DESIGN, SYNTHESIS AND EVALUATION OF DIPEPTIDES AS PROBES FOR STUDIES OF GASTROINTESTINAL TRACT DIPEPTIDE TRANSPORT SYSTEM

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Master of Philosophy

THE ASTON UNIVERSITY

September 2001

ASTON UNIVERSITY

Design, Synthesis and Evaluation of Dipeptides as Probes for Studies of Gastrointestinal Tract Dipeptide Transport System

A thesis submitted by Yan Gao for the degree of Master of Philosophy

Summary: The design of prodrugs suitable for transport by the dipeptide transporter can be a useful strategy for improving the absorption of small polar drugs which exhibit very poor bioavailability.

A series of dipeptides (thirty two dipeptide compounds), in which the C-terminal residue is proline or a proline ester, has been synthesized. The inhibitory effect of these dipeptides on the uptake of the dipeptide Gly-Pro by a Caco-2 cell line has been determined by using tritium labeled substrates.

The results suggest the dipeptide with proline benzyl ester and Glutamic acid benzyl ester has a good inhibitory activity.

Keywords: Prodrug, dipeptide, absorption of oral drug, Caco-2 cell.

ACKNOWLEDGEMENT

I would like to express my gratitude to my supervisor Dr Yongfeng Wang for his support, enthusiasm and encouragement.

I would also express my thanks to Miss Ying Kang. She did brilliant research work about this project in year 1999 and her results is helpful and suggested my project in year 2000.

The assistance given to me by Jonathon Biggs and Professor Willian J. Irwin in Gly-Pro uptake experiment is gratefully acknowledged. Specially, Jonathon Biggs cultured the Caco-2 Cells for the uptake experiment, and help me get the test results.

Thanks go to the staff, Mr. Mike Davis and Mrs Karen Farrow for their technical support and tolerance.

A special thank you to Dr Dan Rathbone for his practical advice and constructive comments.

Finally I would like to thank everyone in the School of Life and Health Sciences for their help during my study at Aston University.

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Abu	2-Aminobutyric acid
ACE inhibitors	Angiotensin-converting enzyme inhibitors
Adi	Adipic acid
Asp	Aspartic acid
ATPase	Adenosine triphosphatase
BBMV	Brush border membrane vesicle
Boc	tert-Butoxycarbonyl
tBu	tert-Butyl
Bzl	Benzyl
dBzl	Diphenylmethyl
CH_2Cl_2	Dichloromethane
Cys	Cysteine
DCC or DCCI	Dicyclohexylcarbodiimide
DCHA	Dicyclohexyl ammonium salt
DMSO	Dimethoxysulfoxide
DTS	Dipeptide transport system
EtOAc	Ethyl acetate
Glu	Glutamic acid
GI tract	Gastrointestinal tract
HOBt	1-Hydroxybenzotriazole
Hyp	Hydroxyproline
IC_{50}	Inhibitory concentration to reduce uptake by 50%
in situ	*Latin* in the normal, natural, original, or appropriate position.

in vitro	*Latin* (of any biological process, reaction, or experiment) occurring or made to occur outside an organism, literally it means 'in glass'.
in vivo	*Latin* (of any biological process, reaction, or experiment) occurring or made to occur within a living organism, literally it means 'in life'.
IR	Infra-red
Met	Methionine
NMR	Nuclear magnetic resonance
P	Significant difference
PABA	ρ-Aminobenzoic acid
Pim	Pimelic acid
Pro	Proline
PTAPT	Transporter Associated Prodrug Therapy
OEt	Ethoxy
OMe	Methoxy
Sbzl	Thiobenzyl
SD	Standard deviation
Sub	Suberic acid
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
THR	Hydrotropin releasing hormone

CHAPTER 1 - INTRODUCTION

Drugs may be introduced into the body in a variety of ways. The oral route is the most common and convenient way for drug administration. However, oral administration of drugs often led to degradation, due to the highly acidic gastric environment, by the enzymes of the mucosa or by the liver before they enter the systemic circulation. Consequently, many highly polar drugs and macromolecular drugs cannot be absorbed through the GI tract because of their insufficient lipophilicity and large molecular weight. These poorly absorbed drugs include hydrophilic antibiotics, such as streptomycin and gentamicin, and peptides and proteins, such as insulin, calcitonin, and thyrotropin releasing hormone (THR) ets.

In order to exploit the convenient of oral administration for most of the drugs, researchers are constantly concentrated on investigation of the effective approaches to improve intestinal absorption, such as effects of various absorption enhancers, protease inhibitors, chemical synthesis of prodrugs and analogues, and the use of various dosage forms.

There are direct and indirect evidences for participation of carrier-mediated membrane transport mechanisms in the GI tract, where several hydrophilic compounds seem to be absorbed efficiently *via* such specialized transporters. Therefore, utilization of the intestinal epithelial transporters to facilitate the absorption of appropriately modified drugs seems to be an attractive strategy for improving the bioavailability of poorly absorbed drugs.

1.1. Anatomy and physiology of the gastrointestinal tract

The gastrointestinal tract is a highly specialized region of the body which functions may be broadly described by the process of secretion, digestion, and absorption[1]. Since all nutrients that are necessary for the body must first be ingested orally, processed by the gastrointestinal tract and then made available for absorption into the blood stream, the gastrointestinal tract represents an important barrier and interface with our environments. Like these nutrients, the gastrointestinal tract also regulates intestinal absorption and digestion of various drugs when administered orally[1].

1.1.1. Stomach

After oral ingestion, food and drug substances are mixed with the acidic contents of the stomach. The primary functions of stomach are storage, mixing, and emptying these contents into the duodenum. While the absorption of drug molecules from the stomach is generally not great when compared with the absorption from the intestine (due to the surface area of the epithelium), gastric emptying is an important physiological event that significantly influences the uptake of drug substances from the intestine[2].

1.1.2. Small Intestine

Since the small intestine is the major site where food and drug absorption takes place, understanding its anatomical organization is crucial in

explaining the different mechanisms of drug absorption. The small intestine, comprising the duodenum, jejunum, and ileum, represents greater than 60% of the length of the gastrointestinal tract, which is consistent with its primary functions. In humans, the length of the duodenum and the jejunum plus ileum is about 0.2 and 2.5m, respectively[2].

A common anatomical feature of the intestine is its four concentric layers. Beginning with the luminal surface, these are the mucosa, submucosa, muscularis layer, and serosa. The mucosa of the intestine consists of epithelial cells, lamina propria, and endothelium. Between mucosa and serosa is the muscularis layer which provides peristalsis to the intestine. Serosa continues to the mesentery, which contains nerves, lymphatics, and blood vessels supplying the gastrointestinal tract.

The surface layer of the intestinal mucosa, the layer in direct contact with the luminal contents, is a continuous sheet of the epithelial cells that lines the villi and their surrounding crypt. The epithelium lining the villi contains (a) large numbers of villus absorptive cells, sometimes called enterocytes, (b) mucus-secreting Goblet cells, (c) a few endocrine epithelial cells, and (d) rare caveolated cells. Additionally, a specialized epithelial cell, the M cell, overlies the apex of Peyer's patches in the ileum[3].

The most important cells in terms of nutrient and drug transport are epithelial absorption cells, as shown in Fig. 1. These cells along with other epithelial cells are arranged in such a way as to considerably enlarge the surface area of what would be a simple cylinder. The organization consists of three levels namely, the folds of Kerckring, villi, and in the case of absorption cells,

microvilli. In general, due to the presence of villi and microvilli, the total surface area of the small intestine in humans is approximately 200m^2, which represents an increase in surface area of 50,000-fold. Such a vast surface increases the efficiency of the absorption of nutrients and medical materials.

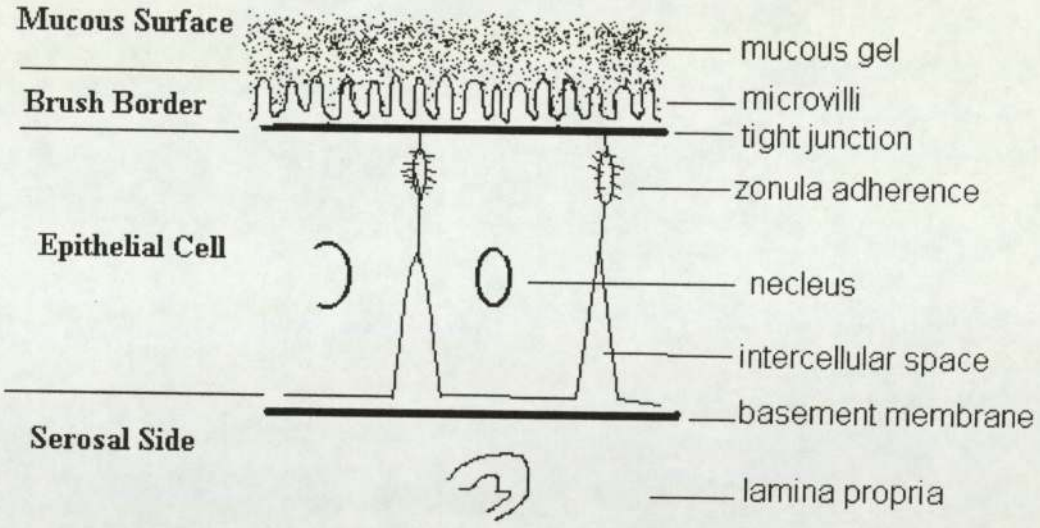


Figure 1.1. Anatomy of intestine epithelial cells

1.1.3. Large intestine

The large intestine in humans has a length of about 1.1m. The large intestinal epithelium is columnar or cuboidal with numerous Goblet cells. However, unlike the small intestinal, the large intestinal epithelium does not contain villi. Consequently, the absorption surface area of the large intestinal mucosa is considerably less than in the small intestine.

The large intestine has two primary functions: (1) the absorption of water and electrolytes, and (2) the storage and elimination of faecal materials.

1.2. Absorption Characteristics of Drugs from the Gastrointestinal Tract

1.2.1. Pathways of Absorption

At the epithelial cell surface, there are two routes which are potentially available to molecules. Transepithelial transport of drugs could be achieved either across the cell (transcellular) or through the junctions that hold the cells together (paracellular) (Fig. 2). Historically, the transcellular pathway has received more attention. Several mechanisms of drug transfer are involved in the transcellular pathway: simple diffusion, facilitated diffusion, carrier-mediated transport.

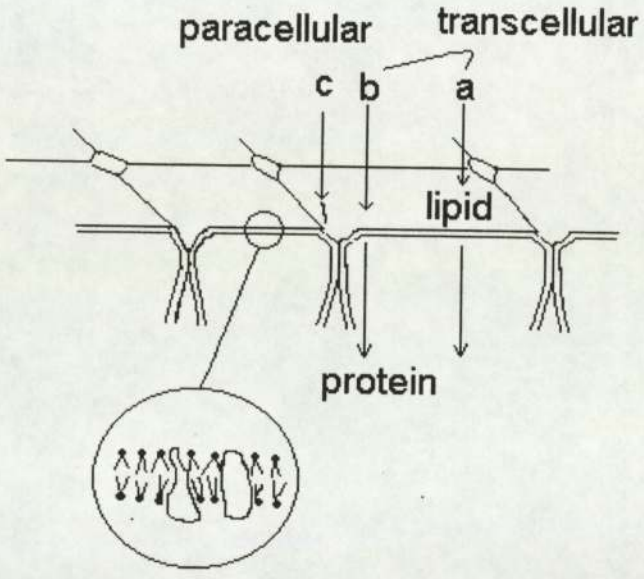


Figure 1.2. Potential routes for crossing the intestine barrier.

Paracellular route

The paracellular route takes advantage of the leakiness of the cell to cell junction. This can vary considerably between epithelia and is often expressed in terms of integrity as measured by a physical parameter such as electrical resistance. In general, absorptive epithelia, for example the small intestine, are found to be leakier than tight epithelia such as that of the bladder. The paracellular pathway is mainly open to ions and small molecules.

Transcellular route

In the transcellular route, drugs are transported by a passive or a carrier-mediated transport system, as explained above. It is essential for a molecule to have characteristics with low molecular size and relatively high lipophilicity in order to pass across the intestine mucosa by this route. The transcellular route is also of importance to an absorbing epithelium which exerts the selective nature of its barrier by a carrier-mediated transport system or endocytosis. These processes show a high degree of selectivity within classes of molecules such as sugars, amino acids, uracil, thymine, and pyrimidines. Overall, to take advantage of the transcellular route, it is necessary to use drugs, which have either the physical properties that support passive absorption or are structural analogues of the naturally transported substrates.

1.2.2. Absorption Mechanisms of Drugs across the Intestinal Mucosa

Various drugs and nutrients are transported across the intestinal mucosa by two major mechanisms. These are passive diffusion and carrier-mediated transport; the latter is further divided into a facilitated diffusion and an active transport system.

Passive diffusion

This process describes the movement of drug molecules from a region of high relative concentration to a region of lower relative concentration[4]. It also includes the movement of ions from a region of high ionic charge of one type to a region of lower charge of the same type or of opposite charge. The transfer of substances by simple diffusion across thin membranes can be generally explained by Equation 1 on the basis of Fick's law.

$$\frac{dQ}{dt} = -DAK(C_0 - C_i)/h \qquad (1)$$

where:

Q = the amount of drug at the absorption

D = diffusion coefficient

A = area of absorption surface

C_0 = concentration of drug in the outer phase of membrane

C_i = concentration of drug in the inner phase of membrane

h = thickness of the membrane

In this case, rates of absorption are determined by physical-chemical properties of the solute and the membrane and by the concentration gradient. The driving force behind diffusion of a molecule through the lipid bilayer and / or the aqueous channels of the membrane is the chemical potential difference of the compound on two sides of the membrane. When some drugs were administered in the gastrointestinal tract the plasma concentration was much lower than the concentration in the gastrointestinal tract due to the rapid removal of the absorbed drug by the circulating blood, making the rate of transport across the membrane proportional to the chemical potential only in the gastrointestinal tract.

Most drug absorption across membrane barriers in the gastrointestinal tract has been assumed to occur through this mechanism[1]. However, in this case, diffusion rate depends on lipophilicity and molecular size of the drugs. The gastrointestinal membrane acts like a lipid barrier which permits the passage of lipid soluble drugs, but across which lipid-insoluble molecules diffuse only with difficulty or not at all. In addition, due to their low diffusion coefficients, macromolecules such as peptides and proteins will not be well absorbed in an intact form from the gastrointestinal tract.

facilitated diffusion

In this transport system, the carrier has been applied to the structure or site in or on the membrane that mediates the transfer of solute by a temporary combination with the solute. The rate of transfer may fall off appreciably when sufficient compound is present in the lumen to saturate the carrier mechanism. Therefore, availability of carrier may become rate-limiting in the absorption process. In addition, an energy source is not required since no transport against the concentration gradient takes place.

Active transport

Active transport is a specialized process which requires the expenditure of energy[4]. The various active transport processes found in the gastrointestinal tract are relatively structure-specific. Many nutrients such as sugars, amino acids, pyrimidines, bile salts, and vitamins are transported across the intestinal membrane by this mechanism[2]. In addition, there is evidence that certain drugs may also be absorbed by these active processes, if their chemical structure are sufficiently similar to the natural substrate. The anticancer drug 5-fluorouracil is an example of an actively transported drug[5,6]. it is similar in structure to the natural substance, uracil, which is absorbed by means of pyrimidine transport process. Recently, many researchers reported that *p*-aminobenzoic acid (PABA)[7], cyclacillin[8], cephadroxil[9], captopril[10] and α-methyldopa[11] were also transported across the intestinal membrane by this mechanism.

This active transport mechanism is similar to facilitated diffusion in that it also requires proteins in the membrane which are specific for the ligands. Active transport, however, is different from facilitated diffusion in that the solutes are transported against a concentration gradient and energy has to be supplied for the transfer. Since active transport involves enzymes, these can be saturated at higher concentration of the drug, and that particular drug can be competed by the same process.

1.3. The finding of dipeptide transporter

Active transport in the intestinal absorption of di- and tripeptides is well known. In 1960s, a hypothesis about protein absorption is that proteins were partially hydrolysed in the intestinal lumen to peptides of various sizes, which then enter mucosal cells and were hydrolysed to amino acids, which then enter the blood stream. To investigate absorption procedure of peptides, a series of peptides, ranging from dipeptides to tetrapeptides, were infused directly into the upper small intestine of human volunteers and their fates were determained[12,13,14,15]. The results of these studies suggested large-scale absorption of dipeptides and tripeptides in intact form.

This suggestion was met with the common belief in 1960s that proteins must be broken down to amino acids in the gut lumen before absorption could occur. The results of absorption studies in patients with genetically impaired transport of certain amino acids showed normal absorption of these amino acids when they were administered in dipeptide form[16,17,18]. This would not have occurred

if dipeptides required hydrolysis before absorption. In fact, studies on distribution of enzymes showed that there is very little or no hydrolase activity against dipeptides in the gut lumen[14], and most of the dipeptidese activity in the mucosal epithelium is located intracellularly[19]. Therefore, the peptide transporter seems to play a major role in the completion of the final step in protein digestion by transferring dipeptides.

The peptide transporter is located in the intestinal brush border membrane. The processes involved in the optimal function of the peptide transporter are shown in Fig. 1.3

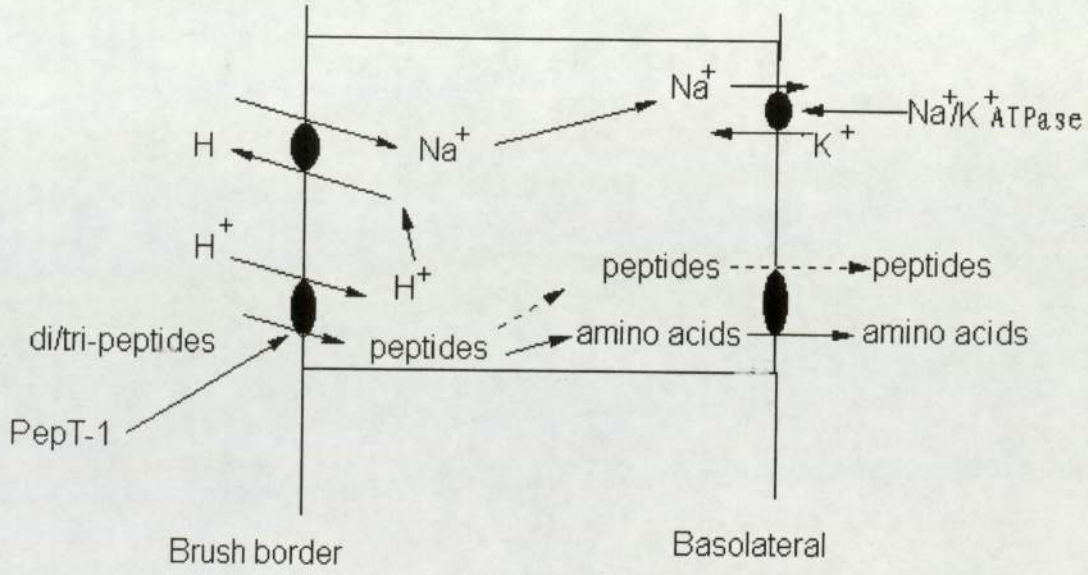


Figure 1.3. The location and the processes involved in the optimal function of the peptide transporter

These include an acidic pH in the microclimate of the intestinal villi, an intracellular alkaline pH maintained by the action of Na^+/H^+ exchanger, and an inside negative membrane potential maintained by the action of Na^+, K^+-adenosine triphosphatase (ATPase) located on the basolateral membrane. Intracellular accumulation of peptides in transporting cells is reduced or eliminated by the action of cytoplasmic peptidases. These enzymes convert peptides to amino acids that are either used by the cell or released into the portal circulation via the amino acid transporters located on the basolateral membrane.

Recent studies have suggested the presence of a peptide transporter in the basolateral membrane. Saito and Inui[20] and Thwaites et al.[21], who compared the characteristics of dipeptide uptake by the apical and basolateral membranes of Caco-2 cells, concluded that peptide transporters on these membranes are different. The phsiological function of the peptide transporter located in the basolateral membrane is probably not for uptake but the exit of dipeptides from enterocytes. This suggestion is supported by observations[22, 23] on tissue accumulation of Gly-Sar after intravenous injection of this dipeptide in rats.

1.4. Absorption of peptidomimetic drugs

Not only intact di- and tripeptides can be taken up by intestinal epithelial cells

via carrier mechanism, several peptidomimetic drugs also rely on the dipeptide carrier for transport across the intestine into the bloodstream.

Peptidomimetic drugs could be referred to as consisting of amino acids or amino acid analogues, and whose synthesis is based on some analogy with natural proteins[24]. The main peptidomimetic drugs are β-lactam antibiotics and angiotensin-converting enzyme inhibitors.

a. β-Lactam Antibiotics

β-lactam antibiotics include penicillin derivatives and many of the cephalosporins. β-lactam antibiotics which possess certain structural features of peptides including a peptide bond with an α-amino group and a free carboxylic acid group (Fig.1.4) are ideal substrates for peptide transport in intact tissue.

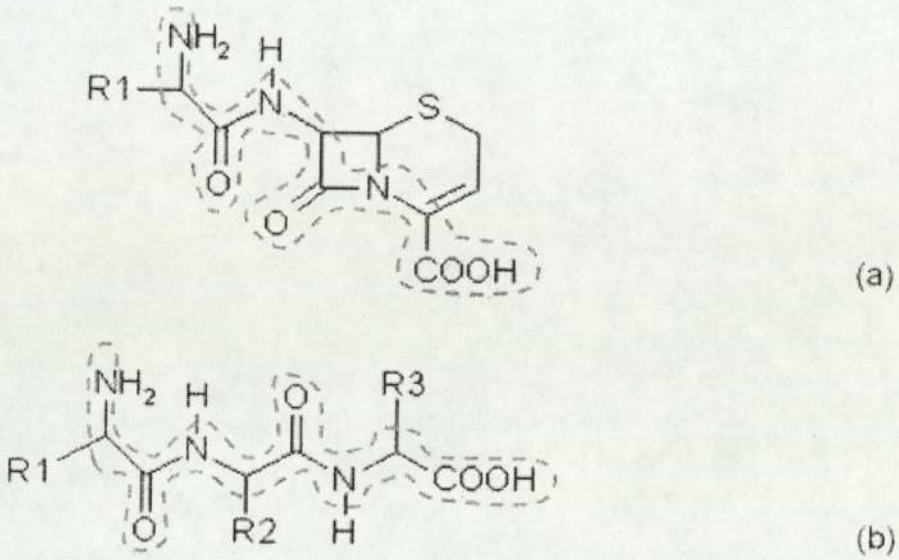


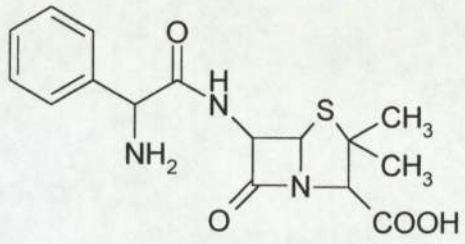
Figure 1.4 structural comparisons of α-amino-β-lactam antibiotics (a) and tripeptides (b).

Saturable absorption kinetics have been reported for amoxicillin in healthy human volunteers[25]. It concluded that the presence of a carrier-mediated system is responsible for the higher than expected bioavailability (about 50%).

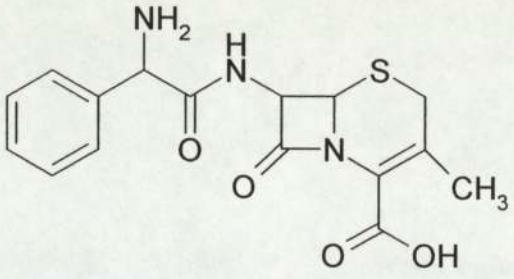
In addition, Okano et al.[26] established that aminocephalosporins were transported *via* a dipeptide transport system that was saturable, Na^+-independent, stimulated by an inwardly directed H^+ gradient, and inhibited by dipeptides including L-carnosine, glycyl-L-proline, glycyl-L-leucine, and phenylalanylglycine, but not by amino acids L-proline, L-phenylalanine, glycine, and histidine. It was suggested that the Na^+-dependence observed in intact tissue was an indirect phenomenon. The presence of Na^+ in the lumen stimulated the Na^+-H^+ antiport, resulting in an inward increase of H^+ flux across brush border membranes. The resulting acidic luminal pH might, in turn, accelerate aminocephalosporin transport.

Cephradine uptake process by brush border membrane vesicles isolated from rat small intestine with an apparent K_m of 9.4 mM was similarly affected. When an inward H^+ gradient and an interior negative K^+ diffusion potential were imposed simultaneously by 9 µM valinomycin, cephradine uptake was enhanced by 23% at 2 minutes[26].

Hori et al.[27] found that there was a good correlation between cephalosporin uptake by intestinal brush border membranes and its absorption in the *in situ* intestinal loop in rats.



Ampicillin



Cephradine

Figure 1.5. Structures of ampicillin and cephradine

b. ACE inhibitors

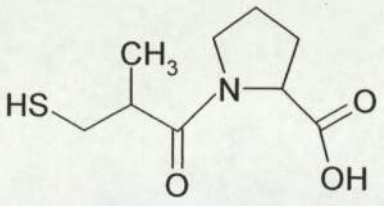
Since the discovery of captopril, angiotensin-converting enzyme (ACE) inhibitors have emerged as an important class of antihypertensive agents for the treatment of high blood pressure and congestive heart failure and act by limiting the conversion of angiotensin I to angiotensin II, a vasoconstrictor, through competition with the natural substrate. Many ACE inhibitors have been approved for medical use or are in various stages of development[28]. These compounds generally belong to three chemical categories: sulphydryl, carboxyl dipeptide, and phosphorus-containing types[29].

Carrier-mediated processes have been implicated in determining the absorption profile of ACE-inhibitors after oral medication in vivo. For example, although the bioavailability of captopril (60-75%[30]) is dose-independent[31], its absorption is reduced markedly in the presence of food[32]. A carrier-mediated absorption pathway has been implicated in an rat intestine luminal perfusion model[10] where the rate of luminal disappearance was concentration-, energy-, pH- and sodium-dependent. Absorption was also inhibited by a range of dipeptides and the β-lactam antibiotic, cephradine.

Active transport has been similarly implicated in the absorption of other ACE inhibitors. Enalapril has a saturable uptake component which is inhibited by the dipeptide L-tyrosylglycine and cephradine, but not by amino acids[33].

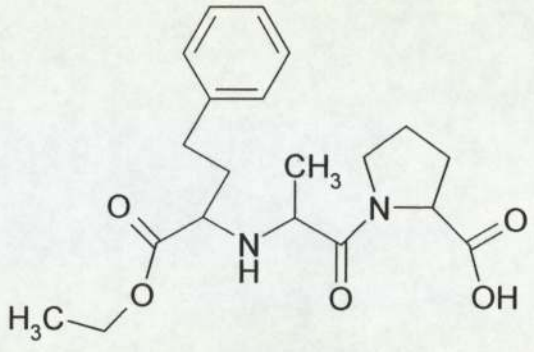
Ceronapril (SQ 29852) is a potent ACE inhibitor in vitro and in vivo following intravenous or oral administration to rats[34]. After oral administration to human volunteers, its plasma appearance occurs slowly (t_{max} ~ 4 h) and, is

dose-dependent[35]. Moreover, its luminal disappearance from the *in situ* rat intestine ring[36] model has both passive and carrier-mediated component; disappearance is also inhibited by L-tyrosylglycine and cephradine, but not by amino acids.



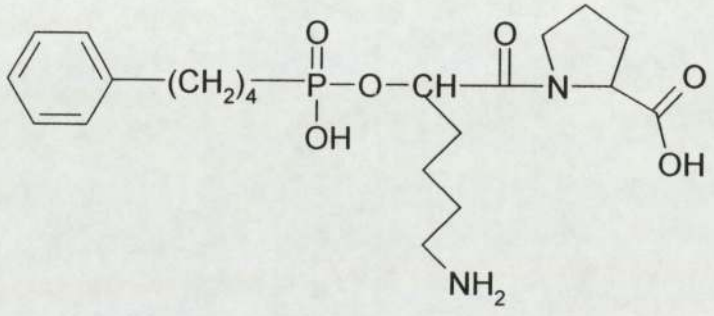
Captopril

Figure 1.6 (I) Structure of captopril.



Enalapril

Figure 1.6 (II) Structure of enalapril



Ceronapril (SQ 29852)

Figure 1.6 (III) Structure of ceronapril (SQ 29852)

1.5. Dipeptide-like prodrugs approach

The oral route is the most convenient route for drug administration concerning patient acceptability, compliance, and convenient. However it is also the route with many variables, which influence the extent and rate of drug absorption (see chapter1).

The possibilities for GI-tract absorption enhancement can be divided into three categories:

— use of GI-tract transport systems

— modification of the drug molecule to be absorbed

— application of absorption enhancers

Therefore, many approaches to enhance the intestinal absorption of poorly absorbed drugs have been developed. These approaches include the use of absorption enhancers, the use of protease inhibitors, of prodrugs and analogues, and of dosage forms such as liposomes and emulsions.

Among these various approaches the prodrug technology appears to be very important for the more traditional drugs, like amino acid containing β-lactacm antibiotics, angiotensin-converting enzyme inhibitors, and L-DOPA. By this bioreversible derivatization technique it is readily feasible to obtain derivatives with increased lipophilicity and, in some cases, metabolic stability.

Although there is no strict universal definition for a prodrug, and the definition may vary from article to article, generally prodrugs can be defined as pharmacologically inert chemical derivatives that can be converted *in vivo* to the active drug molecules, enzymatically or nonenzymatically, to exert a therapeutic effect. Ideally, the prodrug should be converted to the original drug as soon as the goal is achieved, followed by the subsequent rapid elimination of the released derivatizing group[37, 38].

The prodrug approach, a chemical approach using reversible derivatives, can be useful in the optimization of the clinical application of a drug. The major goal in designing prodrugs is to overcome some limitation of the parent drug. This is illustrated in Fig.1.7.

The prodrug approach gained attention as a technique for improving drug therapy in the early 1970s[40]. Numerous prodrugs have been designed and developed since then to overcome pharmaceutical and pharmacokinetic barriers in clinical drug application, such as low oral drug absorption, lack of site specificity, chemical instability, toxicity, and poor patient acceptance (bad taste, odor, pain at injection site, etc.)[39].

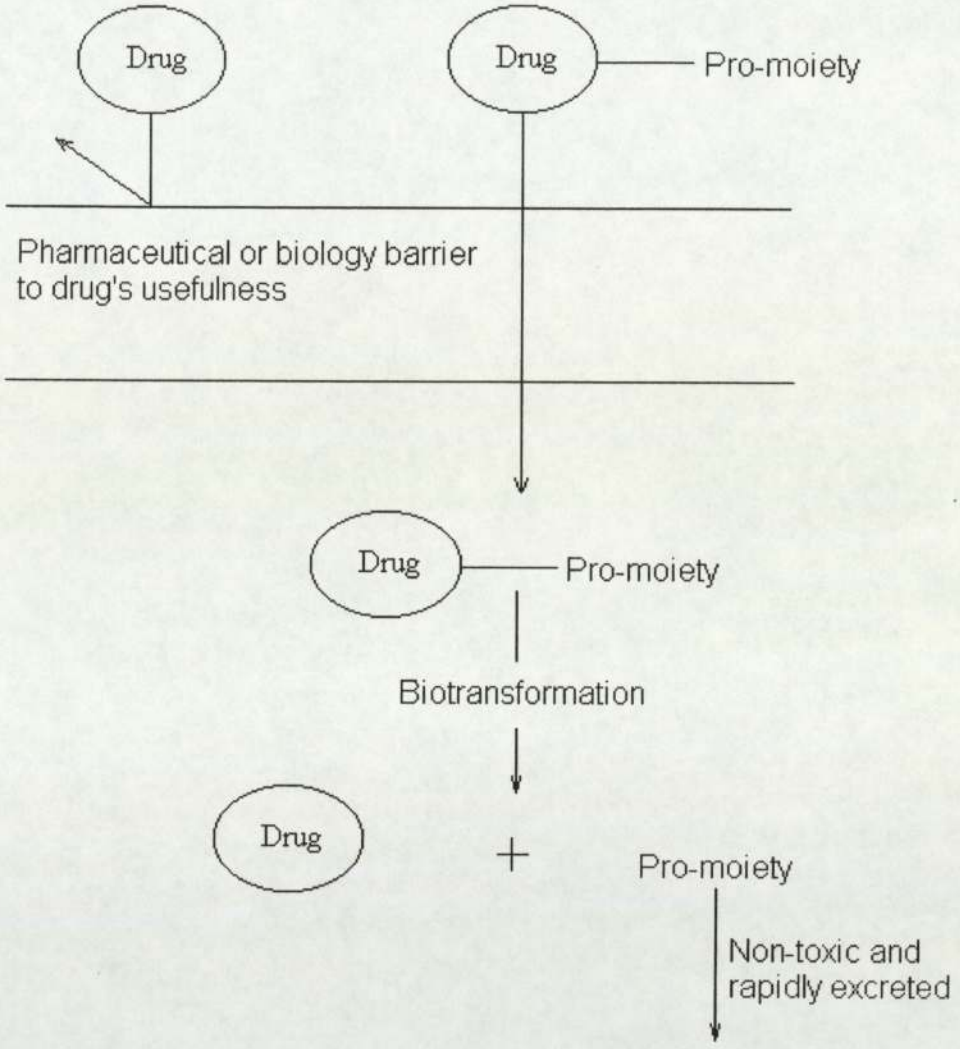


Figure 1.7. Descriptions of the prodrug concept

Although the classical approach to improve membrane permeability of polar drugs uses lipophilic derivatives to increase passive membrane penetration, the targeted prodrug approach uses transporters designed for facilitating membrane transport of polar nutrients such as amino acids and peptides. There is direct and indirect evidence for the participation of carrier-mediated membrane transport mechanisms, where several hydrophilic compounds seem to be absorbed efficiently *via* specific transporters[40]. Therefore, targeting specific membrane transporters is particularly important when prodrugs are polar or charged.

From this point of view, use of intestinal epithelial transporters to facilitate the absorption of appropriately modified drugs seems to be an attractive strategy for improving the bioavailability of poorly absorbed drug molecules. Prodrugs can be designed to resemble the intestinal nutrients structurally and to be absorbed by specific carrier proteins. In this case, prodrugs may have the additional advantage of producing nontoxic nutrient byproducts in which prodrugs are converted to the parent drug molecules. There have been many attempts to improve drug absorption targeting specific membrane transporters, including amino acid, peptide, and glucose transporters.

Among various membrane transporters, peptide transporters are attractive targets in prodrug design to improve oral drug absorption because they have several advantages. First, peptide transporters have broad substrate specificity and high capacity[41]. This characteristic is essential for their normal physiologic function. Theoretically, hundreds of dipeptides and thousands of different tripeptides can be generated from 20 amino acids that are chemically and structurally diverse[41]. Therefore, the physiologic advantage of the broad

substrate specificity of peptide transporters is obvious. Second, peptide transporters have been more extensively studied than other transporters, and considerable information is available. Therefore, structural modifications targeting peptide transporters can be optimised to a much greater degree compared to other transporters. Finally, recent cloning and controlled expression in mammalian cell systems allow us to attempt rational drug design to target the peptide transporters.

Attempts have been made to improve drug absorption by making peptide analogues so the peptide carrier can be used. The intestinal peptide transport system will accept the altered drug as substrate transporting it into its parent form. In this context drugs that are modified in this way with the addition of a part of structure to form dipeptide-like structures that improves absorption from the intestine but is removed before the drug is activated are prodrugs.

As previously described, peptide transporters have broad substrate specificity and are a good target for prodrug development to improve oral drug absorption. As shown in Figure 1.8, Amidon et al have developed the prodrug strategy targeting peptide transporters, defined as Peptide Transporter Associated Prodrug Therapy (PTAPT). A polar drug with low membrane permeability through passive diffusion is converted into a prodrug that is absorbed via the peptide transporter into the cell. This prodrug may still be very polar to assure sufficient solubility in the gastrointestinal lumen but may be well absorbed across the intestinal epithelium via the peptide transporters. Following membrane transport, the prodrug may be hydrolyzed by enzymes in the mucosal cell, blood, or liver.

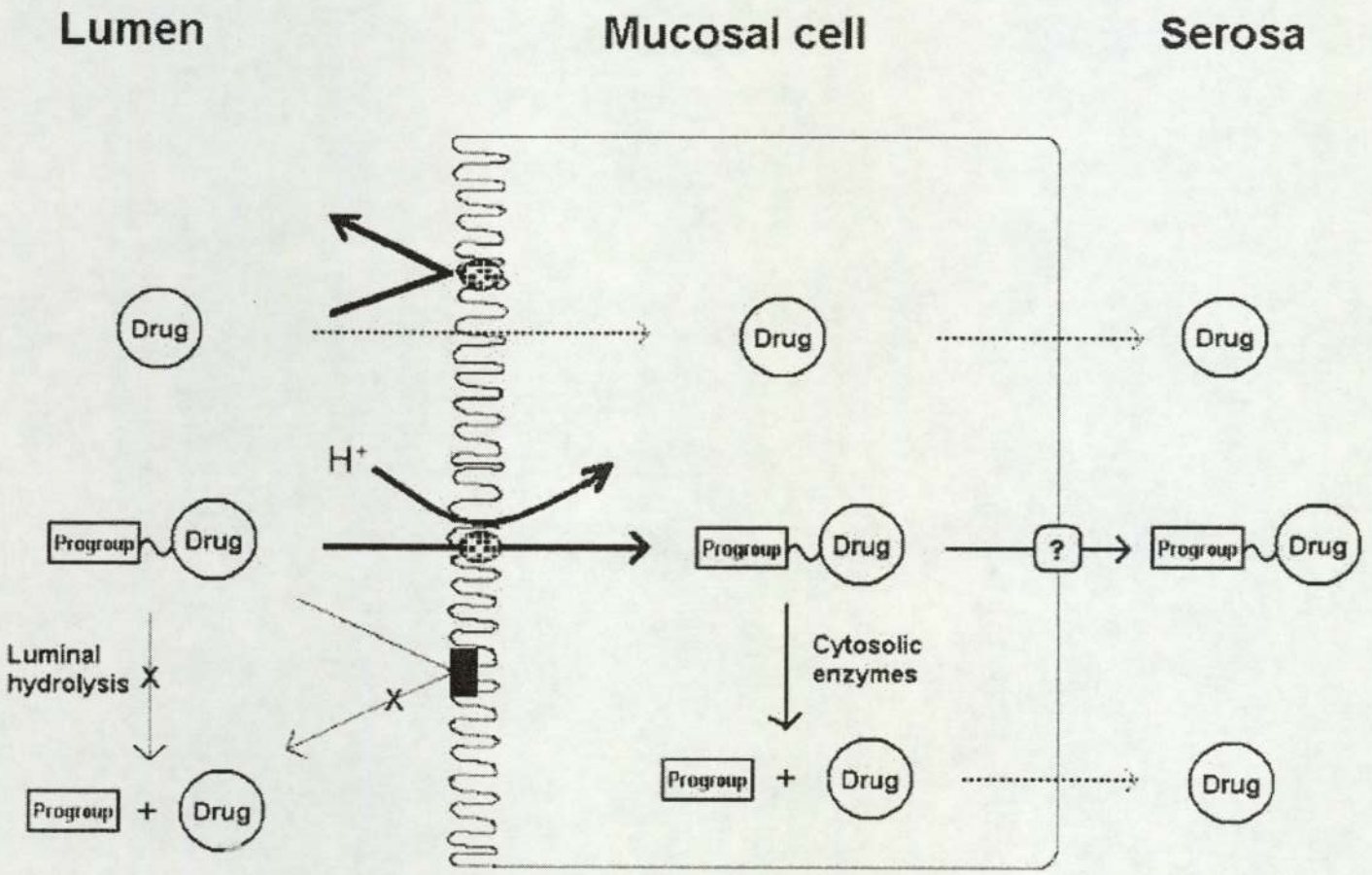


Figure 1.8. Peptide Transporter Associated Prodrug Therapy (PTAPT).

This prodrug strategy has been demonstrated to be effective for improving the membrane permeability and systemic availability of the polar α-methyldopa through peptidyl derivatives[42, 43].

L-α-Methyldopa is a poorly absorbed antihypertensive agent and an amino acid analogue. Its oral bioavailability has been studied in both humans and rats[44,45,46]. Its mechanism of absorption is carrier-mediated *via* an amino acid carrier[47].

Amino acid carriers are structurally restrictive, and there are possible four distinctive carriers. Each carrier is primarily responsible for the transport of a specific type of amino acid[48]. This structural restriction is thought to be the main reason for L-α-methyldopa to be poorly absorbed since the α-Methyl group severely hinders the binding of the substrate to the carrier as in the case of α-aminoisobutyric acid[49].

Attempts have been made to improve the absorption of L-α-methyldopa by the approach of making the peptide analogues prodrug to improve L-α-methyldopa absorption. Three basic reasons underlie the synthesis of these dipeptides: first, the peptide carrier is likely to have a more relaxed structural requirement and be generally more efficient than amino acid carriers[50]; second, most peptides are hydrolyzed to their constituent amino acids before entering the systemic circulation; and third, the derivatizing groups, amino acids, are natural substances with a low toxicity potential.

Intestinal permeabilities of five dipeptidyl devivatives of L-α-Methyldopa were studied by an *in situ* intestinal perfusion method[11]. The results of this analysis

are summarized in Table 1.1.

Compoud	1.0mM	0.1mM	0.01mM
L-α-Methyldopa(l)	0.41 (0.11)	0.4 (0.22)	0.43 (0.14)
Gly-l		4.34 (0.27)	
Pro-l		1.68 (0.23)	
l-pro		5.41 (0.55)	
Phe-l		5.29 (1.57)	
l-phe	4.30 (0.30)	10.22 (0.45)	10.9 (1.8)

Table 1.1. Wall permeabilities ($P_w \pm$ SE) of L-α-methyldopa and Its dipeptidyl derivatives

Comparison with the parent compound, L-α-methyldopa, indicates that the dipeptides showed significant increases in permeabilities, ranging from 4 to 20 times ($P < 0.01$). using a concentration of 50 mM L-gly-L-gly to coperfuse with l-phe or phe-l, it was possible to decrease the permeabilities of these two compounds substantially. The results indicated that the absorption of these dipeptides is by the same carrier system responsible for the absorption of other peptides. Furthermore, the substantial increases in the permeabilities of the dipeptides over that of L-α-methyldopa agree with the current understanding of the intestinal transport of amino acids and peptides and indicatate that the peptide carrier is less structurally specific than the amino acid carriers.

In testing the hydrolysis of the L-α-methyldopa dipeptidyl derivatives, it was found that these dipeptide analogues were more slowly hydrolysed in the test system than gly-phe. However, significant hydrolysis does occur, suggesting that these dipeptidyl derivatives of L-α-methyldopa may be prodrug strategy.

L-Dopa is the drug of first choice to control chronic Parkinson's disease, a disease in which patients deteriorate gradually if dopamine is not continuously supplied in sufficient amount. The major problems of oral L-dopa include low water solubility and extensive degradation in the gut wall[51]. Consequently, though absorbed rapidly and completely, L-dopa has low oral bioavailability.

Many prodrugs of L-dopa have been studied in the past twenty years, including recent efforts on ester prodrugs of L-dopa[52]. Since for L-Dopa, gut-wall metabolism is much higher than hepatic first - pass effect, prodrugs of L-Dopa have to minimize decarboxylation of L-dopa. Further, the prodrug has to be

absorbed effiently in the intestine. Without fulfilling these desired requirements, prodrugs may not offer any advantage. An early, rapid release of L-Dopa from the prodrugs in the lumen and epithelium will result in a similar extent of gut-wall first pass metabolism. Thus, desirable prodrugs should not release L-Dopa until absorbed into the circulation. Since the intestinal peptide transporter has broad substrate specificity and does not require a free N-terminal α-amino group and since a di-/tripeptide lacking an N-terminal α-amino group and having Pro at the C-teminus is relatively resistant to intestinal proteolytic degradation[53,54], it is proposed to design a prodrug of L-Dopa which is absorption into the circulation. Under this principle, pGlu-L-Dopa-Pro was designed[55].

With rats as the animal model, the stability of pGlu-L-Dopa-Pro in intestinal homogenates was determined, and then the transport characteristics of pGlu-L-Dopa-Pro were studied using *in situ* perfusion.

The results indicate that pGlu-L-Dopa-Pro is absorbed efficiently by the intestinal peptide transporter, is relatively stable in intestinal epithelium and is converted to L-Dopa by successive actions of pyroglutamy aminopeptidase I and prolidase. pGlu-L-Dopa-Pro has the desired properties to ensure good absorption and minimal decarboxylation in the gut wall.

In conclusion, Targeting the peptide transporter, with its broad specificity, in order to achieve significant oral efficacy of di- / tripeptide drugs is an appealing strategy. The principal goal of modification to make prodrug is alteration of membrane permeability to improve oral (GI permeability), brain, tumour and cellar delivery of these agents. When these prodrugs are used for improving

oral bioavailability, various issues dealing with GI absorption of drug must be considered. The prodrug should have good chemical stability for formulation, pH stability for dissolution in GI tract, good enzymatic stability for lumenal contents as well as brush border membrane, and good permeability.

The prodrug should revert to the parent drug either in the enterocyte or once absorbed into systemic circulation. The prodrug should have complete enzymatic and chemical stability during the absorption process and in blood but readily revert to the parent compound once it has permeated the targeted cell, thereby 'trapping' the drug in the cell.

CHAPTER 2 - DESIGN OF PRODRUG

2.1. The study of SQ-29852

Since peptide transporters are potential targets for facilitating GI tract absorbing oral drug, a proper parent structure of prodrug is needed.

SQ-29852 (Fig. 2.1) ((S)- 1- (6 – amino – 2 - ([hydroxy – 4 - phenylbutyl] - phosphinyl) oxy) -1- oxo – hexyl – L - proline) is a phosphonic acid, which is a lysyl proline ACE inhibitor. A previous study indicated that it is absorbed by the dipeptide transport system (DTS[56]).

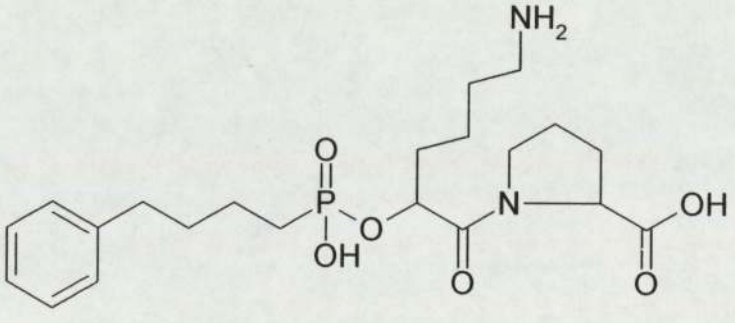


Figure 2.1. Structure of SQ-29852

At low clinically relevant doses, SQ-29852 was well absorbed (> 50 %) in dogs[57]and human[58]. Consistent with a carrier-mediated absorption mechanism, the absorption of SQ-29852 is saturable in these species, with values as low as 4% after administration of high doses.

SQ29852 is an ideal probe for evaluation of DTS because it is chemically and metabolically stable and it is absorbed almost exclusively by the DTS[59] on one hand, on another hand it appears to specific probe for the DTS as the dose-dependent reduction in absorption from about 60% to less than 8% (3 and 3000mg / kg respectively) suggests that at least 85% of an orally administered low dose of SQ-29852 absorbed by a saturable process. Competition studies with SQ-29852 and dipeptides *in situ* in rats indicated that the saturable absorption process for SQ-29852 was indeed the DTS[44]. Thus, the passive diffusion component of the oral absorption of SQ-29852 is minimal (<15%). The poor passive transcellular permeability of SQ-29852 is consistent with its physical-chemical properties[47]: (1) it is highly charged at physiological pH (two negative and one positive charge), (2) it has a molecular weight of 440Da, and (3) it has very low lipophilicity (logP = - 0.77[22]).

The disposition of SQ-29852, a lysyl proline ACE inhibitor, was evaluated *in vivo* in rats, and it was shown to be a stable and specific probe for the DTS. *In vivo* results, obtained with SQ-29852, indicated that the DTS is apparently distributed throughout the entire GI tract of rats, including the colon.

In animals, SQ-29852 is well absorbed (70%) at low doses and its absorption is less than 10% at high doses, suggesting that it has a minimal absorption *via*

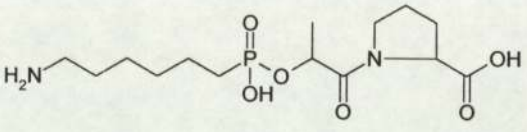
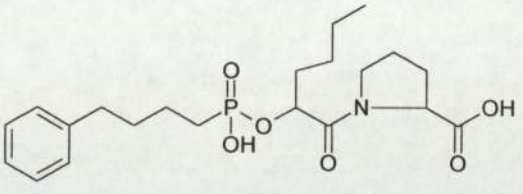
passive diffusion[44]. In this regard, SQ-29852 appears to be a better probe than the β-lactam aminocephalosporins since the latter have been reported to have a significant passive component to their overall absorption[60].

Above reason indicated that SQ-29852 is a better model compound for studying the DTS. SQ-29852 provides consistent results in a variety of *in situ*, *in vitro*, and *in vivo* models.

Because SQ-29852 is a lysyl proline ACE-inhibitor, based on its structure, prolinyl dipeptides were synthesized. The prolinyl dipeptides were tested with caco-2 cells to investigate absorption profile in order to find most suitable probe for dipeptide transport system study.

2.2. Previous work

In the previous studies[61], it has been revealed that the length of alkyl chains at the position α to the carbonyl group in N-acylated proline affects the binding activity towards the dipeptide transporter system (DTS). Such as in (1) and (2), change from methyl to butyl, the binding ability is increased dramatically (Table 2.1).

Compound	Structure	%Inhibition Mean [S.D.]	IC_{50}mM Mean [S.D.]
(1) SQ-30640		-1.15[4.68]	y*
(2) SQ-29907		88.97[2.14]	1.24[0.27]

*y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

Table 2.1. The binding ability of SQ-30640 and SQ-29907

It was also found that the benzyl ester of SQ-29852 showed the better binding ability in the tests with the DTS (Table 2.2).

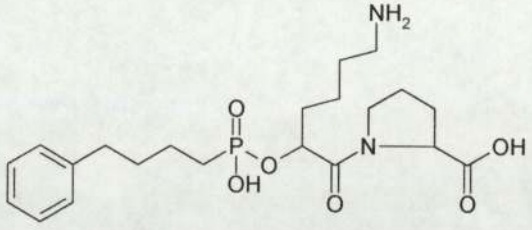
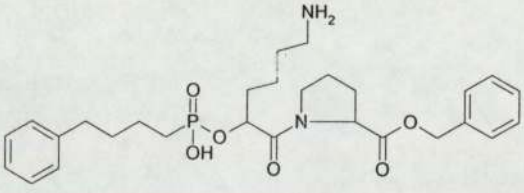
Compound	Structure	%Inhibition Mean[S.D.]	Ic50mM Mean[S.D.]
SQ-29852		60.51 [6.73]	0.68 [0.17]
SQ-31065		90.41[1.90]	0.05 [0.01]

Table 2.2. The binding ability of SQ-29852 and SQ-31065

The study of analogues of SQ-29852 suggested that:

(1). Proline can be as the basic structure (see Fig 2.2) for design of prodrugs that have dipeptide-like structure and is a substrate of the dipeptide transporter in the intestinal mucosa cells.

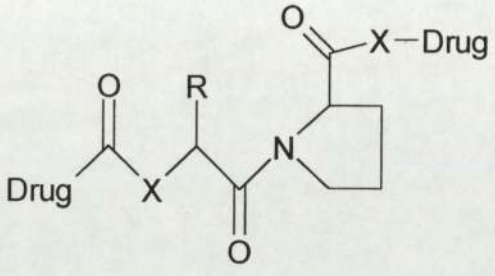


Figure 2.2. The Model of prodrug (1). "X" could be "O" or "NH" depending upon the properties of the drug.

(2). 4-hydroxy proline is also used in prodrug design[61]. (see Fig 2.3).

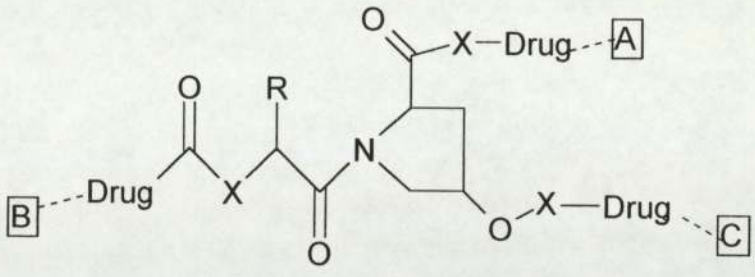


Figure 2.3. Model of prodrug (2)

There are three potential sites for attaching a drug: position A, position B and position C (Figure 2.3). A and B are in the same situation as when proline is used as the basic situation. In the previous studies, it was found that a satisfaction in the 4-hydroxy proline promoted the binding ability of the drugs to

the DTS[61]. Therefore, position C became a new site, thereafter.

In comparison with results of derivatives of SQ-29852, the test results of our year 1999's thirty-three compounds with Caco-2 cells showed us:

1. The complex phosphate N-acyl groups on proline in SQ-29852 and its derivatives seem not essential for the inhibition (Inhibition represents transport ability. The more transport of [^{3}H] Gly-Pro was inhibited in the Caco-2 cells in the experiment, the more amount of tested compound transported into the cells.), because the simple acyl groups such as Glu or Met give good inhibitory activity, apparently better than SQ-29852.

2. In agreement with the observation in the studies of derivatives of SQ-29852, increasing hydrophobility of proline part enhances the inhibition. Most significant is overall the stronger inhibitory activity of proline benzyl esters than its free acid and methyl esters.

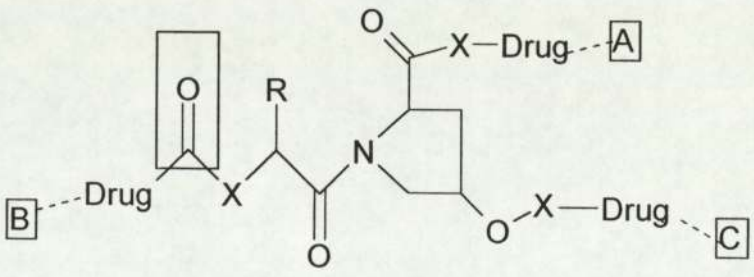
3. Hydrophobicity of the N-acyl chains of proline also influences inhibition. From these data, it tells us longer hydrocarbon chain or higher hydrophobicity from Ala to Nle hydrocarbon chain grows from CH_3 to $CH_3CH_2CH_2CH_2$, IC_{50} is enhanced from 1.829mM to 0.438mM.

2.3. The new intention of the project

1. In light of better inhibition of proline benzyl ester (which is more

hydrophobic), the study of this project focus on the exchange of proline ester group to more hydrophobic ones, such as diphenylmethyl to work out the best for the parent structure of the prodrugs.

2. In line with the finding of importance of a nucleophilic group in acyl chain[61], a group of compounds with different length of the acyl chain was synthesized and tested to determine the desirable structure with a certain length of the acyl chain, which shows the best inhibition ability in the Gly-Pro uptake test.



3. According to previous findings with SQ-29852 derivatives[61], 4-hydroxy proline was used as the parent structure in the synthesis of new derivatives.

4. From the previous research on this project[61], it was found that the synthesized dipeptide samples with the C-terminal protected by benzyl group or methyl group, or with the C-terminal free, showed quite different activities in the biological tests. However, the number of the dipeptide derivatives that have been designed and synthesized so far is far to enough to make any conclusion. Therefore, to get a complete dipeptide derivative sample group and to get more evidence about different uptake activities among the dipeptides with the C-terminal benzyl ester, methyl ester and free carboxylic group, we synthesized all the dipeptides, which have been prepared in the previous research, with the three different C-terminal chemical environments that were

mentioned hereinbefore.

2.4. Test model (Caco-2 cells)

2.4.1. An in vitro model of the intestinal epithelial cell barrier

A number of new cell culture models for drug absorption studies are currently being characterized and validated.

Recently, with the improvement of cell culture systems, intestinal cell line became more and more important for studying the transport of drugs across the epithelia. Among 20 human colon carcinoma cell line tested, Caco-2 cells were the only one to differentiate spontaneously as normal intestinal absorptive cells when grown in standard culture condition. Therefore, Caco-2 cells have become the "work house" for scientists in the field of oral absorption[62, 63, 64].

Caco-2 cells, which are derived from a human colon adenocarcinoma, exhibit morphological and functional characteristics of small intestinal cells. As the Caco-2 cell line displays the most highly differentiated properties under standard culture conditions[65], it appears to be the most relevant *in vitro* system for investigating transepithelial transport processes.

A number of laboratories[64, 66, 67] have demonstrated that Caco-2 cells can be routinely grown as confluent monolayers on microporous filters. The

monolayers develop enterocytic morphology typical of villus cells and a polarity of a number of brush-border enzymes[64, 65, 66]. Depending upon the exact experimental conditions used, full expression of these properties is achieved between approximately 15-20 days in culture[65, 66]. Establishment of the barrier function of the monolayers can be demonstrated by lack of passage of a number of permeability markers[65, 66]. The inability of horseradish peroxidase (mol. wt. 40,000) to cross the tight junctions developed between adjacent Caco-2 cells demonstrates that the barrier properties of the *in vitro* system to this macromolecular probe are similar to those of the small intestine in *vivo*[68].

2.4.2. Dipeptide transporters in Caco-2 Cells

Intestinal cells such as enterocytes are equipped with an array of transport proteins to facilitate uptake of otherwise poorly absorbed compounds. Many of these transport systems, such as the peptide transporters, intrinsic factor, bile salt carrier, and vitamin C, are of major interest for drug discovery and delivery issues, because they might be used to enhance systemic uptake of therapeutics after oral administration. Because screening for drug carrier interactions is not feasible *in vivo,* it was important to the scientific community to use a relevant intestinal cell line. Fortunately, the Caco-2 cell expresses many of the aforementioned carrier proteins.

Studies with brush border membrane vesicles (BBMV) revealed that the electrochemical gradient for H^+, as opposed to Na^+, energizes di/tripeptide absorption[69,70]. This observation was corroborated in Caco-2 monolayers by Thwaites et al.[71,72,73], who investigated the role of the intestinal di/tripeptide carrier in the transepithelial transport of peptides. Furthermore, Ganapathy and

Leibach[74] proposed that the pH gradient across the apical membrane of the enterocyte is maintained during H^+-coupled dipeptide transport by the Na^+, H^+ exchange at the apical surface and by Na^+ extrusion *via* the Na^+, K^+-ATPase at the basolateral membrane.

Saito and Inui[20] demonstrated the existence of a dipeptide transporter on both the apical and basolateral membranes using the dipeptide-like anticancer agent bestatin, confirming results from Thwaites et al. Interestingly, although bestatin uptake, like Gly-Sar uptake, was pH-sensitive from the apical membrane, it was insensitive to pH when accumulation was examined from the basolateral site. This result suggests that bestatin is transported in the basolateral-to-apical direction by a transporter distinct from the H^+-dependent apical transporter.

In another study, Thwaites et al.[70] examined the transport of angiotensin converting enzyme (ACE) inhibitors such as lisinopril, and enalaprilat. Captopril and enalaprilat were able to stimulate H^+-flux, suggesting transport by the di/tripeptide carrier in Caco-2 cells.

It is becoming evident that Caco-2 cells share similarities to small intestinal tissue with respect to the expression of H^+-dependent peptide cotransporters.

For these reasons, a human intestinal cell line (Caco-2 cells) is becoming the standard for investigation of the absorption of drugs intended for oral delivery.

CHAPTER 3 – DISCUSSION OF DIPEPTIDE CHEMISTRY

Peptides and proteins exhibit the largest structural and functional variation of all classes of biologically active macromolecules. The essential structural features of peptides and protein molecules are chains of amino acids linked to one another by amide bonds. (Fig. 3.1)

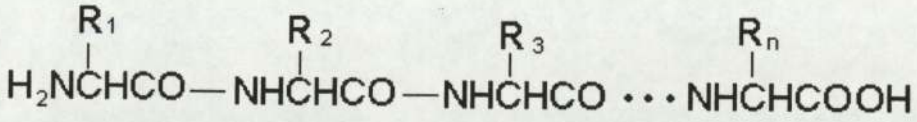


Figure. 3.1 The structure of peptides

3.1. The formation of peptide bond

Formation of an amide bond between two amino acids is an energy-requiring reaction. Carboxylic acids do react with amines at elevated temperatures and amides can be produced this way. For instance, ammonium acetate can be converted to acetamide by heating. The temperature, however, at which such transformations occur far exceed the limits considered safe for complex peptides. In fact, peptide synthesis is usually performed at or below room temperature and the coupling methods which involve heating of the reaction mixture are regarded as not generally useful. Therefore, in order to form a peptide bond, one of the groups that will produce the desired amide, either the carboxyl or the amino group, must be activated.

The methods by which coupling along the lines of Figure 3.2 can be performed fall into three main groups:

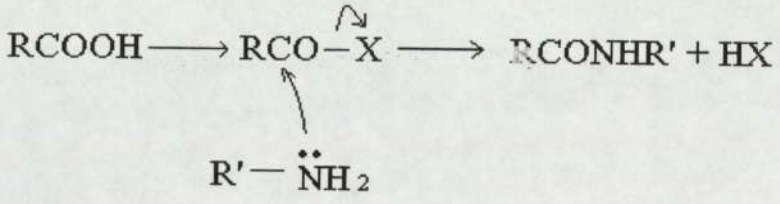


Figure 3.2. The formation of peptide bond (X is a good leaving group)

(1) Those in which a reactive acylating agent is formed from the carboxyl component in a separate step, followed by immediate treatment with the amine component;

(2) Those in which an isolable acylation agent is formed in a separate step and is purified before aminolysis;

(3) Those in which the acylating intermediate is generated in the presence of the amine and carboxyl components by addition of a coupling reagent to a mixture of the amine and carboxyl components ("direct coupling").

It has already been indicated that practically all of the techniques which have ever been used for constructing peptide bonds are of the type outlined in Figure 3.2. A great variety of leaving groups and reactions for attaching them to the electrophilic carbon atom of the carboxyl component have been investigated and recommended, although the number of methods actually in regular use is comparatively small because the criteria which an acceptable method must meet are very demanding. Good yields of easily isolated products with freedom from racemization are required, as well as convenience and economy.

The principal reaction in the synthesis of peptide chains is acylation of the amino group of an amino acid by the carboxyl group of a second amino acid with formation of an amino bond. The presence of several functional groups in amino acid molecules and the necessity to maintain the integrity of the α-carbon chirality centers during makes peptide synthesis enormously more

complicated than simple carboxyamide formation. Therefore, a number of methods have been developed for peptide synthesis under mild conditions.

3.1.1. Other methods review

3.1.1.1.The use of Acyl Halides and Pseudohalides

3.1.1.1.1. Acyl Chlorides

The activation of acylamino acids by conversion to the corresponding acyl chlorides followed by reaction with amino acids or esters under Schotten-Baumann conditions[75] or by treatment with amino esters in organic solvents is, in principle, the most obvious and simple approach to peptide synthesis (e.g., Figure 3.3[75]) and this kind of method[76] played an important role in the early days of peptide synthesis.

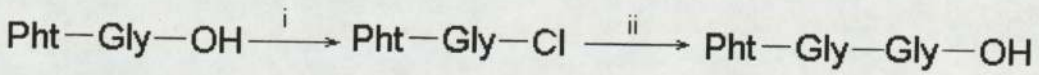
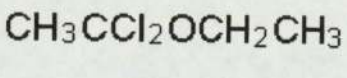
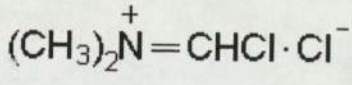


Figure 3.3. The method of Acyl Chlorides to form the peptide bond. (Conditions: i, PCl_5 in benzene (81%); ii, Gly in MgO-aqueous dioxane (90%))

The reagents normally used for acyl chloride formation (thionyl chloride, phosphorus pentachloride, etc.) are too vigorous to be compatible with complex or sensitive substrates and, furthermore, simple acylamino acid chlorides cyclize spontaneously to give oxazolones and hence racemic peptides. Benzyloxycarbonylamino acid chlorides are isolable but unstable, decomposing to N-carboxyanhydrides on warming[77]: although they cyclize rapidly to give benzyloxyoxazolones[78] under basic conditions, optically active peptides can be isolated after reaction with amino esters. The chlorides of trifluoroacetyl-, and tosylamino acids are reasonably stable, although the last decompose under Schotten-Baumann conditions[79]. Milder alternatives to the classical reagents for acid chloride preparation have been devised, such as compounds (Figure 3.4) (**a**) [76] and (**b**)[80], but the acid chloride method must nevertheless be regarded as completely obsolete for conventional peptide synthesis, as many more subtle, convenient and efficient methods are available.



(a)



(b)

Figure 3.4. Milder alternatives to the classical reagents for acid chloride preparation

3.1.1.1.2. Acyl Cyanides

Suitable protected amino acids can be converted *via* their bromides to the corresponding acyl cyanides, which react with amino esters to give peptides[81]. But the lack of mild methods of generating the acyl cyanides has precluded development of the method[82,83].

3.1.1.1.3. Acyl Azides

The activation in the form of acid azides is a powerful and practical approach for the formation of peptide bond, which was introduced by Curtius[84] 75 years ago but is still an important procedure[85].

The original step of this action is hydrazinolysis of alkyl esters and conversion of the hydrazides to acid azides with the help of nitrous acid (Figure 3.5) at around or just below 0℃, which works well in simple cases. But now, the reaction involving dry acid and an organic nitrite ester in a dry organic solvent at a low temperature is preferred[86].

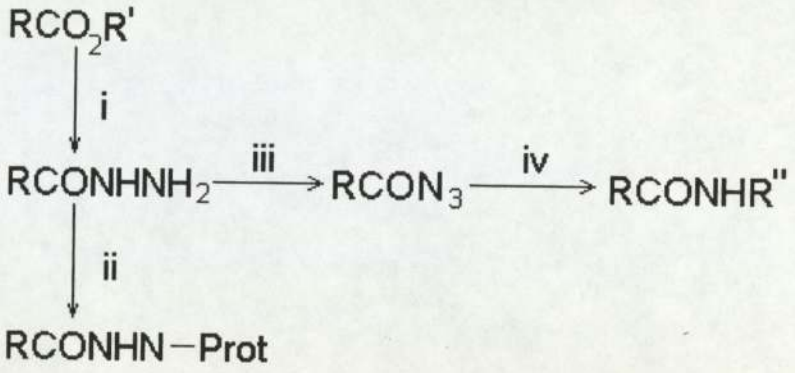


Figure 3.5. The method of Acyl Azides to form the peptide bond. (Conditions: i, NH_2NH_2; ii, deprotection; iii, $NaNO_2$ in aqueous AcOH-HCl or an alkyl nitrite with dry H^+; iv, $R''NH_2$.)

Recently, the direct conversion of carboxylic acids to acid azides by treatment with diphenylphosphoryl azide (Fig. 3.6) has become a viable alternative method[87].

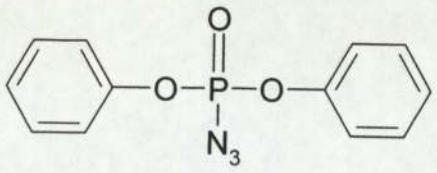


Figure 3.6. Structure of diphenylphosphoryl azide

3.1.1.2. The use of Anhydrides

3.1.1.2.1. Symmetrical Anhydrides

Acylamino acid symmetrical anhydrides can be prepared [88] from the corresponding acylamino acids by use of a variety of reagents including ethoxyacetylene and dicyclohexylcarbodiimide. However, aminolysis of a symmetrical anhydride is a wasteful process, because in this acylation, only one of them is regenerated but usually not recovered (Figure 3.7).

Figure 3.7. The method of Symmetrical Anhydrides to form the peptide bond.

3.1.1.2.2. Mixed anhydrides with carboxylic acid

A mixed anhydride of benzoylglycine with benzoic acid was presumably involved in the classic synthesis of the benzoylglycylglycine (Figure 3.8)[89].

$$PhCOCl + NH_2CH_2CO_2Ag \longrightarrow PhCONHCH_2CO_2H + PhCONHCH_2CONHCH_2CO_2H$$

Figure 3.8. Synthesis of the benzoylglycylglycine

3.1.1.2.3. Mixed anhydrides with carbonic acid

The most generally successful mixed anhydride method involves the generation and aminolysis of a carboxylic-carbonic anhydride as outlined in Figure 3.9[90].

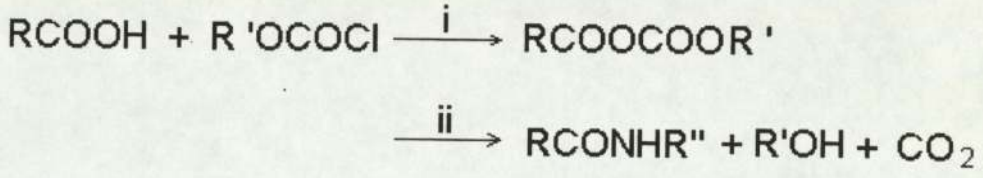


Figure 3.9. The method of mixed anhydrides to form the peptide bond (Conditions: I, 1 equiv tertiary base / unreactive dry solvent / ca -10℃ / a few minutes; ii, R"NH_2)

3.1.1.3. Active Esters

The use of ester aminolysis (Figure 3.10) for peptide synthesis is as old as the subject itself[91].

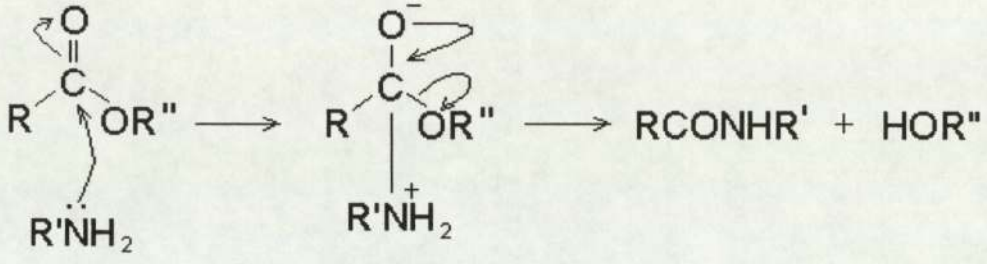


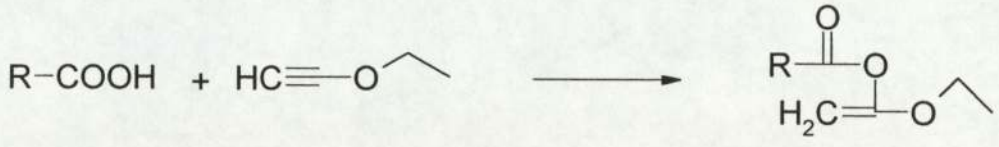
Figure 3.10. The use of ester aminolysis for peptide synthesis.

In the development of peptide synthesis methodology, active esters play an important role, since in the ester, there is only one electrophilic center, no second acylation product can be generated. The reaction can be facilitated by use of a good leaving group, as in esters of phenols and other similarly acidic functionalities. Active esters are most commonly prepared during DCCI-mediated coupling[92] between a protected amino acid and the ester moiety.

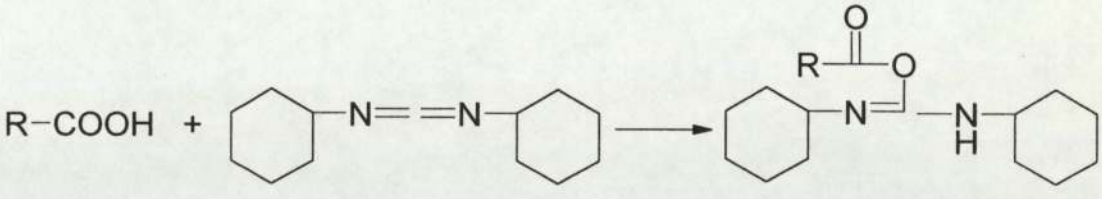
3.1.2. Our approach to form the peptide bond (Coupling Reagents method)

Coupling reagents which can be added to a mixture of both components are more popular in peptide synthesis now. Practically, this has become an easy and effective approach to the formation of the peptide bond.

In 1955 two compounds were simultaneously proposed as coupling reagents for the formation of peptide bonds: ethoxyacetylene[93] by Arens and dicyclohexylcarbodiimide (DCC or DCCI)[93] by Sheehan and Hess. Activation of the carboxyl group occurs through its addition to a triple bond in the acetylene derivative in the case of ethoxyacetylene and to an N=C double bond in carbodiimides: (Figure 3.11)



Ethoxyacetylene



DCC

Figure 3.11. Ethoxyacetylene and DCC to effect the formation of peptide

A characteristic feature of both reagents is the activation of the carboxyl group *in situ* with the presence of the amino-component, such as carbodiimides to form an amide bond (Figure 3.12):

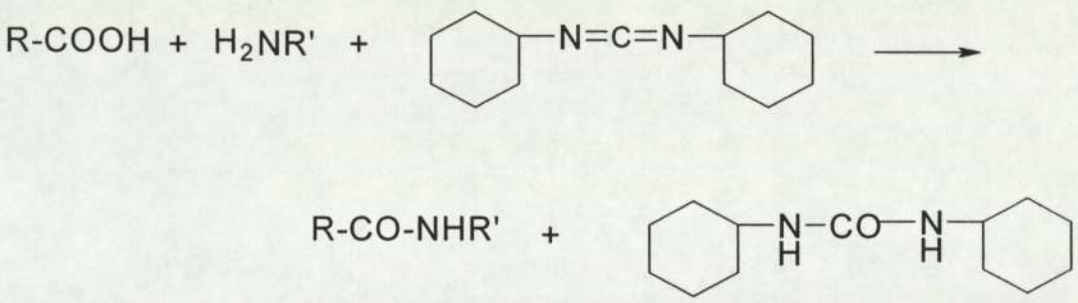
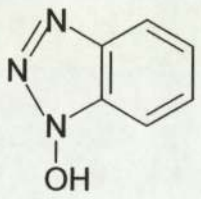


Figure 3.12. Carbodiimides used as coupling reagents.

The by-products of the coupling reactions, N,N'-dicyclohexylurea in DCC-mediated couplings are readily removed from the reaction mixtures. As a result, dicyclohexylcarbodiimide became and still is a mainstay of peptide chemists.

Some shortcomings of the DCC method can be eliminated by the use of "additives". Of these, 1-hydroxybenzotriazole (Figure. 3.14)[94] is the most commonly applied.



HOBt

Figure 3.13. Structure of HOBt

Since the additive, e.g. 1-hydroxybenzotriazole, is usually applied in an amount which is equimolecular with the two components to be coupled, there are two moles of nucleophiles present in the reaction mixture for each mole of carboxyl component or carbodiimide. There is a significant change in the kinetics of the process. Coupling with DCC involves two or more consecutive reactions. The second nucleophile, the additive, present in almost constant

concentration, will accelerate the entire process, but more importantly it converts the overactivated intermediates of the DCC-reaction to the less reactive esters of 1-hydroxybenzotriazole or N-hydroxysuccinimide. The ability of additives to provide multiple pathways to the same product is shown in the following figure:

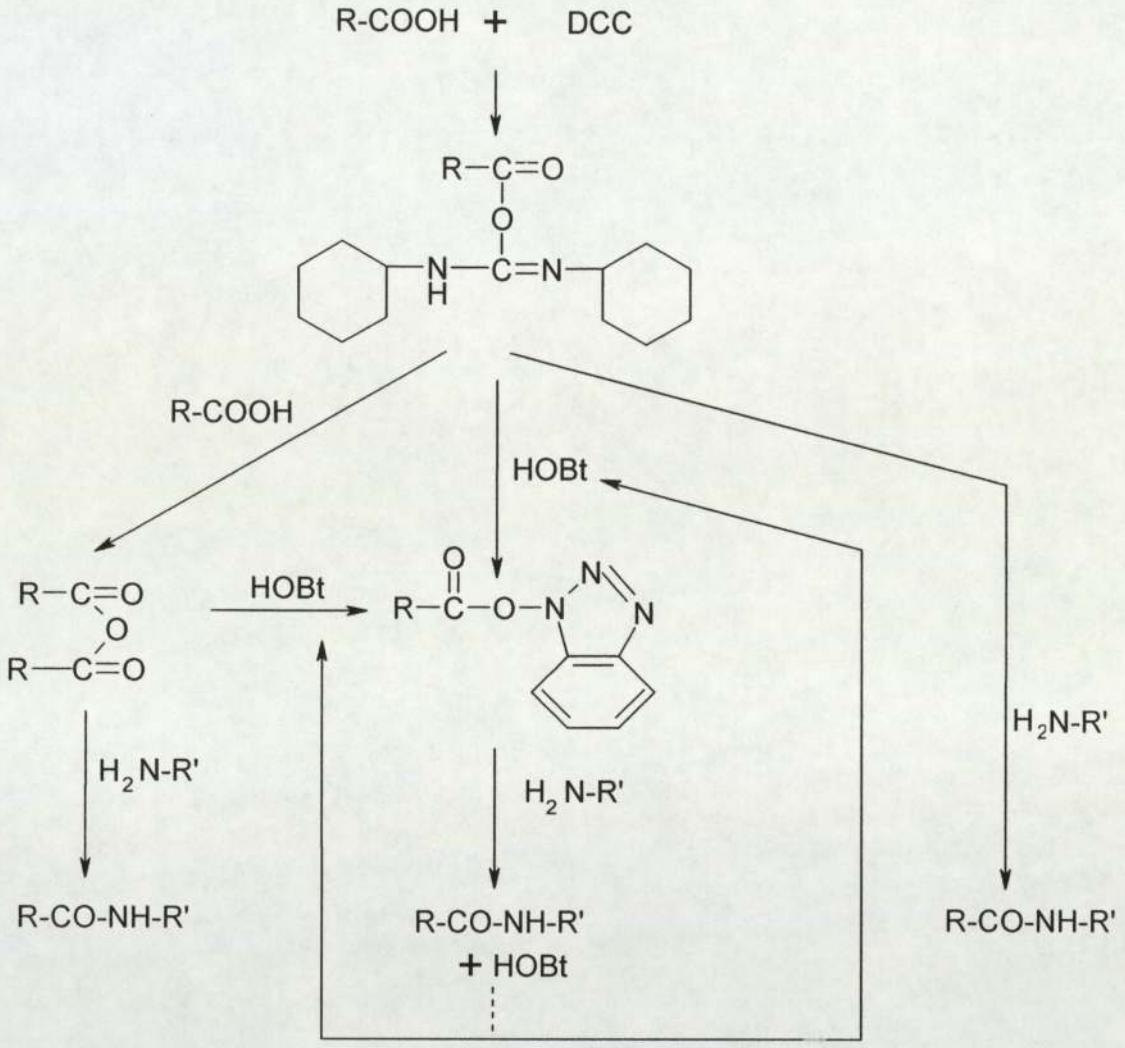


Figure 3.14. Coupling reagents method to form the peptide bond

3.2. The selection of protecting groups

In the preparation of peptide, some functional group should be blocked, for example (Figure 3.15):

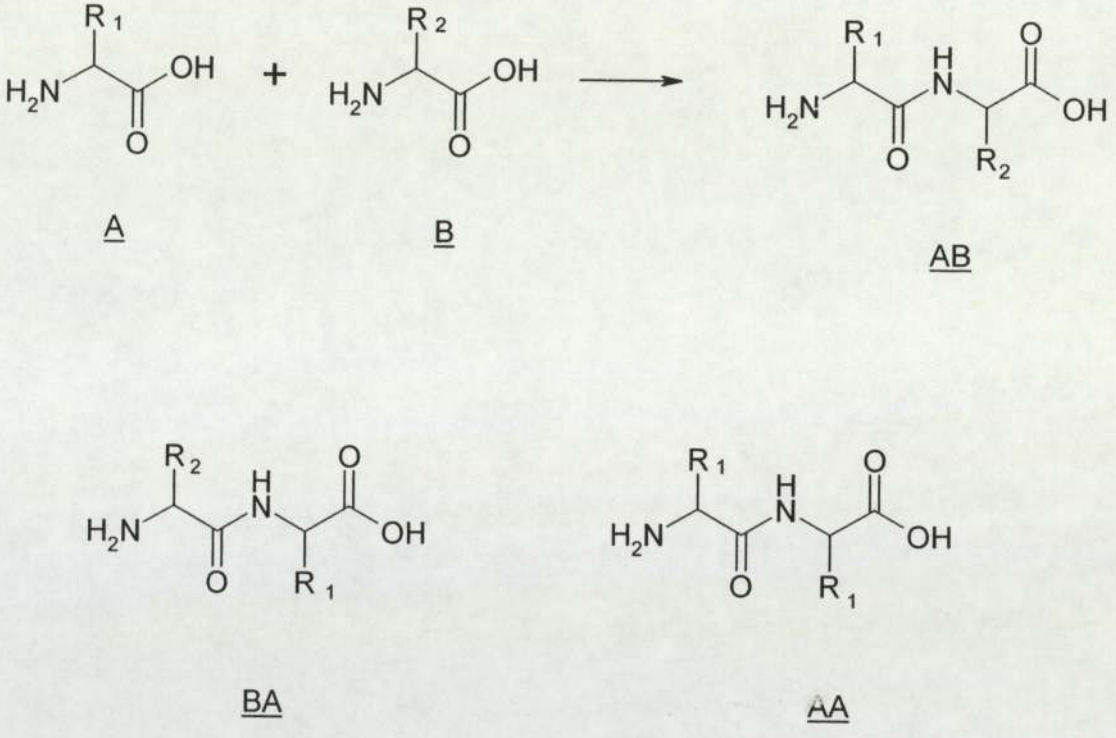


Figure 3.15. Dipeptide AB, BA and AA

In this reaction, when the carboxyl group in A is activated, it will acylate with B and dipeptide AB will produce. However, the $-NH_2$ group in unreacted A could

react with the activated A to form AA (Figure 3.15). Also, there are some other derivatives in this reaction, such as peptide AAB, AABB and so on. Thus, the protection group is necessary in this reaction.

3.2.1. Butoxycarbonyl group (protection of the amino group):

t-Butoxycarbonyl (Boc) group which is introduced by Anderson and Mcgregor in 1957[95]. The group, tert-butoxycarbonyl (Boc), is cleaved with moderately strong acids. Boc group was be removed with trifuloroacetic acid, a reagent that can also play the role of solvent. The ease with which the tert-butoxycarbonyl group is removed from a protected peptide might be the reason for the increasing popularity of this method of protection. Within a short time, complete removal of the teriary butoxycarbonyl group is achieved. As no heating, usually no harm should be done to the peptide chain.

A simple approach to these N-Boc-compounds is offered by the aminolysis of suitable tert-butoxycarbonyl aryl esters. While the yields are low for the phenyl ester and the phenyl thiolester[96], satisfactory results can be obtained with the p-nitrophenyl ester[93] (Figure 3.16):

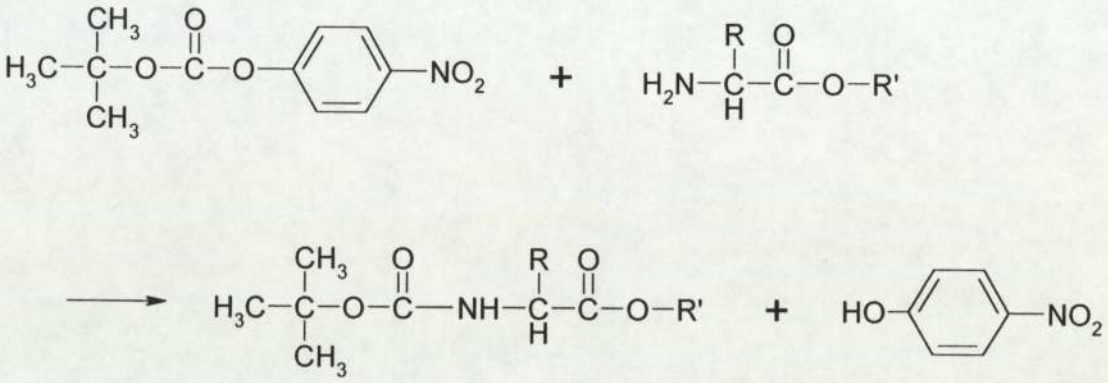


Figure 3.16. The formation of N-Boc-amino acid

Trifluoacetic acid proved to be useful for the cleavage of the tert-butoxycarbonyl group (Figure 3.17).

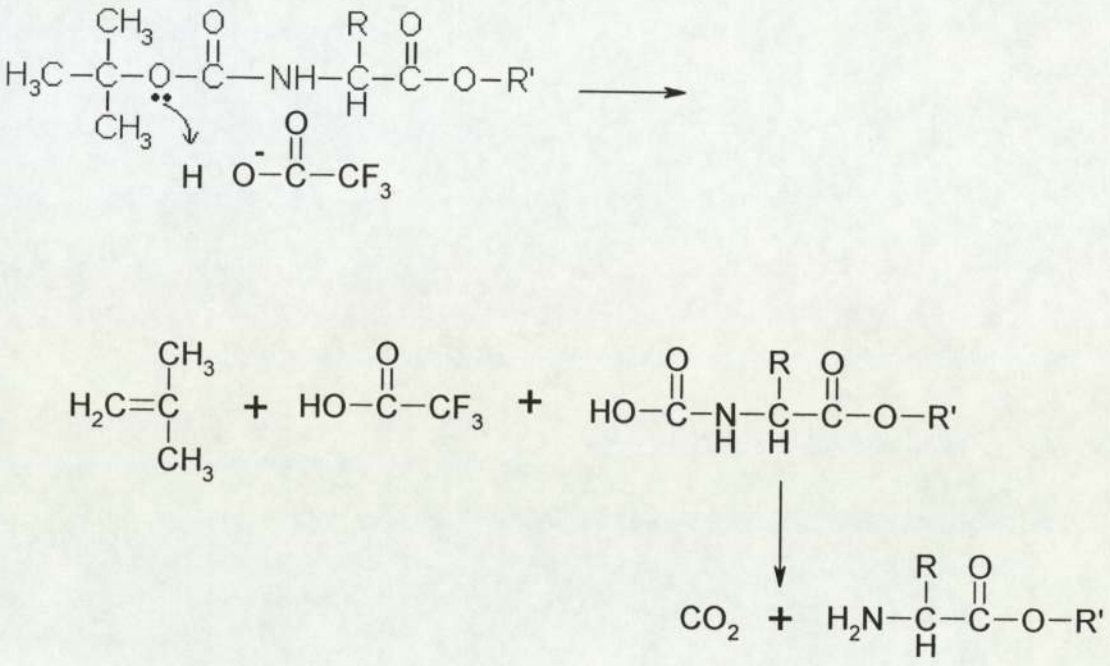


Figure 3.17. The cleavage of the tert-butyloxycarbonyl group

3.2.2. Diphenylmethyl ester (carboxyl protecting group)

In light of better inhibition of proline benzyl ester (which is more hydrophobic), this project focuses on the studies of affect of proline ester group on the hydrophobic ones. So diphenylmethyl ester is selected to synthesis to work out the best for the parent structure of the prodrugs.

The employed method is showed below.

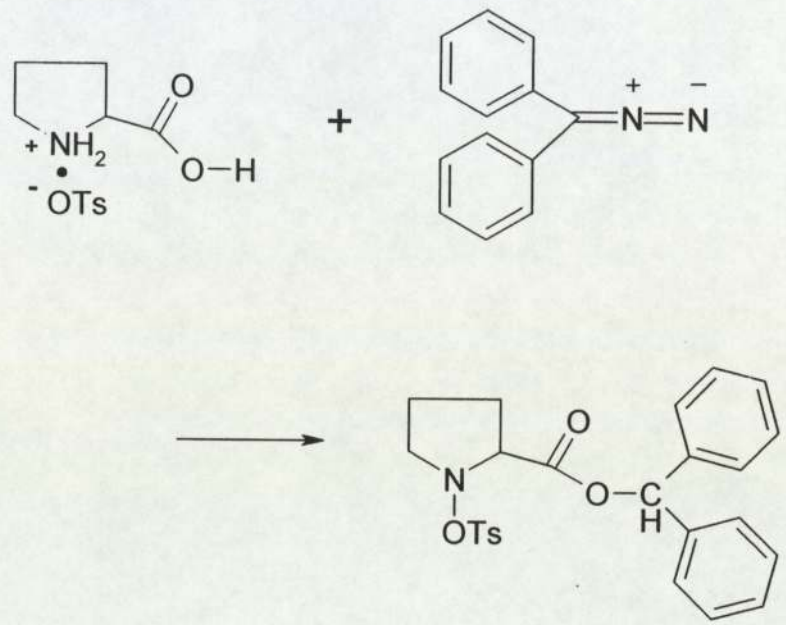


Figure 3.18. The formation of diphenylmethyl ester

Roberts, Watanabe, and McMahon[97] investigated the mechanisms of the two reactions involving benzoic acid and diazodiphenylmethane in ethanol. They concluded that the diphenylmethyl ester was formed from acid and diazodiphenylmethane in a single-step process, for which transition states may be formulated as shown below.

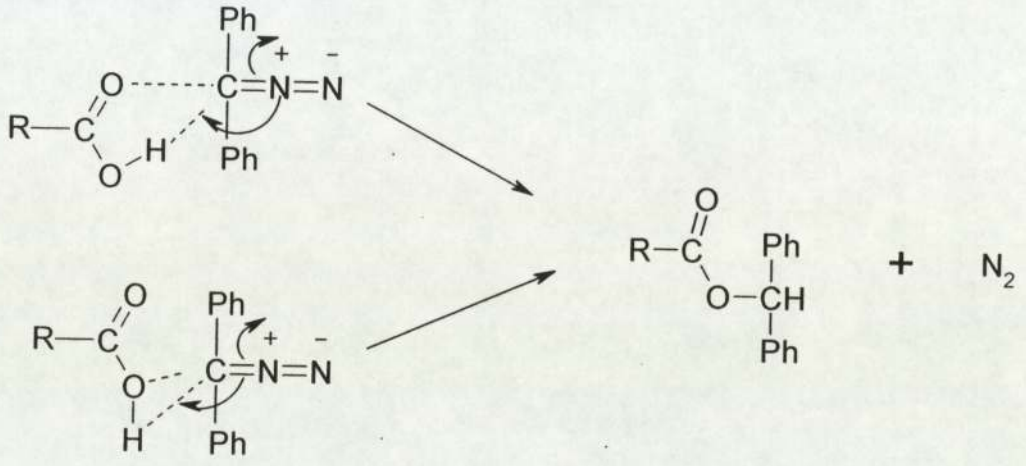


Figure 3.19. The reaction mechanism of the formation of diphenylmethyl ester

Diphenyldiazomenthane has been prepared from benzophenone hydrazone using yellow HgO[98]. (Figure. 3.20)

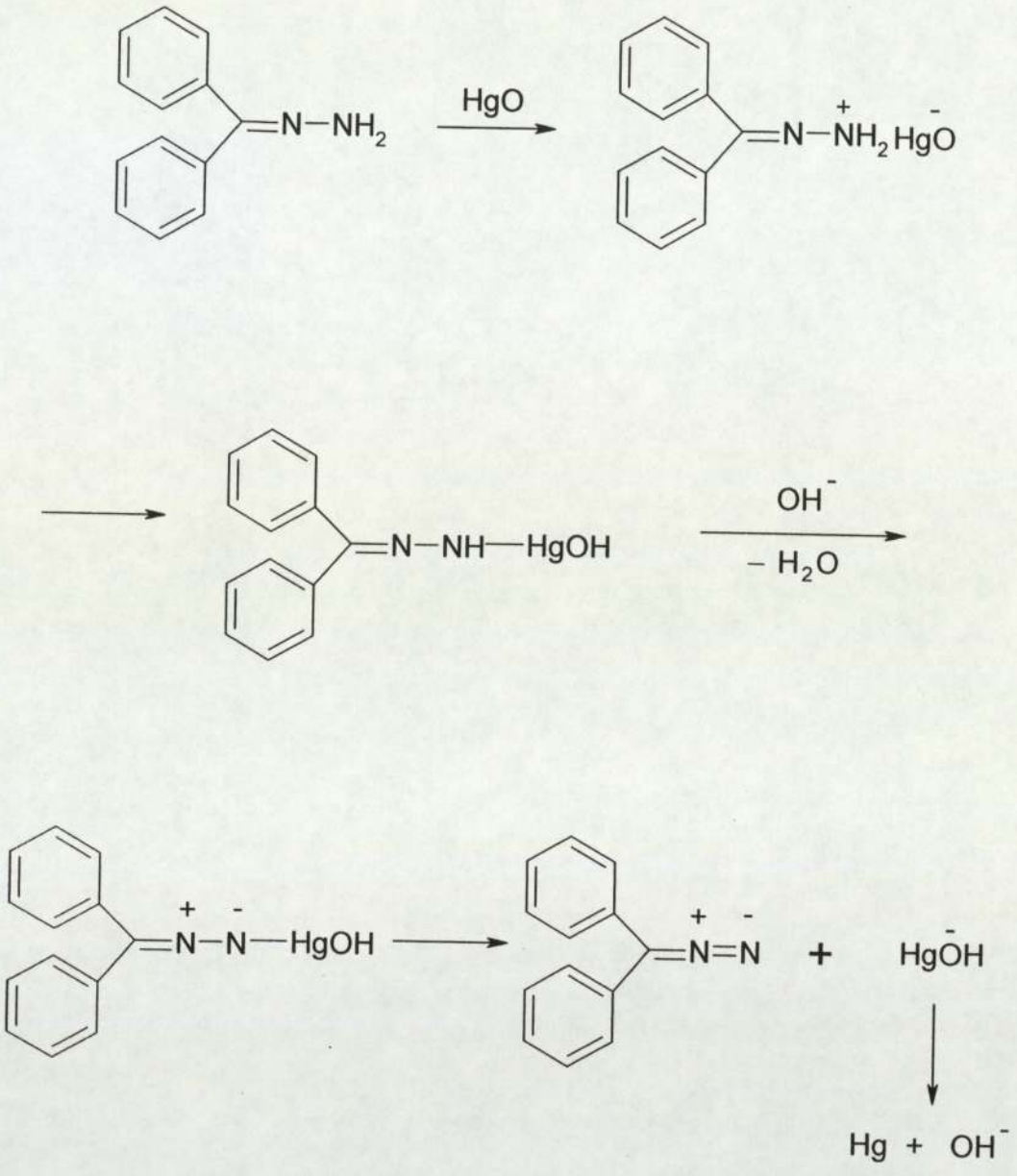


Figure 3.20. The reaction mechanism of the formation of diphenyldiazomethane[98]

CHAPTER 4 - EXPERIMENTAL

4.1. Chemistry

4.1.1. Materials:

In the dipeptide synthetic experiment, all the protected amino acids were purchased from the Novabiochem Company. Adipic acid monomethyl ester and N,N'-dicyclohexylcarbodiimide (DCC) were from Aldrich company. Ethyl hydrogen pimelate and ethyl hydrogen substrate were from Lancaster. N-hydroxybenzotriazole (HOBt) was from Sigma Company. All column chromatographic purifications were accomplished on silica gel 60 (200-400 mesh) with the appropriate solvent gradients.

4.1.2. The Formation of Dipeptide

General method:

A: The Formation of Dipeptide Bond: The cooled mixture (5℃) of an amino acid (1 mmol), the derivative of L-proline (1 mmol), N-hydroxybenzotriazole (HOBt) (1 mmol) and N,N'-dicyclohexylcarbodiimide (DCC) (1 mmol) was treated with triethylamine (TEA) (0.14ml/1mmol the derivative of L-proline hydrochloride) in dried tetrahydrofuran (THF) (2.4ml/1mmol the derivative of L-proline). The reaction was kept stirring in ice bath for three hours. Then the mixture was allowed to warm to room temperature (20℃), being kept stirring

for another one hour. After the reaction was finished, the precipated dicyclohexylurea was filtered, filtrate was diluted with ethyl acetate (EtOAc) and washed with $NaHSO_4$, saturated $NaHCO_3$ and NaCl twice respectively, the organic solution was dried over Na_2SO_4.The solvent of filtrated solution was removed to yield the product as an oil.

B: Deprotection of Boc (and tBu ester) Group: The synthesize dipeptide (with protecting group) (0.8mmol) was dissolved in 1ml of anisole, and 5ml of cool trifluoracetic acid (TFA) was then added. The mixture was stirred at room temperature for 2h, and then the TFA/anisole solution was evaporated to dryness. Solid was dissolved in 30ml of dichloromethane (CH_2Cl_2) and water (1:1), and the aqueous phase was extracted with CH_2Cl_2 (three times) to remove trace amounts of anisole and tert-butyl anisole. Lyophilization of aqueous phase to afford the N-free dipeptide.

C: Deprotection of Boc Group: A solution of protected amino acid (0.5mmol) in diethyl ether (3 ml) and ethanol (3 ml) was treated with *p*-toluenesulphonic acid (1eq). The reaction mixture was stirred at room temperature for 5mins, followed by removal of the solvent in vacuo at 40℃. The residue was repeatedly dissolved in Et_2O (3 ml) and EtOH (3 ml) and solvent evaporated in vacuo, using a warm water bath, until reaction was complete. The residue was partitioned between 0.3M HCl (20ml) and ethyl acetate five time of 15ml. The aqueous layer was freeze-dried to obtain the product.

Procedure D: benzophenone hydrazone (2 g, 10.2 mmol) was mixed with anhydrous sodium sulphate (2.5 g), 30ml ether, saturated potassium hydroxide in ethanol (0.8 ml), and yellow mercuric oxide (5.38 g, 24.8 mmol). The mixture was shaken for 75mins. Solid was filtered off and the solution was washed with water then dried over $MgSO_4$. The solvent of filtrated solution was removed to yield the diphenyl diazomethane.

L-Proline was added into tolune-4-sulphonic acid (1eq). The mixture was solubilized by methanol, and then the solvent was evaporated in vacuo. The residue was dissolved in CH_2Cl_2.

The diphenyldiazomethane was added into above L-proline toluene-4 -sulphonic solution in ice bath drop by drop, till the pink color fade. Stirring was continued at 0℃ for further 3h, basified with saturated $NaHCO_3$ to PH 8-9. The solvent was evaporated, and the residue was purified over silica column (with EtOAc: MeOH =5:1) to yield the L-proline diphenlmethyl ester.

Procedure E: Conversion of a DCHA (dicyclohexyl ammonium) salt to the free acid: Suspended 2.5mmol of Boc-Asp(OtBu)-OH DCHA in 10ml ethyl acetate in a separating funnel. Added 1.2 equivalent (3ml) of ice-cold 2M H_2SO_4 and shaked until dissolved. Removed the top (ethyl acetate) layer and set aside. Added a further 5ml of cold water to the aqueous layer and extract with twice 10ml of ethyl acetate. Combined all the ethyl acetate and washed with 10ml of water twice, dried over $MgSO_4$. Removed the ethyl acetate in a rotary evaporator at no more than 40℃. Completed the solvent removal under high vacuum in a desiccator with fresh NaOH.

Procedure F: To a solution of Hyp (10mmol) and TEA (2.10 ml, 15mmol) in water (6ml) was added dioxane (6ml) and 2-(Boc-oxyimino)-2-phenylacetonitrile (2.71g, 11mmol) at room temperature. The mixture became homogeneous within one hour and stirring was continued for two more hours. After addition of water (15ml) and EtOAc (20ml), the aqueous layer was separated, washed with EtOAc (20ml), acidified with 5% citric acid solution, and extracted with EtOAc. Dried over $MgSO_4$ and removed the ethyl acetate in a rotary evaporator at no more than 40℃. Completed the solvent removal under high vacuum in a desiccator with fresh NaOH to give N-Boc-Hyp.

Procedure G: Boc-Hyp (1.13g, 0.0049mol) in THF (50ml) was treated with NaH (50% oil, 0.50g, 0.0104mol), and the resulting mixture was stirred at room temperature for 1.5 hours. Benzyl bromide (1.70g, 0.010mol) was added, and the resulting mixture was heated under reflux for 5 hours. The reaction mixture was quenched with ice-water and extracted with hexane. The aqueous solution was acidified with $KHSO_4$ and extracted with EtOAc. The dried (MgSO4), EtOAc solution was concentrated in vacuo to give Boc-Hyp(Bzl) as a colorless oil.

Above product in MeOH (15ml) was added to MeOH (15ml) previously treated with $SOCl_2$ (1.5ml) at 0-5℃, and the resulting mixture was stirred at room temperature for 18 hours. The reaction mixture was then concentrated in vacuo, diluted with CH_2Cl_2, and extracted with 1N NaOH, then dried with $MgSO_4$. The solvent was concentrated in vacuo to give Boc-Hyp(Bzl)-OMe.

The chemistry experiment data of dipeptides and their derivatives are shown below:

NMR spectra were recorded on a Bruker AC250 Spectrometer at ^{1}H (250.1 MHz) and ^{13}C (62.9 MHz) and using tetramethylsilane as an internal standard. Mass spectroscopic analysis was carried out on a Hewlett Packard 5989B MS engine with an HP 59987A API Electrospray LC/MS interface; the LC being an HP1100 system, with autosampler. Flash column chromatography was performed using Sorbsil C60 silica gel. TLC was carried out using aluminium backed Merck Silica Gel 60 F_{254} plates and visualized under UV (254nm).

All the intermediates and final products obtained are oils, no melting point were recorded.

Because the compounds synthesized are analogues, the typical IR spectra data are:

(I) In stage one (the formation of peptide bond), the carbonyl group in peptide bond is ~ 1650cm^{-1};

(II) In stage two (deprotection of Boc group), the amine group is ~ 3400cm^{-1};

(III) In stage three (deprotection of C-terminal ester), there is a broad "V" shape peak between 2500 ~ 3000cm^{-1}.

The NMR data of dipeptides and their derivatives:

Pro(OdBzl)[99]: The title compound was prepared using the method D, yield 92.1%. m/z 281 [M^+].

^{1}H NMR (d_6-DMSO): δ 1.85-1.98 (overlapping m, 3H, Pro β -+ γ -CH_2), 2.27-2.34 (m,1H, Pro γ -CH_2), 3.15 (dd, *J*=7.6, 1.8 Hz, 2H, Pro δ -CH_2), 4.40, 4.44, 4.47 (t, *J*=8.8 Hz, 1H, Pro α -H), 6.88 (s, 1H, CH), 7.09-7.49 (overlapping m, 10H, aromatic).

^{13}C NMR (d_6-DMSO): δ 173.31 (C=O), 139.91, 139.60, 139.33, 139.20, 128.55, 128.31, 128.30, 128.14, 127.81, 127.72, 126.92, 126.83, 57.12, 52.59, 37.89, 28.45, 24.17.

Hyp(Bzl)-OMe[100]: The title compound was prepared using the method D, yield 92.1%. m/z 236 [M+H]$^+$.

^{1}H NMR (d_6-DMSO): δ 2.20-2.54 (overlapping m, 2H, Pro β -CH_2), 3.55 (overlapping m, 2H, Pro δ -CH_2), 3.83 (s, 3H, CH3), 4.32 (t, *J*=8.7 Hz, 1H, Pro α -H), 4.43 (m,1H, Pro γ -CH_2),4.54 (overlapping m, 2H, O-CH_2), 7.23-7.33 (overlapping m, 5H, aromatic).

^{13}C NMR (d_6-DMSO): δ 172.77 (C=O), 134.34, 125.81, 120.77, 116.02, 111.26, 106.28, 72.48, 60.25, 53.49, 39.91, 31.72, 22.01.

Boc-Asp(OBzl)-Pro(OBzl):The title compound was prepared using the method A, yield 92.3%. m/z 510 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H, BocCH_3), 2.03 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.20 (m, 1H, Pro γ-CH_2), 2.55-2.78 (overlapping m, 2H, CH_2), 3.48 (t, *J*=7.9 Hz, 2H, Pro-δ-CH_2), 4.53-4.68 (q, *J*=6.9 Hz, 1H, Pro α-H), 4.88 (m, 1H, CH), 5.04-5.18 (overlapping m, 4H, 2BzlCH_2), 5.41 (d, *J*=6.2 Hz, 1H, O=C-NH), 7.27-7.34 (overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.21 (C=O), 171.98 (C=O), 170.62 (C=O), 169.33 (C=O), 135.39, 135.29, 128.52, 128.46, 128.42, 128.42, 128.35, 128.31, 128.20, 128.17, 128.09, 67.52, 67.02, 59.31, 52.04, 48.28, 28.76, 28.31, 24.53, 22.30.

Asp(OBzl)-Pro(OBzl): The title compound was prepared using the method B, yield 78.1%. m/z 410 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.94-2.31 (overlapping m, 4H, Pro β-+ γ-CH_2), 2.68-2.86 (overlapping m, 2H, CH_2), 3.64 (t, *J*=8.0 Hz,2H, Pro δ-CH_2), 4.61 (m, 1H, α-H), 4.69 (m, 1H, CH), 4.95-5.14 (overlapping m, 4H, 2 BzlCH_2), 7.25-7.36 (overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 170.93 (C=O), 170.67 (C=O), 169.64 (C=O), 135.39, 135.29, 128.52, 128.46, 128.42, 128.42, 128.35, 128.31, 128.20, 128.17, 128.09, 67.52, 67.02, 59.30, 52.04, 48.29, 24.50, 22.29.

Adi(OMe)-Pro(OBzl): The title compound was prepared using the method A, yield 87.8%. m/z 347 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.97-2.30 (overlapping m, 4H, Pro β -+ γ -CH_2), 2.31 (overlapping m, 4H, 2 O=C-CH_2), 3.46 (t, *J*=8.1 Hz, 2H, Pro δ -CH_2), 3.49 (s, 3H, CH_3), 4.53 (m, 1H, Pro α -H), 5.07-5.20 (overlapping m, 4H, 2 BzlCH_2), 7.27-7.33 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.81 (C=O), 173.73 (C=O), 171.25 (C=O), 135.35, 135.20, 128.56, 128.16, 127.72, 126.86, 59.54, 58.65, 51.39, 46.84, 33.98, 33.81, 31.36, 28.86, 24.51, 22.52.

Pim(OEt)-Pro(OBzl): The title compound was prepared using the method A, yield 89.1%. m/z 375 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.22 (overlapping m, 3H, CH_3), 1.38, (overlapping m, 2H, -CH_2-), 1.55-1.72 (overlapping m, 4H, –CH_2···CH_2-),1.91-2.19 (overlapping m, 4H, Pro β -+ γ -CH_2), 2.23-2.33 (overlapping m, 4H, 2 O=C-CH_2), 3.59 (overlapping m, 2H, Pro δ -CH_2), 4.10 (q, *J*=6.8 Hz, 2H, -CH2-O), 4.53 (m,1H, Pro α -H), 5.15 (q, *J*=7.0 Hz, 2H, BzlCH_2), 7.33 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.89 (C=O), 172.85 (C=O), 171.23 (C=O), 135.37, 135.21, 128.34, 128.12, 127.35, 126.86, 60.06, 58.46, 52.03, 46.85, 34.12, 31.03, 29.05, 28.85, 24.62, 22.49, 14.11.

Sub(OEt)-Pro(OBzl): The title compound was prepared using the method A, yield 78.9%. m/z 389 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.19 (overlapping m, 3H, CH_3), 1.25, (overlapping m, 4H, -CH_2-CH_2-),1.62 (overlapping m, 4H, –CH_2···CH_2-), 1.93-2.16 (overlapping m, 4H, Pro β -+ γ -CH_2), 2.31 (overlapping m, 4H, 2 O=C-CH_2), 3.58 (overlapping m, 2H, Pro δ -CH_2), 4.10 (q, *J*=7.1 Hz, 2H, -CH_2-O), 4.53 (m, 1H, Pro α -H), 5.15 (q, *J*=7.0 Hz, 2H, BzlCH_2), 7.32 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.89 (C=O), 172.85 (C=O), 171.23 (C=O), 139.92, 139.61, 139.37, 139.21, 128.54, 128.34, 128.30, 128.12, 127.82, 127.35, 127.31, 126.86, 60.06, 58.46, 52.03, 46.85, 34.12, 31.05, 29.09, 28.85, 24.63, 22.49, 14.15.

Boc-Cys(SBzl)-Pro(OtBu): The title compound was prepared using the method A, yield 95.3%. m/z 464 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.39-1.44 (overlapping m, 18H, Boc+tbu CH_3), 1.76 (overlapping m, 3H, Pro β -+ γ -CH_2), 2.12 (m, 1H, Pro γ -CH_2), 2.75 (overlapping m, 2H, -S-CH_2), 3.49 (overlapping m, 2H, Pro δ -CH_2), 3.75 (overlapping m, 2H, ben-CH_2-S), 4.39 (m, 1H, -CH-), 4.57 (m, 1H, Pro α -H), 5.36 (d, *J*=6.9 Hz, 1H, O=C-NH), 7.07-7.29 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 170.79 (C=O), 170.61 (C=O), 169.59 (C=O), 155.26, 136.54, 134.72, 129.14, 128.89, 81.23, 79.64, 60.20, 51.61, 46.84, 36.19, 33.78, 30.81, 28.94, 28.24, 28.19, 27.84, 27.74, 25.49, 24.83,21.00.

Cys(SBzl)-Pro: The title compound was prepared using the method B, yield 95.3%. m/z 308 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.87 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.17 (m, 1H,Pro γ-CH_2), 2.80-2.99 (overlapping m, 2H, -S-CH_2), 3.55 (overlapping m, 2H, Pro δ-CH_2), 3.82 (overlapping m, 2H, ben-CH_2-S), 4.17 (m, 1H,-CH-), 4.29 (m, 1H, Pro α-H), 7.26-7.34 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.24 (C=O), 166.65 (C=O), 136.33, 128.92, 128.76, 128.70, 127.58, 127.42, 59.10, 45.39, 36.33, 35.32, 29.91, 24.22, 22.68.

Boc-Glu(OtBu)-Pro(OBzl): The title compound was prepared using the method A，yield 97.8%. m/z 490 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.38-1.43 (overlapping m, 18H, Boc+tbu CH_3), 1.94-2.22 (overlapping m, 6H, Pro β-+ γ-CH_2 + Glu –CH_2-), 2.35 (overlapping m, 2H, O=C-CH_2), 3.72 (t, *J*=8.2 Hz, 2H, Pro δ-CH_2), 4.49 (m, 1H, Glu-CH-), 4.57 (m, 1H, Pro α-H), 5.14 (dd, *J*=8.1, 2.0 Hz, 2H, BzlCH_2), 5.22 (d, 1H, O=C-NH), 7.32 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.12 (C=O), 171.52 (C=O), 170.80 (C=O), 166.99 (C=O), 154.31,135.49, 128.45, 128.17, 128.05, 80.30, 79.47, 66.73, 58.77, 50.88, 46.81, 30.66, 28.86, 28.21, 27.99, 27.70, 24.81.

Glu-Pro(OBzl): The title compound was prepared using the method B，yield 70.1%. m/z 334 [M^+].

^{1}H NMR (CDCl3): δ 1.92-2.18 (overlapping m, 6H, Pro β -+ γ-CH_2 +Glu

$-CH_2$-), 2.54 (overlapping m, 2H, O=C-CH_2), 3.46-3.63 (t, *J*=8.3 Hz, 2H, Pro δ -CH_2), 4.42 (m, 1H, Glu-CH-), 4.55 (m, 1H, Pro α-H), 5.09 (dd, *J*=7.9, 1.9 Hz, 2H, BzlCH_2), 7.28 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 171.19 (C=O), 168.67 (C=O), 167.89 (C=O), 134.18, 128.55, 128.20,128.04, 67.66, 57.78, 50.76, 48.45, 29.98, 28.77, 28.67, 28.24, 24.55.

Boc-Glu(OtBu)-Pro(OMe): The title compound was prepared using the method A，yield 96.9%. m/z 414 [M^+].

^1H NMR ($CDCl_3$): δ 1.40-1.43 (overlapping m, 18H, Boc+tbu CH_3), 2.00 (overlapping m, 6H, Pro β-+ γ-CH_2 + Glu $-CH_2$-), 2.35 (overlapping m, 2H, O=C-CH_2), 3.72 (overlapping m, 5H, Pro δ-CH_2 +CH_3), 4.49 (overlapping m, 2H, Glu-CH- +Pro α-H), 5.21(d, *J*=6.8 Hz, 1H, O=C-NH).

^{13}C NMR ($CDCl_3$): δ 172.20 (C=O), 170.54 (C=O), 168.33 (C=O), 167.24 (C=O), 81.02, 79.82, 58.67, 52.12, 51.07, 46.23, 32.01, 28.88, 28.24, 27.99, 27.67, 27.21, 24.83.

Glu-Pro(OMe): The title compound was prepared using the method A，yield 83.7%. m/z 258 [M^+].

^1H NMR ($CDCl_3$): δ 1.99-2.23 (overlapping m, 6H, Pro β-+ γ-CH_2 +Glu $-CH_2$-), 2.65 (overlapping m, 2H, O=C-CH_2), 3.56 (t, *J*=8.2 Hz, 2H, Pro δ

-CH_2), 3.69 (s, 3H, CH_3), 4.48 (overlapping m, 2H, Glu-CH- +Pro α -H), 5.21 (d, *J*=6.9 Hz, 1H, O=C-NH).

^{13}C NMR ($CDCl_3$): δ 173.55 (C=O), 171.80 (C=O), 167.18 (C=O), 59.03, 53.67, 50.21, 47.33, 40.45, 38.45, 28.55, 24.71.

Boc-Glu(OBzl)-Pro(OtBu): The title compound was prepared using the method A, yield 97.6%. m/z 490 [M^+].

^1H NMR ($CDCl_3$): δ 1.40-1.43 (overlapping m, 18H, Boc+tbu CH_3), 1.92-2.18 (overlapping m, 6H, Pro β -+ γ -CH_2 +Glu –CH_2-), 2.32 (overlapping m, 2H, O=C-CH_2), 3.45 (overlapping m, 2H, Pro δ -CH_2), 4.33 (overlapping m, 2H, Glu-CH- +Pro α -H), 5.16 (overlapping m, 2H, benzl CH_2), 5.41 (d, *J*=7.0 Hz, 1H, O=C-NH), 7.33(overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.68 (C=O), 171.58 (C=O), 170.52 (C=O), 169.73 (C=O), 135.12, 134.60, 128.52, 128.43, 128.37, 128.25, 67.75, 59.23, 52.37, 48.61, 35.07, 33.71, 28.51, 28.27, 24.51, 22.27.

Glu(OBzl)-Pro[101]: The title compound was prepared using the method B, yield 77.2%. m/z 334 [M^+].

^1H NMR ($CDCl_3$): δ 1.83-2.17 (overlapping m, 6H, Pro β -+ γ -CH_2 +Glu –CH_2-), 2.25 (overlapping m, 2H, O=C-CH_2), 3.48 (overlapping m, 2H, Pro δ -CH_2), 4.13 (overlapping m, 2H, Glu-CH-), 4.23 (m, 1H, Pro α -H), 5.20 (dd,

J=8.7, 2.3 Hz, 2H, benzl CH_2), 7.32 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 171.53 (C=O), 170.51 (C=O), 169.77 (C=O), 135.22, 134.61, 128.57, 128.43, 128.37, 128.25, 67.75, 59.23, 52.37, 48.61, 35.07, 33.71, 24.51, 22.27.

Boc-Abu-Pro(OBzl): The title compound was prepared using the method A, yield 93.5%. m/z 390 [M^+].

^1H NMR($CDCl_3$): δ 0.93 (t, *J*=6.8 Hz, 3H, CH_3), 1.41 (s, 9H, Boc -CH_3), 1.61 (q, *J*=6.1 Hz, 2H, -CH_2-), 1.94-2.03 (overlapping m, 3H, Pro β-+ γ -CH_2), 2.25 (m, 1H, Pro β -CH_2), 3.64 (overlapping m, 2H, Pro δ -CH_2), 4.38 (m, 1H, -CH-), 4.60 (m, 1H, Pro α -H), 5.15 (dd, *J*=7.8, 1.8 Hz, 2H, benzl CH_2), 5.28 (d, *J*=6.9 Hz, 1H, O=C-NH), 7.33 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 169.98 (C=O), 168.21 (C=O), 166.99 (C=O), 128.45,128.20, 128.09, 67.39, 59.66, 53.23, 47.34, 28.87, 28.25, 26.77, 24.83, 9.38.

Abu-Pro(OBzl): The title compound was prepared using the method B, yield 78.8%. m/z 290 [M^+].

^1H NMR ($CDCl_3$): δ 0.95 (t, *J*=6.9 Hz, 3H, CH_3), 1.86-1.93 (overlapping m, 5H, Abu –CH_2- +Pro β-+ γ -CH_2), 2.20 (m, 1H, Pro β -CH_2), 3.63 (overlapping m, 2H, Pro δ -CH_2), 4.16 (m, 1H, -CH-), 4.51 (m, 1H, Pro α -H), 5.12 (dd, *J*=8.8, 2.3 Hz, 2H, benzl CH_2), 5.28 (d, *J*=7.1 Hz, 1H, O=C-NH), 7.33 (overlapping m,

5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 171.17 (C=O), 168.27 (C=O), 135.19, 128.50, 128.37, 128.16, 67.00, 52,34, 46.52, 29.81, 24.97, 23.21, 8.03.

Boc-Cys-Pro(OtBu): The title compound was prepared using the method A, yield 95.4%. m/z 374 [M^+].

^1H NMR ($CDCl_3$): δ 1.39-1.41 (overlapping m, 18H, Boc +tbu CH_3), 1.99-2.13 (overlapping m, 3H, Pro β -+ γ -CH_2), 2.31(m, 1H, Pro β -CH_2), 2.93 (overlapping m, 2H, -S-CH_2-), 3.60 (overlapping m, 2H, Pro δ -CH_2), 4.51 (m, 1H, Pro α -H), 5.23 (d, *J*=6.8 Hz, 1H, O=C-NH).

^{13}C NMR ($CDCl_3$): δ172.73 (C=O), 170.34 (C=O), 165.86 (C=O), 57.91, 52.43, 39.16, 28.98, 28.81, 28.69, 24.33.

Cys-Pro: The title compound was prepared using the method B, yield 67.1%. m/z 218 [M^+].

^1H NMR (d_6-DMSO): δ 1.88-1.98 (overlapping m, 3H, Pro β -+ γ -CH_2), 2.31 (m, 1H, Pro β -CH_2), 2.89 (overlapping m, 2H, -S-CH_2-), 3.41(overlapping m, 2H, Pro δ -CH_2), 4.32 (m, 1H, Pro α -H).

^{13}C NMR (d_6-DMSO): δ 172.89 (C=O), 165.58 (C=O), 58.89, 52.46, 39.38, 28.60, 24.68.

Adi(OMe)-Pro(OdBzl): The title compound was prepared using the method A, yield 71.1%. m/z 423 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.63 (s, 4H, -CH_2-CH_2-), 1.87-1.91 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.17 (m, 1H, Pro β-CH_2), 2.29 (s, 4H, 2 O=C-CH_2), 3.49 (overlapping m, 2H, Pro δ-CH_2), 3.61 (s, 3H, CH_3), 4.60 (m, 1H, Pro α-H), 6.83 (s, 1H, CH), 7.28 (overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.81 (C=O), 173.73 (C=O), 171.25 (C=O), 139.93, 139.61, 139.35, 139.20, 128.56, 128.37, 128.33, 128.16, 127.82, 127.72, 126.92, 126.86, 59.54, 58.65, 51.39, 46.84, 33.98, 33.81, 31.36, 28.86, 24.59, 22.50.

Pim(OEt)-Pro(OdBzl): The title compound was prepared using the method A, yield 73.9%. m/z 451 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.19 (t, J=6.9 Hz, 3H, CH_3), 1.35 (overlapping m, 2H, -CH_2-), 1.59 (overlapping m, 4H, 2 –CH_2-), 1.89 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.15 (m, 1H, Pro β-CH_2), 2.27(overlapping m, 4H, 2 O=C-CH_2), 3.54 (overlapping m, 2H, Pro δ-CH_2), 4.08 (dd, J=8.6, 2.2 Hz, 2H, -CH_2-O), 4.61 (m, 1H, Pro α-H), 6.81(s,1H, CH), 7.27 (overlapping m,10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.89 (C=O), 172.85 (C=O), 171.23 (C=O), 139.92, 139.61, 139.37, 139.21, 128.54, 128.34, 128.30, 128.12, 127.82, 127.35, 127.31, 126.86, 60.06, 58.46, 52.03, 46.85, 34.12, 31.05, 29.09, 28.85, 24.63,

22.49, 14.14.

Sub(OEt)-Pro(OdBzl): The title compound was prepared using the A, yield 73.3%. m/z 456 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.21 (t, J=7.0 Hz, 3H, CH_3), 1.31 (overlapping m, 4H, -CH_2-CH_2-), 1.59 (overlapping m, 4H, 2 –CH_2-), 1.88 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.18 (m, 1H, Pro β-CH_2), 2.30 (overlapping m, 4H, 2 O=C-CH_2), 3.53 (overlapping m, 2H, Pro δ-CH_2), 4.11 (dd, J=8.1, 2.0 Hz, 2H, -CH_2-O), 4.64 (m, 1H, Pro α-H), 6.83 (m, 1H, CH), 7.25 (overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.71 (C=O), 173.83 (C=O), 171.28 (C=O), 139.95, 139.62, 139.31, 139.21, 128.55, 128.36, 128.32, 128.15, 127.82, 127.71, 127.12, 126.93, 60.07, 58.62, 46.88, 33.98, 34.32, 31.37, 28.93, 28.41, 24.71, 22.38, 14.16.

Boc-Asp(OBzl)-Pro(OtBu):The title compound was prepared using the method A, yield 90.7%. m/z 476 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.38 (s, 18H, Boc + tbu CH_3), 1.89(overlapping m, 3H, Pro β-+ γ-CH_2), 2.05 (m, 1H, Pro γ-CH_2), 2.59-2.72 (overlapping m, 2H, CH_2), 3.64 (t, J=6.9 Hz, 2H, Pro δ-CH_2), 4.58 (m, 1H, Pro α-H), 4.82 (m, 1H, CH), 5.06 (overlapping m, 2H, BzlCH_2), 5.43 (d, J=6.8 Hz, 1H, O=C-NH), 7.26 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 170.79 (C=O), 170.11 (C=O), 169.19 (C=O), 166.49

(C=O), 154.97, 135.59, 128.35, 128.28, 128.10, 127.07, 66.45, 60.10, 49.05, 46.75, 38.13, 28.10, 27.73, 25.52, 21.85, 14.01.

Asp(OBzl)-Pro[102]:The title compound was prepared using the method B, yield 78.9%. m/z 320 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.89 (overlapping m, 3H, Pro β-+γ-CH_2), 2.17 (m, 1H, Pro γ-CH_2), 3.01(overlapping m, 2H, CH_2), 3.64 (overlapping m, 2H, Pro δ-CH_2), 4.38 (m, 1H, Pro α-H), 4.68 (m, 1H, CH), 5.05 (overlapping m, 2H, BzlCH_2), 7.26 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 170.31 (C=O), 179.56 (C=O), 164.20 (C=O), 134.98, 134.34, 128.47, 128.33, 67.21, 58.97, 52.23, 45.69, 35.14, 28.17, 22.26.

Boc-Asp(OBzl)-Pro(OMe)[103]:The title compound was prepared using the method A, yield 94.4%. m/z 434 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.38 (s, 9H, Boc CH_3), 1.89 (overlapping m, 3H, Pro β-+γ-CH_2), 2.07 (m, 1H, Pro γ-CH_2), 2.55-2.70 (overlapping m, 2H, CH_2), 3.59 (s, 3H, CH_3), 3.65 (t, J=6.6 Hz, 2H, Pro δ-CH_2), 4.42 (m, 1H, Pro α-H), 4.83 (m, 1H, CH), 5.05 (overlapping m, 2H, BzlCH_2), 5.47 (d, J=7.1 Hz, 1H, O=C-NH), 7.23-7.26 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.27 (C=O), 172.00 (C=O), 170.85 (C=O), 169.48 (C=O), 135.58, 135.47, 128.37, 128.11, 128.08, 128.03, 66.49, 60.15, 52.40,

48.85, 46.79, 37.80, 30.99, 28.82, 28.13, 24.91, 21.93, 14.03.

Asp(OBzl)-Pro(OMe)[104]:The title compound was prepared using the method B, yield 65.3%. m/z 334 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.92 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.15 (m, 1H, Pro γ-CH_2), 3.01 (overlapping m, 2H, CH_2), 3.58 (s, 3H, CH_3), 3.61 (t, J=6.9 Hz, 2H, Pro δ-CH_2), 4.51 (m, 1H, Pro α-H), 4.65 (m, 1H, CH), 5.10 (overlapping m, 2H, BzlCH$_2$), 7.30 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 171.58 (C=O), 170.52 (C=O), 169.73 (C=O), 135.12, 134.60, 128.52, 128.43, 128.37, 128.25, 67.75, 59.23, 52.37, 48.61, 35.07, 33.71, 28.51, 24.54, 22.29.

Adi(OMe)-Pro(OMe): The title compound was prepared using the method A, yield 68.9%. m/z 271 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.58 (overlapping m, 4H, -CH_2-CH_2-), 1.87-1.91 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.01 (m, 1H, Pro β-CH_2), 2.24 (overlapping m, 4H, 2 O=C-CH_2), 3.39 (overlapping m, 2H, Pro δ-CH_2), 3.52 (s, 3H, CH_3), 3.62 (s, 3H, ProCH_3), 4.38 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ 173.75 (C=O), 172.72 (C=O), 171.36 (C=O), 59.19, 52.41, 46.83, 33.79, 32.44, 30.70, 29.03, 26.05, 24,79, 22.37.

Pim(OEt)-Pro(OMe): The title compound was prepared using the method A, yield 70.1%. m/z 299 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.10 (t, *J*=7.3 Hz, 3H, CH_3), 1.26 (overlapping m, 2H, -CH_2-), 1.53 (overlapping m, 4H, 2 –CH_2-), 1.87 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.02 (m, 1H, Pro β-CH_2), 2.19 (overlapping m, 4H, 2 O=C-CH_2), 3.48 (overlapping m, 2H, Pro δ-CH_2), 3.57 (s, 3H, CH_3), 3.98 (dd, *J*=8.9, 2.3 Hz, 2H, -CH_2-O), 4.33 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ 173.37 (C=O), 172.50 (C=O), 171.59 (C=O), 59.88, 58.34, 52.26, 46.75, 33.85, 31.18, 28.95, 24.79, 24.20, 22.31, 13.98.

Sub(OEt)-Pro(OMe): The title compound was prepared using the method A, yield 65.2%. m/z 313 [M^+].

^{1}H NMR($CDCl_3$): δ 1.10 (t, *J*=6.8 Hz, 3H, CH_3), 1.20 (overlapping m, 4H, -CH_2-CH_2-),1.50 (overlapping m, 4H, 2 –CH_2-), 1.86 (overlapping m, 3H, Pro β -+ γ -CH_2), 1.98 (m, 1H, Pro β -CH_2), 2.15 (overlapping m, 4H, 2 O=C-CH_2), 3.48 (overlapping m, 2H, Pro δ-CH_2), 3.57 (s, 3H, CH_3), 3.98 (dd, *J*=8.5, 2.1 Hz, 2H, -CH_2-O), 4.32 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ 173.39 (C=O), 172.66 (C=O), 171.57 (C=O), 59.82, 58.31, 51.79, 46.74, 33.98, 28.94, 24.55, 24.52, 24.11, 13.98.

Adi(OMe)-Pro(OtBu): The title compound was prepared using the method A, yield 68.9%. m/z 313 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.31 (s, 9H, tbu CH_3), 1.58 (overlapping m, 4H, -CH_2-CH_2-), 1.88-1.91 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.03 (m, 1H, Pro β-CH_2), 2.24 (overlapping m, 4H, 2 O=C-CH_2), 3.37 (overlapping m, 2H, Pro δ-CH_2),

3.58 (s, 3H, CH_3), 4.38 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ 173.45 (C=O), 173.40 (C=O), 171.56 (C=O), 59.97, 46.82, 34.02, 31.28, 29.02, 28.67, 28.35, 27.75, 25.54, 24.87, 24.06, 22.03, 19.91.

Adi(OMe)-Pro: The title compound was prepared using the method B, yield 53.2%. m/z 257 [M^+].

^1H NMR ($CDCl_3$): δ 1.66 (overlapping m, 4H, $-CH_2-CH_2-$), 1.87-1.91 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.03 (m, 1H, Pro β-CH_2), 2.32 (overlapping m, 4H, 2 O=C-CH_2), 3.48 (overlapping m, 2H, Pro δ-CH_2), 3.65 (s, 3H, CH_3), 4.53 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ173.47 (C=O), 173.43 (C=O), 171.35 (C=O), 59.97, 46.82, 34.07, 31.28, 29.02, 27.75, 25.51, 24.87, 24.07, 22.13, 18.89.

Pim(OEt)-Pro(OtBu): The title compound was prepared using the method A, yield 70.1%. m/z 341 [M^+].

^1H NMR($CDCl_3$): δ 1.08 (t, J=7.2 Hz, 3H, CH_3), 1.31 (overlapping m, 2H, $-CH_2-$), 1.33 (s, 9H, tbu CH_3), 1.54 (overlapping m, 4H, 2 $-CH_2-$), 1.82 (overlapping m, 3H, Pro β-+ γ-CH_2), 1.99 (m, 1H, Pro β-CH_2), 2.16 (overlapping m, 4H, 2 O=C-CH_2), 3.45 (overlapping m, 2H, Pro δ-CH_2), 3.96 (dd, J=8.7, 2.2 Hz, 2H, $-CH_2-O$), 4.23 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ173.45 (C=O), 172.94 (C=O), 171.39 (C=O), 59.91, 46.82, 34.02, 31.28, 29.03, 28.67, 28.33, 27.75, 25.51, 24.87, 24.51, 24.06, 22.03,

13.91.

Pim(OEt)-Pro: The title compound was prepared using the method B, yield 65.3%. m/z 285 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.21 (t, J=7.1 Hz, 3H, CH_3), 1.51 (overlapping m, 2H, -CH_2-), 1.73 (overlapping m, 4H, 2 –CH_2-), 1.82 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.16 (overlapping m, 4H, 2 O=C-CH_2), 2.32 (m, 1H, Pro β-CH_2), 3.45 (overlapping m, 2H, Pro δ-CH_2), 3.96 (dd, J=8.8, 2.5 Hz, 2H, -CH_2-O), 4.23 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ172.45 (C=O), 172.01 (C=O), 171.13 (C=O), 59.87, 46.83, 34.12, 31.23, 29.02, 27.75, 25.53, 24.87, 24.77, 24.08, 22.11, 13.87.

Sub(OEt)-Pro(OtBu): The title compound was prepared using the method A, yield 61.1%. m/z 355 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.02 (t, J=6.8 Hz, 3H, CH_3), 1.13 (overlapping m, 4H, -CH_2-CH_2-), 1.24 (s, 9H, tbu CH_3), 1.40 (overlapping m, 4H, 2 –CH_2-), 1.74 (overlapping m, 3H, Pro β-+ γ-CH_2), 1.93 (m, 1H, Pro β-CH_2), 2.05 (overlapping m, 4H, 2 O=C-CH_2), 3.39 (overlapping m, 2H, Pro δ-CH_2), 3.89 (dd, J=8.9, 2.4 Hz, 2H, -CH_2-O), 4.13 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ173.42 (C=O), 173.40 (C=O), 171.56 (C=O), 59.92, 46.82, 34.02, 31.28, 29.02, 28.67, 28.35, 28.03, 27.75, 25.54, 24.87, 24.71, 24.06, 22.03, 13.92.

Sub(OEt)-Pro: The title compound was prepared using the method B, yield 37.0%. m/z 299 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.20 (t, J=6.9 Hz, 3H, CH_3), 1.51 (overlapping m, 4H, -CH_2-CH_2-), 1.71 (overlapping m, 4H, 2 –CH_2-), 1.97 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.15 (overlapping m, 4H, 2 O=C-CH_2), 2.25 (m, 1H, Pro β-CH_2), 3.65 (overlapping m, 2H, Pro δ-CH_2), 3.89 (dd, J=9.0, 2.5 Hz, 2H, -CH_2-O), 4.21 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ173.41 (C=O), 173.20 (C=O), 171.35 (C=O), 59.97, 46.82, 34.02, 31.28, 29.02, 28.67, 28.55, 28.35, 27.75, 25.54, 24.87, 24.06, 24.01, 22.06, 13.81.

Boc-Asp(OtBu)-Pro(OBzl):The title compound was prepared using the method A, yield 98.2%. m/z 476 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.35 (s,18H, Boc+tbu CH_3), 1.90 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.03 (m, 1H, Pro γ-CH_2), 2.36-2.59 (overlapping m, 2H, CH_2), 3.67 (t, J=7.0 Hz, 2H, Pro δ-CH_2), 4.48 (m, 1H, Pro α-H), 4.79 (m, 1H, CH), 5.05 (overlapping m, 2H, BzlCH_2), 5.38 (d, J=6.8 Hz, 1H, O=C-NH), 7.24-7.26 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ171.60 (C=O), 170.88 (C=O), 169.78 (C=O), 169.34 (C=O), 135.52, 128.44, 128.18, 128.07, 127.95, 126.69, 67.10, 60.18, 59.36, 49.09, 46.43, 38.62, 31.12, 28.80, 28.16, 27.79, 24.63, 20.85, 14.05.

Pim(OEt)-Pro: The title compound was prepared using the method B, yield 65.3%. m/z 285 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.21 (t, J=7.1 Hz, 3H, CH_3), 1.51 (overlapping m, 2H, -CH_2-), 1.73 (overlapping m, 4H, 2 –CH_2-), 1.82 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.16 (overlapping m, 4H, 2 O=C-CH_2), 2.32 (m, 1H, Pro β-CH_2), 3.45 (overlapping m, 2H, Pro δ-CH_2), 3.96 (dd, J=8.8, 2.5 Hz, 2H, -CH_2-O), 4.23 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ172.45 (C=O), 172.01 (C=O), 171.13 (C=O), 59.87, 46.83, 34.12, 31.23, 29.02, 27.75, 25.53, 24.87, 24.77, 24.08, 22.11, 13.87.

Sub(OEt)-Pro(OtBu): The title compound was prepared using the method A, yield 61.1%. m/z 355 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.02 (t, J=6.8 Hz, 3H, CH_3), 1.13 (overlapping m, 4H, -CH_2-CH_2-), 1.24 (s, 9H, tbu CH_3), 1.40 (overlapping m, 4H, 2 –CH_2-), 1.74 (overlapping m, 3H, Pro β-+ γ-CH_2), 1.93 (m, 1H, Pro β-CH_2), 2.05 (overlapping m, 4H, 2 O=C-CH_2), 3.39 (overlapping m, 2H, Pro δ-CH_2), 3.89 (dd, J=8.9, 2.4 Hz, 2H, -CH_2-O), 4.13 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ173.42 (C=O), 173.40 (C=O), 171.56 (C=O), 59.92, 46.82, 34.02, 31.28, 29.02, 28.67, 28.35, 28.03, 27.75, 25.54, 24.87, 24.71, 24.06, 22.03, 13.92.

Sub(OEt)-Pro: The title compound was prepared using the method B, yield 37.0%. m/z 299 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.20 (t, J=6.9 Hz, 3H, CH_3), 1.51 (overlapping m, 4H, -CH_2-CH_2-), 1.71 (overlapping m, 4H, 2 –CH_2-), 1.97 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.15 (overlapping m, 4H, 2 O=C-CH_2), 2.25 (m, 1H, Pro β-CH_2), 3.65 (overlapping m, 2H, Pro δ-CH_2), 3.89 (dd, J=9.0, 2.5 Hz, 2H, -CH_2-O), 4.21 (m, 1H, Pro α-H).

^{13}C NMR ($CDCl_3$): δ173.41 (C=O), 173.20 (C=O), 171.35 (C=O), 59.97, 46.82, 34.02, 31.28, 29.02, 28.67, 28.55, 28.35, 27.75, 25.54, 24.87, 24.06, 24.01, 22.06, 13.81.

Boc-Asp(OtBu)-Pro(OBzl):The title compound was prepared using the method A, yield 98.2%. m/z 476 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.35 (s,18H, Boc+tbu CH_3), 1.90 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.03 (m, 1H, Pro γ-CH_2), 2.36-2.59 (overlapping m, 2H, CH_2), 3.67 (t, J=7.0 Hz, 2H, Pro δ-CH_2), 4.48 (m, 1H, Pro α-H), 4.79 (m, 1H, CH), 5.05 (overlapping m, 2H, BzlCH_2), 5.38 (d, J=6.8 Hz, 1H, O=C-NH), 7.24-7.26 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ171.60 (C=O), 170.88 (C=O), 169.78 (C=O), 169.34 (C=O), 135.52, 128.44, 128.18, 128.07, 127.95, 126.69, 67.10, 60.18, 59.36, 49.09, 46.43, 38.62, 31.12, 28.80, 28.16, 27.79, 24.63, 20.85, 14.05.

Asp-Pro(OBzl):The title compound was prepared using the method B, yield 46.3%. m/z 320 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.90 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.03 (m, 1H, Pro γ-CH_2), 2.36-2.59 (overlapping m, 2 H, CH_2), 3.67 (t, *J*=7.0 Hz, 2H, Pro δ-CH_2), 4.48 (m, 1H, Pro α-H), 4.79 (m,1H, CH), 5.05 (overlapping m, 2H, BzlCH_2), 5.38 (d, *J*=6.8 Hz, 1H, O=C-NH), 7.24-7.26 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ171.55 (C=O), 170.81 (C=O), 169.70 (C=O), 135.51, 128.44, 128.18, 128.01, 127.92, 126.69, 67.13, 60.11, 59.36, 49.09, 46.43, 38.62, 31.13, 28.81, 24.63, 20.83, 14.11.

Boc-Asp(OtBu)-Pro(OtBu):The title compound was prepared using the method A, yield 95.2%. m/z 442 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.42 (s, 27H, Boc+tbu CH_3), 1.96 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.00 (m, 1H, Pro γ-CH_2), 2.41-2.62 (overlapping m, 2H, CH_2), 3.72 (t, *J*=6.9 Hz, 2H, Pro δ-CH_2), 4.37 (m, 1H, Pro α-H), 4.82 (m, 1H, CH), 5.34 (d, *J*=7.1 Hz, 1H, O=C-NH).

^{13}C NMR ($CDCl_3$): δ171.76 (C=O), 170.38 (C=O), 169.98 (C=O), 169.63 (C=O), 67.10, 60.18, 59.31, 49.09, 46.43, 38.63, 31.12, 28.80, 28.16, 28.01, 27.79, 27.67, 24.63, 20.85, 14.05.

Asp-Pro[105]: The title compound was prepared using the method B, yield 51.7%. m/z 230 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.98 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.07 (m, 1H, Pro γ-CH_2), 2.47-2.71 (m, 2H, CH_2), 3.68 (t, *J*=6.8 Hz, 2H, Pro δ-CH_2), 4.51 (m, 1H, Pro α-H), 4.93 (m, 1H, CH).

^{13}C NMR ($CDCl_3$): δ171.72 (C=O), 169.98 (C=O), 169.43 (C=O), 67.11, 60.13, 59.32, 49.11, 46.52, 38.63, 31.12, 28.80, 28.03, 27.71, 24.63, 20.81, 13.97.

Boc-Asp(OtBu)-Pro(OMe): The title compound was prepared using the method A, yield 89.7%. m/z 400 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.42 (s, 18H, Boc+tbu CH_3), 1.77 (overlapping m, 3H, Pro β-+ γ-CH_2), 1.99 (m, 1H, Pro γ-CH_2), 2.39-2.57 (overlapping m, 2H, CH_2), 3.48 (overlapping m, 3H, CH_3), 3.79 (t, *J*=6.5 Hz, 2H, Pro δ-CH_2), 4.23 (m, 1H, Pro α-H), 4.61 (m, 1H, CH), 5.49 (d, *J*=6.9 Hz, 1H, O=C-NH).

^{13}C NMR ($CDCl_3$): δ172.26 (C=O), 170.78 (C=O), 170.25 (C=O), 169.93 (C=O), 59.31, 49.09, 46.41, 38.63, 31.11, 28.84, 28.16, 28.01, 27.79, 27.67, 24.63, 23.87, 20.85, 14.58.

Asp-Pro(OMe): The title compound was prepared using the method B, yield 65.4%. m/z 244 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.84 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.03 (m,1H, Pro γ-CH_2), 2.41-2.58 (overlapping m, 2H, CH_2), 3.51 (m, 3H, CH_3), 3.78 (t, *J*=6.6 Hz, 2H, Pro δ-CH_2), 4.33 (m, 1H, Pro α-H), 4.67 (m, 1H, CH).

^{13}C NMR ($CDCl_3$): δ 172.19 (C=O), 170.69 (C=O), 169.81 (C=O), 59.95, 52.24, 48.70, 46.27, 38.93, 33.59, 29.32, 24.63, 21.85, 13.89.

Boc-Glu(OBzl)-Pro(OdBzl): The title compound was prepared using the method A，yield 81.3%. m/z 600 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.39 (s, 9H, Boc CH_3), 1.59 (overlapping m, 2H, -CH_2-), 1.90 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.07-2.28 (m, 1H, Pro γ-CH_2), 2.41 (overlapping m, 2H, O=C-CH_2), 3.66 (t, *J*=6.7 Hz, 2H, Pro δ-CH_2), 4.58 (m, 1H, -CH-NH), 4.64 (m, 1H, Pro α-H), 5.10 (s, 2H, -CH_2-ph), 6.85 (s, 1H, CH), 7.24-7.32 (overlapping m, 15H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.19 (C=O), 170.81 (C=O), 170.37 (C=O), 169.61 (C=O), 139.67, 135.51, 128.50, 128.33, 128.26, 128.21, 128.11, 127.96, 127.83, 127.07, 126.66, 126.32, 66.34, 58.23, 48.84, 46.33, 37.16, 30.45, 28.73, 28.50, 28.19, 24.61, 24.43, 14.12.

Glu(OBzl)-Pro(OdBzl): The title compound was prepared using the method B，yield 80.6%. m/z 500 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.60 (overlapping m, 2H, -CH_2-), 1.93 (overlapping m, 3H,

Pro β-+ γ-CH_2), 2.09-2.33 (m,1H, Pro γ-CH_2), 2.57 (overlapping m, 2H, O=C-CH_2), 3.52 (t, *J*=7.0 Hz, 2H, Pro δ-CH_2), 4.14 (m, 1H, -CH-NH), 4.65 (m, 1H, Pro α-H), 5.07 (s, 2H, -CH_2-ph), 6.86 (s, 1H, CH), 7.24-7.38 (overlapping m, 15H, aromatic).

^{13}C NMR ($CDCl_3$): δ 173.54 (C=O), 170.14 (C=O), 165.10 (C=O), 143.81, 135.47, 128.51, 128.43, 128.37, 128.28, 128.23, 128.19, 127.42, 127.11, 126.92, 126.89, 126.45, 66.68, 58.99, 54.81, 45.30, 30.04, 28.05, 24.46, 24.43, 22.57.

Boc-Asp(OBzl)-Pro(OdBzl): The title compound was prepared using the method A, yield 70.1%. m/z 586 [M^+].

^1H NMR ($CDCl_3$): δ 1.40 (s, 9H, Boc CH_3), 1.91 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.06-2.26 (m,1H, Pro γ-CH_2), 2.67 (overlapping m, 2H, O=C-CH_2), 3.71 (t, *J*=6.8 Hz, 2H, Pro δ-CH_2), 4.63 (m, 1H, -CH-NH), 4.84 (m, 1H, Pro α-H), 5.10 (s, 2H, -CH_2-ph), 6.86 (s, 1H, CH), 7.24-7.31 (overlapping m, 15H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.30 (C=O), 170.57 (C=O), 170.25 (C=O), 169.60 (C=O), 139.78, 135.58, 128.59, 128.47, 128.37, 128.21, 128.11, 127.96, 127.81, 127.15, 126.91, 126.67, 66.65, 58.04, 48.93, 46.89, 37.44, 30.23, 28.73, 28.20, 28.17, 24.63, 24.43, 22.78.

Asp(OBzl)-Pro(OdBzl): The title compound was prepared using the method

B，yield 89.3%. m/z 486 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.98 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.05-2.31 (m,1H, Pro γ-CH_2), 2.74 (overlapping m, 2H, O=C-CH_2), 3.46 (t, *J*=6.9 Hz, 2H, Pro δ-CH_2), 4.19 (m, 1H, -CH-NH), 4.48 (m, 1H, Pro α-H), 5.15 (s, 2H, -CH_2-ph), 6.90 (s, 1H, CH), 7.24-7.33 (overlapping m, 15H, aromatic).

^{13}C NMR ($CDCl_3$): δ 170.71 (C=O), 169.62 (C=O), 164.14 (C=O), 135.18, 128.53, 128.47, 128.37, 128.23, 128.11, 127.96, 127.81, 127.15, 126.91, 126.67, 66.93, 58.99, 52.07, 45.89, 35.27, 28.30, 22.39.

Boc-Met-Pro(OdBzl): The title compound was prepared using the method A，yield 77.4%. m/z 330 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.32 (s, 9H, Boc CH_3), 1.91 (overlapping m, 3H, Pro β-+ γ-CH_2), 2.09 (s, 3H, S-CH_3), 2.10-2.26 (m, 1H, Pro γ-CH_2), 2.57 (overlapping m, 4H, -CH_2-CH_2-), 3.71 (t, *J*=7.1 Hz, 2H, Pro δ-CH_2), 4.41 (m, 1H, -CH-NH), 4.68 (m, 1H, Pro α-H), 6.84 (s, 1H, CH), 7.24-7.33 (overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 171.53 (C=O), 170.25 (C=O), 169.61 (C=O), 135.78, 128.53, 128.33, 128.21, 128.16, 127.60, 127.82, 127.16, 126.77, 126.01, 59.61, 54.79, 44.88, 38.42, 31.21, 28.73, 28.21, 28.18, 24.63, 24.51, 22.74, 15.92.

Met-Pro(OdBzl): The title compound was prepared using the method B, yield 71.1%. m/z 230 [M^+].

^{1}H NMR ($CDCl_3$): 1.88 (overlapping m, 3H, Pro β-+γ-CH_2), 2.03 (s, 3H, S-CH_3), 2.10-2.36 (m,1H, Pro γ-CH_2), 2.66 (overlapping m, 4H, -CH_2-CH_2-), 3.56 (t, J=6.9 Hz, 2H, Pro δ-CH_2), 4.06 (m, 1H, -CH-NH), 4.65 (m, 1H, Pro α-H), 6.83 (s, 1H, CH), 7.25-7.35 (overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 170.72 (C=O), 170.33 (C=O), 169.67, 139.77, 135.51, 128.59, 128.47, 128.37, 128.21, 128.19, 127.96, 127.88, 127.15, 126.93, 126.67, 59.65, 54.73, 44.98, 38.44, 31.23, 28.81, 23.41, 15.77.

Boc-Asp(OBzl)-Hyp(Bzl)-OMe: The title compound was prepared using the method A, yield 61.8%. m/z 540 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.35 (s, 9H, BocCH_3), 1.92-2.41 (overlapping m, 2H, Pro β-CH_2), 2.63-2.82 (overlapping m, 2H, Asp –CH_2-), 3.58 (s, 3H, CH_3), 3.55 (overlapping m, 2H, Pro δ-CH_2), 4.11 (t, J=6.3 Hz, 1H, Pro α-H), 4.43 (overlapping m, 2H, O-CH_2), 4.82 (m, 1H, Pro γ-CH_2), 5.07 (overlapping m, 2H, Asp –CH_2-ph), 7.25 (overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.26 (C=O), 170.83 (C=O), 170.08 (C=O), 169.37 (C=O), 137.60, 135.69, 128.40, 128.37, 128.31, 128.21, 128.08, 127.67,

127.53, 127.49, 71.20, 66.47, 60.17, 57.89, 52.60, 52.03, 51.55, 48.67, 37.25, 34.90, 28.18, 28.13, 28.11, 20.85, 14.09.

Asp(OBzl)-Hyp(Bzl)-OMe: The title compound was prepared using the method B, yield 83.7%. m/z 440 [M^+].

^{1}H NMR (d_6-DMSO): δ 1.98-2.40 (overlapping m, 2H, Pro β-CH_2), 2.66-2.81 (overlapping m, 2H, Asp –CH_2-), 3.49 (s, 3H, CH_3), 3.76 (overlapping m, 2H, Pro δ-CH_2), 3.98 (t, J=6.2 Hz, 1H, Pro α-H), 4.27 (overlapping m, 2H, O-CH_2), 4.75 (m,1H, Pro γ-CH_2), 5.02 (overlapping m, 2H, Asp –CH_2-ph), 7.28 (overlapping m, 10H, aromatic).

^{13}C NMR (d_6-DMSO): δ 176.56 (C=O), 175.53 (C=O), 173.99 (C=O), 126.24, 125.71, 120.73, 116.09, 111.46, 106.82, 71.43, 71.25, 69.39, 68.62, 66.97, 64.56, 60.11, 53.89, 52.68, 46.32.

Boc-Glu(OBzl)-Hyp(Bzl)-OMe: The title compound was prepared using the method A, yield 77.2%. m/z 554 [M^+].

^{1}H NMR ($CDCl_3$): δ 1.39 (s, 9H, Boc CH_3), 1.75 (overlapping m, 2H, -CH_2-), 1.89 (overlapping m, 2H, Pro β-CH_2), 2.40 (overlapping m, 2H, O=C-CH_2), 3.57(s, 3H, CH_3), 4.00 (t, J=6.8 Hz, 2H, Pro δ-CH_2), 4.11 (m, 1H, Pro α-H), 4.36 (m, 1H, Pro γ-CH_2), 4.50 (m, 1H, -CH-NH), 4.58 (s, 2H, -O-CH_2-ph), 5.04 (s, 2H, Glu –CH_2-ph), 7.23(overlapping m, 10H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.60 (C=O), 171.96 (C=O), 170.72 (C=O), 169.29 (C=O), 137.58, 135.93, 128.42, 128.37, 128.01, 127.67, 127.51, 70.99, 66.05, 60.11, 57.72, 52.02, 50.71, 34.74, 29.18, 28.15, 27.59, 20.79, 14.07.

Glu(OBzl)-Hyp(Bzl)-OMe: The title compound was prepared using the method B, yield 83.6%. m/z 454 [M^+].

^1H NMR (d_6-DMSO): δ 1.72 (overlapping m, 2H, $-CH_2-$), 1.81 (overlapping m, 2H, Pro β-CH_2), 2.44 (overlapping m, 2H, O=C-CH_2), 3.63(s, 3H, CH_3), 4.08 (t, J=7.1 Hz, 2H, Pro δ-CH_2), 4.29 (m, 1H, Pro α-H), 4.36 (m,1H, Pro γ-CH_2), 4.53 (m, 1H, -CH-NH), 4.59 (s, 2H, -O-CH_2-ph), 5.12 (s, 2H, Glu –CH_2-ph), 7.24 (overlapping m, 10H, aromatic).

^{13}C NMR (d_6-DMSO): δ 172.66 (C=O), 171.96 (C=O), 170.73 (C=O), 137.54, 135.92, 128.44, 128.40, 128.05, 127.66, 127.53, 70.92, 66.12, 60.15, 57.76, 52.09, 50.74, 34.71, 29.12, 28.16, 20.78, 14.13.

Boc-Glu(OtBu)-Hyp(Bzl)-OMe: The title compound was prepared using the method A, yield 69.2%. m/z 520 [M^+].

^1H NMR (d_6-DMSO): δ 1.29 (overlapping m, 18H, Boc CH_3), 1.59 (overlapping m, 2H, $-CH_2-$), 1.89 (overlapping m, 2H, Pro β-CH_2), 2.20 (overlapping m, 2H, O=C-CH_2), 3.54(s, 3H, CH_3), 3.88 (overlapping m, 2H, Pro δ-CH_2), 4.09 (m, 1H, Pro α-H), 4.34 (m, 1H, -CH-NH), 4.36 (m,1H, Pro γ-CH_2), 4.58 (s, 2H, -O-CH_2-ph), 7.15 (overlapping m, 5H, aromatic).

^{13}C NMR (d_6-DMSO): δ 172.01 (C=O), 171.94 (C=O), 170.78 (C=O), 169.77 (C=O), 137.49, 128.23, 128.05, 127.56, 127.43, 127.37, 70.98, 60.02, 57.64, 51.92, 50.73, 34.68, 30.35, 28.09, 27.88, 27.79, 20.72, 13.98.

Glu-Hyp(Bzl)-OMe: The title compound was prepared using the method A, yield 73.7%. m/z 364 [M^+].

^1H NMR (D_2O): δ 1.64 (overlapping m, 2H, -CH_2-), 1.99 (overlapping m, 2H, Pro β-CH_2), 2.31 (overlapping m, 2H, O=C-CH_2), 3.46 (s, 3H, CH_3), 3.66 (overlapping m, 2H, Pro δ-CH_2), 3.86 (m, 1H, Pro α-H), 4.00 (m, 1H, -CH-NH), 4.27 (m,1H, Pro γ-CH_2), 4.82 (s, 2H, -O-CH_2-ph), 7.14 (overlapping m, 5H, aromatic).

^{13}C NMR (D_2O): δ 173.44 (C=O), 171.58 (C=O), 170.16 (C=O), 134.48, 125.84, 120.73, 116.09, 111.44, 106.80, 74.24, 71.17, 69,13, 66.33, 60.05, 55.65, 52.56, 51.30, 40.83, 31.05, 26.65, 22.07.

Boc-Met-Hyp(Bzl)-OMe: The title compound was prepared using the method A, yield 69.2%. m/z 466 [M^+].

^1H NMR ($CDCl_3$): δ 1.33 (m, 9H, Boc CH_3), 1.79 (overlapping m, 2H, Pro β -CH_2), 2.09 (s, 3H, S-CH_3), 2.44 (overlapping m, 4H, -CH_2-CH_2-), 3.57 (overlapping m, 3H, CH_3), 3.95 (overlapping m, 2H, Pro δ-CH_2), 4.12 (m, 1H, Pro α-H), 4.37 (m, 1H, -CH-NH), 4.40 (m,1H, Pro γ-CH_2), 4.50 (s, 2H, -O-CH_2-ph), 7.18 (overlapping m, 5H, aromatic).

^{13}C NMR ($CDCl_3$): δ 172.08 (C=O), 171.91 (C=O), 170.73 (C=O), 137.37, 128.27, 127.64, 127.46, 127.43, 70.95, 60.11, 57.63, 51.92, 50.57, 34.80, 30.02, 29.42, 28.09, 27.88, 20.79, 14.98.

Met-Hyp(Bzl)-OMe: The title compound was prepared using the method A, yield 69.2%. m/z 366 [M^+].

^1H NMR (D_2O): δ 1.69 (overlapping m, 2H, Pro β-CH_2), 2.13 (s, 3H, S-CH_3), 2.71 (overlapping m, 4H, -CH_2-CH_2-), 3.53 (overlapping m, 3H, CH_3), 3.87 (overlapping m, 2H, Pro δ-CH_2), 4.09 (m, 1H, Pro α-H), 4.34 (m, 1H, -CH-NH), 4.41 (m,1H, Pro γ-CH_2), 4.53 (s, 2H, -O-CH_2-ph), 7.24 (overlapping m, 5H, aromatic).

^{13}C NMR (D_2O): δ 177.26 (C=O), 175.59 (C=O), 126.20, 125.82, 120.76, 116.12, 111.48, 106.84, 71.75, 70.65, 69.52, 64.60, 60.11, 53.90, 52.68, 46.32, 41.83, 27.08, 21.70.

4.2. Caco-2 Cell Uptake Experiments

4.2.1. Solutions

The Caco-2 cell uptake experiments required four solutions which play important role in the uptake experiment. They are Transport media, Gly-Pro solution, Stop solution and Detergent solution.

Transport media

The ingredients of transport media is shown as following:

4.88g Hank's Balance Salt Solution (HBSS)

2.44g 2-[N-morpholino]ethanesulphonic acid (MES) buffer

58g Proline

500ml Double distilled water (ddH2O)

The pH of the solution was adjusted to 6.0 by the addition of 2mM NaOH. For the uptake study at different pHs, the solution was adjusted to pH5.0, 5.5, 6.0, 6.5. A new solution containing 2.98g EPES buffer instead of the MES buffer was made and then adjusted to pH7.0 and 7.5.The solution was stored at 4℃.

Caco-2 cells produce prolidase, an enzyme that cleaves Gly-Pro. The presence of this enzyme could mean that [^{3}H] proline is entering the cells not [^{3}H] Gly-Pro. Excess proline (10mM) is added to saturate the amino acid transporter and stop [^{3}H] proline entering the cell. Therefore the entire radioactivity in the cells should be in Gly-Pro.

Gly-Pro solution

Gly-Pro solution is the most important solution. It contains the radiolabel as well as any compounds being tested. It is transport solution with a few additions. The amount of labelled Gly-Pro need to be added to 4ml transport solution was calculated as below.

Activity of Gly-Pro=48.5Ci/mmol, 1mCi/ml

Want 4ml of a 50nM Gly-Pro solution=0.2nmol

48.5 μ Ci=1nmol

9.7 μ Ci=0.2nmol

1μCi=1μl

9.7μCi=9.7μl

Therefore, 9.7μl Gly-Pro was added to 4ml transport solution.

1% DMSO was added to help them dissolve in the test experiment. All compounds had a final concentration of 1mM.

Stop solution

Stop solution contains 0.25 sodium azide dissoved in 500ml PBS. This 0.05% w/v solution is an inhibitor of uptake and so stops any further uptake after the experiment has finished. The solution was stored at 4℃.

Detergent solution

The detergent solution contains 5ml of Triton-X dissolved in 500ml d_2H2O. The 1% v/v solution is used to dissolve the cell membranes. The solution was stored at room temperature.

4.2.2. Protocol

The transport and Gly-Pro solutions were warmed to 37℃, while the stop solution was stored on ice. The culture media from each well was aspirated and replaced by 0.5ml transport media. After 5 minutes, the transport media was aspirated and replaced by 0.5ml Gly-Pro solution. The radioactive solution was left on the cells for 3 minutes before being collected into a scintillation vial (labelled D). The cells were then washed twice with ice-cold stop solution (0.5ml/well) for five minutes. The washings were collected in two vials (labelled W1 and W2). Detergent solution was then added to each well (1ml/well) and the plate was left in an incubator overnight. The resulting solublised cell solution was collected into a vial (labelled C). Each vial had 5ml scintillation

fluid added to it and then had its radioactivity counted. Each well had four vials associated with it (D, W1, W2 & C).

4.2.3. Calculation

4.2.3.1. Radioactivity in cells

Each experiment was repeated four times, so a mean and standard deviation result can be calculated.

The radioactivity in each vial was used to calculate the concentration of [^{3}H] Gly-Pro in each well. Then the mean concentrations could be used to calculate control percentage and IC_{50} values. The example calculation is shown below (Table 4.1).

Vial	**Well 1**	**Well 2**
D (dpm)	1548976.0	1535718.0
W1 (dpm)	62927.7	56335.3
W2 (dpm)	4019.2	4012.7
C (dpm)	17669.6	15171.4
Total (dpm)	1633592.0	1611237.0
Corrected C (dpm)	17669.6	15381.9

Table 4.1. An Example Calculation

Because the C value is larger than the W2 value, it can be concluded that the radioactivity in vial C came from inside the cells and was not just left on the surface of the cells. As the total amount of radioactivity each well receives is different, the radioactivity in the cells for each well is corrected to the value. The corrected value is calculated from the following equation.

$$\text{Corrected well 2C} = \left(\frac{\text{Total Well 1}}{\text{Total Well 2}} \right) \times (\text{Well 2C})$$

4.2.3.2. Amount of Gly-Pro in cells

When the radioactivity in the cells in each well is calculated, the amounts need to be used in the following equation and calculate the amount of Gly-Pro.

$1\text{dpm} = 4.545 \times 10^{-13}\text{Ci}$

$48.5\text{Ci} = 1 \times 10^{-3}\text{mol}$

$4.545 \times 10^{-13}\text{Ci} = 9.371 \times 10^{-13}\text{mol}$

Therefore, $1\text{dpm} = 9.371 \times 10^{-6}\text{pmol}$

4.2.3.3.The Calculation of percentage of control, inhibition and IC_{50}

$$\text{Control \%} = \frac{\text{(well pmol)}}{\text{(mean control pmol)}} \times 100\%$$

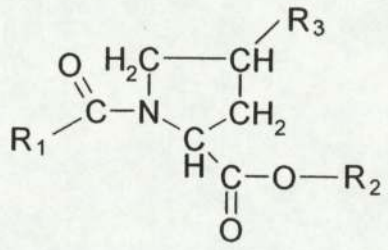
$$\text{Inhibition\%} = 100\% - \text{Control \%}$$

$$IC_{50} = \frac{\text{(inhibitor concentration mM)}}{\left[\frac{\text{(mean control pmol)}}{\text{(well pmol)}}\right] - 1}$$

CHAPTER 5 - RESULTS AND DISCUSSION

5.1. Result of Gly-Pro uptake

The test experiments were performed to calculate the IC_{50} and percentage of control for dipeptide derivatives. The results are shown in the Tables and Figures below.



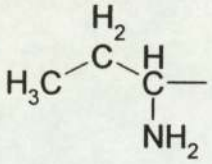
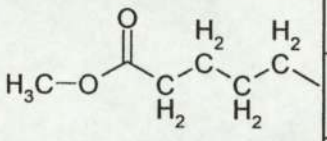
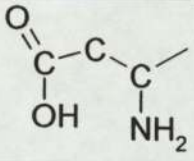
	R_1	R_2	R_3	% Control	SD	P**
Abu-Pro		H	H	78.9	5.0	<0.0001
Abu-Pro (OBzl)		Bzl	H	30.0	6.8	**
Adi(OMe)-Pro		H	H	84.8	10.2	
Adi(OMe)-Pro (OBzl)		Bzl	H	68.6	7.9	0.0008
Adi(OMe)-Pro (OdBzl)		dBzl	H	81.1	4.1	**
Adi(OMe)-Pro (OMe)		Me	H	96.8	2.9	
Asp-Hyp(Bzl)-OMe		Me	OBzl	104.9	3.6	

Table 5.1. Dipeptide Derivative Uptake Results (%Control) as at 16/10/00

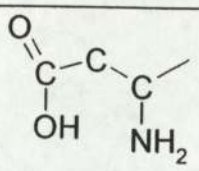
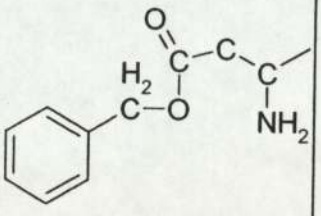
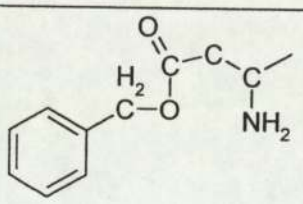
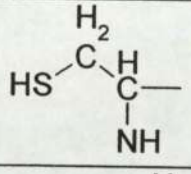
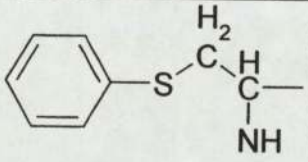
Asp-Pro		H	H	51.6	1.6	
Asp-Pro (OBzl)		Bzl	H	44.0	11.2	0.0782
Asp-Pro (OMe)		Me	H	56.1	1.7	ns
Asp(OBzl)-Hyp(Bzl)-OMe		Me	OBzl	46.2	6.3	
Asp(OBzl)-Pro		H	H	78.8	5.0	
Asp(OBzl)-Pro(Obzl)		Bzl	H	49.6	1.9	<0.001
Asp(OBzl)-Pro (OdBzl)		dBzl	H	78.3	3.3	**
Asp(OBzl)-Pro(OMe)		Me	H	42.5	4.6	
Cys-Pro		H	H	78.0	2.8	
Cys-(SBzl)-Pro		H	H	39.4	3.8	

Table 5.1. Dipeptide Derivative Results as at 16/10/00 (continued)

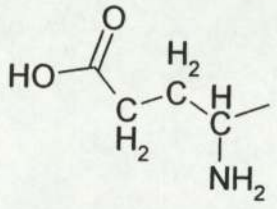
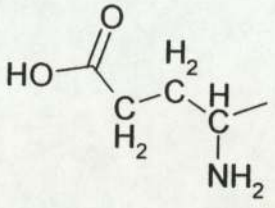
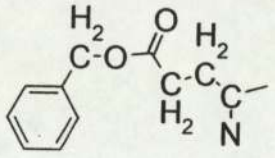
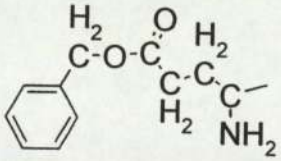
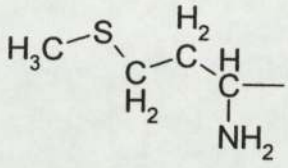
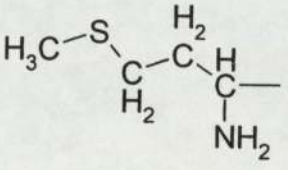
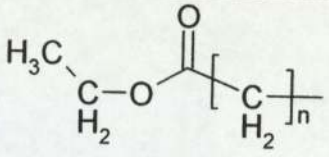
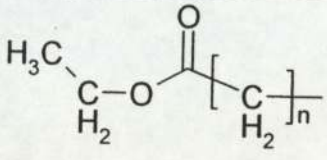
Glu-Hyp(Bzl)-OMe		Me	OBzl	83.0	8.8	
Glu-Pro(OBzl)		Bzl	H	52.2	2.4	0.8371
Glu-Pro(OMe)		Me	H	51.2	9.0	ns
Glu(OBzl)-Hyp(Bzl)-OMe		Me	OBzl	59.9	4.9	
Glu(OBzl)-Pro		H	H	64.2	10.3	
Glu(OBzl)-Pro(OBzl)		Bzl	H	34.6	7.6	0.0016
Glu(OBzl)-Pro(OdBzl)		dBzl	H	52.2	4.5	**
Met-Hyp(Bzl)		Me	OBzl	61.2	8.1	
Met-Pro		dBzl	H	64.3	5.5	

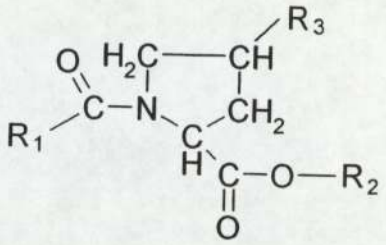
Table 5.1. Dipeptide Derivative Results as at 16/10/00 (continued)

Pim(OEt)-Pro	 (n=5)	H	H	59.9	11.7	0.0520
Pim(OEt)-Pro (OBzl)		Bzl		75.4	4.1	ns
Sub(OEt)-Pro (dBzl)	 (n=6)	dBzl	H	56.6	5.4	0.0101
Sub(OEt)-Pro (OMe)		Me	H	80.0	8.9	*
Sub(OEt)-Pro (OBzl)		Bzl	H	70.7	10.0	

* Probability that the means and SDs are the same. P<0.05 is significant difference.

3 means = One way ANOVA, 2 means = Student's t-test

Table 5.1. Dipeptide Derivative Results as at 16/10/00 (continued)



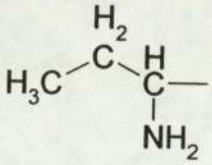
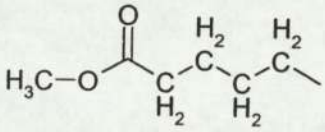
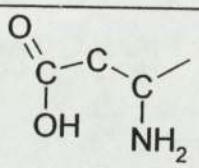
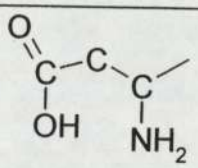
	R_1-	R_2	R_3	IC50 mM	SD	p*
Abu-Pro		H	H	3.949	1.317	0.0018
Abu-Pro (OBzl)		Bzl	H	0.438	0.132	**
Adi(OMe)-Pro		H	H	7.503	4.444	
Adi(OMe)-Pro (OBzl)		Bzl	H	2.319	0.728	0.8088
Adi(OMe)-Pro (OdBzl)		dBzl	H	4.499	1.329	ns
Adi(OMe)-Pro (OMe)		Me	H	-21.209	92.556	
Asp-Hyp(Bzl)-OMe		Me	OBzl	-39.229	36.964	
Asp-Pro		H	H	1.066	0.068	
Asp-Pro (OBzl)		Bzl	H	0.856	0.461	0.1147
Asp-Pro (OMe)		Me	H	1.282	0.088	ns

Table 5.2. Dipeptide Derivative Uptake Results (IC_{50}) as at 16/10/00

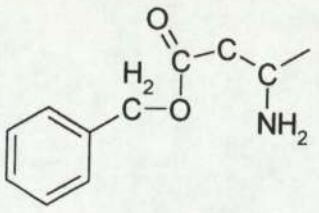
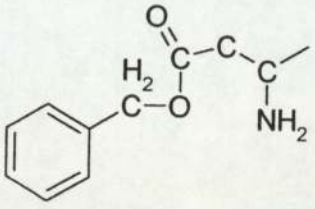
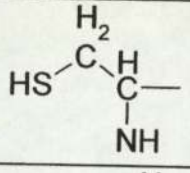
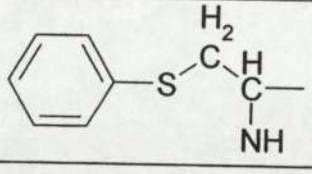
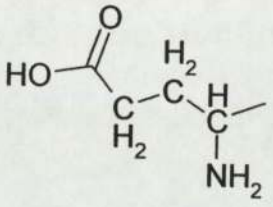
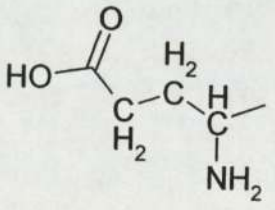
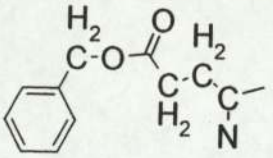
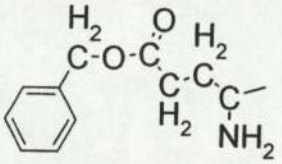
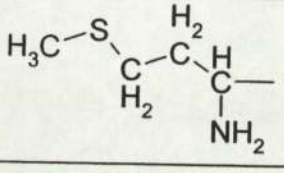
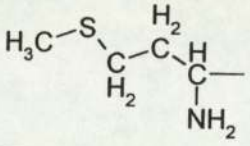
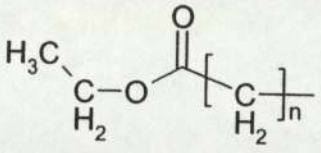
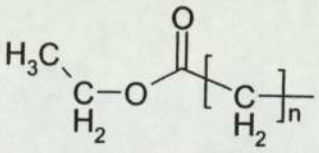
Asp(OBzl)-Hyp(Bzl)-OMe		Me	OBzl	0.878	0.218	
Asp(OBzl)-Pro		H	H	3.941	1.207	
Asp(OBzl)-Pro(Obzl)		Bzl	H	0.986	0.073	<0.0001
Asp(OBzl)-Pro (OdBzl)		dBzl	H	3.695	0.733	**
Asp(OBzl)-Pro(OMe)		Me	H	0.748	0.145	
Cys-Pro		H	H	3.596	0.551	
Cys-(SBzl)-Pro		H	H	0.655	0.106	
Glu-Hyp(Bzl)-OMe		Me	OBzl	6.460	4.242	
Glu-Pro(OBzl)		Bzl	H	1.094	0.101	0.8722
Glu-Pro(OMe)		Me	H	1.101	0.372	ns

Table 5.2. Dipeptide Derivative Uptake Results (IC_{50}) as at 16/10/00(continued)

Glu(OBzl)-Hyp(Bzl)-OMe		Me	OBzl	1.520	0.295	
Glu(OBzl)-Pro		H	H	1.962	0.771	
Glu(OBzl)-Pro(OBzl)		Bzl	H	0.546	0.195	0.0069
Glu(OBzl)-Pro(OdBzl)		dBzl	H	1.105	0.200	**
Met-Hyp(Bzl)		Me	OBzl	1.663	0.571	
Met-Pro		dBzl	H	1.851	0.480	
Pim(OEt)-Pro	 (n=5)	H	H	2.269	0.405	
Pim(OEt)-Pro(OBzl)		Bzl	H	1.624	0.678	0.0231
Pim(OEt)-Pro(OdBzl)		dBzl	H	3.153	0.666	*
Pim(OEt)-Pro(OMe)		Me	H	2.336	0.548	
Sub(OEt)-Pro(dBzl)	 (n=6)	Bzl	H	1.331	0.290	0.0747
Sub(OEt)-Pro(OMe)		dBzl	H	4.956	2.942	ns
Sub(OEt)-Pro(OBzl)		Me	H	2.815	1.618	

* Probability that the means and SDs are the same. P<0.05 is significant difference.

3 means = One way ANOVA, 2 means = Student's t-test

Table 5.2. Dipeptide Derivative Uptake Results (IC50) as at 16/10/00 (continued)

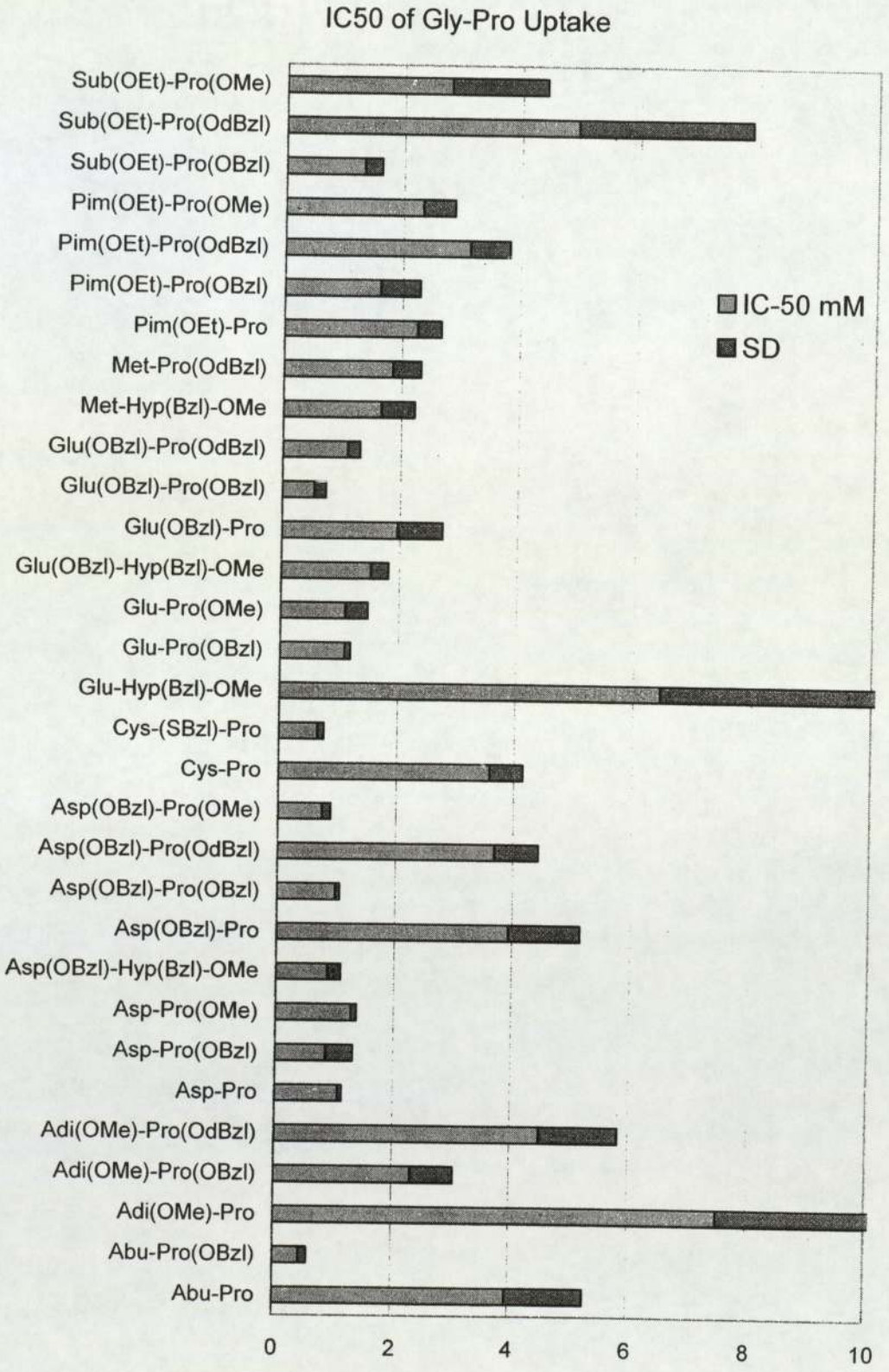


Figure 5.1. IC_{50} of Gly-Pro Uptake of 32 dipeptides (synthesised in year 2000)

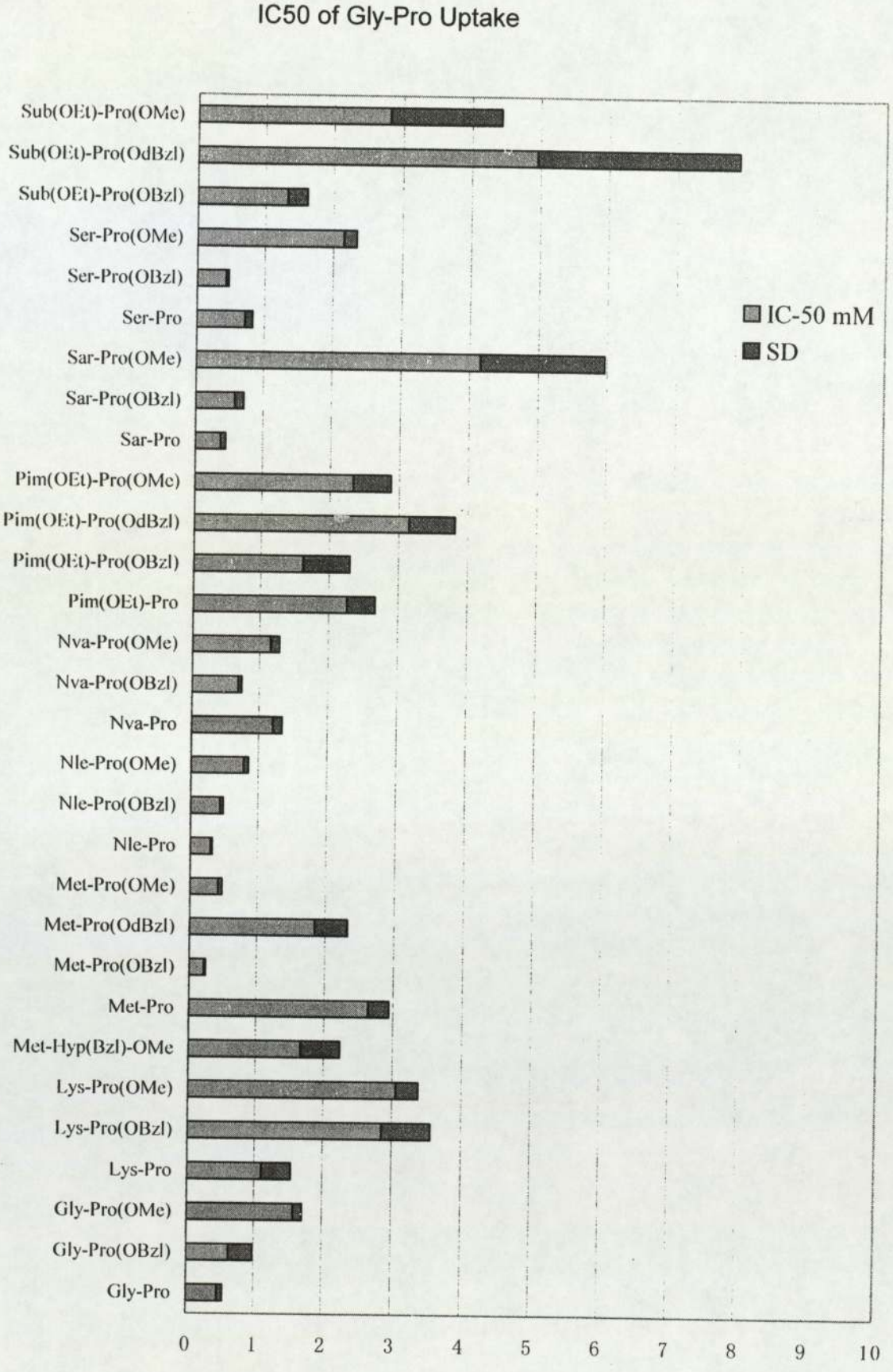


Figure 5.2. IC_{50} of Gly-Pro Uptake of total 65 dipeptides (synthesised in year 1999-2000)

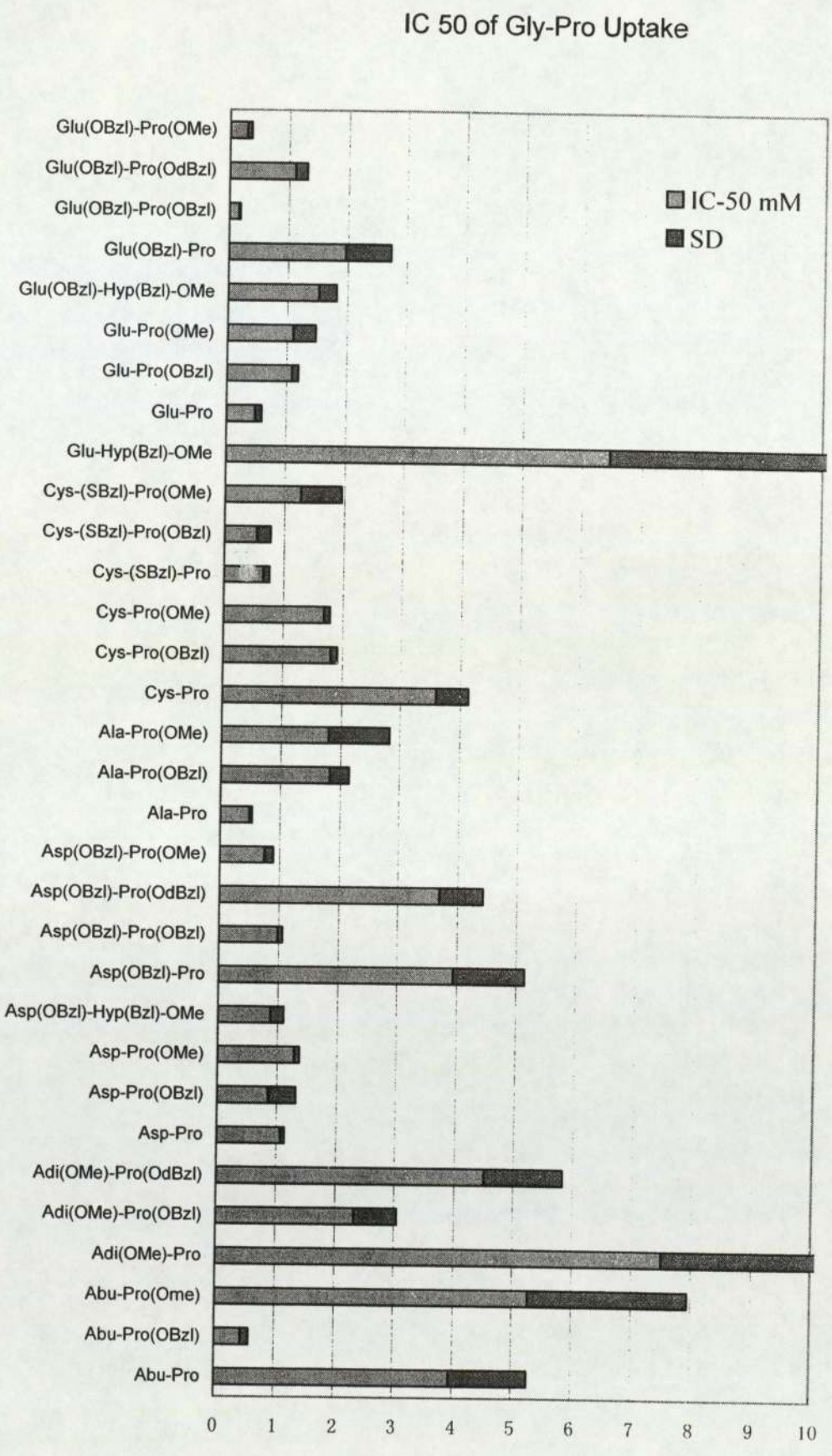


Figure 5.2. IC_{50} of Gly-Pro Uptake of total 65 dipeptides (synthesised in year 1999-2000) (continued)

5.2. The effect of DMSO on uptake

Dipeptide derivatives can hardly be dissolved in aqueous solution. However, they could be dissolved in the aqueous test media properly, therefore, a small amount of DMSO was added in to the solution to help in the Gly-Pro solution. However, DMSO itself is toxic to Caco-2 cells and so could reduce uptake by killing cells. Therefore, the concentration of DMSO has to be lower than its toxic limit. Then, different concentrations of DMSO are necessary to be tested before the experiment. The results are shown in figure 5.3.

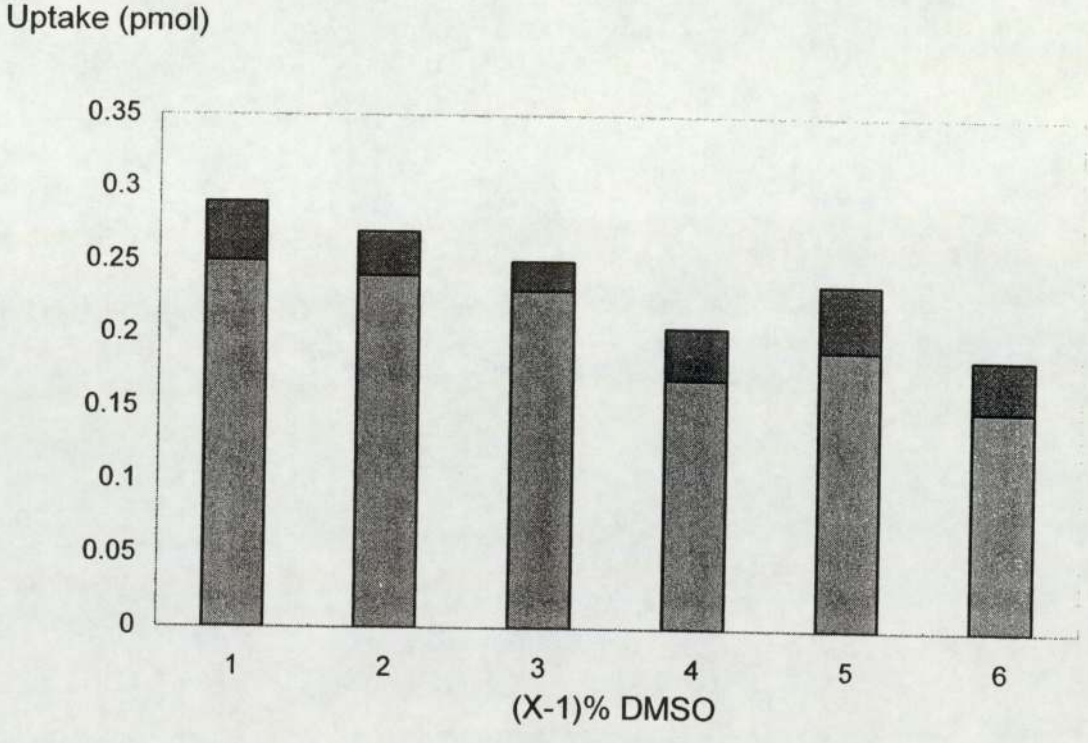


Figure 5.3. Effect of DMSO Concentration on Gly-Pro Uptake (n=4)

There is no significant difference between 0% DMSO (control cells) and 1 or 2% DMSO. Therefore, in most uptake experiment, 1% DMSO was in to increase dipeptide derivatives solubility. The higher concentration of DMSO, the more toxic it is to Caco-2 cells. It is concluded that the concentration of DMSO being lower than 2% could be used in the uptake test experiment.

5.3. The effect of pH on the experiment

In Gly-Pro uptake experiment, pH is one of the effects affecting the result of experiment. Gly-Pro uptake at six different pH levels was tested. The result was shown in Figure 5.4.

There is no significant between the uptake pH 6.0 and either pH 5.5 or 6.5. However, the standard deviation of pH 6.0 is smaller than other two pH values. The pH 6.0 was chosed to be as the optimum pH. All the experiments were performed at pH 6.0.

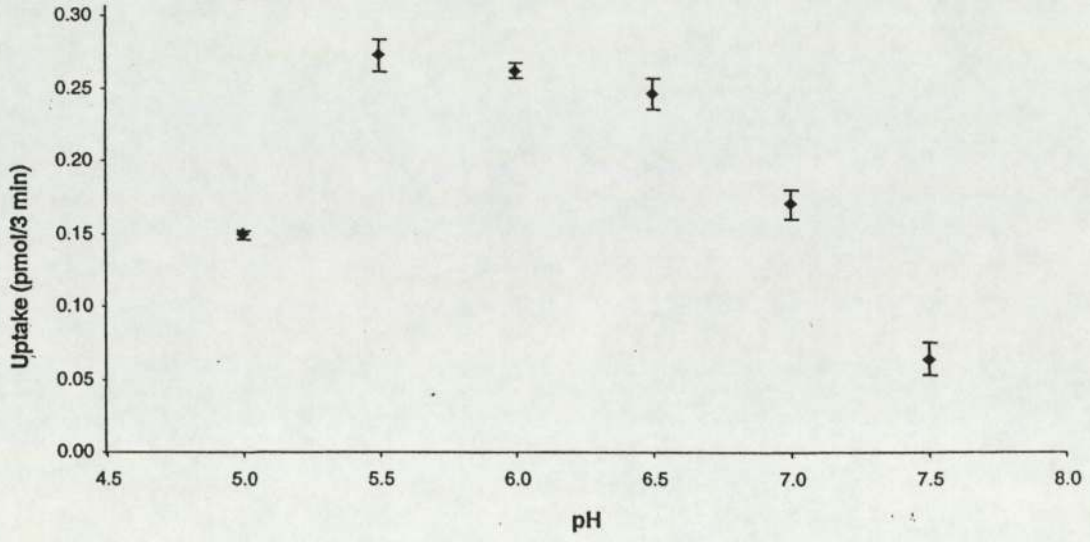


Figure 5.4. The effect of PH on the experiment

5.4. Conclusion

In the peptide chemistry section, from the NMR spectra, it has been shown that the methods used in peptide bond formation and deprotection worked very well.

From the results, four possible options have been drawn to minimise racemization during the formation of the peptide bond:

(I) The use of nonpolar solvents (THF, DMF and so on).

(II) A minimum of base (TEA).

(III) Low reaction temperatures (ice bath).

(IV) Carbonate protective groups (benzyl ester and methyl ester)

The aim of this research is to synthesis a good inhibitor as novel parent structures of the dipeptide-like prodrugs to improve the oral drugs absorbality. The main conclusions can be drawn from the Gly-Pro uptake.

(I) Our year 1999 results[61] showed among all proline dipeptide esters, methyl, ethyl, and benzyl, the dipeptide with benzyl ester has the strongest inhibitory activity. This seemed imply the ester group in dipeptides may play a big role in influence of affinity of substrates to receptors. Following this indication, we synthesised diphenylmethyl ester dipeptides in year 2000 in order to understand the importance of hydrophobicity of the ester group in this prodrug concept. The results of inhibitory tests of diphenylmethyl esters

showed a reduced activity in comparison with the benzyl ester dipeptides. Therefore the best ester group for the prodrug probably is benzyl ester.

(II) The experiment results of a series of compounds with dicarboxylic acid groups (Fig 5.5) suggested that the distance between two carboxylic acid groups could not be more than two methylenes (n=2). Since when n>2, the inhibitory activity was decreased greatly.

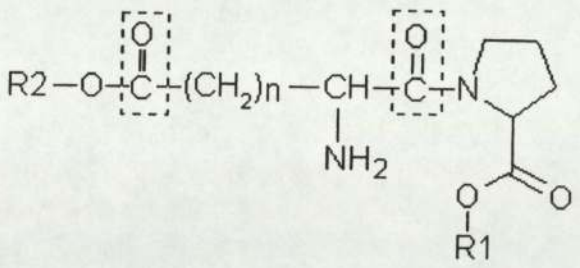


Figure 5.5. The position of carboxylic acid group in dipeptide compound

(III) When introducing a big phenylmethyloxy group on 4-position of proline, no significant change of inhibitory activity was observed. This implies that this position could be an anchor for a small molecular drug.

(IV) The best three inhibitors synthesized so far are Glu(OBzl)-Pro(OBzl) (IC_{50} is 0.546mM), Abu-Pro (OBzl) (IC_{50} is 0.438mM), Asp(OBzl)-Pro(OMe) (IC_{50} is 0.748mM).

Moreover, the concentration range of DMSO and pH range have been tested again. The recommended should be lower than 2% and the optimum pH is 6.0.

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