DESIGN, SYNTHESIS AND EVALUATION OF DIPEPTIDES AS PROBES FOR STUDIES OF GASTRO-INTESTINAL TRACT DIPEPTIDE TRANSPORT

Ying Kang

Master of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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In loving memory of my father, Zhentian Kang.

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Summary

The use of peptides and proteins as drugs is limited in part by their poor absorption by the gastro-intestinal tract. A knowledge of the mechanics by which this absorption occurs may lead to the design of more effective peptide-based drugs, and work presented here is an initial study into the relationship between simple peptides and their uptake.

A series of dipeptides, in which the C-terminal residue is proline or a proline ester, has been synthesised. The inhibitory effect of these dipeptides on the uptake of the dipeptide Gly-Pro by a Caco-2 (the human colorectal carcinoma cell line) cell line has been determined by using tritium labelled substrates. Structural features, which lead to increase the inhibitory potency with various series of inhibitor synthesis were identified.

Keyword: peptide, gastro-intestinal tract, dipepitde, inhibitor, Gly-Pro, caco-2

INDEX

ABBREVIATION

DTS	dipeptide transport system
GI tract	gastro-intestinal tract
ACE inhibitor	angiotensin-converting enzyme inhibitor
DCCI	dicyclohexylcarbodiimide
DCU	N,N'-dicyclohexylurea
HOBt	1-hydroxybenzotriazole
Bop	benzotriazolyloxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
PyBop	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
Cbz(Z)	benzyloxycarbonyl
Boc	*t*-butoxycarbonyl
TFA	trifluoroacetic acid
NMR	nuclear magnetic resonance
IR	infra-red
Caco-2	the human colorectal carcinoma cell line
DCHA	dicyclohexyl ammonium salt
DMSO	dimethylsulfoxide
IC50	inhibitory concentration to reduce uptake by 50%
SD	standard deviation
P	significant difference
THF	tetrahydrofuran
TEA	triethylamine

CHAPTER 1 - GENERAL INTRODUCTION

1. Absorption Barriers

For chronic therapies in all therapeutic areas, the most desirable method of drug administration remains the oral route. This route of administration presents formidable challenges to the development of effective, safe and commercializable delivery systems for peptides and proteins because of the minimal bioavailability (**Table 1.1**) [1]. This is due to their instability in the gastro-intestinal tract (GI tract), poor absorption and rapid biotransformation in the body. With more and more therapeutic peptides, proteins and peptide-like drugs being discovered in the pharmaceutical area, much attention has been paid to improve their oral bioavailability from less than 1% (**Table 1.1**) to at least 30-50% [1].

Conventional tablet formulations are used for a few drugs that are structural mimics of di- and tripeptides, i.e. some β-lactam antibiotics and ACE-inhibitors. The high oral bioavailability of some of these drugs is probably achieved through serendipitous use of dietary peptide transporters in the gut. However almost all drugs that are given orally are absorbed across the intestinal mucosa by passive diffusion; in order to be absorbed, a drug has to diffuse across a series of separate barriers.

Table1.1 Percentage of Dose Absorbed from Various Routes of Administration [1]

Percent of Dose Absorbed		
Route	**Insulin(M.W.6,000)**	**Leuprolide (M.W. 1,210)**
Oral	0.05	0.05
Nasal	30	2–3
Buccal	0.5	----------
Rectal	2.5	8
Vaginal	18	38
Subcutaneous	80	65

1.1. Intestinal Barriers

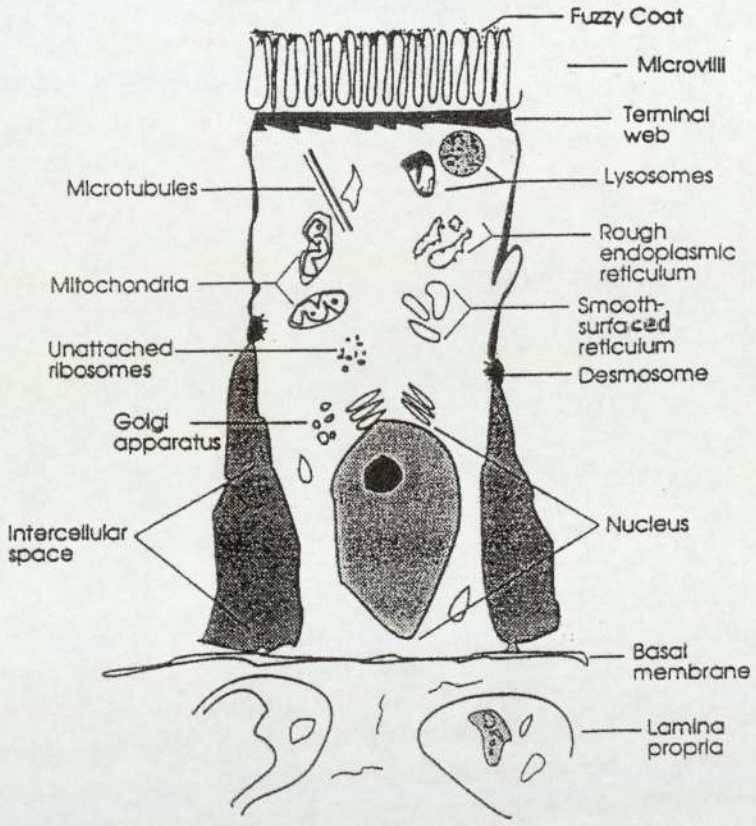
After ingestion, drugs mix with the acidic contents of the stomach but, due in part to the surface area and contact time, there is relatively little absorption from this organ. Gastric emptying passes the contents of the stomach into the small intestine, which is the major site of food and drug absorption [2].

In an average adult human, the small intestine is about 2.8m long which is divided into three parts: duodenum (30cm), jejunum (120cm), and the ileum (130cm). The total digestive surface area is approximately $200m^2$, owing to the circular folds of the villi and microvilli [3].

Villi are folds in the surface of the intestine (mucosa), about 1mm long. Closer examination of the cells that make up the villi show that 90% are the same

type: enterocytes (**Fig. 1.1**) [2]. These cells form the most important anatomical barrier against drug absorption [2].

Fig. 1.1 The Structure of Enterocyte [2]



Adapted from Figure 4, Kararli, 1989

Enterocytes are produced through mitotic division and maturation of the cells called Crypts of Lieberkuhn, which are located at the bottom of the villi. The maturing enterocytes continuously migrate up to the tips of the villi, where they are finally sloughed off into the intestine [2]. The most distinctive feature of the enterocyte is its apical brush-border membrane, which is composed of tightly packed microvilli (about 1μm long). Next to the microvilli is the "fuzzy coat" which is about 0.1μm thick. Fuzzy coat is made from glycoproteins secreted from the enterocytes. The apical membrane contains digestive

enzymes such as sucrase, oligoaminopeptidases, maltase, and alkaline phosphatase. In addition to enterocytes, lymphocytes, goblet, and endocrine cells are also found in the intestine mucosa [2].

The mucosa of small intestine consists of three main layers [3]:

--The muscularis mucosa.

It is a continuous sheet of smooth muscle. This muscle layer may help the functioning of the intestine and facilitate the emptying of the crypt luminal contents [3].

--The middle layer, or lamina propria.

It is the connective tissue inside the villus and surrounding the crypt epithelium. Lamina propria performs a variety of functions, such as immunological functions. In addition, it is the structural and nutrients support for the epithelial cells. Numerous defensive cells, such as lymphocytes, plasma cells and macrophages, are found in this layer, where they react with the foreign substances that enter this layer through the GI tract [3].

--The epithelial cells of the small intestine [4].

There are other cell types (**Table 1.2**) that are found in the epithelium of the small intestine.

Table 1.2 Different Type of Intestinal Epithelial Cells [4]

NAME	SITE	FUNCTION
Paneth's cells	The bases of the crypts	Protein secreting, regulation of the microbial flora
Enteroendocrine cells	In the crypts and on villi	Intestinal motility, pancreatic secretion and gall-bladder contraction
Goblet cells	Scattered among the enterocytes in the villi	Excrete an acidic glycoprotein which forms a protective layer on the glycocalyx of the microvilli of the enterocytes
M (microfold) cells	Overlying Peyer's patches	Involved in transporting macromolecules from the lumen of the intestine to lymphocytes (migrating cells in epithelium) where responses to foreign antigens can be undertaken [5]

After passing through the intestine, the remaining nutrients and drugs enter the colon, in the form of chyme. The colon is 1.1m long. There are no villi on the surface of the colon. It is the important place to absorb water, sodium and other minerals. The microvilli are less tightly compact and the enterocytes here are slightly different to those in small intestine. There are hundreds of microorganisms in the colon. Most of them are anerobic and are involved in

reductive reactions. The colon converts chyme into faeces, which are then excreted from the body [2].

1.2 Enzymatic Barriers to Peptide and Protein Drug Delivery

Peptide and protein drugs are subject to degradation by numerous enzymes or enzyme systems throughout the body. Therefore, enzyme becomes the major barrier which limits the amount of peptide and protein drugs from reaching their targets [6].

It is widely accepted that an enzymatic reaction acts by lowering the energy of the transition-state intermediates of the biological reaction [6]. Certainly, proteases are the same. Their proteolysis activity is defined by their affinity for substrates. Consequently, it is important that the activity of this kind of enzyme be stringently controlled in many biological events [7].

Numerous proteases are initially synthesised as precursors called zymogens without enzymatic activity. Zymogens are subsequently activated at the functional site. Proteases are essentially hydrolases; hence they are able to cleave peptide bonds with the addition of water. Any protease has the potential to hydrolyze more than one substrate [7]. Actually, proteases are proteins as well, they are between 20,000 and 60,000 daltons [6].

Proteases are classified as exo- or endopeptidases. Exopeptidases include amino- and carboxypeptidases and dipeptidases. They act on the N- or C-end termini of a protein which is not blocked by any substitution. Endopeptidases include serine proteinase, cystein proteinase, aspartic

proteinase and metalloproteinase. They cleave the peptide bonds internally in accordance with their mechanism rather than substrate specificity [8].

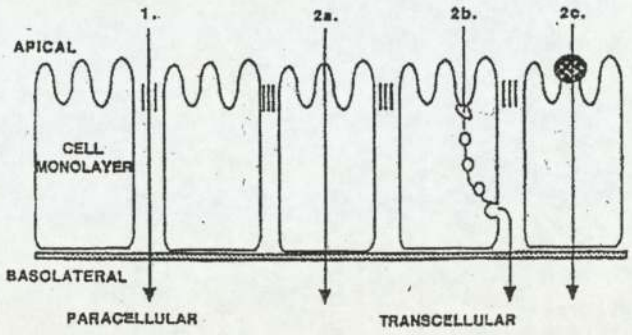
2. Drug absorption

The determination of oral absorption is an important part in the preformulation of drug entities. One principal difficulty using peptides as therapeutic agents is the requirement of parenteral administration, since systemic bioavailability is usually very low after oral administration. For the rational design of orally active drugs, an understanding of the mechanism of absorption from the gastrointestinal tract is necessary.

2.1. Mechanisms of Absorption

There are two main methods of drug absorption into cells: transcellular and paracellular. The drug absorption route through the cell membranes is called the transcellular route, while the parallel route through the tight junctions between the cells is called the paracellular route [9]. The mechanism by which peptides and proteins traverse intestinal epithelial cells is transcellular, which includes: endocytotic, carrier-mediated and passive progress (**Fig 1.2**) 10].

Fig. 1.2 Diagram of routes and mechanisms for the transport of drugs and delivery systems across cellular barriers [10].



(1) Paracellular

(2) Transcellular (2a. passive progress; 2b. endocytosis; 2c. carrier-mediated)

2.1.1. Passive Transport [2]

Passive transport refers to the transport of solute along a concentration and electrical gradient where the solute does not interact with the structural elements of the membrane. Most unionised molecules, such as O_2, CO_2, can easily cross the plasma membrane.

2.1.2. Carrier-Mediated Transport [2]

In addition to passive transport, there are specific carrier-mediated systems in the intestine to facilitate absorption of nutrients. Carrier-mediated transport normally requires at least three steps: binding, transfer across the membrane and release. This type of transport can be divided into facilitated transport and active transport.

Facilitated transport interacts with the membrane protein, which is different from passive transport. The protein involved in transport is capable of combining reversibly with specific substrates. Since these specific substrates

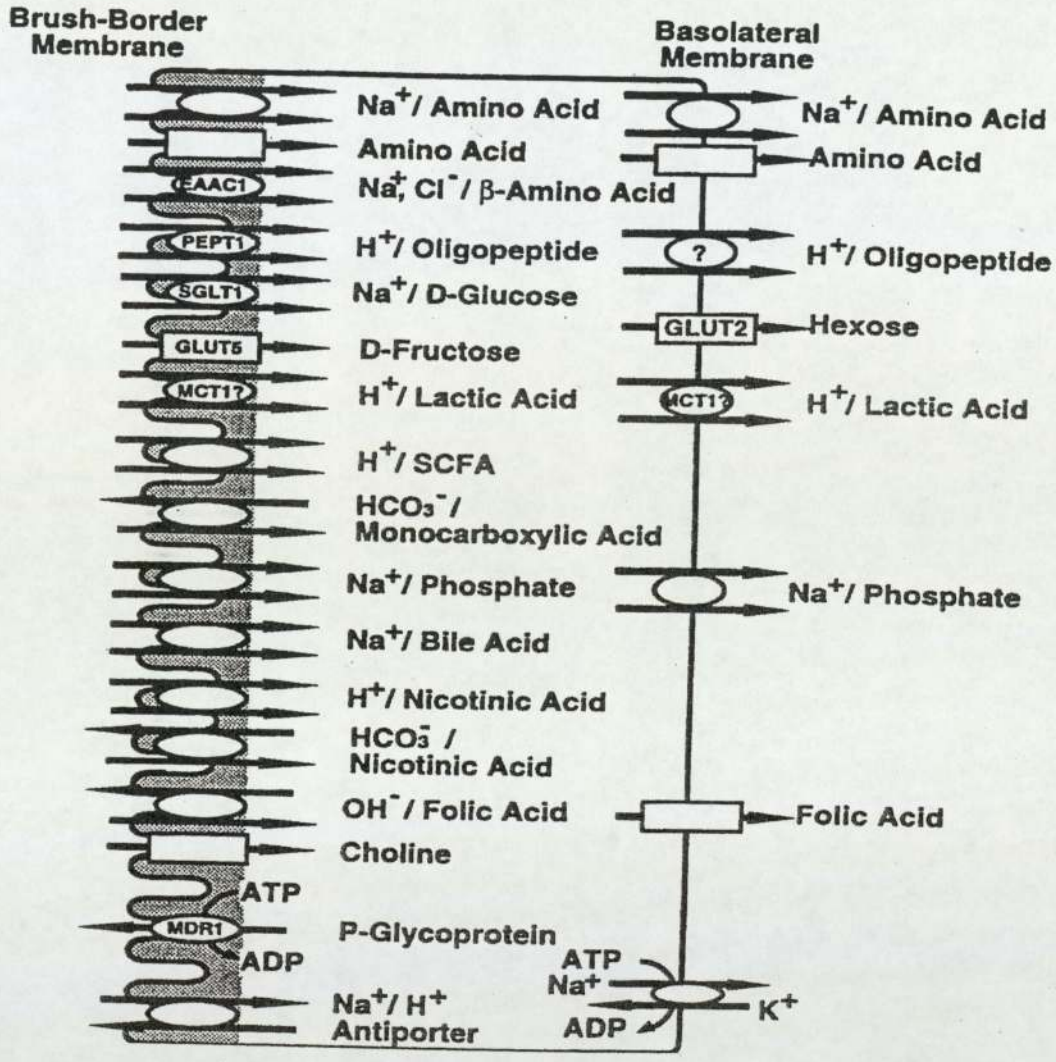
could move along the concentration gradient, they do not need energy source.

Opposite to facilitated transport, active transport requires an energy source, which is supplied in two ways. The first is the hydrolysis of adenosine triphosphate (ATP). The transporters in this way are known as pumps, e.g. Na^+/K^+ATPase pump. The absorption of many compounds is relative to the electrochemical gradient of Na^+ and H^+; both of them have inward directed concentration gradients which are maintained by pumps. The second is the use of these gradients to transport other compounds [2]. **Figure 1.3** below identifies a number of intestinal transporters in both the apical and basolateral membranes [11].

2.1.3. Endocytosis [2]

A small portion of the plasma membrane can progressively enclose materials near the surface of the cell. The membrane first invaginates and then pinches off to form an intracellular vesicle, containing the ingested materials. The vesicles can then merge with other cellular membranes to transport the materials. The vesicles can merge with lysosomes, in which case the material is degraded. The vesicles can merge with the basolateral membrane as well, in which case the material enters the blood stream.

Fig. 1.3 Summary of intestinal epithelial transporters [11]



2.2. Peptide Absorption

Free amino acids are absorbed into the enterocyte across the brush border membrane via group specific amino acid transport systems [12]. Since the absorption of intact dipeptides into the portal vein was first observed, it has been found that small peptides that consist of two or three amino acids are transported by a specific peptide transport system. This peptide transport system is localised in the brush border membrane of the enterocyte, and transports di-/ tri-peptides into the cell. This is followed by intracellular

hydrolysis to amino acids. The enterocytes are one of the richest sources of peptidase activity against small peptides [12].

2.2.1. Absorption of Peptide

Intact di- and tripeptides can be taken up by intestinal epithelial cells by a carrier mechanism which is distinct from the amino- acid transport system [12].

Absorption of di- and tripeptides is proposed to involve a proton-coupled uptake across the apical cell membrane via a carrier-mediated process and exit across the basolateral cell membrane by a mechanism, which may also be carrier-mediated [13].

Following carrier-mediated peptide uptake, intracellular hydrolysis of these peptides generally results in reduced amounts of intact peptides exiting across the basolateral cell membrane. Efficiency of absorption of intact di- and tripeptides can be increased by structural modifications which decrease metabolism, such as inclusion of proline, hydroxyproline or D-amino-acid residues [14].

Structure absorption data indicate that peptide transport is reduced by methylation or acetylation of the N-terminal amino group or by esterification of the C-terminal carboxyl group [12].

2.2.2. Driving Forces for Transport of Peptides

The peptide transport is electrogenic both in intact epithelium and in intestinal brush border membrane, resulting in transfer of positive charge across the membrane [15, 16]. The study of the ion responsible for the charge transfer first came from studies in which peptide transport was stimulated by an inwardly directed H^+ gradient [13]. The human colon carcinoma cell line caco-2 expresses the H^+-peptide co-transport system. Substrate-induced H^+ flow was also observed with a number of dipeptides that are relatively resistant to hydrolysis including Gly-Pro, Gly-Gly, Pro-Gly, Gly-Sar and carnosine [17].

The Na^+-H^+ exchanger is localised in the brush border membrane. The exchanger catalyses the entry of Na^+ from lumen into the cell in exchange for the exit of H^+ from the cell into the lumen [17].

According to Leibach's hypothesis [13], the Na^+-H^+ exchanger generates and maintains the inward proton gradient, while the Na^+/K^+ ATPase in the basolateral membrane maintains a low intracellular sodium concentration.

Inhibition of the basolateral Na^+/K^+ ATPase or the luminal Na^+-H^+ exchanger reduced peptide carrier mediated transport across rabbit ileum epithelium [18, 19]. Thus, Na^+ is only indirectly involved in the process and the H^+ gradient, along with the membrane potential, provides the driving force for the peptide transport system [19].

Utilisation of two different driving forces, a Na^+ gradient for active absorption of amino acids and a H^+ gradient for absorption of peptides, is advantageous to the organism since this removes competition between amino acids and

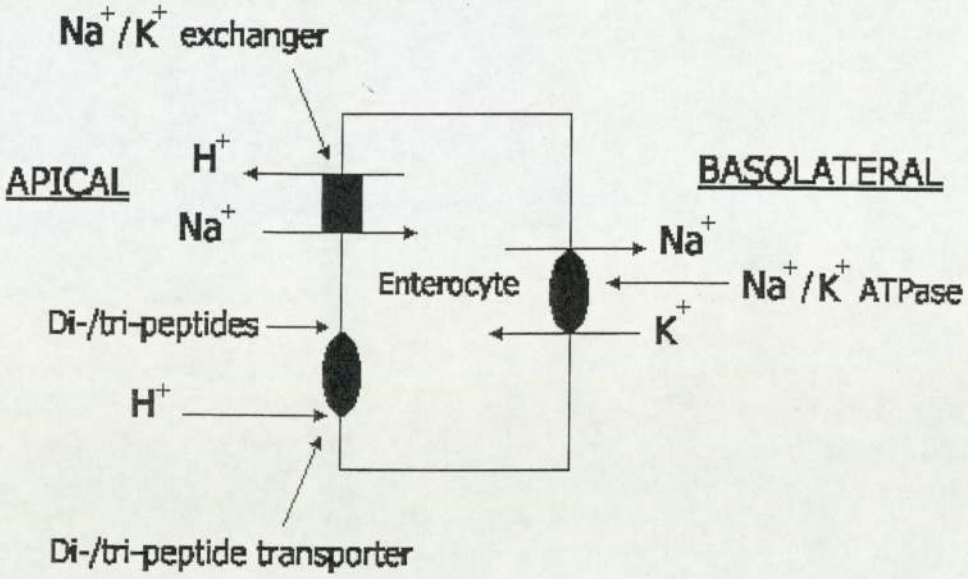
peptides for the energy source and allows these absorptive processes to operate in parallel [20].

2.2.3. The Effect of Ion Concentration on Peptide/Peptide-like Drugs Uptake

In the 1960s, a hypothesis formulated about protein absorption is that proteins are partially hydrolysed in the intestinal lumen to peptides of various sizes, which then enter mucosal cells and are hydrolysed to amino acids, which then enter the blood stream. In 1968, it was reported that amino acids could be more rapidly absorbed from small peptides than from the equivalent mixture of free amino acids [21].

The uptake of peptides and peptide-like drugs is coupled with an influx of protons [13]. The low intracellular concentration of H^+ and an inwardly directed proton gradient across the apical membrane are maintained by the Na^+/H^+ exchanger in the brush-border membrane. This acts to pump intracellular protons out in the apical compartment in exchange for Na^+ ions. The physiological intracellular concentration of Na^+ ions was, in turn, maintained by Na^+/K^+ ATPase pump at the basolateral membrane when Na^+ is exchanged for K^+. **Figure 1.4** below shows the location of di-/tripeptide transporter [13].

Figure 1.4 the location of the di-/tri-peptide transporter [13]



Intracellular accumulation of peptides is reduced by the action of cytoplasmic peptidases. These enzymes hydrolyse the peptides into amino acids. Then, the amino acids are used by the cells or excreted out of the cells via amino acid transporters located on the basolateral membrane. There is another peptide transporter in the basolateral membrane as well because the examination of bestatin shows that it is inhibited in both directions (apical to basolateral and vice versa) by dipeptides [21].

The optimum pH of uptake from the apical surface is 6.0. The basolateral surface, however, is not sensitive to pH. Kinetic parameters indicated that the apical and basolateral transporters could be distinguished from each other. Intravenous injection of a dipeptide led to accumulation of the dipeptide in the kidney (where there is another transporter) but not the intestine. This implies that the basolateral transporter does not absorb dipeptides from the blood

[22].

2.3. Peptidomimetic Drugs

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 [23]. The main peptidomimetic drugs are β-lactam antibiotics and angiotensin-converting enzyme inhibitors.

2.3.1 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. It is also deactivates the endogenous depressor substance bradykinin [24]. Many ACE-inhibitors have been approved for medical use. These compounds generally belong to three chemical catagories, viz., sulfhydryl, carboxyalkyl dipeptide, and phosphorus-containing types (**Fig 1.5**) [24]

Fig. 1.5a The Relevant Structure of Sulfhydryl Type

Captopril

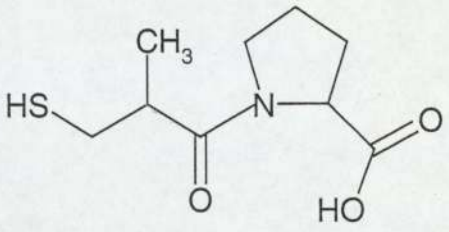
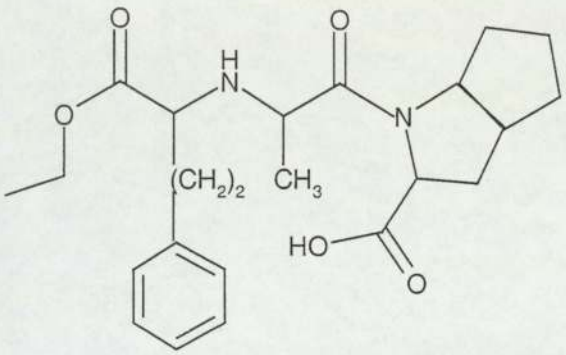


Fig. 1.5b The Relevant Structure of Carboxyalkyl Type

Enalapril



Enalaprilat

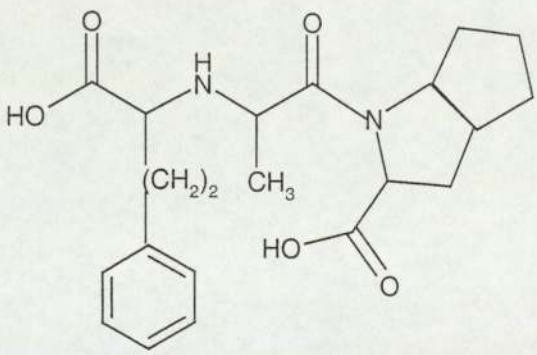
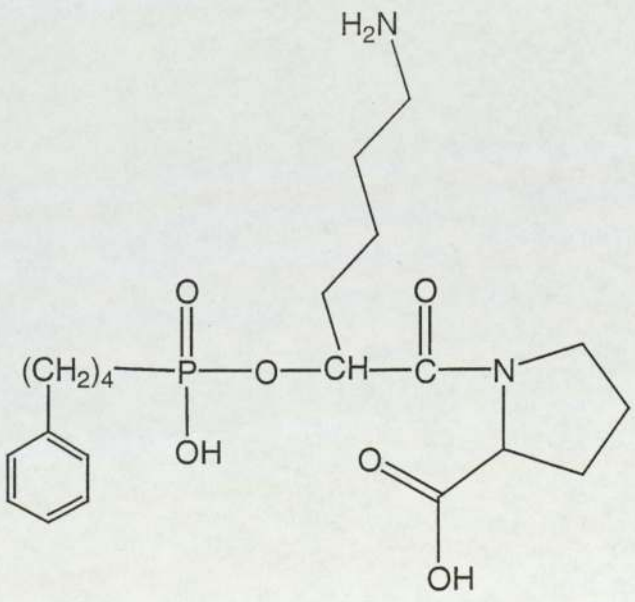


Fig. 1.5c The Relevant Structure of Phosphorus-Containing Type

Ceronapril (SQ 29851)



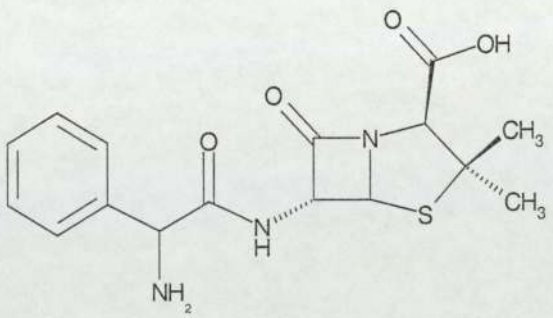
2.3.2. β-Lactam Antibiotics

Amino β-lactam antibiotics include penicillin derivatives and cyclosporin derivatives. Some of structures are shown in the **Fig 1.6**.

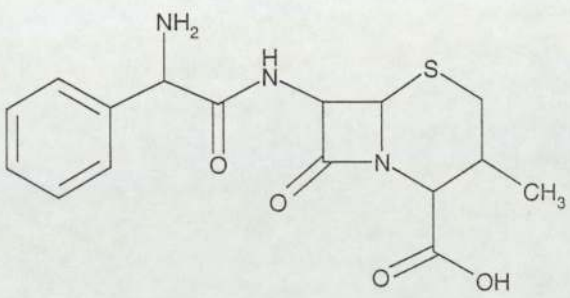
β-Lactam antibiotics which possess certain structural features of peptides including a peptide bond with an α-amino group and a free carboxylic acid group, are ideal substrates with which to characterize peptide transport in intact tissure with complications due to hydrolysis by peptidases or metabolism.

Fig. 1.6 β-Lactam Antibiotics Structure

Ampicillin



Cephradine



2.4 Absorption of Peptidomimetic Drugs

Absorption of some peptidomimetic drugs, e.g. prodrug esters (will be introduced in next chapter) such as pivampicillin, appears to correlate with lipid solubility and presumably results from passive diffusion [25].

For β-lactam antibiotics or ACE inhibitors with zwitterionic properties and low lipid solubility, absorption via a diffusional process will be low. However, because of molecular size and physical characteristics, some peptidomimetic

drugs have oral bioavailability grater than would be expected. Part of the explanation for this phenomenon appears to be that these molecules are transported by a di-/tripeptide carrier [26]. From the studies in situ, in vitro, and in vivo, the uptake of these peptidomimetic drugs appears to be a proton-dependent, saturable process which can be inhibited by di-/tripeptide or by metabolic inhibitors, but not by amino acids or organic acids [27].

These studies provide a mechanism for peptidomimetic drug uptake at the apical cell membrane. A carrier that transports peptides across the basolateral membrane was reported by J. Dayer [28].

The studies (in situ, in vivo, in vitro) support the hypothesis that apical membrane uptake is the rate-limiting step in absorption of molecules which can be transported on the di-/tripeptide carrier mechanism.

2.5 Classification of Various Substrates for the Intestinal Peptide Carrier

Based on three-dimensional structural mutualities, Swaan has prepared a classification table (**Table 1.3**) of various substrates for the intestinal peptide transport [29].

The table was divided into three classes [29]:

(Ia). The compounds here bind to the transporter and are transferred across the membrane. This class contains actively transported compounds.

(Ib). A prodrug group contains ACE-inhibitor ester prodrugs. These compounds are actively transported by the peptide transporter, but undergo biotransformation to their parent compound after intestinal transport in order

to exert their pharmacological activity. This group also contains the ester prodrug carficillin, an antibiotic drug, whose parent drug carbenicillin is not transported by peptide carrier.

(Ic). The group contains a second carboxylic function, like diacid ACE inhibitors, but still can be shown to be transported actively.

(II). In contrast to their parent compounds, the ester prodrugs in this group are recognized by the peptide carrier, and are actively transported. Obviously, the negative carboxylic acid moiety must be responsible for the prevention of transport to the other side of the membrane. Thus, the conversion of negative carboxylic function into an ester function can re-establish affinity for the carrier. In conclusion, a negative group is not compatible with transport.

(III). The compounds in this group are structurally similar to the cephalosporins and penicillins in group I, but exert no affinity for the carrier.

(IIIa). The C-terminal carboxylic acid function is esterfied with a side chain.

(IIIb). This group includes compounds that contain side chains with positively charged or hydrogen-acceptor groups in the proximity of the pharmacophoric carboxylate.

2.6. Dipeptide Transport System (DTS)

Absorption of di-/tripeptides from the GI-tract of mammals occurs via the DTS, located in the brush of small intestinal enterocytes. It has also been suggested that the H^+-coupled dipeptide transporter plays an important role in

the oral absorption of peptide-like drugs, such as β-lactam antibiotics, like cepharadine, cefixine, and cefroxadine [30]. A complementary DNA clone encoding small intestinal H^+/dipeptide transporter (Pep T_1) has also been isolated and its involvement in the transport of the oral β-lactam antibiotics determined [31]. Like these antibiotics, the ACE inhibitors, e.g. enalapril, captopril, lisinopril, have also been suggested to be transported, at least partly, by the dipeptide carrier. Studies with brush border membrane vesicles [32, 33] and more recently with caco-2 cells [34] have indicated that DTS is (a) energy-dependent, (b) Na^+-independent, and (c)stimulated by an inward H^+gradient. Studies with natural dipeptides in humans and rats and immunohistochmical evaluation in rats indicated the presence of the DTS in the lower GI tract [34]; The different absorption might be explained by the specificity and stability of the various drugs that have been used as probes of the DTS.

Table 1.3: A Classification of Various Substrates for the Intestinal Peptide Transport [29]

	Cephalosporins	Penicillin	ACE-inhibitors	Others
Ia	Cefaclor Cephalexin Cefadroxil Cephradine SCE-100 Cefatrizine Cephalotin Cefroxadine Cefdinir Loracarbef	Amoxicillin Ampicillin Cyclacillin Phenoxy-methyl-penicillin Propicillin	Captopril	Alafosfalin Bestatir Camosine
Ib		Carfecilllin	Enalapril Quinapril Benazepril Fosinopril	
Ic	Cis-Ceftibuten FK089 Cefixime		Lisinopril SQ 29852	
II	Latamoxef Trans-Ceftibuten	Carbenicillin	Enalaprilat Quinaprilat Benazeprilat Fosinoprilat	
III a	Pivcefalexine	Bacampicillin Pivampicillin		
III b	Cefazolin Cefoperazone Cefoxitin Cefotiam Cefmetazole			

The intestinal peptide transporter has a broad substrate specificity apart from its natural substrates. It transports a wide variety of hydrophilic peptide mimetic drugs; some of them are shown in **Fig 1.7** below [35]. Orally absorbed antibiotics, including penicillins and cephalosporins, and also ACE-inhibitors are drugs taken up by the peptide transporter [36].

Most of the studies concerned the effects of structural modification on peptide uptake by examining the inhibition of peptide transport by chemically modified molecules. The molecules to be used as probes for the examination of the inhibition of peptide transport will be introduced in the discussion of DTS.

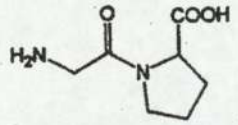
3. Prodrug

A major obstacle for clinically useful peptide and peptidomimetic drugs is their poor delivery characteristics. Most peptides are rapidly metabolised by proteolysis at most routes of administration. Generally, they are non-lipophilic compounds showing poor biomembrane penetration characteristics. They possess short biological half lives due to rapid metabolism and clearance.

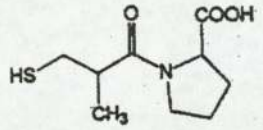
Many different strategies have been employed to overcome the penetration and enzymatic barriers associated with the delivery of peptide and protein drugs, such as synthesis of analogues, use of protease inhibitors, absorption enhancers and the development of new dosage forms.

Fig 1.7: chemical structures of selected di-/tripeptides and peptide-like drugs

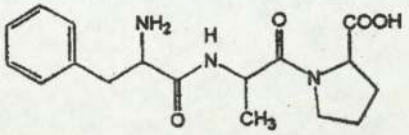
Gly-Pro



captopril

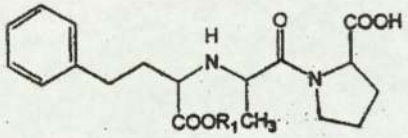


Phe-Ala-Pro



enalapril

enalaprilat



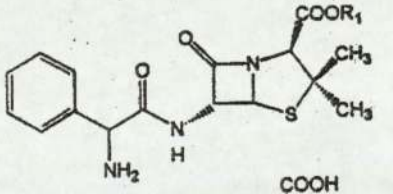
R_1:

$-C_2H_5$

$-H$

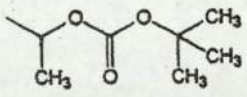
ampicillin

pivampicillin

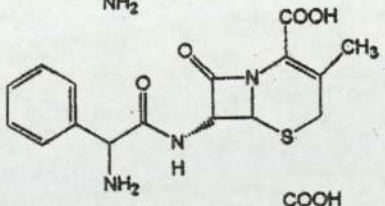


R_1:

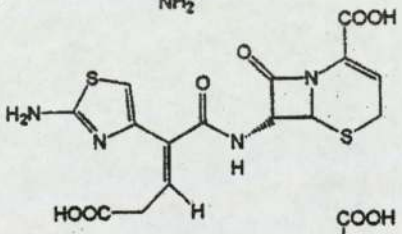
$-H$



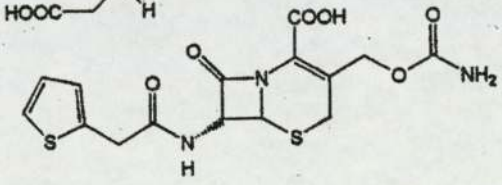
cephalexin



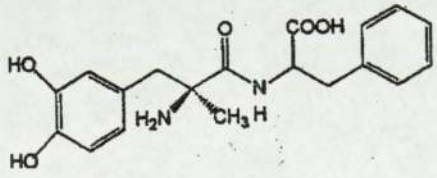
ceftibuten



cefoxitin



L-alpha-methyldopa-Phe

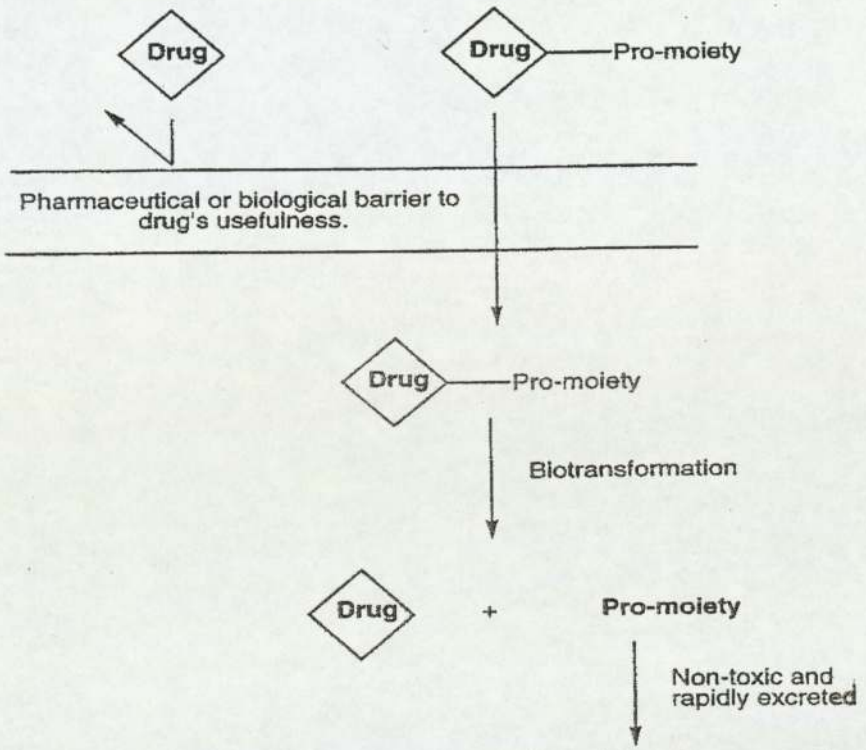


Among these various approaches the prodrug technology appears to be very important for the more traditional drugs of the peptide- or amino acid-type, like amino containing β-lactam antibiotics, angiotensin-converting enzyme inhibitors, and L-DOPA. By this bioreversible derivatization technique it is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

This approach to delivery problems may be derivatization of the bioactive peptides to produce prodrugs or transport-forms which possess enhanced physicochemical properties in comparison to the parent compounds with regard to delivery and metabolic stability. Thus, on one hand, such derivatization may protect small peptides against degradation by enzymes present at the mucosal barrier; on the other hand, this type of derivatization could render hydrophilic peptides more lipophilic and hence facilitate their absorption.

To be a useful approach, however, the derivatives should be capable of releasing the parent peptide spontaneously or enzymatically in the blood following their absorption [37]. The non-selective prodrug approach appears to be well suited for highly polar drugs that are to be administered locally at the response site as less polar prodrugs, which can rapidly generate the parent compound following the penetration through the limiting barrier [35]. A major requirement for the prodrug approach to be successful is the need for reliable conversion of prodrug to the parent drug, either through enzymatic or non-enzymatic catalysed reactions, once the barrier to delivery has been circumvented (**Fig 1.8**) [38].

Fig.1.8 Schematic representation of the prodrugs concept to circumvent various pharmaceutical and biological barriers to drug delivery.



Dipeptide analogues (β-lactam antibiotics and angiotensin-converting enzyme inhibitors), and amino acids analogue (L-methyldopa) will be introduced as the example for the prodrugs.

3.1. Prodrugs of β-Lactam Antibiotics

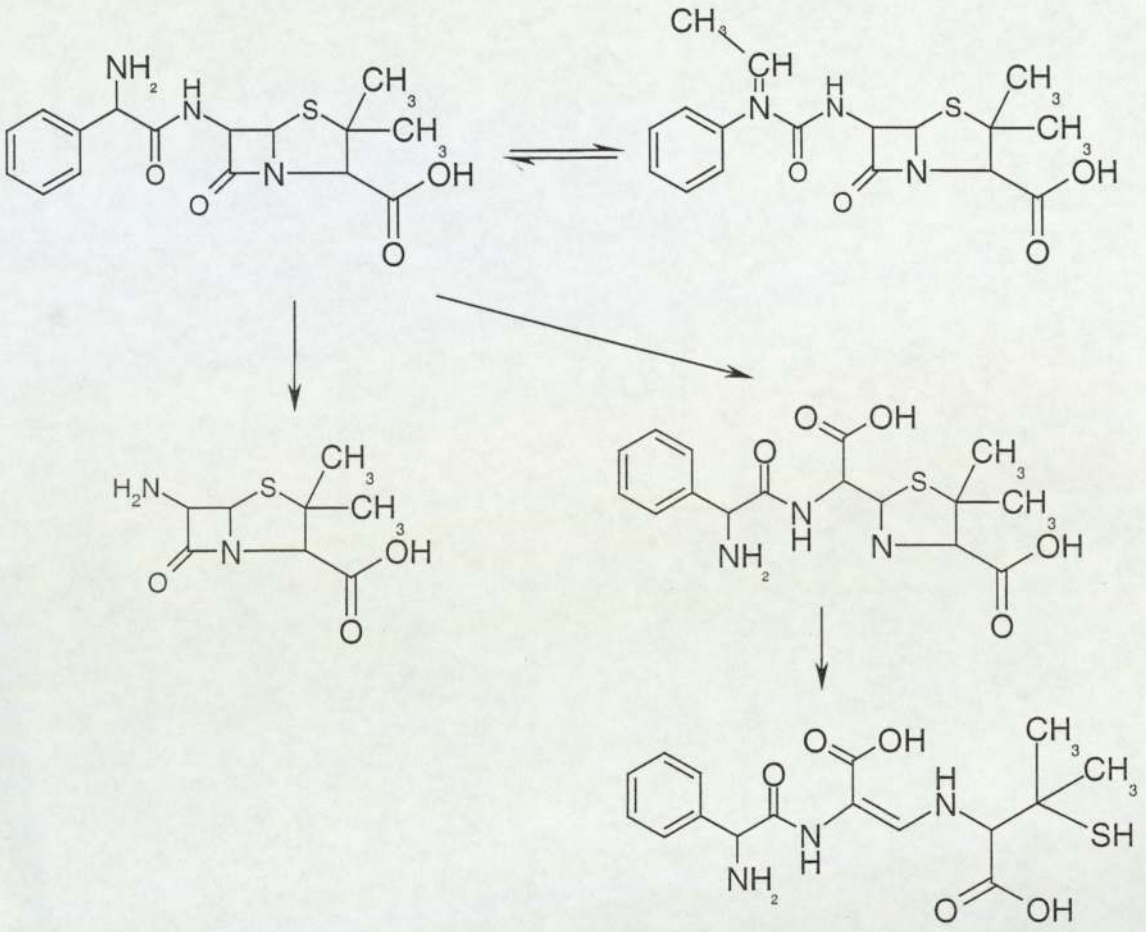
The successful treatment of intracellular bacterial infection requires the use of antibiotics capable of accumulating to a sufficient extent in the host cells (*i.e.* above the minimal inhibitory concentration (MIC) of the drug) [39]. β-Lactam antibiotics have this property, but they are not sufficiently effective in practice due to their poor bioavailability, despite their high pharmacological activities. It is reasonable to assume that this is because the polar character of the drug and that a transient masking of the carboxyl group could improve its oral

absorption. The prodrug approach — esterification is an ideal method to overcome such difficulties; for example, some ampicillin esters are used clinically as prodrugs (pivampicillin, talampicillin, and bacampicillin[40]). Ampicillin and its prodrugs will be introduced as examples for β-lactam antibiotics in the following section.

3.1.1. The Reason for the Prodrugs of Ampicillin

Known products of ampicillin biotransfermation are summarised in **Figure 1.9**. About 10% of an oral dose is excreted in the urine as the penicilloic acid[41, 42], while α-aminobenzylpenamaldic acid has been tentatively identified as a urinary metabolite in man [43]. A less polar substance has been identified by Murakawa *et al* [44] as Schiff base quantities in kidney, liver, serum, and urine.

Fig. 1.9 Biotransformation of Ampicillin

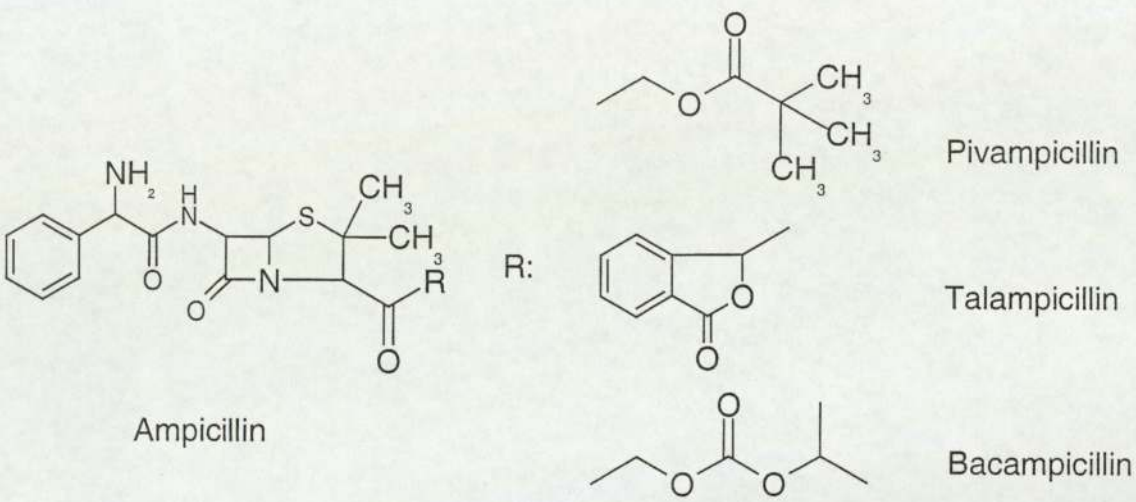


Serum antimicrobial activity declines biexponentially with a terminal half-life of 1.0-1.5hr after intravenous administration of ampicillin in man. Serum clearance is about 350ml/min and that about 85% of the dose is excreted in the urine [45, 46].

The amount of ampicillin excreted in bile is negligible [41]. Ampicillin can be efficiently absorbed after intramuscular injection. Loo *et al* [46] estimated that 87% of a dose of sodium ampicillin is absorbed, most of it during the first 3 hours. Absorption of orally administered ampicillin is incomplete (32-53%), although it is relatively stable to acid [41]

The fact is that much higher blood level and urinary recovery are attained after parenteral than after oral administration [47]. A number of prodrugs of ampicillin have been made in attampts to improve its aqueous solubility, chemical stability, and/or oral absorbability. **Figure 1.10** below shows some ester prodrugs for ampicillin.

Fig. 1.10 The structures of ester prodrugs of ampicillin



These three ester prodrugs do not have intrinsic antimicrobial activity; however, after absorption from the GI-tract, they are rapidly hydrolysed by nonspecific esterases into ampicillin. The advantage these esters have over ampicillin is their more complete and faster absorption from the GI-tract.

3.1.2. Pivampicillin

The bioavailability of ampicillin following oral dose of pivampicillin has been estimated to be 82-92%[47]. It is the pivaloyloxymethyl ester which readily hydrolyzes to ampicillin, formaldehyde and pivalic acid in the presence of non-specific esterases. Half life for hydrolysis in homogenates of human gastric and intestinal mucosa is about 5 minutes [48].

Because of the difference in bioavailability, serum activity after pivampicillin tends to be 2-3 times higher that after an equivalent oral dose of ampicillin, while urinary recoveries tend to approach those following a parenteral dose of ampicillin [49].

3.2. Prodrugs of Angiotensin-Converting Enzyme Inhibitors

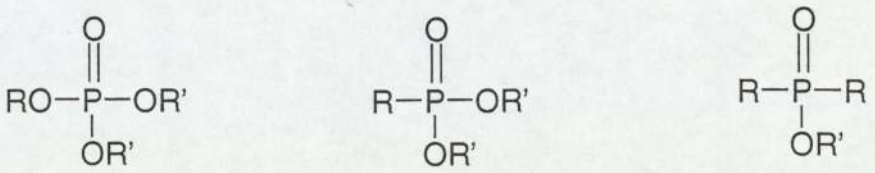
Angiotensin-converting enzyme inhibitors are traditional dipeptide analogues, the bioavailability of their oral administration is very low, which has been introduced above. The ACE-inhibitors emerged as an important class of antihypertensive agents for the treatment of high blood pressure and congestive heart failure. Furthermore, the improvement of the bioavailability of this type of drugs is very important. Enalapril and phosphorus-containing drugs will be introduced in the following section.

3.2.1. Prodrugs of Phosphates, Phosphonates and Phosphinates

The delivery problem of drugs containing a phosphate, phosphonate, or phosphinate-containing functional [50] group exists in the absorption. The general structures of phosphate, phosphonate, and phosphinate-containing drugs are shown below (**Fig. 1.11**) [50].

Fig. 1.11 General structure of phosphate, phosphonate, and phosphinate drugs.

Phosphate phosphonate phosphinate



Drug: R= organic residue; R'= H or (-)

Prodrug: R= organic residue; R' = promoeity

In phosphorus-containing type of angiotensin-converting enzyme inhibitors, there are two important drugs fosinopril (**Fig. 1.12**) and ceronapril (**Fig. 1.5c**) are phosphinate and phosphonate types respectively.

Fig. 1.2 Phosphinate Drug — Fosinopril

Fosinopril

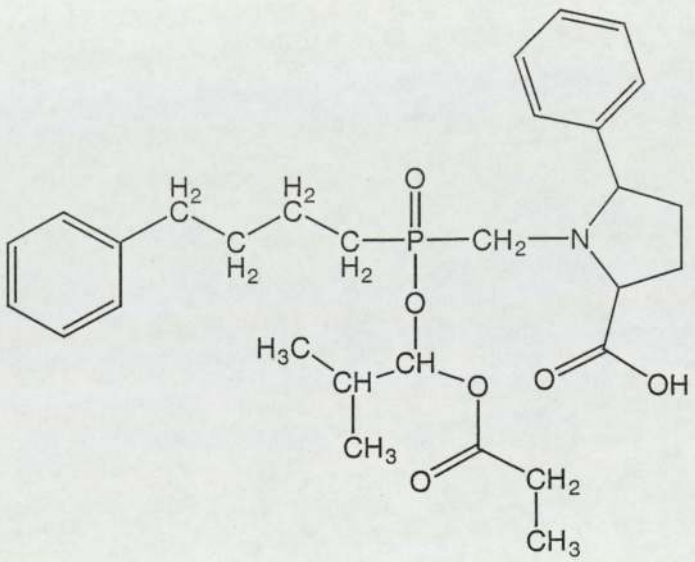
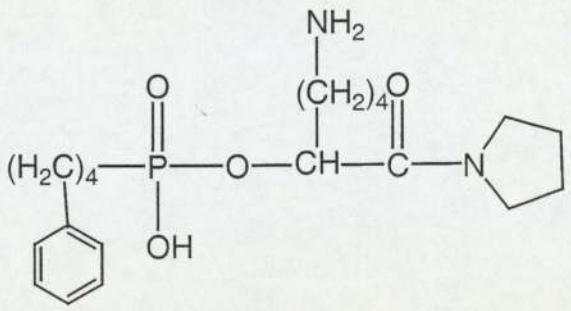


Fig.1.5c Phosphonate Drug — Ceronapril (SQ 29851)

Ceronapril



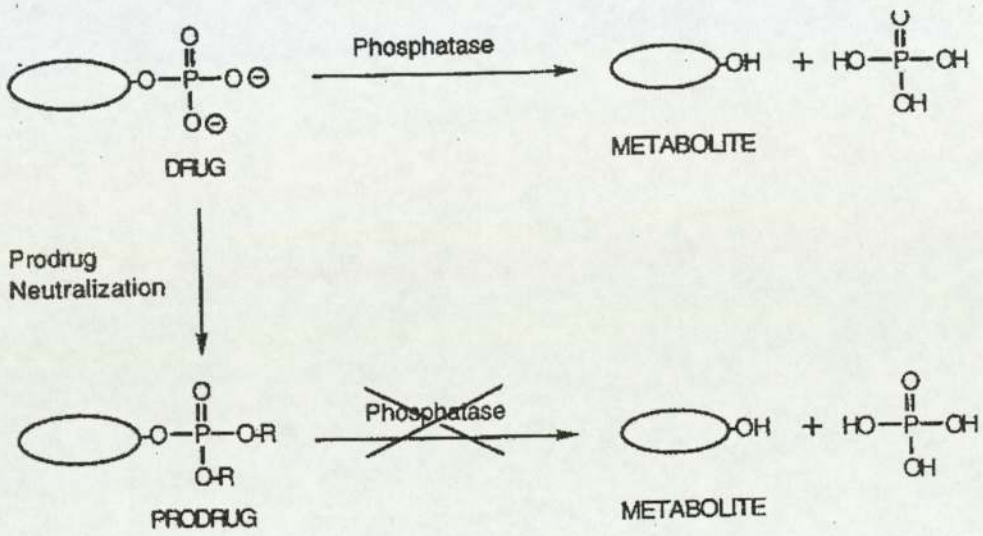
Phosphate, phosphonate and phosphinate groups carry an anionic charge (mono- or di-) at nearly all physiological pH values making them very polar. These highly ionised species do not readily undergo passive diffusion across cellular membranes and tissues. Possibly, the non-modified molecules alter the distribution/elimination pattern of the parent drug.

However, the neutralised chemical derivatization of ionizable phosphate, phosphonate and phosphinate-containing functional groups could overcome these shortcomings. Generally, the phosphorus-coupled oxygen(s) is neutralised to form neutral ester(s). The molecules with neutral esters have better access to cells and tissues because of the increased lipophilicity.

Phosphates, phosphonates, and phosphinates-containing agents exhibit a low volume of distribution, because, they tend to be subject to polarity. In addition to renal clearance, phosphates containing drug, primary alcohols and phenols are substrates for many phosphorylases present in the body which readily clip the phosphate group from the drug; phosphonates and phosphinates have the advantage of being more chemically stable and showing essentially no enzymatic liability [50]. The neutral ester drug is very important for

phosphates because it could disguise the phosphate from enzymes thereby altering the apparent elimination and half-life (**Fig. 1.13**).

Fig. 1.13 Scheme showing the possible advantages of prodrugs in altering the phosphatase cleavage of phosphate-containing drugs [50].



3.2.2. Enalaprilat and Its Prodrug — Enalapril

Enalapril was the second orally active angiotensin converting enzyme inhibitor drug (captopril is the first orally active ACE inhibitor) to become widely available for therapeutic use. Enalapril is administered as a maleate salt and is the monoethyl ester of enalaprilat, an active ACE-inhibitor which is poorly absorbed (only about 10%) from the GI tract [51].

Enalapril (**Fig. 1.5b**) is a lipid-soluble and relatively inactive prodrug with good oral absorption (60-70%) from the gastrointestinal tract [51], a rapid peak plasma concentration (1 hour) [51] and rapid clearance (undetectable by 4 hours) by de-esterification in the liver to a primary active diacid metabolite—enalaprilat [51]; this further differentiating feature is that enalapril

is a prodrug. The parent drug has an elimination half-life of about 2 hours [51]. Unchanged enalapril and enalaprilat are excreted both in urine and faeces, with the urinary route predominating [52].

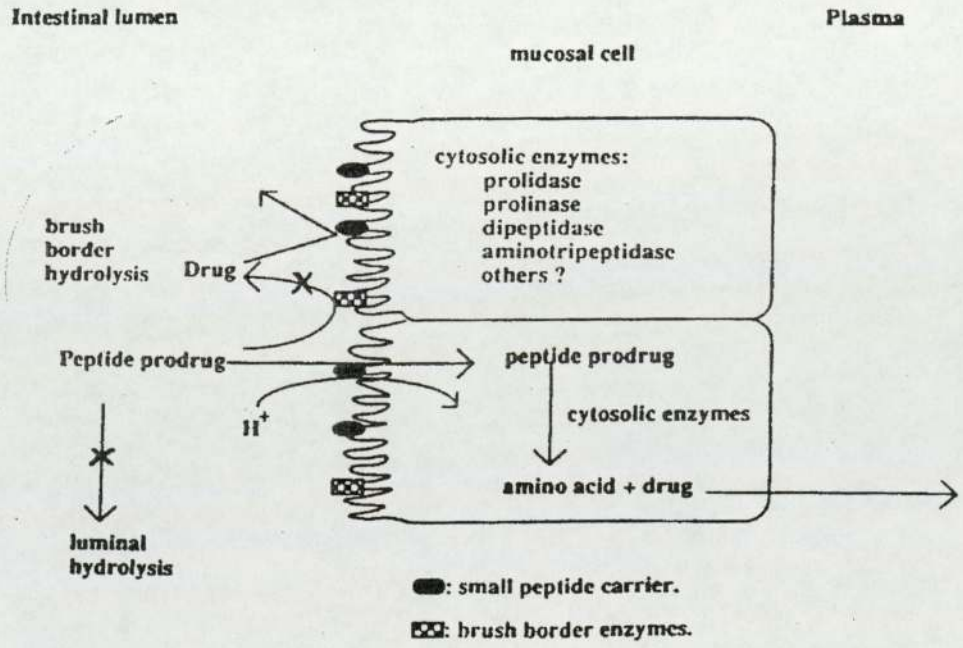
The oral bioavailability of enalapril is about 53-74% (based upon urinary recovery of enalapril and enalaprilat), whereas the bioavailability for enalaprilat is about 36-44% when intravenous enalapril is used as the reference standard [53, 54].

3.3. L-α-Methyldopa

L-α-Methyldopa is a poorly absorbed antihypertensive agent and an amino acid analogue. High inter- and intra-subject's variation (8-62%) in oral bioavailability was observed [55]. Due to its structural similarity to amino acids, the low and variable bioavailability was thought to be due to its complicated absorption via various amino acid-mediated transport systems in the intestine, and amino acid carriers are structurally restrictive [56]. The α-methyl group severely hinders the binding of the substrate to the carrier (similar to α-amino-isobutyric acid). It has been revealed that certain dipeptide-mediated carrier transport systems are responsible for the intestinal absorption of orally absorbable amino-β-lactams [57]. Most of amino-β-lactams are tripeptide mimetics containing D-phenylglycine or D-P-hydroxyphenylglycine. Since the carrier systems showed broad specificity with less structural requirement for the substrates [36], it is therefore rational to speculate that other di- or tripeptide mimetics containing these amino acids will also be absorbed via the transport systems.

Amino acid carriers are structurally restrictive, so peptide analogues were made to improve L-α-methyldopa's absorption, which could use the peptide carrier (**Fig 1.14**) [20]. The further reasons of this synthesis are: firstly, the peptide carrier has less structural requirement, and generally, it is more efficient than the amino acid carrier(s); secondly, most peptides are hydrolyzed to their constituent amino acids before entering the systemic circulation; and thirdly, the derivatizing groups, amino acids, are natural substances with low toxicity potential [58].

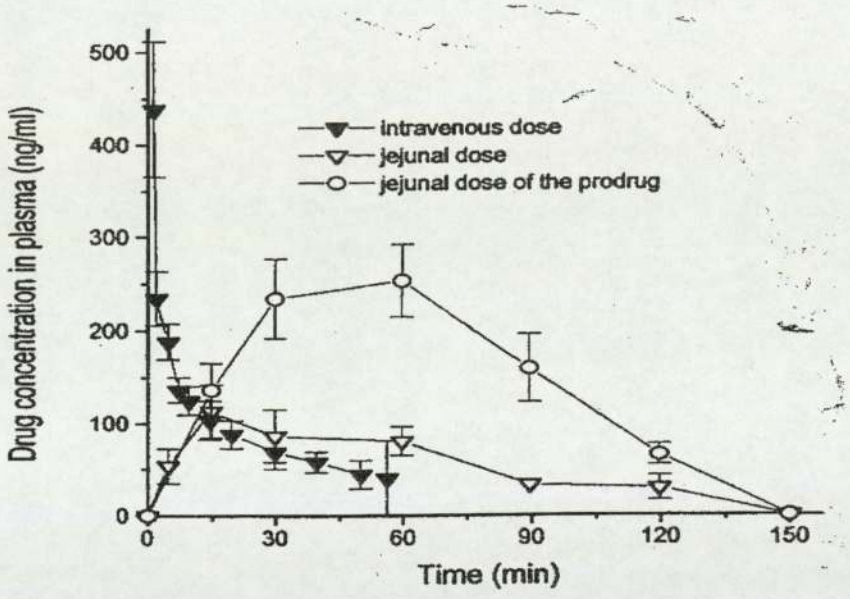
Fig. 1.14 Schematic representation of a peptide prodrug strategy for improving oral absorption [20].



Permeabilities of the dipeptide prodrugs were more than 10 times higher compared to the parent compound, L-α-Methyldopa [58]. The uptake of L-α-Methyldopa-Phe and Phe- L-α-Methyldopa was inhibited by addition of dipeptides and cephradine [58, 59]. The parent compound is released in homogenates after intestinal hydrolysis of dipeptidyl prodrugs. Other studies

showed that there was an increase rate of conversion of L-α-Methyldopa after administrating the prodrugs in rats (**Fig. 1.15**) [60]

Fig.1.15 Plasma profile of L-α-methyldopa following intravenous dose of L-α-methyldopa and jejunal dose of L-α-methyldopa-phenylalanine and L-α-methyldopa (n=6-7).



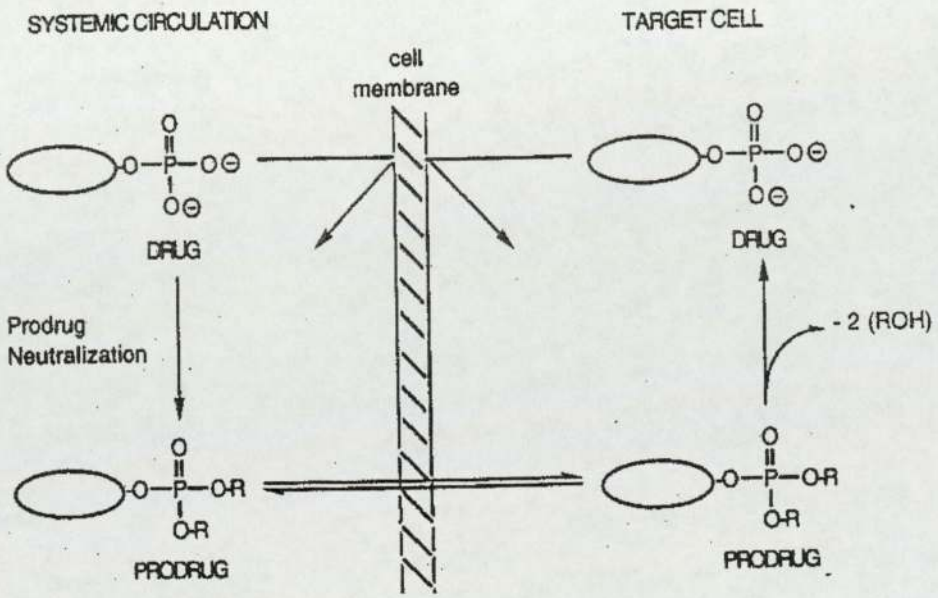
L-α-Methyldopa-Pro is absorbed via the peptide carrier and hydrolyzed by cytosolic prolidase, a potential peptide-prodrug converting enzyme [61].

In conclusion, Targeting the peptide transporter, with its broad specificity, in order to achieve significant oral efficacy of di-/tripeptide drugs is an appealing stategy [62]. The principal goal of modification to make prodrugs is alteration of membrane permeability to improve oral (GI permeability), brain, tumour and cellular delivery of these agents [48]. 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 being suitable for various pH environments in the GI

tract, adequate solubility for dissolution in the 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. Even better, 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 (**Fig 1.16**) [50]. The figure takes phosphate-containing drug as an example.

Fig. 1.16 Scheme. Proposed scheme illustrates the potential advantages of prodrugs over the parent molecule for intracellular targeting. [49]



Moreover, the rate of bioreversion is a very important factor in prodrugs for improved oral delivery and prodrugs for improved cell targeted delivery. Because on one hand, if bioreversion is very fast and non-specific, prodrug reversion may take place before the limiting barrier is overcome. On the other

hand, if reversion is slow and inefficient at all sites, the prodrug may readily reach the site of action but never release enough parent drug to elicit a pharmacological response.

CHAPTER 2 –DISCUSSION OF PEPTIDE CHEMISTRY

The main orally active peptidomimetic drugs are the widely used, β-lactam antibiotics and angiotensin-converting enzyme inhibitors (ACE-inhibitors). Both of these peptidomimetic drugs possess certain structural features of a peptide, including the peptide bond and free carboxylic group. β-Lactam antibiotics and ACE-inhibitors are both subject to carrier-mediated transport, and the dipeptide transport system is involved in their transport. Some of these drugs are used as probes to study the dipeptide transport system. Among these drugs, Ceronapril (SQ-29852) which is phosphinyloxyacyl proline is an ideal probe to study dipeptide transport (will be introduced in the biological introduction). Therefore, based on this result, more prolinyl dipeptides were synthesised to find which are the best inhibitors.

In this chemical introduction, the formation of the dipeptidyl bond, and the protection of functional groups will be introduced respectively.

1. Formation of Peptide Bond

Peptide bond forming procedures involving the generation and aminolysis of reactive carboxy derivatives (**Fig. 1**) have been employed in peptide synthesis.

Activation-*i.e.* The attachment of a leaving group to the acyl carbon of the carboxy component, to enable the nucleophilic attack of the amino group (of the amino acid to be acylated) is necessary, because ordinary carboxylic acids simply form salts with amines.

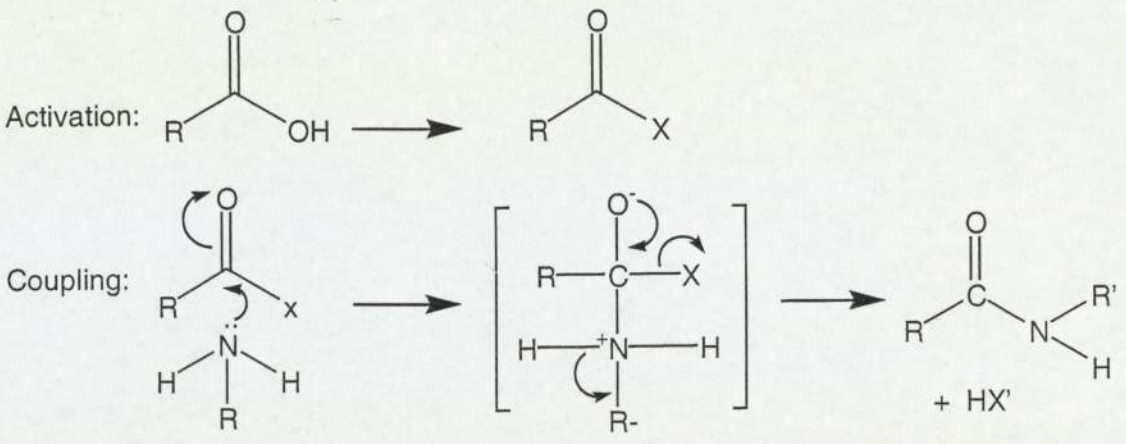


Fig. 1

There are three different ways of coupling along the lines of **Figure 1**. [63]

(I). A reactive acylating agent is formed from the carboxy component in a separate step or steps, followed by immediate treatment with the amino group;

(II). An isolatable acylating agent is formed separately;

(III). The acylating intermediate is generated in the presence of the amino group, by the addition of an activating agent to a mixture of the two components.

The activation in (I), (II), and (III) is usually achieved by reaction of the carboxy component with an electrophilic reagent, either by addition [64] or by substitution [65].

Different ways (activation and coupling) to make the peptide bond will be introduced in the following section.

1.1. The Acid Chloride (Fluoride) Method

The chlorine atom becomes an obvious choice for the activation of acylamino acids by conversion to corresponding acyl chlorides due to its electron-

withdrawing ability. The traditional reagents used for acyl chloride formation (thionyl chloride, phosphorus pentachloride) are too vigorous, so this method remained limited:

(I). Phosphorus pentachloride is chosen to react with the carboxyl group of an amino acid in the cold (**Fig. 2**) [66]. While the HCl generated readily escapes, the acid chloride mixes with the less volatile phosphorus oxychloride [66].

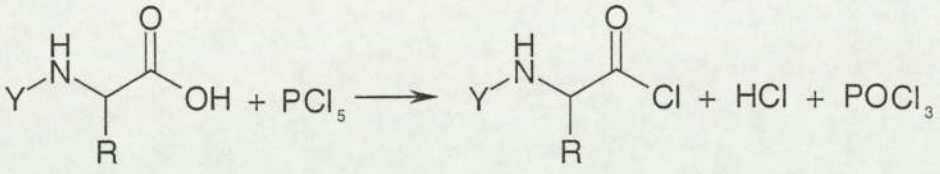


Fig. 2

(II). The use of thionyl chloride ($SOCl_2$) seems better since both by-products (HCl and SO_2) are volatile (**Fig. 3**) [67].

These two reagents are too vigorous to be compatible with complex or sensitive substrates and, furthermore, intramolecular nucleophilic attack results in acylamino acid chlorides cyclizing to give 'oxazolones' (**Fig. 4**) and hence racemic peptides. Z-Amino acid chlorides are isolable but unstable, decomposing to N-carboxyanhydrides. But the oxazolone formation is not easy when the acyl substituent is an alkoxycarbonyl protecting group. Furthermore, the alkoxyoxazolones (**Fig. 5**) are both less easily racemized and more easily aminolysed than are the oxazolones (**Fig. 4**) derived from simple acylamino acids. The discovery of Fmoc amino acid chlorides and also fluorides altered the position of this method [68]. These reagents are easily prepared and stable. Acyl fluorides are more stable than acyl chlorides to

neutral oxygen nucleophiles, and tertiary bases, but both of them show high reactivity to the amino group.

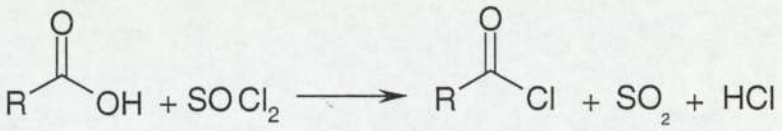


Fig.3

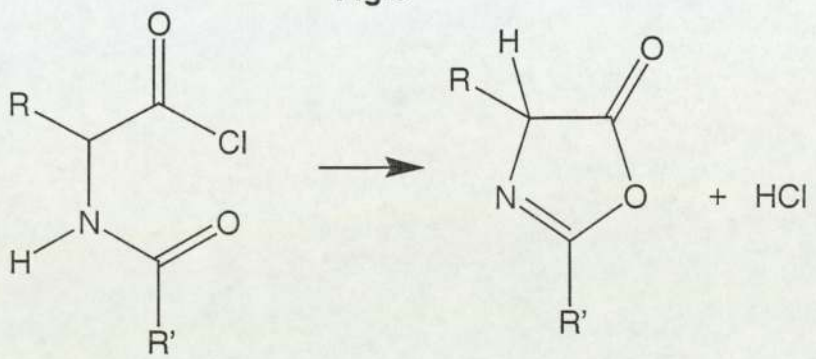


Fig. 4

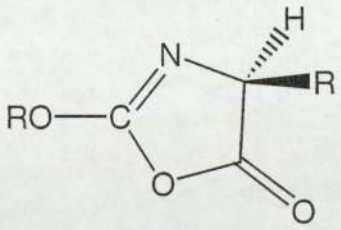


Fig. 5

1.2. The Acid Azide Method

The activation in the form of acid azides is a powerful and practical approach for the formation of a peptide bond, which was introduced by Curtius [69] at the turn of this century, but is still an important procedure [70, 71].

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 (**Fig. 6**) at around or just below 0°C, 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 [72].

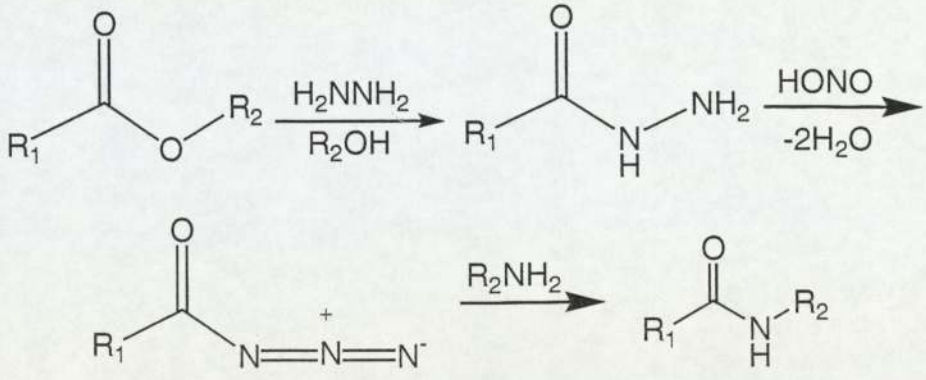


Fig. 6

Recently, the direct conversion of carboxylic acids to acid azides by treatment with diphenylphosphoryl azide (**Fig. 7**) has become a viable alternative method.

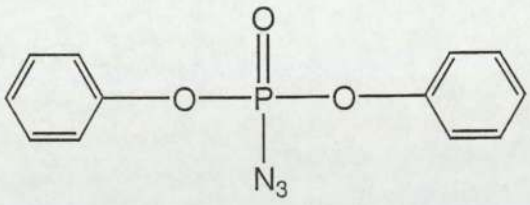


Fig. 7

1.3. Anhydrides Method

Symmetrical Anhydrides, acylamino acid symmetrical anhydrides can be prepared with corresponding symmetrical anhydrides of carboxylic acid by use of a variety of reagents, including DCCI (dicyclohexylcarbodiimide) (see section on coupling reagents) [73]. However, aminolysis of a symmetrical anhydride is a wasteful process, because in this acylation, only one of two molecules of carboxylic acid is incorporated into the product while the other is regenerated but usually not recovered (**Fig. 8**).

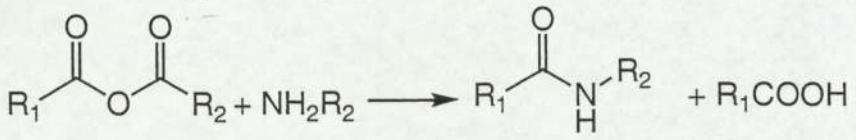


Fig. 8

Mixed anhydrides with carboxylic acid was presumbably involved in the classic synthesis of benzoyglycylglycine by Curtius [74]. The problem of this method is that the anhydride has two similar electrophilic sites and can therefore undergo aminolysis ambiguously. However, the selectivity could be improved by steric hindrance (**Fig. 9**), e.g. $-CH_2CH(CH_3)_2$, and inductive depression of electrophilicity, so as to direct the attack to the carboxy component carbonyl [75].

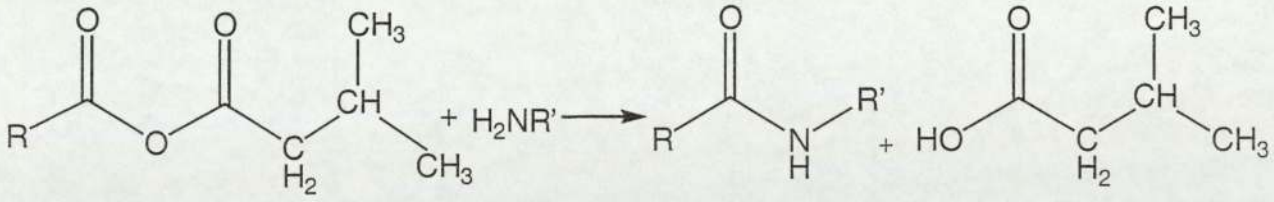


Fig. 9

The second possible acylation product, an isovalerylamide (**Fig. 10**) is often produced in negligible amount, and can be isolated as crystalline materials.

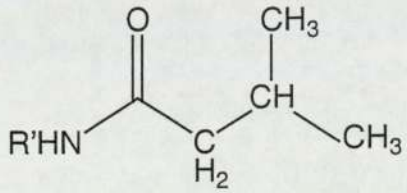


Fig. 10

Mixed anhydrides with carbonic acids, the most generally successful mixed anhydride method involves the generation and aminolysis of a carboxylic-carbonic anhydride [76], as outlined in **Figure11**.

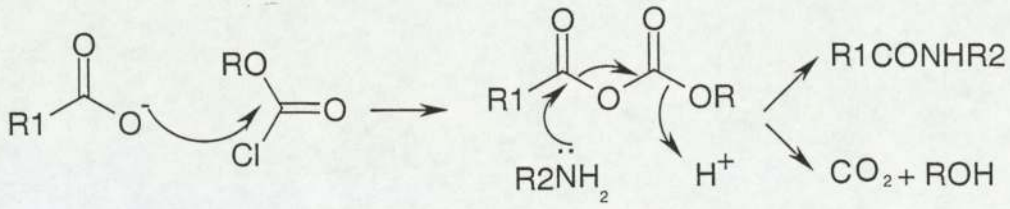


Fig. 11

A further enhancement of combined steric-electronic effects is found in trimethylacetic acid (pivalic acid) mixed anhydrides (**Fig. 12**). Ethylcarbonic acid mixed anhydrides were used in the peptide synthesis, but many prefer isobutylcarbonic acid mixed anhydrides.

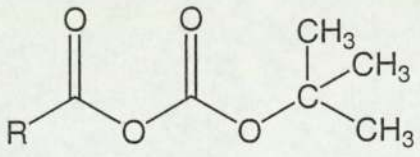


Fig. 12

One of the carbonyl groups in the activated intermediate is flanked by two oxygen atoms which diminishes its reactivity, so the nucleophilic attack is directed toward the carbonyl of the original carboxy component. Alkoxycarbonyl derivatives of the amino component are not usually formed in more than trace amounts, except with hindered components [77].

1.4. Active Esters

In the development of peptide synthesis methodology, active esters play an important role, since in the ester, there is only one electrophilic centre, and 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 by DCCI-mediated coupling [78] between a protected amino acid and the ester moiety.

The introduction of the negative group in the ester renders the carbon from the carbonyl group more electrophilic. Therefore it is easily attacked by the nucleophiles (the amino group from the amine component). For example, methyl esters could be activated by the electron-withdrawing group—cyanomethyl ester (**Fig. 13**) [79].

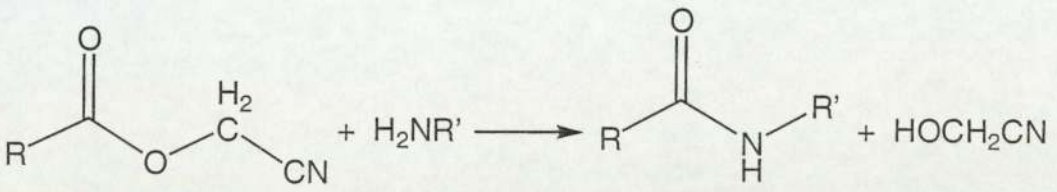


Fig. 13

In 1973, the major improvement of the discovery of the catalytic effect of 1-hydroxybenzotriazole on the aminolysis of active esters has become more important [80]. The reaction could be rationalised by the assumption of a ternary complex among active ester, amine and catalyst (**Fig. 14**) [80].

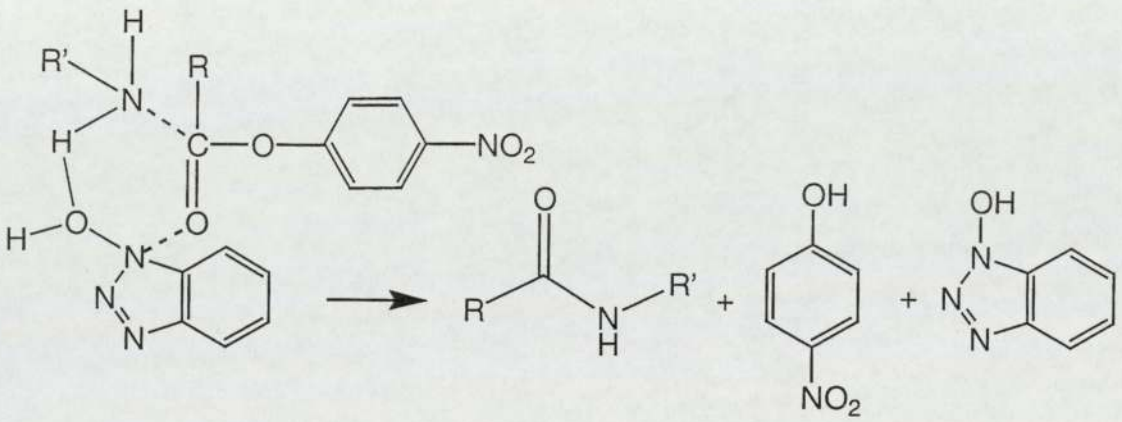


Fig. 14

The addition of tertiary amines suggests that base catalyzed trans-esterification occurs(**Fig. 15**).

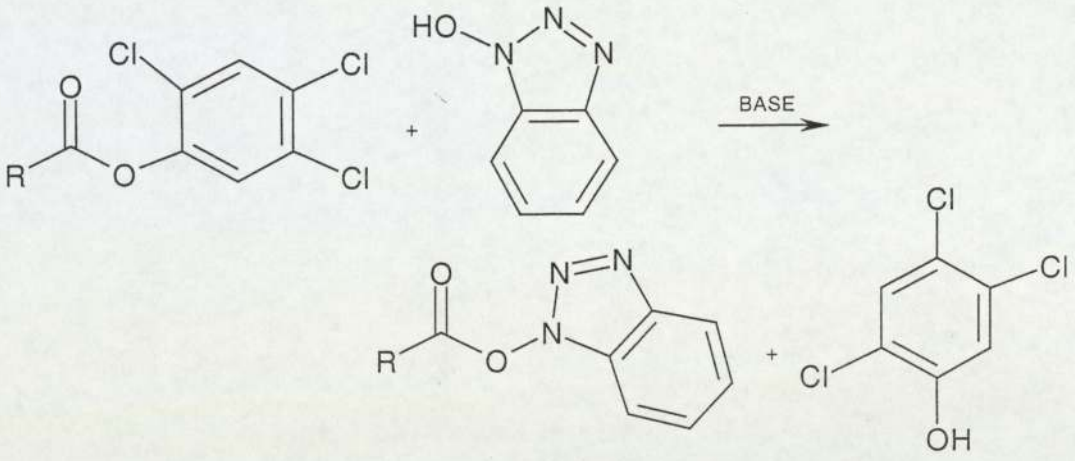


Fig. 15

Esters of N-hydroxysuccinimide ester are highly reactive and the desired peptide easy to get (**Fig. 16**). Since N-hydroxysuccinimide is very water-soluble, the purification for the product is facilitated [81].

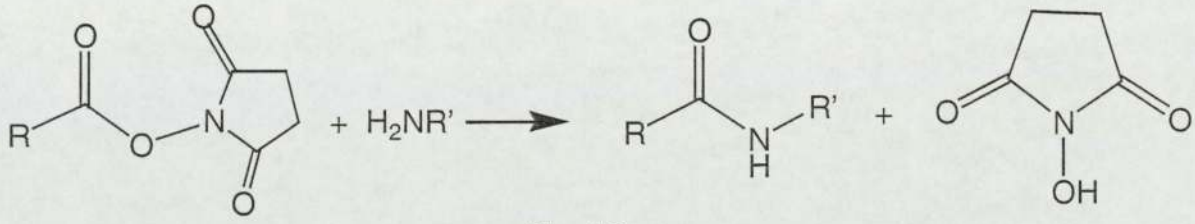


Fig. 16

1.5. Coupling Reagents

Dicyclohexylcarbodiimide (DCC, DCCI) has been the important reagent for activating carboxy groups in the peptide synthesis since Sheehan and Hess reported their results in 1955 [82]. The coupling reagents were added to the mixture of the carboxyl component and the amine component. Amines react with carbodiimides (yielding guanidine derivatives) slowly (**Fig. 17**), but the reaction rate of this could be negligible compared with the rapid rate of the

addition of carboxylic acids to carbodiimides (**Fig. 17**) to give O-acyl-isoureas which are potent acylating agents. O-Acylisourea leads to peptide by immediate aminolysis or symmetrical anhydride. The reason is that the N=C group provides powerful activity leading to coupling with the concomitant formation of dicyclohexylurea (**Fig. 18**).

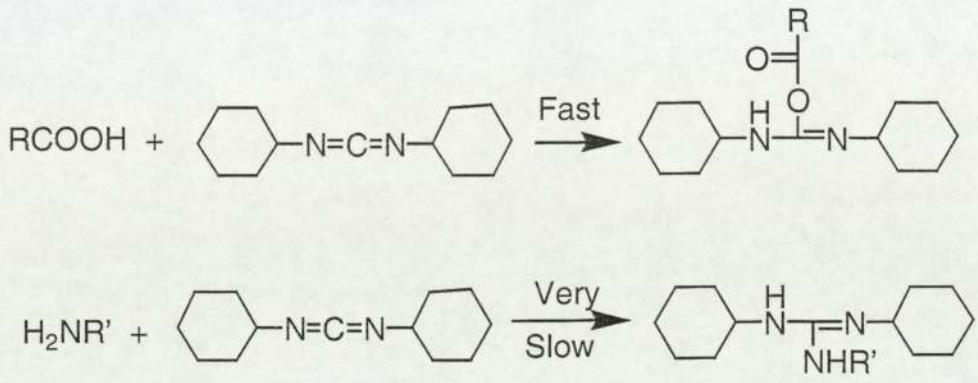


Fig. 17

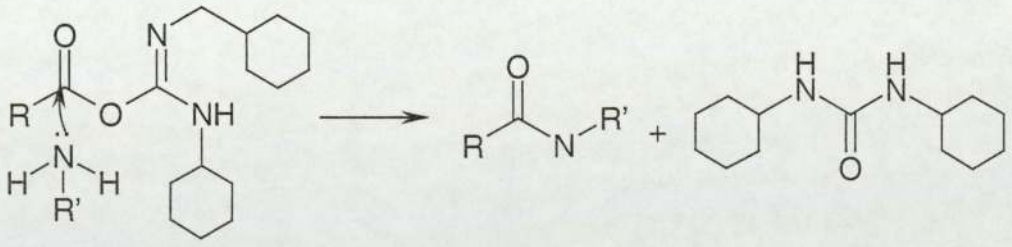


Fig. 18

There are two obvious advantages in this method, one is the speedy execution of activation, the other one is the insoluble by-product, N, N'-dicyclohexylurea (DCU) which can be removed simply by straight-forward filtration in solution synthesis. However, the nucleophilic centre on O-acylisoureas competes with the amine component for intramolecular acyl transfer. This competition leads to the formation of inactive by-product, N-acylureas (**Fig. 19**), reducing the yield; this can be formed by an intermolecular reaction between the urea and a symmetrical anhydride in DMF.

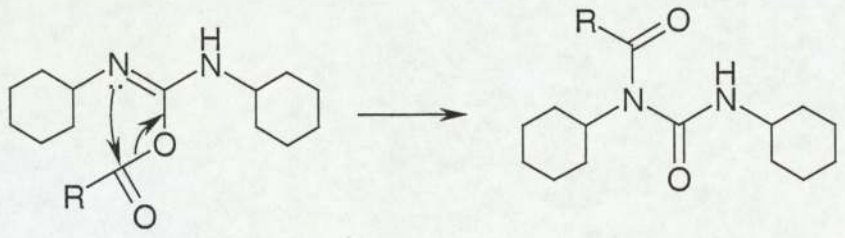


Fig. 19

Both racemization and N-acylurea formation can be suppressed by the addition of auxiliary nucleophiles which are able to react very rapidly with the O-acylisourea before side-reaction happens. Numerous possible additives can be used, *N*-hydroxysuccinimide was the first [81], but 1-hydroxybenzotriazole (**HOBt**) has been the one most regularly used so far [83]. The scheme for this reaction is shown in **Figure 20**. The presence of a second nucleophile in the reaction reduces the concentration of *O*-acylisourea. It should be noted that HOBt is regenerated during acylation, hence its concentration remains almost constant during coupling.

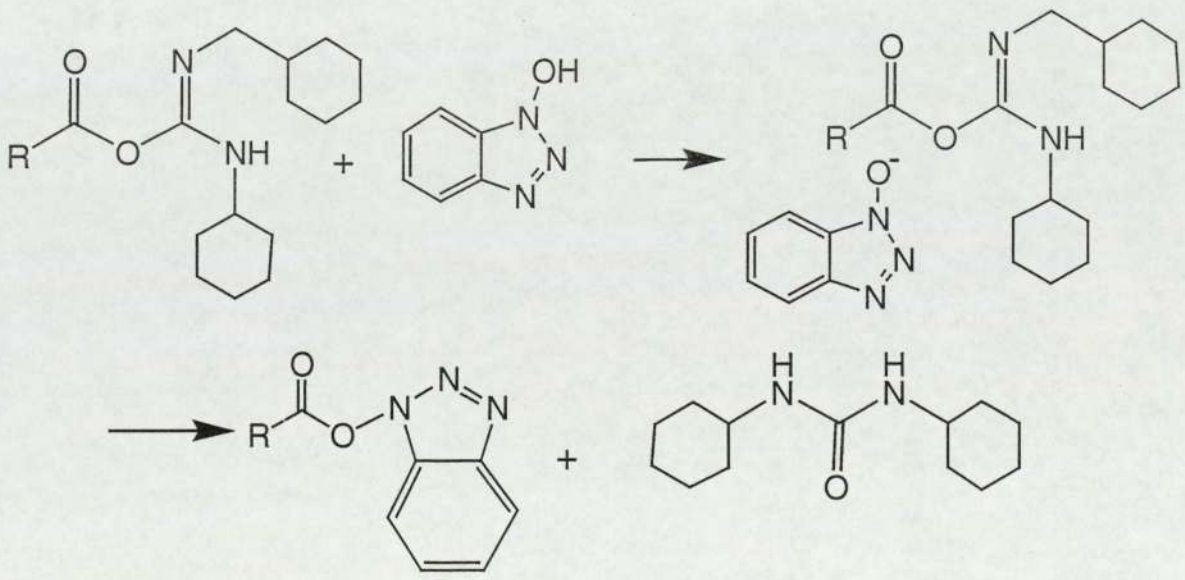


Fig. 20

1.6. Phosphonium Reagents

Acyloxyphosphonium species, which can be generated by the attack of carboxylate anions on suitable phosphonium cations, react readily with nucleophiles at the acyl carbon (**Fig. 21**) [84].

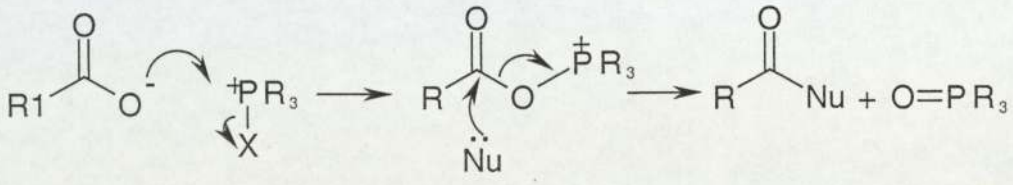


Fig. 21

The most important of these reagents is Castro's [85] benzotriazolyloxy-tris-(dimethylamino)-phosphonium hexafluorophosphate, (Bop reagent) (**Fig. 22**). Acyloxyphosphonium species react with nucleophiles at the acyl carbon, and salts of such cation could be used as direct coupling reagents. For coupling, an equivalent of the reagent is simply added to a 1:1 mixture, in a suitable inert solvent, of the amino and carboxy components, together with tertiary base, to ensure that the latter is in its anionic form.

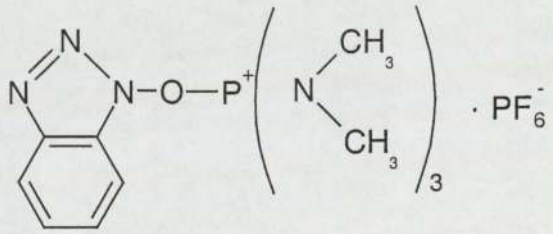


Fig. 22

The important reaction for the use of this reagent is via the ester of HOBt (**Fig. 23**) [63]. As the initial step is anion-cation reaction, it is favoured in the less polar solvents. There is a safety consideration about BOP, in that its co-product is hexamethylphosphoramide, a highly toxic substance. For this

reason, the closely related salt, the PyBop reagent (**Fig. 24**) was developed by Castro [86].

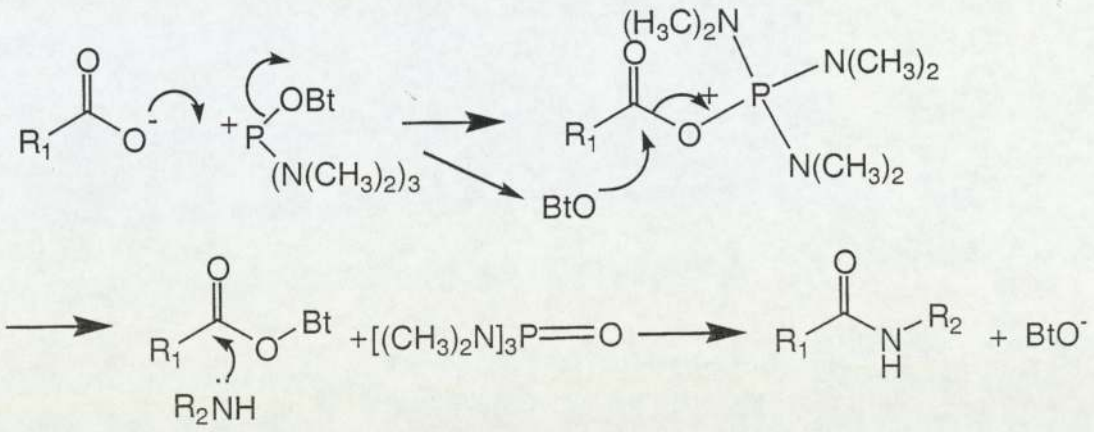


Fig. 23

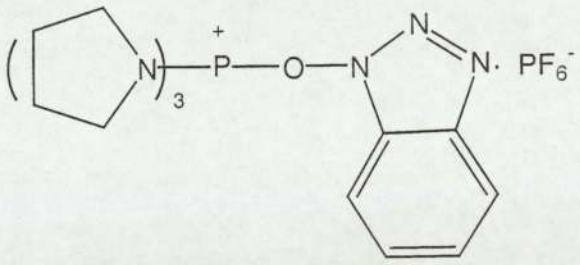


Fig. 24

In short, there are some criteria for a coupling reagent.

(i) It should be inert toward the amine component,

(ii) It should not generate the reactive intermediate containing a nucleophilic centre, because this can compete with the amine component for the acyl group,

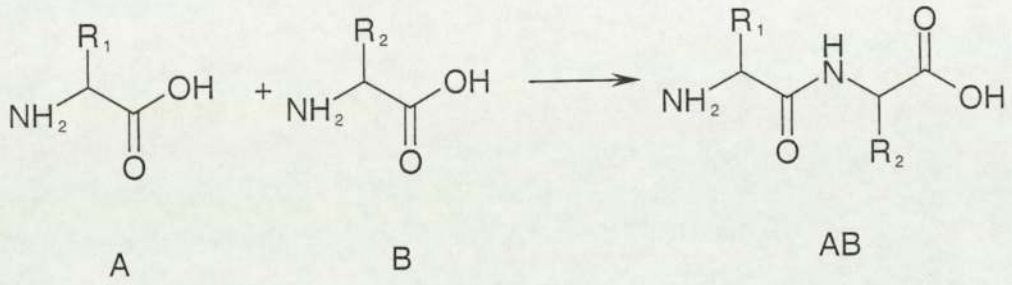
(iii) It should not cause over activation which could lead to side reactions and hence to by-products.

In conclusion, an ideal chemistry would allow peptide bond formation to be carried out rapidly and quantitively under mild conditions, avoiding side-

reactions, without challenging the integrity of adjacent chiral centres, and generating easily removed co-products.

2. Functional Group Protection

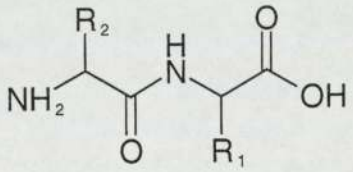
In the preparation of peptides, some functional groups should be blocked, for example (**Fig. 25**):



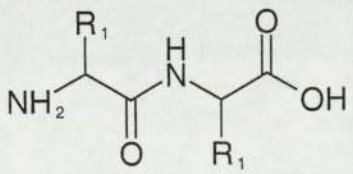
A B AB

Fig. 25

In this reaction, when the carboxyl group in A is activated, it will acylate with B and dipeptide AB will be produced. However, the -NH_2 group in unreacted A could react with the activated A to form AA (**Fig. 26**). 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.



BA



AA

Fig. 26

2.1. The Protection of the Amino Group

It is necessary to block the amino functional group of the amino acid during the activation of the carboxyl group participating in the subsequent coupling. It is equally important to ensure that the group is easily removed, otherwise the formed peptide bond is endangered. Normally, blocking of the α-amino function is generally transient while that of all other functions mostly semipermanent (**Fig. 27**). Chemists realised that the amine-protecting group from organic chemistry is not suitable for peptide chemistry. For example, acylation and benzoylation of amine groups is impractical, because the vigorous hydrolysis for deacylation cleaves a peptide bond as well. Therefore, the protecting group must stay firmly at its post as long as it is needed; and when the protection is done, it should leave the amino group quietly and quickly.

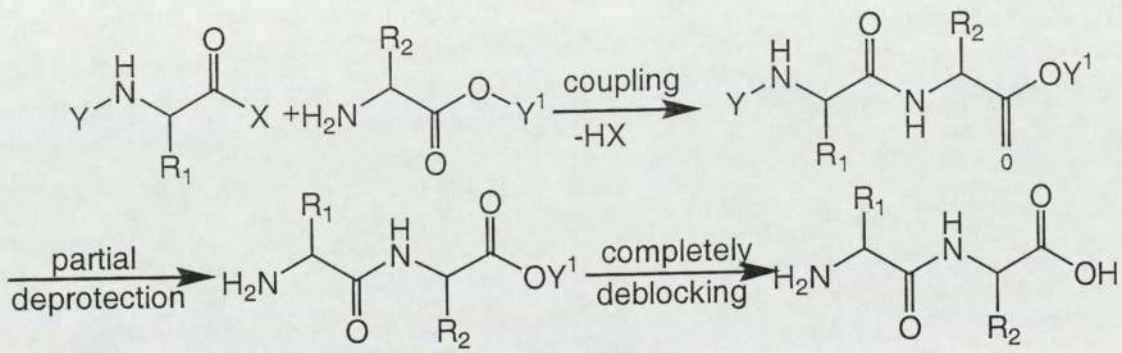


Fig. 27

2.1.1. Benzyloxycarbonyl (*Z*) protection

It was an important invention when in 1932 Bergmann and Zervas proposed the replacement of ethoxycarbonyl group by the benzyloxycarbonyl (***Cbz or Z***)

(**Fig. 28**) group [87], as the latter is easily and specifically removed by hydrogenation (**Fig. 29**).

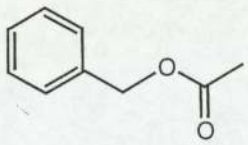


Fig. 28

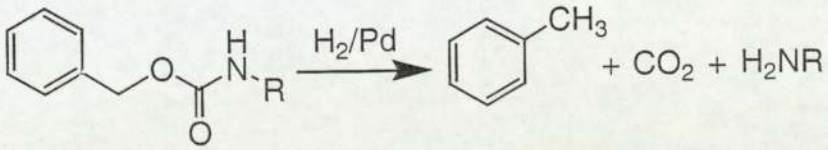


Fig. 29

Hydrogenolysis of the benzyl ester is catalyzed by palladium. Normally, it is applied as palladium black or palladium on charcoal or palladium on barium sulfate. The by-products produced, toluene and carbon dioxide, are removed easily. Thus, this classical method of hydrogenation in the presence of palladium on charcoal still remains popular in many laboratories.

In the introduction of benzyloxycarbonyl (***Z***) group, the classical procedure is acylation with the appropriate chloroformate (**Fig. 30**) [88].

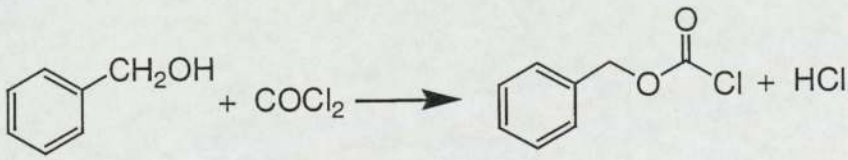


Fig. 30

Then, the purified benzyl chlorocarbonate is added into the aqueous solution of the amino acid in the presence of alkali (**Fig. 31**) and the blocked amino acids are obtained by acidification followed by purification.

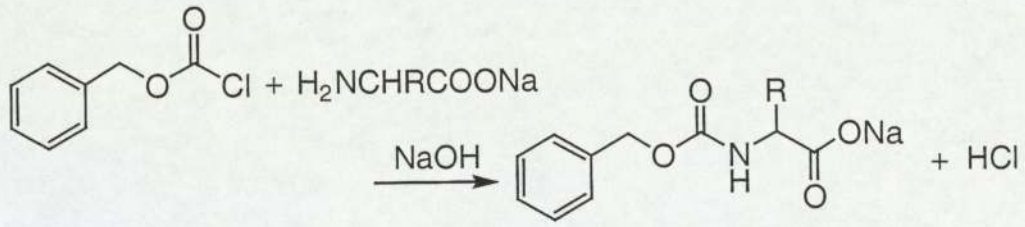


Fig. 31

There are some other reductive methods which could be used as well, such as sodium in liquid ammonia.

The benzyloxycarbonyl (***Z***) group can also be removed by acidolysis, with HBr in acetic acid [89]. It is very easy to carry out, because most protected peptides are soluble with ease in acetic acid, especially in the presence of a high concentration of HBr. In the heterolytic fission of carbon-oxygen, benzyl cation is important in facilitating the decomposition of the protonated intermediate (**Fig. 32**).

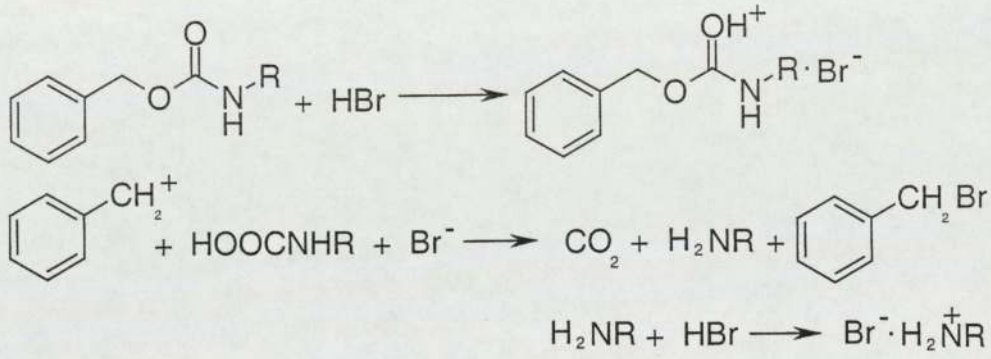


Fig. 32

Furthermore, increasing the acidity and/or polarity accelerates the cleavage, liquid HF and HBr in trifluoroacetic acid both remove Z groups rapidly [63]. The sensitivity of the benzyloxycarbonyl group can be increased by some modification of the benzyl group. Electron releasing substituents in the

aromatic ring by increasing the stability of benzyl cations, can also affect the rate, accelerating the cleavage; hence the *p*-methoxybenzyloxycarbonyl group (**Fig. 33**) could be cleaved by weaker acids, e.g. diluted solution of HCl in acetic acid and also by trifluoroacetic acid [90].

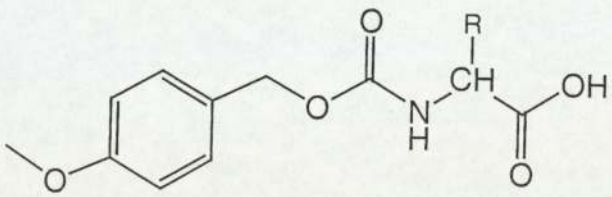


Fig. 33

Catalytic hydrogenolysis (**Fig. 34**) of ***Z*** groups could be achieved with a variety of catalysts and conditions of which 80% acetic acid/ ambient temperature and pressure/10% palladium on charcoal is one. But catalyst-poisoning by divalent sulphur is a problem [63]

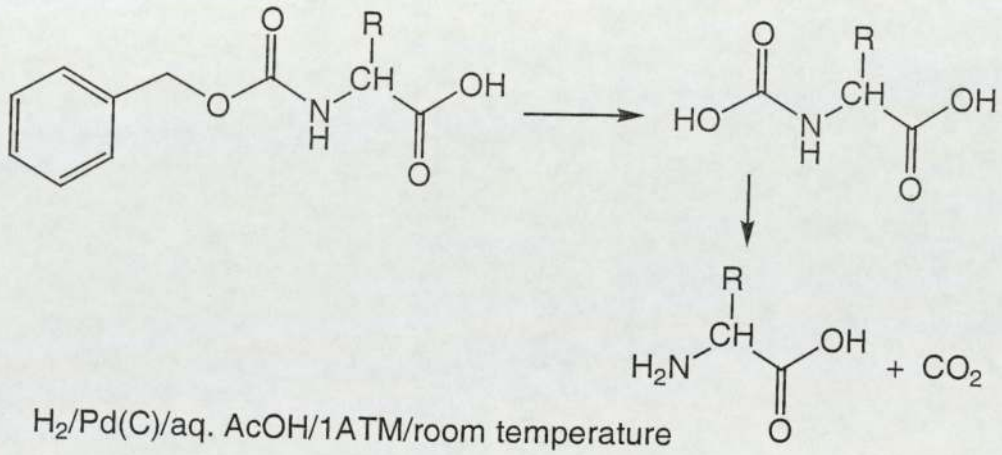


Fig. 34

2.1.2. *t*-Butoxycarbonyl (*Boc*) Protection

The *tert*-butyloxycarbonyl group (**Fig. 35**) [91], source of the stable *tert*-butyl cation has become a very popular blocking group for the amine function. The

Boc group is completely stable to catalytic hydrogenolysis conditions. Basic and nucleophilic reagents have no effect on the Boc group, it is better than that of the ***Z*** group.

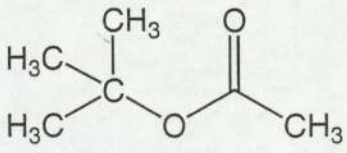


Fig. 35

In the preparation of Boc-blocking amino acid, *tert.*-butyl-pyrocarbonate, usually described as *tert.* butyl dicarbonate or *Boc*-anhydride, is successful, mainly because the by-products formed during the introduction of *Boc* group, are easily removed (**Fig. 36**), and *tert*-butyl chloroformate is unstable.

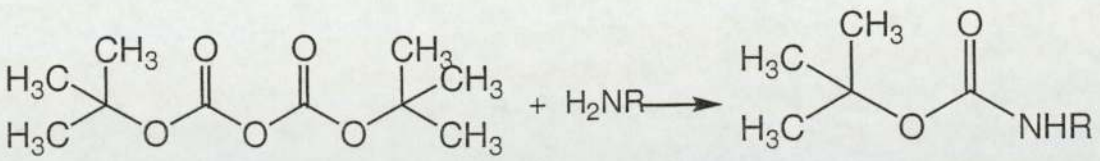


Fig. 36

The Boc group removal is conveniently carried out by trifluoroacetic acid, either neat or diluted with dichloromethane followed by flooding with ether or evaporation; these are mild and reliable conditions (**Fig. 37**).

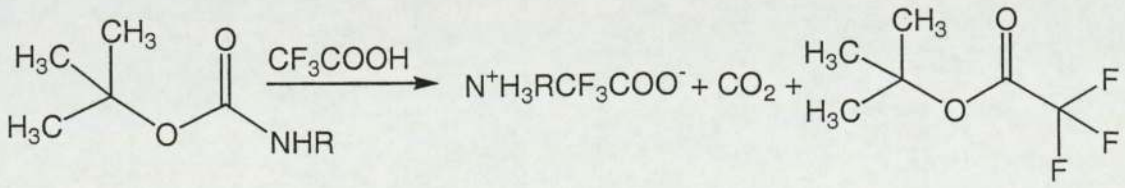


Fig. 37

2.1.3. 2-(4-Biphenylyl)-isopropoxycarbonyl (*Bpoc*) Protection

Furthermore, biphenylylisopropyloxycarbonyl (*Bpoc*) [92] (**Fig. 38**) group is even more acid-labile than Boc, because the corresponding carbonium ion is not only tertiary but is also further stabilised by the biphenyl substituent. It can be removed by chloroacetic acid-dichloromethane mixture at ambient temperature-conditions. Bpoc is stable to bases and nucleophiles, but, like the Z group, it is cleaved by catalytic hydrogenolysis.

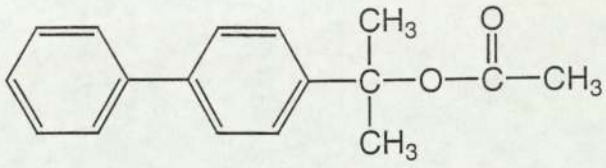


Fig. 38

2.1.4. 9-Fluorenylmethoxycarbonyl (*Fmoc*) (Fig. 39) Protection

The *Fmoc* group [93] is very stable to acidic reagents, but is cleaved under certain basic conditions. Piperidine (20% in DMF) is the routine reagent, and the deprotection only takes a few seconds at room temperature [94]. Deprotection with piperidine happens quickly. This deprotective procedure does not affect ***Z*** or ***Boc*** groups.

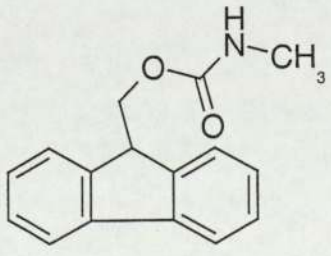


Fig. 39

2.2. Protection of the Carboxyl Group

There are two important reasons to block the carboxyl group.

(I) Transient carboxyl protection of the amine component is necessary in the coupling stage, since the free carboxyl group might serve as the carboxyl-component in the subsequent coupling of segments.

(II) Semipermanent blocking should be used if the carboxyl belongs to the C-terminal residue of a target molecule, or this group could react with the amine group in the amine-component. Furthermore, the masking group is removed only in the concluding 'final' deprotection.

This is why the side chain carboxyls of aspartyl and glutamyl residues need protection, and have to remain blocked until the peptide bond forms.

The general approach for carboxyl protection is esterification. Methyl and ethyl ester formation is a simple way which is suitable for semipermanent blocking. This method of carboxyl protection remains in the practice of peptide synthesis. Methyl esters provide good carboxyl-protection; they are not affected by HBr/AcoH, trifluoroacetic acid, catalytic hydrogenolysis conditions, thiols, or amines in organic solvents, so the selective removal of amino-protecting groups from peptide methyl ester derivatives presents no difficulty. But the problem in the dipeptide stage is that dipeptide methyl ester free base cyclize to diketopiperazines. Saponification is normally a satisfactory method of removal. In addition to methyl and ethyl ester, benzyl ester as the blocking group for carboxyl group is often used as well. Benzyl esters are cleaved by catalytic hydrogenation and also by acidolysis, HBr in acetic acid or HF, but not by TFA. Electron-withdrawing substituents, e.g. p-nitrobenzyl esters,

destabilize the benzyl cation and render the benzyl group, even more resistant to acids. In contrast, some moderately strong acids, like diluted HCl in acetic acid or trifluoroacetic acid, is effective for the acidolytic cleavage of p-methoxybenzyl ester.

In 1984 [95], the allyl ester was introduced as the blocking group for carboxylic acids. The cleavage by HX is complicated by the co-current saturation of double bond but, in the presence of palladium-complexes, the allyl group is smoothly transferred to morpholine or other weakly basic nucleophiles (**Fig. 40**).

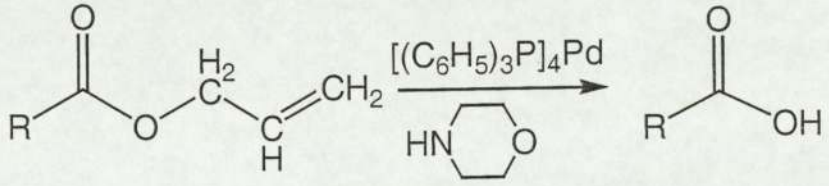


Fig. 40

2.3. Protection of Side Chain

The presence in the side chain of functional groups, such as SH (cysteine), OH (serine, tyrosine), and amine (lysine), or -COOH (glutamic acid), it is necessary to use the transient blocking groups to prevent unwanted reactions.

2.3.1. Blocking of Side Chain Amine Group

The side chain of lysine (and ornithine) residues, ε-amino group, should be blocked, in order to avoid the side reaction. The fact is that the side chain amino group is the more basic and nucleophilic because there is an electron-withdrawing group next to the α-amino group. The blocking group is expected to remain in the chain building process, hence it must resist the reagent of

deprotection of α-amino group and should be cleaved in the completion of the peptide synthesis. The principal ε- protecting groups are ***Boc*** and ***Z***, which have cleavage characteristics much the same as α-***Boc*** and α-***Z*** respectively [96]. After coupling, the deprotection of ***Boc*** groups is preceded by catalytic hydrogenation. The reason is that the Boc group is unaffected in the hydrogenolysis; the Boc group could be cleaved in trifluoroacetic acid, e. g. (**Fig. 41**):

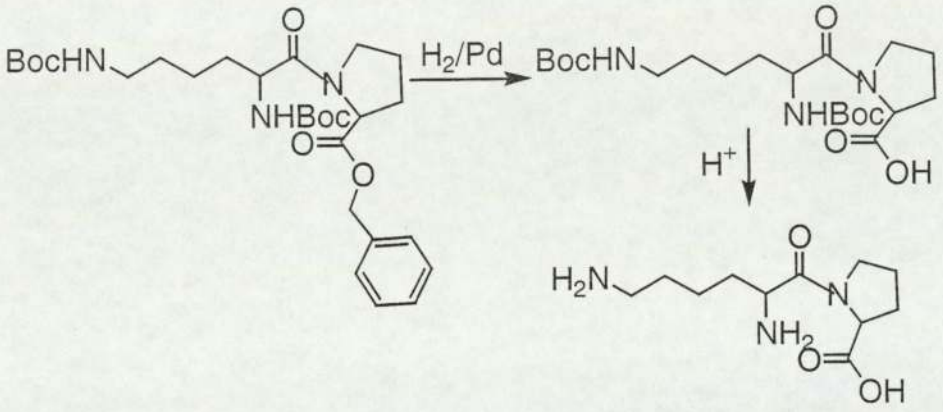


Fig. 41

If ***Boc*** deprotection is before the cleavage of benzyloxycarbonyl(***Z***) group, it will not be perfect, because ***Z*** group is sensitive to the acid as well, part of the ***Z*** group is lost in the cleavage of ***Boc*** group. The quantity might be small, but this is a source of by-product. A remedy for this problem is to destabilize the benzyl cation with electron-withdrawing group and thus make the ***Z***-group more acid-resistant. The modified ***Z***-groups (**Fig. 42**) require very strong acid, such as hydrogen fluoride or trifluoromethanesulfonic acid, for their removal in the final step.

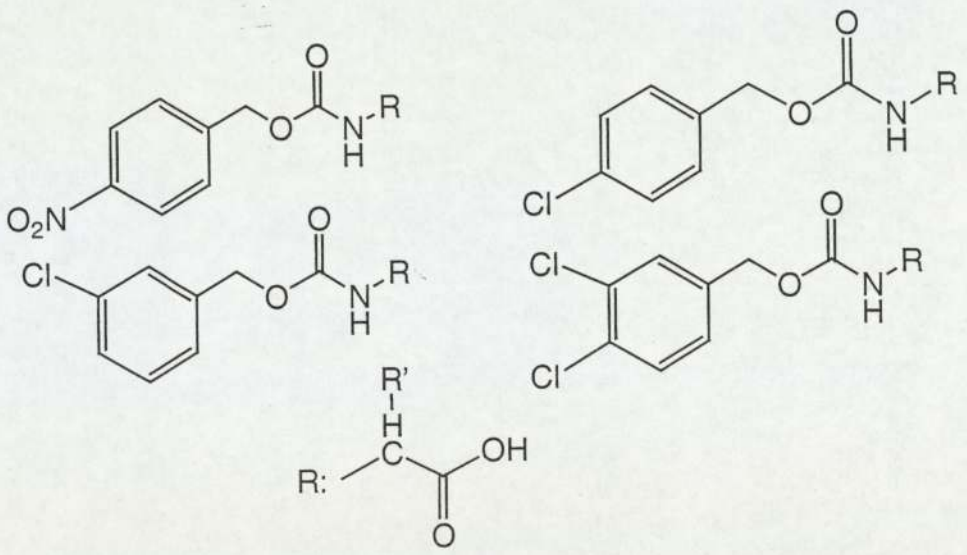


Fig. 42

2.3.2. Protection of Hydroxyl Group

There are three amino acids with a side chain hydroxyl group, threonine, tyrosine, and serine(**Fig. 43**). Hydroxy groups, especially phenolic hydroxy groups (tyrosine), react with acylating agents, and therefore they are protected in peptide synthesis. But sometimes they are left unprotected when the carboxy-activation is mild. In threonine nucleophilic character of the secondary hydroxyl is less than that of the amino group and it is further decreased by steric hindrance of the nearby methyl group. Hence, the blocking for threonine side chain is not mandatory.

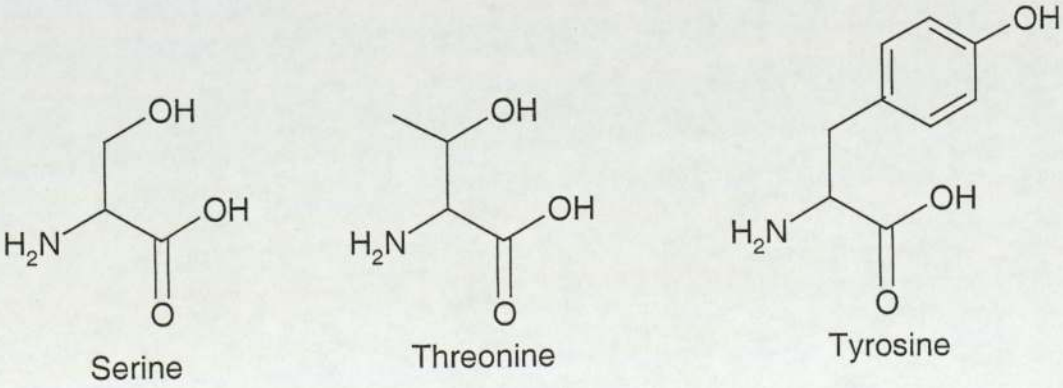


Fig. 43

The situation is different with the primary hydroxyl group in serine residues. Excess of the activated carboxyl derivatives will cause appreciable O-acylation in the presence of a base even with moderately active reagents. If the benzyl group is chosen to protect the side chain of serine, normally it is removed by hydrogenation.

2.3.3 Protection of Sulfhydryl Group

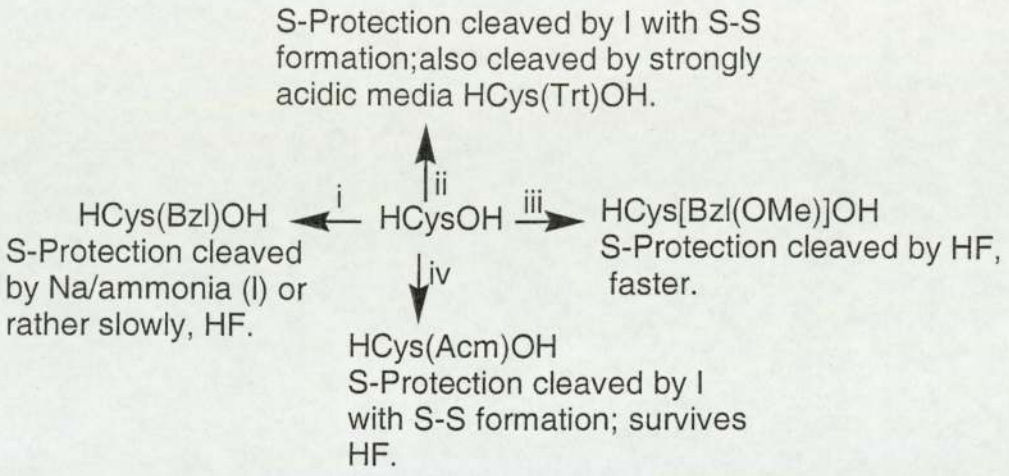
In peptide synthesis, cysteine presents a unique synthetic problem. The sulfhydryl group of cysteine needs to be masked, because its nucleophilic character can not be ignored in reactions.

Because of their soft nucleophilic reactivity, the S-protection can be facilitated by direct alkylation of cysteine itself (**Fig44**) [97]. Apart from the thioether-type groups of graded cleavability, some of which are shown in **Figure 42**, the only other significant approach is mixed disulphide formation with *t*-butyl mercaptan [97]. None of the S-protecting groups indicated is compatible with ordinary catalytic hydrogenolysis, because sulfyl group in cysteine can poison the catalyst, but $H_2/Pd/NH_3$ (liquid) may be used for removing ***Z*** from Cys(Bzl) derivatives [98].

2.3.4 Protection of Thioether in Methionine

In peptide synthesis, the thioether group in the side chain of methionine is quite inert. Only some slight oxidation to the sulfoxide can be observed. But there is a more important side reaction which happens in acidolytic removal of protecting groups, such as benzyl and *tert*- butyl and in analogous cleavage of the benzyloxycarbonyl and *tert*-butyloxycarbonyl group. The reason is that the

thioether is an excellent acceptor of carbocations. Also, it is quite sensitive to alkylation by the by-products of acidolytic fission namely benzyl bromide and *tert*-butyl trifluoroacetate [99] (Fig. 45). The thioether side-chain interferes with catalytic hydrogenation [**Fig. 46**]. The *tert.* butyl group is readily removed from sulfonium by mild heating or longer storage at room temperature.



Conditions: i. BzlBr/aq. NaOH/5°C;

ii. TrtOH/AcOH/BF_3*Et_2/60°C;

iii. Bzl(OMe)Cl, cf. i;

iv. $AcNHCH_2OH$ (i.e. AcmOH)/TFA/20°C/0.5hr. All except Cys(Bzl) may also be cleaved.

In some cases with direct disulfphide formation, by reagents such as sulphenyl chlorides, $Hg(OAc)_2$, and $Tl(CF_3CO_2)_3$.

Fig. 44

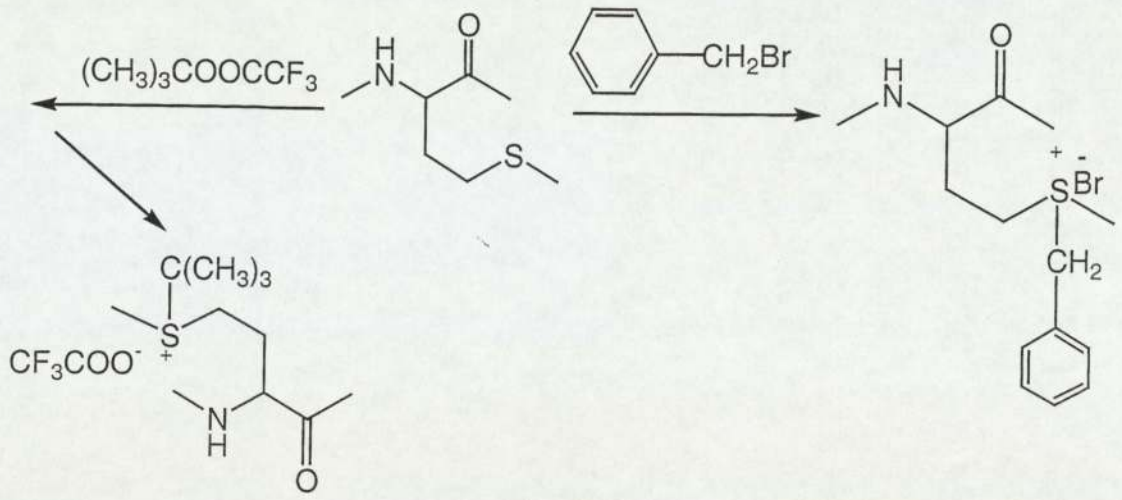


Fig. 45

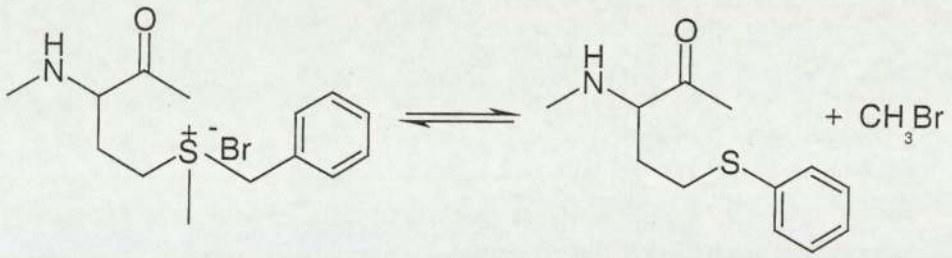


Fig. 46

CHAPTER 3 – DISCUSSION OF DTS STUDY

1. Probe Study of the Dipeptide Transport system

Since peptide transporters are potential targets for improving oral drug absorption, there has been much interest in the mechanisms through which absorption from the GI-tract is mediated. Therefore, choosing the proper probe in the study of dipeptide transport system is very important. Enalapril and ceranapril which both are ACE-inhibitors have been studied.

1.1. The Study of SQ-29852

SQ29852(**Fig3.1**) ((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) [29].

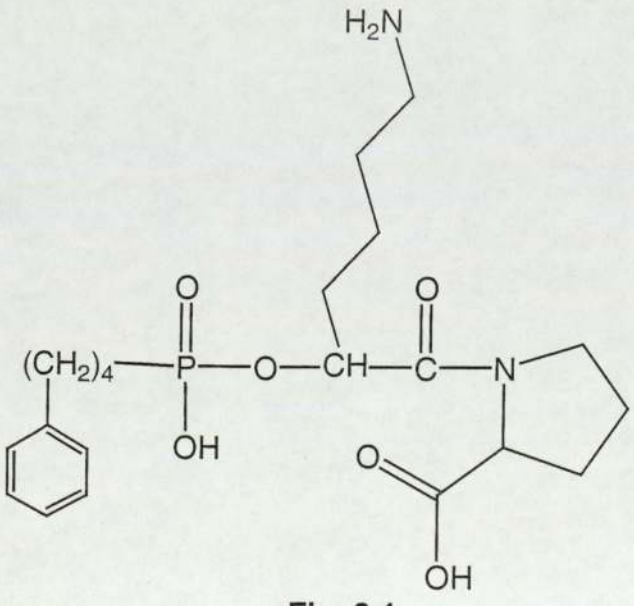


Fig. 3.1

At low clinically relevant doses, SQ 29852 was well absorbed (>50%) in dogs [100] and humans [101]. 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 [102]. SQ 29852 appears to be a specific probe for the DTS because the dose-dependent reduction in absorption from about 60% to less than 8% (3 and 3000mg.kg^{-1} respectively) suggests that at least 85% of an orally administered low dose of SQ-29852 is absorbed by a saturable process. Competition studies with SQ29852 and dipeptides in *situ* in rats indicated that the saturable absorption process for SQ-29852 was indeed the DTS [29]. 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 [102]: (1) it is highly charged at physiologic 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).

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.

1.2. The Study of Enalapril

Enalapril maleate (Fig3.2) is an ester prodrug (bioavailability 60-70%) [51] that is converted in vivo to enalaprilat, which is the active ACE inhibitor. Friedman et al [26], in a report, based on in situ intestinal perfusion in rats, concluded that the intestinal absorption of enalapril was a saturable carrier-mediated process via the dipeptide transport system (DTS).

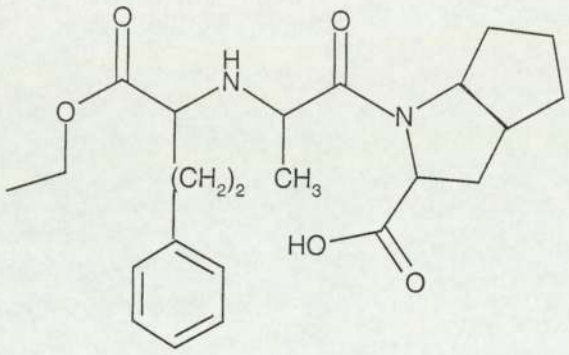


Fig. 3.2

1.2.1 Permeability of Enalapril Across Caco-2 Cells

The presence of the DTS was demonstrated by evaluation of the transport of Gly-Pro across Caco-2 cells; gly-L-pro transport was concentration- and temperature-dependent and inhibited by gly-sar, cephradine, SQ29852. The caco-2 cells permeability of enalapril was not inhibited by 10-fold molar excess of several DTS substrates (SQ29852, cephradine, and gly-L-pro) (**Fig3.3**) [103], whose structure are shown in **Figure 3.4**. Caco-2 cell permeability of enalapril was a passive, non-saturable process, and was not inhibited by compounds previously demonstrated to absorb via the DTS.

Fig. 3.3 Permeability of enalapril across Caco-2 cells (Mean ± SD; n=3) at an initial concentration of 0.3mM in the presence of compounds known to absorbed via the DTS: SQ-29852, gly-L-pro, and cephradine. ANOVA indicated that these DTS substrates did not inhibit enalapril permeability ($P > 0.05$). Permeability of methoxy-inulin was measured simultaneously in each well [103].

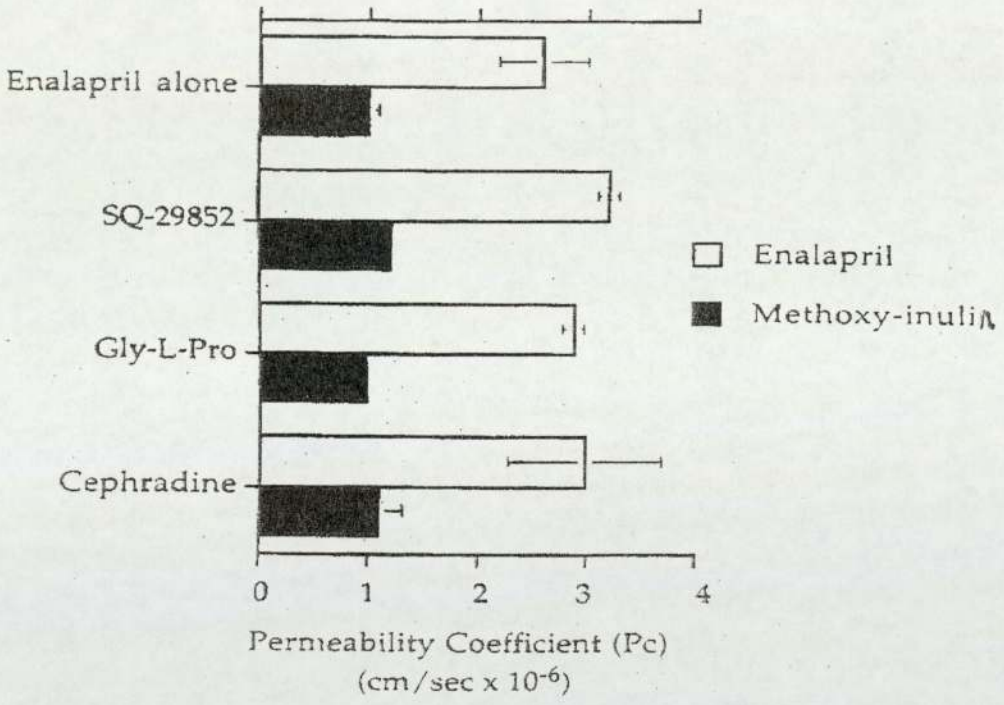
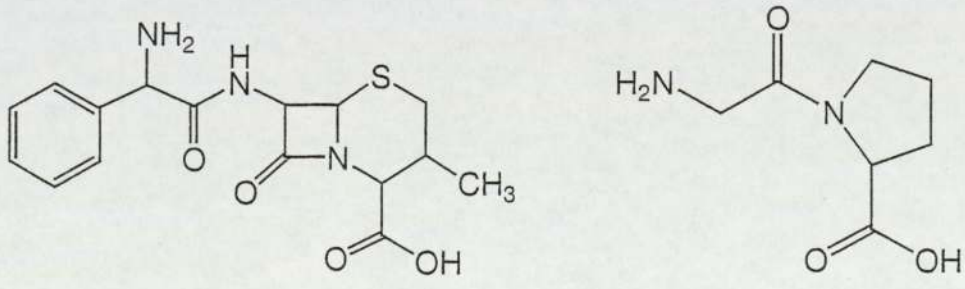


Fig. 3.4 The structure of cephradine and Gly-Pro



Cephradine Gly-Pro

1.2.2. A Wide Range of Doses of the Oral Absorption of Enalapril

The oral absorption of enalapril in rats or dogs was not saturated even at relatively high doses of 50mg/kg or 6.0mg/kg, respectively. This corresponds to about 50-fold (rats) or 20-fold (dogs) higher than the typical therapeutic dose in humans (10mg) when normalized for the inter-species difference in body surface area [103].

1.3 Comparison of SQ-29852 and Enalapril as A Probe of the DTS

It was shown that SQ-29852 is an ideal model compound for studying the DTS because it is: (1) a stable and specific probe of the DTS (*i.e.* negligible absorption via passive diffusion), (2) provides consistent results on the DTS in a variety of in *vitro* [104], in *situ* [29], in *vivo* models [102].

Contrary to what was observed for enalapril, the oral absorption of SQ-29852 was saturable and clearly dose dependent in rats [102], dogs [100] and humans [101]. 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 [29]. In this regard, SQ-29852 appears to be a better probe than the β-lactam amino-cephalosporins since the latter have been reported to have a significant passive component to their overall absorption [105].

1.4. Conclusion

The current *in vitro* and *in vivo* results indicate that enalapril is absorbed by non-saturable, passive diffusion process, without involvement of the DTS. A comparison of enalapril with another ACE-inhibitor, SQ-29852, indicate 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 synthesised. The prolinyl dipeptides are transported in caco-2 cells to investigate which is most suitable probe for dipeptide transport system study.

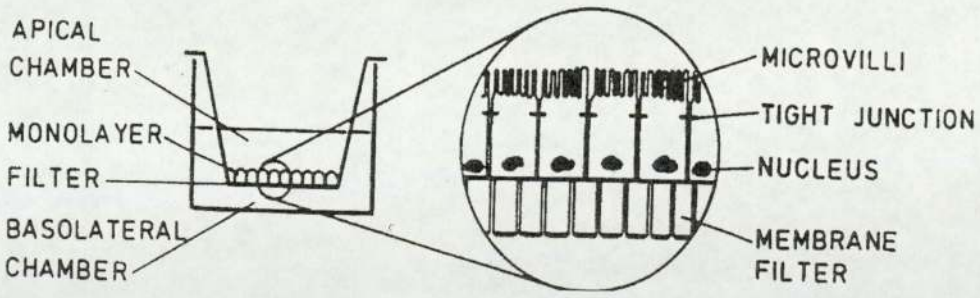
To facilitate such studies, caco-2 cell monolayers, a cell line derived from a human colon carcinoma which demonstrates enterocyte morphology and absorptive behaviour, is now used extensively as a model for the study of intestinal drug transport. The expression of a dipeptide-transporter in culture was demonstrated with D-cephalexin, which has pH-dependent uptake. In sodium free conditions, D-cephalexin uptake was inhibited by a range of dipeptides, but not by amino (and imino) acids [40].

2. Caco-2 Cells

Usually, oral administration is the easy and ideal route of choice for drug delivery. The design of a new therapeutic drug requires optimisation to increase potency, achieve the desired pharmacokinetic profile, and assure transport across biological membranes. Therefore, direct and accurate biological studies are required.

A number of new cell culture models for drug absorption studies are currently being characterised and validated [106]. Epithelial or endothelial mono- or multilayers are cultivated in permeable cell culture inserts. The inserts are placed in wells in normal cell culture plates where the cells are allowed to grow and differentiate. During this period, the cells form confluent layers with barrier properties (**Fig 3.5**) [106].

Fig 3.5 Diagram of chamber for growing intestinal epithelial cells on membrane filters. The cells form confluent monolayers of well-differentiated enterocyte-like cells after a few weeks in culture [106].



The cell culture models have many advantages compared to conventional drug absorption models. The advantages are listed by Audus et al [107] and included: (i). rapid evaluation of the permeability and metabolism of a drug; (ii). the opportunity to study mechanisms of drug absorption under controlled conditions; (iii). rapid evaluation of methods of improving drug absorption, e.g. by the use of prodrugs, absorption enhancers, or other pharmaceutical additives; (iv). the opportunity to perform studies on human cells; and (v). the opportunity to minimise time consuming, expensive, and sometimes controversial animal studies.

2.1. The Choice of Cells.

The most studied cell culture models are those of the intestinal epithelium. These models, especially those based on the human colorectal carcinoma cell line, caco-2, are currently widely used in investigation [108, 109].

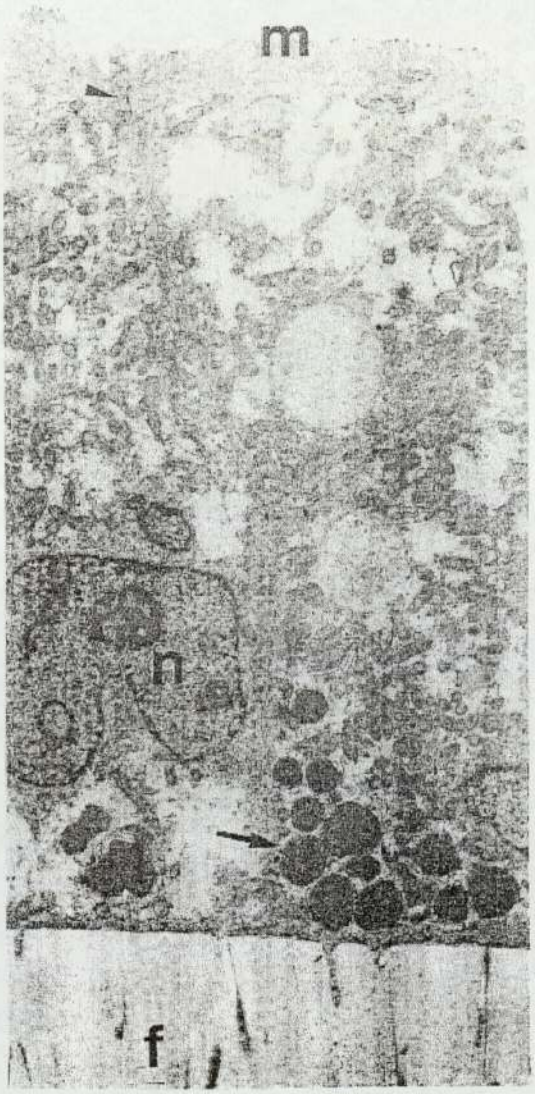
Caco-2 cells undergo spontaneous differentiation under normal cell culture conditions: polarization of the cells with formation of domes and well-developed apical brush borders with several hydrolases. The polarized appearance of caco-2 cells grown on porous support is demonstrated in **Fig3.6** [106].

Caco-2, which differentiates spontaneously, is preferred in most cases. Caco-2 cells are easy to cultivate and show a high degree of differentiation. In caco-2 cells, the activity of the peptide metabolising enzyme dipeptidylpeptidase IV is higher than the cell lines which do not differentiate spontaneously, like HT29, HCT GEO, and HCT EB, respectively [106].

Caco-2 cells also expressed higher levels of amino peptidase N, and this was the only cell line that expressed sucrase-isomaltase and lactase. However, the caco-2 hydrolases have molecular forms typical of fetal colon. Thus, although caco-2 cells have a morphological appearance resembling that of small intestinal enterocytes, the brush border hydrolase pattern indicates that these cells are similar to human fetal colonocytes [106].

Fig3.6 Caco-2 cells grown on polycarbonate filters. The cells form monolayers of well-differentiated columnar cells with apical microvilli

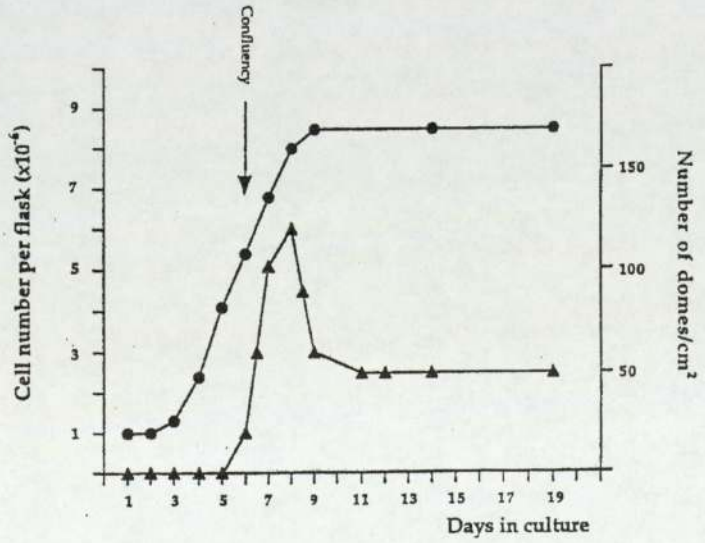
(m) and tight junctions (arrowhead). The nuclei (n) are generally located in the basal part of the cells as are lipid droplets (arrows). The cells are grown on a porous polycarbonate filter (f). Apical part of the Caco-2 monolayer with microvilli (m), tight junctions (arrowheads), desmosome (d), and intercellular spaces. Adapted from Artursson, P., 1991 [106].



2.2. The Development of Caco-2 Cells

During differentiation, caco-2 monolayers grown on plastic display domes characteristic of transepithelial ionic transport [111]. Domes are randomly distributed over the monolayer. The number of domes reaches a peak at day 8 and decreases to a steady level after day 10 (**Fig 3.7**) [112]. Dome size, however, tends to increase over time. Dome formation results from the accumulation of secreted compounds at the basolateral domain. In caco-2 cells, Na^+/K^+ ATPase is not involved in dome maintenance in this cell line [113]. Caco-2 cells are able to differentiate as mature enterocytes. Differentiation is evidenced by the development of microvilli and the formation of tight junctions, which separate basolateral and apical domains. Differentiation is complete after 25-30 days in culture. During differentiation, enzymatic activities are gradually increased, becoming maximal 15-21 days after confluency [114].

Fig.3.7 Kinetics of cell proliferation (•) and the dome formation (▲) in Caco-2 monolayers grown in 25 cm^2 plastic flasks [106]



After differentiation, caco-2 cells are very similar to normal enterocytes with regard to their mophological characteristics [114]. At confluency, half the cells form a thick brush border with a high density of microvilli and the rest form clusters [112].

Typical brush border microvilli exhibit characteristics of morphological polarization of the cells, as well as the presence of junctional complexes constituted by tight junctions and desmosomes. Cell dimensions vary slightly showing the heterogeneity of caco-2 cells. The morphology of epithelial cells is characterized by tight junctions that seal the epithelial layer. Tight junctions regulate movement of solutes and immune cells between epithelial and endothelial domains [115].

In conclusion, like epithelial cells, they are characterized by the presence of tight junctions and the development of apical and basolateral domains and brush border cytoskeleton.

2.3. The Use of Caco-2 Cells

Caco-2 cells have been used in the assessment of the cellular permeability of potential drugs, formulation strategies designed to enhance membrane permeability, and potential effects on the biological barrier, the determination of optimal physicochemical characteristics for passive diffusion of drugs, and the elucidation of pathways of drug transport. The caco-2 cell can also be used to study presystemic drug metabolism due to its expression of various cytochrome P450 isoforms and phase II enzyme [116].

2.4. Conclusion

Some human intestinal epithelial cell lines differentiate to enterocyte-like cells in culture. These cell lines can be grown as monolayers with characteristics similar, but not equal to, those of normal enterocytes, but the results are encouraging: good correlations between cell culture and *in vivo* studies have been established. Caco-2 cells are useful in studies of the mechanisms of passive as well as active drug and peptide absorption. They can be also used in the effects of different pharmaceutical additives (including absorption enhancers) on epithelial integrity and drug absorption.

CHAPTER 4 - EXPERIMENTAL

1. Design of Experiments

The aim of this research was to design, synthesize, and test novel DTS (Dipeptide Transport System) probes in an attempt to improve their specificity, and stability in biological systems, as determined in a Gly-Pro inhibition test.

Enalapril and Ceronapril (SQ29851) have been used as probes for DTS in several previous studies in the Drug Delivery department in Aston University. Both of these two drugs are prolinyl (C-terminal) compounds. However, there are differences between these two drugs, the structures of which are shown on page 16:

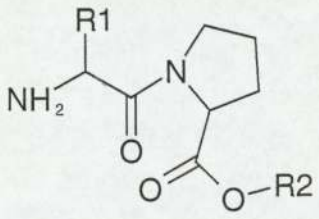
(i). Enalapril is a carboxyalkyl dipeptide, and ceronapril (SQ29851) is a phosphonic acid;

(ii). The polarity of ceronapril is higher than enalapril (lipophilicity is lower);

(iii). There is a secondary amine in enalapril, but in ceronapril, it is primary amine.

As discussed on page 71 ceronapril showed a much greater efficacy as a DTS probe than enalapril. The previous work, however, had not elucidated the factors determining the relative potency of the two drugs. These results and the advantage of ceronapril over enalapril became the stimulus of the present study. This investigation involved synthesising a large number of dipeptide analogues of the ceronapril and enalapril, consising of an amino acid and

proline (**picture below**), with proline as the C-terminal part in the synthesised dipeptide.



R1= Various functional groups

R2= H, CH_3, CH_2Ph

The inhibitory effect of Gly-Pro uptake was established of varying the length of amino acids' carbon chain between one carbon and five carbons, the basicity and the acidity of amino acids' side chain. Finally, sarcosine, with an imine group which is similar to enalapril was used to prepare Sar-Pro to investigate the effect of the imine.

2. Chemistry

2.1. Materials and Instruments Used

In the dipeptide synthetic experiment, all the protected amino acids were obtained from the Novabiochem company. Hydroxybenzotriazole (HOBt) and palladium 10%wt. on activated carbon were obtained from the Aldrich company. Triethylamine (TEA), dicyclohexylcarbodiimide (DCC), dichloromethane (CH_2Cl_2), N,N'-dimethylformamide (DMF), and trifluoroacetic acid (TFA) were available in the laboratory.

^1H NMR spectra were obtained using at 250 MHz on a Bruker AC-250 or at 300 MHz on a Bruker AMX-300 instrument, ^{13}C NMR spectra were recorded on the same instrument at 62.5MHz with proton decoupling. Thin layer

chromatography was carried out using Whatman silica gel precoated aluminium plates. IR spectra were recorded on a Mattson 3000 instrument. Because the compounds synthesised 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}.

2.2. The Formation of Dipeptide

2.2.1. The Formation of Dipeptide Bond

1. The cooled mixture (5°C) of a protected amino acid (1 equivalent), L-proline benzyloxycarbonyl (methyl) ester hydrochloride (1 equivalent) and triethylamine (TEA) (0.14ml/1mmol proline ester hydrochloride) suspended in dried tetrahydrofuran (THF) (2.4ml/1mmol proline benzyl ester hydrochloride) was treated with N-hydroxybenzotriazole (HOBt) (1 equivalent) and N, N'-dicyclohexylcarbodiimide (DCC) (1 equivalent). The reaction was kept stirring in an ice bath for three hours. Then the mixture was allowed to warm to room temperature (~20°C), with stirring continued for another one hour. T.l.c. (thin layer chromatography) was used to chase the completion of reaction. The insoluble white solid---- dicyclohexylurea was filtered and the filtrate was washed twice with ethyl acetate. The diluted solution was partitioned by 5% $NaHSO_4$, saturated $NaHCO_3$ and saturated NaCl twice respectively. All the inorganic layers were combined

and washed by ethyl acetate, then the organic layer was dried over Na_2SO_4, filtered. The solvent was removed under reduced pressure to give an oil.

In one preparation, in which the TEA was replaced by NMM (4-methylmorpholine N-oxide monohydrate), no pure product was obtained. A similar poor result was obtained when DMF was substitured for dichloromethane.

2.2.2. Deprotection of Boc Group

2. The synthesised protected dipeptide (with protecting group) (1mol) was dissolved in the dried dichloromethane (CH_2Cl_2) (2ml/1mmol dipeptide). The mixture was cooled to 0°C, then the trifluoroacetic acid (TFA) (2ml/1mmol dipeptide) was added in one portion. The reaction was kept stirring at 0°C for 1 hour, then allowed to attain room temperature (~20°C) and kept stirring for an additional 2 hours. The solvent was removed under the pressure to give a dark brown oily compound. The proper system was used to purify the compound by flash column. The tubes containing the product were collected and the solvent was concentrated to give a light coloured oily compound.

2.2.3. Removal of Methyl Ester

3. After deprotection of butoxycarbonyl group, part of the compound (1mmol) was dissolved in methanol: H_2O (1/1:v/v, 24.7ml/1mmol dipeptide). Aqueous lithium hydroxide (LiOH) (9.6%) was dropped in portions to keep the pH 12~13, and the reaction was kept stirring at this pH value for 1.5

hour. After 3.5hr, the pH of solution was adjusted to 7 with 1N hydrochloric acid. The solution was concentrated and the residue was dissolved in the methanol to give the inorganic insoluble solid (LiCl). The mixture was filtered, the solvent was removed under pressure to give the compound which was purified by the flash column.

2.2.4. Removal of Benzyl Ester

4. Dipeptide (without Boc group) (1mmol) was dissolved in dried ethanol (20.5 ml/1mmol dipeptide). Then were added acetic anhydride (0.1ml/ 1mmol dipeptide) and palladium 10%wt. on activated carbon (Pd/C) (0.1g/ 1mmol dipeptide). The reaction was left in room temperature (20°C) and 60psi under hydrogen for 15 hours. After the reaction stopped, the mixture was filtered through celite, washed twice by ethyl acetate and all the organic solution was combined. The solvent was removed under pressure to give a viscous oil. The residue was purified by proper solvent mixture under flash column.

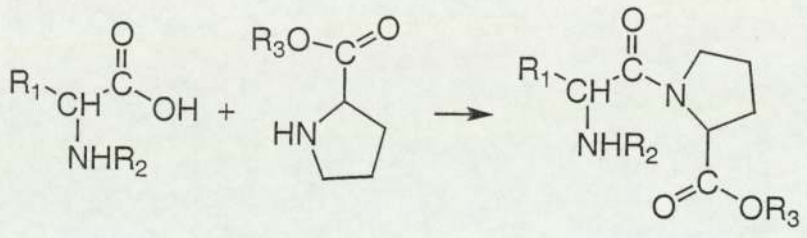
2.2.5 Conversion of DCHA Salt

5. The amino acid with DCHA salt (1mmol) was suspended in ethyl acetate (4ml/1mmol dipeptide) in a separating funnel. 1.2 equivalent (6ml) of ice-cold 2M H_2SO_4 was added and shaken until the amino acid salt was dissolved. The removed top layer (ethyl acetate) was set aside. 10ml cold water was added further to the aqueous layer and extracted twice with 5ml of ethyl acetate. All the organic layers were combined and washed twice with 5ml of water. The organic layer was dried over magnesium sulfate (Mg_2SO_4). After filtration, the ethyl acetate was removed in a rotary

evaporator at not more than 40°C. The solvent removal was completed under high vacuum in a desiccator with fresh NaOH.

2.3. Result

Table (4.1): the equation below is the basic route for the synthesis of dipeptides, the structures of starting material were described in the following table, and the corresponding product number.



(I) (II) (III)

(I)			(III)		
R_1	R_2	Name (I)	R_2	R_3	No.
H	Boc	N-α-t-Boc-L- glycine	Boc	Bzl Me	25 23
				Bzl Me	26 24
				H	27
CH_3		N-α-t-Boc-L-alanine	Boc	Bzl Me	7 5
				Bzl Me	8 6
				H	9
CH_2CH_3	Z	Z-γ-CBZ-γ-aminobutyric acid	Z	Bzl Me	3 1
				Me	2
				H	4
$(CH_2)_2CH_3$	Boc	N-α-t-Boc-L-norvaline	Boc	Bzl Me	45 43
				Bzl Me	46 44
				H	47
$(CH_2)_3CH_3$		N-α-t-Boc-	Boc	Bzl	40

		L-norleucine		Me	38
				Bzl Me	41 39
				H	42
$NH(Boc)(CH_2)_4$		N-α,ε-di-t-Boc-L-lysine	Boc	Bzl Me	30 28
				Bzl Me	31 29
				H	32
$CO(OBzl)(CH_2)_2$		N-α-t-Boc-L-glutamic acid γ-benzyl ester	Boc	Bzl Me	20 18
				Bzl Me	21 19
				H	22
$CH_3S(CH_2)_2$		N-α-t-Boc-L-methionine	Boc	Bzl Me	35 33
				Bzl Me	36 34
				H	37
$HSCH_2$		N-α-t-Boc-L-cysteine	Boc	Bzl Me	16 14
				Bzl Me	17 15
$(Bzl)SCH_2$		N-α-t-Boc-S-benzyl-L-cysteine	Boc	Bzl Me	12 10
				Bzl Me	13 11
$HOCH_2$		N-α-t-Boc-L-serine	Boc	Bzl Me	53 55
				Bzl Me	54 56
				H	57
$CH_3N(Boc)CH_2COOH$	Boc	N-α-t-Boc-L-sarcosine	Boc	Bzl Me	50 48
				Bzl Me	51 49
				H	52

The NMR data of dipeptides and their derivatives are shown below:

All the intermediates and final products obtained are oils, no melting points were recorded. The compounds marked with "*" do not have record in Beilstein database.

Z-Abu-Pro-OMe (No1.): 0.52g, yield 71%.

^{1}H NMR ($CDCl_3$): δ 7.28 (5H, m), 4.98-5.12 (2H, t), 4.38-4.51 (1H, dd), 3.65 (3H, s), 3.64-3.68 (1H, t), 3.58-3.61 (2H, m), 2.15-2.17 (2H, m), 1.91-1.95 (2H, m), 1.78, 1.81 (2H, m), 0.96 (3H, t).

^{13}C NMR ($CDCl_3$): δ 172.1, 170.7, 155.9, 136.5, 136.4, 128.3, 128.2, 127.9, 127.8, 66.4, 60.1, 53.3, 46.7, 31.0, 28.7, 25.4, 22.0, 20.4.

Abu-Pro-OMe (No.2*): 0.11g, yield 63%.

^{1}H NMR ($CDCl_3$): δ 6.82 (NH_2, bs, after D_2O shake), 3.94-4.11 (1H, t), 3.68 (3H, s), 3.62 (1H, dd), 3.57 (2H, m), 2.28-2.34 (2H, m), 1.93-2.00 (2H, m), 1.79-1.88 (2H, m), 0.94 (3H, t).

^{13}C NMR ($CDCl_3$): δ 170.7, 165.4, 58.9, 56.3, 52.1, 45.2, 30.8, 28.2, 22.5, 20.8.

Z-Abu-Pro-OBzl (No.3): 0.50g, yield 70%

^{1}H NMR ($CDCl_3$): δ 7.36 (5H, m), 5.10-5.18 (2H, t), 4.34-4.64 (1H, t), 3.74-3.78 (1H, dd), 3.63-3.68 (2H, m), 2.20-2.24 (2H, m), 1.92-2.00 (2H, m), 1.81-1.87 (2H, m), 0.92. 0.94, 0.96 (3H, t).

^{13}C NMR ($CDCl_3$): δ 171.6, 170.8, 156.0, 2(136.4, 128.6, 128.6, 127.9, 127.9, 127.4), 2(66.6), 58.8, 53.4, 46.8, 28.8, 25.7, 24.8, 21.5.

Abu-Pro-OH (No.4) [117]: 0.13g,yield 85%.

^{1}H NMR (DMSO): δ OH (8.26, bs, after D_2O shake), NH_2 (6.72, bs, after D_2O shake), 4.16 (1H, t), 3.64 (1H, dd), 3.30-3.34 (2H, m), 2.11 (2H, m), 1.97-2.03 (2H, m), 1.80-1.83 (2H, m), 0.80, 0.83, 0.85 (3H, t).

^{13}C NMR ($CDCl_3$): δ 174.7, 168.4, 61.8, 50.8, 46.3, 32.7, 31.0, 22.3, 20.7.

Boc-Ala-Pro-OMe (No.5): 0.30g, yield 63%.

^{1}H NMR ($CDCl_3$):δ 4.42-4.49 (1H, q), 3.68 (3H, s), 3.58-3.65 (1H, dd), 3.17-3.18 (2H, m), 2.15-2.18 (2H, m), 1.97-2.03 (2H, m), 1.78 (3H, d), 1.25-1.28 (9H, s).

^{13}C NMR ($CDCl_3$): δ 171.9, 171.3, 155.6, 78.9, 61.3, 54.9, 51.3, 45.9, 35.2, 28.0, 27.9, 27.8, 21.0, 19.7.

Ala-Pro-OMe (No.6)[118]: 0.09g, yield 74%.

^{1}H NMR ($CDCl_3$): δ 8.00 (NH_2, bs, after D_2O shake), 4.49 (1H, q), 3.69 (3H, s), 3.50-3.60 (1H, dd), 3.47 (2H, m), 2.27 (2H, m), 2.17 (2H, m), 1.98, 1.97(3H, d).

^{13}C NMR (d^5-Pyridine): δ 170.8, 167.0, 60.1, 59.4, 51.2, 45.3, 33.8, 22.7, 20.6.

Boc-Ala-Pro-OBzl (No.7): 1.53g, yield 85%.

^{1}H NMR ($CDCl_3$): δ 7.29-7.32 (5H, m), 5.03-5.19 (2H, t), 4.40-4.57 (1H, q), 3.64-3.67 (1H, dd), 3.53-3.57 (2H, m), 2.15-2.19 (2H, m), 1.95-1.99 (2H, m), 1.90, 1.92 (3H, d), 1.26 (9H, s).

^{13}C NMR ($CDCl_3$): δ 172.0, 170.9, 157.0, 136.7, 128.3, 128.2, 127.9, 127.6, 126.9, 79.1, 60.2, 58.9, 52.6, 46.2, 33.8, 27.9, 27.9, 27.9, 21.9, 20.8.

Ala-Pro-OBzl (No.8) [119]: 0.63g, 85%

^{1}H NMR ($CDCl_3$): δ8.20 (NH_2, bs, after D_2O shake), 7.27-7.37 (5H, m), 5.00-5.20 (2H, t), 4.24, 4.60 (1H, q), 3.59 (1H, dd), 3.44 (2H, m), 2.20 (2H, m), 1.96 (2H, m), 1.41,1.43 (3H, d).

^{13}C NMR ($CDCl_3$): δ 171.0, 168.8, 135.6, 128.5, 128.3, 128.3, 127.6, 127.5, 67.0, 59.2, 48.0, 46.6, 24.5, 22.6, 21.0.

Ala-Pro-OH (No.10) [120]: 0.18g, yield 63%.

^{1}H NMR (d^5-Pyridine): δ 9.55 (OH, after D_2O shake), 6.54(NH_2, bs, after D_2O shake), 4.67-4.73 (1H,m), 3.56-3.57 (1H,m), 3.35-3.41 (2H,m), 2.02-2.05 (2H,m), 1.83-1.88 (2H,m)), 1.56, 1.59 (3H, d).

^{13}C NMR (d^5-Pyridine): δ 173.5, 171.9, 59.0, 50.8, 44.9, 29.9, 28.0, 22.4.

Boc-Cys(SBzl)-Pro-OMe (No.10): 0.88g, yield 65%.

^{1}H NMR ($CDCl_3$): δ 7.17-7.31 (5H, m), 4.89 (1H, t), 4.39-4.52 (1H, dd), 3.65-3.72 (2H, t), 3.61 (3H, s), 3.38-3.52 (2H, m), 2.93 (2H, d), 2.45 (2H, m), 2.06-2.12 (2H, m), 1.36-1.37 (9H, s).

^{13}C NMR ($CDCl_3$): δ 172.0, 169.8, 155.2, 137.8, 128.9, 128.4, 128.4, 127.0, 126.9, 70.4, 60.2, 58.8, 44.4, 37.8, 33.9, 28.8, 28.8, 28.8, 22.2, 20.9.

Cys(SBzl)-Pro-OMe (No.11*): 0.65g, yield 81%.

^{1}H NMR ($CDCl_3$): δ 8.00 (NH_2, bs, after D_2O shake), 7.20, 7.27, 7.30, 7.32, 7.36 (5H, m), 4.50 (1H, t), 4.33 (1H, dd), 3.71-3.78 (2H, t), 3.59 (3H, s), 3.26-3.47 (2H, m), 2.88 (2H, d), 2.15 (2H, m), 1.89 (2H, m).

^{13}C NMR ($CDCl_3$): δ 171.6, 169.8, 136.9, 128.9, 128.7, 128.6, 127.4, 127.3, 59.3, 59.1, 53.5, 50.8, 45.3, 31.0, 24.4, 22.4.

Boc-Cys(SBzl)-Pro-OBzl (No.12): 2.46g, yield 77%.

^{1}H NMR ($CDCl_3$): δ 7.18-7.32 (10H, m), 5.14 (2H, t), 4.81 (1H, t), 4.48 (1H, dd), 3.66-3.73 (2H, t), 3.53 (2H, m), 2.67-2.75 (2H, d), 2.10-2.14 (2H, m), 1.80-1.92 (2H, m), 1.39 (9H, s).

^{13}C NMR ($CDCl_3$): δ 171.4, 169.8, 155.2, 137.8, 135.5, 129.0, 129.0, 128.5, 128.3, 128.2, 128.2, 126.9, 126.8, 72.1, 69.8, 60.3, 58.9, 46.4, 37.9, 31.9, 28.2, 28.2, 28.2, 22.2, 20.9.

Cys(SBzl)-Pro-OBzl (No.13*): 1.57g, yield 65%.

^{1}H NMR ($CDCl_3$): δ 7.89 (NH_2, bs, after D_2O shake), 7.18-7.29 (10H, m), 5.14 (2H, t), 4.81 (1H, t), 4.08-4.26 (1H, dd), 3.76 (2H, t), 3.42 (2H, m), 2.80-2.82 (2H, d), 2.03-2.12 (2H, m), 1.86 (2H, m).

^{13}C NMR ($CDCl_3$): δ 171.0, 166.8, 140.9, 137.1, 135.3, 128.8, 128.8, 128.6, 128.6, 128.4, 128.4, 127.3, 127.0, 126.9, 67.0, 59.4, 55.6, 46.9, 37.4, 31.1, 28.6, 24.5.

Boc-Cys-Pro-OMe (No.14): 0.52g, yield 62%.

^{1}H NMR ($CDCl_3$): δ 4.50-4.64 (1H, dd), 3.73-3.82 (1H, t), 3.71 (3H, s), 3.25 (2H, m), 2.77-2.86 (2H, d), 2.18-2.25 (2H, m), 1.85-1.99 (2H, m), 1.38-1.44 (9H, s).

^{13}C NMR ($CDCl_3$): δ 172,2, 169.2, 158.8, 79.9, 60.8, 58.8, 52.5, 47.2, 33.7, 28.9, 28.2, 28.2, 24.8, 22.5.

Cys-Pro-OMe (No.15*): 0.28g, yield 77%.

^{1}H NMR (DMSO): δ 6.72 (NH_2, bs, after D_2O shake), 4.30-4.40 (1H, dd), 3.68-3.70 (1H, t), 3.62 (3H, s), 3.42 (2H, m), 2.86-2.92 (2H, d) 2.16-2.20 (2H, m), 1.87-1.92 (2H, m).

^{13}C NMR (DMSO): δ 177.3, 171.8, 59.8, 53.2, 50.1, 46.7, 33.1, 24.3, 21.6.

Boc-Cys-Pro-OBzl (No.16): 1.10g, yield 64%.

^{1}H NMR ($CDCl_3$): δ 7.31 (5H, m), 5.15 (2H, t), 4.55-4.67 (1H, dd), 3.72-3.80 (1H, t), 3.25 (2H, m), 2.71-2.81 (2H, d), 2.16-2.22 (2H, m), 1.97-1.99 (2H, m), 1.37-1.44 (9H, s).

^{13}C NMR ($CDCl_3$): δ 171.5, 169.3, 155.1, 140.5, 128.5, 128.5, 128.3, 128.1, 128.1, 79.9, 63.9, 58.9, 53.3, 47.3, 33.7, 28.9, 28.2, 28.2, 24.8, 22.3.

Cys-Pro-OBzl (No.17*): 0.76g, yield 91%.

^{1}H NMR ($CDCl_3$): δ 5.69 (NH_2, bs, after D_2O shake), 7.30-7.31 (5H, m), 5.21 (2H, t), 4.56-4.70 (1H, dd), 3.65 (1H, t), 3.50 (2H, m), 2.97 (2H, d), 2.16 (2H,

m), 1.96 (2H, m).

^{13}C NMR ($CDCl_3$): δ 171.2, 168.2, 135.3,128.5, 128.3, 128.1, 128.1, 128.0, 66.9, 59.2, 51.7, 46.9, 30.4, 24.7, 22.2.

Boc-Glu(OBzl)-Pro-OMe (No.18): 1.33g, yield 72%

^{1}H NMR($CDCl_3$): δ 7.25-7.29 (5H, m), 5.43-5.46 (2H, t), 4.42-4.50 (1H, t), 4.33 (1H, dd), 3.65 (3H, s), 3.60 (2H, m), 2.40-2.49 (2H, m), 2.09-2.12 (4H, m), 1.87-1.95 (2H, m), 1.35 (9H, s).

^{13}C NMR ($CDCl_3$): δ 176.6, 171.7, 171.7, 155.5, 135.4, 128.5, 128.4, 128.2, 128.0, 126.7, 72.3, 66.7, 59.4, 55.6, 51.7, 46.7, 28.1, 28.1, 28.1, 27.5, 27.3, 22.4, 20.9.

Glu(OBzl)-Pro-OMe (No.19*): 0.54g, yield 96%

^{1}H NMR($CDCl_3$): δ 8.60 (NH_2, bs, after D_2O shake), 7.30-7.32 (5H, m), 5.00-5.16 (2H, t), 4.36-4.54 (1H, t), 4.29 (1H, dd), 3.71 (3H, s), 3.45-3.50 (2H m), 2.59-2.68 (2H, m), 2.15 (4H, m), 1.89-1.99 (2H, m).

^{13}C NMR ($CDCl_3$): δ 173.7, 172.6, 172.5, 135.4, 128.5, 128.4, 128.4, 128.2, 128.2, 66.7, 58.4, 52.9, 51.2, 46.7, 27.8, 27.6, 22.7, 20.3.

Boc-Glu(OBzl)-Pro-OBzl (No.20): 2.12g, yield 91%

^{1}H NMR ($CDCl_3$): δ 7.32-7.35 (10H, m), 5.04-5.27 (4H, m), 4.53-4.56 (1H, t), 4.26 (1H, dd), 3.64-3.67(2H, m), 2.44-2.47 (4H, m), 2.02 (2H, m), 1.93-1.95 (2H, m), 1.38 (9H, s).

^{13}C NMR ($CDCl_3$): δ 172.5, 171.6, 171.4, 155.5, 135.8, 135.5, 128.2, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.9, 127.8, 74.3, 74.3, 70.8, 58.7, 53.5, 46.7, 28.7, 28.7, 28.7, 27.6, 27.4, 24.7, 22.6.

Glu(OBzl)-Pro-OBzl (No.21)*: 1.58g, yield 98%

^{1}H NMR ($CDCl_3$): δ 5.78(NH_2, bs, after D_2O shake), 7.33 (10H, m), 5.03-5.20 (4H, m), 4.42 (1H, dd), 3.74 (2H, m), 3.56 (1H, t), 2.54-2.72 (2H, m), 2.06-2.17 (4H, m), 1.91 (2H, m).

^{13}C NMR ($CDCl_3$): δ 176.8, 172.8, 172.8, 135.4, 135.3, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 128.1, 128.0, 72.5, 72.5, 66.7, 58.4, 50.7, 47.1, 27.8, 27.2, 22.2, 20.9.

Glu-Pro-OH (No.22)*: 0.24g, yield 42%

^{1}H NMR (DMSO): δ 11.7, 10.9 (2OH, bs), 6.57(NH_2, bs), 4.74-4.83 (1H, dd), 3.65 (1H t), 3.40 (2H, m), 2.53 (2H, t), 2.48 (2H, m), 1.98(2H, m), 1.78-1.85 (2H, m).

^{13}C NMR (DMSO): δ 174.2, 170.3, 170.2, 58.8, 53.6, 46.7, 29.6, 28.7, 24.3, 22.3.

Boc-Gly-Pro-OMe (No.23): 1.03g, yield 79%.

^{1}H NMR ($CDCl_3$): δ 4.20-4.25 (1H, dd), 3.66-3.70 (2H, s), 3.44 (3H, s), 3.23-3.35 (2H, m), 2.17 (2H, m), 1.90-1.96 (2H, m), 1.37 (9H, s).

^{13}C NMR ($CDCl_3$): δ 171.7, 167.4, 155.4, 78.8, 58.1, 55.2, 52.3, 42.5, 27.8, 27.8, 27.8, 21.2, 20.5.

Gly-Pro-OMe (No.24*):0.37g, yield 67%.

^{1}H NMR ($CDCl_3$): δ 8.25 (NH_2, bs, after D_2O shake), 4.55 (1H, dd), 3.75 (2H, s), 3.69 (3H, s), 3.47-3.58 (2H, m), 2.20 (2H, m), 2.04-2.06 (2H, m).

^{13}C NMR ($CDCl_3$): δ 172.0, 171.3, 59.2, 52.8, 40.5, 33.4, 23.4, 21.3.

Boc-Gly-Pro-OBzl (No.25): 0.98g, yield 82%.

^{1}H NMR ($CDCl_3$): δ 7.33-7.40 (5H, m), 5.23 (2H, t), 4.57-4.61 (1H, dd), 3.57-3.65 (2H, s), 3.46-3.49 (2H, m), 2.19-2.24 (2H, m), 2.00-2.08 (2H, m), 1.46 (9H, s).

^{13}C NMR ($CDCl_3$): δ 171.6, 167.3, 155.7, 135.4, 128.6, 128.5, 128.5, 128.5, 126.4, 79.6, 67.4, 58.9, 49.0, 42.9, 28.8, 28.8, 28.8, 24.5, 22.1.

Gly-Pro-OBzl (No.26)[121]: 0.36g. yield 67%.

^{1}H NMR ($CDCl_3$): δ 8.32 (NH_2, bs, after D_2O), 7.30-7.37 (5H, m), 5.19 (2H, t), 4.45-4.60 (1H, dd), 3.89 (2H, s), 3.40-3.48 (2H, m), 2.13-2.19 (2H, m), 1.93 (2H, m).

^{13}C NMR ($CDCl_3$): δ 171.3, 165.5, 135.0, 128.5, 128.5, 128.3, 128.2, 128.2, 67.0, 59.2, 45.9, 40.5, 28.6, 24.2.

Gly-Pro-OH (No.27)[122]: 0.11g, yield 53%.

^{1}H NMR (d^5-Pyridine): δ 9.84 (OH, bs), 6.48 (NH_2, bs), 4.42-4.63 (1H, dd), 3.60 (2H, s), 3.12-3.18 (2H, m), 2.20 (2H, m), 1.86-1.90 (2H, m).

^{13}C NMR (d^5-Pyridine): δ 170.8, 165.8, 59.9, 49.4, 39.3, 24.5, 22.4.

Boc-Lys-Pro-Ome(No. 28): 0.64g, yield 84%

^{1}H NMR ($CDCl_3$): δ 4.22-4.37 (1H, dd), 3.54 (1H, t), 3.46 (2H, m), 3.51 (3H, s), 2.90 (2H, m), 2.15 (2H, m), 1.97-2.00 (2H, m), 1.80-1.82 (2H, m), 1.52 (2H, m), 1.27-1.38 (2H, m), 1.21 (18H, s).

^{13}C NMR ($CDCl_3$): δ 176.9, 171.8, 171.7, 155.3, 78.2, 78.3, 60.0, 55.3, 51.3, 46.5, 39.8, 31.8, 28.1, 28.1. 28.1, 28.1, 27.9, 25.1, 24.3, 21.7, 20.6.

Lys-Pro-OMe (No. 29)[123]: 0.31g, yield 98%.

^{1}H NMR (DMSO): δ 7.83, 8.21 ($2NH_2$, bs, after D_2O shake), 4.37-4.41 (1H, dd), 3.69 (1H, t), 3.62 (3H, s), 3.46 (2H,m), 2.75 (2H, t), 2.22 (2H,m), 1.91 (2H,m), 1.71 (2H, m), 1.55 (2H, m), 1.34 (2H, m).

^{13}C NMR ($CDCl_3$): δ 176.8, 167.4, 58.7, 52.0, 50.6, 40.5, 40.2, 33.4, 32.6, 24.6, 22.5, 20.6.

Boc-Lys-Pro-OBzl (No.30): 1.15g, yield 86%

^{1}H NMR ($CDCl_3$): δ 7.32 (5H, m), 5.19 (2H, t), 4.29-4.35 (1H, dd), 3.45-3.69 (1H, t), 3.02-3.04 (2H, m), 2.69 (2H, t), 2.34 (2H, m), 2.00 (2H, m), 1.69-1.70 (2H, m), 1.51-1.54 (2H, m), 1.24-1.26 (2H, m), 1.39 (18H, s).

^{13}C NMR ($CDCl_3$): δ 176.6, 171.0, 170.8, 155.8, 139.4, 128.4, 128.1, 128.0, 127.9, 126.6, 78.5, 78.3, 58.6, 55.5, 50.4, 43.7, 43.6, 34.7, 32.1, 28.7, 28.7, 28.7, 28.7, 28.6, 28.6, 21.9, 21.3, 20.8.

Lys-Pro-OBzl (No.31*): 0.38g, yield 80%.

^{1}H NMR ($CDCl_3$): δ 8.28, 5.36 ($2NH_2$, bs, after D_2O shake), 7.32-7.40 (5H, m), 5.06-5.18 (2H, t), 4.41-4.47 (1H, dd), 3.68-3.74 (1H, t), 3.33-3.47 (2H, m), 2.69

(2H, t), 2.23-2.29 (2H, m), 1.81-1.89 (2H, m), 1.65-1.78 (2H, m), 1.49 (2H, m), 1.34-1.39 (2H, m).

^{13}C NMR (DMSO): δ 171.2, 167.5, 135.8, 128.5, 128.5, 128.2, 127.8, 127.8, 66.1, 58.8, 50.6, 46.8, 45.6, 29.5, 28.6, 26.6, 24.8, 20.8.

Lys-Pro-OH (No.32)[123]: 0.15g, yield 53%.

^{1}H NMR (DMSO): δ 10.91 (OH, bs), 6.78, 5.45 ($2NH_2$, bs), 4.29 (1H, dd), 3.64 (1H, t), 3.31 (2H, m), 2.74 (2H, t), 2.49 (2H, m), 2.05 (2H, m), 1.79 (2H, m), 1.50 (2H, m), 1.31 (2H, m).

^{13}C NMR (DMSO): δ176.0, 169.8, 59.0, 53.8, 43.7, 42.8, 35.9, 33.5, 22.9, 20.9, 19.8.

Boc-Met-Pro-OMe (No.33): 1.35g, yield 94%

^{1}H NMR ($CDCl_3$):δ 4.17-4.31 (1H, dd), 3.63 (3H, s), 3.59 (1H, t), 3.47 (2H, m), 2.41 (2H, t), 2.32 (2H, m), 2.16 (2H, m), 2.07 (3H, s), 1.88-1.91 (2H, m), 1.48 (9H, s).

^{13}C NMR ($CDCl_3$): δ 175.8, 170.3, 155.1, 78.7, 58.2, 51.6, 50.4, 46.5, 34.8, 29.3, 27.7, 27.7, 27.8, 23.5, 20.4, 14.8.

Met-Pro-OMe (No.34*): 0.62g, yield 79%

^{1}H NMR ($CDCl_3$): δ 8.39 (NH_2, bs, after D_2O shake), 4.52-4.56 (1H, dd), 3.69 (1H, t), 3.50 (2H, m), 3.41 (3H, s), 2.59-2.74 (2H, t), 2.18-2.25 (2H, m), 2.07(2H, m), 2.04 (3H, s), 1.89 (2H, m).

^{13}C NMR ($CDCl_3$): δ 175.2, 169.7, 59.3, 53.7, 50.7, 44.1, 34.8, 29.8, 22.5, 20.9, 14.9.

Boc-Met-Pro-OBzl (No.35): 1.51g, yield 86%

^{1}H NMR ($CDCl_3$): δ 7.32-7.36 (5H, m), 5.08-5.22 (2H, t), 4.58-4.63 (1H, dd), 3.78 (1H, t), 3.56 (2H, m), 2.53-2.59 (2H, t), 2.17-2.28 (4H, m), 2.09 (3H, s), 1.95-2.05 (2H, m), 1.43 (9H, s).

^{13}C NMR ($CDCl_3$): δ 175.6, 168.6, 155.4, 135.6, 128.5, 128.5, 128.3, 127.6, 127.3, 79.4, 67.1, 58.4, 53.6, 44.8, 37.3, 29.2, 28.7, 28.7, 28.5, 23.7, 20.6, 15.7.

Met-Pro-OBzl (No.36*): 0.67g, yield 79%

^{1}H NMR ($CDCl_3$): δ 6.72 (NH_2, bs, after D_2O shake), 7.34-7.37 (5H, m), 5.04-5.23 (2H, t), 4.41-4.67 (1H, dd), 3.60 (1H, t), 3.43 (2H, m), 2.60 (2H, t), 2.24 (2H, m), 2.13 (2H, m), 2.08 (3H, s), 1.99 (2H, m).

^{13}C NMR ($CDCl_3$): δ 174.9, 169.6, 135.2, 128.4, 128.3, 128.0, 127.9, 127.9, 69.2, 58.9, 53.4, 46.9, 37.8, 30.1, 24.6, 21.8.

Met-Pro-OH (No.37)[124]: 0.12g, yield 98%

^{1}H NMR (DMSO): δ 11.3 (OH, bs), 6.40 (NH_2, bs), 4.17 (1H, dd), 3.72-3.74 (1H, t), 3.27-3.50 (2H, m), 2.48-2.49 (2H, t), 2.00-2.20 (4H, m), 1.97 (3H, s), 1.82-1.88 (2H, m)

^{13}C NMR ($CDCl_3$): δ 176.7, 175.4, 57.5, 55.8, 45.1, 33.0, 29.2, 21.7, 20.8, 14.5.

Boc-Nle-Pro-OMe (No.38): 0.42g, yield 84%

^{1}H NMR ($CDCl_3$): δ 4.12-4.19 (1H, t), 3.67 (1H, dd), 3.50 (3H, s), 2.07-2.17 (4H, m), 1.92 (2H, m), 1.87 (2H, m), 1.40 (9H, s), 1.22-1.28 (4H, m), 0.87, 0.89, 0.91 (3H, t).

^{13}C NMR ($CDCl_3$): δ 172.1, 170.3, 156.7, 78.9, 59.1, 54.3, 51.7, 45.9, 33.6, 29.9, 28.7, 28.7, 28.7, 25.8, 24.1, 22.0, 19.7.

Nle-Pro-OMe (No.39*): 0.18g, yield 83%

^{1}H NMR ($CDCl_3$): δ 8.19 (NH_2, bs, after D_2O shake), 4.16-4.48 (1H, dd), 3.68 (1H, t), 3.51 (3H, s), 2.21 (2H, m), 2.19 (2H,m), 1.95 (2H,m), 1.81 (2H, m), 1.22-1.32 (4H, m), 0.83, 0.86, 0.89 (3H, t).

^{13}C NMR ($CDCl_3$): δ 175.9, 172.7, 59.1, 52.1, 51.7, 46.9, 33.4, 30.2, 25.9, 25.3, 24.6, 22.2.

Boc-Nle-Pro-OBzl (No.40): 0.79g, yield 83%

^{1}H NMR ($CDCl_3$): δ 7.32-7.35 (5H, m), 5.07, 5.12, 5.15, 5.20 (2H, t), 4.39-4.60 (1H, dd), 3.72 (1H, t), 3.57 (2H, m), 2.23 (2H, m), 1.90-1.99 (2H, m), 1.65-1.73 (2H, m), 1.40-1.44 (9H, s), 1.20-1.36 (4H, m), 0.83, 0.86, 0.89 (3H, t).

^{13}C NMR ($CDCl_3$): δ 171.6, 167.3, 155.7, 135.4, 128.6, 128.5, 128.5, 126.4 126.4, 79.6, 67.4, 58.9, 49.0, 42.9, 33.7, 31.3, 28.8, 28.8, 28.8, 25.5, 24.8, 24.5, 22.1.

Nle-Pro-OBzl (No.41*): 0.49g, yield 82%.

^{1}H NMR ($CDCl_3$): δ 5.73 (NH_2, bs after D_2O shake), 7.28 (5H, m), 5.17 (2H, t), 4.53-4.56 (1H, dd), 3.62 (1H, t), 3.47 (2H, m), 2.14-2.19 (2H, m), 1.92-1.93 (2H,m),1.77 (2H, m), 1.21-1.34 (4H, m), 0.77, 0.80, 0.83 (3H, t).

^{13}C NMR ($CDCl_3$): δ 171.2, 171.1, 135.1, 128.6, 128.5, 128.4, 128.4, 128.2, 67.2, 59.4, 52.7, 47.3, 35.7, 30.5, 28.6, 24.7, 22.1, 20.2.

Nle-Pro-OH (No.42)[125]: 0.17g, yield 70%

^{1}H NMR ($CDCl_3$): δ 11.8 (OH, bs), 6.75 (NH_2, bs), 4.22-4.24 (1H, dd), 3.63-3.66 (1H, t), 3.32-3.41 (2H, m), 2.14 (2H, m), 2.03 (2H, m), 1.88 (2H, m), 1.68 (2H, m), 1.23 (2H, m), 0.81, 0.85, 0.89 (3H, t).

^{13}C NMR (DMSO): δ 175.2, 168.6, 61.6, 59.9, 46.4, 30.9, 26.0, 24.4, 22.3, 22.0, 21.0.

Boc-Nva-Pro-OMe (No.43): 0.78g, yield 69%

^{1}H NMR ($CDCl_3$): δ 4.19-4.30 (1H, dd), 3.51-3.56 (1H, t), 3.45 (3H, s), 3.39-3.41 (2H, m), 1.96-2.00 (2H, m), 1.80-1.83 (2H, m), 1.76-1.78 (2H, m), 1.64 (2H, m), 1.18 (9H, s) 1.00-1.03 (3H, t).

^{13}C NMR ($CDCl_3$): δ 172.0, 169.3, 154.7, 78.5, 59.8, 58.8, 52.3, 45.9, 35.6, 30.9, 27.6, 27.4, 27.4, 21.8, 20.5, 18.2.

Nva-Pro-OMe (No.44*): 0.48g, yield 89%

^{1}H NMR ($CDCl_3$): δ 8.26 (NH_2, bs, after D_2O shake), 4.21-4.54 (1H, dd), 3.67 (3H, s), 3.54 (1H, t), 3.48 (2H, m), 2.20 (2H, m), 1.98-2.04 (2H, m), 1.83 (2H, m), 1.76 (2H, m), 0.93 (3H, t).

^{13}C NMR ($CDCl_3$): δ 170.8, 168.2, 60.3, 59.0, 51.7, 46.9, 32.5, 30.9, 24.5, 22.3, 20.9.

Boc-Nva-Pro-OBzl (No.45): 0.30g, yield 66%.

^{1}H NMR ($CDCl_3$): δ 7.28-7.33 (5H, m), 5.14 (2H, t), 4.55-4.59 (1H, t), 3.69 (1H, dd), 3.60 (2H, m), 2.00-2.02 (2H, m), 1.93-1.97 (2H, m), 1.64 (2H, m), 1.33-1.44 (9H, s), 1.22 (2H, m), 0.84, 0.87, 0.90 (3H, t).

^{13}C NMR ($CDCl_3$): δ 171.6, 167.3, 155.7, 135.4, 128.6, 128.5, 128.2, 128.0, 126.4, 72.6, 67.4, 58.8, 55.0, 42.9, 33.7, 31.9, 28.9, 28.9, 28.9, 23.7, 22.5, 21.0.

Nva-Pro-OBzl (No.46)[126]: 0.12g, yield 98%.

^{1}H NMR ($CDCl_3$): δ 10.3 (NH_2, bs, after D_2O shake), 7.32-7.39 (5H, m), 5.13, (2H, t), 4.00-4.08 (1H, dd), 3.81 (1H, t), 3,53-3.58 (2H, m), 2.12-2.18 (2H, m), 1.98 (2H, m), 1.61 (2H, m), 1.30 (2H, m), 0.85-0.91 (3H, t).

^{13}C NMR ($CDCl_3$): δ 175.9, 168.7, 136.4, 127.8, 127.6, 127.5, 126.3, 126.1, 67.3, 59.8, 48.7, 42.9, 32.3, 31.2, 23.9, 22.8, 21.5.

Nva-Pro-OH (No.47)[127]: 0.12g, yield 56%.

^{1}H NMR (d^5-Pyridine): δ 10.89 (OH, bs), 5.67 (NH_2, bs), 4.55-4.59 (1H, dd), 3.84 (1H, t), 3.40 (2H, m), 2.33 (2H, m), 2.16 (2H, m), 1,97 (2H, m), 1.84 (2H, m), 0.88, 0.91, 0.94 (3H, t).

^{13}C NMR (d^5-Pyridine): 175.9, 170.8, 59.4, 51.2, 44.8, 34.9, 31.7, 28.9, 23.5, 21.0.

Boc-Sar-Pro-OMe (No.48): 0.84g, yield 88%.

^{1}H NMR ($CDCl_3$): δ 4.46-4.48 (1H, dd), 3.67 (3H, s), 3.50 (2H, m), 3.48 (2H, s), 2.89 (3H, s), 2.10-2.14 (2H, m), 1.94-2.02 (2H, m), 1.40 (9H, s).

^{13}C NMR ($CDCl_3$): δ 174.2, 172.0, 157.6, 72.6, 58.9, 54.6, 49.7, 43.1, 33.5, 27.9, 27.9, 27.9, 23.9, 19.8.

Sar-Pro-OMe (No.49)[128]: 0.19g, yield 93%.

^{1}H NMR (DMSO): δ 4.34-4.37 (1H, dd), 3.61 (3H, s), 3.40-3.48 (4H, m), 2.50 (3H, s), 2.12-2.18 (2H, s), 1.89-1.94 (2H, m).

^{13}C NMR ($CDCl_3$): δ 172.7, 172.1, 58.5, 52.1, 50.7, 45.8, 32.6, 22.7, 21.2

Boc-Sar-Pro-OBzl (No.50): 1.61g, yield 81%.

^{1}H NMR ($CDCl_3$): δ 7.19 (5H, m), 5.20 (2H, t), 4.32 (1H, dd), 3.67 (3H, s), 3.50 (2H, m), 3.47 (2H, s), 2.49 (3H, s), 2.26 (2H, m), 1.90-2.01 (2H, m), 1.42 (9H, s).

^{13}C NMR ($CDCl_3$): δ 171.9, 170.5, 156.1, 135.6, 128.6, 128.4, 128.4, 128.4, 128.4, 79.8, 66.7, 58.9, 50.6, 46.0, 35.3, 28.8, 28.8, 28.8, 24.8, 22.1.

Sar-Pro-OBzl (No.51*): 1.03g. yield 88%.

^{1}H NMR ($CDCl_3$): δ 9.44 (NH_2, bs, after D_2O shake), 7.20 (5H, m), 5.21 (2H, t), 4.42-4.54 (1H, dd), 3.64 (3H, s), 3.47 (2H, m), 3.43 (2H, s), 2.56, 2.69 (3H, d), 2.11-2.20 (2H, m), 1.84-1.98 (2H, m).

^{13}C NMR ($CDCl_3$): δ 172.1, 170.1, 135.3, 128.7, 128.5, 128.3, 128.3, 128.1, 79.2, 58.6, 54.2, 46.2, 33.0, 24.4, 22.2.

Sar-Pro-OH (No.52*): 0.42g, yield 60%.

^{1}H NMR (DMSO): δ 4.18 (1H, dd), 3.48 (2H, s), 3.38 (2H, m), 2.48 (3H, s), 2.34 (2H, m), 1.81 (2H, m).

^{13}C NMR (DMSO): δ 176.6, 172.5, 58.9, 54.6, 42.8, 34.7, 23.5, 20.6.

Boc-Ser-Pro-OBzl (No.53): 0.328, yield 50%

^{1}H NMR ($CDCl_3$): δ 1.36 (9H, s), 1.87-1.96 (2H m), 2.34 (2H m), 3.34 (2H, m), 3.60 (1H, t), 4.10 (2H, d), 4.36 (1H, dd), 5.16 (2H, t), 7.29 (5H m).

^{13}C NMR (CDCl3): δ 176.1, 170.1, 155.5, 135.2, 128.1, 128.1, 127.9, 127.8, 127.8, 79.9, 72.5, 67.2, 59.6, 57.9, 43.1, 28.7, 28.7, 28.7, 23.5, 21.6.

Ser-Pro-OBzl (No.54)[129]: 0.16g, yield 63%

^{1}H NMR ($CDCl_3$): δ 7.20 (5H, m), 5.21 (2H, t), 4.40 (1H, dd), 4.00 (2H, d), 3.59 (1H, t), 3.42 (2H, m), 2.34 (2H, m), 1.97 (2H, m).

^{13}C NMR ($CDCl_3$): δ 176.8, 168.0, 134.8, 129.1, 128.5, 128.5, 128.1, 128.1, 72.9, 67.3, 61.0, 58.9, 45.2, 23.8, 20.4.

Boc-Ser-Pro-OMe (No.55): 0.34g, yield 38%

^{1}H NMR($CDCl_3$): δ 4.26-4.32 (1H, dd), 4.05 (2H, d), 3.65 (3H, S), 3.58-3.63 (1H, t), 3.40-3.43 (2H m), 2.16 (2H, m), 1.83-1.97 (2H m),

13C NMR (CDCl3): δ 172.2, 170.1, 155.5, 79.7, 66.1, 59.7, 58.5, 52.5, 43.9, 28.8, 28.7, 28.7, 24.5, 22.3.

Ser-Pro-OMe (No.56)[130]: 0.14g, yield 71%

^{1}H NMR (DMSO): δ 8.97 (OH, bs), 5.65 (NH_2 bs), 4.33 (2H m), 4.07 (2H, d), 3.69 (3H, s), 3.60 (1H, t), 3.47 (2H, m), 2.19-2.25 (2H, m), 1.78-1.95 (2H, m).

^{13}C NMR (DMSO): δ 172.8, 170.8, 70.2, 62.5, 59.4, 51.2, 44.7, 24.8, 21.3.

Ser-Pro-OH (No.57)[131]: 0.13g, yield 62%

^{1}H NMR(DMSO): δ 11.9 (CO*OH*, bs), 8.79 (OH, bs), 6.01(NH_2, bs), 4.23-4.30 (1H, dd), 4.10 (2H, d), 3,67 (1H, t), 3.37 (2H, m), 2.45 (2H, m), 1.92 (2H, m).

^{13}C NMR (DMSO): δ 172.5, 171.6, 66.9, 60.9, 57.9, 44.6, 23.0, 20.4.

3. Solubility

In the following Gly-Pro uptake experiment, all the dipeptides and their derivatives are required to be soluble in water-buffer. However, most of dipeptides and dipeptide derivatives are not water-soluble.

Because dimethoxysulfoxide (DMSO) is a very powerful solvent to dissolve insoluble chemicals, DMSO could be used to help dipeptides and dipeptide derivatives dissolve in the buffer. However DMSO is very toxic to caco-2 cells used in the Gly-Pro uptake experiment; furthermore, if the concentration is higher than the required concentration, the caco-2 cells could be killed. Therefore, solubility experiments were necessary, in order to find the optimum percentage of DMSO in buffer, which would allow insoluble chemicals to be dissolved in buffer; but which was harmless to caco-2 cells.

It was already known that, for caco-2 cells, the highest concentration of DMSO in a buffered solution of dipeptides and their derivatives must be no more than 5%.

3.1. Buffer

According to the buffer to be used in Gly-Pro uptake experiment, two different types of buffer were made, which will be introduced as discussed below.

1> HBSS/MES Buffer

0.98g of Hank's Balanced Salt Solution (HBSS).

0.488g of 2-[N-Morpholino]-Ethanesulphonic Acid (MES) whose useful pH range is 5.5-6.7.

100ml of Double Distilled Water (dd H_2O).

2> HBSS/HEPES Buffer

0.98g of Hank's Balanced Salt Solution (HBSS).

0.5958g of N-[2-Hydroxyethyl]Piperazine-N'-[2-Ethanesulfonic Acid] (HEPES) whose useful pH range is 6.8-8.2.

100ml of Double Distilled Water (dd H_2O)

The pH value of HBSS/MES and HBSS/H.EPES buffers was adjusted to pH6, 6.5 and 7, 7.5 respectively by addition of 1mM NaOH.

3.2. The Operation of Solubility

The dipeptide Gly-Pro-OMe is taken as an example to introduce the solubility experiment. Gly-Pro-OMe(0.37g) was dissolved in 20ml of

dichloromethane to get 1.85×10^{-2}g/ml solution. A syringe was used to draw 0.1ml of this solution diluted by 10ml of dichloromethane to get 0.185mg/ml Gly-Pro-OMe solution. 1.8ml of this solution was drawn by syringe and the solvent was removed under reduced pressure.

From the effect of DMSO uptake test, there is not much difference between 1% and 2%. The concentration should be controlled around 1% because of the toxicity of DMSO to caco-2 cells. The result of the solubility experiment of Gly-Pro-OMe is shown below (**Table 4.2**) and is typical of other dipeptides and their derivatives.

Apart from the cysteinyl derivatives, all the water-insoluble dipeptides and their derivatives could be dissolved in biological buffer by the addition of DMSO, which is no more than 1%. In solubility experiment with cysteinyl derivatives, even if 2% DMSO was used, they still can not dissolve very well, which might be due to the sulfur(S) in the chemical structure.

4. Gly-Pro Uptake Experiment

The 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.

Table 4.2 The example of solubility experiment

pH VALUE	THE QUANTITY OF CHEMICAL	DMSO%	Gly-Pro-OMe%
pH 6	DMSO 0.10ml buffer 11.8ml Gly-Pro-OMe 0.3045mg	0.8	2.5
pH 6.5	DMSO 0.12ml buffer 12.6ml Gly-Pro-OMe 0.3502mg	0.9	2.7
pH 7	DMSO 0.10ml buffer 10.7ml Gly-Pro-OMe 0.3045mg	0.9	2.8
pH 7.5	DMSO 0.11ml buffer13.7ml Gly-Pro-OMe 0.3200mg	0.8	2.3

4.1. The Medium Used in Uptake Experiment

4.1.1. Transport media

The ingredients of transport media are shown as following:

4.88g Hank's Balanced Salt Solution (HBSS)

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

0.58g Proline

500ml Double distilled water (dd H_2O)

The pH of solution was adjusted to 6.0 by the addition of 1mM NaOH. For the uptake study at different pHs, the solution was adjusted to pH 5.0, 5.5, 6.0, 6.5. A new solution containing 2.98g HEPES buffer instead of the MES buffer was made and then adjusted to pH 7.0 and 7.5. The solution was stored at 4°C.

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 all the radioactivity in the cells should be in Gly-Pro.

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 needed 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

48.5μCi=1nmol

9.7μCi=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.

4.1.2 Stop solution

Stop solution contains 0.25g sodium azide dissolved 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°C.

4.1.3 Detergent Solution

The detergent solution contains 5ml of Triton-X dissolved in 500ml dd H_2O. This 1% v/v solution is used to dissolve the cell membranes. The solution was stored at room temperature.

4.1.4. Protocol

The transport and Gly-Pro solutions were warmed to 37°C, 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. 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 4.3**)

Table 4.3 The Example Calculation

Vial	Well 1	Well 2
D (dpm)	1548976	1535718
W1 (dpm)	62927.7	56335.3
W2 (dpm)	4019.15	4012.74
C (dpm)	17669.6	15171.4
Total (dpm)	1633592	1611237
Corrected C (dpm)	17669.6	15381.89

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 it would have been if the well had received the total amount of radioactivity Well1 did. The corrected value is calculated from the following equation. The corrected value is calculated from the following equation.

Corrected Well 2C=((Total Well1)/(Total Well2)) * (Well 2C)

4.3. 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 to calculate the amounts of Gly-Pro;

$1dpm=4.545x10^{-13}$ Ci

$48.5Ci=1*10^{-3}$ mol

$4.545x10^{-13}Ci=9.371x10^{-18}$ mol

Therefore, $1dpm=9.371x10^{-6}$ pmol

4.4. The Calculation of Percentage of Control, inhibition and IC50

Control%=(well pmol)/(mean control pmol)*100%

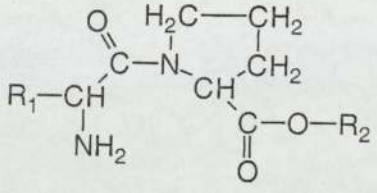
Inhibition%=100%-Control%

IC_{50} mM=(Inhibitor concentration mM) / {[(mean control pmol) / (well pmol)]-1}

5. Result of Gly-Pro uptake

Inhibitor effects of dipeptide derivatives on uptake. The test experiments were performed to calculate the IC_{50} and percentage of control for thirty-three dipeptide derivatives. The results are shown in the **Table 4.4,** and **Figures 4.1, 4.2,** and **4.3** are the different comparison of the dipeptides and dipeptide derivatives.

Table4.4 Dipeptide Derivative Results as at 13/8/99



R_1	R_2	IC50 mM	SD	p*
H-	H	0.458	0.079	
Gly-Pro	Bzl	0.627	0.355	0.0018
	Me	1.575	0.137	
CH3-	H	0.487	0.042	
Ala-Pro	Bzl	1.829	0.320	0.0632
	Me	1.798	1.030	

CH3-CH2-	H	0.779	0.180	
Abu-Pro	Me	5.268	2.679	0.0443
CH3-CH2-CH2-	H	1.208	0.136	
Nva-Pro	Bzl	0.686	0.054	0.0024
	Me	1.161	0.131	
CH3-CH2-CH2-CH2-	H	0.299	0.027	
Nle-Pro	Bzl	0.438	0.044	0.0001
	Me	0.786	0.071	
NH2-CH2-CH2-CH2-CH2-	H	1.111	0.427	
Lys-Pro	Bzl	2.853	0.708	0.0065
	Me	3.058	0.322	
CH3-S-CH2-CH2-	H	2.637	0.307	
Met-Pro	Bzl	0.218	0.023	0.0001
	Me	0.415	0.060	
HOOC-CH2-CH2-	H	0.483	0.107	
Glu-Pro	Bzl	0.157	0.027	0.0395
CO(OBzl)-CH2-CH2-	Me	0.294	0.074	

Glu(OBzl)-Pro				
Bzl-S-CH2-	Bzl	0.545	0.229	0.1545
Cys(SBzl)-Pro	Me	1.274	0.683	
HS-CH2-	Bzl	1.802	0.113	0.2538
Cys-Pro	Me	1.686	0.100	
HO-CH2-	H	0.723	0.106	
Ser-Pro	Bzl	0.431	0.040	0.0001
	Me	2.156	0.184	
CH3-NH-CH2-COO-Pro	H	0.377	0.073	
Sar-Pro	Bzl	0.583	0.136	0.0074
	Me	4.161	1.804	

A number of compounds were not very soluble but most dissolved in 1% DMSO. However, the cysteinyl derivatives did not dissolve much even using 2% DMSO.

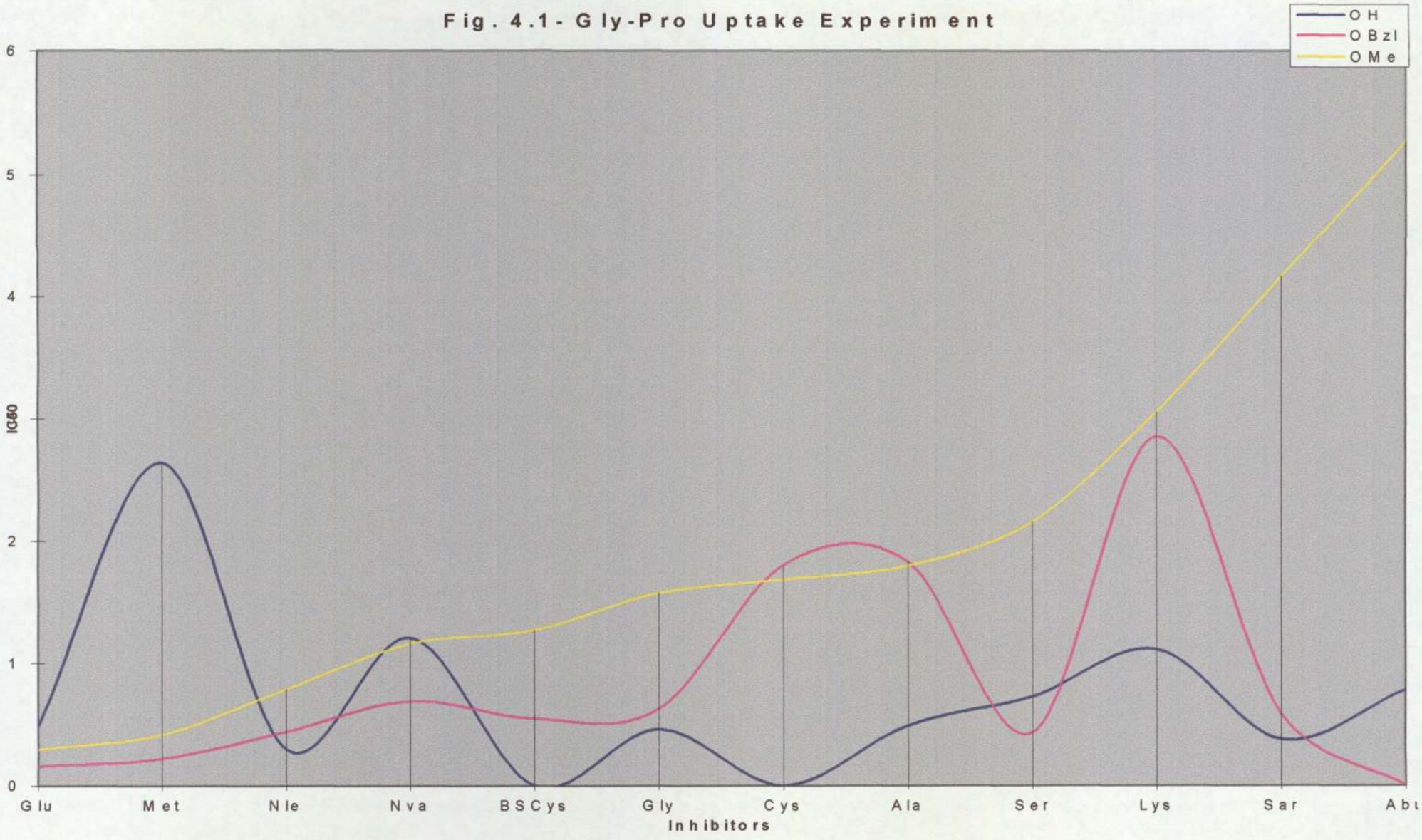


Fig. 4.1- Gly-Pro Uptake Experiment
O H
O Bzl
O Me
6
5
4
2
1
0
IC50
Glu
Met
Nle
Nva
BSCys
Gly
Cys
Ala
Ser
Lys
Sar
Abu
Inhibitors

Fig.4.3- Graph showing IC50 of gly-pro uptake for dipeptides grouped as proline free acids, benzyl esters and methyl esters

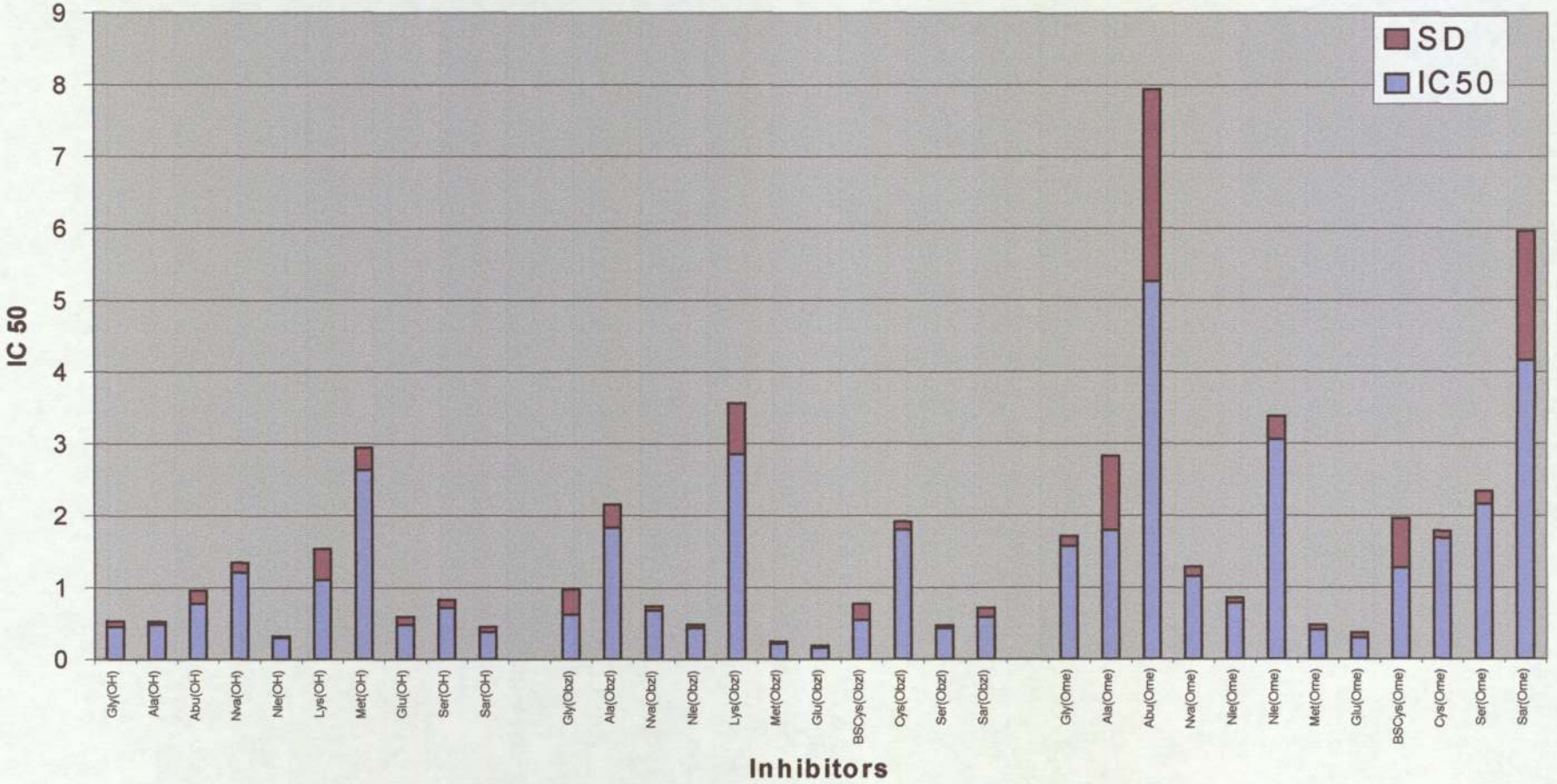
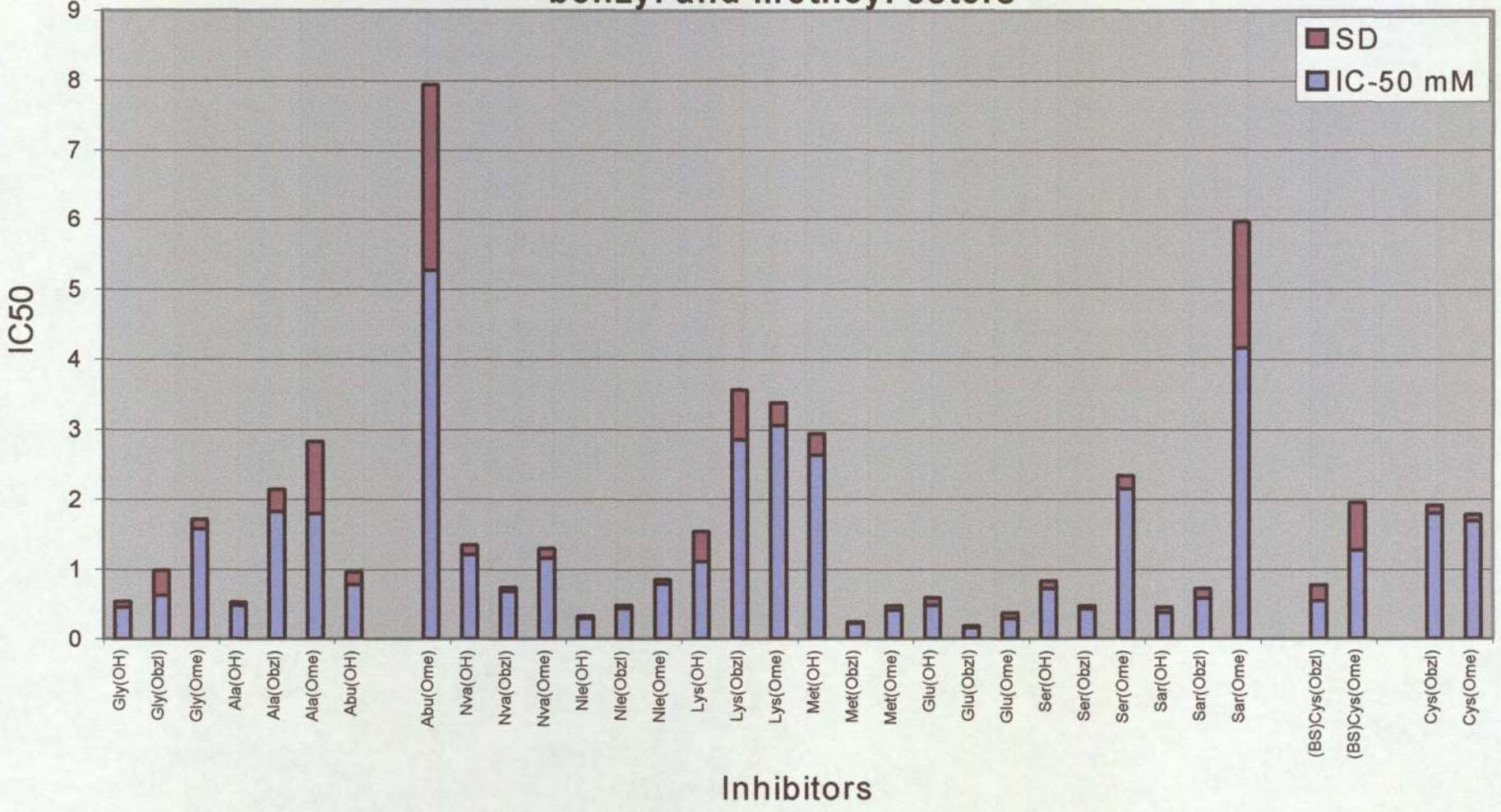


Fig.4.2 - Graph showing IC50 of gly-pro uptake for diepptides grouped as N-terminal amino acids and corresponding proline free acids, benzyl and metheyl esters



5.1. The Influence of Dipeptide Structure

The relationship between the chemical structure of the various dipeptides and their ease of transport into the cell will be discussed in the following section.

5.1.1. The Change of Carbon Chain of Dipeptide

Glycine, alanine, aminobutyric acid, norvaline and norleucine were incorporated into dipeptides with proline. These five amino acids do not have special groups, so, the length of their chain should be the major factor which influence dipeptide uptake into caco-2 cells. The results indicated that norleucine-containing dipeptide (IC50 is 0.299) and its derivatives (methyl ester IC50 is 0.786, benzyl ester IC50 is 0.438) are the best in this group. It is concluded that the longer the chain of this amino acid is, the better inhibitor this dipeptide is. However, sometimes it is not the necessary factor, for example, the chain of norvaline is longer than glycine, but in the result of uptake experiment, IC50 of Nva-Pro-OH (with free carboxylic group) 1.208 which is higher than that of Gly-Pro-OH, (IC50 is 0.458). The IC50 of Gly-Pro-OBzl (0.627) and Gly-Pro-OMe (1.575) are both higher than Nva-Pro-OBzl (0.686) and Nva-Pro-OMe (1.161).

5.1.2. Dipeptide with Active Side Chain

The results of the uptake experiment shows that the dipeptides containing lysine were least effective as inhibitors for Gly-Pro uptake, i.e. the basic side chain had a detrimental effect. Lys-Pro-OH which has free carboxylic group with an IC50 of 1.111 was better than its derivatives with benzyl ester group (IC50 is 2.853) and methyl ester group (IC50 is 3.058).

Compared with Lys-Pro and its derivatives, the acidic amino acid, glutamic acid is more ideal which has two free carboxylic groups. Glu-Pro-OH, has free acidic group, whose IC50 is 0.483. However, it is interesting that this dipeptide's derivatives, with two benzyl ester groups or one benzyl and methyl ester group, IC50 are 0.157 and 0.294 respectively), are obviously better. Therefore, a dipeptide with a free carboxylic group in the side chain is not necessarily the best inhibitor, e.g. the IC50 of Glu-Pro-OH which has two free carboxylic groups is worse than that of Glu(OBzl)-Pro-OBzl where the two carboxylic groups are replaced by two benzyl ester groups.

Cysteine and serine have similar structures, the only difference between these two amino acids is the polar group, thiol group (-SH) in cysteine and alcohol group (-OH) in serine. Ser-Pro-OBzl was compared with Cys-Pro-OBzl, the IC50 of Ser-Pro-OBzl is 0.723 which is better than latter (IC50 is 1.802). In order to find the effect of benzyl group further, Cys(SBzl)-Pro-OBzl and Cys(SBzl)-Pro-OMe were produced. After the uptake experiment, both of their IC50s are better than Cys-Pro-OBzl and Cys-Pro-OMe without protection on thiol. And Cys(SBzl)-Pro-OBzl, IC50 is 0.545, which is worse than Ser-Pro-Obzl (IC50 is 0.431), but better than Ser-Pro-OMe (IC50 is 2.156).

Sarcosine does not have the free amide group, but after the uptake experiment, Sar-Pro-OH with IC50 of 0.377 is a good inhibitor, which is better than Sar-Pro-OBzl whose IC50 is 0.583. However, the other derivative of Sar-Pro-OH with methyl ester group is a poor inhibitor with an IC50 of 4.161.

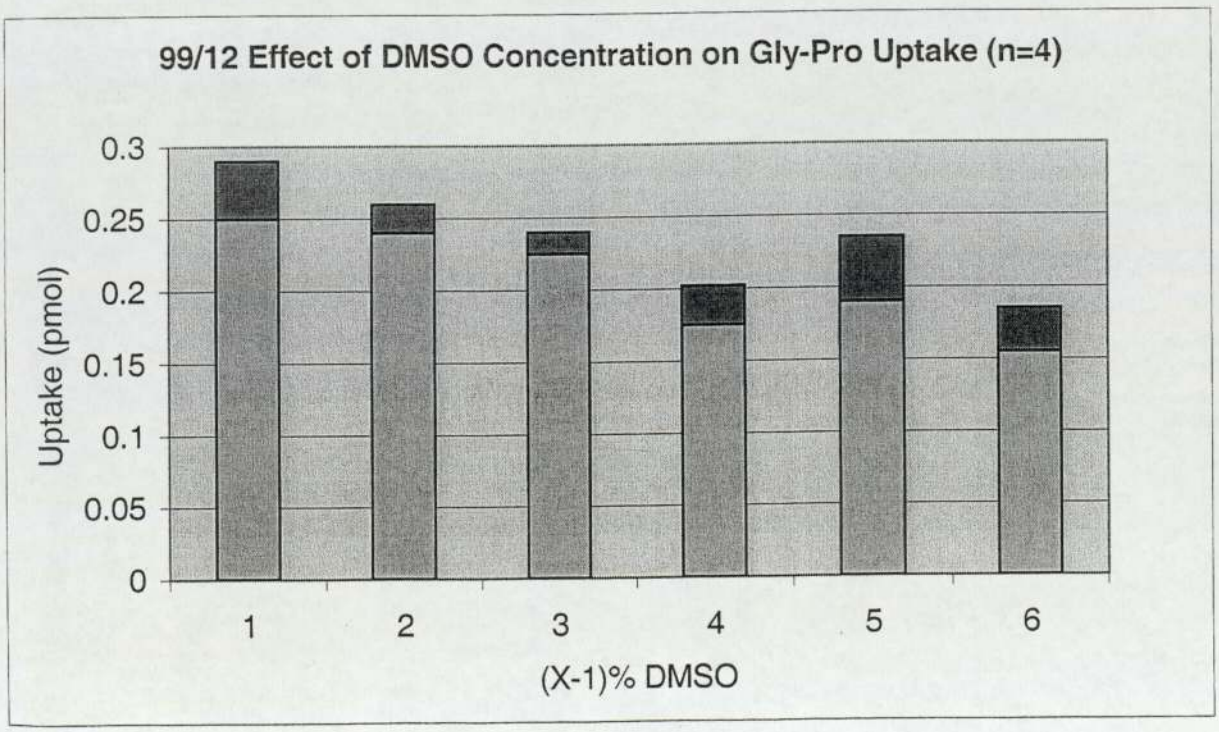
From the result of the uptake experiment, dipeptide with free carboxylic group is not necessarily the best inhibitor. In contrast, dipeptide derivatives with

benzyl ester show very good inhibitor's ability, for example, the two best inhibitors are both benzyl esters Met-Pro-OBzl with an IC50 of 0.218 and Glu(OBzl)-Pro-OBzl with an IC50 of 0.157. However, dipeptide derivatives with methyl ester are poor inhibitors, e.g. the two poorest inhibitors are both methyl esters Abu-Pro-OMe (IC50 is 5.268) and Sar-Pro-OMe (IC50 is 4.161).

5.2. The Effect of DMSO on Uptake

In the Gly-Pro uptake test experiment, DMSO was added in to the solution to help dipeptide derivatives be dissolved in the Gly-Pro solution. However, DMSO is toxic to Caco-2 cells and so could reduce uptake by killing cells. Therefore, the concentration of DMSO is limited. Then, different concentrations of DMSO are necessary to be tested before the experiment. The results are shown in **Figure 4.4**.

Fig.4.4 Effect of DMSO Concentration on Gly-Pro Uptake (n=4)



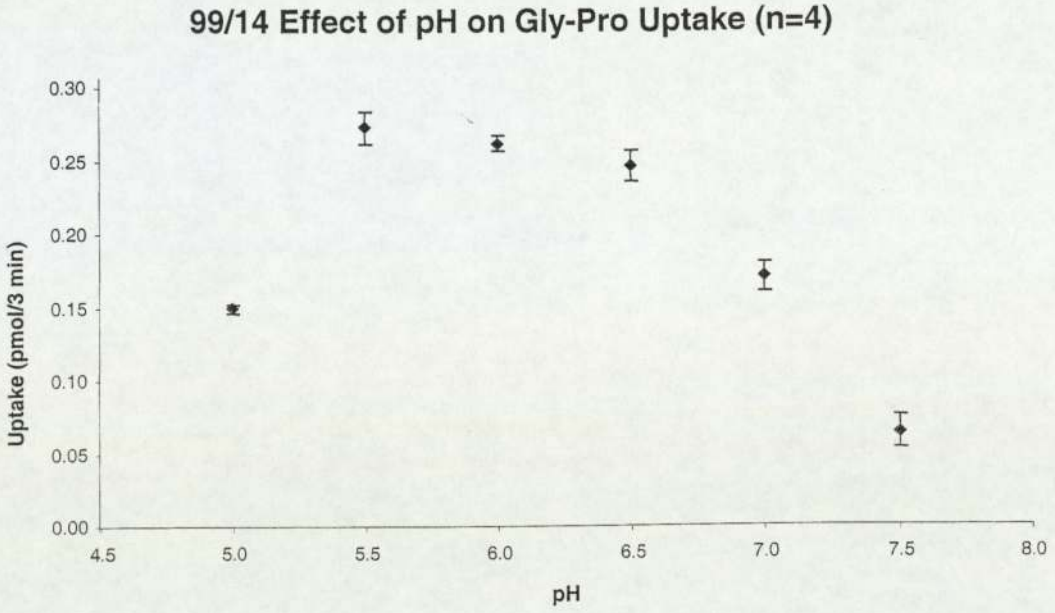
There is no significant difference between 0% DMSO (control cells) and 1 or 2% DMSO. Therefore, in most uptake experiments, 1% DMSO was used to increase dipeptide derivatives solubility. 2% DMSO only was used in cysteinyl derivatives, because of its very poor solubility. The higher concentration of DMSO, the more toxic it is to caco-2 cells. Actually, cysteinyl derivatives still can't be dissolved completely in the solution with 2% DMSO, probably the group of -SH has effect on the solubility. However, at higher concentrations, the toxic effect becomes more significant. 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 the Gly-Pro uptake experiment, pH is one of the factors affecting the result of experiment. Gly-Pro uptake at six different pH levels was tested. The result was shown in **Figure 4.5**.

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

Fig. 4.5 – The effect of pH on the experiment:



CHAPTER 5- CONCLUSION

1. 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, though the NMR results for Cys(SBzl)-Pro-OBzl and Cys-Pro-OBzl were disappointing as deprotection of the benzyl group was not achieved. One possible reason for these anomalous results is that pallalium on carbon could be poisoned by the sulfur existing in cysteine, which makes the catalytic hydrogenation a non-viable option in these cases.

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, DCM).

(ii) A minimum of base (TEA).

(iii) Low reaction temperatures (ice bath).

(iv) Carbonate protective groups.

The aim of this project was to find a good inhibitor, furthermore, the main conclusions can be drawn from the Gly-Pro uptake.

(i) In 1981, Adibi S. A. [12] concluded that the peptide transport is reduced by the esterification of the C-terminal carboxy group. However, from my work, it has been shown that C-terminal benzyl ester dipeptides are sometimes better inhibitors than C-terminal free acid dipeptides. From the data

obtained in the uptake experiments, even the methyl ester dipeptide (Met-Pro-OMe, IC50 0.415) is better than its free acid dipeptide (Met-Pro-OH, IC50 2.637).

(ii) The best three inhibitors synthesized are Glu(OBzl)-Pro-OBzl (IC50 is 0.157), Met-Pro-OBzl (IC50 is 0.218), Glu(OBzl)-Pro-OMe (IC50 is 0.294).

Moreover, the concentration range of DMSO and pH range was tested. The ideal concentration should be lower than 2% and the optimum pH is 6.0.

2. Further Work

Based on the result of the Gly-Pro uptake experiment, the best inhibitors identified may be sufficiently potent to act as probes in future investigation.

The surprising results given by the benzyl ester dipeptides imply that the benzene ring could be introduced into the synthesis of di-/tripeptides. The inhibitory effect of di-/tripeptides with a different number of benzene rings incorporated into the structure could be compared.

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