased knowledge gained of the aminopeptidase active site enables us to set up the following four requirements for future inhibitor design. In particular, these compounds should possess a free unsubstituted α-amino group, a hydrophobic side chain, a powerful zinc ligand, and possibly a second hydrophobic group. One should be very careful with designing the location of this second non-polar moiety in order to avoid reducing the binding affinity of the zinc ligand. Presently, the most potent inhibitors for aminopeptidase are the thiols which can inhibit enzymes in the nanomolar range. Bartlett and Marlowe (1983) reported the use of phosphonamidates as transition state analogue inhibitors of thermolysin. The design of future aminopeptidase inhibitors can be focussed on functional group manipulation of a phosphonamidate structure. One of the examples is illustrated as follows:



R_1 and R_2 are an isobutyl group and a hydrophobic side chain respectively which can interact with the H_1 and H_2 subsites of Figure 9. The N-terminal will bind to the A_1 subsite while the phosphonamido group will probably coordinate with the zinc atom. Since we now know that one of the important factors affecting the

rate of acceleration is enzyme-transition state complementarity, the possibility of these phosphonamidates being transition state analogue inhibitors of aminopeptidase is extremely promising. The proposed synthetic scheme for these phosphonamidates is illustrated in Figure 10.

In conclusion, although we do not succeed in developing highly potent inhibitors in this work for future analgesics, phosphonamidate is highly recommended to serve as the future trend for developing these drugs.



R = any hydrophobic side chain

Figure 10: Proposed Synthetic Scheme of Phosphonamidates (Bartlett and Marlowe, 1983; Jacobsen and Bartlett, 1981).

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