A LIPIDIC α-AMINO ACID BASED ADJUVANT/CARRIER SYSTEM FOR PEPTIDE DELIVERY AND ENHANCING PEPTIDE IMMUNOGENICITY

by

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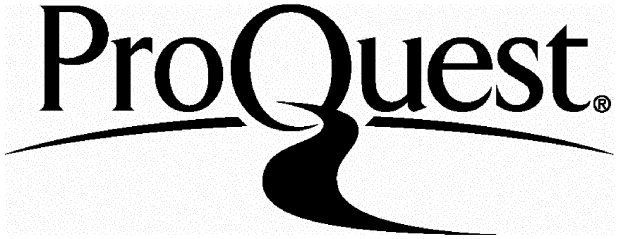

To Mum and Dad,
thankyou

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ABSTRACT

Many pharmacologically active drugs are restricted in their use due to their instability in biological environments and poor oral absorption. In particular, with the recent advances in molecular biology, peptide and protein drugs show vast potential yet fail from the point of view of their intact delivery to the required site of action. This dissertation attempts to approach these problems by means of chemical modification of poorly absorbed, unstable peptides.

The α-amino acids with long alkyl side chains, the so-called lipidic amino acids and their homo-oligomers, the lipidic peptides, represent a class of compounds which combine structural features of lipids with those of amino acids and peptides. These novel compounds provide an excellent means of amplifying peptide lipophilicity to enhance membrane transport and also increase the biological stability of the peptide by protecting it from enzymatic degradation.

This phenomenon has been demonstrated with the poorly absorbed, enzymatically labile peptides thyrotropin releasing hormone (TRH) and luteinizing hormone releasing hormone (LHRH), in Caco-2 cell studies, with both homogenates and intact monolayers. The novel conjugates developed have also been absorbed and detected after oral administration and appear to be stable for a considerable time *in vivo*.

The lipidic amino acids have also been used to help increase the lipophilicity of polylysine which was developed to produce high antibody responses to synthetic peptides. To attain higher antibody responses, the antigen must be anchored to the cell membranes, however the polylysine system is not lipophilic enough to fulfil this requirement. Thus by incorporating lipidic amino acids to the polylysine system, the membrane binding effects and the metabolic stability of this novel Lipid-Core-Peptide (LCP) system is enhanced, allowing the system to anchor down to the cell membrane, and improve the antibody response to the immunogen.

Studies with a selection of peptide epitopes from *Chlamydia trachomatis*, FMDV and

β-haemolytic M-protein, attached to the LCP system are presented and show the LCP system to act as a possible peptide vaccine carrier/adjuvant system, which elicits high antibody responses over a long duration without the need for other toxic adjuvant preparations.

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CHAPTER 1

INTRODUCTION

Ever since the commercial introduction of insulin and thyroid hormone, peptides and proteins have been investigated as potential drug candidates. For a new drug to be clinically effective it should be a highly active compound, exerting its pharmacological properties with minimal side effects and should reach the required site of action in therapeutic concentrations. In recent years, the rapid progress made in molecular biology has given us the opportunity to identify target molecules and to design drugs of a highly specific nature to tackle poorly controlled diseases. However, even though numerous peptides with great therapeutic promise are known, an intense effort is still required if these potential drug molecules are to be delivered to their active site. In particular, peptides and proteins must negotiate a multitude of barriers if they are to be administered by the oral route. With the goal of oral peptide delivery in mind, this thesis exploits the use of a novel group of compounds, the lipidic amino acids, not only for their potential in peptide delivery, but also their possible use as immunoadjuvants for synthetic peptide vaccines.

1.1 Drug absorption

Other than a drug being introduced directly into the systemic circulation by intravenous administration, a drug molecule must undergo some form of absorption for it to pass from the site it is administered to the site at which it exerts its biological action. The absorption process depends upon the ability of the of the drug to cross cell membranes, which can be considered to be bilayers of phospholipid interspersed with proteins and studded with water filled pores (Singer and Nicholson 1972).

Drug absorption studies began with observations that acidic drugs, being unionised at low pH, were well absorbed from the stomach while basic drugs, ionised at low pH, were poorly absorbed (Schanker *et al.* 1957, Hogben *et al.* 1957). The fact that a correlation also existed between the lipid solubility of a compound and its rate of transfer across the gut (Schanker 1959) led to the theory that absorption proceeded *via* diffusion across the lipid membranes of cells and would be favoured by unionised,

lipophilic compounds. Thus, the interrelationship between a compound's dissociation constant, lipid solubility and the pH of the environment formed the basis of the pH-partition hypothesis of drug absorption (Shore *et al.* 1957).

Although the pH-partition theory is consistent with current knowledge of cell membrane structure and can explain many aspects of drug absorption, its does not take into account the anatomical differences seen within the gastrointestinal (GI) tract. Observations that absorption of weak acids and bases takes place more rapidly in the proximal intestine than in the stomach appears to contradict the pH-partition hypothesis. However, it does not take into account the much greater surface area offered by the intestine (Crouthamel *et al* 1971) or the presence of an unstirred mucus layer close to the gut lumen with both different pH and permeability to the bulk intestinal contents (Gibaldi 1979). Further inconsistencies are observed in the fact that compounds having similar partition coefficients can be absorbed at markedly different rates (Martin 1981).

1.2 Delivery of peptides and proteins

Several challenges confront the delivery of peptide and protein drugs. A major challenge in using peptides and proteins as drugs is preservation of their structural integrity until they reach their sites of action, which are often remote from the site of administration. For some peptides, such inherent metabolic instability has been overcome by developing analogues that are metabolically more stable and that, at the same time, are either as potent or more potent than their parent peptides. Another challenge in peptide and protein delivery is to comprehend the magnitude of the enzymatic barrier against peptides that are administered parenterally, orally, buccally, rectally, nasally, transdermally, ocularly or vaginally, only then can stable analogues or conjugates be developed. The final challenge is to overcome the resistance of the mucosal membranes to material of poor permeability and high molecular weight, thus allowing the penetration of peptide and protein drugs and leading to the oral delivery of peptide drugs.

1.2.1 Routes of peptide delivery

Presently, a wide variety of routes of administration and delivery systems exists for drug substances, some of these systems can be employed directly for peptide delivery, however, others cannot be used in their present form and require extensive modification.

1.2.1.1 Parenteral administration

At present most of the new peptide pharmaceuticals are administered be parenteral routes such as intravenous (IV), intramuscular (IM) and subcutaneous (SC), though this form of delivery has traditionally been poorly accepted by patients, except for those suffering from life-threatening diseases. Approaches to alleviate this problem include alternative routes such as nasal, pulmonary, rectal, buccal, vaginal, transdermal and ocular routes (Siddiqui and Chien 1987). However, absorption from these routes is much less than after parenteral administration

IV administration has the advantage of rapid onset of action, although it also offers selective distribution of particles to different organs depending on size (Illum and Davis 1982), this approach may be useful for targeting peptide drugs. A further advantage is that the route affords maximum bioavailability allowing small doses to be used (Audhya and Goldstein 1983), which in turn reduces the risk of toxicity and cost. Disadvantages of the parenteral route include local reactions at the site of administration such as extravasation and tissue necrosis, while systemic effects can account for infection, embolism, hypersensitivity reactions and speed shock (Martis 1986).

1.2.1.2 Transdermal administration

Continuous IV infusion is seen as the superior mode of drug delivery, but requires hospitalisation of the patients. There is now a growing awareness that IV drug infusions can be duplicated by continuous delivery through transdermal route (Shaw

et al. 1976).

The transdermal route has distinct advantages in that it lacks proteolytic enzymes (Pannatier *et al.* 1978) which would degrade peptide drugs and it avoids hepatic first pass metabolism (Chien 1983). Nevertheless, the peptides are still large hydrophillic molecules and will have difficulty in diffusing through the stratum corneum and other skin layers, and would require active help from a delivery system. Iontophoresis has been developed for just this reason and involves the use of an electric current to induce migration of charged molecules, in an electrolyte medium (Parsramppuria 1991, Chien *et al.* 1987 Srinivasan *et al.* 1989).

1.2.1.3 Nasal administration

Due to the large surface area available for absorption and its highly vascularised bed of mucosa, coupled with the fact that the nasal cavity appears to have very little metabolising capacity, absorption across the nasal membrane seems a feasible route for peptide delivery. Indeed peptides have already been delivered successfully by the nasal route, some of which are of high molecular weight, although a relationship exists between the size of the molecule and its extent of nasal absorption. Peptides include luteinizing hormone releasing hormone (LHRH) (Anik *et al.* 1984), adrenal corticotrophic hormone (ACTH) (Paulson and Nordstrom 1952) and oxytocin (Hoover 1971).

Passage of the peptides across the nasal membrane proceeds by passive diffusion (Su *et al.* 1985) and seems in most cases to afford between 1-20% absorption of the peptide, depending on the molecular weight (Harris 1986). Efforts to facilitate nasal absorption have led to the use of promoters which can be classified as surfactants, chelators, bile salts and saponins (Hirai *et al.* 1981, Lasch and Brigel 1926, Meyer 1935). These agent are employed to modify pH, prevent peptide aggregation, help micelle formation and even create disorder in the structural integrity of the nasal mucosa.

1.2.1.4 Oral administration

Maximising the bioavailability of orally administered peptide and protein drugs has been an ongoing, yet elusive, goal for many years, since by design, the GI tract is very efficient at preventing the uptake of intact peptides and proteins. Although many workers have reported oral activity of these drugs, the doses required are often excessive compared to the parenteral dose, for example LHRH requires a dose 3000 times that of the IV dose for it to show oral activity (Sandow and Petri 1985).

To be successfully delivered by the oral route, a peptide or protein must be stable to the low pH found in the stomach and also withstand proteolytic degradation by enzymes present in the gut lumen and brush border. Furthermore, peptides do not normally exhibit characteristics that lend themselves to good transport across biological membranes, since they are large in size and tend to be polar in nature. Even if the macromolecule is taken up by the cell intact, it may be stored and metabolised without being passed into systemic circulation (Gabev and Foster 1983, Lecce 1984). Assuming the molecule manages to reach the systemic circulation intact, it may well be metabolised by the liver (first pass effect) or be excreted into the bile (Renston *et al.* 1980).

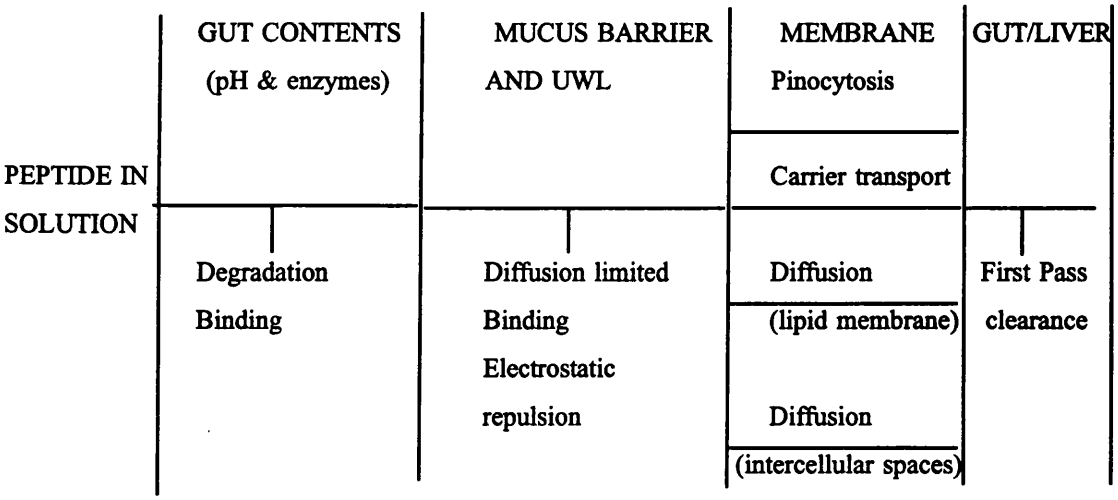


Figure 1. Barriers to the oral absorption of peptides

UWL - unstirred water layer

The absorption of peptide drugs has been reported to occur to varying extents. Some of the mechanisms of passage of peptides and proteins across the mucosal barrier are:-

1) PARACELLULAR ROUTE: movement between the tight junctions of cell and/or across the spaces formed when cells are extruded into the intestinal lumen.

2) TRANSCELLULAR ROUTE: passage through the membrane by direct permeation of the lipid bilayer or by channels (aqueous pores or otherwise) or pinocytosis. The latter two routes probably involving carrier-mediated transport.

1.2.1.4.1 Physical barriers to absorption of peptides

The intestinal mucosa represents a complex barrier to the absorption of peptides. The membrane surface of the microvillus consists of the common lipid bilayer interspersed with protein. The outer membranes of adjacent cells are fused at the basal membrane, creating "tight junctions", which form an effective barrier between intestinal lumen and the intercellular spaces. The surface of the apical membrane sports a uniform layer of filamentous glycoprotein known as the glycocalyx (Egberts *et al.* 1984), which has a negative charge at physiological pH largely due to the presence of sialic acids at the terminal of the carbohydrate chain. Superimposed on this surface is a layer of mucus (5μm) and further still is an unstirred water layer (UWL) which is approximately 400μm thick (Thomson and Dietschy 1984). The movement of peptides through this unstirred layer will involve simple diffusion and can be rate-limiting to the absorption of hydrophobic molecules. For polar molecules the mucus and glycocalyx surface coat, may represent a diffusional barrier because of its viscosity and electronegative charge (Smithson *et al.* 1981, Esposito *et al.* 1983).

Certain anatomical and physiological differences can be seen along the GI tract and one such area of interest are patches in the intestinal tract belonging to the gut-associated lymphoid tissue (GALT), known as Peyer's patches (Ten Cate 1969). Their

thinner than in other places in the intestine and the patches are notably deficient in lysosomes and so are seen as a possible site to target drugs whose action is within the lymphatic system, including immunomodulating drugs.

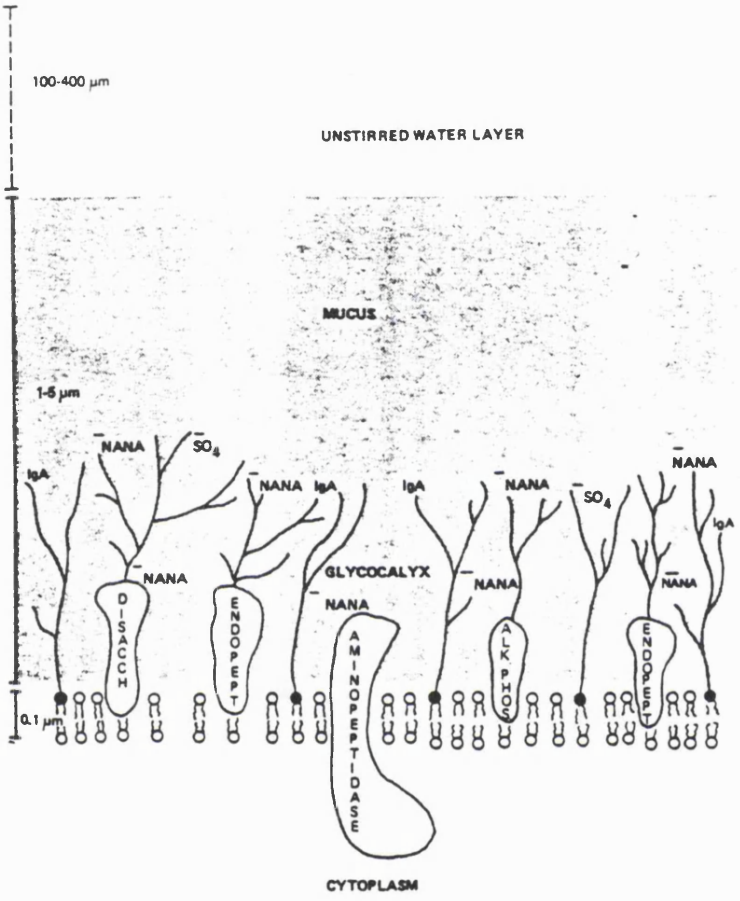


Fig. 2.Intestinal mucosal barrier (NANA-N-acetyl neuramic acid)

1.2.1.4.2 Enzymatic barriers to oral peptide delivery

The enzymatic barrier is by far the most important of the multitude of barriers limiting the absorption of peptide and proteins from the GI tract, efficiently digesting proteins to a mixture of amino acids and small peptides of two to six residues, prior to their appearance in the portal circulation (Adibi and Mercer 1973). Hydrolysis can occur at several sites, both luminally at the brush border and intracellularly (Silk *et al.* 1976, Adibi 1971).

Protein digestion is initiated in the gastric juice by a family of aspartic proteinases (pepsins), which are active at pH 2-3 and become inactive above pH 5, however, they

(pepsins), which are active at pH 2-3 and become inactive above pH 5, however, they do not generally degrade peptides to amino acids, but just break larger proteins up into smaller polypeptides. The resultant partial digest is then subjected to pancreatic proteases in the duodenum, which consists of the endopeptidases trypsin, chymotrypsin and elastase and the exopeptidase carboxypeptidase A. The endopeptidases work to cleave most of the internal links within the peptide and function maximally at pH 8 (Naughton and Sanger 1961), while the exopeptidase cleaves residues with a free terminal carboxyl group at the C-terminal.

Although the pancreatic peptidases are very efficient at digesting proteins, their activity towards small peptides is very much restrained. The bulk of luminal activity against peptides is derived from either the brush border or the cytoplasm of the enterocyte (Silk *et al.* 1976). These proteases consist of aminopeptidases A and N, diaminopeptidase IV, endopeptidase 24.11, angiotensin-converting enzyme, Gly-Leu peptidase and Zn-stable Asp-Lys peptidase (Louvard *et al.* 1976, Kenny and Maroux 1982, Tobey *et al.* 1985). Typically, they are anchored in the apical membrane with the active site essentially in an extracellular environment (Feracci *et al.* 1982, Hussain 1985). Although the brush border peptidases are capable of hydrolysing peptides of up to ten amino acids, they tend to prefer tri- and tetrapeptides and prefer peptides whose N-terminal residue possesses a lipophilic side chain (Matthews and Payne 1980). The cytosolic proteases, by contrast, tend to prefer dipeptides to larger oligopeptides (Kim *et al.* 1972), hence, these proteases work through the whole range of possible peptides to compliment each other.

Besides the brush border and cytosol, lysosomes and other organelles are also potential sites of peptide degradation and although their role has been neglected in many studies to date, one would expect them to assume greater importance as a larger fraction of orally administered peptides are engineered to survive luminal and brush border hydrolysis. The case of GI absorption of epidermal growth factor (EGF) serves as a good example. Less than 1% of the administered dose was seen in plasma after 3 hours, despite there being little degradation (16%) in the lumen of the stomach and small intestine (Thornburg *et al.* 1984). Further work indicated association of

radiolabelled EGF with lysosomal vacuoles and eventually revealed that degradation took place in the lysosomes (Gonnella *et al.* 1987).

1.3 Strategies for peptide delivery

Enhancement of membrane transport and cellular uptake of drugs can be divided into two broad categories, physical methods and chemical methods. The physical strategies encompass the pharmaceutical formulation of the drug where the drug is not actually chemically modified. Such methods include entrapment of the active species in liposomes, soluble and biodegradable polymers, microspheres and the use of solubility and penetration enhancers. Chemical methods involve the chemical modification of the drug itself. This particular approach may result in a modification that is either irreversible, in which case the new compound is intended to be active in its own right or reversible, whereby the active compound is expected to be regenerated from the "prodrug" *in vivo* after absorption. An introduction to the two methods is presented below.

1.3.1 Physical methods

1.3.1.1 Liposomes

Liposomes were first proposed as potential drug carrying agents two decades ago (Gregoriadis and Ryman 1972) and have received wide interest in their application as a controlled drug/peptide delivery system (Gregoriadis 1980, Juliano 1981). They consist of one or more phospholipid bilayers surrounding an aqueous internal phase and are formed spontaneously when amphipathic lipids are dispersed in excess water (Leserman and Barbet 1982), thus can be considered synthetic relatives of cell membranes (Gruner 1987). Their design allows water soluble drugs to be encapsulated in the aqueous core of the liposome and hydrophobic/lipophilic drugs to be incorporated within the phospholipid bilayer.

Following IV administration, the majority of liposomes appear to be taken up by

phagocytic cells of the reticuloendothelial system (RES) such as the liver and spleen (Gregoriadis *et al.* 1974), which would limit their use as general drug carrying agents. Thus, the major biological interest in liposomes has been their potential to target active compounds to cells of the RES for the treatment of parasitic, fungal and bacterial macrophage-related diseases (Schroit *et al.* 1983), viral diseases (Koff and Fidler 1985) and cancer metastases (Fidler 1985). They have also been investigated for their use as a means of affording protection of insulin (Weingarten *et al.* 1985) and other peptides and proteins (Adrian and Huang 1979) against enzymatic degradation. Most recently, liposomes have been utilised by immunologists for use with synthetic peptide vaccines to act as non-toxic adjuvants and also as a possible carrier system for orally active vaccines, since they are well received by Peyer's patches which are the gateway to the lymphatic system (Michalek *et al.* 1987, Childers *et al* 1988).

However, despite their favourable properties as a drug carrier, results of studies where liposomes have been administered orally are far from encouraging. Many liposomes appear to be degraded in the gastro-intestinal environment when given orally (Rowland and Woodley 1980) and those which are resistant to degradation fail to pass through the intestinal tissue (Schwinke *et al.* 1984). On the other hand, liposomes have shown some promise in delivery to the nose and lungs as aerosol formulations (Juliano *et al.* 1978).

1.3.1.2 Mucoadhesive polymers

With the exception of the ears, mucus covers all the internal tracts and orifices of the body, presenting as a continuous unstirred gel layer over the mucosal epithelium. this layer provides a site for adhesive polymers to attach and deliver a drug to the epithelial surface. the desirable features of such a system are:-

1) localisation of the dosage form in specified regions to improve and enhance bioavailability of drugs.
2) promotion of intimate contact of the formulation with the underlying absorbing

surface to allow modification of tissue permeability for absorption of macromolecules.

3) prolonged residence time of the dosage form to permit maximal absorption and allow once-a-day dosing.

The polymers used should be non-toxic and non absorbable, adhere rapidly to wet tissue and able to release the incorporated drug in a controlled manner. The drug is released from the polymer network as it swells in the aqueous environment forming a gel and as the degree of hydration increases then so does the mucoadhesive strength of the polymer. Polymers used include poly acrylic acid, hydroxypropylcellulose and sodium carboxymethylcellulose.

1.3.1.3 Microspheres

Microspheres are described as small particles ranging from tens of nanometres up to one hundred microns in size. Microcapsules are similar in many respects but comprise small spheres that have an outer layer or membrane enclosing a core material that could be the drug itself (Tomlinson 1983).

Microspheres intended for drug delivery can be prepared from a variety of different materials and are of different physical characteristics, depending on the intended site of action, the nature of drug to be delivered and the duration of action (Davis and Illum 1986, Juni and Nakano 1987). Materials include albumin (Fujimoto *et al.* 1985), fibrinogen (Miyazaki *et al.* 1986) and polyacryl starch (Artursson *et al.* 1984). Several peptides have been successfully delivered using microsphere and microcapsule controlled release technology, although most are designed for parenteral administration. These include the LHRH analogue nafarelin and the anti-prostatic cancer drug tryptorelin, both prepared using poly (*d, l*-lactic-co-glycolic) acid (PLGA), a biodegradable polyester (Vickery *et al.* 1986, Parmar *et al.* 1985).

1.3.1.4 Penetration enhancers

Penetration enhancers are substances that facilitate the absorption of solutes across biological membranes. There are five major types of enhancers:-

1) chelators such as EDTA, citric acid, salicylates, *N*-acyl derivatives of collagen and enamines (*N*-amino acyl derivatives of β-diketones) (Shiga *et al.* 1985).
2) surfactants such as sodium lauryl sulphate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Miyamoto *et al.* 1983).
3) bile salts such as sodium deoxycholate, sodium glycocholate and sodium taurocholate (Hayakawa *et al* 1988).
4) fatty acids such as oleic acid, caprylic acid and capric acid, and their derivatives such as acylcarnitines and mono- and diglycerides (Palin *et al.* 1986, Ueda *et al.* 1983).
5) nonsurfactants such as cyclic ureas and 1-alkyl- and 1-alkenylazacycloalkanone derivatives (Wang *et al.* 1988).

Enhancers are thought to act by one of three mechanisms. The first, which has received the most attention, is that of compromising the integrity of the mucosal membrane by acting either at the apical cell membrane (transcellular pathway) or the tight junctions between cells (paracellular pathway). The other less researched mechanisms are the inhibition of proteolysis and increasing the thermodynamic activity of peptide drugs (Touitou *et al.* 1987). However, due to the nature of their action, penetration enhancers have raised concerns as to the irritation and extent of mucosal damage they cause, and so the nature of enhancer used must be chosen carefully depending on the site at which the enhancer is required to act (Peters *et al.* 1987, Nishihata *et al.* 1981).

1.3.2 Chemical methods

1.3.2.1 N- and C-terminal modification

If it is known that a peptide is degraded by aminopeptidases, the first step is to introduce an N-terminal modification that will prevent the enzyme from interacting

with the peptide. Some of the modifications that have been utilised are: N^{α}-acetyl, formyl, pyroglutamic acid, N^{α}-alkylation, chain extension with other amino acids and removal of the α-amino group (Morley *et al.* 1981).

The same is true of the C-terminal with relation to carboxypeptidase degradation. The carboxylic acid may be masked by converting it to an ester, or even converting the C-terminal residue to an amide, indeed many of the synthetic enkephalin peptide analogues possess a C-terminal primary amide and have shown significantly longer half-lives *in vivo* (Hansen and Morgan 1984). Metkephamid, Tyr-D-Ala-Gly-Phe-MeMet-NH_2, is an example of a peptide that contains both N- and C-terminal modifications designed to limit proteolytic degradation. It contains an N^{α}-methylation and a carboxamide, as well as a D alanine substitution. While the parent peptide, Methionine enkephalin, has an *in vivo* half life of seconds, metkephamid has a half-life approaching 1hour.

Another method that has been used to prevent carboxypeptidase-mediated degradation is to covert the C-terminal amino acid to an amino alcohol which was also successful with the enkephalin peptide, wherein the C-terminal methionine was converted to a methioninol residue (Roemer *et al.* 1977).

The modifications described above also impart an increased lipophilicity to peptides, especially at physiological pH (pH 7.4) where carboxylic acids would be ionised while esters, amides and alcohols are neutral, thus adding to the possibility of better membrane transport. Further modifications are methyl ketones, nitriles, alkyl amides, tetrazoles and decarboxy amino acids (Spatola 1983, Morley 1969).

1.3.2.2 Cyclisation

One modification that addresses the aminopeptidase and carboxypeptidase degradation problems simultaneously is the conversion of the linear peptide to a cyclic analogue. In its simplest form this modification will create a peptide that no longer possesses a free amino or carboxy terminus, which may normally be susceptible to proteolysis.

However, conversion to the cyclic form will result in a major conformational change, which in turn may affect the biological activity of the molecule. But, as long as sufficient conformational information is known about the parent, then a cyclic analogue can be designed which maintains the critical parameters required for biological activity. One potential success is the cyclic hexapeptide [cyclo (MeAla-Tyr-D-Trp-Lys-Val-Phe)], an analogue of somatostatin which is a linear 14-mer with one disulphide bridge. This cyclic analogue was found to possess a high degree of metabolic stability and exhibited a duration of action greater than 4 hours in dogs, after oral administration (Veber *et al.* 1984).

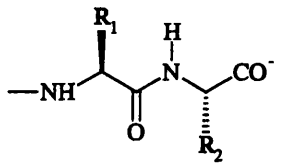
1.3.2.3 Intra chain modifications

In many cases the cleavage sites of peptides are located internally rather than at the termini of the sequence. Since many of the peptidases recognise the naturally occurring L-amino acids, replacement with D-residues at the labile bond in the peptide will enhance its resistance to proteolytic degradation. This approach is only viable so long as the residue replaced is non-essential for activity. Many successful peptide analogues have been designed which incorporate D-amino acids at susceptible positions in the sequence, LHRH is a typical case. The main cleavage site of the decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) which results in activation is the Tyr^5 -Gly^6 bond. Analogues were developed with L-Gly^6 replaced by D-Ala (Monahan *et al.* 1973) and D-Ser (t-Butyl) (Clayton and Shakespear 1978).

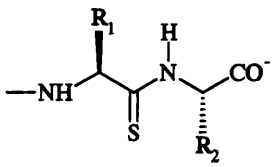
In those cases where the chirality of one or both of the residues that flank the enzymatically labile bond is known to be required for biological activity, an alternative strategy is to modify the peptide bond by N^{α}- or C^{α}-alkylation. Generally, a methyl substitution is adequate to stabilise the amide bond as well as increasing lipophilicity. This method has been employed with enkephalin peptides where N^{α}-methylation of a phenylalanine residue successfully extended the *in vivo* half-life (Hansen and Morgan 1984). A similar modification was made to the α-carbon of a tyrosine residue in an angiotensin II analogue, which resulted in stability of the peptide to chymotrypsin for 3 hours (Khosla *et al.* 1983).

1.3.2.4 Amide bond replacement

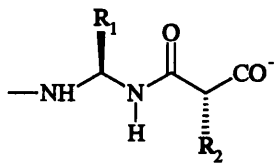
Replacement of the susceptible amide bond within a peptide chain has been shown to confer protection on the peptide from enzymatic degradation by peptidases. However, the resulting compound containing the modified backbone is, strictly speaking, no longer a peptide, but a "pseudopeptide" (Spatola 1983, Samanen 1985), some examples of which are listed below:-



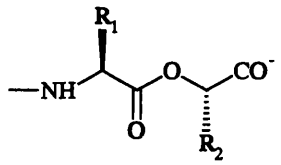
Amide



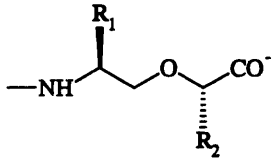
Thioamide



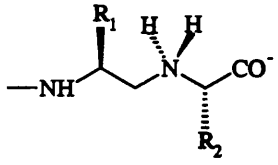
Retro Inverso



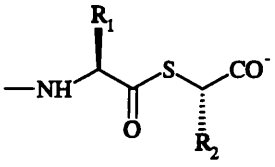
Ester



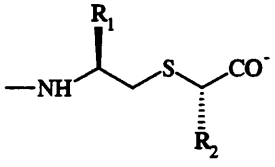
Ether



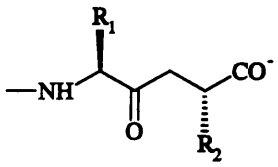
Reduced carbonyl



Thioester



Thioether



Ketomethylene

Obviously, there are many types of linkages that may be used, thus, the ultimate goal needs to be considered before a choice is made. Parameters such as oral availability, structure, hydrogen bonding biological activity and required lipophilicity must be taken into account before the amide bond is replaced.

1.3.2.5 Prodrugs

Chemical modification of peptide drugs to tackle biological instability and poor

membrane transport may result in undesired loss of biological activity. A potentially useful approach to solve this problem may be bioreversible derivatisation of the bioactive peptides to produce prodrugs. The basic requisite for the application of the prodrug approach is the ready availability of chemical linkages which allow reconversion to the parent drug *in vivo*. This conversion may take place before absorption, during absorption, after absorption or at the specific site of drug action, depending upon the specific goal for which the prodrug is designed. Prodrugs designed to overcome solubility problems in formulating intravenous injection solutions should preferably be converted to the parent drug immediately after injection, so that the concentration of circulating prodrug would rapidly become insignificant in relation to the active drug. Conversely, if the objective of the prodrug is to produce a sustained drug action, the rate of conversion should not be too rapid.

The conversion or activation of prodrugs to the parent molecule can take place by a variety of reactions. The most common prodrugs are those requiring hydrolytic cleavage mediated be enzymatic catalysis, yet in other cases the active compound is regenerated from the prodrug by biochemical reductive or oxidative processes.

1.3.2.5.1 Prodrugs minimising proteolytic degradation

Successful prodrugs have been designed that are resistant to the proteolytic action of aminopeptidases by reversible modification of the amino terminus. A novel prodrug concept developed by Amsberry and Borchardt (1990) involves the coupling of the amino function of a peptide to bioreversible derivatives of 3-(2'-hydroxy-4'-6'-dimethylphenyl)-3-dimethylpropionic acid (i) or 3-(3', 6'-dioxo-2', 4'-dimethyl-1', 4'-cyclohexadiene)-3, 3-propionic acid (ii) (Fig. 3). The derivatised hydroxy amides (i) and (ii) are initially converted to (iii) by enzymatic catalysis (step 1), followed by the non-enzymatic lactonisation of (iii), leading to the generation of the free peptide and the lactone (iv). Step 1 may be catalysed by hydrolytic or reductive mechanisms (Amsberry and Borchardt 1991, Amsberry *et al.* 1991).

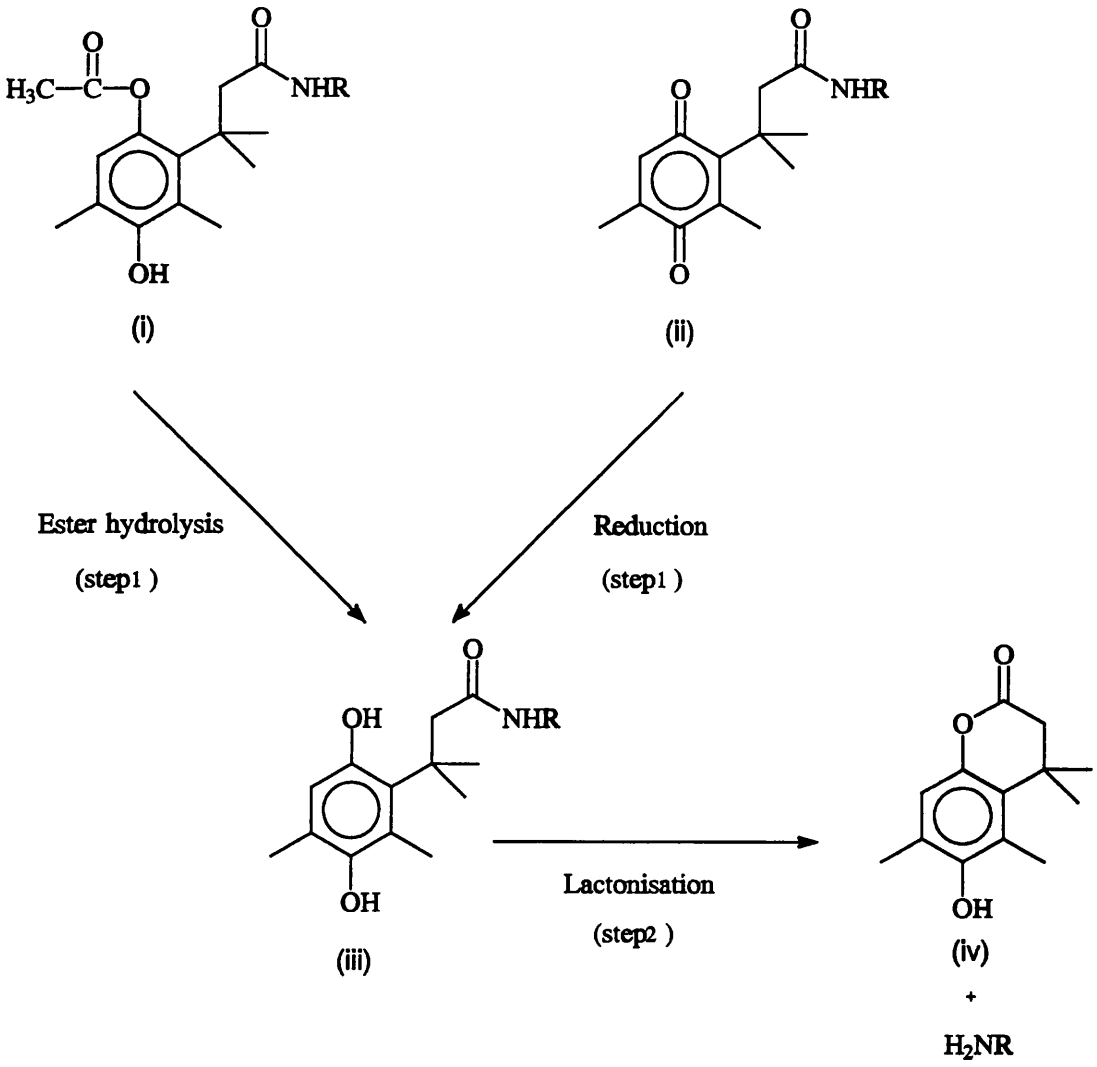


Fig. 3. Bioreversible peptide prodrug derivatives

The enkephalins are rapidly hydrolysed *in vivo* by two well defined enzymes, neutral endopeptidase and aminopeptidase N. Aminopeptidase-resistant prodrugs of Leu-enkephalin and Met-enkephalin have been developed by condensing the enkephalins with various aldehydes and ketones to form a series of 4-imidazolidinone derivatives (Rasmussen and Bundgaard 1991). These derivatives are converted to the parent enkephalin *via* non-enzymatic hydrolysis and the rates of conversion are significantly influenced by the steric characteristics of the 2-substituents on the terminal amino acid (Fig. 4).

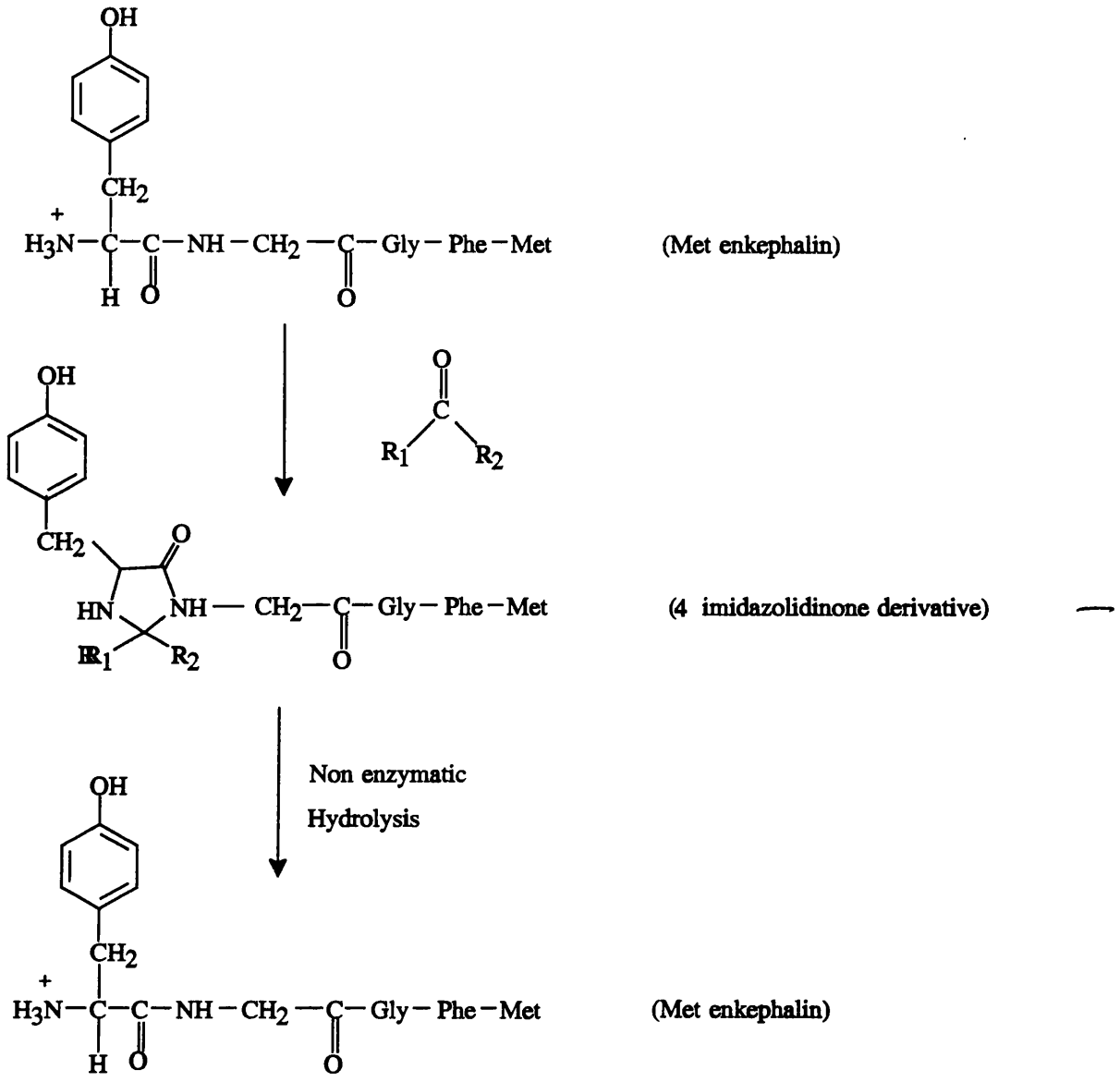


Fig 4. 4-imidazolidinone prodrug of met-enkephalin

Bundgaard and Rasmussen (1991) prepared a series of prodrugs resistant to carboxypeptidase A, based on the fact that the rate of proteolysis is significantly influenced by the nature of the neighbouring amino acid (Hanson and Smith 1948, Snoke and Neurath 1949). These N-α-hydroxyalkyl derivatives were prepared by hydrolysis or aminolysis of various N-acyl 5-oxazolidinones, and were found to display a much greater stability against carboxypeptidase A than the parent peptide. The same concept may be applied to protect peptides against endopeptidases like

chymotrypsin and trypsin, by reversible alkylation of the endo peptide bond susceptible to hydrolysis. Under physiological conditions, the N-α-hydroxyalkyl derivatives are non-enzymatically converted to the parent peptide, with the conversion rate depending on both polar and steric factors within the acyl and N-α-hydroxyalkyl moieties (Bundgaard and Rasmussen 1991). In an attempt to alter the chemical reactivities of the N-α-hydroxyalkyl prodrugs, further modification produced N-acyloxyalkyl prodrugs (Fig. 5) (Bundgaard and Rasmussen 1991).

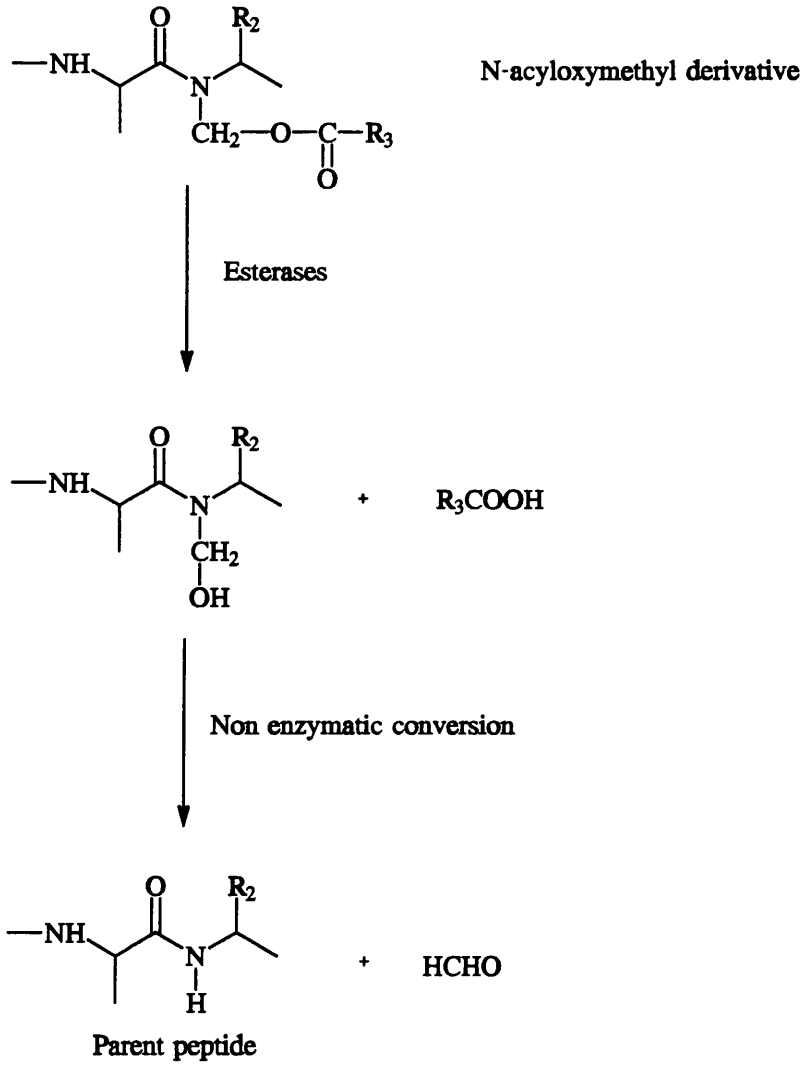


Fig. 5. N-acyloxyalkyl prodrugs

1.4 Synthetic peptide vaccines

The motivation for developing synthetic peptide vaccines stems from the need to overcome several problems incurred with the use of conventional vaccines, consisting of either killed or live attenuated disease-causing organism. Notwithstanding the tremendous achievements obtained with such vaccines in reducing both incidence and morbidity of many infectious diseases, considerable obstacles that interfere with their effective application are often encountered. These include high restriction to strain and type specificity of the infectious agent due to continuous antigenic variations in viruses and parasites; MHC restriction of the immune response; difficulties in tissue culture growing of the organism as well as production and storage of many vaccine preparations; biohazard in both production and use of vaccines against lethal infections. Because of these complications there are still many viral and parasitic diseases for which no effective vaccines exists and approaches are sought to circumvent the problems of conventional vaccines.

The use of synthetic peptides for vaccination is one alternative that is being explored (Arnon 1991). Its attractiveness is the simplicity of the approach, the information it brings to the molecular understanding of the immune response required for protection and the considerable practical advantage that such products could offer. These vaccines contain a relatively small peptide or peptides, which have been chosen because they possess the necessary epitopes of the organism that elicit a protective immune response. Furthermore, as they are chemically defined and do not contain infectious material, they should be devoid of any biohazard factors.

1.4.1 Immunoadjuvants

A major difficulty encountered during development of modern vaccines is their weak immunogenicity. This problem has been traditionally overcome by using adjuvants. Adjuvants are compounds or associations of compounds able to increase or modulate the antigen-specific immune response. It is now recognised that their biological properties depend upon their ability to activate selectively one of the two $CD4^+$ T-cell

subpopulations, T_H1 or T_H2, that control the major features of specific immune responses (Mosmann and Coffman 1989).

The most frequently used adjuvant in experimental animals is complete Freund's adjuvant (CFA), which consists of a water-in-oil emulsion containing killed mycobacteria. Although very effective at evoking high level and long lasting immunity, this adjuvant is not suitable for humans because it induces local reactions and granulomas, inflammation and fever due to the slowly metabolisable oil and the mycobacteria. The only approved adjuvant for human use to date is alum, but it is not active with all immunogens and stimulates only humoral responses. For development of new vaccines, the availability of improved adjuvants suitable for clinical use is crucial. Such adjuvants should display several different profiles of activity since an appropriate adjuvant must not only enhance the immune response but should also drive this response to achieve the appropriate type of protective immunity required.

1.4.1.1 Non-ionic block polymer surfactants

Non-ionic block polymer (NBP) surfactants are copolymers consisting of hydrophobic polyoxypropylene and hydrophillic polyoxyethylene. NBP surfactants have mostly been studied following administration in oil-in-water emulsions, for their stimulating activity on the humoral responses against peptides, proteins and polysaccharides (Hunter *et al.* 1991). NBP surfactants can bind proteins to the surface of oil drops, and these complexes act on macrophages to enhance the expression of MHC class II molecules on their surface and also enhance the molecules' ability to present antigens to T-cells (Howerton *et al.* 1990).

1.4.1.2 Monophosphoryl lipid A

Monophosphoryl lipid A (MLA) is a less toxic, adjuvant-active derivative of lipopolysaccharide (LPS) (Ribi *et al.* 1984). MLA promotes interferon-γ (IFN-γ) production by T-cells (Tomai and Johnson 1989) and so indirectly aids in the production of antibodies. MLA is also known to stimulate tumour necrosis factor

(TNF) production by macrophages (Ulrich *et al.* 1991), thus the combination of MLA and released TNF could also stimulate natural killer cells to produce IFN-γ. IFN-γ in association with macrophages could induce the differentiation and selection of T_H1 cells (Gajewski *et al.* 1989).

1.4.1.3 Muramyl dipeptides

The smallest subunit of the mycobacterial cell wall, found in complete Freund's adjuvant, that retains immunoadjuvant activity is *N*-acetyl muramyl-L-alanyl-D-isoglutamine, called muramyl dipeptide (MDP) for short (Ellouz *et al.* 1974). Hundreds of MDP derivatives have been synthesised and they display a large range of immunomodulatory activities (Warren *et al.* 1986). Administered in saline, MDPs induce mainly humoral responses and, in water-oil emulsions, they increase both antibody and cell mediated immunity (Warren *et al.* 1986). A particular combination of threonyl-MDP with a squalene emulsion, termed "Syntex adjuvant" (Allison and Byars 1991) and shown to be efficient at improving humoral and cell-mediated responses.

MDPs act on several types of immune cells, including macrophages and B- and T-cells. They induce the secretion of interleukin 1 (IL-1), which may contribute to their immunoadjuvant activity *in vivo* and are able to co-stimulate T-cells *in vitro* (Damais *et al.* 1978). An MDP derivative, muramyl tripeptide phosphatidylethanolamine, acts synergistically with IFN-γ to increase MHC class II antigen expression on macrophages (Landmann *et al.* 1988), while the same derivative is very active when administered with a microfluidised emulsion (Van Nest *et al.* 1992).

1.4.1.4 Cytokines as adjuvants

Molecular biology has provided a range of pure recombinant cytokines, which have been tested as modulators of specific immune responses. The first cytokine to be used as an adjuvant, IL-1, enhanced the secondary antibody response to bovine serum albumin (Staruch and Wood 1983). Although IL-1 enhances IL-2 production, the

adjuvant activity of IL-1 is independent of the effect on IL-2 production (Reed *et al.* 1989). IFN-γ administered with antigens can activate helper T-cells for antibody and delayed-type hypersensitivity responses (Heath and Playfair 1992). IFN-γ also enhances the release of IL-1 and increases MHC class II expression on antigen presenting cells, Properties that may explain the adjuvant activity of IFN-γ (Heath and Playfair 1992).

1.4.2 Vaccine carriers

In most cases synthetic peptides less than 12-15 residues long are not good immunogens by themselves, even when administered in potent adjuvants. Therefore, the usual procedure is to attach them to macromolecular carriers such as proteins, synthetic polyamino acids, erythrocytes and inert particulate beads to enhance their immunogenicity, however the method of conjugation may influence the type of antibody obtained (Soutar and Palfreyman 1986). Nonresponsiveness to a peptide in an immunised animal may sometimes be overcome by attaching the peptide to another peptide that induces helper T-cell activity (Francis *et al.* 1987). Common protein carriers used are bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH) (Lerner 1982, Bittle *et al.* 1982), however, non-specific antibody is often raised to the carrier protein as well as the peptide.

An approach that avoids the formation of antibodies to the carrier protein and allows several peptides such as different B- and T-cell epitopes to be associated in a single construction is the multiple antigen peptide (MAP) system, introduced by Tam (1988). The MAP system is constructed on a small, immunogenically inert core matrix of lysine residues bearing radially branched peptides. The advantage of the system is that the antigen is chemically defined and may contain either multiple copies of a single peptide epitope or different antigenic peptides attached to the same macromolecule which may be used as such for vaccination. It has been suggested that the MAP system is particularly suitable for eliciting antipeptide antibodies that cross react with the parent viral or microbial antigens and thus for acting as potential synthetic vaccines (Munesinghe *et al.* 1991).

1.4.2.1 Vaccine carriers which combine adjuvant properties

Delivery systems such as emulsions, liposomes, nanoparticles and microspheres are widely regarded as carriers with immunoadjuvant properties. Most of these delivery systems exhibit adjuvant properties due to their ability to release the antigen over a longer period than when the antigen is delivered in the free form, while others are capable of modulating the immune system in addition to their sustained or controlled release properties.

1.4.2.1.1 Liposomes

Following the first report by Allison and Gregoriadis (1974) on the adjuvant activity of liposomes, considerable attention has been focused on their possible utility as antigen carriers. Liposomes serve as carriers of antigens and adjuvants, as depots for slowly releasing antigen and as targeting agents for delivery of novel antigens and adjuvants to antigen presenting cells (Alving 1991). Several factors affect the adjuvant properties of liposomes. These include the net charge of the liposomal bilayers and the antigen to be delivered, the position of the antigen either entrapped within the liposome or adsorbed on the surface, the type and fluidity of liposomal lipids and their phase transition temperature, the type of liposomes (unilamellar or multilamellar) and their sizes and the phospholipid-to-antigen ratios (Latif and Bachhawat 1984, Gregoriadis 1990).

An interesting observation has been noted, that booster doses of antigens in liposomes after a priming dose with the liposome-associated antigen do not show any adjuvant effect. Rabbits primed with liposome-associated human serum albumin (HSA) injected intravenously were reported not to have an enhanced secondary immune response after booster injection compared to that when the booster dose of HSA was administered free in solution (van Rooijen *et al.* 1984).

1.4.2.1.2 Nanoparticles and microspheres

Particulate delivery systems such as nanoparticles and microspheres also possess adjuvant activity when used as vehicles for antigens. The adjuvant effect of microspheres made of poly (D,L-lactide-co-glycolide) (DL-PLG) copolymer containing staphylococcal enterotoxin B (SEB) and subcutaneously injected into mice was comparable to CFA, but in contrast to CFA, the microspheres did not induce inflammation and granulomata in the mice (O'Hagan *et al.* 1991).

The adjuvant activity of biodegradable microspheres during primary responses is explained by their rapid uptake by the mononuclear cells in the reticulo-endothelial system (RES) following intravenous injection, resulting in high local concentrations of antigen in these cells and also by their ability to be degraded rapidly in the lysosomal milieu of the macrophage. The size of the microspheres also plays a role in their adjuvant activity, microspheres of 1-10 μm exhibit a stronger adjuvant effect than those with a diameter greater than 10 μm (Eldridge *et al.* 1991). It has also been suggested that microspheres of 1-10 μm are suitable for oral immunisation since they can target the Peyer's patches. Three peroral immunisations with 1-10 μm microspheres induced both secretory (mucosal IgA) and circulating (IgM, IgG and IgA) antitoxin antibodies, in contrast to free antigen administered perorally, which did not stimulate any antibodies (Eldridge *et al.* 1990).

1.4.2.1.3 Iscoms

Iscoms (immune stimulating complex) are spherical structures of about 40 nm, composed of a matrix consisting of the saponin adjuvant Quil A in a complex with cholesterol and phospholipids, in which antigens are incorporated by hydrophobic interactions (Morein *et al.* 1984). Protein antigens incorporated into iscoms have been shown to be highly immunogenic *in vivo*, inducing strong humoral and cellular responses (Morein *et al.* 1987).

1.4.2.1.4 Lipopeptides

Lipids have been conjugated to peptide epitopes in order to potentiate the immunogenicity of the sequence by acting as an adjuvant as well as a carrier. The mechanism responsible for the adjuvant effect of lipids is not well understood, but the general hypothesis is that the lipid retains the antigen at the site of injection performing a depot function, protects the antigen from rapid destruction by proteolytic enzymes, facilitates phagocytosis and stimulates the cells of the immune system.

A successful system developed by Jung *et al.* (1985) is based on tripalmitoyl-S-glycerylcysteinylserine (Pam_3Cys-Ser) which is derived from the N-terminal of lipoprotein from the outer cell membrane of *Escherichia coli* and contains three long chain fatty acids. The system has been shown to activate macrophages (Hoffmann *et al.* 1988) and is a potent stimulator of B-lymphocytes (Metzger *et al.* 1991). The system also utilises polar/hydrophillic amino acids as a linker between the lipid and the peptide epitope in order to increase the water solubility of the system.

The lipopeptide-antigen conjugates have been shown to induce antigen-specific antibodies *in vivo*, in particular to an epitope from the foot and mouth VP1 viral protein (Weismuller *et al.* 1989). It has also been shown that lipopeptide vaccines carrying killer cell epitopes can effectively prime virus specific cytotoxic T-lymphocytes *in vivo* (Deres *et al.* 1989). It is proposed that the lipopeptide has the ability to serve as a membrane anchor, allowing the peptide antigen to be expressed on the surface of antigen presenting cells or for viral protection, to divert the peptide into the endogenous processing pathway, allowing association with the class I MHC molecules and presentation at the surface of the cell, while at the same time increasing the half life of the construct by enhancing its biological stability and thus, maximising and prolonging the immune response to the peptide.

Another class of lipopeptidic immunostimulants have recently been proposed by Deprez *et al.* (1995) which are based on murein-derived lauroyl peptides. The "desmuramyl peptidolipid" adjuvants, the lauroyl-L-alanyl-D-glutamyl-L,L-2,6-

diaminopimeloylglycine (pimelautide) and the lauroyl-L-alanyl-D-glutamyl-L,L-2,6-diaminopimelic acid (trimexautide), stimulate phagocytic cells and T-lymphocytes and *in vivo*, these compounds exert an adjuvant effect on antibody production (Baschang 1989). The compounds were evaluated with a peptide epitope derived from the third hypervariable domain of the HIV-1 envelope glycoprotein gp 120. The trimexautide linked peptide was able to induce a virus specific CTL response, while the pimelautide linked peptide induced a strong antibody response, suggesting that small structural modifications can bring about selective stimulation of subpopulations of immunocompetent cells (Deprez *et al.* 1995).

CHAPTER 2

CHEMISTRY

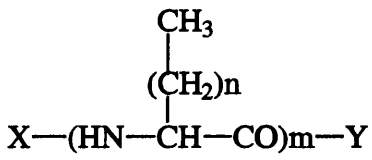
2.1 Lipidic α-amino acids and peptides

The α-amino acids with long alkyl side chains, the so-called lipidic amino acids represent a class of compounds which combine structural features of lipids with those of amino acids. These lipidic amino acids may be oligomerised to form lipidic peptides, which can take several forms. They include linear homo-oligomers, hetero-peptides where the peptide chain may include either coded amino acids or substituted lipidic amino acids and finally cyclic structures produced as a result of backbone or side chain cyclisation, yielding a compound which is similar in appearance to that of micelles. One would expect the chimeric nature of these compounds to be reflected in their physical properties; they should be highly lipophilic due to the long alkyl side chains, yet show polar and conformational behaviour characteristic of amino acids and peptides. In addition, the structure may be varied in order to modify the physical nature of the compounds, in particular the length of the alkyl side chain can be changed in order to increase or decrease the lipophilic character of the lipidic amino acid.

The potential uses of the lipidic amino acids have already been explored, and include lubricants (Takino 1987), polishes (Sagawa and Takehara 1987a), cosmetics (Kitamura 1987) and as surface improving agents for ceramics (Sagawa and Takehara 1987b). Lipidic amino acids and peptides may also be used as detergents and water-resistant and biocompatible films (Gibbons 1988). However, of greater significant interest is the possible use of lipidic amino acids as an aid to drug delivery and drug formulation (Toth *et al.* 1991a). Because of their bifunctional nature, the fatty amino acids and peptides have the capacity to be chemically conjugated to drugs with a wide variety of functional groups. The linkage between drug and lipidic unit may either be biologically stable (ie. a new drug is formed) or possess biological or chemical instability (ie. the conjugate is a pro-drug). In either case, the resulting conjugates would be expected to possess a high degree of membrane- like character, which may be sufficient to facilitate their passage across membranes.

2.1.1 Unsubstituted lipidic α-amino acids

There are two reported synthetic routes for preparing the lipidic amino acids. One pathway involves the appropriate α-bromoalkanoic acid being stirred with ammmonium hydroxide for several days to yield the the amino acid in one step (Takino et al. 1989, Gerencevic *et al.* 1966, Kimura 1962, Birnbaum *et al.* 1953). Albertson (1946) reported an alternative method for synthesising the lipidic amino acids which which was adopted for the preparation of the required unsubstituted compounds **1a-d** (Gibbons *et al.* 1990).

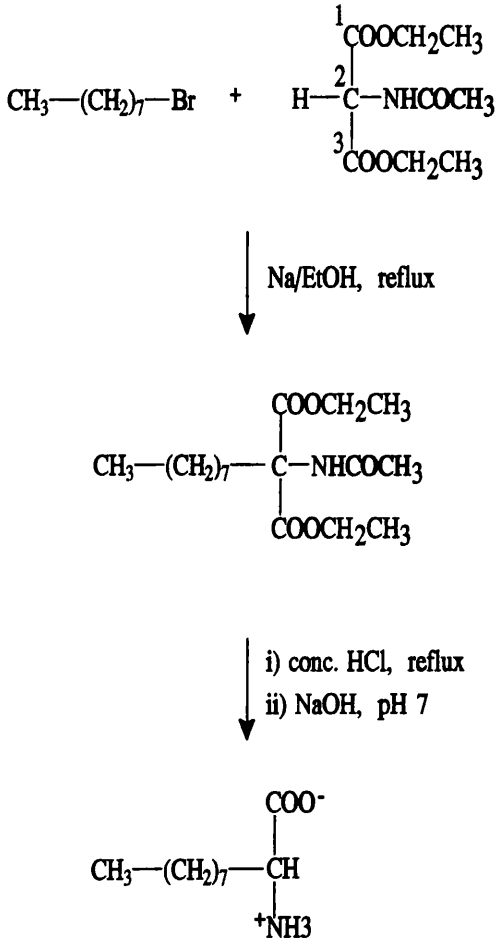


1

1	X	Y	n	m
a, b c, d	H	OH	7, 9, 11, 17	1
e, f, g, h	Boc	OH	7, 9, 11, 17	1

The ten carbon atom amino acid **1a** was synthesised by refluxing 1-bromooctane with diethyl acetamidomalonate in the prescence of sodium ethoxide (scheme 1). The proton in position 2 of the malonate ester is weakly acidic because of the neighbouring electronegative functions and dissociated in the strongly basic conditions used. The resulting nucleophile (carbanion) can attack the brominated carbon of bromooctane, which is electron deficient due to the electron withdrawing effect of bromine, forming the octyl-substituted diethyl acetamido-octylmalonate. Hydrolysis of the intermediate by refluxing with concentrated mineral acid, cleaved the amide and two ester bonds. The unstable geminal diacid, 2-amino-2-carboxyl decanoic acid thus formed, underwent spontaneous decarboxylation to yield the α-aminodecanoic acid **1a**.

The amino acids **1a**-**1d** were prepared in racemic form using the same procedure as for **1a**, starting with the appropriate 1-bromoalkane. All lipidic conjugates were then synthesised from the racemic amino acid mixtures (Scheme 1).



Scheme 1

2.1.2 Homo- and hetero-oligomers

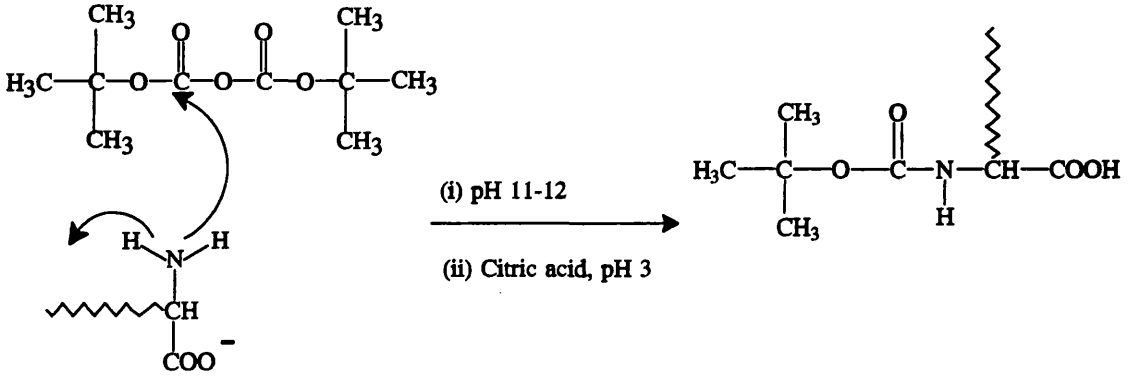
The lipidic peptides are formed by condensation of the lipdic amino acids with themselves or other natural or unnatural amino acids. This condensation requires the amino group of one amino acid to be coupled to the carboxyl group of another, and

so to enable correct synthesis of the desired peptide, the amino or carboxyl function must be protected when not required to take part in the reaction. Since solid phase peptide synthetic methods were used to couple the lipidic amino acids to themselves and other amino acids, it is only necessary to protect the amino group of the incoming amino acid as the C-terminus of the peptide is anchored to the solid support and can take no part any reaction.

2.1.2.1 Protection of the amino group

There are a wide range of protecting groups known for the amino function of amino acids. They include; i) the acyl groups formyl (Fischer and Warburg 1905), chloroacetyl (Holley *et al.* 1952) and phthalyl (Kid and King 1948), ii) the alkyl groups triphenylmethyl (trityl) (Helferich *et al.* 1925) and benzyl (Velluz *et al.* 1954), iii) p-toluenesulphonyl (Tos) (Schonheimer 1926) and iv) the urethanes which include carbobenzoxy (Z) (Bergmann and Zervas 1932), tertiary butyloxycarbonyl (t-Boc) (Carpino 1957) and 9-fluorene methoxycarbonyl (Fmoc) (Carpino and Han 1957). Due to the ease and economy of the synthesis, and the relatively simple procedures required for its removal, the t-Boc group was chosen for α-amino protection during peptide synthesis. Also the t-Boc protected lipidic amino acids have excellent solubility in the organic solvents used for peptide synthesis.

The t-Boc protected unsubstituted lipidic amino acids **1e**-**1h** were synthesised in good



Scheme 2

yields, by reacting the symmetrical anhydride ditertiary butyldicarbonate ($(Boc)_2O$) (Tarbell *et al.* 1972) with the corresponding lipidic amino acid. Nucleophilic attack of the α-amino group of the lipidic amino acid on one carbonyl group of the $(Boc)_2O$ resulted in the formation of the N-protected lipidic amino acid (Scheme 2).

2.2 Solid phase peptide synthesis

The peptides synthesised were done so utilising solid phase peptide synthetic techniques which were originally elaborated by Merrifield (1963). In short, the carboxyl group of the C-terminal amino acid of the target peptide is linked to a derivatised solid support. Amino acids are then added in a stepwise manner until the desired protected peptide chain is formed. The peptide is then cleaved from the solid support and the side chain protecting groups removed, in the "deprotection" step, leaving a crude product which requires purification.

The solid supports (resins) used were the aminoacyl (4-carboxamidomethyl)benzyl ester resin (Pam resin) (Mitchell *et al.* 1978), and the *p*-methylbenzhydrylamine (*p*MBHA) resin (Matsueda and Stewart 1981), both derivatives of a suspension copolymer of styrene-(1% w/w) divinylbenzene. Cleavage with a strong acid from the Pam resin results in a peptide with a free alpha-carboxyl group, while cleavage of the peptide from the *p*MBHA resin yields an alpha-carboxamide. It is important that the resins are free from reactive functionalities such as aldehyde or hydroxymethyl groups in order to minimise side reactions (Kent and Clark-Lewis 1985).

2.2.1 Peptide bond formation

Formation of an amide bond between two amino acids is an energy requiring reaction, and since carboxylic acids tend to react successfully with amines only at elevated temperatures, one of the groups that will produce the desired amide must be activated. At present activation of the caboxyl group (*C-activation*) remains the underlying principle of all coupling methods in use. Conversion of carboxylic acids to powerful acylating agents is achieved by substitution of the hydroxyl group for an electron

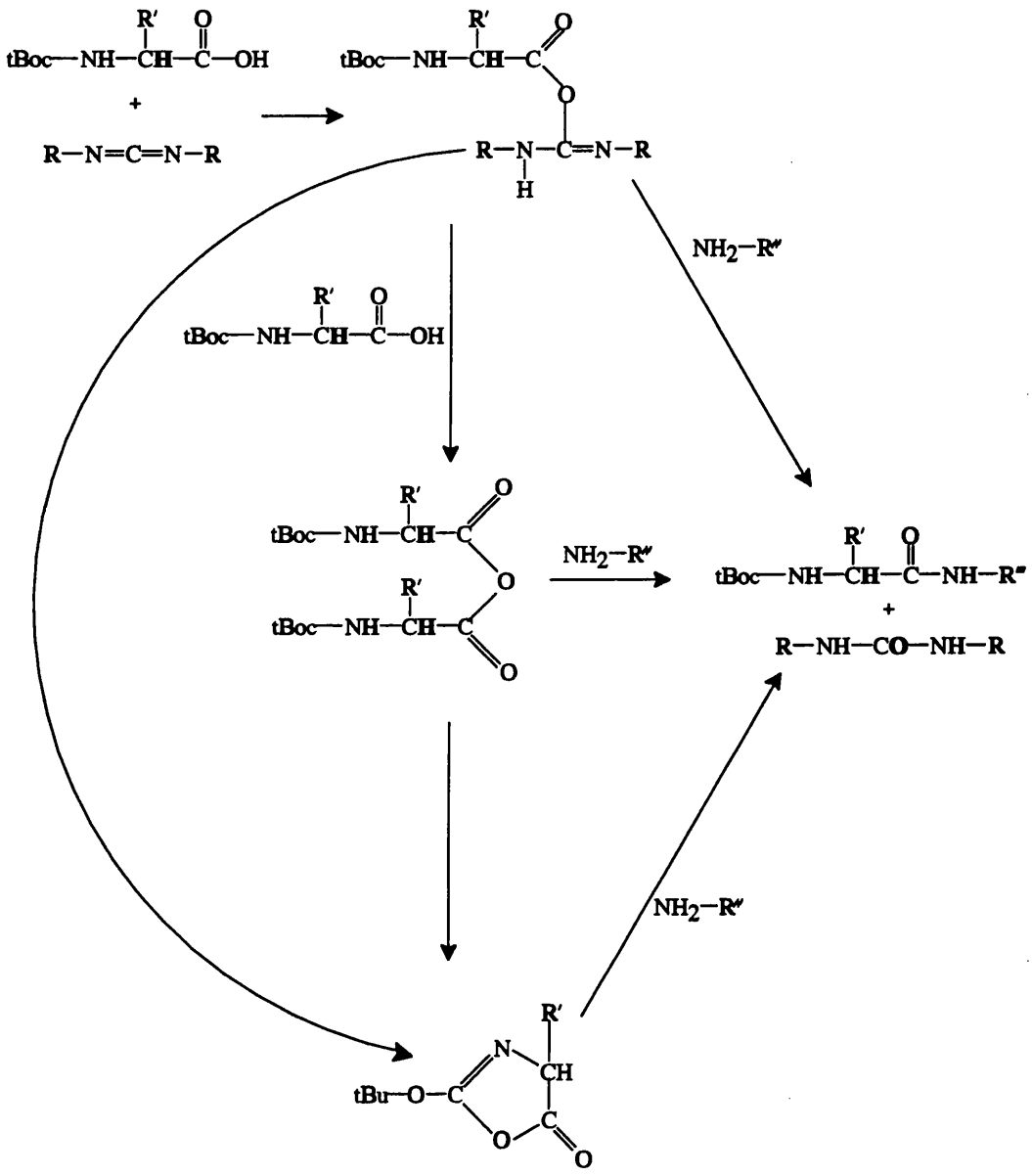
withdrawing substituent which polarises the carbonyl group and renders its carbon atom sufficiently electrophilic to facilitate the nucleophilic attack by the amino group. A tetrahedral intermediate is formed and is stabilised by the elimination of the electron withdrawing substituent (X), which is usually a good leaving group.

The most widely used activating/coupling reagent used is dicyclohexylcarbodiimide (DCC), introduced by Sheehan and Hess (1955), and was chosen because of its simplistic use. Activation of the carboxyl group occurs through its addition to an N=C bond in the carbodiimide and proceeds very rapidly. The amino group will react with the carbodiimide to form guanidine derivatives (Muramatsu *et al.* 1963), but this reaction takes place extremely slowly and is unable to compete with the rapid addition of the carboxyl group. Carbodiimide mediated coupling can proceed through three major pathways:

Addition of the carboxyl group to an N=C double bond in the carbodiimide results in the formation of an O-acylisourea derivative. This reaction occurs relatively quickly and the peptide bond is then formed by nucleophilic attack of the amino component on the carbonyl adduct of the O-acylisourea. The O-acylisoureas do have some basic character, so it would be reasonable to assume that the high reactivity observed in aminolysis may be as a result of base catalysis. The major by-product of the DCC mediated acylation is N,N'-dicyclohexylurea which is readily removed by washing the peptidyl resin with organic solvents, an operation which also removes unreacted starting materials.

An alternative mechanism can be brought about by modifying the carbodiimide process to generate symmetrical anhydrides. This is readily accomplished by changing the ratio of protected amino acid to carbodiimide from equimolar, as in O-acylisourea formation, to 2:1, and enables a carboxylate anion from a second protected amino acid to attack the reactive carbonyl function of the O-acylisourea yielding the symmetrical anhydride, which is a potent acylating agent. The amide bond is then created by nucleophilic attack of the amino component on either carbon atom of the reactive carbonyl groups in the symmetrical anhydride.

A third mechanism which probably plays a minor role in DCC mediated peptide bond formation, is the generation of 5(4H)-oxazolones via intramolecular rearrangement of O-acylisourea. These oxazolones are good acylating agents but are prone to racemisation, since the acidic proton can be easily abstracted by bases from the chiral centre due to resonance stabilisation of the carbanion intermediate generated.



Scheme 3. Summary of DCC reaction mechanism

2.2.1.1 Suppression of side reactions with the auxilliary nucleophile 1-hydroxybenzotriazole (HOBt)

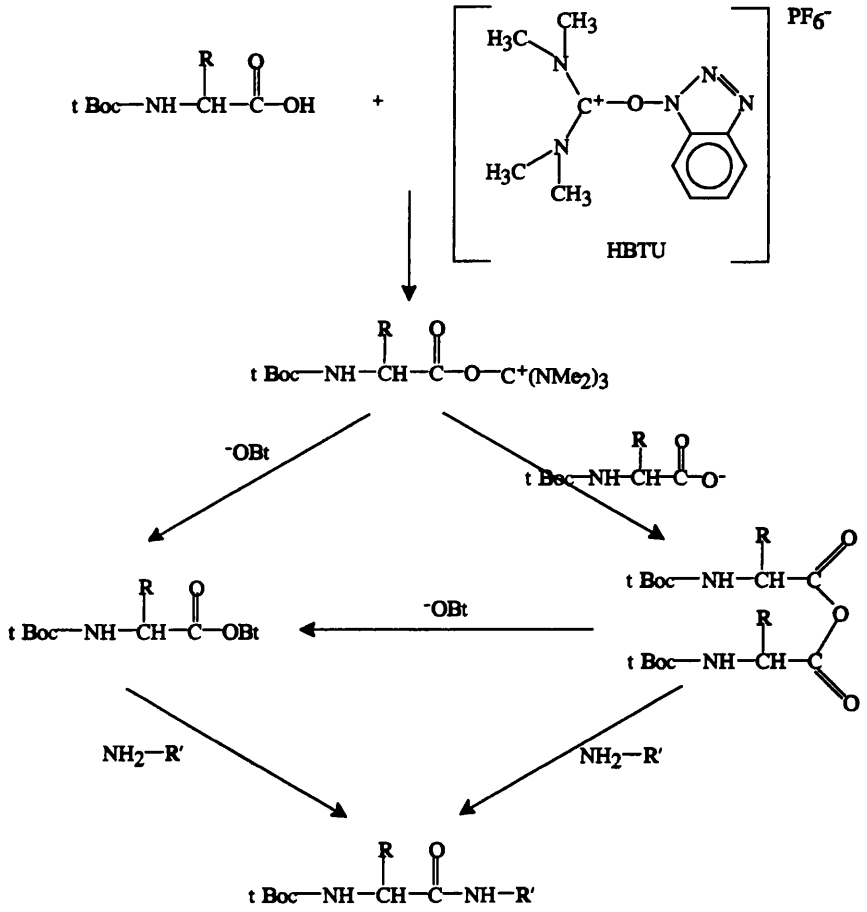
Carbodiimide mediated coupling does not proceed without some side reactions, in particular the intramolecular rearrangement of the O-acylisourea derivative. The activated carbonyl function undergoes nucleophilic attack from the nearby NH group present in the O-acylisourea. This results in an O → N shift yielding an N-acylurea derivative as a by-product. These ureides not only contaminate the reaction mixture but also represent a loss of valuble carboxyl component since they are unreactive. Racemisation is also a problem in DCC mediated coupling leading to loss of chiral purity.

Both of these side reactions can be minimised with the use of the auxilliary nucleophile 1-hydroxybenzotriazole (HOBt) (König and Geiger 1970). Attack by HOBt on the activated intermediates, yields the O-acyl-1-hydroxybenzotriazole. Thus, the lifetime of highly reactive intermediates, such as O-acylisoureas, symmetrical anhydrides or oxazolones, is considerably reduced. In contrast, the concentration of the additive hardly changes during the coupling reaction as it is continuously regenerated. Hence the entire process is accelerated and the overactivated intermediates of the DCC reaction are converted to the less reactive esters of 1-hydroxybenzotriazole. The active esters, formed *in situ* are less likely to undergo O → N shift, but are still sufficiently reactive to allow satisfactory acylation. Secondly, since HOBt is weakly acidic it is able to suppress base catalysed proton abstraction from the chiral carbon and so maintain chiral purity.

2.2.2.2 Uronium salts as alternative coupling reagents

Acyloxyuronium species, which can be generated by the attack of carboxylate anions on suitable uronium cations, react readily with nucleophiles at the acyl carbon. They are intermediates of the so-called oxidation-reduction condensation (Mukaiyama *et al.*, 1979), and a number of uronium and phosphonium salts have been developed for use as direct coupling reagents. Initially the phosphonium salt benzotriazolyloxy-tris-

(dimethylamino) phosphonium hexafluorophosphate (BOP) (Castro *et al.*, 1975) was used but, due to the generation of the highly toxic co-product hexamethylphosphoramide, BOP has been superseded by the safer PyBOP reagent (Martinez *et al.*, 1988). Of the uronium salts, 2-(1H-Benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) and 2-(1H-Benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium tetrafluoroborate (TBTU) (Knorr *et al.*, 1989) are widely used and provide excellent yields, with few side reactions.



Scheme 4

For coupling, one equivalent of reagent is used, together with a tertiary base to ensure the carboxy component is in its anionic form. Coupling can proceed via a number of different pathways (Scheme 4), all accumulating in a highly efficient coupling yield. The initially formed highly reactive acyloxyuronium intermediate can react rapidly

with the amino acid carboxylate to form the symmetrical anhydride, or alternatively can be converted to the effective benzotriazolyl active ester. Both intermediate species then undergo nucleophilic attack by the free amino group to give the coupled peptide. In a simpler case, the amino component and the acyloxyuronium derivative can react directly yielding the desired product. Interestingly, the liberated ionised hydroxybenzotriazole can react slowly with the symmetrical anhydride resulting in its conversion to the benzotriazolyl active ester. In order to completely supress racemisation, additional HOBt is often added, forcing the reaction to go predominantly via the active ester pathway (Bernatowicz *et al.*, 1989).

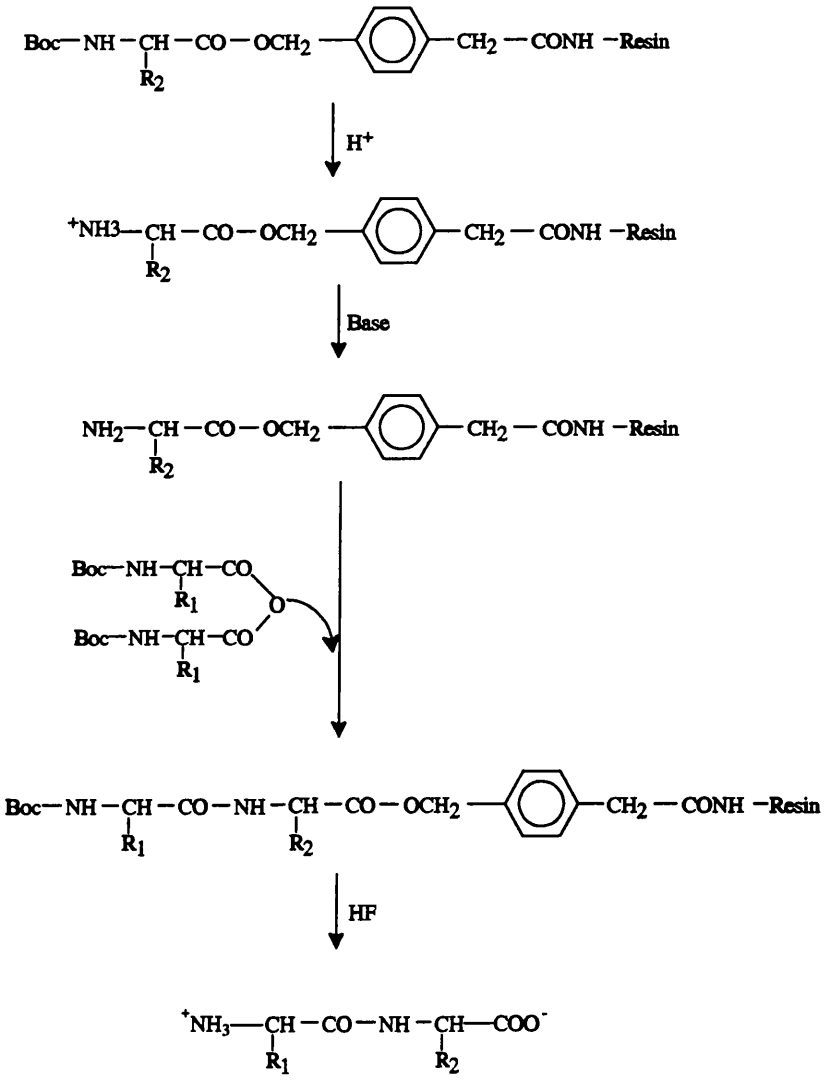
2.2.3 Semipermanent side chain protection

In the assembly of peptide chains by chemical means it is necessary to protect reactive side chain functionalities, which are not involved in peptide bond formation. The protection must be stable to the reaction conditions required during chain assembly but must also be easily removable once the peptide chain is completed. The type of side chain protection used depends upon the nature of N^{α}-protection employed. The N^{α}-Boc/benzyl based strategy relies on the principle of graduated acid lability of the protecting groups, the N^{α}-Boc group is removed by trifluoroacetic acid (TFA) to allow chain elongation while the side chain protection is resistant to TFA cleavage but is removed, in most cases, by anhydrous hydrogen fluoride (HF) which also cleaves the assembled peptide chain from the insoluble rein support. Histidine and tryptophan residues employ protecting groups which are not labile HF labile and require further treatment to remove the dinitrophenol and formyl protecting groups from histidine and tryptophan respectively and apart from these two residues, satisfactory protecting groups exist for all amino acids (Barany and Merrifield 1980, Tam *et al.* 1979, Yajima *et al.* 1978).

The Fmoc based strategy uses different mechanisms for removal of the N^{α}- and side chain protecting groups, a secondary amine for the N^{α}- protecting group and the side chain protection being labile to TFA. Fmoc based solid phase peptide synthesis was first introduced by Meienhofer (1978) and so less work has been done on the side

reactions and protecting groups than for Boc/benzyl chemistry, however, in the last decade satisfactory progress has been made, making a full range of Fmoc-amino acids derivatives available (Ramage and Green 1987, Sieber and Riniker 1987). The major problem seen with the Fmoc approach has been the limited solubilities of the activated Fmoc-amino acids in the solvents commonly used in peptide synthesis, leading to reports of extraordinary slow coupling kinetics (Dryland and Sheppard 1986).

2.2.4 Peptide chain assembly



Scheme 5

In order to make effective use of the solid phase approach of Merrifield (1963), it is necessary to understand the fundamental aspects of solid phase chemistry:

1) the peptide is covalently linked to a solid support.
2) once swollen in the appropriate solvent, the solid support provides an interpenetrating polymer network, within which synthesis occurs.
3) the covalently attached peptide chains are effectively in solution.
4) peptide and resin mutually enhance one another's solvation.
5) reactions are rapid and obey normal kinetics.

Chain assembly occurs on polymer chains randomly scattered throughout the interior of the swollen bead (Merrifield and Littau 1968). Because the polymer is only lightly cross-linked, it is highly solvated and the covalently attached peptide chains are effectively in solution (Live and Kent 1982, Erickson and Merrifield 1976). The choice of organic solvent in which the resin is swollen is therefore very important in order for maximum solvation to occur and also to allow rapid coupling of each amino acid, solvents most commonly used are CH_2Cl_2 and DMF.

The chemical steps involved in the addition of an amino acid to a preloaded PAM resin are shown above in Scheme 5. The peptide chain is assembled by repetition of these steps (excluding the final deprotection). First the t-Boc protecting group is removed from the α-amino group of the resin-bound amino acid using 50-65% (v/v) TFA in CH_2Cl_2, this reaction is rapid ($t_{½}$~20secs) and goes essentially to completion within 15 minutes. The resulting trifluoroacetate salt of the peptide resin is then neutralised with the hindered tertiary amine diisopropylethylamine (DIEA), the use of a highly purified tertiary amine helps minimise side reactions, especially those involving the aspartic acid side chain (Tam *et al.* 1979). The rationale for the coupling procedures that are routinely used has already been described, yet it is important to point out the role of the organic solvent in the coupling reaction. The use of the polar solvent DMF improves coupling efficiency and can often overcome the problem of difficult couplings sometimes experienced when CH_2Cl_2 is used as a solvent (Kent and Merrifield 1981).

2.2.4.1 Documentation of chain assembly

In solid phase synthesis, purification consists only of filtration and washing of the resin-bound intermediates and because of this, it is necessary to drive all reactions involved in the chain assembly to completion. It is important to have accurate information on the yields at all steps of the chain assembly so that the chemistry can be optimised for the synthesis, documentation of the efficiency of chain assembly is also the first step in ensuring the production of a peptide of high purity and of known structure.

The simplest method of monitoring coupling efficiency is to use the quantitative ninhydrin reaction (Sarin *et al.* 1981) to measures residual uncoupled amine at the end of each cycle of amino acid addition. The method is sensitive and accurate, giving reliable data down to less than 0.1% residual amine (>99.9% coupling) with a reproducibility of +/- 0.05%. The conditions of the ninhydrin reaction are sufficiently vigorous (5-10 min at 100°C in pyridine) to reveal amine that was unavailable for coupling, due possibly to aggregation or Schiff's base formation (Kent 1984).

Quantitative Edman degradation of the protected peptide-resin product can also be used to document the efficiency of chain assembly in Boc chemistry (Kent *et al.* 1982) and also provides confirmation of the correct sequence of amino acids in the product.

2.2.4.2 Side reactions

Over the past two decades, intensive efforts have resulted in the identification and correction of side reactions occurring at every cycle of solid phase Boc chemistry, regardless of amino acid sequence (Kent *et al.*1979, Mitchell *et al.*1978, Kent and Merrifield 1983). In many instances, the side reactions were caused by the presence of on the resin support of extraneous functional groups that participated in the chemistry (Kent 1984). The problems were eliminated by the use of chemically defined, clean peptide-resins that were stable to the conditions of chain assembly (Mitchell *et al.*1978).

Residue or sequence specific side reactions have also been the subject of extensive investigation in Boc chemistry. Thus, the mechanisms of two common side reactions, diketopiperazine formation for Pro-containing dipeptide resins and pyrrolidone carboxylic acid formation from N-terminal glutamine, have been elucidated and conditions sought to minimise their occurrence (Gisin and Merrifield 1972, Dimarchi *et al.* 1982). In the both cases, the recommended conditions for avoiding these side reactions coincide with the preferred conditions for optimised chain assembly, namely deprotection with high concentrations of TFA, rapid washing and coupling with separately activated amino acids in polar solvents.

2.2.4.3 Difficult sequences

The most serious problem in stepwise solid phase synthesis is incomplete peptide bond formation, giving rise to peptides with one or more internal amino acids missing but with properties similar to the target peptide. Even after the elimination of chronically occurring side reactions, incomplete coupling can still occur and is more prevalent in some sequences than in others. The primary source of the problem has been shown to be the sequence-dependent tendency of the resin-bound peptide to form intermolecular aggregates (Live and Kent 1984) and the use of high resin loadings exaggerates the phenomenon (Kent and Merrifield 1981). Strategies to overcome this problem include the use of a dipolar aprotic solvent like DMF and resins of low substitution (Kent 1985), the use of heat in the coupling step (Tam 1985) and the conversion of residual unreacted resin-bound peptide chains to nonreactive (terminated) species (Barany and Merrifield 1980). This latter method of "capping", in combination with suitable purification steps to select out only the full length peptide chain, can eliminate the problem posed by deletion peptides.

2.2.4.4 Deprotection and cleavage from the resin

The final steps in the generation of the synthetic peptide involve the removal of the side chain protecting groups and cleavage from the resin support. However, before cleavage the N^{α}-Boc group is removed with TFA to prevent alkylation of any

methionine side chains by tertbutyl cations generated in the cleavage reaction conditions (Noble *et al.* 1976). In general, the side chain protecting groups and the peptide anchoring bond are designed to be labile to strong acids, such as HF and trifluoromethanesulphonic (TFMSA). HF has many desirable characteristics for deprotection of synthetic peptides (Sakakibara 1971), however, treatment with HF results in the generation of highly reactive carbonium species, since acidolysis proceeds by an S_N1 reaction mechanism (Tam *et al* 1983). To prevent the carbonium by-products alkylating side chains of the peptide, scavengers such as p-cresol, p-thiocresol and anisole are added to the reaction mixture to mop up these reactive species.

An alternative HF procedure designed to avoid generation of the carbonium ion intermediates has been devised (Tam *et al* 1983). In the first stage, removal of most protecting groups takes place by S_N2 acidolysis, at low temperature in a low-acidity function medium consisting primarily of dimethyl sulphide and either HF or TFA/TFMSA. The peptide-resin bond is stable to this step. After removal of the coproducts and reagents, the remaining protecting groups are removed and the peptide cleaved by means of S_N1 acidolysis at low temperature, in a high-acidity function medium. All the side chain protecting groups are labile under these strong acid conditions except for the dinitrophenyl (DNP) group which must be removed from histidine by thiolysis (Shaltiel and Fridkin 1970) while the peptide is both resin-bound and before removal of the N^{α}-Boc group.

2.3 Lipidic peptide conjugates

The lipidic amino acids and peptides represent a class of compounds which combine stuctural features of lipids with those of amino acids (Gibbons *et al.* 1990) and have potential as a drug delivery system (Toth *et al.* 1991a). Because of their bifunctional nature, the lipidic amino acids and peptides have the capacity to be chemically conjugated to or incorporated into poorly absorbed drugs or peptides. The resulting conjugates possess a high degree of membrane-like character and the resulting permeability coefficient of the drug moiety may increased to such an extent that its

passage across the hydrophobic membrane of the GI tract is facilitated. Toth *et al.* (1994c) conjugated lipoamino acids to a herpes simplex ribonucleotide reductase inhibitor of five amino acid residues, addition of one lipidic unit raised the octanol:water partition coefficient of the peptide from approximately -8.5 to +1.9 while two lipoamino acids afforded a further increase to +4.8. While Toth *et al.* (1994a) recently showed that the oral uptake of the anti-inflammatory compound, benzoquinolizine, could be greatly improved by the conjugation of lipoamino acids. The long alkyl side chains also have the additional effect of affording protection to a labile peptide drug from enzymatic degradation (Toth *et al.* 1994b).

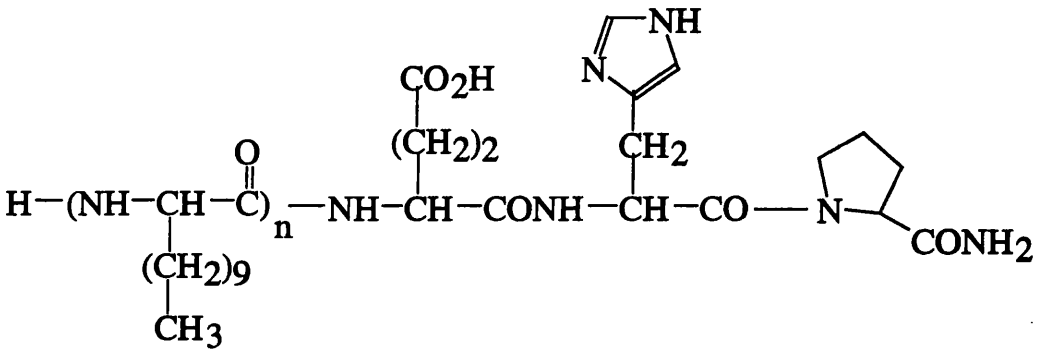
In order to ascertain whether or not the lipidic amino acids provide a means of delivering peptides effectively while simultaneously protecting them from proteolysis, thyrotropin releasing hormone (TRH) and luteinising hormone releasing hormone (LHRH), were chosen as model peptides for metabolic and oral absorption studies. Both peptides were chosen simply because their absorption and metabolism are already well documented, providing valuable information for the design of the lipidic conjugates and the experimental protocols.

2.3.1 Lipidic conjugates of TRH

The tripeptide thyrotropin releasing hormone (TRH), is a hypothalmic hormone that stimulates the release of thyrotropin, prolactin and growth hormone from the pituitary and is used as a diagnostic agent in the evaluation of hyperthyroid and hypothyroid states. In clinical practice, TRH is presently administered parenterally because of low brain-levels following oral administration. Since TRH is relativley resistant to proteolytic degradation in the GI tract, its poor oral activity is probably due to poor absorption and rapid clearance in the bloodstream. TRH is rapidly degraded following first-order kinetics, with a half life in humans of approximately 5 min following i.v. administration. Two different enzymes are responsible for the catabolism of TRH: prolyl endopeptidase (generating deamino-TRH) and pyroglutamyl amino peptidase (generating the products pGlu and His-Pro-NH_2).

2.3.1.1 Synthesis of TRH conjugates

The tripeptide TRH **2a** was synthesized by the solid phase technique of Merifield (1963), where pyroglutamic acid (pyrGlu) was replaced with glutamic acid (Glu) and extended on the N-terminus with one or two 2-amino-dodecanoic acid (**1b**) resulting in compounds **2b** and **2c**. All couplings were performed using the DCC/HOBt strategy described in **2.2.2**. The 2-*tert*-butoxycarbonylamino-dodecanoic acid was synthesised from 1-bromododecane as described in **2.1.1**and **2.1.2.1**.



2

2	**n**	**Configuration**
a	0	-
b	1	**1b**-TRH
c	2	**1b**-**1b**-TRH

Because the lipidic amino acid was racemic, coupling it to TRH to form a "monomer" conjugate resulted in a diastereomeric mixture of **2b**. Similarly the "dimer" conjugate, obtained by coupling two lipidic amino acids to TRH, resulted in four diastereomers of **2c**. After cleavage from the resin, the lipidic peptide conjugates **2b** and **2c** were dissolved in 90% acetic acid, while the unconjugated TRH (**2a**) was soluble in 10%

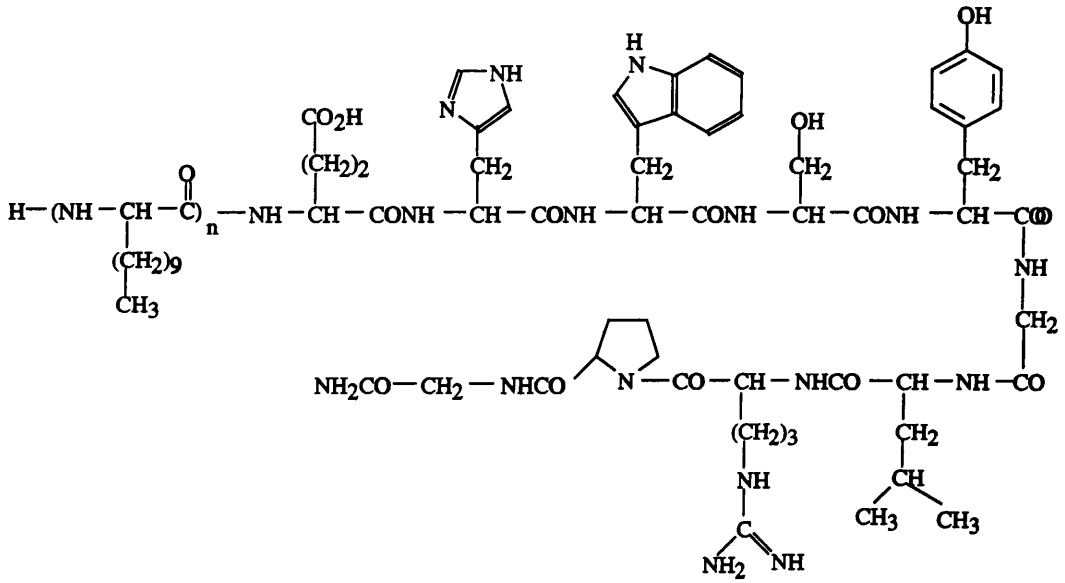
acetic acid, **2a**, **2b** and **2c** were then lyophilised after dilution. The compounds were purified by reverse-phase high performance liquid chromatography (RP-HPLC) using acetonitrile and water as solvents and obtained in moderate yield, however, the diastereomers were not separated and were examined as a diastereomeric mixture. The primary structures of the compounds were verified by MS and ^{1}H NMR.

2.3.2 Lipidic conjugates of LHRH

The decapeptide LHRH, a hypothalmic hormone that regulates the secretion of lutenizing hormone (LH) and follicle-stimulating hormone (FSH), is used in medicine for the treatment of infertility. The molecule is too large (mol. wt. 1200) and too hydrophilic to cross the GI tract mucosa; the peptide is also highly susceptible to enzyme degradation. Degradation proceeds by a variety of routes, of particluar importance is the degradation mediated by endopeptidases on the Tyr^5-Gly^6 bond, in combination with enzymes that hydrolyse the pyroGlu^1 or cleave on the carboxyl side of Pro. Due to the physical and chemical barriers afforded by the GI tract, the oral administration of LHRH required a dose 3000-times higher than that of the parenteral route (Sandow and Petri, 1985). The half-life for LHRH in the circulation is extremely rapid (2-8 min) and involves the mixing of the peptide throughout the vascular and extracellular fluid spaces.

2.3.2.1 Synthesis of LHRH conjugates

The decapeptide LHRH (**3a**) was synthesised in the same manner as the TRH (**2a**) on solid phase utilising DCC/HOBt strategy. As before, the pyrGlu was replaced with Glu to allow extension of the N-terminus with one or two 2-amino-dodecanoic acid (**1b**). Again, compound **1b** was racemic and so diastereomeric mixtures of the lipidic conjugates were obtained, but in this case the individual diastereomers were separated by RP-HPLC, although their absolute configurations were not determined. An extra step was required, before HF cleavage, to remove the formyl protecting group from the tryptophan side chain, this was carried out with piperidine in DMF.



3

3	**n**	**Configuration**
a	0	-
b	1	L-**1b**-LHRH
c	1	D-**1b**-LHRH
d	2	L-**1b**-L-**1b**-LHRH
e	2	D-**1b**-D-**1b**-LHRH
f	2	L-**1b**-D-**1b**-LHRH
g	2	D-**1b**-L-**1b**-LHRH

HPLC and MS data for the conjugates is summarised below in table 1.

Compound	Fraction No.	Retention time (min)	MS $[M+H]^+$
3		12.36	1200
3b, 3c	fr.1	17.34	1398
	fr.2	18.13	1398
3d-3g	fr.1	21.25	1596
	fr.2	21.83	1596
	fr.3	23.73	1596
	fr.4	23.97	1596
2a		6.66	380
2b	Racemic	15.56, 15.97	577
2c	Racemic	21.77, 22.12 22.66, 23.71	776

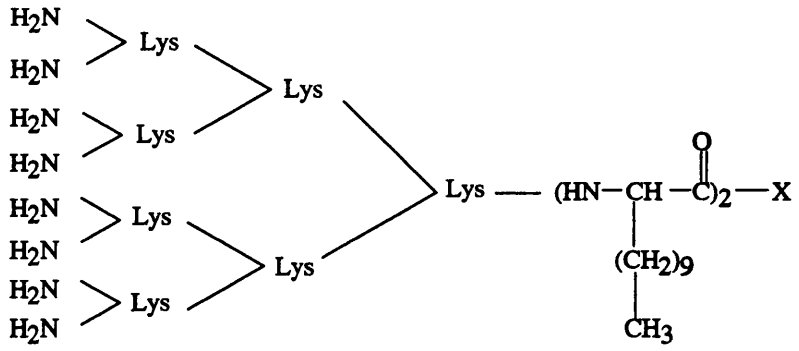
Table 1.

2.3.3 Lipidic-polylysine conjugates of TRH and LHRH

The major priority in trying to improve the transmembrane absorption of poorly absorbed compounds was, in our laboratory, to impart them with increased membrane-like character by conjugation to lipoamino acids and their oligomers (Toth *et al.* 1991a, Hughes *et al.* 1991, Toth *et al.* 1991b, Toth *et al.* 1991c). However the resultant conjugates often showed reduced water solubility and attempts using hydrophillic moieties were made to address this problem, including the conjugation of sugar molecules to the lipidic derivatised compounds (Toth *et al.* 1992). The tetra- and octameric forms of poly-L-lysine have been utilised by immunologists as a means of presenting multiple copies of peptide epitopes to the cells of the immune system,

in order to maximise the immunogenic response to the peptide (Tam 1988). Since the construct is of a highly hydrophillic nature, it was thought that conjugation of the lipidic TRH and LHRH conjugates to the C-terminus of the polylysine system would enhance the solubility of the conjugates in aqueous media. Water solubility is an important consideration in both the formulation of any drug molecule and the way in which it exerts its biological effect, however, for a peptide to be orally absorbed, it must retain some lipophilic character to permit passive diffusion across biological membranes. Thus, the resulting conjugates should possess a higher degree of water solubility than compounds **2b** and **c** and compounds **3b-g**, while at the same time exhibiting an increased lipophilicity.

2.3.3.1 Synthesis of lipidic-polylysine TRH and LHRH conjugates



4

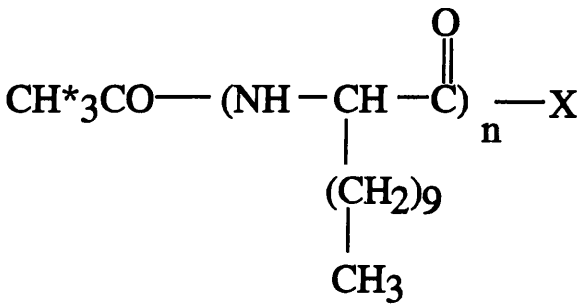
4	**X**
a	$EHPNH_2$
b	$EHWSYGLRPGNH_2$

The respective lipidic peptide chains were built up on solid phase using DCC/HOBt methodology, as described for compounds **2c** and **3d-g**. However instead of cleaving the peptide chain at this point, an octameric form of the polylysine construct was built on the N-terminal of each peptide, yielding compounds **4a** and **4b**. Construction of the polylysine scaffold was achieved using a lysine residue which was Boc protected at both its α- and ε-amino functions, thus, after being coupled to the peptide chain and after Boc deprotection with TFA, the unprotected lysine residue allowed coupling to continue *via* both its α- and ε-amino functions. Successive coupling of the double Boc protected lysine afforded a branched lysine system, with eight amino functional groups.

The lipidic-polylysine peptide conjugates were then cleaved from the resin and lyophilised. At this point it was noted that conjugates **4a** and **4b** were soluble in 5% acetic acid, whereas the conjugates **2b** and **c** and **3b-g** required 90% acetic acid in which to dissolve, indicating that addition of the polylysine system had produced a dramatic increase in aqueous solubility. The conjugates were then purified by RP-HPLC and were used as racemic mixtures.

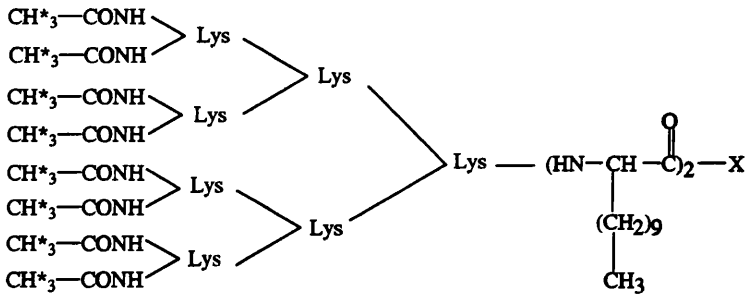
2.3.4 Radiolabelled conjugates of TRH and LHRH

In order that the conjugates described thus far could be traced or detected in biological assays, it was necessary to synthesise radiolabelled forms of the compounds described thus far. Tritium was chosen to label the compounds because of its long half-life, its relatively low cost and its widespread availability. The tritium radiolabel was introduced by acetylating the N-terminal of each compound with tritiated acetic anhydride in DMF in the presence of the strong base DIEA, while the conjugates were still resin-bound, resulting in **5a-f**, **6a** and **6b**.



5

5	**n**	**X**
a	0	$EHPNH_2$
b	1	$EHPNH_2$
c	2	$EHPNH_2$
d	0	$EHWSYGLRPGNH_2$
e	1	$EHWSYGLRPGNH_2$
f	2	$EHWSYGLRPGNH_2$



6

6	X
a	$EHPNH_2$
b	$EHWSYGLRPGNH_2$

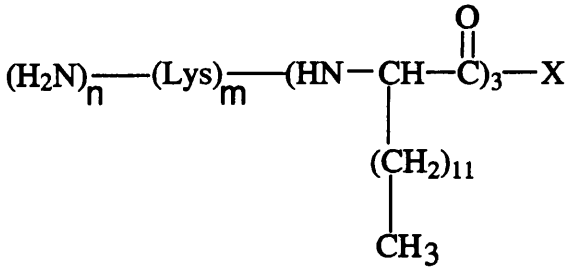
2.4 The lipid-core peptide

Synthetic peptides are widely used to generate antibodies. To induce high antibody response, the low molecular weight peptide must be conjugated to a carrier protein (KLH, BSA). Triple-chain lipopeptides also constitute efficient low molecular weight carrier/adjuvant systems, which can be linked to antigens to yield immunogens for antibody production without further additive (Prass *et al.* 1987). High antibody response was observed, when the peptides were incorporated into polylysine to form Multiple Antigenic Peptide (MAP) (Tam 1988). To attain higher antibody response, the antigen must be anchored to the cell membranes, however the polylysine system is not lipophilic enough to fulfil this requirement. A novel Lipid-Core-Peptide (LCP) system has been developed by incorporating lipidic amino acids into the polylysine system to enhance lipophilicity and membrane binding effects and the metabolic stability of the LCP system (Toth *et al.* 1993). This system has been designed as a combined adjuvant-carrier-vaccine system, which can induce a maximal immune

response to a peptide epitope over a prolonged period.

2.4.1 Synthesis of the lipid-core peptide

The LCP system was synthesised by solid phase methods on three different resins, in order to find the support which provided the most efficient environment for the synthesis of lipidic polylysine construct (7). Boc-protected lipidic amino acid **1g** was coupled to MBHA resin using DCC/HOBt automated solid phase peptide synthetic methods. After the necessary N-deprotection, two more lipidic amino acids **1g** were coupled to the peptide-resin, followed by the sequential addition of Boc-Lys(Boc). The first level of coupling of Boc-Lys(Boc) produced two amino ends (**7a**), the second level of coupling resulted in four (**7b**) and the third level of coupling yielded eight



7

7	**X**	**m**	**n**
a	NH_2	1	2
b	NH_2	3	4
c	NH_2	7	8
d	L-Ala	7	8
e	L-Pro	7	8

amino groups (**7c**). Similar syntheses of **7d** and **7e** were carried out on Boc-Ala-PAM and Boc-Pro-PAM resins, using lipidic amino acid **1g** as an anchor, in order to compare the coupling efficiencies on the different resins.

Using a double coupling procedure with DCC/HOBt strategy (section **2.2.2**) the coupling efficiencies (Table 2) on MBHA resin, which were evaluated by quantitative ninhydrin testing, were satisfactory, while the coupling efficiencies on PAM resins were lower and in order to obtain a satisfactory coupling yield, triple couplings had to be carried out.

Coupling step	Double coupling efficiency (%)		
	MBHA resin	BocAlaPam resin	BocProPAM resin
1	99.7	99.1*	99.5
2	99.5	99.4	99.3*
3	99.7	99.7	99.4
4	99.6	99.0*	99.6
5	99.7	98.0*	99.3*
6	99.5	99.1*	99.4

*for minimum 99.4% coupling efficiency triple coupling was employed

Table 2. Synthesis of LCP system. Comparison of coupling efficiency on different resins.

The lipidic conjugating unit gives a great flexibility in the physico-chemical properties of the coupled compound because of the vast number of variables which can be modified, (i) the length of the peptide chain can be increased, (ii) the length of the alkyl chain is variable, (iii) the free substituent on the C-termini of the conjugating units allows further conjugation eg. (a) sugars, to increase hydrophilicity, (b)

antibodies, to target the conjugates, (c) fluorescent and radio-labels, for tracer studies. Another novel aspect of this project is that the spacer-lipid-core-peptide system can be synthesised using an automated peptide synthesizer, without isolating the intermediates.

Various peptide epitopes which have been identified as potential vaccine candidates, were synthesised on the LCP system and the structures immunogenicity evaluated.

2.4.2 A peptide vaccine candidate for *Chlamydia trachomatis*

Trachoma is an ocular infection principally caused by three serovars of *Chlamydia trachomatis* (A, B/Ba, and C) (Grayston and Wang 1975). Shortly after *C. trachomatis* was established as the etiologic agent of trachoma, large scale human trials of vaccine prevention using whole purified bacterial cells were performed. The best of the trials established that vaccine prevention of trachoma could produced by parenteral immunisation (Grayston and Wang 1978). However, vaccine induced immunity decayed rapidly and required very high concentrations of antigen to elicit a response. Further study of whole cell vaccination for trachoma prevention was halted because of the difficulties in preparing sufficient antigenic mass and because of vaccine induced hypersensitivity reactions to breakthrough infections.

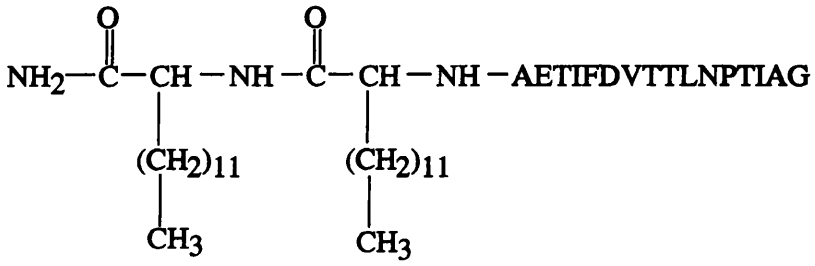
Enthusiasm was rejuvenated when the *C. trachomatis* major outer membrane protein 1 (Momp 1) was established as the serotyping antigen and was also identified as the major target of neutralising antibodies (Caldwell *et al.* 1981, Zhang *et al.* 1987). Elucidation of the genetic basis for Momp 1 antigenic variation revealed four variable domains (VD), two of which were immunodominant and surface exposed (VD I and VD IV). A sequence from VD IV of serovar B, designated as P_2, was found to contain both a T-helper cell epitope and a B-cell epitope. Also a 12 residue sequence from VD I of serovar C was found to neutralise chlamydial infectivity in an *in vitro* neutralisation assay (Zhong *et al* 1993). These possible vaccine candidates were combined with the lipidic α-amino acids and the LCP system and their immunological potential evaluated.

2.4.2.1 Synthesis of lipidic P_2 and LCP-P_2 vaccine candidates

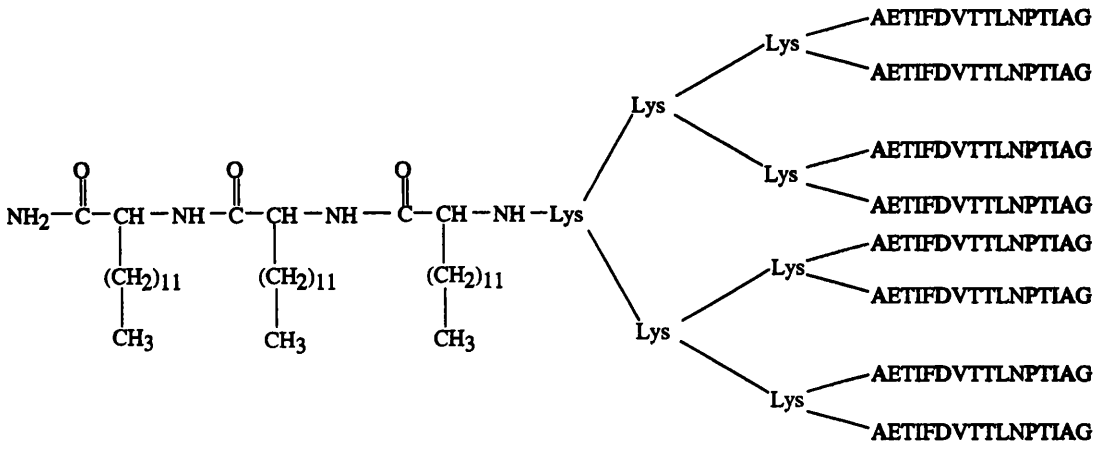
Peptides were synthesised in a stepwise manner, on solid phase using MBHA resin, on an automated peptide synthesizer. The P_2 sequence **9** was synthesised both as a dimeric lipidic conjugate from the Boc protected lipidic amino acid **1g** to give compound **10** and as an LCP octameric construct yielding structure **11**.

AETIFDVTTLNPTIAG

9



10



11

Both compounds **10** and **11** were synthesised using DCC/HOBt double coupling protocols with activation being carried out in CH_2Cl_2 and the coupling reaction completed in DMF. Peptides were cleaved from the resin by treatment with HF in the presence of p-cresol and p-thiocresol. Purification was conducted initially by gel filtration and then further purified by RP-HPLC. Since, as before, **1g** was racemic, compounds **10** and **11** were present as a mixture of diastereomers.

2.4.2.2 Synthesis of lipidic H1 and LCP-H1 vaccine candidates

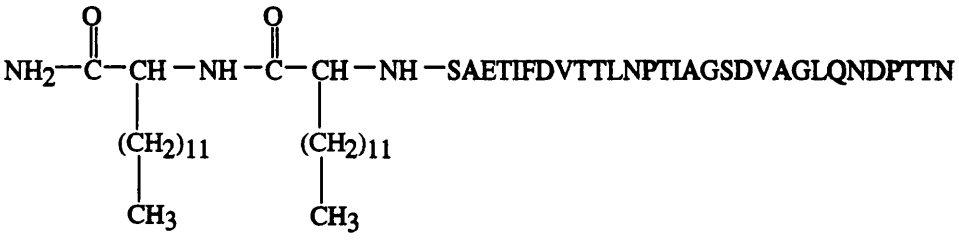
The P_2 sequence **9** and the twelve residue sequence from VD I of Mompl of serovar C were synthesised together and the new sequence **12** was designated H1.

SAETIFDVTTLNPTIAG**SDVAGLQNDPTTN**

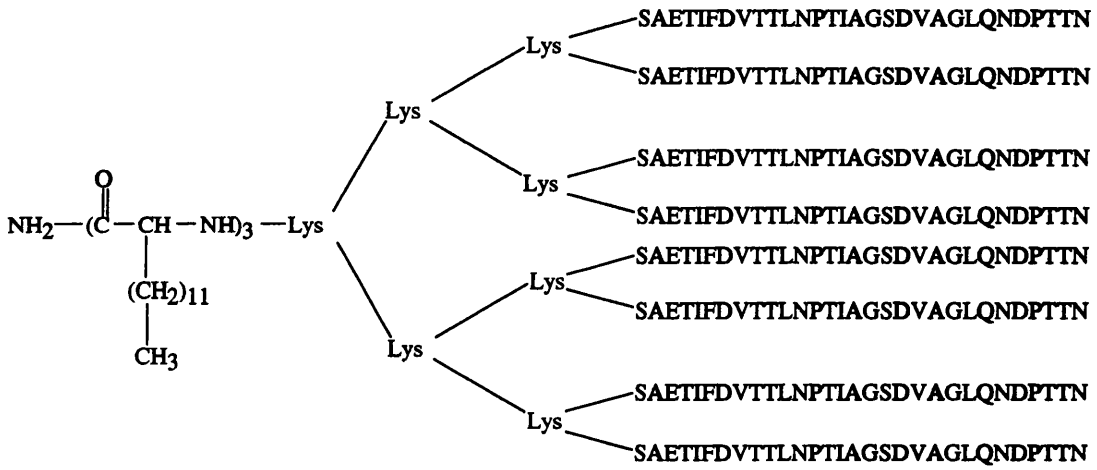
from B mompl VD IV **from C mompl VD I**

12

Compound **12** was synthesised both with **1g** to form the dimeric lipidic peptide conjugate **13** and also on the LCP system, again using the racemic lipidic amino acid **1g**, yielding compound **14**. The coupling and purification protocols were the same as those used for compounds **10** and **11** (Zhong *et al.* 1993).



13



14

2.4.3 Investigation of a peptide vaccine for Foot and Mouth Disease Virus

Foot and Mouth disease virus (FMDV) is a picornavirus that causes an economically important disease of farm animals. The virus conforms to a quasispecies genetic structure in that FMDV populations consist of fluctuating distributions of genetic and antigenic variants (Sobrino *et al.* 1983, Mateu *et al.* 1988). Seven distinct serotypes of FMDV (termed A, O, C, Asia 1, SAT 1, SAT 2 and SAT 3) have been characterised and each serotype includes a large but indeterminate spectrum of subtypes and variants (Pereira 1977) which often co-circulate during disease outbreaks (Domingo 1992). This antigenic heterogeneity represents one of the major difficulties in controlling FMDV by vaccination, since immunity conferred by vaccination against one serotype leaves the animals susceptible to infection by the other six. Moreover, the antigenic variation within a serotype, can be so great that immunity against the homologous strain of virus may not necessarily ensure protection against infection by other viruses within that serotype.

Currently used inactivated virus vaccine induces solid protection, however, immunity is short and multiple vaccination is necessary to protect livestock. Additionally, the escape of viruses from vaccine production centres or use of improperly inactivated

vaccines present a constant risk of new outbreaks (Brown 1992). A further worry is the need, for those preparing the vaccines, to handle large quantities of the hazardous material and thus requires stringent safety procedures. To overcome these problems, synthetic vaccines for FMDV have been under study for more than a decade. Two major immunogenic areas are thought to exist on VP1, one of the four capsid proteins (Brown 1988). In particular, experimental immunogens containing residues 140-160 and 200-213, have resulted in the induction of neutralising antibody and the protection of both experimental and natural hosts (Brown 1992).

The induction of T-cell response in animals immunised with inactivated FMDV has proven to be essential in eliciting an effective neutralising antibody reaction and for protection in experimental hosts (Francis *et al.* 1988, Piatti *et al.* 1991) and studies have shown the presence of B- and T-cell epitopes within the VP1 capsid polypeptide, specifically within residues 135-160 (Zamorano *et al.* 1994). Work carried out on the protection mechanism also suggests the involvement of the reticuloendothelial system in conferring protection against the virus on the host (McCullough *et al.* 1992). It has been proposed that a low concentration of antibody may still provide a neutralising response by forming complexes with the virus particle for which macrophage have a high affinity. The complex is then engulfed by the macrophage and the virus particle destroyed by lysosomal degradation.

In collaboration with Professor Fred Brown at the Animal Disease Center, Plum Island, USA, the peptide sequence 141-160 from VP1 of serotype A, subtype 12, which represents the variable loop of the VP1 capsid protein (Acharya *et al.*1989), was synthesised and evaluated for its potential to induce neutralising antibody and so be a possible candidate for an FMDV synthetic peptide vaccine. The peptide was shown to have neutralising ability when coupled to KLH.

2.4.3.1 Synthesis of FMDV vaccine candidates

The linear sequence 141-160 of VP1 from the A12 strain **15**, was synthesised on MBHA resin using HBTU as the coupling reagent, as described in **2.2.2.2**. A C-

terminal cysteine residue was added to allow conjugation of the peptide chain to the carrier KLH, for immunisation purposes.

$G^{141}SGVRGDFGSLAPRVARQLP^{160}$-Cys

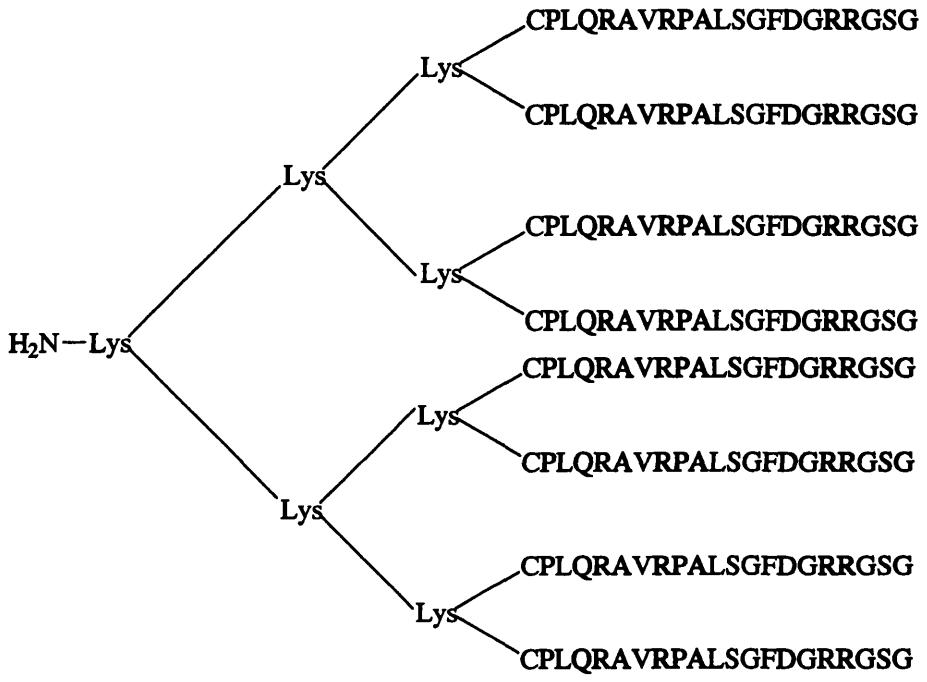
15

Fast coupling cycles of approximately 15 minutes per residue were utilised and employed *in situ* neutralisation of the resin bound peptide. *In situ* neutralisation in Boc chemistry SPPS was first used by Castro *et al.* (1987) in combination with BOP/DIEA activation of the amino acids. The coupling step was carried out in the presence of base without prior neutralisation of the TFA salt of the deprotected amino group of the growing peptide chain. The method that was followed for this synthesis was based on that of Schnolzer *et al.* (1992).

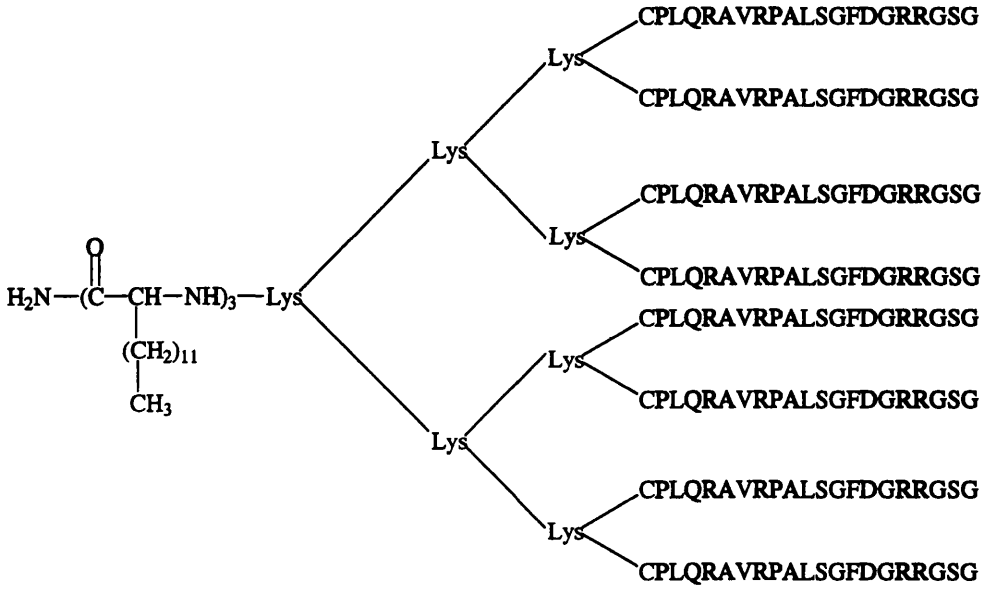
Boc amino acids were activated using one equivalent of HBTU and an amount of DIEA which was sufficient for the neutralisation of the Boc amino acid, plus two equivalents relative to the protonated resin. Thus, the entire amount of DIEA was already added to the preactivated mixture of Boc amino acid and HBTU before addition to the resin, whereas with carbodiimide activation the resin is neutralised in a separate step after TFA deprotection of the terminal Boc protecting group. Furthermore, Boc deprotection was achieved by brief treatment with 100% TFA, which is an excellent solvent for protected peptide chains and may have the effected of disrupting previously formed secondary structures which could hinder further coupling steps.

2.4.3.1.1 Synthesis of FMDV-MAP and FMDV-LCP vaccine candidates

In order to compare and evaluate the immunogenic potential of the MAP and LCP vaccine carrier systems, the peptide epitope **15** was synthesised on an octameric branched MAP resulting in compound **16** and on an octameric LCP system, yielding structure **17**. Both compounds were synthesised manually on a low substituted MBHA resin using the fast coupling HBTU method described above. The LCP construct was


CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
H2N—Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG

16


CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
H2N—(C—CH—NH)3—Lys
O
(CH2)11
CH3
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG
Lys
CPLQRAVRPALSGFDGRRGSG

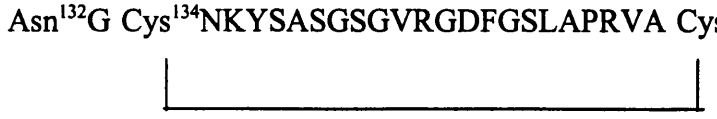
17

2.4.3.2 A cyclic FMDV peptide vaccine candidate

In many studies involving synthetic peptide vaccines, short linear sequences have failed to induce neutralising antibodies, whereas a longer sequence containing the same epitope can provide partial protection (Shapira *et al.* 1985). This seemed to indicate that the length of the peptide could be crucial for enforcing the correct folding required to mimic the native structure. Thus, many workers have attempted to mimic the native structure of immunogenic peptide sequences by either chemical means (Plaue 1990) or by inducing secondary and tertiary structure with the help of an external peptide sequence (Schulze-Gahmen *et al.* 1986).

Chemical methods of inducing secondary structure generally involve cyclisation of a peptide to mimic loop structures in the native protein. Ring closure can be performed between N- and C-termini, side chain functional groups, a combination of side chain functionalities and N- or C-terminus or by the use of cysteine residues to form disulphide bonds. All of these methods constrict the peptide to a rigid conformation, but variations can be introduced by altering the distance between N- and C-termini if spacer molecules are inserted in the bridging sequence. A disulphide linkage was chosen to form the cyclic peptide in this case.

In the case of the FMDV peptide, it was decided that in addition to the epitope being synthesised as a MAP and LCP construct, a cyclic version would be prepared to mimic the variable loop in the native viral particle. A slightly longer sequence was used for the cyclic peptide from residue 132-160, with Thr^{134} and Arg^{157} replaced by cysteine residues to allow the formation of a disulphide bridge **18**.



18

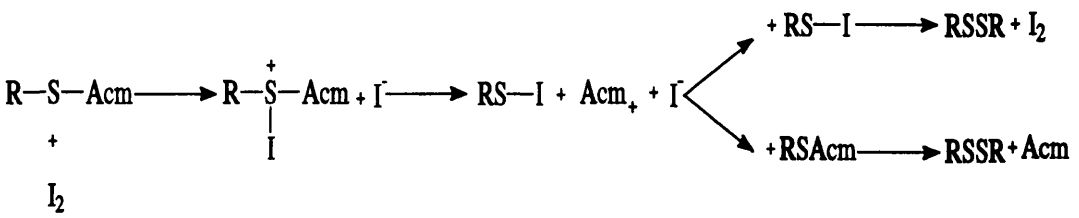
A C-terminal cysteine residue was again included to allow the peptide to be conjugated to KLH for immunisation purposes.

2.4.3.2.1 Disulphide bond formation of cyclic peptides

Many methods have been evaluated and used with some success in the pursuance of cyclic peptide synthesis. Aerial oxidation and the glutathione redox system (Saxena and Wetlaufer 1970) in buffer at pH 8 are traditionally the most commonly used methods for oxidation of sulphahydryl groups to disulphide bonds. Despite the many years for which they have been used, both systems still suffer from a lack of specific guidelines for their use.

Disulphide bonds do not form spontaneously, but require an electron acceptor to be present, many acceptors have been with the classic being O_2. The reaction for air oxidation is apparently dependent on metal ions such as Cu^{2+} which transiently bind the -SH group. Other metal ions (Mn^{2+}, Hg^{2+}, Fe^{2+}) have been shown to inhibit the oxidation (Huxtable 1986). General conditions for air oxidation include buffer at pH 7.5-8.5 and stirring at room temperature, exposed to air. The peptide concentration has traditionally been kept in the μM range (Rivier *et al.* 1987, Chatrenet and Chang 1992), but more recent work has shown good results at mM concentrations (Alewood *et al.* 1991). The choice of solvent is obviously driven by the necessity of having the peptide in solution for oxidation to occur. Basic peptides are often hydrophobic and may require the addition of guanidine or urea to solubilise them in basic conditions.

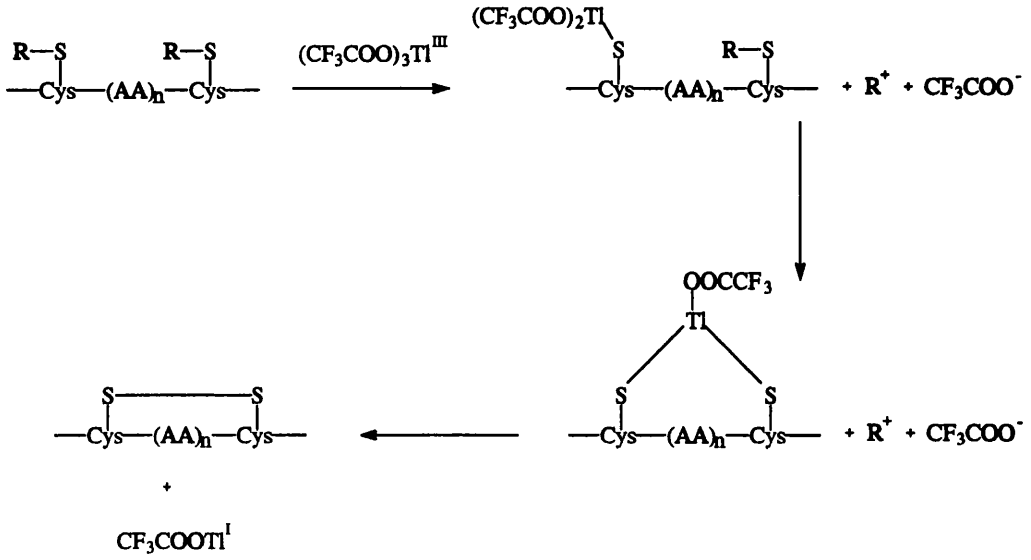
The use of iodine for the deprotection and oxidation of protected peptides has been popular in solution phase peptide synthesis (Jones *et al.* 1973). It has also proven to be a useful method for use with solid phase peptide synthesis, where it has carried out the role of deprotection and subsequent oxidation of the cysteine acetamidomethyl



Mechanism for the action of iodine as an oxidant

(Acm) side chain protecting group used in Boc or Fmoc SPPS. Because the Acm group is stable in HF treatment, the crude Acm protected peptide can be purified prior to deprotection and oxidation with iodine, resulting in a cleaner product.

There are, unfortunately, two main problems associated with the use of iodine for deprotection and oxidation of Cys-S- groups. The oxidation of the thiol function can proceed to the sulphonic acid (which is irreversible) and iodination of histidine, tryptophan and tyrosine residues can occur during treatment with iodine. Formation of the sulphonic acid can be prevented by controlling the conditions of the deprotection/oxidation and then rapidly removing the iodine from contact with the product. Aqueous acetic acid solutions of 80-90% and DMF have been shown to stop at the desired cystine product, while removal of the iodine by extraction into carbon tetrachloride from the aqueous solution has proven reliable (Wade *et al.* 1986). Prevention of iodination of susceptible residues is simple if the iodine is used while the side chain protection is still in place ie. the peptide is uncleaved and still attached to the resin, however, if iodine is used post cleavage solvent choice is critical and acetic acid or aqueous acetic acid are the only suitable options.



Mechanism for thallium(III)trifluoroacetate oxidation

Another agent that has been used for deprotection/oxidation of cysteine is thallium(III)trifluoroacetate [Tl(III)TFA] which acts as a soft acid (Klopman 1968) and then as an oxidant (Uermura *et al.* 1977).

Tl(III)TFA cleaves most S-protecting groups with Cys(Bzl) being the exception, thus, this method of cystine formation would not be applicable to syntheses where multiple disulphide bonds are required, since selective deprotection of differing cysteine side chains would not be possible. It is also important to note that the use of TFA as the solvent in this reaction is critical, since in experiments carried out by Albericio *et al.* (1991) with Cys(Acm) protected peptides, other solvents (DMF, acetic acid, propionic acid, CH_2Cl_2) failed to completely remove the Acm protection. Both iodine and Tl(III)TFA have been used to cyclise peptides while still resin bound (Albericio *et al.* 1991).

Both methods were evaluated in our laboratory and in this particular case iodine oxidation was the method chosen to form peptide **18** while still resin bound. Acm was the chosen S-protecting group, while an aqueous acetic acid solution was employed as the solvent. The iodine was extracted into carbon tetrachloride and the resin was then flow washed with DMF. After HF cleavage, the peptide was purified by RP-HPLC.

2.4.4 A vaccine for rheumatic fever

Group A β-haemolytic streptococci are responsible for pharyngitis and skin infections, but the etiologies of two nonsuppurative inflammatory conditions that can follow these infections, rheumatic fever and its complications and acute glomerulonephritis, are not well understood. Acute rheumatic fever follows a throat infection with certain serotypes of group A β-haemolytic streptococci. This disease, and the recurrences that can follow further streptococcal throat infections, expresses its most serious pathology in the heart. It has been suggested that the disease represents an autoimmune illness initiated as a cross-reactivity between the M protein of streptococci and cardiac tissue (Cunningham *et al.* 1986, Dale and Beachey 1986). Despite the etiology of the disease, it has been suggested that a subunit vaccine based on the M protein may prevent rheumatic fever. Antibodies against the polymorphic end amino-terminus of the protein can kill streptococci in a type specific manner (Beachey *et al.* 1984, Beachey and Seyer 1986) and recently immunisation with a conserved segment of the M protein was shown to afford some protection against colonisation (Bessen and Fischetti 1990).

The difficulties associated with designing such a subunit vaccine, however, relate to the presence of potentially deleterious epitopes (cross-reactive with heart tissue) and to the fact that the amino-terminal half of the protein is highly variable. A subunit vaccine that contains B-cell epitopes that are targets of bactericidal antibodies, but does not contain heart cross-reactive epitopes is required. Thus, because of this variability, attention moved from the amino-terminus to the highly conserved carboxy-terminus of M protein. As a result an epitope was defined within a 20 amino acid sequence **19** of the carboxy-terminus which could opsonise streptococci of different M types (Pruksakorn *et al.* 1992). The sequence, labelled peptide 145, was considered as a possible vaccine candidate for rheumatic fever.

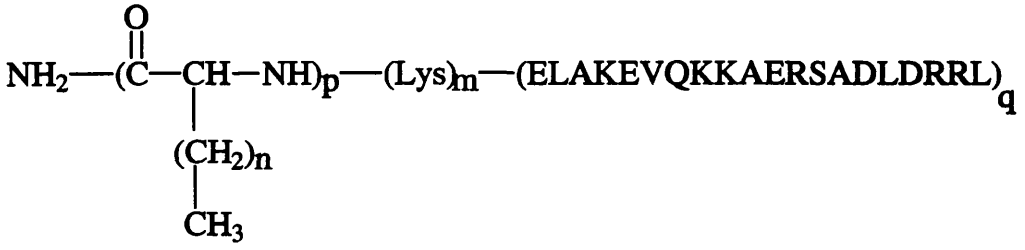
LRRDLDASREAKKQVEKALE

19

In collaboration Professor Michael Good at the Molecular Immunology Unit, Queensland Institute of Medical Research, Brisbane, peptide **19** was synthesised on varying LCP constructs, to enhance its ability to produce bactericidal antibodies to group A streptococci.

2.4.4.1 Synthesis of LCP-145 constructs

In order to ascertain whether variations in the LCP construct would increase its ability to boost the immunogenicity of immunodominant peptides, the peptide sequence **19** was synthesised initially on three different LCP variants. These variants differed by either the number of lipidic units in the fatty tail of the system or whether a tetrameric or octameric branched lysine system was employed, resulting in compounds **20a**, **b** and **c**.



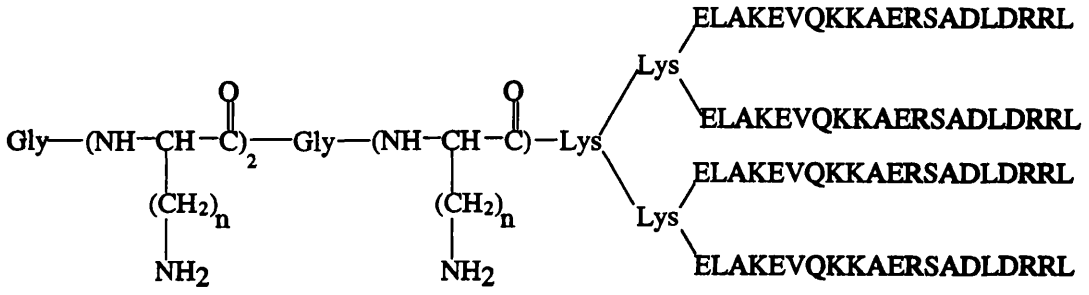
20

20	**n**	**p**	**m**	**q**
a	11	3	7	8
b	11	2	3	4
c	11	3	3	4

Synthesis of the compounds was carried out on solid phase with a low substituted

MBHA resin using the Boc protected lipidic amino acid **1g** for the lipidic tail of the systems. The fast coupling HBTU/*in situ* neutralisation chemistry described in **2.4.3.1** was employed for chain elongation. Cleavage from the resin was by HF and purification was carried out by using gel filtration and RP-HPLC.

Further optimisation of the LCP construct was carried out by investigating the effect of varying the length of the alkyl side of the lipidic amino acids in the fatty tail, and the use of spacers. Four different side chain lengths were chosen and so four tetrameric LCP-145 constructs were synthesised in a similar manner to compounds **20a**, **b** and **c** described above. The resulting compounds **21a**, **b**, **c** and **d** were synthesised from the appropriate Boc protected lipidic amino acids **1e**, **1f**, **1g** and **1h** respectively.



21

21	**n**
a	7
b	9
c	11
d	17

All the LCP-145 constructs were extremely water soluble including compound **21d**

which incorporates three twenty carbon lipoamino acids in its construction. It is important to note that when using the HBTU chemistry described above, to couple asparagine or glutamine residues, the resin must be flow washed with both DMF and then CH_2Cl_2 before Boc removal with 100% TFA in order to avoid chain termination caused by cyclisation of the terminal Asn or Gln.

2.5 Design of lipophilic peptide inhibitors of human neutrophil elastase

Human neutrophil elastase (HNE; EC 3.4.21,37) plays physiological functions in host defense against bacterial infections and extracellular matrix (ECM) remodelling following tissue injury (Takahashi *et al*,1989). During acute inflammatory disorders, its local concentrations probably exceeds those of its natural inhibitors as α_1-proteinase inhibitor, leading to ECM degradations. The implication of HNE is emphysema, adult respiratory distress syndrome, glomerular nephritis, rheumatoid arthritis and periodontitis is well documented (Mittman, 1972, Janoff, 1972a, Janoff, 1972b, Janoff, 1985, Burchardi *et al.*, 1984, Stein *et al.*,1985, Miyano *et al.*, 1982, Digenis *et al.*, 1986). HNE inhibitor imbalance can be restored by the administrations of natural inhibitor (α_1P_i) on low molecular weight inhibitors as peptidic reversible or irreversible inhibitors (Trainor, 1987, Kettner and Shenvi, 1984, Hassal *et al.*, 1985, Yasutake and Powers 1981).

Peptidic HNE inhibitors have a common hydrophobic peptide sequence, which partially mimics certain amino acid sequences found in elastin. Ala-Ala-Pro-Val sequence fits the P_4-P_1 sub-sites of elastase and inhibits HNE competitively with K_i approx.10^{-4}M. The covalent coupling of lipophilic moieties as long chain cis unsaturated fatty acids was found to considerably increased the inhibitory capacity of the substance, in keeping with the presence of an unusual hydrophobic binding site in HNE located near its active centre (Hornebeck *et al.*, 1985, Boduier *et al.*, 1991). In order to get a better insight of the size of this hydrophobic pocket, a series of lipopeptides of increasing lipophilic character were synthesized keeping constant the peptide moiety (Ala-Ala-Pro-Val) and analyzed for their HNE inhibitory capacities.

These lipidic peptides combine the physico-chemical and biological properties of peptides and proteins with those of lipids and membranes and can also confer to biologically labile compounds protection against enzyme degradation (Toth *et al.*, 1994). In that sense, they behave as bifunctional inhibitors.

2.5.1 Synthesis of lipophilic peptide inhibitors of HNE

A series of inhibitors of HNE was synthesised based on the core tetrapeptide sequence **22** (Toth *et al.* 1995). The N-terminal was modified by the addition of one, two or three 2-aminotetradecanoic acid moieties yielding **22a**, **b** and **c**. A fourth conjugate was synthesised with three N-terminal lipidic moieties and with the C-terminal carboxylic acid being replaced by a methyl ester yielding **22d**.

X-L-Ala-L-Ala-L-Pro-L-Val-Y

22

22	**X**	**Y**
a	$(CH_3)_3COCO$-D,L-$NH[CH(CH_2)_{11}CH_3]$	OH
b	$(CH_3)_3COCO\{$D,L-$NH[CH(CH_2)_{11}CH_3]CO\}_2$	OH
c	$(CH_3)_3COCO\{$D,L-$NH[CH(CH_2)_{11}CH_3]CO\}_3$	OH
d	$(CH_3)_3COCO\{$D,L-$NH[CH(CH_2)_{11}CH_3]CO\}_3$	OCH_3

All conjugates were synthesised by solution phase peptide synthesis in a stepwise manner, using carbodiimide mediated coupling and the N-terminal lipidic units were incorporated by using the Boc protected lipidic amino acid **1g**. C-terminal protection of the peptide was afforded by a methyl ester of the carboxyl function, while for N^{α}-protection, Boc protection was employed.

Initially, Boc protected proline was coupled with valine methyl ester hydrochloride

yielding the Boc-Pro-Val OMe dipeptide. The synthesis was continued by removal of the Boc group by acid hydrolysis (HCl/MeOH) and the next Boc protected amino acid coupled via the carbodiimide procedure. This was repeated until the desired sequence had been obtained, with the final Boc protecting group being left in place to mask the amine. For all conjugates, except **22d**, the C-terminal methyl ester was removed by alkali saponification (KOH/EtOH) to yield the free carboxylic acid. Throughout the syntheses all reactions were monitored by thin layer chromatography (TLC) and purification of the required peptides was carried out using silica flash chromatography and RP-HPLC using a C_4 column and 80% acetonitrile as the organic modifier.

A further series of HNE inhibitors, which were variations of peptide **22c**, were synthesised using solid phase methodology on PAM resin, to yield the free carboxylic acid. In this case it was necessary to modify the N-terminal amine and so permanent N^{α}-Boc protection was not required. The aim of the second series of conjugates was to ascertain the effects of:

i) Acetylating the amino terminal.
ii) Replacing the N-terminal amino group with a carboxyl moiety.
iii) Further increasing the lipidic nature of the N-terminus while removing all ionic functions at this end terminus.

Acetylating of the N-terminus was carried out with acetic anhydride in the presence of the strong base DIEA, a tritiated version of the compound was also synthesised (for radiolabel tracer studies) from tritiated acetic anhydride. This resulted in compounds **22e** and **22f**.

In order to position a carboxylic acid at the N-terminus, it was decided to first add a glutamic acid residue and then acetylate the end amino function. After cleavage with HF, a free carboxylic acid would be afforded from the glutamic acid side chain, while the amino function remained masked by the acetyl group, thus creating compound **22g**, which possessed a carboxyl function at both termini.

An increase in N-terminal lipophilicity was achieved by coupling the carboxyl function of palmitic acid with the free amino group of the peptide whilst still resin bound. This was performed using DCC coupling protocols (section **2.2.2**). HF cleavage afforded compound **22h**.

All modifications to the peptides were carried out whilst the peptides were resin bound to aid final purification. After HF cleavage, 90% acetic acid was required to dissolve the lipidic peptide conjugates, since the compound were extremely lipophilic, as was to be expected.

22	**X**	**Y**
e	$CH_3CO\{_{D,L}\text{-}NH[CH(CH_2)_{11}CH_3]CO\}_3$	OH
f	$C^3H_3CO\{_{D,L}\text{-}NH[CH(CH_2)_{11}CH_3]CO\}_3$	OH
g	$CH_3CONH[CH(CH_2)_2COOH]CO\{_{D,L}\text{-}NH[CH(CH_2)_{11}CH_3]CO\}_3$	OH
h	$CH_3(CH_2)_{14}CO\{_{D,L}\text{-}NH[CH(CH_2)_{11}CH_3]CO\}_3$	OH

Throughout the syntheses, coupling efficiencies were monitored by ninhydrin testing, while purification of these further conjugates was carried out as for those HNE inhibitors described previously.

CHAPTER 3

BIOLOGY

3.1 Biological assessment of TRH and LHRH lipidic conjugates

The chemical modification of the enzymatically labile model peptides, LHRH and TRH, with a novel class of compound, the lipidic amino acids, was thought to improve the intestinal transport of these normally hydrophillic peptides across the intestinal epithelium (Toth *et al.* 1994a, Flinn *et al.* 1996). It was also postulated that the long unsubstituted alkyl side chains of the lipidic amino acids would be able to stabilise the peptides in a biological environment by protecting them from the proteolytic enzymes known to degrade the compounds, thus extending the biological half-lives of the peptides (Toth *et al.* 1994b). Various studies were performed using these conjugates to ascertain the effect of conjugation of peptides to lipidic amino acids, with the ultimate goal of developing a delivery system for the oral administration of biologically active peptide compounds. The experimental work was designed to provide information about the intestinal absorption, the biological stability and the distribution of the lipidic conjugates within a biological system.

3.1.1 Biological stability of TRH and LHRH lipidic conjugates in the presence of degrading enzymes

In order to determine if the lipidic amino acids were capable of conferring enhanced metabolic stability on the peptides, their respective degradation profiles were studied following incubation with Caco-2 cell homogenates. The Caco-2 cell culture model is the most commonly used cell line in drug and peptide absorption studies. This cell line forms confluent monolayers and differentiates to cells with an enterocyte-like morphology under standard cell culture conditions (Chantret *et al.* 1988). The Caco-2 cell homogenates possess the brush border enzymes typical of the small intestinal enterocyte (Artursson 1990), providing a viable indication of metabolic stability in the GI tract.

The tripeptide TRH (**2a**) and the decapeptide LHRH (**3a**) were extended on the N-

terminus with one or two 2-amino-dodecanoic acid (**1b**) resulting in compounds **2b**-**2g** and **3b**-**3g**.

Because the lipidic amino acid was racemic, coupling it to LHRH to form a "monomer" conjugate resulted in a diastereomeric mixture: compounds **3b** and **3c**. These monomer conjugates were separated by HPLC (fraction 1 and fraction 2, Table 1), but the absolute configurations were not determined. Similarly the "dimer" conjugate, obtained by coupling two lipidic amino acids to LHRH, resulted in four compounds (**3d**-**3g**), which were separated by HPLC (fractions 1-4, Table 1). The "monomer" conjugate of TRH (conjugation of a single lipidic amino acid to TRH) resulted in a diastereomeric mixture, **2b** and. This diastereomeric mixture was used without HPLC separation; the TRH "dimer" conjugate **2c** was also used as a diastereomeric mixture.

Peptides **2a-c** and **3a-g** were dissolved in phosphate buffer, with the help of DMSO (4% final) and were incubated at 37°C with the Caco-2 cell homogenate solution. Samples were taken in this time course assay and the enzymatic degradation reaction

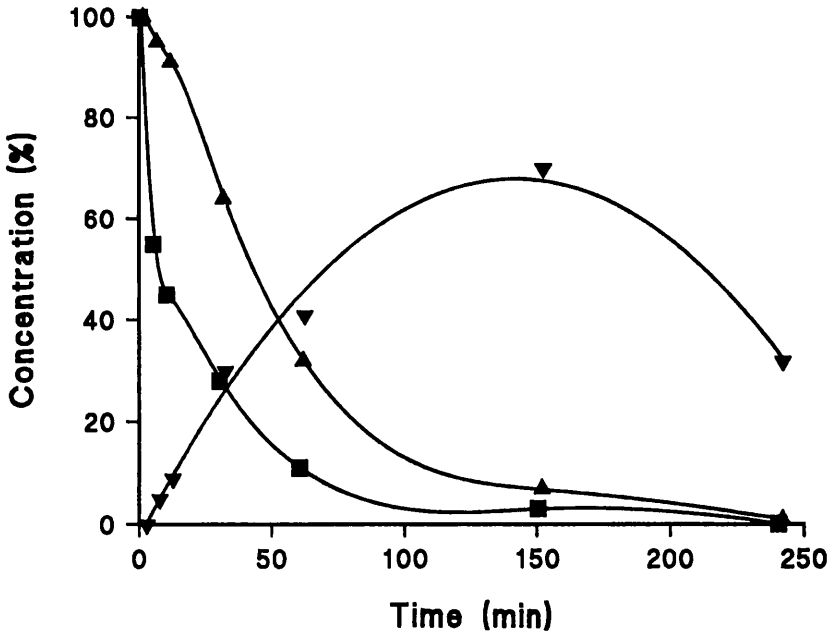


Fig. 6. CaCo-2 enzyme degradation ■=LHRH, ▲=fr. 1 of **3b**, **3c**, ▼=released LHRH

terminated by the addition of TFA. The amount of peptide conjugate present was determined by RP-HPLC, while the peptide or peptide conjugate degradation products were isolated by semipreparative RP-HPLC and their identity determined by FAB-MS.

LHRH (**3a**) incubated with Caco-2 cell homogenates degraded rapidly, the half life of the peptide was about 5 min. The monomer conjugates of LHRH (**3b** and **3c**) showed an increased half life of 43 min (Fig. 6.). Interestingly, fraction 1 of the monomer conjugate released the parent LHRH peptide. The released LHRH showed a substantially enhanced stability, being present in the cell homogenate mixture even after 4 hours (Fig. 6).

The enzyme degradation of fraction 2 of the monomer conjugate showed a similar half-life, but LHRH release was not observed. Conjugation of LHRH with two lipidic amino acids yielded compounds **3d**-**3g** (fractions 1-4). The rate of enzyme degradation of the four compounds was different. Fraction 1 was not degraded by Caco-2 enzymes, even after 6 hours incubation. Fraction 2 degraded rapidly, the half life of the conjugate was about 5 min, but the monomer conjugate and the parent LHRH were released (Fig. 7). The released monomer conjugate and LHRH remained in the cell homogenate mixture for more than four hours.

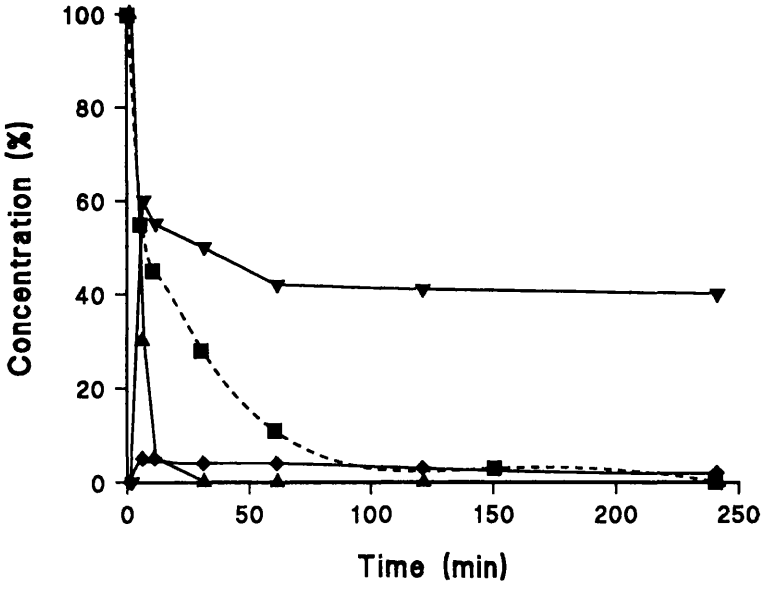


Fig. 7. CaCo-2 enzyme degradation ■=LHRH, ▲=fr.2 of **3d**-**3g**, ▼=released **3b** or, **3c**, ◆=released LHRH

Fraction 3 degraded slowly, the half life of the conjugate was more than 6 hours, with LHRH release beginning after 4 h, so that 5 % LHRH was present after 6 hours incubation (Fig. 8). The half life of fraction 4 was about 3 hours; the compound degraded to a monomer conjugate (either **3b** or **3c**) (Fig. 9), however LHRH release was not observed.

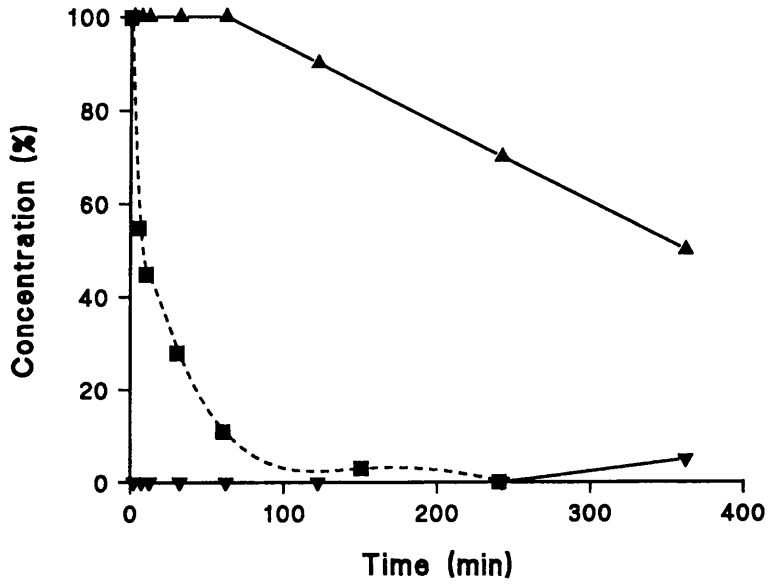


Fig. 8. CaCo-2 enzyme degradation ■=LHRH, ▲=fr.3 of **3d**-**3g**, ▼=released LHRH

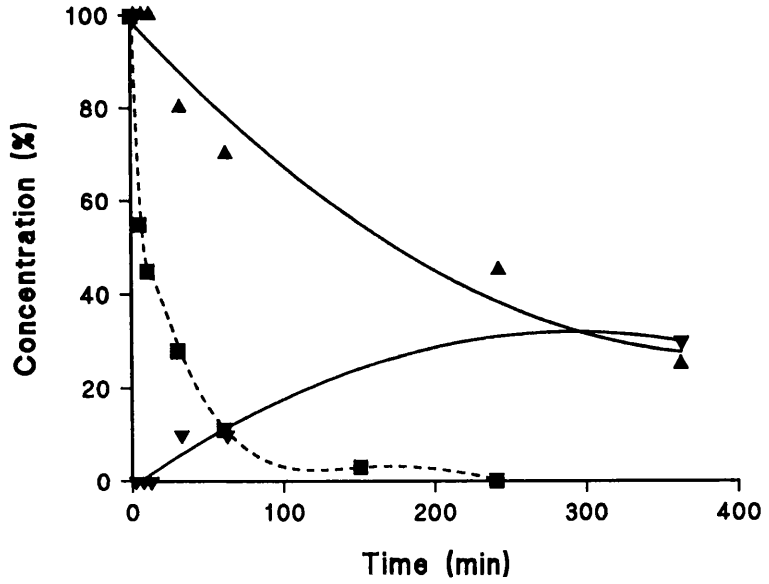


Fig. 9. CaCo-2 enzyme degradation ■=LHRH, ▲=fr.4 of **3d**-**1g**, ▼=released **3b** or **3c**

Caco-2 cell homogenate degradation of TRH (**2a**), monomer conjugate (**2b**) and dimer conjugate (**2c**) were studied using the diastereomeric mixtures. The TRH (**2a**) and the monomeric conjugate (**2b**) showed a similar degradation profile with Caco-2 enzyme as that of the corresponding LHRH compounds. TRH degraded rapidly, the half life of the compound was about 3 min. The half life of the monomeric conjugates **2b** was 30 min, and about 5 % of the conjugates was still detectable after 4 hours incubation. Parent TRH was released from the monomer conjugates, reached a maximum concentration after 1 hour incubation and was present even after 4 hours (Fig. 10).

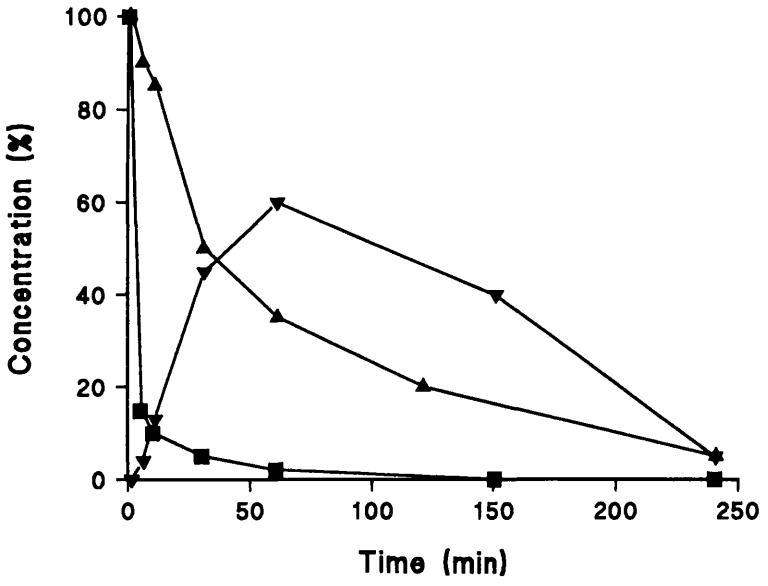


Fig. 10. CaCo-2 enzyme degradation ■=TRH, ▲=**2b**, ▼=released TRH

The half life of the dimer conjugate **2c** with Caco-2 enzymes was about 2.5 hours and although the amount present gradually decreased, there was still 5 % of the conjugate present even after 4 hours incubation. Parent TRH and monomer conjugate amounts during the 4 hours incubation (Fig. 11).

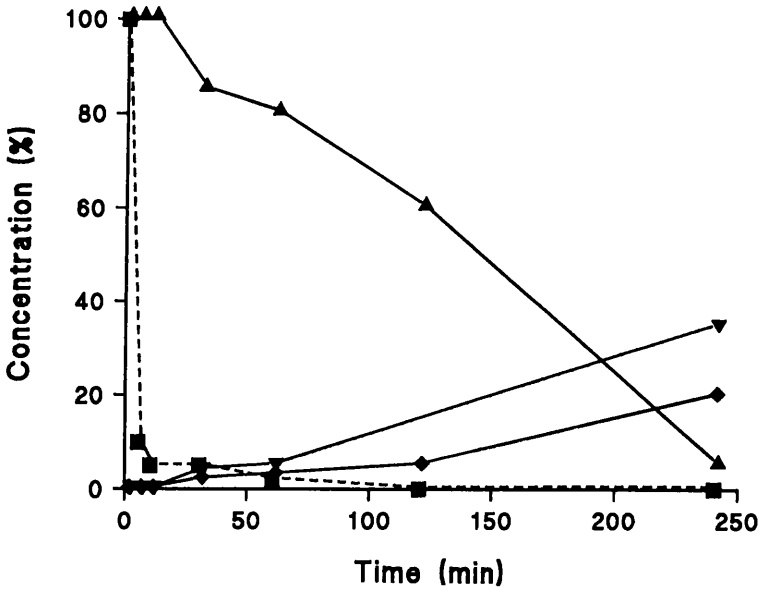


Fig. 11. CaCo-2 enzyme degradation ■=TRH, ▲=2c, ▼=released 2b, ◆=released TRH

3.1.2 Transport of TRH and LHRH lipidic conjugates through Caco-2 cell monolayers

Caco-2 cell monolayers were employed to assess the transport of the lipidic peptide conjugates through an intestinal epithelial barrier. Caco-2 cells form confluent monolayers and present a barrier whose morphology and enzyme content is similar in nature to human intestinal epithelium. The increase in lipophilicity of the peptide conjugates should enhance the ability of the conjugates to diffuse passively through the monolayer.

3.1.2.1 TRH lipidic conjugate transport profiles

Radiolabelled TRH conjugates **5a**, **b**, **c** and **6a** were dissolved in DMSO and the solutions were diluted with Hepes buffer to final concentrations of 1.0-2.0 x 10^{-4} M. The final concentration of DMSO, which was 0.5-0.75%, did not affect the epithelial integrity of the monolayer, which was tested by measurement of transport of [^{14}C] mannitol and transepithelial electrical resistance, described by Anderberg *et al.* (1992).

The [^{14}C] mannitol permeability of the monolayers was also studied in the presence of the TRH conjugates. The conjugate solutions were added to the apical side of the Caco-2 cell monolayers and samples were withdrawn from the basolateral chamber and analyzed by liquid scintillation. The apparent permeability coefficients (P_{app}) of the TRH conjugates were calculated by the method of Artursson (1990).

No clear differences in the transport of the TRH-conjugates (**5b**, **5c** and **6a**) as compared to the transport of TRH analogue (**5a**) could be observed in the Caco-2 monolayers (Table 3). Similarly, no significant differences in [^{14}C] mannitol permeability could be observed after incubation with the TRH-conjugates ($p>0.05$). Previous studies have shown that TRH is transported passively across Caco-2 monolayers by the paracellular route 20-40 times more rapidly than reported in this study (Lundin *et al.* 1991; Thwaites *et al.* 1993). This may be a result of that different cell populations and/or growth conditions were used in the different studies. Differences in passage number, cell clone and/or cell culture medium may all have an effect on the paracellular permeability and TRH analogue **5a** permeability. This variability does not affect the interpretation of the transport experiments in the Caco-2 monolayers.

Compound	P_{app} (TRH) (cm/s) ($x10^7$)	P_{app} (Mannitol) (cm/s) ($x10^7$)
--	--	2.4 ± 1.8
5a	0.5 ± 0.1	2.6 ± 0.7
5b	0.3 ± 0.1	13.5 ± 5.4
5c	0.8 ± 0.1	6.46 ± 3.1
6a	1.5 ± 0.8	2.0 ± 0.9

Table 3. Apparent permeability coefficients of TRH conjugates and [^{14}C] mannitol

However, since not all of the initial radioactivity could be accounted for, either in the

apical and basal medium or by association with the filter, the cells themselves were examined. It was found that almost 60% of **6a** was associated with the cells after 4 hours, while the other conjugates also showed cell association of between 10% and 20% (Fig.12). Whether the compounds are adsorbed onto the surface of the cell or internalised is not yet clear, but in either case this result shows a significant change in the permeability profile of TRH.

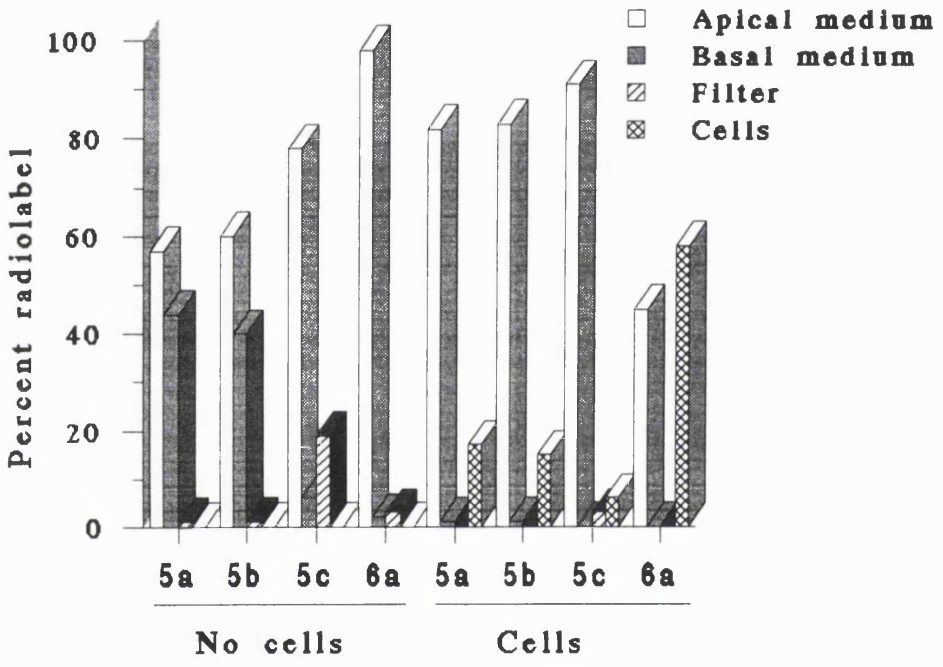


Fig. 12. Association of radiolabel after incubation of tritiated TRH conjugates in transwell inserts with and without Caco-2 cells after 4 hours incubation.

3.1.2.2 LHRH lipidic conjugate transport profiles

Radiolabelled LHRH conjugates **5d**, **e**, **f**, and **6b** were dissolved in Hepes buffer as described above for the TRH conjugates, to give solutions of final concentrations of 2, 10 and 50μM. These solutions were then administered to the Caco-2 cell monolayers in a similar manner to that described above. The degree of radiolabel transport was again assessed using liquid scintillation counting and the extent of

binding of the lipidic LHRH conjugates to the polycarbonate wells and filters, as well as cell association, was determined.

Cmpd	Conc. (μM)	Percentage of radiolabel detected						
		Apical chamber	Basal chamber	Cells	Filter	Insert walls	Washings	**Total**
5d	2	95.38	0.25	0.44	0.42	2.16	0.58	**99.23**
	10	94.65	0.23	0.15	0.11	0.85	0.64	**96.63**
	50	96.24	0.27	0.05	0.03	0.58	0.79	**97.96**
5e	2	89.22	0.38	1.19	0.82	5.60	1.20	**98.41**
	10	88.26	0.15	0.43	0.30	2.98	1.63	**93.75**
	50	93.67	0.29	0.43	0.18	2.55	1.83	**98.95**
5f	2	81.34	0.23	2.17	0.64	9.05	1.06	**94.49**
	10	94.95	0.23	1.27	0.28	4.03	1.21	**101.97**
	50	95.13	0.20	0.59	0.10	2.40	0.81	**99.23**
6b	2	94.08	1.78	1.15	0.55	1.17	2.30	**101.03**
	10	93.65	0.49	0.51	0.19	3.54	1.47	**99.85**
	50	96.32	0.69	0.10	0.06	1.61	0.76	**99.54**

Table 4. Association of radiolabel after incubation of tritiated LHRH conjugates i n Transwell inserts with Caco-2 cells.

Indeed, although some transport of the conjugates was seen in the apical to basal direction, a significant amount of the compounds was involved in either cell or filter association and for the compound to adhere to the filters it must first have passed

through the cell monolayer. Therefore, the results seen for complete transport of the conjugates through the Caco-2 cell monolayer must be viewed along with the results for the binding of the radiolabel to the polycarbonate filters. Also it is possible that any radiolabel unaccounted for could possibly have been internalised within the cell and lysis of the cell would be required to test this theory.

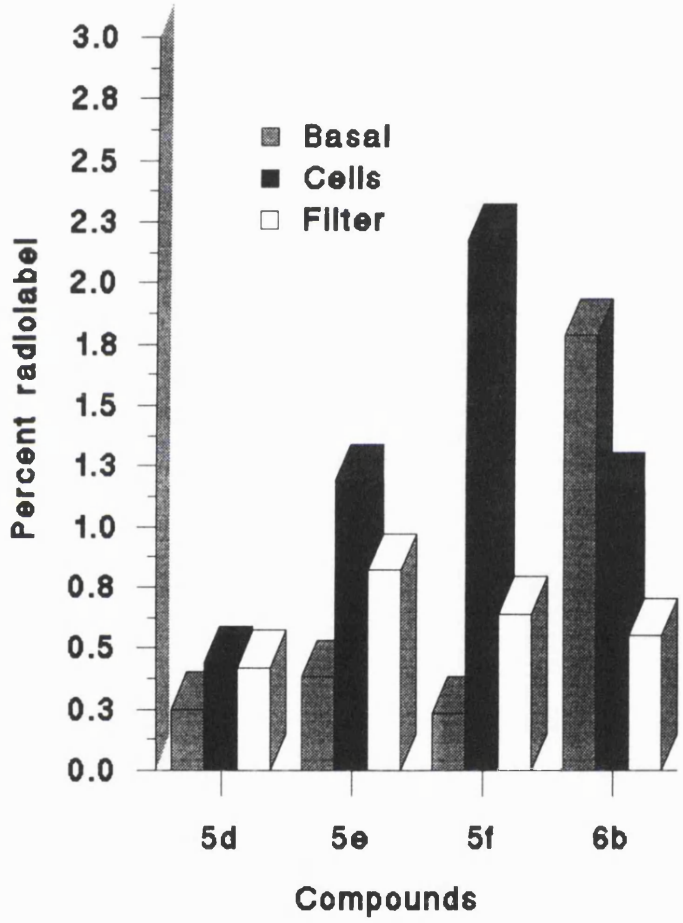


Fig. 13. Association of radiolabel with Caco-2 cells in Transwell inserts after 2 hours incubation with a 2μM solution of lipidic LHRH conjugates.

After 2 hours incubation with a 2μM solution of the compounds it can be seen that compound **6b** has the greatest basal presence and that the amount of adhesion of the compound to the filter is reduced (Fig. 13), compared with the other lipidic conjugates **5e** and **f**. Taking into account both the basal presence and the filter association of the

compounds, all of the lipidic LHRH conjugates show greater transport through the Caco-2 cell monolayer than the unconjugated LHRH compound **5d** and values vary from approximately 0.7% for **5d** to 2.4% for **6b**, which represents a 3-4 fold increase in transport across the monolayer.

Another very important observation, is the trend seen in the association of the radiolabel with the Caco-2 cells themselves. Fig. 13 quite clearly shows that as the lipophilicity of the compound is increased, then so is its association with the cell, with **5f** having over 2% association with the cells which is again a 4 fold increase

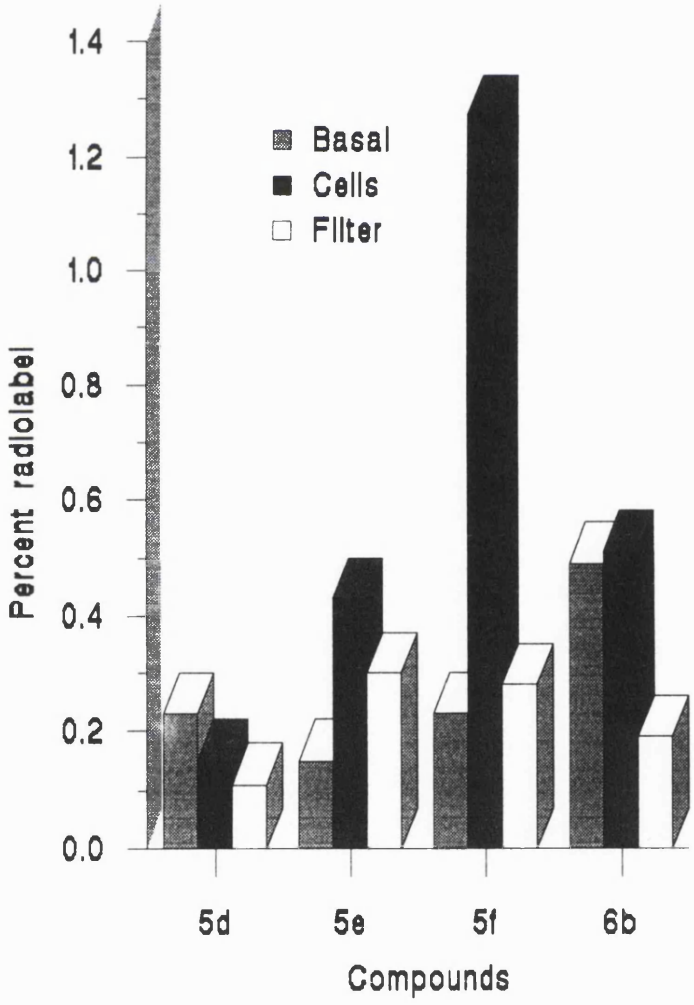


Fig. 14. Association of radiolabel with Caco-2 cells in Transwell inserts after 2 hours incubation of with a 10μM solution of lipidic LHRH conjugates.

compared with the value for the free unconjugated LHRH.

The same trends are observed when the concentration of the incubation solution is increased to 10μM (Fig. 14), with compound **6b** showing the greatest transport in the apical to basal direction while the most lipophilic conjugate, **5f**, again showing the greatest association with the Caco-2 cells. All lipidic conjugates showed transport

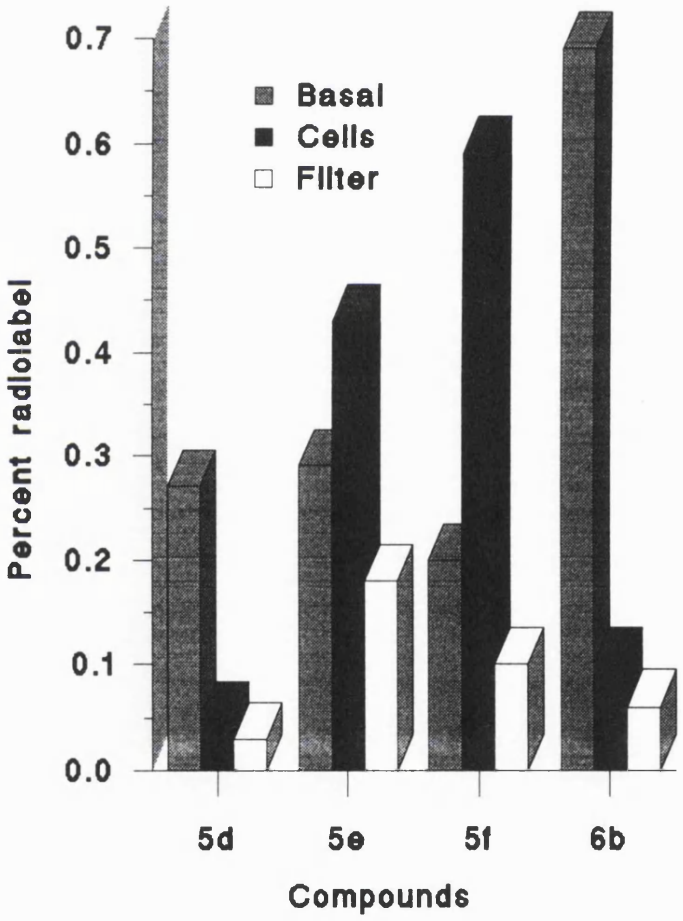


Fig. 15. Association of radiolabel with Caco-2 cells in Transwell inserts after 2 hours incubation with a 50μM solution of lipidic LHRH conjugates.

profiles greater than that of the unconjugated LHRH compound **5d**, when taking into account the filter association of the compounds as well as their basal presence.

Finally, the study was repeated with 50μM solutions of the compounds (Fig. 15), and again a similar relationship was seen between lipophilicity and cell transport as well as that of lipophilicity and cell association.

Throughout the study compound **6b** consistently showed the greatest transport across the Caco-2 cell monolayer. The design of the conjugate is such that the lipidic units provide the necessary increase in lipophilicity, while the N-terminal polylysine construct increases the water solubility of the compound and the results from this study reinforce the need to create an optimum balance between the water solubility and lipophilic nature of a compound, if successful delivery is to be achieved across the intestinal epithelium.

In a separate study the lipidic LHRH conjugates **5d-f** and **6a** and **b** were incubated with Caco-2 cell monolayers for periods of 6 and 12 hours to ascertain the affect of time on the transport profiles of the conjugates. The data from this study was expressed as the mass (in μg) of the compounds associated with the cells and filter of the Transwell inserts (Fig. 16).

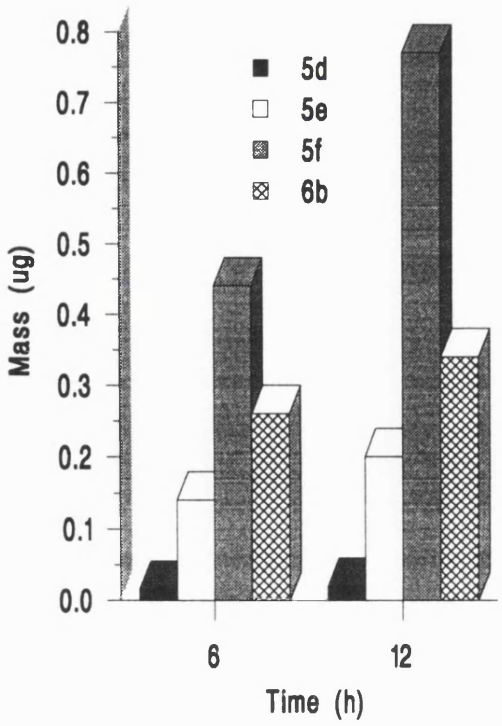


Fig. 16. Association of radiolabel with cells and filter after incubation of lipidic LHRH conjugates with Caco-2 cell monolayers after 6 and 12 hours

Fig. 16 demonstrates that the lipidic LHRH conjugates show an 8-30 fold increase in their transport profiles after 12 hours, compared with that of the unconjugated cmpound **5d**.

3.1.3 Oral absorption of lipidic TRH and LHRH conjugates

The lipoamino acids and their oligomers provide an excellent means of enhancing peptide lipophilicity and also increase the biological stability of the peptide by protecting it from enzymatic degradation as has already been shown. It was now decided that in order to assess whether this approach was a viable method of improving the oral uptake of poorly absorbed peptides, radiolabelled lipidic conjugates of TRH and LHRH were administered orally to rats and the uptake examined.

3.1.3.1 Assessment of the oral absorption of TRH and LHRH lipidic conjugates

The (TRH), is relatively resistant to proteolytic degradation in the G.I. tract, its poor oral activity is probably due to poor absorption and rapid clearance in the bloodstream (Yokohama *et al.*, 1984) TRH is rapidly degraded following first-order kinetics, with a half life in humans of approximately 5 min following i.v. administration (Leppaluoto, *et al.*, 1977).

LHRH is too large and too hydrophilic (partition coefficient 0.0451; Banks and Cession, 1985) to cross the GI tract mucosa, it is also highly susceptible to degradation by enzymes and has a very short half-life in the circulation of approximately 3-6 minutes (Handelsman and Swerdloff, 1986 and Sandow *et al.*, 1981). Attempts have been made to stabilise LHRH against proteolytic cleavage and include the use of D amino acids at the sixth position and modification of the C-terminus of the peptide chain, which have resulted in superanalogues such as buserelin and leuprolide. Approaches to enhance the oral delivery of LHRH and its analogues include association with colloidal carriers, entrapment in polymeric matricies and liposomes and the use of absorption/penetration enhancers.

Radiolabelled TRH and LHRH conjugates **5a-f** were administerd orally to rats by gavage using a blunt tipped feeding needle, as a 1mg/ml solution containing 1-2% DMF to aid solubilisation. The uptake of the conjugates was examined in the blood, liver, spleen, kidneys, small intestine and large intestine after administration by liquid scintillation counting, RP-HPLC and MS. The the non lipidic **5a** and **5d** rapidly degraded in the stomach and gut and only the uptake of radioactive metabolites was observed since the intact **5a** or **5d** could not be detected by HPLC-MS.

The overal uptake of tripeptide TRH analogues **5b** and **5c** was higher than the overal uptake of decapeptide LHRH analogues **5e** and **5f** which is to be expected considering the size difference between the peptides. It was also seen that within the same series, compounds with higher lipophilicity were taken up to a greater extent (uptake of **5c** > uptake of **5b**, and uptake of **5f** > uptake of **5e**), clearly showing the importance of lipophilicity (Fig.17, Fig.18).

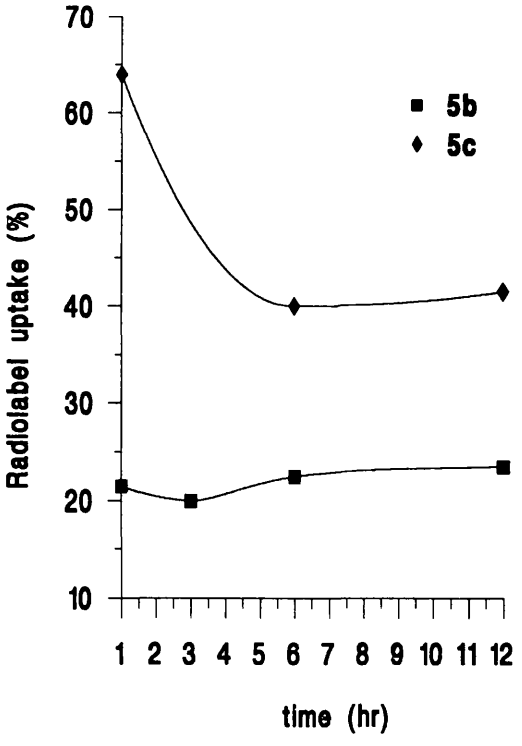


Fig. 17.Total radiolabel uptake of **5b** and **5c** in all organs

Due to the nature curve fitting programme used in generating Figs. 17-32, maximal values on certain graphs (Figs. 25, 26, 27, 28 and 32) may be slightly misleading and might not provide a completely accurate representation of the experimental data obtained.

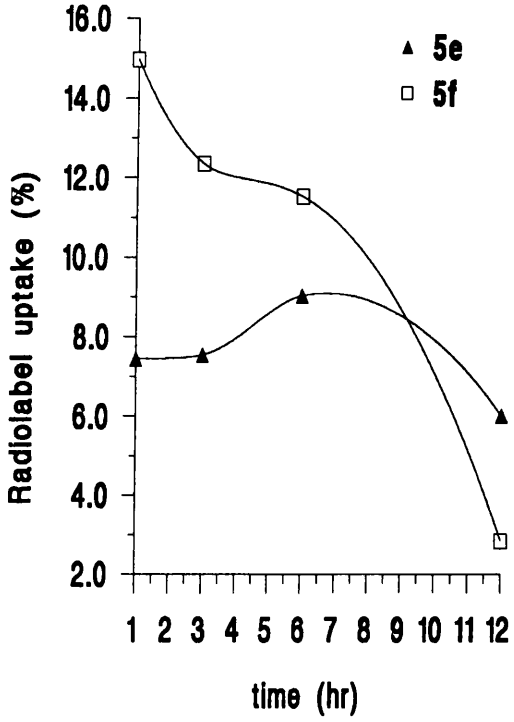


Fig. 18. Total radiolabel uptake of **5e** and **5f** in all organs

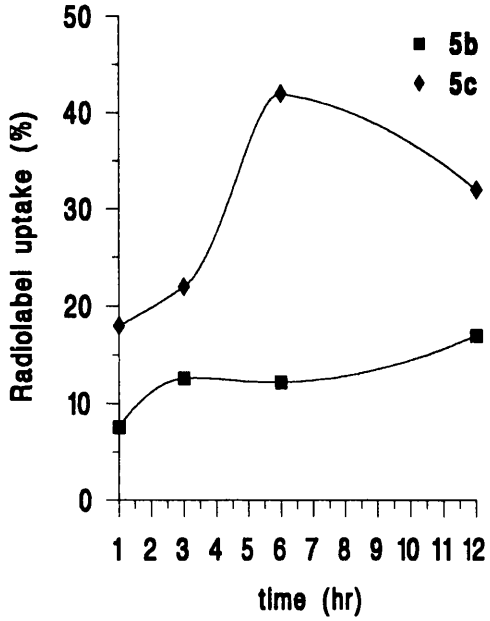


Fig. 19. Radiolabel uptake of **5b** and **5c** in blood

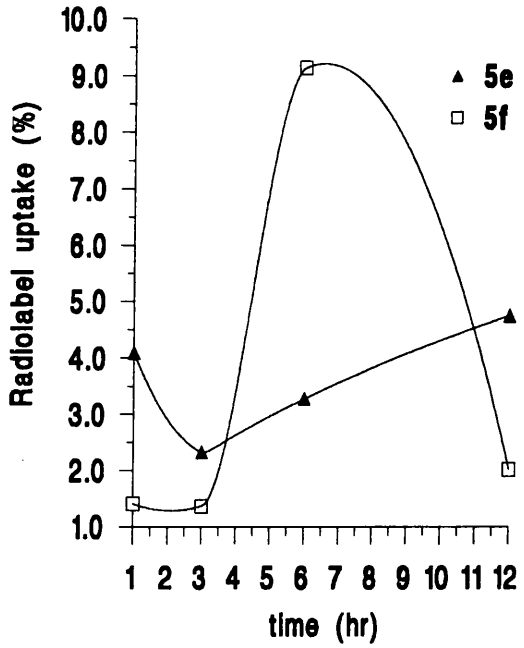


Fig. 20. Radiolabel uptake of **5e** and **5f** in blood

The blood (Fig. 19 and Fig. 20), stomach (Fig. 21 and Fig. 22), small intestine (Fig. 23 and Fig. 24), large intestine (Fig. 25 and Fig. 26) and spleen (Fig. 27 and Fig. 28) uptake of TRH and LHRH analogues **5b**, **5c** and **5e**, **5f** were in good correlation.

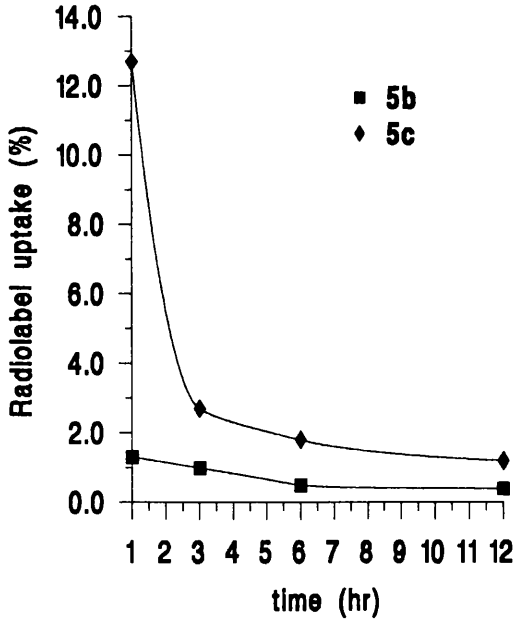


Fig. 21. Radiolabel uptake of **5b** and **5c** in stomach

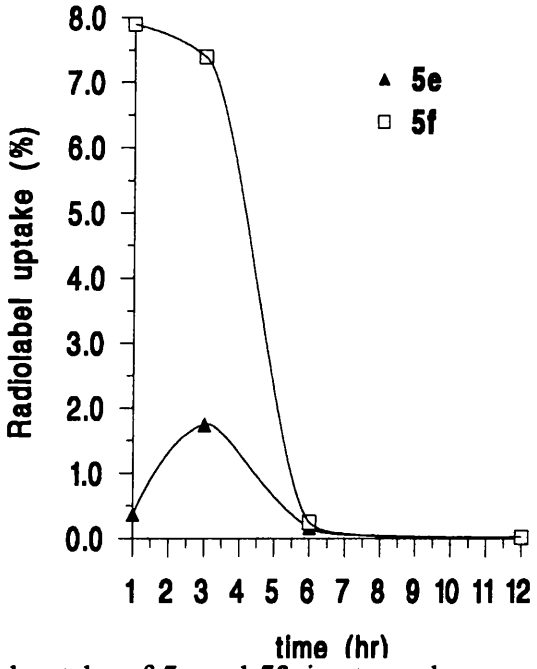


Fig. 22. Radiolabel uptake of **5e** and **5f** in stomach

The more lipophilic **5c** and **5f** showed higher uptake than the less lipophilic **5b** and **5e** in the blood, stomach, small intestine and large intestine and lower uptake in the spleen.

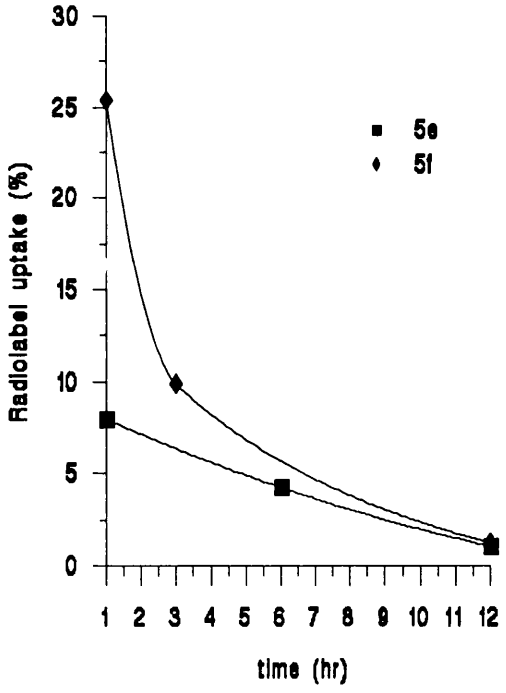


Fig. 23. Radiolabel uptake of **5b** and **5c** in small intestine

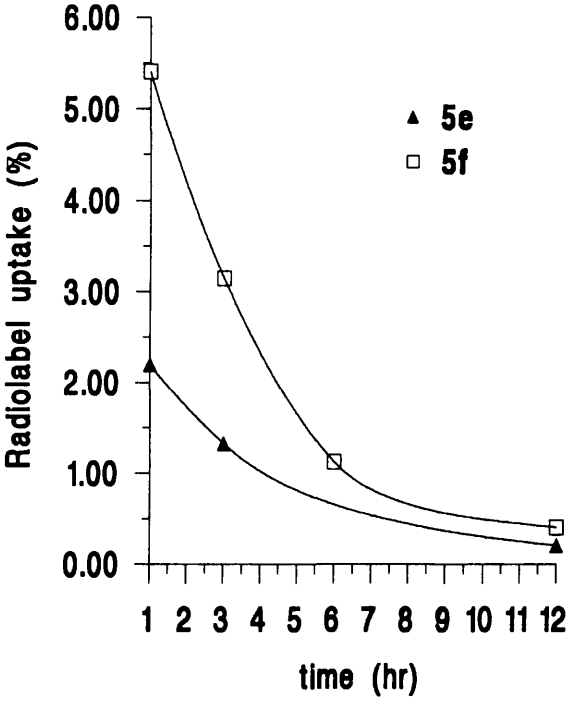


Fig. 24. Radiolabel uptake of **5e** and **5f** in small intestine

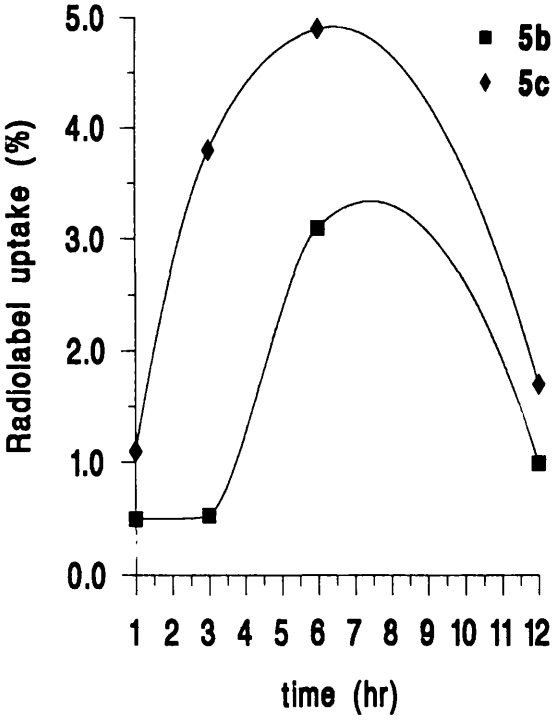


Fig. 25. Radiolabel uptake of **5b** and **5c** in large intestine

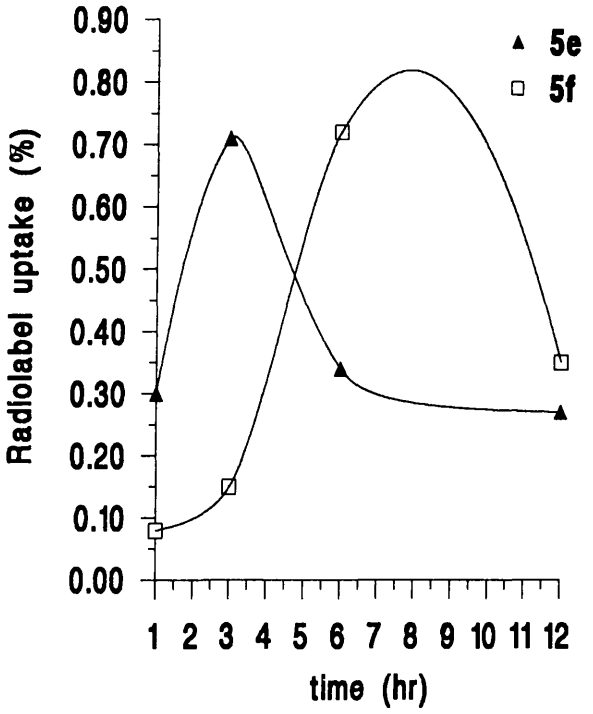


Fig. 26. Radiolabel uptake of **5e** and **5f** in large intestine

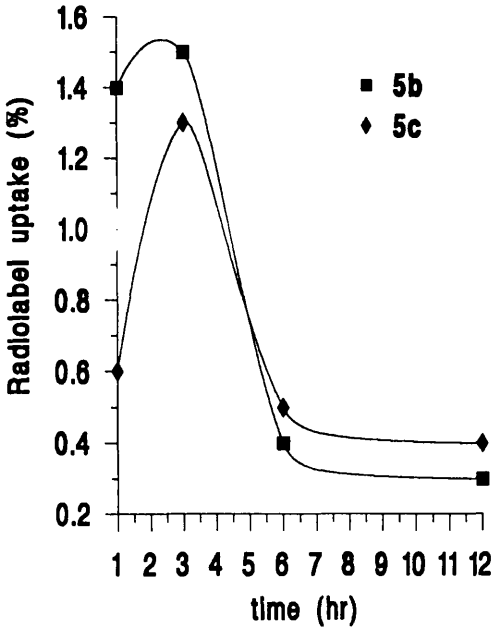


Fig. 27. Radiolabel uptake of **5b** and **5c** in spleen

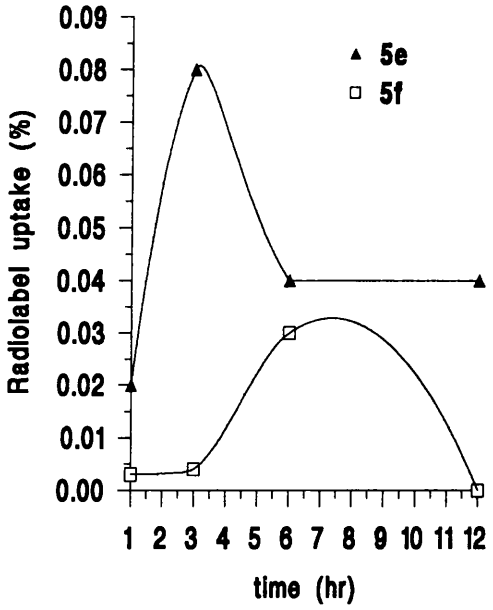


Fig. 28. Radiolabel uptake of **5e** and **5f** in spleen

The liver and kidney uptake (Figs.29, 30, 31, 32) showed a different trend. The more lipophilic TRH conjugate **5c** was in higher concentration in the liver and kidney than the less lipophilic TRH cojugate **5b**, while the more lipophilic LHRH analogue **5f** was in lower concentration than the less lipophilic analogue **5e** in these organs, showing a slower secretion of compound **5f**. Interestingly all compounds were present and detectable even after 10 h post administration.

In order to confirm the identity of the detected radiolabel, compounds **5b**, **5c**, **5e** and **5f** were extracted from the blood 3 h post administration. The extracted compounds were then purified by HPLC, characterised by MS and requantified by liquid scintillation counting. The separation of tripeptide TRH conjugates **5b** and **5c** required multiple HPLC and capillary electrophoresis purification (the crude extract contained a large amount of lipoprotein fragments). We were able to detect the presence of intact **5b** and **5c** and obtain mass spectra of the products, but the method was not suitable for quantitation.

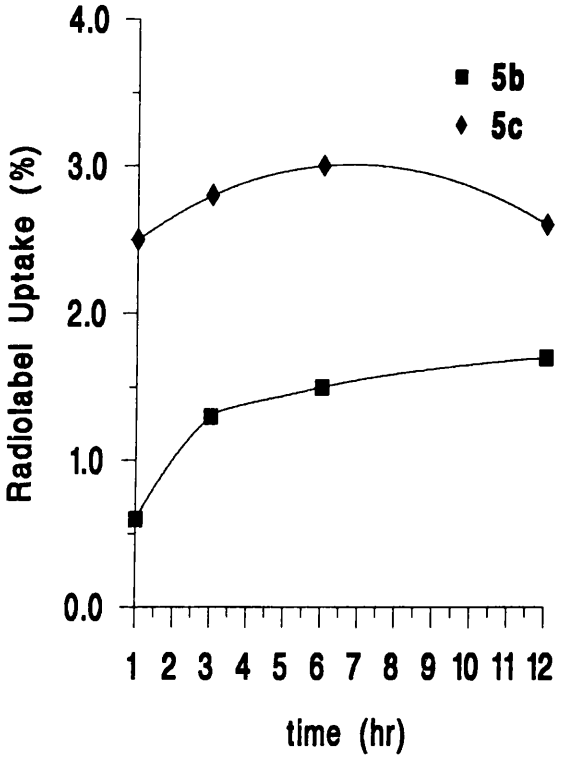


Fig. 29. Radiolabel uptake of **5b** and **5c** in liver

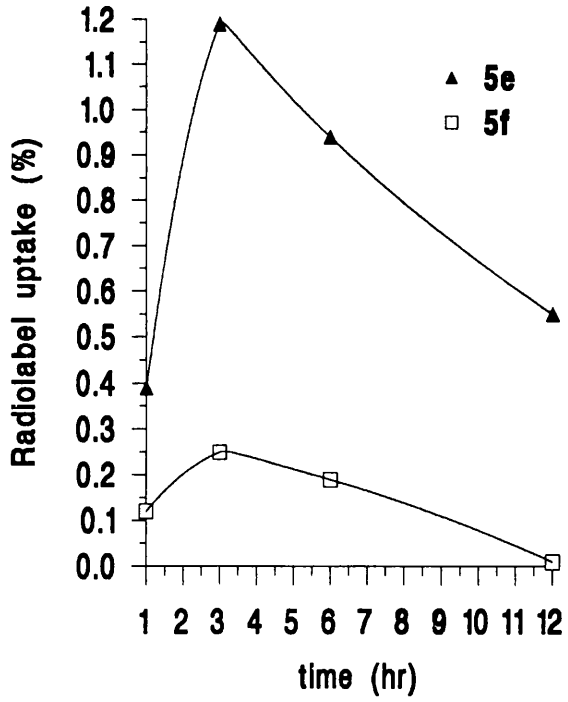


Fig. 30. Radiolabel uptake of **5e** and **5f** in liver

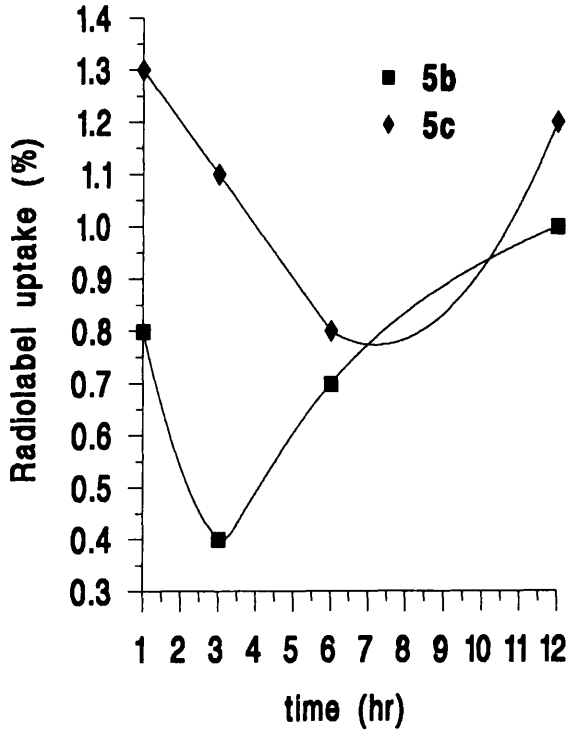


Fig. 31. Radiolabel uptake of **5b** and **5c** in kidney

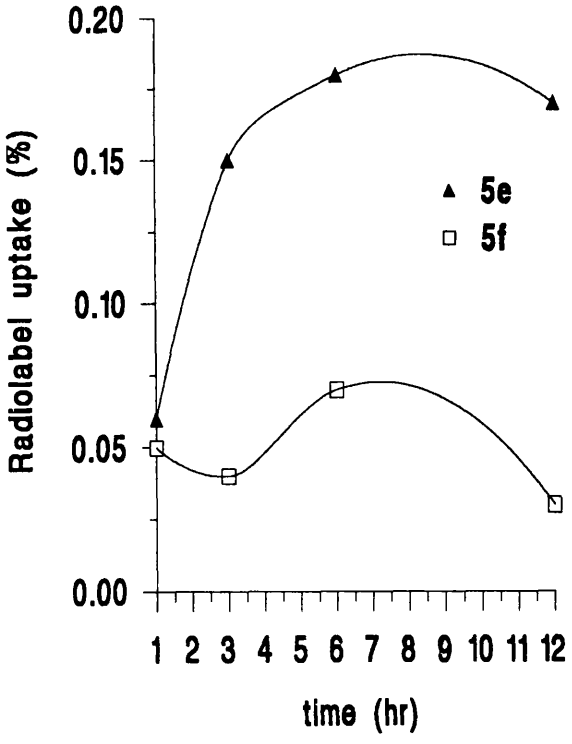


Fig. 32. Radiolabel uptake of **5e** and **5f** in kidney

The purification of decapeptide analogues **5e** and **5f** required less chromatography, so the amount of radiolabel detected in the purified conjugates **5e** and **5f** was in agreement (Table 5) with the detected radiolabel (Fig. 20) from the initial scintillation counting of the crude homogenates.

Compound	Blood uptake (%)	
	Crude homogenate	Purified extract
5e	2.3	1.7
5f	1.2	1.0

Table 5. Comparison of blood uptake of compounds 5e and 5f

In summary, the novel conjugates developed have been absorbed and detected after oral administration and appear to be stable for a considerable time *in vivo.*

3.2 Immunogenic evaluation of an LCP based peptide vaccine for *Chlamydia trachomatis*

To induce high antibody response to synthetic peptides, successful approaches have involved incorporation of the peptides into a polylysine core to form a multiple antigen peptide (Wang *et al.* 1991, Tam 1988). A novel improvement has been developed, using a lipidic moiety at the C-terminal of the polylysine system (lipid-Polylysine-Core peptide, LCP) (Toth *et al.* 1993).

3.2.1 Effects of polymerisation on immunogenicity of P_2 peptide

Since the P_2 peptide **9** contains both B-cell epitopes and T-helper sites (Zhong *et al.* 1993), the effects of polymer construction on immunogenicity of the P_2 sequence was evaluated, resulting in the synthesis of dimeric (**10**) and octameric (**11**) forms of the P_2 peptide. Thus, **10** and **11** were used to immunise four congenic strains of mice and

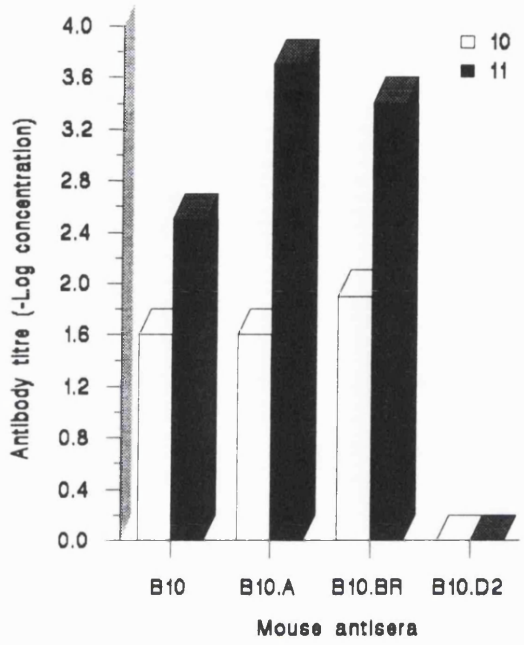


Fig. 33. Titration of IgG antibodies raised with **10** and **11** in four mouse strains.

the antisera were assayed against serovar B elementary body (EB) whole organisms in ELISA (Fig. 33). The immunological evaluation was carried out out in collaboration with Professors Robert Brunham and Ron Reid at the Universities of Alberta and B.C., Canada. Peptide **10** elicited anti-EB antibodies with relatively low titres, whereas the LCP-P_2 construct **11**, was able to raise antibodies in high titres in B10.A (H-2^a) and B10.BR (H-2^k) mice and intermediate titres in B10 (H-2^b) mice. B10.D2 (H-2^d) was a nonresponder to **10** and **11**, which is consistent with the H-2^d restriction of T-cell immune response observed with the P_2 peptide **9** (Zhong *et al.* 1993). These observations suggest that the P_2 peptide **9** is a potential candidate sequence for providing a T-helper cell site and B-cell sites covering B serogroup serovars and that the LCP preparation significantly enhanced immunogenicity of the P_2 sequence (**9**).

3.2.2 Immunogenicity of the 30-mer chimeric peptide H1

Monoclonal antibodies to VD I of the outer membrane protein of serovar C are able

to bind to native organisms of various strains in the C serogroup and neutralise chlamydial infectivity in an *in vitro* neutralisation assay (Zhong *et al.* 1993). Thus a chimeric peptide (**12**), incorporating the P_2 sequence (**9**) and a 13 amino acid sequence from VD I of C Omp 1 (SDVAGLQNDPTTN), was constructed as the dimeric lipopeptide **13** and the octameric LCP peptide **14**. These compounds were then used to immunise various strains of mice using the inflammatory complete Freund's adjuvant (CFA) and a new non-inflammatory adjuvant NAGO (Zheng *et al.* 1992).

Immunogen	Antigen	B10 (H-2^b)	B10-A (H-2^a)	B10.BR (H-2^k)	B10D2 (H-2^d)	SJL (H-2^s)
13 + CFA	**13**	1:160	1:160	1:320	1:80	
	A EB	1:160	1:160	1:160	1:80	
	B EB	1:80	1:160	1:160	1:80	
	C EB	1:160	1:320	1:320	1:80	
14+ CFA	**14**	1:512,000	1:128,000	1:64,000	1:800	1:1,024,000
	A EB	1:25,600	1:3,200	1:3,200	<1:100	1:51,200
	B EB	1:12,800	1:3,200	1:1,600	<1:100	1:25,600
	C EB	1:25,600	1:12,800	1:1,600	<1:100	1:25,600
14+ NAGO	**14**	1:256,000				
	A EB	1:12,800				
	B EB	1:12,800				
	C EB	1:25,600				

Table 6. The ELISA titre was expressed as the highest dilution of antisera which gave an OD value 4-fold above background.

As shown in Table 6, both **13** and **14** induced peptide reactive and EB reactive antibodies. B10.D2 (H-2^d) strain displayed the lowest antibody response to the 30-mer sequence **12** consistent with the H-2^d haplotype restriction of immune responses to the P_2 sequence (**9**), as seen in Fig. 33. The anti-peptide antibody titres in sera raised with the LCP peptide **14** were 10- to 3200-fold higher than those raised with the dimeric lipopeptide **13**. Antisera were able to bind to whole EB of serovar A, B and C coated on ELISA plates. All three serovars were equally well recognised by each sera. However, the anti-EB antibody titres were 20- to 40-fold lower than the anti-peptide antibody titres. Also, from the Table 6 it can be seen that the antibody titres from the B10 (H-2^b), raised using peptide **14** with NAGO, compare very favourably with those titres gained using CFA.

3.2.2.1 Immunogenic evaluation of liposome entrapment of LCP-H1

Liposomes have recently been used as adjuvant/carrier systems for peptide vaccine candidates (Gregoriadis 1990). In the work by Zhong *et al.* (1993), only the total IgG was measured against peptide **14**. The immunogenic potential of peptide **14** was compared with a liposome entrapped peptide **14** preparation either alone or together with interleukin-2 (IL-2) to see whether this might improve the antibody titers and also the IgG subclass responses. The entrappment and evaluation was carried out in collaboration with Professor Gregoriadis at The School of Pharmacy, University of London. Mice were immunised with 30 and 50 μg of free peptide **14** (30F and 50F), liposome entrapped peptide **14** (30E and 50E) or peptide **14** co-entrapped in a liposome with either a low (30CoEL and 50CoEL) or high dose (30CoEH and 50CoEH) of IL-2. Primary and secondary anti-peptide antibody titers were determined by ELISA along with long term responses. The liposomes used were formed from egg phosphatidylcholine and cholesterol according to the method described by Kirby and Gregoriadis (1984). Figs 34-39 represent the anti-peptide IgG_1, IgG_{2a} and IgG_{2b} primary and secondary responses, respectively. With the 30 and 50 μg doses used, none of the groups differed significantly in terms of primary (all subclasses) and IgG_{2a} secondary responses.However, liposomal peptide alone or in combination with IL-2 (both low and high doses) were superior to the free peptide in boosting IgG_1 titers

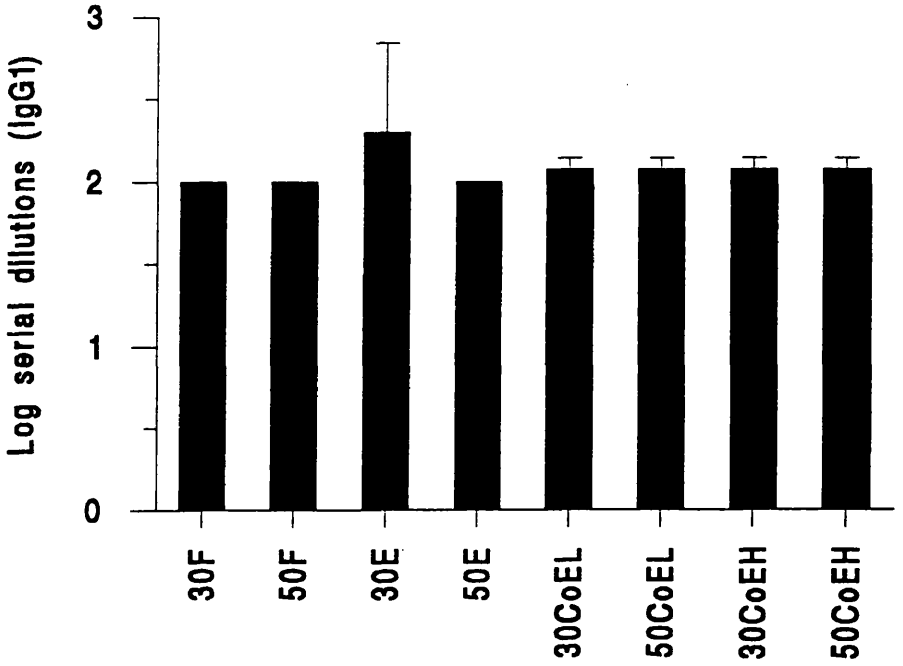


Fig. 34. IgG1 primary responses to free or liposomal peptide 14 with or without IL-2

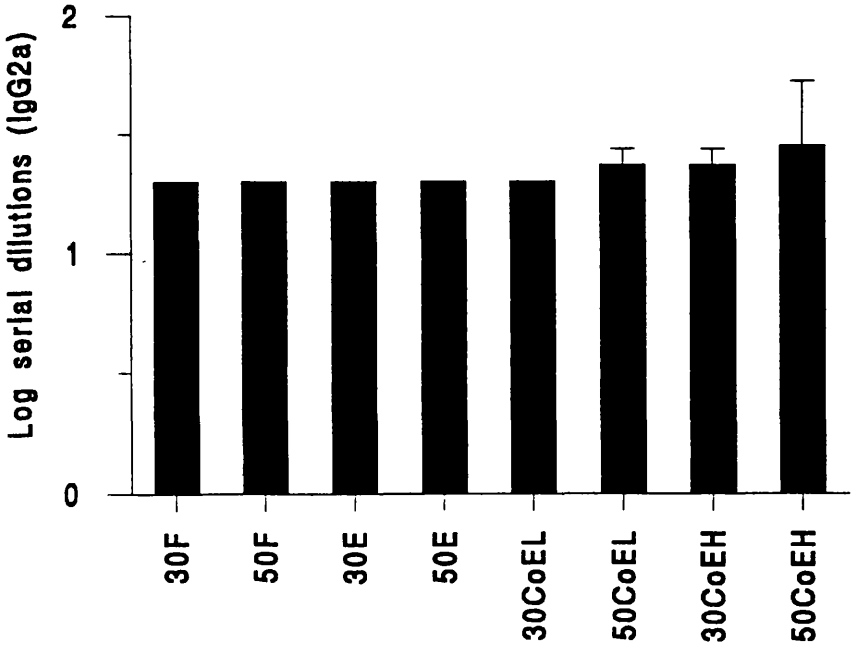


Fig. 35. IgG2a primary responses to free or liposomal peptide 14 with or without IL-2

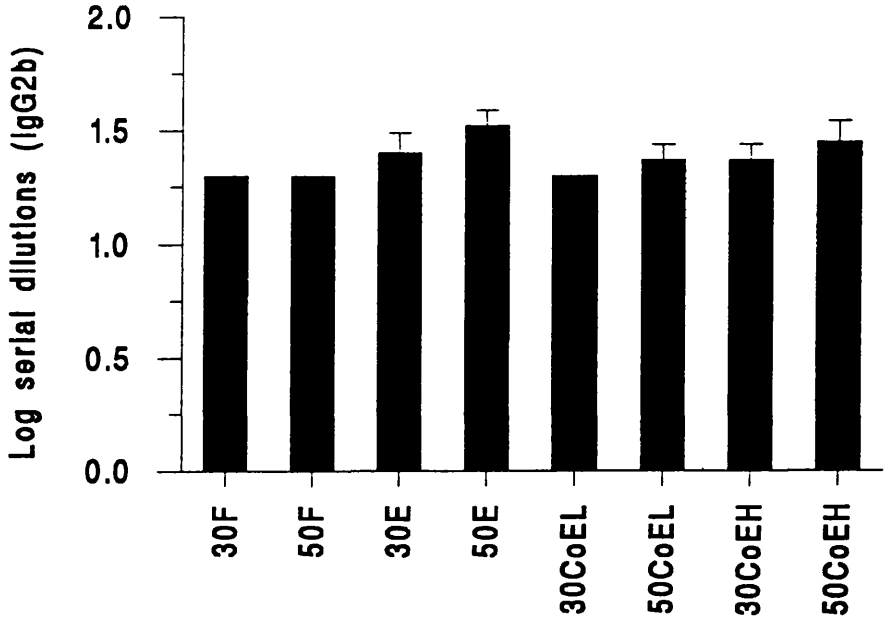


Fig. 36. IgG2b primary responses to free or liposomal peptide 14 with or without IL-2

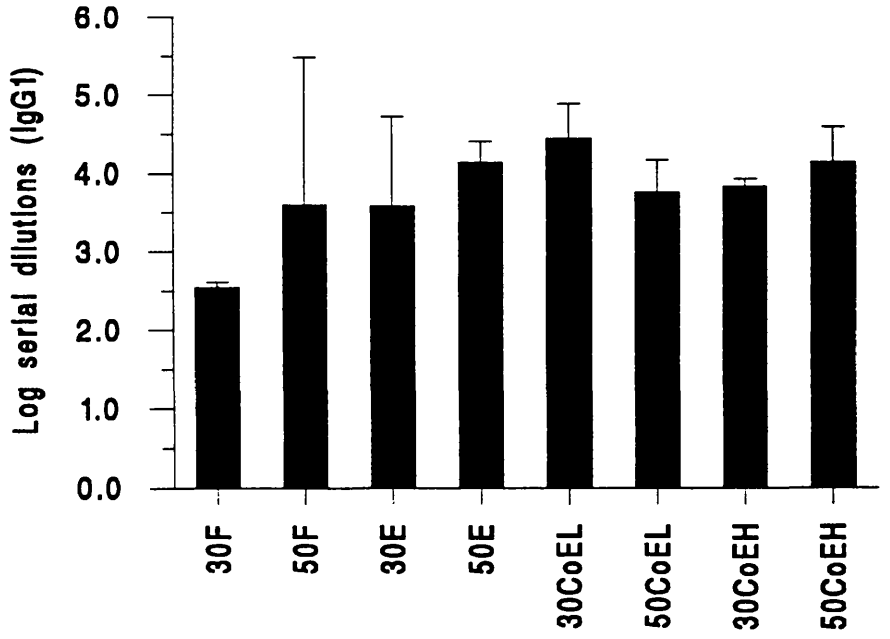


Fig. 37. IgG1 secondary responses to free or liposomal peptide 14 with or without IL-2

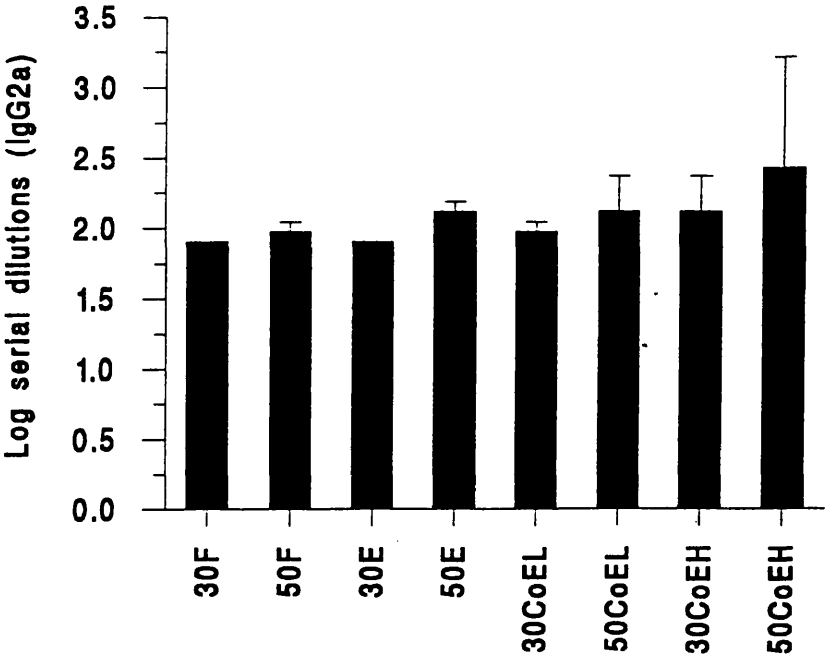


Fig. 38. IgG2a secondary responses to free or liposomal peptide 14 with or without IL-2

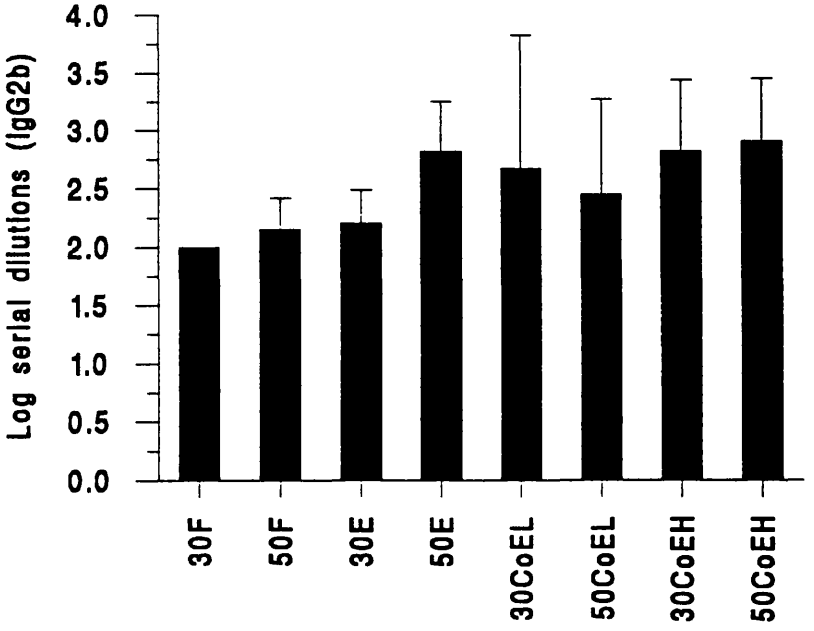


Fig. 39. IgG2b secondary responses to free or liposomal peptide 14 with or without IL-2

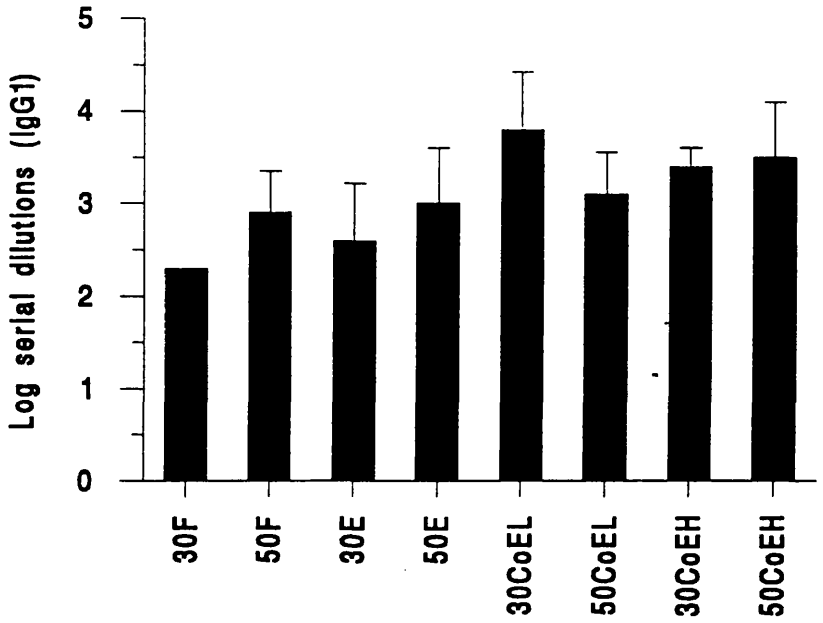


Fig. 40. IgG1 long term responses to free or liposomal peptide 14 with or without IL-2

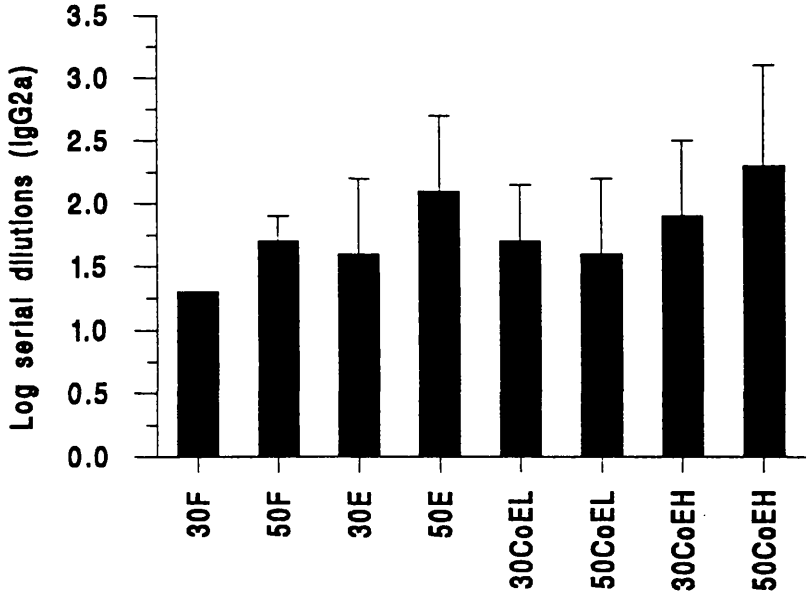


Fig. 41. IgG2a long term responses to free or liposomal peptide 14 with or without IL-2

with an immunisation dose of 30 μg. The 50 μg dose of free peptide **14** was equally effective as the liposomal preparations even when they contained IL-2 as the co-adjuvant. Only the 30 μg co-entrapped formulation (high IL-2 dose) increased the IgG_{2b} secondary responses significantly when compared to the free peptide. No significant differences were observed between the entrapped and co-entrapped preparations.

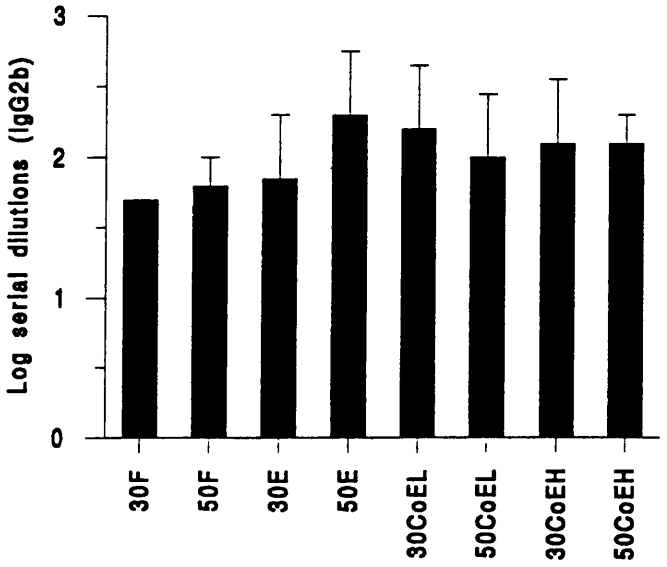


Fig. 42 IgG2b long term responses to free or liposomal peptide 14 with or without IL-2

Although the antibody titers declined slightly three months after the booster injection when compared to the secondary responses, there were still significantly high amounts maintained, indicating that the peptide could evoke memory responses as well. The long term subclass responses are presented in Figures 40-42. Evaluation of IgG_1 titers showed that only the 30 μg co-entrapped formulations were still superior to the free peptide. The same was true for the IgG_{2b} long term responses with the 30 μg co-entrapped formulations being superior to the free peptide **14**. There were no differences between the groups in terms of IgG_{2a} long term responses.

3.2.2.1.1 Conclusions

Over the range of different peptide doses used in this study, it was found that immunoadjuvant effect of liposomes was seen when an intermediate peptide dose (30 μg) was administered to the animals. Above this amount, the free peptide was equally effective. However, incorporation of IL-2 together with the peptide antigen in the same liposomal preparation still maintained the antibody titers and boosted them in the case of IgG_{2a} secondary responses when the 30 μg peptide dose was used.

Although in the case of bacterial toxins antibodies of any subclass with sufficiently high binding affinities can protect the host, for protection against many microorganisms, antibodies of isotypes that can activate complement and bind to receptors on antibody-dependent effector cells are required. In mouse, IgG_{2a} is the isotype that induces better protection against infectious agents (Allison and Byars 1986). The presence of IgG_{2a} specific for the infectious agent confers greater specificity for the cytolytic activity of natural killer cells by mediating antibody dependent cellular cytotoxicity (Yuan *et al.* 1994). Since *C. trachomatis* is an organism adapted to both intracellular and extracellular survival, antibodies of a protective nature such as IgG_{2a} become of the utmost importance in providing protection against the pathogen. This fact is well demonstrated with the influenza virus vaccines which cannot induce immunity in mice comparable to that seen following live virus infection, due to very low levels of IgG_{2a} antibodies induced by the vaccine.

3.3 An LCP based peptide FMDV vaccine which requires no additional adjuvants

The use of carriers and adjuvants are commonplace and often essential in order to raise antibody to a specific peptide antigen. The peptides are often conjugated to carrier proteins to increase the molecular size and weight of the potential immunogenic preparation. Additionally, adjuvants are often required to help provoke or heighten an immune response, but these are frequently toxic and precluded from

use in humans.

It is hypothesised that the LCP system can act as both a vaccine carrier and an adjuvant. The repeated amino terminal groups allow multiple copies of a peptide antigen to be incorporated into the structure, thus, negating the need for a carrier protein to increase molecular weight, while the lipidic tail of the system may have the effect of replacing the chemical adjuvants which are often themselves lipidic in nature. The lipidic tail may also play a further role in protecting the peptide from proteolytic degradation (Toth *et al.* 1994b) and aiding cell association of the preparation to cell

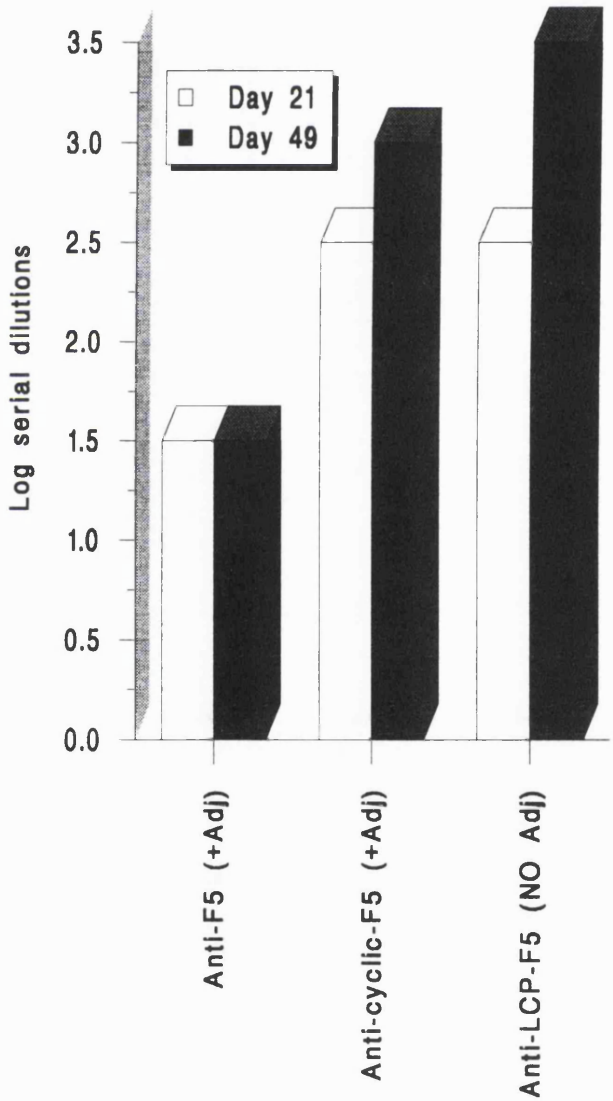


Fig. 43. Comparison of anti-17 serum raised without adjuvant, to anti-15 and anti-18 sera raised using adjuvant.

membranes, allowing effective internalisation of the preparation by antigen presenting cells.

3.3.1 Immunogenic evaluation of an LCP based FMDV peptide vaccine

In order to assess the effectiveness of the LCP structure as an adjuvant/carrier system for peptide vaccines, guinea pigs were immunised with either the linear FMDV peptide epitope **15**, a cyclic preparation of the same epitope (**18**) or the peptide epitope constructed on the LCP system (**17**). Peptides **15** and **18** were conjugated to the carrier protein KLH and administered in a mineral oil adjuvant, while peptide **17** was administered alone in phosphate buffered saline (PBS) without any carrier protein or adjuvant. The immunogenic evaluation was carried out in collaboration with Professor Fred Brown at the Animal Disease Center, Plum Island, USA.

The animals were bled on days 21 and 49 post immunisation and boosted with a second injection on day 22. The anti-peptide antibody titers were assessed by ELISA.

From fig. 43 it can be seen that even without adjuvant, the primary response to the LCP construct **17** is equal to that of the primary response of the cyclic peptide **18** with carrier and adjuvant. The response to the linear peptide **15** is lower than the cyclic form of the epitope, indicating that its native loop structure provides a more specific antigen for recognition. However, the secondary response to the LCP peptide is superior to that of the cyclic peptide epitope **18**, while no change is seen in the linear peptide **15**.

In a further study the above procedure was repeated but with an extra compound, peptide **16**, representing the epitope attached to just the MAP system alone, in order to compare the immunogenic ability of the LCP system with the MAP system. Unfortunately the data is unavailable for comment as yet.

3.3.2 Conclusions

The above data shows that the LCP system does not require any further adjuvants or carriers in order to provide a neutralising antibody response. It is also clear that the response evoked is long lasting and is increasing after nearly 50 days. It seems as though the lipid tail of the LCP system may replace the need for a toxic inflammatory adjuvant such as Freund's which is precluded from clinical use. It is highly likely that the lipophilic anchor aids internalisation and prolongs the immune response by reducing "antigenic drift".

The fact that the cyclic peptide also gave a reasonably good response would indicate the need for the epitope to be presented in the conformation it would appear if it were as the native protein which in this case is a loop structure. The obvious approach to improving the present data would be to present the epitope as the cyclic structure but incorporated into the LCP system.

3.4 Assessment of a vaccine for rheumatic fever

A number of requirements that must be addressed for peptide-145 (**19**) to become a successful streptococcal vaccine and include the ability to induce antibodies to a multiple of serotypes, the capacity for antigen presentation in the context of multiple HLA class II alleles and the stimulation of high titered IgG. A group of studies have therefore been undertaken, to significantly increase the immunogenicity of peptide-145.

One approach to induce a high titered antibody response was to incorporate the peptide-145 (**19**) into a polylysine core to form a multiple antigenic peptide. A further refinement to this approach has been to build the peptide **19** into the LCP system, to enhance membrane binding effects and metabolic stability of the peptide.

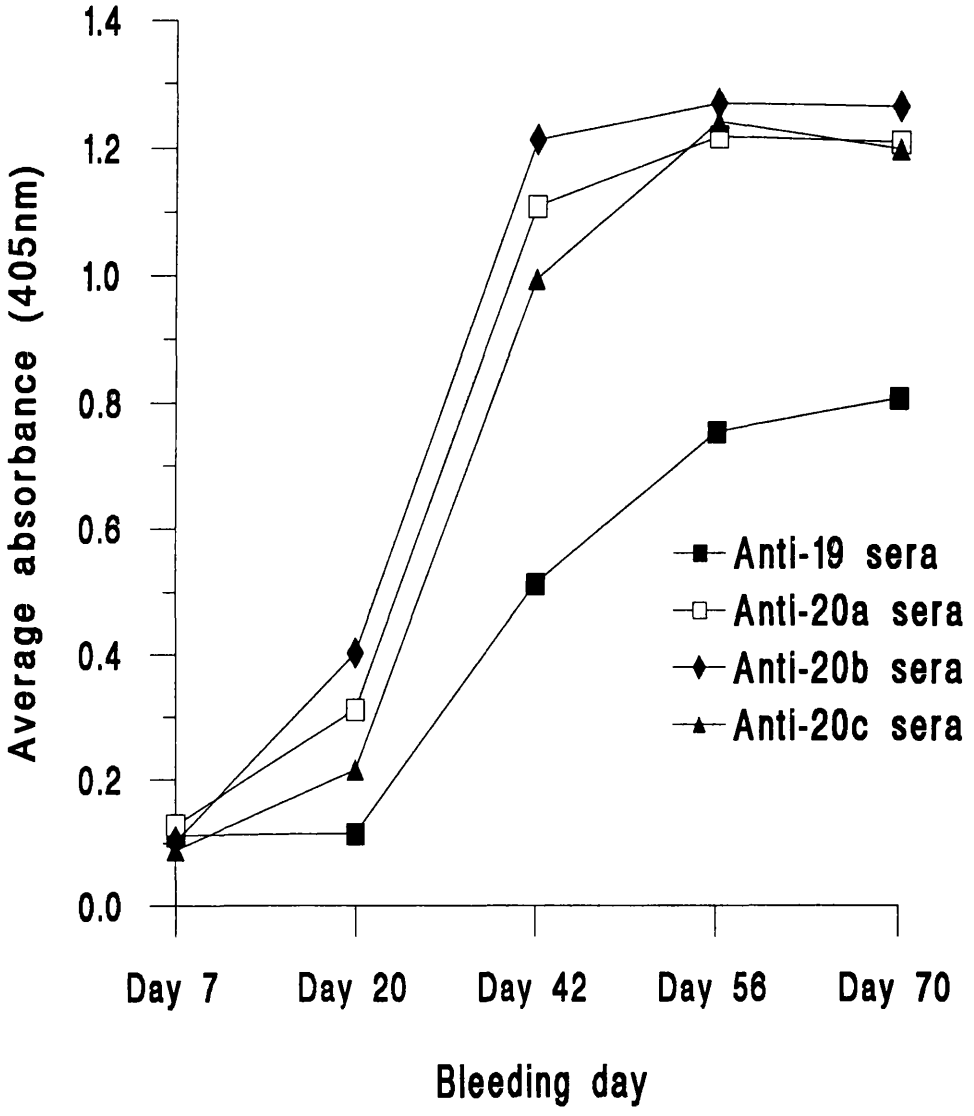


Fig. 44. Average absorbance (at 405nm) of anti-**19**, anti-**20a**, anti-**20b** and **20c** mouse sera at a dilution of 1:100

3.4.1 Immunogenic evaluation of differing LCP-145 peptide constructs in B10.BR mice

Three LCP-145 variants, **20a-c**, were synthesised as described in **2.4.4.1** and mice from a B10.BR congenic background were immunised with 30 μg of peptides **19**, **20a**, **20b** and **20c** by subcutaneous injection at the tail base, in CFA. All boosts were given at the same concentration in sterile PBS. All immunological evaluations were carried out in collaboration with Professor Michael Good at the Queensland Institute of Medical Research, Brisbane.

The immunisation protocol was as follows:

Pre-bleed

⇓

Immunisation

⇓ (bled on day 7, 14, 20)

1° Boost

⇓ (bled on day 28, 34)

2° Boost

⇓ (bled on day 42, 48)

3° Boost

⇓ (bled on day 56, 62)

4° Boost

⇓

Bled on day 70

Antibody production was monitored in each mouse over a 70 day period by ELISA.

It was observed that the LCP-145 constructs were more immunogenic than the peptide-145 monomer (**19**) alone in B10.BR mice (Fig. 44). They produced a more rapid antibody response and in addition generated a higher titer (102400-204800 on day 70) than the peptide-145 monomer (12800-51200 on day 70). It was also observed that the LCP peptides generate a strong antibody response in 100% of the immunised

mice, whereas immunisation with peptide-145 monomer generates a response in 0-75% of the mice only.

3.4.2 Immunogenic evaluation of differing LCP-145 constructs in mice of varying congenic backgrounds

In this study 30 μg of the peptides **19**, **20a**, **20b** and **20c** were administered as above in three different strains of mice, in order to see if the LCP constructs were capable of overcoming the genetic restriction seen previously in the peptide-145 monomer.

The immunisation protocol was as follows:

Pre-bleed
⇓
Immunisation
⇓ (bled on day 7, 14, 20)
1° Boost
⇓ (bled on day 28, 34)
2° Boost
⇓ (bled on day 42, 48)
3° Boost
⇓ (bled on day 69)
4° Boost
⇓
Bled on day 88

Antibody production was monitored over an 88 day period in each mouse by ELISA.

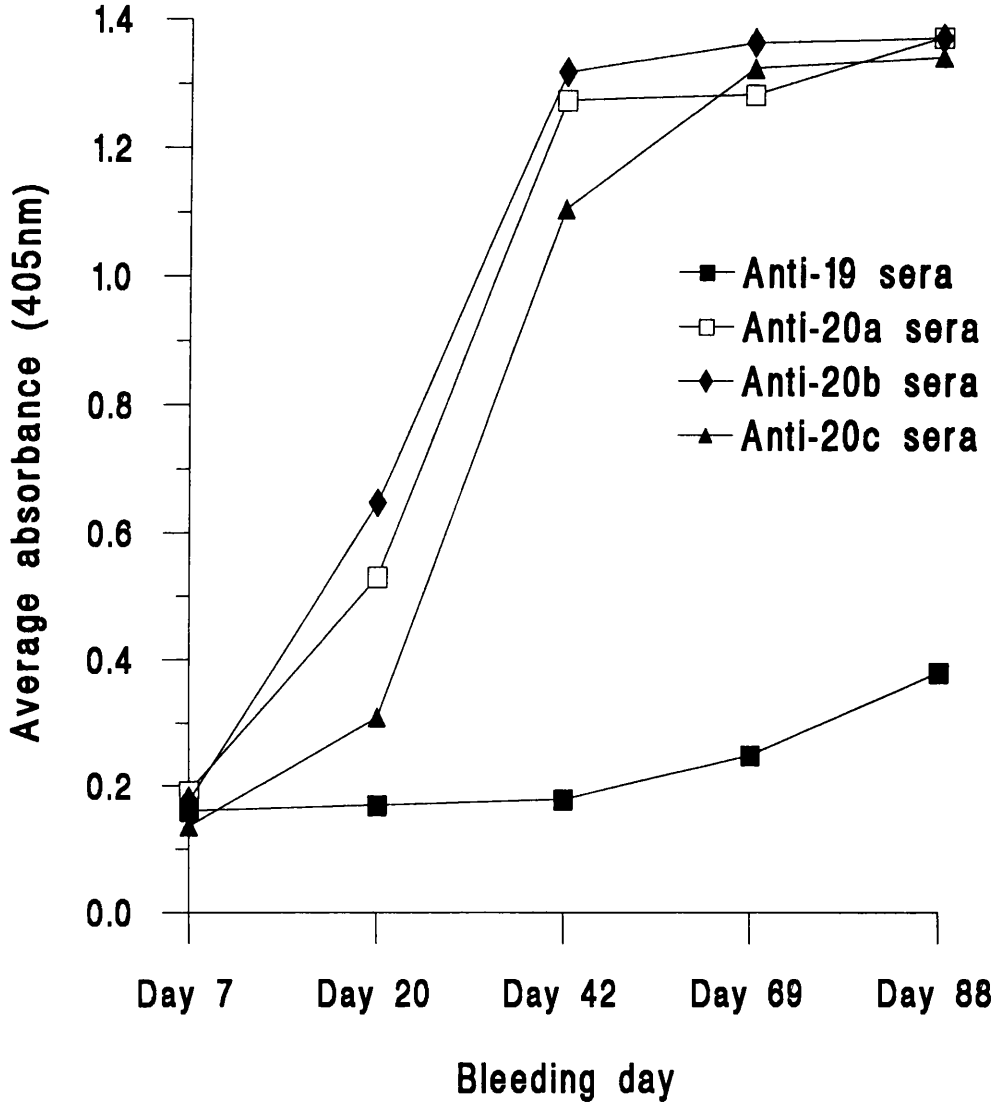


Fig. 45. Average absorbance (at 450nm) of anti-**19**, anti-**20a**, anti-**20b** and anti-**20c** B10.BR mouse serum at a dilution of 1:100

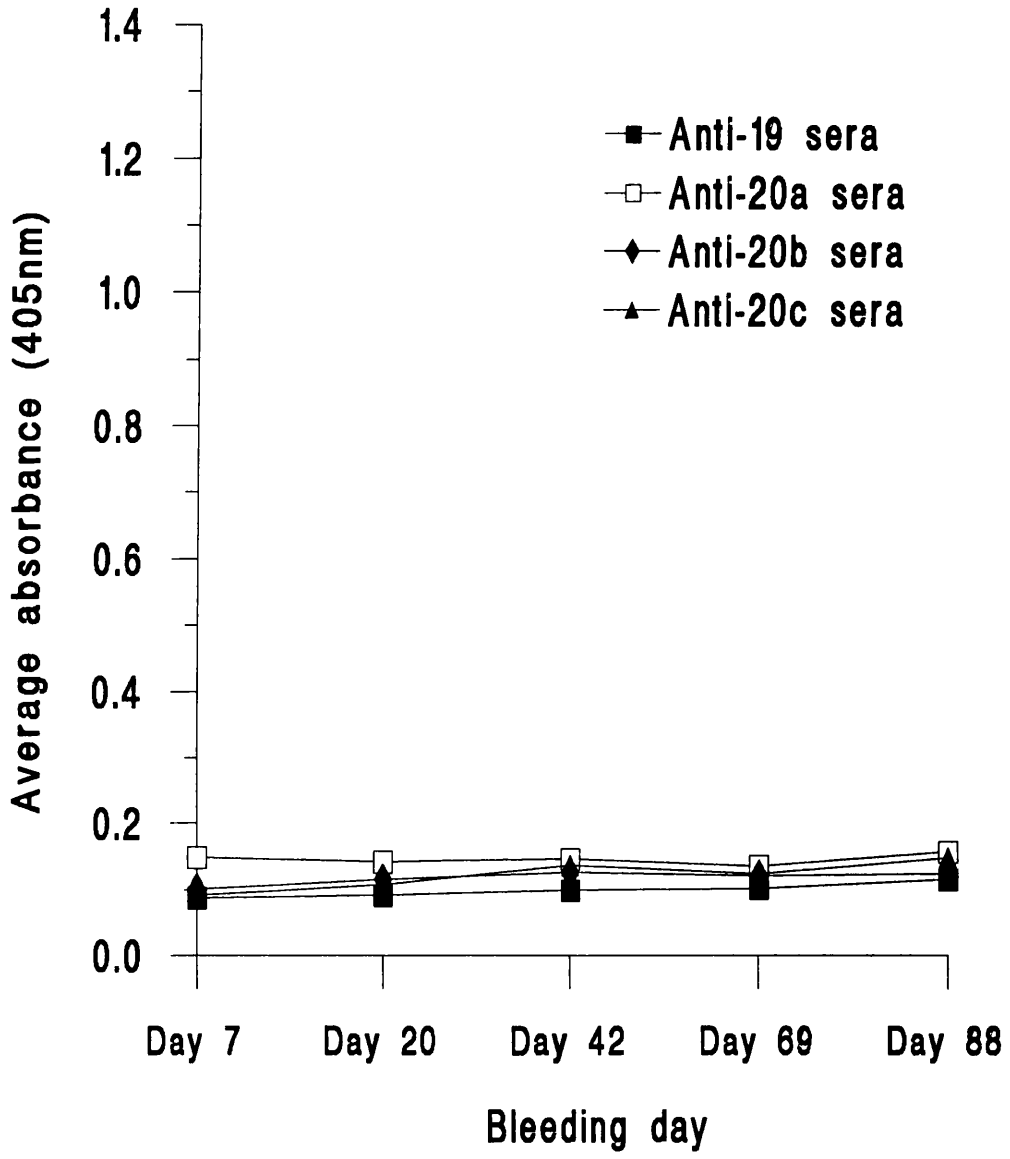


Fig. 46. Average absorbance (at 405nm) of anti-**19**, anti-**20a**, anti-**20b** and anti-**20c** B10.D2 mouse serum at a dilution of 1:100

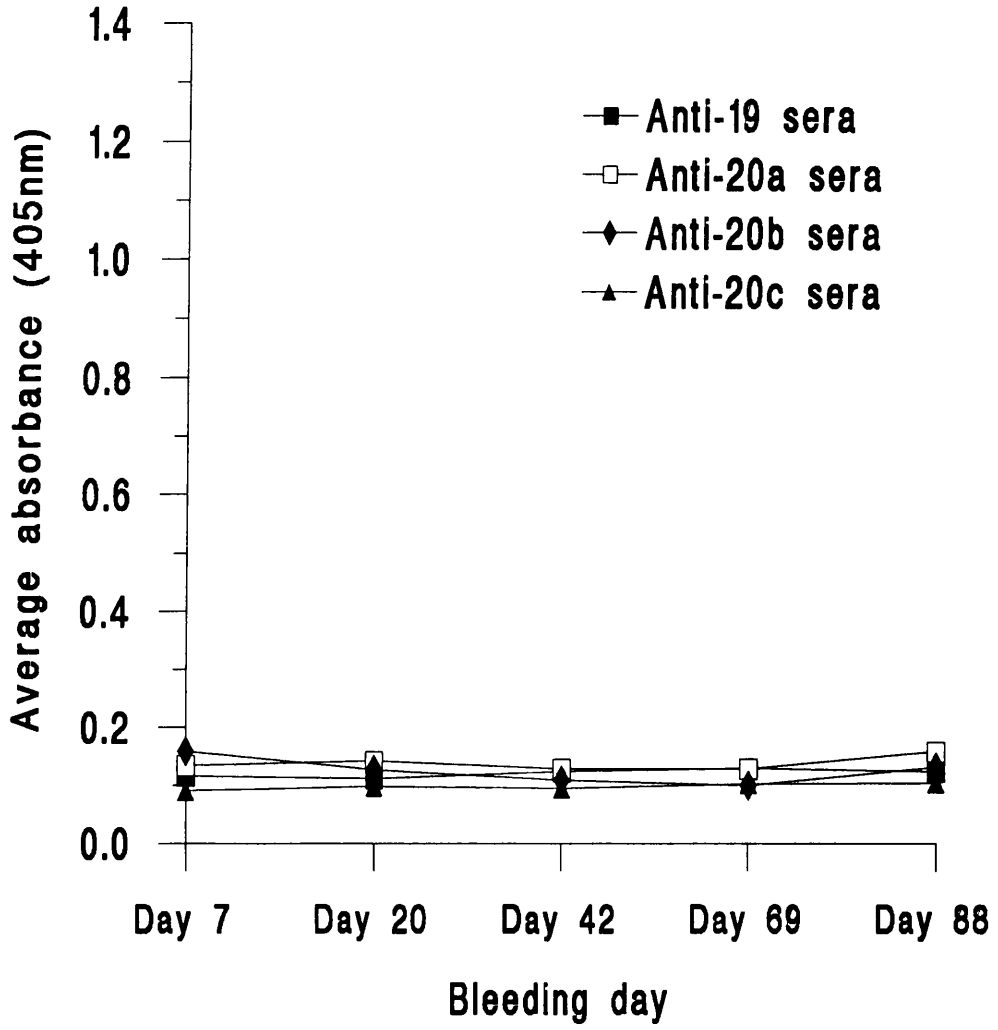


Fig. 47. Average absorbance (at 405nm) of anti-**19**, anti-**20a**, anti-**20b** and anti-**20c** C57BL/10 mouse serum at a dilution of 1:100

It was observed that the lipid-core-peptides were unable to break the genetic restriction of peptide **19** (the B10.BR mice respond to all peptides, Fig. 45, but the B10.D2 and

C57BL/10 mice do not respond to any) (Figs. 46 and 47 respectively).

3.4.3 Optimisation of alkyl side chain length of the lipidic amino acids within the LCP-Streptococcal M-protein construct

In order to assess the effect of the degree of lipophilicity within the fatty tail of the LCP construct, compounds **21a-d** were synthesised and consisted of peptide **19** on a tetrameric LCP construct, but the lipidic amino acids used were of differing side chain length.Thus, the extent of the lipophilicity in the fatty tail was controlled by varying the number of carbon atoms in each lipidic unit side chain.

The immunisation protocol was the same as that in **3.4.2**. However, the study is still in progess and the protocol is not yet complete and as a result no data is available at present, but will be reported in a future communication.

3.4.4 Conclusions

The LCP constructs were all able to produce high antibody responses and much more rapidly than the peptide **19** alone and little difference was seen between the terameric and octameric constructs in their ability to induce antibody, or indeed the rapidity of the response.

The most interesting aspect of the results shown is the capability of the LCP peptides to maintain very high antibody titers even after almost 3 months. In a previous study carried out using an octameric MAP construct with peptide **19**, similar antibody titers were obtained to those shown for the LCP peptides above, but lacked the long term responses seen with the LCP system (W. Hayman, *personal communication*).

This preliminary evidence would suggest that the fatty tail of the LCP construnts plays an important role in enhancing and protecting the immunogenic peptide sequence, so enabling high titer, long term responses to be obtained.

3.5 In vitro and in vivo studies of HNE lipophilic-peptide inhibitors

3.5.1 In vitro studies

The inhibitory capacity of lipopeptides **22a-d** towards HNE amidolytic activity was determined as reported (Hornebeck et al., 1985) using Succinoyl-Alanyl-Alanyl-Alanine paranitroanilide as substrate. Percent inhibitory refers as velocity ratios in presence and absence of inhibitor. IC_{50} is defined as the concentrations of lipopeptide required for achieving 50% inhibitions of enzyme.

In vitro inhibition of HNE amidolytic activity towards $Suc(Ala)_3NA$ by lipopeptides

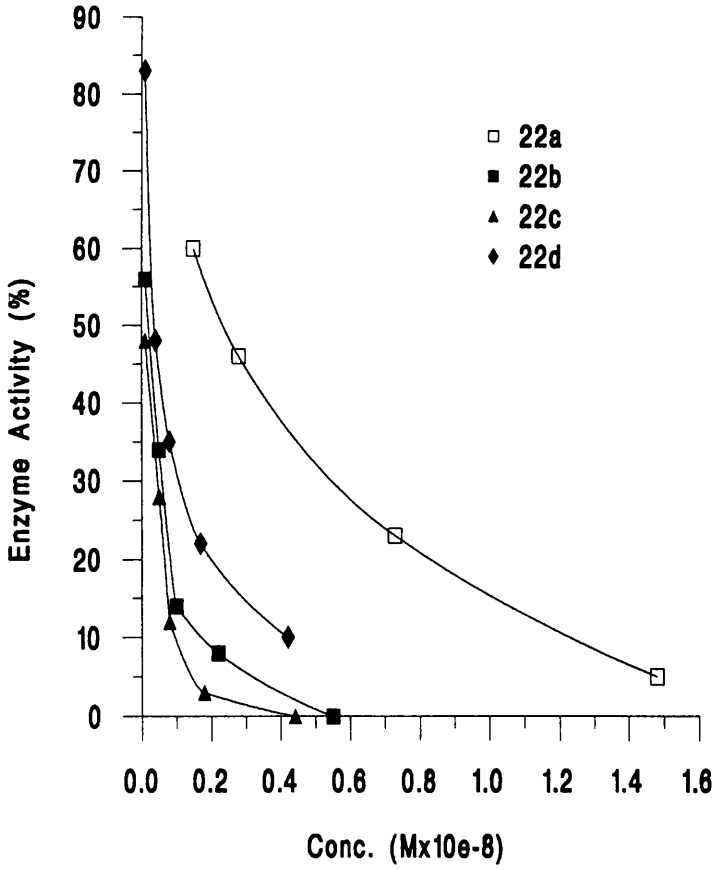


Fig 48. In vitro HLE inhibitory activity of **22a**, **22b**, **22c** and **22d**

22a, **22b**, **22c** and **22d** is shown in Fig. 48 and Table 7.

Table 7. IC_{50} values (giving 50% inhibition of HLE activity) of compounds **22a**, **22b**, **22c** and **22d** (evaluated from the dose response curves).

Inhibitor	IC_{50} (mmol ml^{-1})
22a	2.9×10^{-9}
22b	2.8×10^{-10}
22c	1.8×10^{-10}
22d	4.1×10^{-10}

Enzyme inhibition increased with increased lipophilicity of the substances; all compounds were more potent HNE inhibitors than N-oleoyl-$(Ala)_2$Pro Val-OH. Compound **22c** inhibited HNE with $IC_{50} = 1.8 \times 10^{-10}$ M as compared with $IC_{50} = 10^{-6}$ M (Hornebeck *et al.*, 1985) for the peptidic moiety N-oleoyl-$(Ala)_2$Pro Val-OH above.

Such difference indicated, in keeping with the size of the lipidic part of the molecule, that the extended hydrophobic subsites of HNE could accommodate large hydrophobic molecules. Modifmg the end carboxylic group of the lipopeptide to an ester (**22d**) led to decreased inhibitory capacity of the compound (Fig 48, Table 7). This substantiates earlier studies suggesting a primordial site in ionic interactions between ARG^{217} in HNE and the free carboxylate of the enzyme.

3.5.2 In vivo studies

The *in vivo* HNE inhibitory capacity of **22c** as well as its protective functions against elastolysis by elastin was further investigated, in collaboration with Professor W. Hornebeck at CNRS, France.

Hair from the back of male white Bouscat rabbit (weighing approx. 1500 g) was first removed and the following solutions were injected intradermally at 6 different sites.

(1) 200 µL of phosphate buffered saline (PBS) pH 7.4.

(2) 500 μg of lipopeptide **22c** in 1:1 (v/v) EtOH/PBS.

(3) 7.6 μM HNE in 200 μL PBS.

(4) 250 μg of lipopeptide **22c** dissolved in 1:1 (v/v) EtOH/PBS followed 15 minutes later by administration at the same site of 7.6 μM of HNE (100 μL solution in PBS).

(5) 7.6 μM HNE (100 μL solution in PBS) followed 15 minutes later by local administration of 250 μg of lipopeptide **22c** dissolved in EtOH/PBS 1:1 (v/v).

(6) Finally, 7.6 μM HNE preincubated for 15 minutes with 250 μg of lipopeptide **22c** and injected intradermally.

Skin biopsies were performed 3 hr post injections. Biopsies were then fixed, dehydrated and embedded in paraffin using routine procedures. Serial sections (5 μm) were performed and elastic fibres were stained using the (+) Catechin dye, a technique

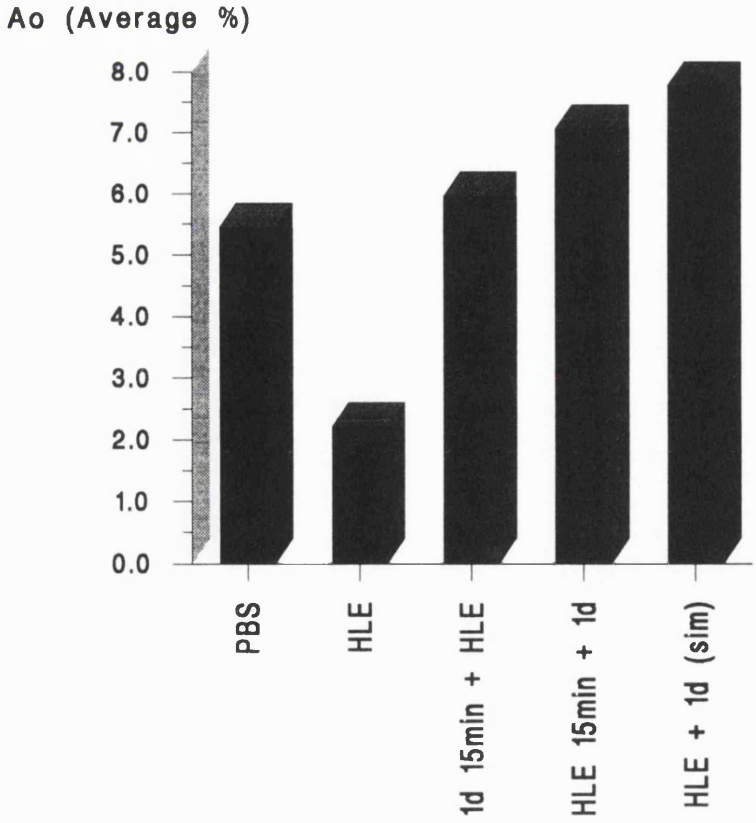


Fig. 49. In vivo enzyme inhibitory activity of compound **22c**

which allowed to individualise the elastic fibres system with colourless background, appropriate for morphometric analysis. Automated image analysis for appreciating the elastolytic potential of a protinase and/or the inhibitory capacity of an HNE inhibitor has been previously thoroughly desribed A_O % refers to the Average Area fraction occupied by elastic fibres in rabbit skin (Fig. 49).

Intradermal injections of lipopeptide **22c** did not modify A_O% of rabbit skin elastic fibres, as compared to animals where only PBS was administered (Photo 1). HNE administrations, in similar conditions, resulted in a significant reductions of dermal elastic fibres levels (Photo 2). When **22c** was administered prior, (15 min) with or after HNE, it could protect elastic fibre degradations induced by the enzyme (Photo 3). Such results extend previous studies demonstrating the *in vitro*, *ex vivo* and *in vivo* bi-functionality of lipopeptides acting as potent elastase inhibitors and elastic fibre protectors against elastolysis.

3.5.3 Effect of N-terminal modifications on the inhibitory activity of lipidic-peptide HNE inhibitors

To ascertain whether modifying the N-terminal of lipidic-peptides would effect their capacity to inhibit HNE, peptide **22c** was resynthesised in such a way as to produce compounds **22e-h**, as described in section **2.5.1**.

These peptides were subjected to the same *in vitro* and *in vivo* assays as described above for peptides **22a-d**. However, the study is incomplete at present and the data is not yet available, but will be reported in a future communication.

3.5.4 Conclusions

An extremely potent lipidic-peptide inhibitor has been synthesised with an IC_{50} value of approximately 10^{-10} M. This is a vast improvement on the previous inhibitor reported, whose IC_{50} value is aproximately 10^{-6} M (Hornebeck *et al.* 1985). However, it is unclear whether the enhanced inhibition of these novel lipidic-peptide inhibitors

is due to increased binding at the active site of the enzyme via a hydrophobic pocket or the enhanced metabolic stability of the peptide due to protection from proteolysis, by the lipidic amino acids (Toth *et al.* 1994b), or a combination of the two.

Photo. 1.

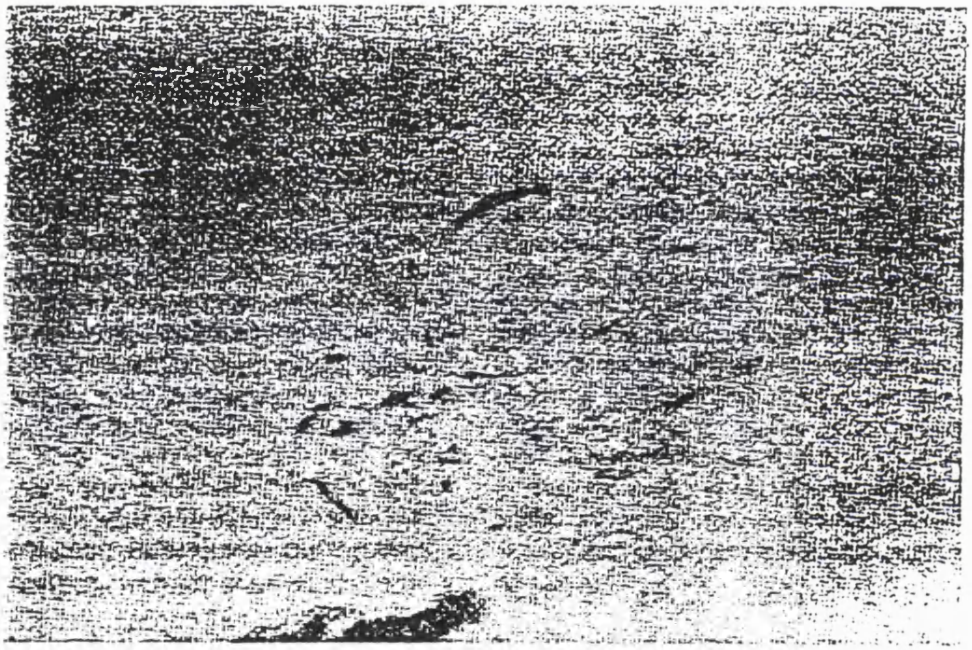


Photo. 2.

Photo. 3.

CHAPTER 4

CONCLUSIONS

The aims of the project were two-fold. Firstly, to synthesise a series of lipidic amino acids and conjugate them to poorly absorbed and biologically unstable peptides. It was envisaged that the lipidic amino acids would enhance the lipophilicity and membrane-like character of the selected peptides, helping them to cross biological membranes. In addition, the long alkyl side chain of the lipidic amino acids was expected to improve the metabolic stability of the peptides by shielding the peptides from enzymatic attack, making them less susceptible to proteolytic degradation.

The second aspect of the project was to incorporate the lipidic amino acids into a polylysine system to form lipid-core peptide (LCP), a novel carrier/adjuvant system for enhancing the immunogenicity of peptide epitopes, moving towards the possibility of a universal presentation system for synthetic peptide vaccine candidates.

4.1 Lipidic peptide conjugates

Unsubstituted lipidic amino acids **1a-d** were prepared which possess a single unfunctionalised, alkyl side-chain.

The peptides chosen to be conjugated with the lipidic amino acids were the tripeptide, TRH and the decapeptide, LHRH and were chosen because both were well documented in the literature as being biologically unstable and poorly absorbed by the oral route. The lipidic peptide conjugates were synthesised using solid phase methodology from the appropriate Boc-protected lipidic amino acid. TRH conjugates **2a-c** and LHRH conjugate **3a-g** were synthesised using the boc protected lipidic amino acid **1f**.

To evaluate the metabolic stability of the conjugates, the lipidic peptides were incubated with Caco-2 cell homogenate. All lipidic conjugates showed a much greater resistance to degradation compared with the degradation profile of the unconjugated parent peptide. A very clear trend was seen in which an increase in the lipophilicity

of the conjugates was accompanied by an increase metabolic stability of the compounds. Also significant was the fact that the dimer conjugates (two lipidic conjugating units) released the monomer conjugate (one lipidic conjugating unit) which in turn released the free peptide through the action of the degrading enzymes. This suggests the possibility of a prodrug type delivery system, which is able to protect the peptide from proteolytic degradation for a period of hours, probably due to the presence of the long alkyl side chains, and then release the free peptide.

Radiolabelled conjugates of TRH and LHRH (**5a-f**, **6a** and **6b**) were then synthesised in order to follow the lipidic conjugates in biological systems.

The ability of the conjugates to cross biological membranes was first assessed using Caco-2 cell monolayers to mimic the epithelial barrier of the gut. Again, a similar correlation was seen, with an increase in lipophilicity providing an enhanced transport profile along with an increased cell association, with larger quantities of lipidic conjugates being seen to cross the monolayer compared with the free TRH peptide.

The final study carried out was the assessment of the uptake of the lipidic conjugates **5a-f** in rats after oral administration. The same trend was seen as in all the previous studies, the greater the lipophilicity, the greater the uptake and metabolic stability of the conjugate. High levels of uptake were seen, for the lipidic conjugates, in stomach and small intestine with the smaller TRH conjugates showing the greatest absorption compared to the larger, bulkier LHRH conjugates. It is possible that the TRH conjugates utilise existing tripeptide transporter mechanisms as well as diffusing passively through the epithelial membrane. A further possibility is the use of Peyer's patches by the lipidic conjugates to pass from the small intestine, since uptake of lipidic compounds is known to be favoured by lymphoid tissue. Intact conjugates were successfully extracted from the rat blood, after oral administration, and their identity confirmed by HPLC, mass spectrometry and capillary electrophoresis

Overall, novel lipidic conjugates of TRH and LHRH were synthesised, which showed greatly enhanced metabolic stability *in vitro* and *in vivo* and a marked increase in their

ability to be taken up, intact, after oral administration. However, an optimum lipophilicity exists which needs to be balanced with the water solubility for each individual case, as too great a lipophilicity may hinder the transport of the conjugate through biological membranes.

4.2 Lipid-core peptide

The Lipid-Core-Peptide (LCP) system which incorporates lipidic amino acids to the polylysine system to enhance lipophilicity and membrane binding effects of a potential peptide-vaccine is an excellent means for enhancing antigenicity. Different spacer-(lipidic amino acid)$_n$-(L-Lys)$_m$ systems (**7a-e**) were synthesised on solid phase, with a fully automated procedure, using Boc strategy.

The lipidic conjugating unit gives a great flexibility in the physico-chemical properties of the coupled compound because of the vast number of variables which can be modified, (i) the length of the peptide chain can be increased (n>1), (ii) the length of the alkyl chain (p) is variable, (iii) the free substituent on the C-termini of the conjugating units allows further conjugation eg. (a) sugars, to increase hydrophilicity, (b) antibodies, to target the conjugates, (c) fluorescent and radiolabels, for tracer studies. Another novel aspect of this project is that the spacer-lipid-core-peptide system can be synthesised using an automated peptide synthesizer, without isolating the intermediates.

4.2.1 An LCP-based vaccine for *Chlamydia trachomatis*

A peptide vaccine for the prevention of chlamydial infection based on sequences from the variable segments of the *C. trachomatis* Omp 1 incorporated into an LCP construct (**14**), has yielded promising results. The essential goal for such a vaccine will be the production of neutralising antibodies, in a mucosal environment, to multiple *C. trachomatis* serovars.

The incorporation of the epitope sequence **12** into an LCP construct significantly enhanced immunogenicity (100-fold) in comparison to monomeric peptide **13** alone. However, although the anti-**14** antibodies were able to neutralise serovars A, B and C, poor cross reactivity was seen with the native antigenic site on the chlamydial EB. Thus, since the anti-**14** antibodies bound more to the peptide than to whole EB, important conformational constraints probably exist in the antigenic sites on chlamydial EB, which are not maintained in the LCP peptide construct. It is speculated that cyclisation of the peptide sequence may improve cross-reactivity between the peptide structure and the native antigenic site.

Entrapment of peptide **14** in a liposome formulation was of no real benefit, but the long term study showed that the LCP peptide construct could still elicit a high antibody response three months after the booster injection.

4.2.2 A foot and mouth disease vaccine candidate

Peptides **15**, **16**, **17** and **18** were synthesised in order to evaluate the potential of using the LCP, without any other adjuvant, to enhance the immunogenicity of an epitope from the VP1 region of FMDV.

The LCP-peptide **17** elicited a very high titer of neutralising antibody, while the cyclic peptide **18** also gave a good response. However, the most significant element of the study was that The LCP-peptide **17** gave the highest antibody response without the use of any immunoadjuvants, while the other constructs all required an adjuvant to elicit a reasonably good neutralising antibody response. This result is of great importance, since most adjuvants currently used in vaccine research are prohibited in humans because of their toxicity and inflammatory nature and the identification of a non-toxic adjuvant/carrier system would greatly improve the possibility of the use of synthetic peptide vaccines in the clinic. A further advantage is that the system is a known molecular entity which is chemically defined and which also possess chemical stability which negates the need for elaborate storage and handling of the product.

Again, as with the chlamydial vaccine, cyclisation of the epitope on the LCP system would appear to be the ideal construct to elicit a maximal neutralising response.

4.2.3 A vaccine candidate for rheumatic fever

Peptides **19** and **20a-c** were synthesised to evaluate the ability of the LCP system to potentiate the immunogenicity of a peptide epitope from the streptococcal M-protein.

As with all the other examples quoted thus far, the LCP system had a dramatic effect on the ability of peptide **19** to stimulate the immune system and obtain opsonised antibodies to the peptide sequence. The LCP linked peptide was able to produce a response more rapidly than the free peptide in CFA and was able to maintain the response over a long duration, but was unfortunately unable to breach the genetic restriction in H-2^d mice.

The size and nature of the LCP system was also assessed and results showed that a tetrameric construct was as effective as the octameric construct and again a balance probably exists between lipophilicity and water solubility.

Further constructs **21a-d** were assessed, which contained lipidic amino acids with differing alkyl side chain lengths, in order to optimise the lipidic tail of the LCP system.

4.3 Lipophilic-peptide inhibitors of HNE

Lipophilic-peptide conjugates **22a-d** were synthesised and assessed for their inhibitory activity against HNE.

In vitro, all lipophilic conjugates **22b-d** showed a much increased inhibitory activity towards HNE, than the inhibitor **22a** described in the literature. The most successful inhibitor required a high lipophilic nature while at the same time possessing a free carboxylic function at the C-terminus of the peptide. These results were in keeping

with the assumption of the presence of a large hydrophobic pocket within the binding site of the HNE. It would also seem that an ionic interaction involving the carboxyl moiety is of some importance.

In vivo, the inhibitor **22c** showed excellent inhibitory activity and protected elastic fibres in rabbit skin from degradation by HNE.

Further peptides **22e-h** were synthesised to further investigate the contribution of the functional end moieties towards the inhibitory activity of the lipophilic peptide HNE inhibitors.

Thus, novel amino lipidic amino acids have been successfully conjugated to poorly absorbed peptides and enhanced their oral absorption and metabolic stability *in vitro* and *in vivo*, suggesting that lipidic amino acids provide an effective means of delivering and protecting peptide drug candidates. These extraordinarily versatile compounds have been found to have a pronounced effect on enhancing peptide immunogenicity, when incorporated within a polylysine construct, yielding a novel adjuvant/carrier system for synthetic peptide vaccines.

CHAPTER 5

EXPERIMENTAL:Chemistry

Mass spectra were obtained on a VG ZAB-SE double focusing instrument, operating at 8kV accelerating voltage, and a VG 11/250 J data system. An Ion Tech gun (Ion Tech Ltd.) operating with Xe atoms at 8kV was used to bombard samples loaded on a stainless steel target with 2µl of glycerol/thioglycerol or m-nitrobenzoic acid (m-NOBA) matrix. Mass spectra were also obtained on a Fisons Matrix assisted laser desorption time of flight spectrometer, with a N_2 laser operating at 337nm and 4µl of sinapinic acid or alpha cyano as matrix. ^{1}H-NMR spectra were obtained on Varian XL-300 and Bruker AM500 instruments operating at frequencies of 300 and 500 MHz respectively; chemical shifts are reported in ppm downfield from internal TMS. UV readings were recorded on a Perkin Elmer Lambda 15 instrument. Reaction progress during synthesis of the lipidic amino acids was monitored by thin layer chromatography (TLC) on Kieselgel PF_{254} using CH_2Cl_2:MeOH 10:1 as the mobile phase, unless otherwise stated. Solvents were evaporated under reduced pressure with a rotary evaporator. Purity of compounds was assessed using two methods i)by reverse phase high pressure liquid chromatography (RP-HPLC) on a 25cm Vydac C_4 or C_{18} column with a 5µm pore size and 4.6mm internal diameter. HPLC grade acetonitrile (Aldrich) and water were filtered through a 25 µm membrane filter and degassed with helium flow prior to use. Analytical separation was achieved with a solvent gradient beginning with 0 % acetonitrile, increasing constantly to 60 % acetonitrile at 30 minutes, staying at this concentration for 20 minutes and decreasing steadily to 0 % acetonitrile for 10 minutes at a constant flow of 1 ml min^{-1}. ii)by capillary electrophoresis (CE), using a Millipore Quanta 4000 instrument. Separation was achieved on a fused silica column with an effective length of 60cm and 50mM phosphate buffer at pH X over 30 minutes using an applied voltage of 20kV, unless otherwise stated. Preparative scale purification was carried out by RP-HPLC using a 25cm TSK-GEL preparative C_{18} column with 10µm pore size and 2.5cm internal diameter. Preparative separation achieved with a solvent gradient beginning with 0 % acetonitrile, increasing constantly to 60 % acetonitrile at 180 minutes, staying at this concentration for 60 minutes and decreasing steadily to 0 % acetonitrile for 30 minutes at a constant flow of 8 ml min^{-1}. The gradient was effected by two

microprocessor-controlled Gilson 302 single piston pumps. Compounds were detected with a Holochrome UV-VIS detector at 214 nm (analytical) and 230 nm (preparative). Chromatographs were recorded with an LKB 2210 single channel chart recorder or a Spectra Physics Integrator.

α-Amino-eicosanoic acid (1d) [A]

In a 500ml round bottom flask equipped with reflux a condenser, sodium (2.5g, 0.11mol) was dissolved in EtOH (85ml). Diethyl acetamidomalonate (24.3g, 0.11mol) and 1-bromo-octadecane (50g, 0.15mol) were added to the viscous solution and the reaction mixture heated under reflux for 24 hours. Upon cooling, the mixture was poured onto ice-water (160ml) and the resulting precipitate filtered and washed with water. The crude solid was placed into a 500ml round bottom flask, concentrated hydrochloric acid (180ml) and DMF (20ml) added and the mixture refluxed for 24 hours. The mixture was allowed to cool, poured into a solution of EtOH:water (3:1) and neutralised with conc. NH_4OH. THe precipitate was collected by filtration and washed with EtOH:water.

Yield: 34.9g (97%) (Gibbons *et al.* 1990)

α-Amino-decanoic acid (1a), α-amino-dodecanoic acid (1b) and α-amino-tetradecanoic acid (1c)

The compounds were synthesised from the appropriate 1-bromo-alkanes, using the method descibed in [A].

Yields:**1a** 85%, **1b** 89%, **1c** 95% (Gibbons *et al.* 1990)

N-tert.butoxycarbonyl-α-amino-eicosanoic acid 1h [B]

Amino acid **1d** (15g, 46mmol) was suspended in a 2:3 mixture of tert.butanol:water (180ml) and 8M sodium hydroxide added dropwise to pH 13. Ditert.butyl-dicarbonate (15g, 69mmol) in tert.butanol (30ml) was added at room temperature, the pH adjusted to 11-12 and the reaction mixture stirred for 2 hours. Following dilution of the reaction mixture with water (50ml), solid citric acid was added to pH 3 and the oil extracted with EtOAc. After drying ($MgSO_4$), the organic layer was evaporated, the residue triturated with acetonitrile and the product filtered.

Yield: 8.2g (93%)

NMR ($CDCl_3$): 10.2 (1H,bs,OH), 5.1 (1H,d,NH), 4.2 (1H,m,α-CH), 2.2 (2H,m,β-CH_2),

1.4 (9H,s,$(CH_3)_3$), 1.24 (32H,m,16xCH_2), 0.9 (3H,t,CH_3).

MS m/z (%): 473 (23), 472 $[M+2Na-H]^+$ (86), 450 $[M+Na]^+$ (33), 393 (15), 373 (22), 372 (100), 370 (23), 282 (23), 282 (14), 176 (23), 119 (10), 57 (26).

Anal. $C_{25}H_{49}NO_4$ (427.65)

Calc. C 70.21 H 11.05 N 3.27

Found C 70.19 H 11.59 N 3.09

N-tert.butoxycarbonyl-α-amino-decanoic acid (1e), N-tert.butoxycarbonyl-α-amino-dodecanoic acid (1f) and N-tert.butoxycarbonyl-α-amino-tetradecanoic acid (1g)

The compounds were synthesised from amino acids **1a**, **1b** and **1c** using the method described in [B].

1e Yield: 93% mp. 64-66°C.

NMR ($CDCl_3$): 4.95 (1H, s, OCONH), 4.29 (1H, m, α-CH), 1.85-1.67 (2H, m, β-CH), 1.45 (9H, s, $C(CH_3)$), 1.27 (12H, m, 6xCH_2), 0.88 (3H, t, CH_3).

MS m/z (%): 333 (17), 332 $[M+2Na-H]^+$ (100), 311 (18), 310 $[M+Na]^+$ (99), 254 (30), 232 (58), 188 (12), 186 (14), 142 (17), 57 (41).

Anal. $C_{15}H_{29}NO_4$ (287.39)

Calc. C 62.68 H 10.17 N 4.87

Found C 62.87 H 10.14 N 4.59

1f Yield: 72% mp. 62-64°C

NMR ($CDCl_3$): 4.98 (1H, s, OCONH), 4.28 (1H, m, α-CH), 1.84-1.67 (2H, m, β-CH), 1.46 (9H, s, $C(CH_3)$), 1.26 (16H, m, 8xCH_2), 0.87 (3H, t, CH_3).

MS m/z (%): 361 (18), 360 $[M+Na]^+$ (100), 339 (13), 338 $[M+H]^+$ (66), 282 (20), 260 (48), 57 (10).

Anal. $C_{17}H_{33}NO_4$ (315.44)

Calc C 64.72 H 10.54 N 4.44

Found C 64.65 H 10.55 N 4.41

1g Yield: 89% mp. 62-64°C

NMR ($CDCl_3$): 4.95 (1H, s, OCONH), 4.27 (1H, m, α-CH), 1.67 (2H, m, β-CH), 1.42 (9H, s, $C(CH_3)$), 1.21 (20H, m, 10xCH_2), 0.9 (3H, t, CH_3).

MS m/z (%): 389 (21), 388 $[M+2Na-H]^+$ (100), 367 (12), 366 $[M+Na]^+$ (60), 310 (18), 288 (57), 57 (30).

Anal. C_{19} H_{37} NO_4 (343.5)

Calc C 6 H 10.54 N 4.44

Found C 64.65 H 10.55 N 4.41

Synthesis of TRH peptide 2a [C]

The synthesis of the peptide was accomplished automatically by a stepwise solid phase procedure on p-methylbenzhydrylamine (MBHA) resin (Novabiochem) (substitution 0.48mmol/1g resin). The synthesis of the peptide was achieved using double coupling with dicyclohexylcarbodiimide (DCC) and a 4 times excess of N-Boc amino acids (2mM), in CH_2Cl_2 for the first coupling and CH_2Cl_2 (20ml)/N-methylpyrrolidone (5ml), for the second. The protecting groups for the synthesis of the peptide were Boc groups for the α-amino-termini, OBzl for the Glu and DNP for the His (all supplied by Novabiochem). In all the couplings the coupling efficiency was more than 99.4% as indicated by quantitative ninhydrin testing. After the second coupling, deprotection of the N-termini was performed in 65% triflouroacetic acid (TFA) in CH_2Cl_2 (20ml for 1min. then another 20ml for 10 min). The deprotected resin peptide was neutralized with 10% diisopropylethylamine (DIEA) in CH_2Cl_2. The resin peptide was carefully washed between and after the deprotection and neutralisation steps. The DNP protecting group from the His was removed with 20% mercaptoethanol-5% diisopropylethylamine in DMF. The peptide was removed from the resin support with high HF method (1.5ml cresol, 1.5ml thiocresol, 15ml HF) to yield the crude peptide, which was precipitated with ethylacetate and redissolved in 6M guanidine HCl-0.1M TRIS solution (20ml) for purification.

Yield: 133 mg

MS m/z (%): 380 $[M+H]^+$ (100), 363 (12), 340 (15), 309 (6), 293 (7), 277 (7), 249 (4), 217 (32), 201 (67), 185 (59), 109 (14).

HPLC retention time (min): 6.66

Synthesis of TRH peptide conjugate 2b [D]

The TRH lipidic conjugate **2b** and was synthesised as described in method [C], with the addition of one 2-amino dodecanoic acid residue, which was introduced as the racemic Boc protected compound **1f** at the N-terminal of the resin bound peptide chain, using the double coupling procedure with DCC and a 4M excess of **1f** in CH_2Cl_2. After cleavage from the resin (as described in [C]), the crude peptide was precipitated with ice cold ether and redissolved in 90% acetic acid (20ml). The solution was then diluted to 100ml with distilled water and lyophilised to give a white solid.

Yield: 152 mg

MS m/z (%): 641 (13), 599 $[M+Na]^+$ (100), 577 $[M+H]^+$ (38), 554 (4), 457 (6), 413 (35), 329 (24), 176 (57), 171 (15), 154 (14), 136 (12).

HPLC retention time (min): 15.56, 15.97 (2 diastereomers)

Synthesis of TRH peptide conjugate 2c [E]

The TRH lipidic conjugate **2c** was synthesised as described in method [D], but with two 2-amino dodecanoic acid residues at the N-terminus.

Yield: 119 mg

MS m/z (%): 797 $[M+Na]^+$ (100), 775 $[M+H]^+$ (18), 752 (5), 655 (8), 518 (4), 461 (5), 170 (42), 136 (4), 110 (12).

HPLC retention time (min): 21.77, 22.12, 22.66, 23.71 (4 diastereomers)

Synthesis of LHRH peptide 3a

LHRH peptide 3a was synthesised as described in method [C], the only exception being the coupling of Boc-substituted-Asn which was mediated by the preformed 1-hydroxybenzotriazole ester in CH_2Cl_2 / N-methylpyrrolidone for both couplings. The protecting groups for the synthesis of the peptide were Boc groups for the α-amino-termini, Br-Z for the Tyr, Tos for the Arg, formyl for the Trp, DNP for the His, OBz for Glu and Bzl for the Ser (all supplied by Novabiochem). The formyl group was removed from the Trp prior to HF cleavage, with 10% piperidine in DMF, stirring at 0°C for 2 hours.

MS m/z (%): 1399 $[M+H]^+$ (100), 1340 (9), 1199 (6), 1043 (6), 715 (8), 497 (9), 426 (14), 382 (26), 312 (19), 267 (24).

HPLC retention time (min): 12.36

Synthesis of LHRH peptide conjugates 3b and 3c

The lipidic LHRH conjugates **3b** and **3c** were synthesised according to method [D].

MS m/z (%): 1596 $[M+H]^+$ (100), 1523 (8), 1397 (9), 1072 (8), 907 (8), 820 (9), 717 (10), 662 (14), 554 (13), 499 (20), 440 (19), 382 (58), 312 (35), 267 (50).

HPLC retention time (min): 17.34,18.13

Synthesis of LHRH peptide conjugates 3d-g

The lipidic LHRH conjugates **3d-g** were synthesised according to method [E].

HPLC retention time (min): 21.25, 21.83, 23.73, 23.97

Synthesis of polylysine conjugates 4a and 4b [F]

The TRH- and LHRH-polylysine conjugates **4a** and **4b** were synthesised according to method [E], with the addition of a branched octameric lysine construct at the N-terminus of the peptides. The polylysine construct was added to the growing peptide chain using the same coupling procedures as before, but by using lysine that was Boc protected at both α- and ε-amino functions, two amino functions were available for coupling by the next residue. Thus, by sequentially adding Boc-Lys (Boc) (Novabiochem), the first level of coupling of Boc-Lys (Boc) produced two amino ends, the second level of coupling resulted in four and the third level of coupling eight amino ends. Each coupling of the Boc-Lys (Boc) was carried out in DMF:CH_2Cl_2 (1:1) and after each coupling the molar excess of Boc-Lys (Boc) and quantity of coupling reagent was doubled since the number of available functional groups had doubled.

Synthesis of radiolabelled TRH and LHRH peptides and their lipidic conjugates 5a-f, 6a and 6b

TRH and LHRH peptides **2a-g**, **3a-g**, **4a** and **4b** were synthesised as described in methods [C]-[F], with the addition that before cleavage from the resin the N-termini of the peptides were acetylated with [^{3}**H**] acetic anhydride. 25mCi of [^{3}**H**] acetic anhydride (Amersham) was diluted with DMF (30ml) and 3ml of the solution was added to the appropriate peptide resins along with DIEA (0.5ml), after the N-terminal Boc protection had been removed (as previously described). The reaction vessel was shaken and the reaction terminated when the level of tritium in free solution no longer decreased and became constant (as determined by liquid scintillation counting). This indicated that no further acetylation was taking place. The peptide resin was then completely acetylated using an excess of cold acetic anhydride with DIEA, with the extent of capping being assessed by quantitative ninhydrin testing, thus yielding compounds **5a-f**, **6a** and **6b**.

Compound **5a**
Yield: 110 mg

MS m/z (%): 423 $[M+H]^+$ (100), 379 (4), 363 (7), 337 (6), 309 (4), 294 (6), 282 (4),

252 (5), 236 (7), 223 (4), 207 (6), 185 (75), 114 (36), 110 (73), 96 (4).

Compound **5b**
Yield: 126 mg

MS m/z (%): 620 $[M+H]^+$ (100), 604 (6), 575 (4), 553 (7), 539 (4).

Compound **5c**
Yield: 115 mg

MS m/z (%): 862 $[M+2Na]^+$ (15), 840 $[M+Na]^+$ (100), 818 $[M+H]^+$ (23), 795 (7), 698 (14), 639 (5), 605 (4), 560 (16), 502 (25), 476 (5), 431 (9), 407 (4), 362 (4), 328 (15), 305 (10), 274 (14), 234 (15), 212 (27), 192 (12).

Compound **5d**
Yield: 160 mg

MS m/z (%): 1243 $[M+H]^+$ (100), 1428 (60), 1201 (32), 1167 (27), 1137 (37), 1089 (35), 1056 (36), 1008 (23), 989 (24), 964 (27), 933 (32), 914 (36), 909 (26).

Compound **5e**
Yield: 154 mg

MS m/z (%): 1489 $[M+2Na]^+$ (23), 1466 $[M+Na]^+$ (100), 1442 $[M+H]^+$ (63), 1426 (44), 1360 (16), 1297 (11), 1242 (7), 1185 (8), 1126 (9), 1072 (8), 1027 (6), 990 (7), 918 (13), 823 (7).

Compound **5f**
Yield: 139 mg

MS m/z (%): 1660 $[M+Na]^+$ (86), 1637 $[M+H]^+$ (100), 1623 (100), 1592 (54), 1565 (44), 1394 (67), 1382 (56), 1334 (53), 1285 (53), 1270 (46), 1198 (51), 1168 (51).

Compound **6a**

Yield: 122 mg

MS m/z (%): 2063 (42), 2009 $[M+H]^+$ (100), 1966 (20), 1920 (20), 1839 (21), 1796 (19), 1738 (13), 1670 (22), 1622 (13), 1555 (11), 1482 (11), 1398 (20), 1342 (11), 1270 (10), 1229 (14).

Synthesis of Lipid-core peptides 7a-e [G]

The synthesis was accomplished on an MBHA (substitution 0.48 mmol/1g) (Novabiochem) resin starting from the Boc protected lipidic amino acid **1g**. The synthesis of the first and every subsequent level of the peptide construction was achieved using 4 M excess of N-Boc amino acids. For all residues the coupling, monitored by quantitative ninhydrin assays, was done with the preformed (DCC) symmetrical anhydride or with the addition of hydroxybenzotriazole in dichloromethane (15 ml), the second coupling was performed in dichloromethane, DMF or dichloromethane/N-methylpyrrolidone. When the coupling efficiency was below 99.4%, a third coupling was carried out as described for the second one. After the second (or third) coupling deprotection of the N-termini was performed in 65 % TFA in dichloromethane. The deprotected resin peptide was neutralized with 10 % diisopropylethylamine in dichloromethane. The peptide was removed from the resin support with the high (1.5 ml cresol, 1.5 ml thiocresol, 20 ml HF) to yield the crude peptide, which was precipitated with ether or ethylacetate and redissolved in 50 % acetic acid (20 ml).

Purification was achieved by RP-HPLC. Analytical HPLC separation was carried out on a Vydac C_{18} 5 RAC column. HPLC grade acetonitrile (Aldrich) and water were filtered through a 25 μm membrane filter and degassed with helium flow prior to use. Analytical separation was achieved with a solvent gradient beginning with 0 % acetonitrile, increasing constantly to 60 % acetonitrile at 30 minutes, staying at this concentration for 20 minutes and decreasing steadily to 0 % acetonitrile for 10 minutes at a constant flow of 0.7 ml min^{-1}. For preparative separation a TSK-GEL

semipreparative C_{18} column was used, and separation achieved with a solvent gradient beginning with 0 % acetonitrile, increasing constantly to 60 % acetonitrile at 180 minutes, staying at this concentration for 60 minutes and decreasing steadily to 0 % acetonitrile for 30 minutes at a constant flow of 7 ml min^{-1}. The gradient was effected by two microprocessor-controlled Gilson 302 single piston pumps. Compounds were detected with a Holochrome UV-VIS detector at 218 nm (analytical) and 230 nm (preparative). Chromatographs were recorded with an LKB 2210 single channel chart recorder. The crude peptides were purified by semipreparative HPLC method.

Compound **7a**

Anal $C_{54}H_{108}N_6O_4$ (905.46)

Calcd. C 71.62 H 12.02 N 9.28

Found C 71.55 H 11.97 N 9.02

MS m/e (%) = 905.6 $[M+H]^+$ (30), 652.4 (100)

Compound **7b**

Anal. $C_{66}H_{132}N_{10}O_6$ (1161.8)

Calcd. C 68.05 H 11.42 N 12.02

Found C 67.87 H 11.40 N 11.86

MS m/e (%) = 1162.5 $[M+H]^+$ (37), 908.8 (23), 780.8 (17), 652.6 (15), 369.2 (31), 277.3 (100)

Compound **7c**

Anal. $C_{90}H_{180}N_{18}O_{10}$ (1674.52)

Calcd. C 64.55 H 10.83 N 15.06

Found C 64.74 H 11.00 N 14.87

MS m/e (%) = 1674 $[M+H]^+$ (8), 1290 (11), 1162 (100), 1034 (17), 909 (80)

Compound **7d**

Anal. $C_{81}H_{160}N_{18}O_{12}$ (1578.27)

Calcd. C 61.64 H 10.22 N 15.99

Found C 61.55 H 10.12 N 15.59

MS m/e (%) = 1579 $[M+H]^+$ (100), 790 (40), 391 (38)

Compound **7e**

Anal. $C_{83}H_{162}N_{18}O_{12}$ (1604.31)

Calcd. C 62.13 H 10.18 N 15.72

Found C 62.27 H 10.18 N 15.55

MS m/e (%) = 1604 $[M+H]^+$ (100), 1492 (24), 1442 (22), 1284 (16), 1212 (21), 744 (28)

Synthesis of *Chlamydial* peptides 10, 11, 13 and 14

The Chlamydial peptides were synthesised from **1g** as described in method [G]. Protecting groups used were OBzl for Glu and Asp, pMeBzl for Cys and Bzl for Ser and Thr (Zhong *et al.* 1993).

Incorporation of *Chlamydial* peptide 14 into liposome

Multilamellar liposomes (MLV) were prepared from egg phosphatidylcholine (PC) and cholesterol (16 μmoles of each). The lipid mixture in chloroform was dried to a thin film at 20°C by evaporation of the solvent at a low speed in a rotary evaporator. In order to ensure the complete removal of the solvent, the lipid film obtained was left under a nitrogen stream for 5 min. Rehydration and formation of the MLV was afforded by the addition of 2ml of distilled water. The liposomes were allowed to anneal at 20°C for one hour.

Small unilamellar vesicles (SUV) were then produced by sonicating the MLV in an ice bath, with a 1 min on 30 sec off cycle fashion (x 10), to obtain a clear solution. The SUV produced were then centrifuged at 1000 x g for 5 min to spin down the titanium particles from the probe of the sonicator.

Peptide **14** was then introduced into the SUV by mixing and then freezing at -20°C or by immersion in liquid nitrogen. The solution was then freeze dried overnight until a powder was obtained. Rehydration of the powder in a controlled fashion resulted in the formation of dehydration-rehydration vesicles (DRV), with peptide **14** having been encapsulated. The rehydration was accomplished with 0.1ml of distilled water at 20°C for 30 min, after which PBS (0.1ml, 0.1M, pH 7.4 containing NaCl) was added and incubated for a further 30 min. Free peptide was separated from its entrapped counterpart by ultracentrifugation at 20,000 rpm for 20 min.

Synthesis of FMDV peptides 15, 16 and 17 [H]

The peptides were synthesised manually on MBHA resin (substitution 0.48mmol/g), starting from **1g**, in a reaction vessel consisting of a sintered glass filter, a tap to waste (under vacuum) and a screw cap. N-Boc amino acids (2mM) were dissolved in 0.5M HBTU/DMF solution (4ml), DIEA (460µl) was then added and the mixture was vortexed for 30 seconds to activate the free carboxyl group of the amino acid. An excess of DIEA is necessary since it was required to neutralise the resin *in situ*, removing the need for any separate neutralisation steps. After introduction to the resin, the reaction mixture was shaken for 10 minutes before the extent of coupling was assessed by quantitative ninhydrin testing. Amino acids not surpassing 99.4% coupling efficiency were subjected to a second coupling step using diisopropylcarbodiimide (DIC), in DMF. Deprotection of the N^{α}-Boc group was carried out by shaking with 100% TFA (2x1 minute), with the peptidyl resin being washed carefully in DMF, before and after this step. If Asn or Gln were the residues being deprotected then the resin was also washed with CH_2Cl_2 before and after the TFA cycle, to prevent any cyclisation of the N-terminal Asn or Gln, which had been reported when just DMF alone was used to wash the resin. Protecting groups used in the synthesis were OBzl for Glu, Bzl for Ser, Tos for Arg, pMeBzl for Cys and OcHxl for Asp. Cleavage from the resin was carried out as described in method [C].

Purification of the peptides was carried out by gel-filtration on Sephadex G-25 or G-50 and by RP-HPLC as described in method [G]. Molecular weight was determined

by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Synthesis of FMDV cyclic peptide 18

The cyclic peptide **18** was synthesised from **1g** as in method [H]. The protecting group for the C-terminal Cys was pMeBzl, while the side chain protection used for the Cys residues involved in the disulphide bridge formation was Acm groups. Once the peptide chain was completed, cyclisation was acomplished on the resin by treating the resin with 10 equivalents of iodine in 4:1 acetic acid : H_2O solvent for 2 hours. The iodine was then extracted by washing with CCl_4 (4x5ml) and then washing vigorously with DMF. The peptide was then cleaved and purified as in method [C].

Synthesis of streptococcal M-protein vaccine candidates 20a-c

The peptides **20a-c** were synthesised from **1g** as in method [H]. The side chain protecting groups used were OBzl for Glu, Cl-Z for lysine in the epitope sequence, Boc for lysine in the polylysine construct, Tos for Arg, Bzl for Ser and OcHxl for Asp. Molecular weight was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), broad envelope peaks were obtained on HPLC.

Synthesis of streptococcal M-protein vaccine candidates 21a-d

The peptides **21a-d** were synthesised from **1e**, **1f**, **1g** and **1h** respectively, as in method [H]. The side chain protecting groups used were OBzl for Glu, Cl-Z for lysine in the epitope sequence, Boc for lysine in the polylysine construct, Tos for Arg, Bzl for Ser and OcHxl for Asp. Molecular weight was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), broad envelope peaks were obtained on HPLC.

Synthesis of lipophilic HNE inhibitor 22a-d

General methods of solution phase peptide synthesis:

DCC mediated coupling

In a round bottom flask equipped with magnetic stirrer and drying tube, 1 equivalent of base is added to N^{α}-Boc protected and C-terminal methyl ester protected amino acids and HOBt (1 equivalent of each), dissolved in DCM or EtOAc. The reaction mixture was cooled to -10°C in an ice/salt bath and DCC (1.1 equivalent) was added to the reaction, which was allowed to stir overnight. The precipitated DCU was filtered and the solvent evaporated *in vacuo*. EtOAc (20 ml) was added to the residue, any precipitate filtered and the filtrate washed with 10% citric acid (5 ml), $NaHCO_3$ (sat) (5 ml) and brine (5 ml). The organic layer was dried over (anh) Na_2SO_4 and evaporated.

N^{α}-Boc deprotection

The Boc/methyl ester protected peptide was dissolved in 1M HCl/methanol and refluxed for 15 mins or until TLC showed no starting material remaining. The solvent was removed at room temperature and the addition of ether gave a yellow oil.

Ester hydrolysis

Removal of the ester groups used for C-terminal protection was carried out by alkali hydrolysis. Three equivalents of alcoholic potassium hydroxide (KOH) solution (1:1 ethanol-water) were used to one equivalent of peptide. The solution was stirred at room temperature for 15 mins with the reaction being followed by TLC. When hydrolysis had been completed the pH of the reactants was reduced to 3 using 1N HCl and the solution was extracted with EtOAc. The EtOAc solution was then dried over NA_2SO_4 and evaporated *in vacuo* to give the required compound.

Compound 22a

Yield: 2.15g (93%)

NMR ($CDCl_3$): 0.84 (9H,m,CH_3 2xval, C_{14}); 1.26 (20H,s,$(CH_2)_{10}$ C_{14}); 1.35 (6H,m,CH_3

alax2); 1.46 (9H,s,$(CH_3)_3$ Boc); 1.59 (1H,m,β-CH C_{14}); 1.98 (2H,m,β-CH pro, γ-CH pro); 2.15 (2H,m,β-CH val, γ-CH pro); 2.33 (1H,m,β-CH pro); 3.58 (1H,m,δ-CH pro); 3.66 (1H,m,δ-CH pro); 4.06 (1H,bs, α-CH C_{14}); 4.45 (1H,q,α-CH val); 4.58 (1H,m,α-CH ala); 4.68 (1H,m,α-CH pro); 4.77 (1H,m,α-CH ala); 4.98 (0.5H,bs,NH C_{14}); 5.12 (0.5H,bs,NH C_{14}); 6.68 (1H,m,NH ala); 7.30 (2H,m,NH val, NH ala).

MS m/z (%): 726 $[M+2Na]^+$ (24.8), 704 $[M+Na]^+$ (100), 682 $[M+H]^+$ (2.9), 626 (16), 604 (17.7), 508 (12.7), 341 (10.3), 215 (39.5), 198 (23.6), 149 (21.7), 85 (10.3), 71 (21.1), 57 (40.1).

Compound 22b

Yield: 0.68g (96%)

NMR ($CDCl_3$): 0.88 (12H,m,CH_3 2xval, 2xC_{14}); 1.28 (40H,m,$(CH_2)_{10}$x2 2C_{14}); 1.35 (6H,m,CH_3 alax2); 1.46 (9H,m,$(CH_3)_3$ Boc); 1.67 (2H,m,β-CH_2 C_{14}); 1.88 (2H,m,β-CH_2 C_{14}); 2.05 (2H,m,γ-CH pro,β-CH pro); 2.15 (2H,m,β-CH val, γ-CH pro); 2.36 (1H,m,β-CH pro); 3.59 (1H,m,δ-CH pro); 3.68 (1H,m,δ-CH pro); 4.01 (1H,bs,α-CH BocC_{14}); 4.46 (2H,m,α-CH alax2); 4.69 (1H,m,α-CH val,α-CH pro); 4.98 (1H,m,NH Boc C_{14}); 6.57 (1H,d,NH ala); 6.72 (1H,d,NH C_{14}); 6.86 (1H,bs,NH ala); 7.36 (1H,b,NH val).

MS m/z (%): 930 $[M+Na]^+$ (72.6), 830 (18.5), 734 (16.9), 633 (15), 205 (38.9), 198 (100), 71 (17), 57 (20.4).

Compound 22c

yield: 0.28g (97%)

NMR ($CDCl_3$): 0.88 (15H,m,CH_3 2xval, 3xC_{14}); 1.27 (60H,m,$(CH_2)_{10}$x3 3C_{14}); 1.34 (6H,m,CH_3 alax2); 1.48 (12H,m,$(CH_3)_3$ Boc,CH_3 ala); 1.67 (6H,bm,β-CH_2 3xC_{14}); 2.10 (5H,bm,γ-CH pro,β-CH pro, β-CH val); 3.61 (2H,m,δ-CH_2 pro); 4.06 (1H,bs,α-CH BocC_{14}); 4.21 (1H,m,α-CH ala); 4.29 (1H,m,α-CH ala); 4.46 (1H,m,α-CH val); 4.82 (3H,bm,α-CH C_{14}x2, α-CH pro); 5.13 (1H,bs,NH Boc C_{14}); 6.58 (1H,bs,NH C_{14}); 6.70

(1H,bs,NH C_{14}); 6.83 (1H,bs,NH ala); 7.50 (1H,m,NH ala); 7.70 (1H,m,NH val).

MS m/z (%): 1155 $[M+Na]^+$ (100), 1055 (19.4), 959 (17.1), 930 (17.8), 859 (9.4), 830 (10), 796 (5.7), 728 (5.4), 648 (6.0), 490 (14.6), 447 (6.3).

Compound 22d

Yield: 0.3g (100)

NMR ($CDCl_3$): 0.88 (15H,m,CH_3 2xval, 3xC_{14}); 1.29 (60H,m,$(CH_2)_{10}$x3 3C_{14}); 1.30 (6H,m,CH_3 alax2); 1.45 (12H,m,$(CH_3)_3$ Boc,CH_3 ala); 1.67 (6H,bm,β-CH_2 3xC_{14}); 2.11 (5H,bm,γ-CH pro,β-CH pro, β-CH val); 3.63 (2H,m,δ-CH_2 pro); 3.71 (3H,m,CH_3 Me ester); 4.05 (1H,bs,α-CH BocC_{14}); 4.21 (1H,m,α-CH ala); 4.30 (1H,m,α-CH ala); 4.42 (1H,m,α-CH val); 4.78 (3H,bm,α-CH C_{14}x2, α-CH pro); 5.13 (1H,bs,NH Boc C_{14}); 6.58 (1H,bs,NH C_{14}); 6.69 (1H,bs,NH C_{14}); 6.87 (1H,bs,NH ala); 7.51 (1H,m,NH ala); 7.70 (1H,m,NH val).

MS m/z (%): 1169 $[M+Na]^+$ (100), 1069 (22.4), 973 (13.9), 944 (27.8), 873 (38.9), 844 (5.1), 810 (7.0), 742 (6.4), 648 (4.7), 490 (14.9), 447 (4.6).

Synthesis of lipophilic HNE inhibitors 22e and 22f

The peptides were synthesised on PAM-Val-OH resin (0.58 mmole/g) (Novabiochem) by method [C], with the N-terminus being extended with three lipidic residues through the addition of the Boc protected residue **1g**. Before cleavage from the resin, the peptides were acetylated at the N-terminus using acetic anhydride (5ml) (a solution of tritiated acetic anhydride in DMF was used to acetylate **22f**) in the presence of DIEA (1ml) until the ninhydrin assay gave a result indicating complete acetylation. Since no protecting groups were required during the syntheses, it was not necessary to use any scavengers in the cleavage reaction and also the peptides were dissolved in 90% acetic acid (25ml) without prior precipitation with ether. The solution was then diluted to 100ml and lyophilised to yield a white solid.

Compound **22e**
Yield: 195 mg

MS m/z (%): 1073 $[M]^+$ (18), 1059 (66), 1055 (9), 1049 (7), 1043 (8), 1035 (43), 1033 (100), 1031 (25), 1029 (9), 1014 (7), 1010 (4), 1003 (5).

Compound **22f**
Yield: 188 mg

MS m/z (%): 1073 $[M]^+$ (10), 1059 (34), 1033 (54), 987 (4), 926 (5), 845 (12), 819 (6), 774 (7), 703 (4), 676 (4), 630 (4), 548 (6), 477 (25), 449 (100), 421 (30), 402 (26).

Synthesis of lipophilic HNE inhibitor 22g

Peptide **22g** was synthesised as for peptide **22e** but with the further addition of a glutamic acid residue, as Glu (OBzl), before acetylation of the N-terminal. Thus upon HF cleavage the C-terminal residue contained both an acetyl group and a free carboxylic acid.

Compound **22g**
Yield: 203 mg

MS m/z (%): 1226 $[M+Na]^+$ (100), 1203 $[M]^+$ (22), 1181 (9), 1097 (9), 1033 (8), 990 (21), 918 (10), 885 (6), 847 (11), 766 (13), 687 (12), 658 (11), 613 (71), 582 (16), 548 (12).

Synthesis of lipophilic HNE inhibitor 22h

Peptide **22h** was synthesised as for peptide **22e** but with the further addition of a palmitic acid moiety, which was coupled using standard DCC protocols [method C].

Compound **22h**

Yield: 160 mg

MS m/z (%): 1395 (34), 1293 $[M+Na]^+$ (33), 1270 $[M]^+$ (20), 1240 (20), 1150 (100), 1105 (12), 1033 (56), 986 (17), 915 (91), 885 (9), 844 (59), 808 (34), 773 (30), 743 (14), 690 (18), 639 (16), 582 (20), 547 (21), 519 (34), 449 (59), 421 (72).

EXPERIMENTAL:Biology

TRH and LHRH lipidic conjugate-CaCo-2 cell homogenate enzyme assay

Caco-2 cells were obtained from the American Type Culture Collection, Rockville, MD. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 1% nonessential amino acids, benzylpenicillin (10 IU/ml) and streptomycin (10μg/ml) in tissue culture flasks for 14 days. Cell culture media and tissue culture flasks were from Costar, Badhoevedorp, The Netherlands. Cells of passage 97 were used. After washing the monolayers with ice-cold isotonic 0.01 M phosphate buffered saline (PBS, pH 7.4), cells were scraped with a rubber policeman, placed in 50ml centrifuge tubes and resuspended and lysed in hypotonic 0.01 M PBS, pH 7.4 at 4 ^{0} C. Aliquots of the cell lysates (5 x 10^{6} cells/ml) were stored at -70^{0} C.

The respective peptides and peptide conjugates (0.1 μmol) were dissolved in 100 mM phosphate buffer (1ml, pH 7, containing EDTA and DTT) and incubated at 37°C with the CaCo-2 enzyme homogenate (1 ml). Samples (100 μl) were taken at various time intervals and the enzyme degradation reaction terminated by the addition of TFA (5 μl). The amount of peptide or peptide conjugate was determined by HPLC. The peptide or peptide conjugate degradation products were isolated by semipreparative HPLC and their identity determined by FAB-MS.

TRH and LHRH lipidic conjugates-Caco-2 cell monolayer experiments

The Caco-2 cells were cultivated on polycarbonate filters (Transwell, Costar, Badhoevedorp, The Netherlands) for transport experiments. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 1% non-essential amino acids, benzylpenicillin (10 IU/ml) and streptomycin (10μg/ml). Cells of passage 101 were used. The integrity of the cell monolayers was tested after 24-27 days in culture by measurement of transport of [^{14}C]mannitol and transepithelial electrical resistance. The mature monolayers had a mannitol permeability of <3 x 10^{-7}

cm/s and a transepithelial electrical resistance > 200 ohm cm2. The [^{14}C] mannitol permeability of the monolayers was also studied in the presence of unlabelled TRH-conjugates.

The cell monolayers were used in transport experiments after 27 days. All transport experiments were performed in Hank's balanced salt solution containing 25 mM Hepes (HBSS). The 3H-labelled TRH-conjugates (T1-T4) were dissolved in DMSO and the solutions were diluted in HBSS to final concentrations of 1.0-2.0 x 10^{-4} M. The final concentration of DMSO was 0.5 - 0.75%. DMSO at this concentration did not change the epithelial integrity. The solutions were added to the apical side of the Caco-2 monolayers. At the required time point, the cell culture inserts were transferred rapidly to new basolateral chambers and samples were withdrawn and analyzed in a liquid scintillation counter as described previously. Apparent permeability coefficients (Papp) were calculated. The filters were washed rapidly three times with cold PBS Dulbecco's and the filter associated radioactivity was determined.

Oral absorption studies of TRH and LHRH lipidic conjugates

Oral dosing: The respective peptide conjugates were dissolved in 50mM phosphate buffer (pH 3, containing 2% DMF) to a concentration of 1mg/ml. 1ml of the solution was then administered to male Wistar rats (approx. 220g) by oral gavage with a blunt tipped feeding needle. The animals were then sacrificed at 1, 3, 6 and 12 hours and the liver, stomach, kidneys, spleen and small and large intestine were removed along with a sample of blood (4ml). All digestive matter was removed from the organs and they were thoroughly washed in phosphate buffered saline (PBS). The organs were then homogenised to a fine slurry and 0.5ml of the homogenate was added to 2ml of Scintran tissue solubiliser, this was also done for blood. The samples were left for five days and allowed to dissolve with the aid of gentle shaking, after which the samples were decolourised using H_2O_2 (30%) and the radiolabel detected by liquid scintillation counting.

Extraction and characterisation of conjugates from blood: Rats were dosed with the

conjugates as described above and sacrificed after 3 hours, using a recommended humane method, blood samples were taken and the protein denatured using TFA (final concentration 10%). The denatured blood was then centrifuged (13000 rpm) and the pellet obtained was resuspended in DMF by vortexing. After further centrifugation the supernatant was removed and the DMF evaporated under reduced pressure in the presence of xylene. The resultant solid was dissolved in 50mM phosphate buffer (pH 3, containing 20% methanol) and run on HPLC using a C_4 Vydac column with acetonitrile/water as solvents. The target peak was then identified, collected and its identity confirmed by mass spectrometry. Furthermore for compound separation a Waters Quanta 4000 Capillary Electrophoresis System was employed a 50 mM phosphate running buffer containing 10 % methanol (pH 2.5) and for structure elucidation a Fisons matrix assisted time of flight laser desorption mass spectrometer was used.

Immunological studies with *Chlamydia* vaccine candidates [I]

Organisms and animals: C. trachomatis serovar A (G17/OT), B (TW5/OT) and C (TW3/OT) were grown in Hela 229 cells and EB were purified from infected cells by density gradient centrifugation (Peeling *et al.* 1984). The following strains of female mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and used at 7-9 weeks of age: C57BL/10snj ($H\text{-}2^b$), B10.A/sgsnj ($H\text{-}2^a$), B10.D2/j ($H\text{-}2^d$), B10.BR/sgsnj ($H\text{-}2^k$), SJL/j ($H\text{-}2^s$), Balb/cj ($H\text{-}2^d$) and CBA/j ($H\text{-}2^k$).

Immunization of mice: Antisera were raised to serovar B or C EB in mice as described by Zhong and Brunham (1992). Briefly, 5 x 10^6 inclusion forming units (IFU) of *C. trachomatis* EB per injection were used. The first injection was given in CFA by i.p. injection, the second injection in IFA by i.p. injection and the third and fourth booster injections were given intravenously in PBS 14 and 28 days later. Anti-peptide antisera were raised in groups of 4-6 mice from each strain injected with 50 µg of each peptide preparation by i.p. injection, emulsified in CFA on day 0 and boosted with 50 µg of the same antigen in IFA on days 14 and 28. Sera were collected 14 days after final injection. Sera from each group were pooled, alliquoted and stored at -20°C until use.

A combination of neuraminidase and galactose oxidase (NAGO) was recently reported to act as a noninflammatory adjuvant to enhance the immunogenicity of synthetic peptides (Zheng *et al.* 1992). Its potency was tested in B10 mice. NAGO adjuvant was prepared as follows: 1unit of neuraminidase (Sigma Chemical, St. Louis, MO) and 5 units of galactose oxidase (Sigma Chemical) in 100 µl of PBS containing 50 µg of peptide were mixed immediately before injection. The mixture was administered subcutaneously (tail base) with 50 µg peptide per injection per mouse. The injection was repeated twice at 14-day intervals. Sera were collected on the 14th day after each injection. Normal mouse sera were collected from each strain and used for control.

ELISA: Microtiter plates (Immulon 2, 96 round U well, Dynatech Laboratories, Alexandria, VA) were coated overnight at 4°C with 10^6 IFU/well of native EB in 100µl of sucrose-phosphate-glutamate (SPG) or 1 µg/well of peptides in 100 µl of PBS. The plates were washed with 200 µl of SPG or PBS once and blocked with 200µl/well of blocking solution at 37°C for 90 min. Serial dilutions of antisera were made in incubation solution and added to the plates at 100 µl/well. The plates were incubated at 37°C for 60 min. After washing four times with 200 µl/well of wash solution (0.05% Tween 20 in PBS, pH 7.4), goat anti-mouse IgG-peroxidase conjugate (Pierce, IL) at 1:4000 dilution in incubation solution was added at 100 µl/well and the plates incubated as before. After washing as before, 100 µl of substrate [2.2-azino-bis-(3-ethyl-benzthiazoline-6 sulphonate) in citrate buffer (pH 4.5)in the presence of H_2O_2] was added to each well. The enzymatic reaction was developed for 15 min at room temperature and the absorbance was measured at 405nm on a Titertek Multiskan plus version 1.43 automatic ELISA reader (EFLAB, Finland).

Immunisation protocols for Liposomal entrapped peptide 14

In all of the experiments male CD-1 mice (20-25g) were used in groups of four or five. For immunisation, intramuscular injections of 0.1ml in volume were given in the hind left legs, on days zero and 29. Blood samples were collected on day 28 for the primary response and 10 days after the booster injection for the secondary response. Where long term antibody responses were checked, samples were taken three months

after the booster injection. Blood was sampled from the tail veins (50 µl) using heparinised capillaries and these were transferred into eppendorfs containing PBS (0.45ml).

Immunisation protocol for FMDV peptides

Guinea pigs of approximately 400g were immunised with 30 µg of peptide in a mineral oil, except the LCP peptide 17 which was given in PBS. Injections were given intramuscularly, on days zero and 22 and blood samples were taken on day 21 for the primary response and day 49 for the secondary response. Antibody titers were evaluated by ELISA, using the method stated for the Chlamydial peptides, described earlier.

Immunisation protocols for Streptococcal M-protein peptides.

Peptides were administered subcutaneously at the tail base of mice of 5-6 weeks in age, in complete Freund's adjuvant, at a concentration of 30 µg. After initial immunisation, boosts were given at the same concentration in sterile PBS, on days 21, 35, 49 and 70. While animals were bled on days, 7, 20, 42, 69 and 88. Antibody titers were evaluated by ELISA, using the method stated for the Chlamydial peptides, described earlier.

***In vitro* human leucocyte elastase inhibitory assay**

The *in vitro* human leucocyte elastase (HLE) inhibitory activity of the compounds was determined using a method described by Hornebeck *et al.* The inhibitor (0-50 µg/ml) and substrate (succinyl trialanine para-nitroanilide, 0.78 µg/ml) were first incubated and the enzyme (2 µg/ml) added. The amount of substrate unaffected by the enzyme was then measured and the inhibition quoted as a percentage.

***In vivo* human leucocyte elastase inhibitory assay**

The *in vivo* HLE inhibitory studies were performed on Rabbit skin. Hairs were removed from the back of a male white Bouscat rabbit weighing 1500 g and the following solutions were injected intradermally at six different sites: (1) 0.2 ml of phosphate buffered salve pH 7.4 (PBS), (2) 500 μg of inhibitor in a 1:1 mixture of EtOH and PBS, (3) 7.6 μM HLE in 0.2 ml PBS, (4) 250μg of inhibitor in 0.1 ml of a 1:1 mixture of EtOH-PBS, followed in 15 minutes by 7.6 μM of HLE in 0.1 ml of PBS, (5) 7.6 μM of HLE in 0.1 ml PBS followed in 15 minutes by 250 μg of inhibitor in a 1:1 mixture of EtOH-PBS, (6) 7.6 μM HLE and 250 μg of inhibitor were injected together in a mixture of PBS and 50μl of EtOH. Skin biopsies were taken 3 h after the injections from the regions 1-6. After fixing, dehydration and embedding in paraffin in several sections of 5-6 μm thick, visualisation of the elastic fibres were achieved by staining with Orcein of Unna. Morphometric analysis was performed on the stained sections. The results were compared with those obtained without injection of the inhibitor or of the enzyme.

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LIST OF ABBREVIATIONS

Acm	Acetamidomethyl
ACTH	Adrenal corticotrophic hormone
$(Boc)_2O$	ditertiary Butyl dicarbonate
BOP	Benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CFA	Complete Freund's adjuvant
DCC	Dicyclohexylcarbodiimide
DIEA	Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNP	Dinitrophenol
DRV	Dehydration-rehydration vesicles
DTT	Dithiothreitol
EB	Elemental body
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetra-acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbant assay
EtOAc	Ethyl acetate
EtOH	Ethanol
FMDV	Foot and mouth disease virus
Fmoc	9-Fluorenylmethoxycarbonyl
FSH	Follicle stimulating hormone
GALT	Gut associated lymphoid tissue
^{1}H-NMR	Proton nuclear magnetic resonance
HBSS	Hepes buffer

HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HF	Hydrogen fluoride
HIV	Human immunodeficiency virus
HNE/HLE	Human neutrophil elastase/human leucocyte elastase
HOBt	1,Hydroxybenzotriazole
I.M.	Intramuscular
I.P.	Intraperitoneal
I.V.	Intravenous
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon-γ
IFU	Inclusion forming units
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-2	Interleukin-2
KLH	Keyhole limpet haemocyanin
LCP	Lipid-core peptide
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LPS	Lipopolysaccharide
m-NOBA	meta-Nitro benzoic acid
MAP	Multiple antigen peptide
MDP	Muramyl dipeptide
MeOH	Methanol
MHC	Major histocompatability complex
MLA	Monophosphoryl lipid A
MLV	Multilamellar vesicles
MS	Mass spectrometry
NAGO	Neuraminidase and galactose oxidase
NBP	Non ionic block polymer

p-MBHA	para-Methylbenzhydralamine
PAM	4-Hydroxymethylphenylacetamidomethyl
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PLGA	Poly (D,L-lactic, co-glycolic) acid
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
RES	Reticulo-endothelial system
RP-HPLC	Reverse phase-high performance liquid chromatography
S.C.	Subcutaneous
SEB	Streptococcal enterotoxin B
SPPS	Solid phase peptide synthesis
SUV	Small unilamellar vesicles
t-Boc	tertiary Butyloxycarbonyl
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethane sulphonic acid
TLC	Thin layer chromatography
TMS	Ttrimethylsilane
TRH	Thyrotropin releasing hormone
UWL	Unstirred water layer
VD	Variable domain
Z	Benzyloxycarbonyl