New Methodology for Solid Phase Peptide Synthesis

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This thesis is submitted in part fulfillment of the requirements of the degree of Doctor of Philosophy in the University of Edinburgh. Unless otherwise stated the work described is original and has not been previously submitted, in whole or in part, for any degree at this or any other university.

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To my parents.

This is what the **Lord** says, He who made the earth, the **Lord** who formed it and established it-the **Lord** is His name:

"Call to me and I will answer you and tell you great and unsearchable things you do not know".
Jeremiah 33:2,3

All things were created by **Him** and for **Him**. **He** is before all things and in **Him** all things hold together.
Colossians 1:16,17

ABSTRACT

A novel linker for the Merrifield solid phase synthesis of peptide amides is described.

This linker, based on 2-alkoxydibenzocycloheptadiene, enables release of the peptide derivative under very mild conditions and is compatible with base labile N^{α} protecting groups.

The efficacy of this linker in generating C-terminal peptide amides has been demonstrated in the synthesis of the natural products, little gastrin, big gastrin, substance P and bombesin.

Modification of the linker allows peptide hydrazides to be prepared. The use of the linker in producing unprotected peptide hydrazides has been exemplified by the synthesis of the ubiquitin fragments (43-47) $NHNH_2$ and (67-76) $NHNH_2$. Use of the linker for the synthesis of fully protected peptide hydrazides requires further development.

Initial experiments indicate that the linker, when substituted appropriately, may also be useful for the solid phase synthesis of fully protected peptide free acids to be used in fragment condensation.

Abbreviations.

AA	Amino acid
AcOH	Acetic acid
Boc	*t*-Butyloxycarbonyl
CM	Carboxymethyl
DCM	Dichloromethane
DCC	N,N-Dicyclohexylcarbodiimide
DIC	N,N-Diisopropylcarbodiimide
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulphoxide
EI	Electron impact ionization
EMS	Ethyl methyl sulphide
ether	Diethyl ether
FAB	Fast atom bombardment
Fmoc	9-Fluorenylmethyloxycarbonyl
HOBt	1-Hydroxy-1,2,3-benzotriazole
HPLC	High performance liquid chromatography
hrms	High resolution mass spectrometry
i-Pr	isopropyl
IR	Infrared
Mbh	4,4-Dimethoxybenzhydryl
n.m.r.	Nuclear magnetic resonance
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulphonyl
ROESY	Rotating frame nuclear overhauser enhancement spectroscopy
R_t	Retention time
SPPS	Solid phase peptide synthesis
t-Bu	tertiary butyl
TFA	Trifluoroacetic acid

TFMSA	Trifluoromethanesulphonic acid
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TOCSY	Total correlation spectroscopy
UV	Ultraviolet

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Chapter 1.
INTRODUCTION

1.1. Hormones and the Endocrine System.

The endocrine system[1] in humans and other higher organisms is a complex and highly efficient system through which multifarious bodily functions and metabolic processes are regulated. This system of biochemical administration consists of a number of glands and organs organized together which synthesise and secrete the chemical messengers known as hormones (hormone is derived from the Greek *hormao* meaning to excite or arouse). The hormone producing glands and organs include the hypothalamus, the pituitary gland, the thyroid gland, the pancreas, the intestine, the adrenals and also the gonads.

The hypothalamus is believed to be of fundamental importance in the regulation of the endocrine system. It is located within the brain and itself receives messages from other parts of brain. These signals initiate a number of events which ultimately result in other glands increasing or decreasing production and release of hormones. The hypothalamus secretes a number of hormones whose primary function is to regulate the release of other hormones from the pituitary gland. The pituitary gland in turn releases chemical messengers which elicit responses from other glands within the endocrine system and thus is responsible for the control of numerous physiological processes within the body.

The pituitary gland is divided into two separate parts, the anterior and posterior regions. The posterior region is responsible for the secretion of vasopressin and oxytocin which control water retention and uterus contraction respectively. These

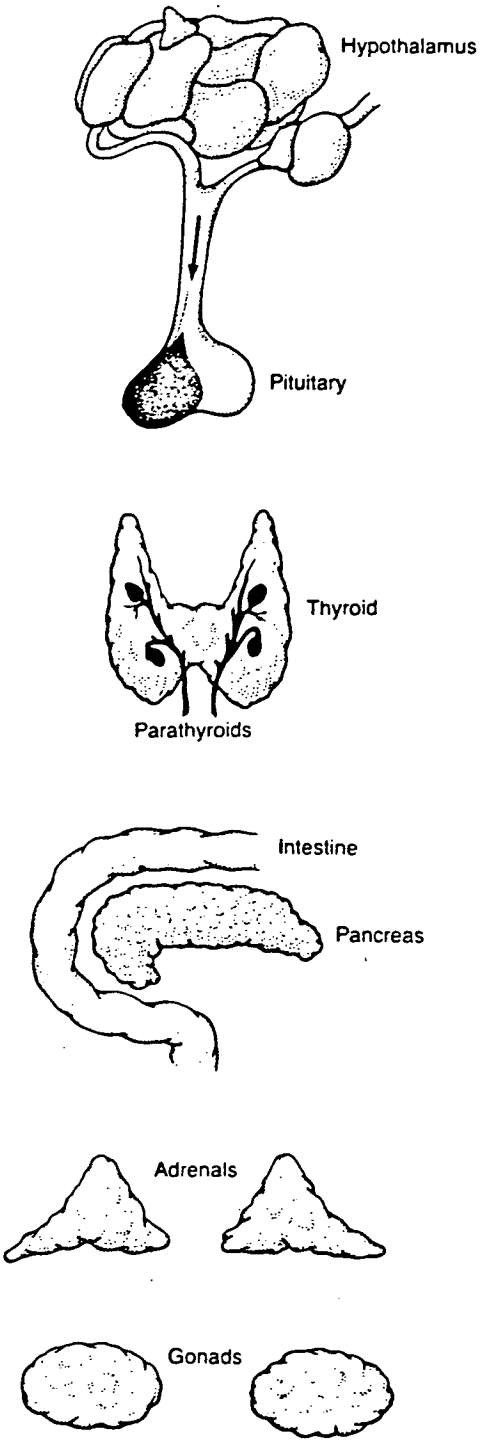


Figure 1.1 The major hormone producing regions of the human body.[2]

hormones are manufactured in the hypothalamus, packaged into storage granules and then moved down through nerve axons into the posterior pituitary. They are stored in axon terminals and then are released into cirulation upon demand. The anterior region of the pituitary is responsible for the release of a number of hormones which regulate body functions and these include growth hormone (GH), thyroid stimulating hormone (TSH), and follicle stimulating hormone (FSH). These hormones are synthesised in the pituitary itself and are then secreted directly into the bloodstream upon requiral. The release of anterior pituitary hormones is regulated by hormones produced in the hypothalamus which are then released into the hypothalamic-pituitary portal circulation. These include thyrotropin releasing hormone (TRH) which controls the release of TSH, luteinising hormone releasing hormone (LHRH) which controls the release of luteinising hormone and growth hormone releasing hormone which mediates in the secretion of GH.

The thyroid gland is responsible for the release of thyroxine which is a messenger that controls the rate of metabolism in a myriad of cell types. Thyroxine release is a prime example of the cascade of events which occurs under hormonal control. This cascade begins with the synthesis and release of TRH by the hypothalamus. TRH then travels to the pituitary gland *via* the portal circulatory system where it initiates the release of TSH. TSH is then secreted into the bloodstream through which it travels until it binds to its corresponding receptor in the thyroid gland and causes the release of thyroxine. Thyroxine is then distributed around the body where as mentioned it regulates physiological metabolism in a variety of cells. The level of thyroxine in serum also serves to regulate the production and secretion of TRH and TSH through feedback inhibition. Low levels of thyroxine actuate an increase in TRH and TSH levels through synthesis and release whereas high levels bring about the opposite effect.

The adrenal glands are responsible for secretion of steroid hormones including cortisol, aldosterone and epinephrine which regulate body preservation, salt conservation and stress response respectively. Hormone release from these glands is controlled by adrenocorticotropic hormone (ACTH) which is released from the pituitary and again is an element in the cascade which is initiated in the hypothalamus.

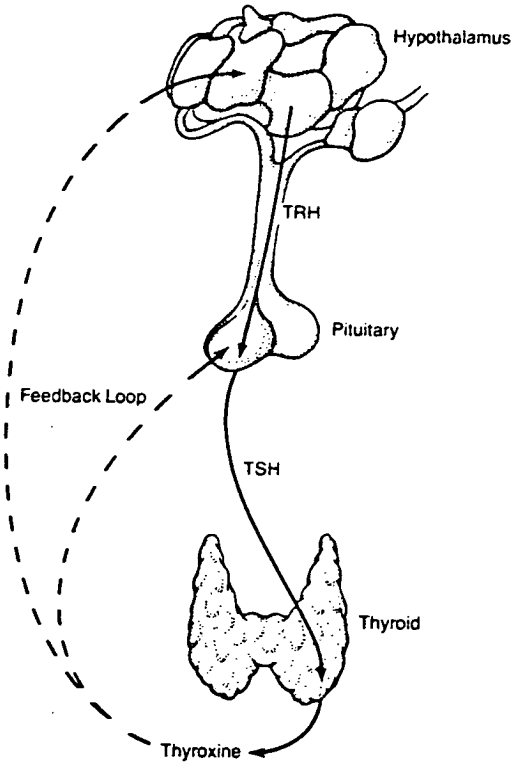


Figure 1.2 Events involved in the thyroxine cascade.[3]

The gonads are glands which produce and secrete the sex hormones estradiol, progesterone and testosterone. These steroid hormones are responsible for reproduction and for the development and maintenance of sexual characteristics.

Hormones are also responsible for controlling the rate of assimilation of food. These chemicals are synthesised for the most part in the intestine and pancreas and

then released into circulation. Insulin and glucagon are manufactured and released by the pancreas and function in the regulation of carbohydrate, fat and protein metabolism. Gastrin is a hormone, produced in the gut, which exists in a number of forms and has an intrinsic role in the regulation of gastric secretion, pancreatic secretion and gastro-intestinal tone and motility[4]. Pure gastrin was first isolated in 1964 by Gregory and Tracy[5] and its primary stucture determined soon after. Upon anticipation or ingestion of food, gastrin is released from the antral region of the stomach into the bloodstream through which it travels to the fundic region and effects secretion of gastric acid. In humans, gastrin is synthesised in nascent form as a 101 amino acid precursor known as preprogastrin[6]. This precursor then undergoes a number of post-translational modifications before its advent as the active hormone. These include several proteolytic cleavages at pairs of basic residues i.e. Arg-Arg or lys-lys, conversion of the C-terminal glycine extended peptide to its des-glycine amide and sulphation of tyrosine.

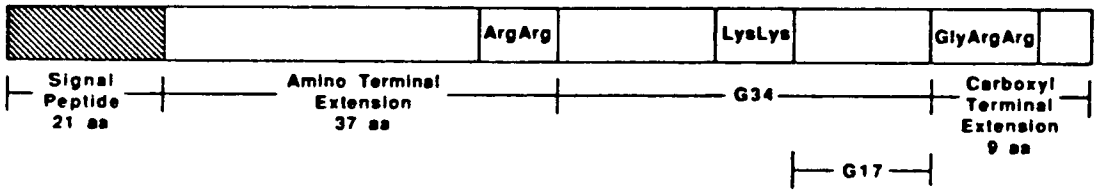


Figure 1.3 Schematic representation of preprogastrin showing cleavage sites. [7]

Gastrin as a biologically active hormone exists as two peptides known as big gastrin (G34) consisting of 34 amino acids and little gastrin (G17) which has 17 residues. Little gastrin is approximately five times more active than big gastrin[8] and is produced by proteolytic cleavage of G34 on the C-terminal side of a pair of lysine residues, followed by cyclisation of the N-terminal glutamine to pyroglutamic acid. Both G17 and G34 exist in sulphated and unsulphated forms and this modification is

believed to protect the peptide from degradation[9]. It has been shown through synthetic studies that the C-terminal tetrapeptide amide of little gastrin exhibits complete physiological activity *in vivo* .[10] This is the minimum size in order for complete manifestation of biological activity.

Glp-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

Glp-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu-Val-Ala-Asp-Pro-Ser-Lys-Lys-
Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

Figure 1.4 Primary structure of Little and Big Gastrin.

Gastrin releasing peptide (GRP) has been shown to stimulate the release of gastrin[11] and apparently acts directly on the gastrin producing cell. GRP is the mammalian analogue of bombesin, a peptide first isolated from frog skin.[12] The bombesin family of peptides have a wide spectrum of biological activity acting as neurotransmitters, autocrine hormones and thermoregulators.[13] In the stomach of mammals, bombesin like peptides (i.e. GRP and its derivatives) are found for the most part in the nerve fibres of stomach mucosa.[14] No bombesin like peptides have been found in the endocrine cells indicating that these peptides are part of the autocrine system.

Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-
Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

Figure 1.5 Sequence of human GRP-homology with bombesin is underlined.[13]

It can be seen that the endocrine system is paramount in the cohesive maintenance of physiological functioning. In the treatment of disorders of the endocrine system, it may be relevant to intervene as early as possible in the cascade of events which occur in the regulation of many physiological processes. This may lead to more efficient

remedies and fewer unwanted side effects. As almost all known hormones which initiate these cascades are peptide hormones, this provides the impetus for the chemical synthesis and study of these biologically significant molecules.

1.2. Neuropeptides.

With the discovery of enkephalins by Hughes and Kosterlitz[15] in 1975, a revolution in brain chemistry had its beginning. They isolated two small peptides from pig brain extracts which exhibited potent agonism of opioid receptors. From the study of enkephalins (designated leucine and methionine enkephalin), it became apparent that these peptides were localised in nerve endings.[16] This was an indication that these compounds were acting as neurotransmitters, a role previously assumed to be performed only by monoamines and amino acids. It was also demonstrated that enkephalin is released in a calcium dependant fashion[17], thus giving further corroboration of its role as a neurotransmitter. Until this time, it had not been known that peptides were involved in the propagation of nerve signals and the discovery of Hughes and Kosterlitz initiated a whole new sphere of interest in neuropeptides.

At present over one hundred neuropeptides have been identified and it has been estimated that the total number may be over two hundred. This group of neuropeptides embodies several other peptides which are an integral part of the endocrine system. These include ACTH, LHRH, TRH, oxytocin, cholecystokinin and gastrin.[18] The discovery of these hormones in the brain has dispelled the theory that peptide hormones have a discrete function and has paved the way for research into their multiple role in mammalian systems.

Among the peptides discovered in the brain which have a primary role in the central nervous system are the enkephalins, the endorphins, the dynorphins, substance P and neurotensin. Of these, substance P (SP) is one of the most extensively studied.

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 Substance P

It was originally isolated in 1931 from extracts of horse intestine[19] however it was not until 1970 that SP was isolated in pure form and shown to be a peptide amide of 11 residues.[20] SP is prevalent in approximately 20% of dorsal root ganglia which have extensions to the skin and spinal cord.[18] This distribution supports the assertion of SP's role as a sensory nerve transmitter i.e. in transmission of pain signals. This conjecture has been further validated through experiments demonstrating that SP is released from sensory nerves upon stimulation and that it causes powerful excitation of certain spinal cord neurones.[21] SP has also been found in small diameter nerve fibres which transmit nociceptive information to the central nervous system.[22a] Although this evidence may be circumstantial, it adds further weight to the putative role of SP as a pain transmitter.

Substance P is one of many neuropeptides which exist in the brain. Due to improved methods of isolation, the number of neuropeptides which have been isolated and characterised is constantly increasing. The elucidation of the structure and biological role of these peptides should lead to a better understanding of the many neurological disorders which occur and may conceivably result in efficacious treatments for these maladies.

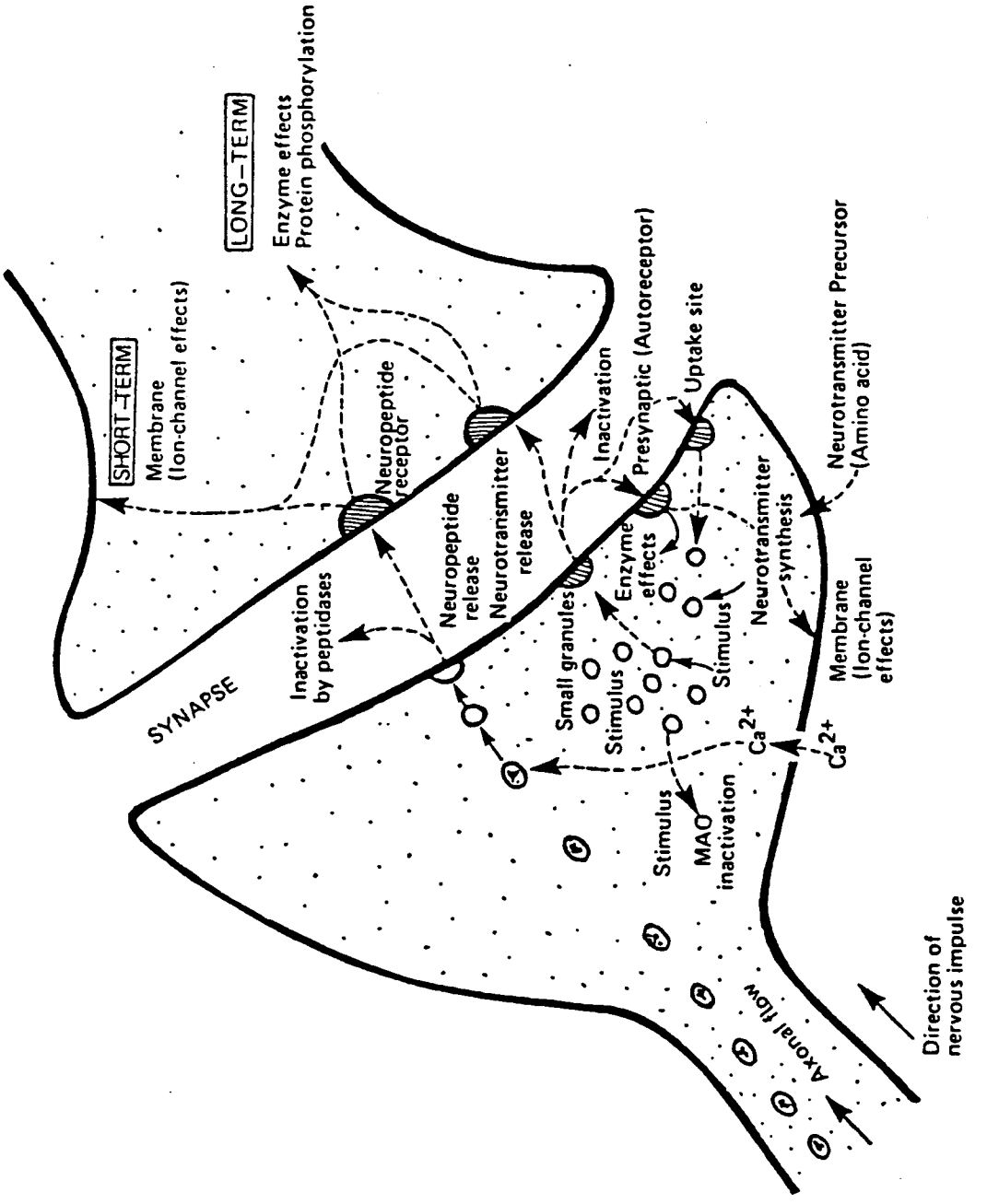


Figure 1.6 Events involving neurotransmitters at a neurone synapse[22b]

1.3. Post-translational processing of peptide precursors.

Peptide hormones and neurotransmitters are seldom if ever synthesised in their biologically active form. Bioactive peptides are produced on ribosomes as dormant precursors which in the case of hormones are known as preprohormones. These large polypeptides are then subjected to a number of processing steps before emerging as their corresponding fully active peptide. It is very likely that this processing plays a significant role in the regulation of the physiological activity of the final secretory product.

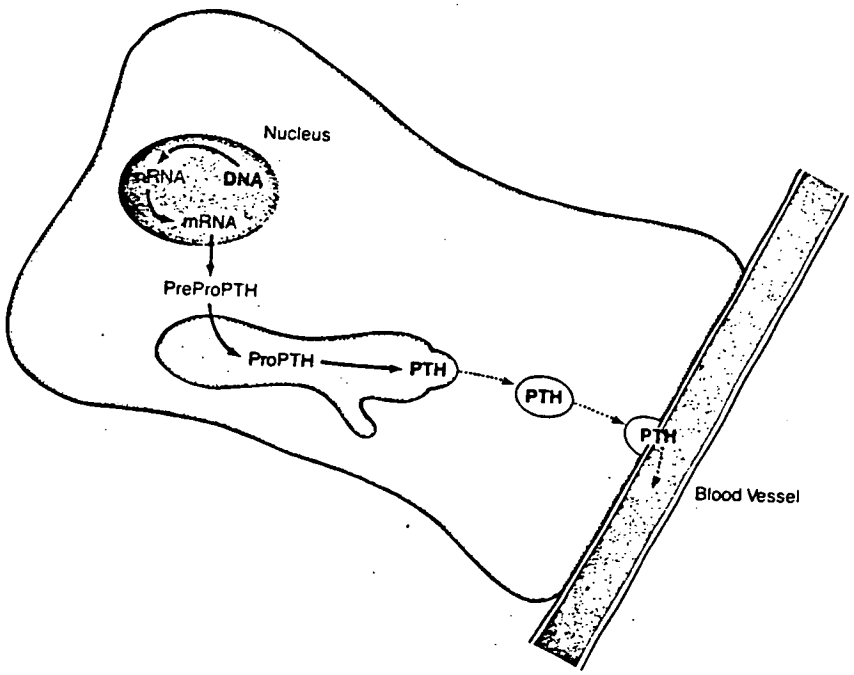


Figure 1.7 Schematic representation of post-translational processing of parathyroid hormone (PTH).[23]

1.3.1. Proteolytic processing.

In most cases, precursors contain initially a sequence of amino acids at their amino terminus which is later removed. This is known as the signal sequence and

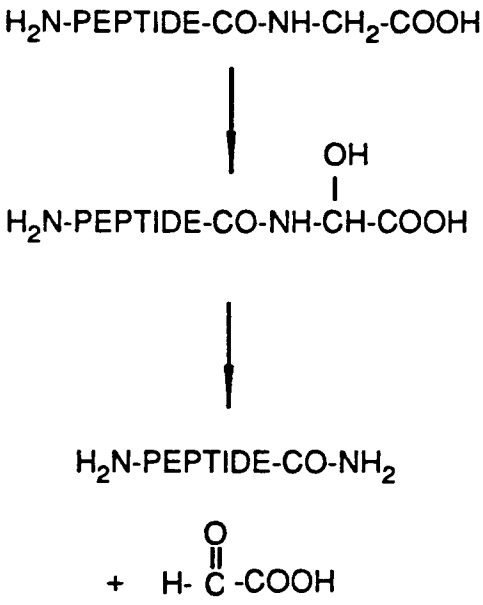
serves in translocation of the precursor across the membrane of the endoplasmic reticulum.[24] Once the peptide has been translocated, it is submitted to a trypsin-like proteolytic cleavage at a basic residue such as arginine or lysine[25] and the signal sequence is thus removed. This precursor may then undergo several more proteolytic cleavages before ending as the ultimate bioactive molecule. These transformations mostly occur through proteolysis of an amide bond usually on the C-terminal side of a pair of basic residues. This trypsin-like cleavage may then be followed if necessary by a carboxypeptidase-like cleavage of the two basic residues if they occur on the C-terminal side of the active molecule.[24] In the case of many endocrine hormones, the bioactive molecule is packaged into storage granules subsequent to proteolysis where it is stored until it is required to be released into circulation.[26] This final proteolytic step often occurs only after other post-translational modifications which may include sulphation, acetylation, phosphorylation and C-terminal amidation.[25]

1.3.2. C-terminal amidation.

It has been estimated that approximately half of the known peptide hormones and neurotransmitters have a C-terminal amide functionality[27] in contrast to the carboxylic acid function which is found with the majority of peptides and proteins in nature. This feature is found almost exclusively with neuropeptides and endocrine peptides and in most cases is essential for the biological activity of the secretory product.[28] It is plausible that the amide function is involved in receptor recognition and binding and that it contributes stability towards C-terminal exopeptidases.[29]

The mechanism through which α-amidation occurs has been demonstrated to involve the generation of a precursor to the bioactive peptide which is extended by a glycine residue at the C-terminus.[29] The glycine extended peptide is then subjected to transformation into the C-terminal amide through involvement of a class of enzymes

known as peptidyl α -amidating monooxygenases.[30] These enzymes have been shown to require copper, O_2 and ascorbate[31] and they also appear to have a requirement for a neutral amino acid in the position next to the C-terminal glycine.[32] This is consistent with the fact that most natural peptide amides indeed have a neutral amino acid C-terminal residue.



Scheme 1. Proposed mechanism of C-terminal amidation.

The above mechanism has been verified through the use of substrates containing C-terminal radiolabelled glycine.[33] The enzyme mediated generation of the amide occurs with accompanying formation of glyoxylate. Use of substrates labelled with ^{14}C-glycine resulted in incorporation of the radiolabel into glyoxylate. Incubation of the enzyme with substrates labelled with ^{15}N-glycine led to incorporation of the label into the C-terminal amide function. As further evidence, the amidation reaction has been shown to be unaffected by the presence of ammonium ions, glutamine or asparagine.[33] Peptidyl α -amidating monooxygenases have been shown to be present in secretory granules[31] which may indicate that α -amidation plays a role in the

regulation of physiological activity although no direct evidence has been obtained to substantiate this assertion.

1.3.3. Other post-translational modifications.

Other modifications of inactive or active precursors of hormones and neuropeptides include acetylation, phosphorylation and sulphation.

Acetylation of the amino terminus of bioactive peptides is common however its physiological role is not well understood. Acetylation of α-melanotropin greatly increases its skin darkening capability however conversely, acetylation of β-endorphin markedly decreases its opiate potency.[34]

Phosphorylation has been demonstrated to occur shortly after synthesis of peptide precursors and has been observed in ACTH.[35] Many other bioactive peptides have potential sites for phosphorylation, i.e. serine residues, however less is known about these.

Sulphation of tyrosine residues has been observed in the various forms of cholecystokinin (CCK) and gastrin.[25] Sulphation of CCK is essential for its functions i.e. stimulation of gall bladder emptying and pancreatic secretion.[36] Sulphation of gastrin is not a prerequisite for its physiological activity although it is believed it may prevent its degradation by enzymes.[9]

1.4. Solid phase peptide synthesis.

1.4.1. Overview.

As a consequence of the innovation of Merrifield[37] and the subsequent developments of many researchers[38], the synthesis of peptides has in many respects become routine. The solid phase technique lends itself especially well to peptide synthesis and has been applied successfully countless times in the assembly of many naturally occurring molecules and their analogues. Solid phase peptide synthesis (SPPS) is conducive to automation due to the repetitive nature of chain elongation and currently there are several automatic peptide synthesisers available. Each set of reactions and procedures which constitute the coupling of one amino acid is designated as a cycle. These reactions include deprotection of the N^{α} amine, coupling of the next amino acid and capping of any unreacted amine function.

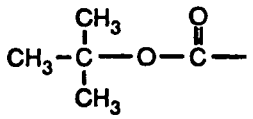
The solid support most commonly used today is the original Merrifield resin which is polystyrene crosslinked with divinylbenzene.[37] Many other resins have been evaluated for their swelling properties and usefulness as solid supports. One other support in widespread use is the polyamide resin developed by Sheppard.[39]

In order to attach an amino acid to a solid support for the purpose of synthesising a desired peptide, the resin must first be functionalised. This is accomplished through use of a linker or handle. The linker is itself designed so as to facilitate removal of the completed peptide from the resin under conditions which are compatible with other facets of peptide synthesis such as N^{α} and side chain deblocking. The linker can thus be varied depending upon the requirements of the overall synthetic strategy. One conventional linker which is widely used is the *p* -alkoxybenzylalcohol resin developed by Wang.[40] (1)

Solid phase synthesis is currently accomplished mainly using either one of two approaches. These are the Fmoc and Boc methodologies.

1.4.2. Boc methodology.

The Boc approach[41] involves the use of graded acidolysis for the removal of N^{α} and side chain protection and cleavage of the peptide-resin link. This involves the use of a linker and side chain protecting groups which are stable to the acidolytic conditions used for removal of the N^{α} protection which is Boc or *t*-butyloxycarbonyl (2).



(2)

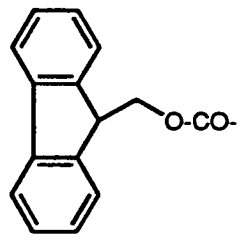
The side chain protecting groups employed with Boc syntheses are for the most part benzyl based derivatives however histidine and arginine differ in that they are usually protected with the tosyl group. The indole group of tryptophan is usually protected with the formyl group in order to preclude alkylation of the indole ring. These groups require treatment with hydrogen fluoride for removal and thus are stable to trifluoroacetic acid which is used for repetitive removal of N^{α} protection.

The linkers commonly used in Boc methodology, as with side chain protection, are based on benzyl derivatives with the peptide C-terminus forming a benzyl ester with the solid support. Tam[42] has developed the high-low HF or TFMSA procedure for the final removal of side chain protection and cleavage of the peptide-resin link. Using this protocol, the side chain protection is removed with low HF concentrations

and thus the side reactions which are likely to occur at this stage are minimized. The side reactions which may occur during HF cleavage include anisylation of glutamic acid, β-aspartamide formation, N-O shift in serine and threonine, alkylation of tryptophan, tyrosine and cysteine and oxidation of cysteine.[43] The low HF or TFMSA cleavage results in S_n2 cleavage of benzyl blocking groups and thus obviates reactions between side chain functionality and benzyl carbenium ions.[44] The high HF or TFMSA cleavage involves the use of a high concentration of these acids to release the resin bound peptide.

1.4.3. Fmoc methodology.

The Fmoc method involves an orthogonal approach to SPPS as N^{α} and side chain protection are base and acid labile respectively. The N^{α} protection used is the urethane, 9-fluorenylmethyloxycarbonyl (Fmoc) (3) developed by Carpino[45] and exemplified in peptide synthesis by Sheppard.[46] One other base labile N^{α} protecting group developed recently by Ramage[47,48] is the bis-nitrophenylethyloxycarbonyl (Bnpeoc) group (4). These groups are removed by piperidine through a E1cB β-elimination mechanism.



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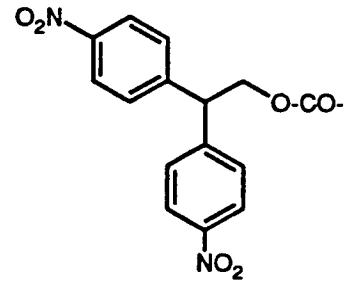


(4)

The side chain protecting groups currently employed with the Fmoc strategy are mostly *t* -butyl based with the exception of histidine and arginine where recent advances[49,50] have done a great deal to resolve the complications associated with these residues. All of these protecting groups are removed with trifluoroacetic acid.

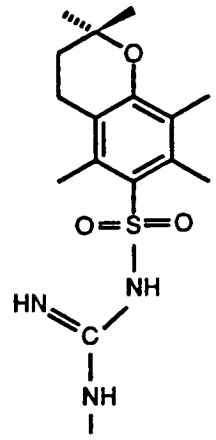
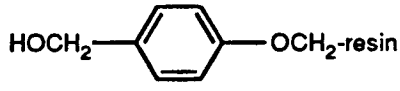


Figure 1.8 The pentamethylchromansulphonyl group for arginine N^G protection. The chroman ring holds the oxygen electron lone pair in the plane of the aromatic ring and thus allows delocalisation into the d-orbitals on sulphur. This facilitates acidolysis of the S-N bond, broken in the rate determining step.

The Wang linker[40] (1) is extensively used in Fmoc chemistry and effects release of the resin bound peptide under conditions similar to those involved in side chain deprotection. The *p* -alkoxy group of this linker facilitates acidolysis by stabilising the incipient carbonium ion involved in the release of the resin bound peptide.



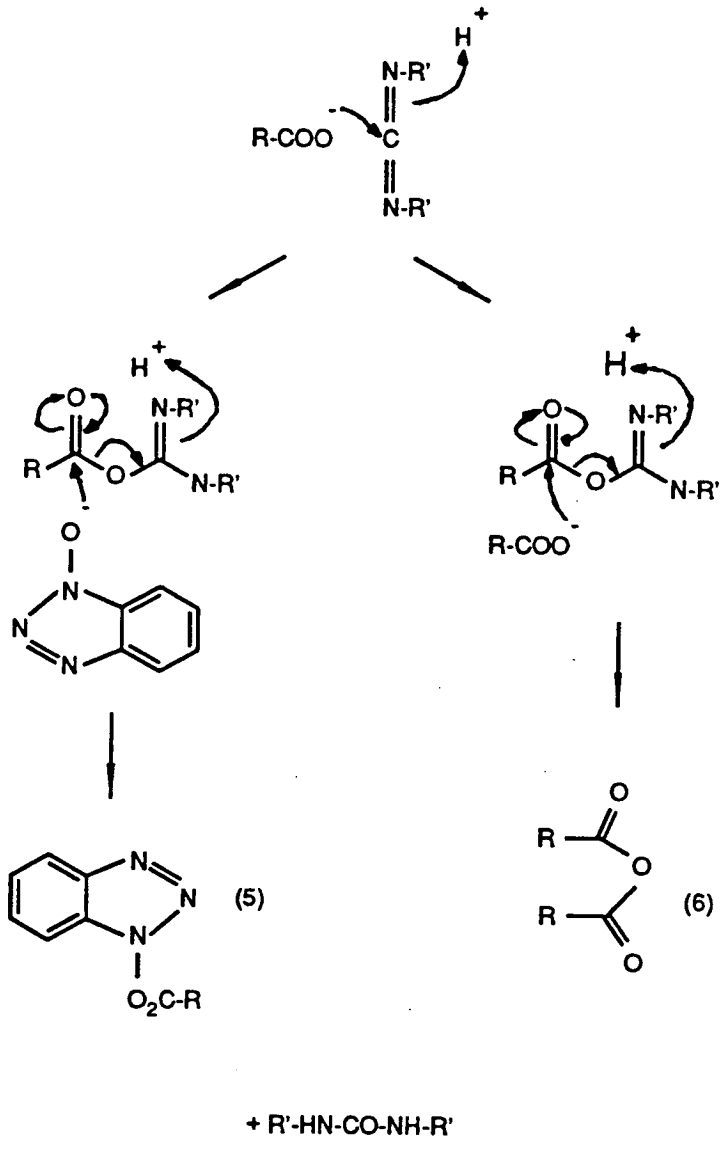
(1)

Amino Acid	Fmoc methodology	Boc methodology
Arg	Pmc	Tosyl
Asp,Glu	O^{t}Bu	OBzl
His	Bum, Trt	Bom, Dnp
Lys	Boc	2Cl-Z
Ser,Thr	^{t}Bu	Bzl
Tyr	^{t}Bu	BrZ
Trp	none	Formyl
Cys	Trt, Acm	MeBzl, Acm

Table 1. Commonly used side chain protecting groups in Fmoc and Boc chemistry[38]

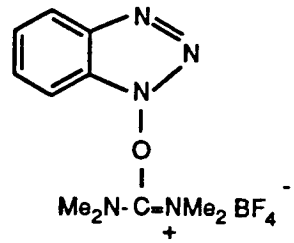
1.4.4. Activation of amino acids.

In effecting peptide bond formation during chain assembly, there is a plethora of reagents which have been developed and used.[51] Despite this abundance, there are only a few such coupling reagents which are in widespread use. The mainstay of these activating agents is the preformed symmetrical anhydride (6). These are formed by the treatment of a protected amino acid with a carbodiimide such as diisopropylcarbodiimide or dicyclohexylcarbodiimide and have been demonstrated to give reliably efficient amide bond formation.[52] Another extensively used activated species is the N-hydroxybenzotriazole ester of a protected amino acid (5) which also undergoes efficient condensation with the N^{α} amine of the growing peptide.[53]

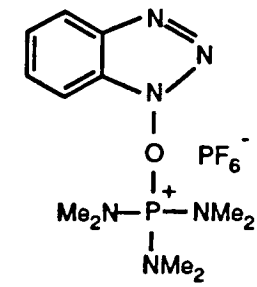


Scheme 2. Mechanism of formation of HOBt active ester and symmetrical anhydride.

More recent additions to the growing number of coupling reagents include the HOBt - tetramethylurea derivative TBTU (2-(1H-benzotriazol-1yl)-1,1,3,3-tetra methyluronium tetrafluoroborate) (7) and its hexafluorophosphate analogue HBTU[54] and the HOBt-phosphoramide derivatives BOP[55] (benzotriazol -1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate) (8) and PyBOP (the tris pyrrolidino derivative of BOP). These compounds have been shown to be extremely efficient at generating the HOBt active ester and thus have found extensive use in SPPS.



(7)



(8)

Capping is performed subsequent to coupling to preclude the occurrence of deletion peptides. This involves blocking irreversibly any unreacted free amine of the N-terminus of the growing peptide. It is usually accomplished using a vast excess of acetic anhydride. This reagent has less steric hindrance at the electrophilic carbonyl than does an activated amino acid and thus may be able to react more readily with any free amine. The acetylated peptide will then be terminated and also should have different chromatographic properties from the desired final product.

It can be seen that the concept of peptide synthesis on a solid support is both feasible and desirable. As a consequence of the repetitive nature of peptide synthesis and the large excesses of reagents used in each step of chain elongation, it is desirable to alleviate the problems associated with purification at each stage. Thus by immobilizing the peptide upon a solid support, any excess reagents can simply be

washed away, therefore circumventing the need for intermediate purification. It is this methodology which has streamlined peptide synthesis and greatly facilitated the technique as a tool in the study of multifarious biologically significant and active molecules.

1.5. The synthesis of C-terminal amides on a solid phase.

Due to the widespread incidence of natural peptides terminating with a carboxamide function, these compounds are interesting both in their structure and in their use as biological probes. As mentioned previously, peptide amides almost exclusively function as endocrine hormones and as neurotransmitters. Also, in the study and use of peptides, it is often desirable to remove the charge forming capability of the C-terminal free acid. Fragments of proteins to be used for immunological purposes will have an 'unnatural charge' at their C-terminus which may have an undesirable affect on the immune response. By synthesising these peptides as their corresponding amide, the C-terminus will be neutral and thus be similar to the amide backbone of the parent molecule. In conformational studies of peptides which are subunits of larger molecules, it may be desirable to again synthesise these molecules as their C-terminal amides since the free acid may contribute an undesired effect to their folding and stability. These factors along with their significance in nature, give impetus to the chemical synthesis of C-terminal amides and their use in the study of biological systems.

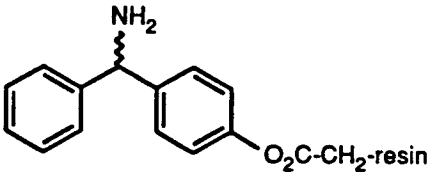
The first milestone in the synthesis of peptide hormones came with the synthesis of oxytocin by du Vigneaud and colleagues.[56] Since this time, the methodology in peptide synthesis has been progessively refined and the emphasis has shifted from solution to solid phase synthesis. Solid phase synthesis has facilitated rapid and efficient synthesis of peptides while advances in chromatography and other

purification techniques have led to the isolation of peptides in very high purity.

In the synthesis of C-terminal peptide amides by SPPS, the rational approach is to functionalise the linker so as to generate the C-terminal amide upon cleavage of the peptide-resin link.

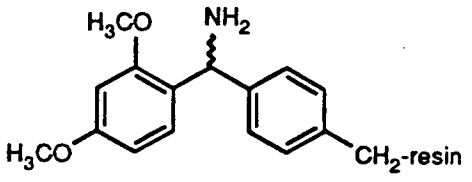
Several linkers have been developed that afford peptide amides upon lysis of the peptide-resin link and these employ a variety of conditions to accomplish this including ammonolysis and varying degrees of acidolysis. Ammonolysis of a benzyl ester linkage has been used by Sheppard and Atherton[57] in the synthesis of oxytocin derivatives. The rate of cleavage however, has been shown to be slow at hindered amino acids and this is undesirable as prolonged exposure of peptides to ammonia may cause deleterious side reactions such as partial racemisation, β -aspartimide formation and lysis of amide bonds.[58]

Linkers which liberate peptide amides upon acidolysis are mostly based upon benzylamine or benzhydrylamine derivatives. Tam[59] has reported the synthesis of peptide amides using the *p* -acyloxybenzhydrylamine (9) resin. This resin liberates the peptide amide upon treatment with HF or TFMSA and thus is compatible with the Boc methodology. Pedroso and coworkers[60] have reported the use of a benzylamine resin in the synthesis of LHRH using Boc chemistry.

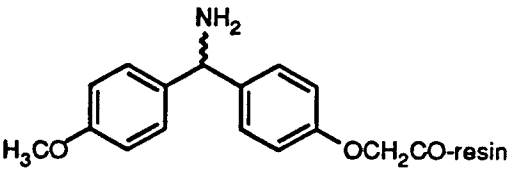


(9)

With the widespread use of Fmoc chemistry in recent years, there developed a need for an amide linker whose cleavage conditions were compatible with the different conditions used for side chain deblocking with this approach. One of the first such linkers was the dimethoxybenzhydrylamine resin (10) developed by Penke and Rivier.[58] The electron donating methoxyl groups facilitate acidolysis of the peptide-resin link and thus enable cleavage of the peptide amide in trifluoroacetic acid. A similar methoxyl substituted benzhydrylamine system (11) was used by Breipohl[61] and its use was demonstrated by the synthesis of oxytocin and LHRH.

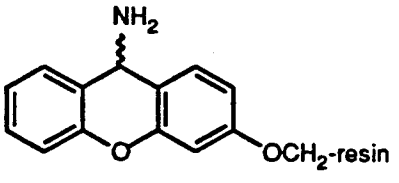


(10)



(11)

Several amide linkers have been reported that exhibit high acid lability. These typically employ low concentrations of TFA in a chlorinated solvent in order to effect release of the peptide amide from the solid support. The Sieber handle[62] based on the alkoxyxanthenyl system (12) allows cleavage of the peptide-resin link in 2% TFA in 1,2-dichloroethane. The Rink linker[63] for the synthesis of protected peptides has been shown to manifest acid lability under very mild conditions. It would seem probable that the amide linker variant of this molecule[63] (13) would exhibit high acid sensitivity although the cleavage conditions have not been optimised.



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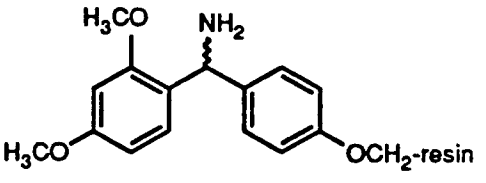
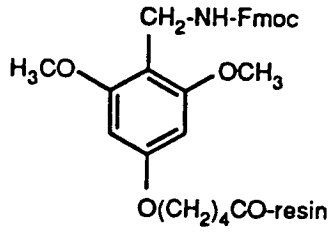


(13)

A recent comparison of acid labile linkage agents for the synthesis of C-terminal amides[64] reports that the PAL linker (14) developed by Albericio *et al.*[65] exhibits the fastest rate of cleavage of the peptide-resin link among those compounds studied. The linkers described also included those developed by Rink[63] (13) and Yajima.[66] (15) This study was accomplished by treatment of each Fmoc-valine loaded resin with TFA containing 5% phenol by weight for various times and then quantifying the remaining protected amino acid left on the resin.



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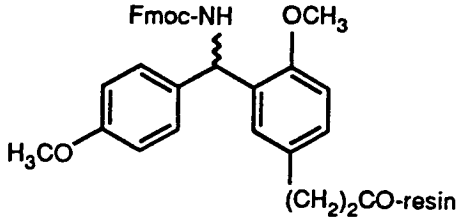
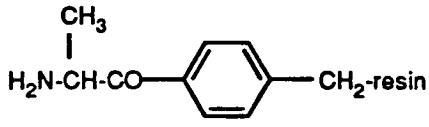


(15)

Another approach to the synthesis of C-terminal amides incorporates the use of photolabile linkers. One such handle is the α-methylphenacylamido resin (16) proposed by Ajayaghosh and Pillai[67] which affords the peptide amide upon photolysis.



(16)

It can be seen that C-terminal amides are desirable substances to synthesise from a chemical and biological perspective and that many approaches utilising a variety of conditions have been used in their synthesis. Despite the array of methods available, there still exists scope for improvements in the methodology for the solid phase synthesis of these important peptides.

1.6. Fragment condensation in peptide and protein synthesis.

In the total synthesis of peptides and proteins, there are generally two principal methods which are used. These are solid phase stepwise elongation and fragment (segment) condensation. Neither method can be said to be consummate in peptide synthesis since depending upon the requirements of an individual synthesis either one or both may have inadequacies. The advantages of fragment condensation lie in the convergent nature of this type of synthesis. Subunits of the target molecule can be synthesised quickly and efficiently, purified with relative ease and then covalently attached in the appropriate manner to obtain the desired product. One limitation of stepwise elongation on a solid phase is the size of the peptide to be synthesised. With current methodology, stepwise synthesis is routine for peptides up to about 40 residues. In larger molecules, purification may become intractable due to side reactions and the generation of by-products with similar chromatographic properties to the desired compound. In the synthesis of proteins it can thus be expedient to synthesise substituent fragments of the required molecule 30 to 40 amino acids in length in a stepwise manner, purify them and then condense them to generate the larger molecule.

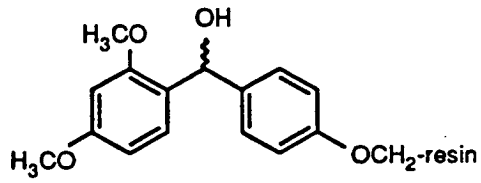
1.6.1. Synthesis using maximally protected fragments.

In order to prevent the occurrence of deleterious side reactions during fragment

condensation, it is desirable to protect amino acid side chains. These include the ε-amino group of lysine, the guanidino group of arginine, the hydroxyls of serine, threonine and tyrosine, the carboxyls of aspartic and glutamic acid and the sulphydryl group of cysteine.

In the solid phase synthesis of protected peptide fragments, it is necessary to release the completed peptide from the solid support without removal of side chain blocking groups. This inevitably involves orthogonal conditions for the removal of N^{α} protection, side chain protection and the cleavage of the peptide-resin link. The two main approaches to this have been graded acidolysis and the use of fluoride ion to release the peptide from the resin.

In the graded acidolysis approach, several linkers have been developed which allow cleavage of the peptide-resin link under very mildly acidic conditions. With base labile Fmoc N^{α} protection, *t* -butyl based esters, ethers and urethanes are typically used to protect side chain functionality. Since *t* -butyl blocking groups require treatment in 50% TFA/DCM for 15 minutes, criteria must be selected in the design of the peptide-resin link which allow a greatly increased rate of acidolysis. A linker developed by Rink[63] (17) fulfills these requirements in that it allows release of the peptide in 10% acetic acid in DCM and thus obviates removal of side chain protecting groups.



(17)

Another linker useful in the preparation of protected fragments by graded acidolysis employs the chlorotrityl group.[68] (18)

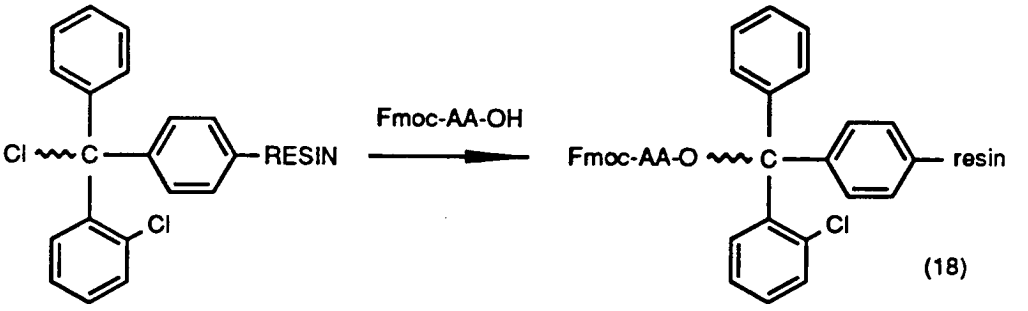
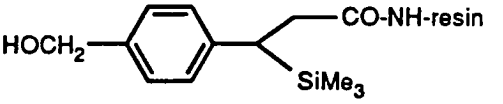


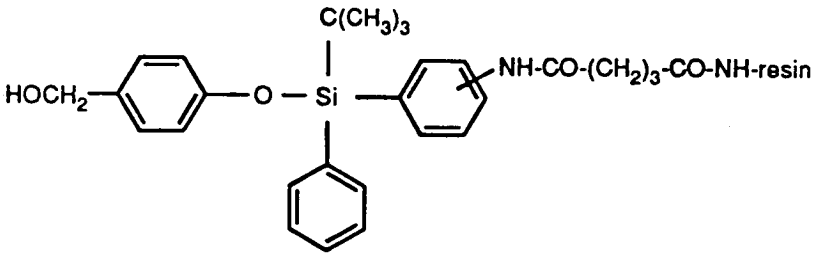
Figure 1.9 Loading of the first amino acid onto chlorotrityl resin.

Fluoride ion cleavage of the peptide-resin link is another orthogonal approach which has been applied successfully to the synthesis of protected fragments. Linkers which allow this type of cleavage are generally derivatised with silicon which is particularly susceptible to attack by the fluoride ion. One such linker has been developed by Ramage[69] and is based on a trimethylsilyl derivative of methyl 4-methylcinnamate (19). This handle affords fully protected peptide fragments upon treatment of the resin bound peptide with tetrabutylammonium fluoride in DMF for 5 minutes and is compatible with base labile N^{α} protection.



(19)

Another variation of this concept was devised by Barany[70] (20). This linker utilizes a *t*-butyldiphenylsilyl group and thus facilitates cleavage with the fluoride ion.



(20)

Other approaches to the solid phase synthesis of fully protected fragments include the use of photolabile linkers[71] and handles cleaved by nucleophiles such as hydrazine[72] and N-hydroxypiperidine.[73]

1.6.2. Synthesis using minimally protected fragments.

A major drawback to the fragment condensation approach is the insolubility of fully protected peptides in aqueous and organic systems. This is assuredly a consequence of the non polar, non ionic nature of protected fragments. One approach which has been used to circumvent this problem involves the use of minimal protection of fragments.[74,75] In this strategy, only the residues with the most reactive side chains are protected while all the others are left free. With the acyl azide method of coupling,[76] the C-terminus can be activated without affecting other side chain carboxylic acids. This method therefore gives scope to the coupling of fragments with unprotected aspartic and glutamic acid residues. Serine, threonine and tyrosine residues can also be left unprotected as their side chain hydroxyls are not nucleophilic enough to react at an appreciable rate under normal coupling conditions. Arginine

can be effectively protected simply by protonation[77] however cysteine is normally protected mainly to prevent dimerisation during purification and to allow selective disulphide formation. Lysine protection is essential in almost all circumstances due to the high nucleophilicity of the ε-amino group. Typical protecting groups which have been used include formyl, Boc and chlorobenzyloxycarbonyl depending on the requirements of the synthesis. Development of a lysine protecting group which does not confer hydrophobicity to a peptide would be a desirable asset in fragment condensation.

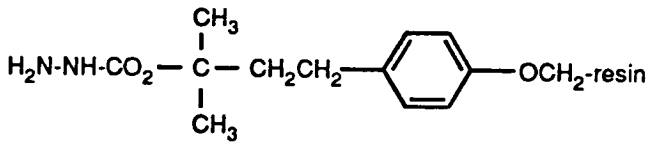
It can be seen that partial protection of peptide fragments should help to alleviate the problems of solubility of protected fragments although in any individual synthesis this will obviously be sequence dependant. Side chain functionality such as the guanidinium group of arginine and the carboxyls of aspartic and glutamic acid will be free to ionize and thus should add hydrophilicity to the fragment. This will make it more amenable to aqueous based purification methods as well as increasing solubility in organic solvents such as DMF used in coupling reactions.

1.6.3. Activation of the fragment C-terminus.

In the coupling of peptide fragments to generate larger peptides or proteins, the C-terminus of the requisite fragment must first be activated. This has been done by a number of methods which include the use of acyl azides[78], DCC/HOBt[79], DCC/ethyl-2-(hydroxyamino)-2-cyanoacetate[73] (EACNO) and N-hydroxy succinimidyl esters.[80] Each of these methods have been shown to give low racemisation with high coupling yields and have been exemplified in a number of syntheses.

The acyl azide method is perhaps one of the most extensively used and has been applied with a high degree of success. The azide is generated through a hydrazide

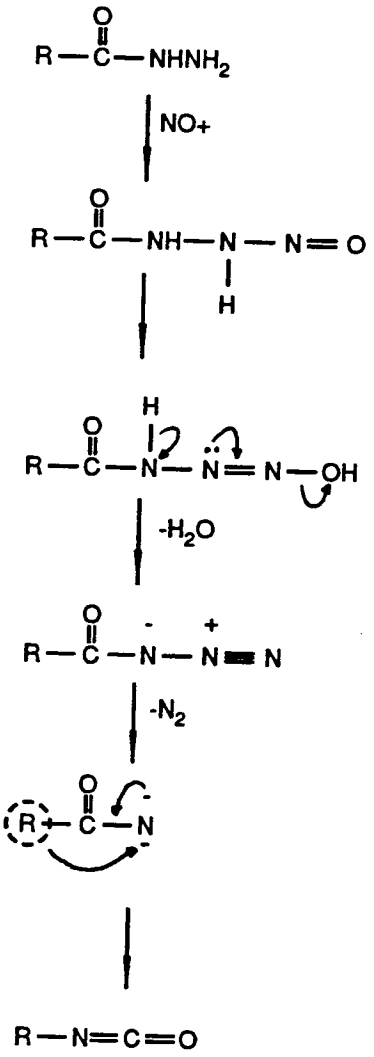
intermediate[76,82] which itself can be produced in a variety of ways by solid phase methods. These include hydrazinolysis of the peptide-resin link[72] and also the use of hydrazide resins. One such hydrazide resin which has been developed is the 3-(*p* -benzyloxyphenyl)-1,1- dimethylpropyloxycarbonylhydrazide resin.[81] (21) This resin employs a *t* -butyl based linker and treatment of the resin bound peptide with 50% TFA in DCM liberates the free peptide hydrazide.



(21)

1.6.4. Coupling of peptide fragments.

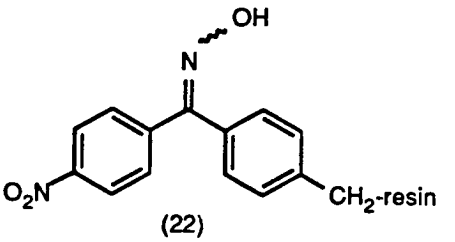
As mentioned previously, a number of types of activation have been used in the condensation of fragments. The azide method was used by Denkewalter and coworkers[75] in the synthesis of ribonuclease-S-protein (104 residues). This group prepared 19 constituent fragments of this enzyme and condensed them through acyl azides to give material which exhibited significant ribonuclease activity. The success of this synthesis was undoubtedly in part attributable to Honzl and Rudinger.[82] These workers developed methodology allowing azide coupling reactions to be performed under anhydrous conditions at low temperatures thus minimizing side reactions such as the Curtius rearrangement.[83] Significantly, this synthesis was achieved using minimal side chain protection as only cysteine and lysine were protected.



Scheme 3. Mechanism of azide formation and Curtius rearrangement.

In the synthesis of subunits of a lysozyme analogue by Ramage and coworkers[80], fragments were successfully coupled using DCC/N-hydroxysuccinimide methodology.

Kaiser[73] has reported the use of oxime resin (22) in the synthesis of protected peptide fragments and the coupling of segments while attached to the solid phase.



(22)

The C-terminal fragment was coupled to the oxime resin using DCC/HOBt or DCC/EACNO and then subsequent fragments coupled to this using the same reagents. A 44 residue model peptide of apolipoprotein A-1 was successfully prepared by this methodology in a convergent manner in which a 21 residue fragment was coupled to a 22 amino acid fragment attached to the solid phase. This was then liberated from the solid phase by nucleophilic displacement with the C-terminal amino acid and following removal of side chain blocking groups and purification, the desired peptide was obtained in good yield. Coupling of the fragments on the solid phase is advantageous as excess reagents can simply be washed away and thus purification is simplified. This method also allows excess reagents to be recovered if necessary.

It can be seen that fragment condensation when used in conjunction with solid phase stepwise elongation can offer greater scope to the synthesis of large peptides and small proteins. The limitations can in some instances be severe however with further improvements in methodology, these will undoubtedly be overcome in the near future.

1.7. Conjugation of peptides with proteins.

In many areas of biological science, there is a demand for efficient coupling of synthetic peptides and proteins to larger proteins which often serve as carriers. This is especially true in the field of immunology where synthetic peptides are conjugated to a large carrier protein (such as bovine serum albumin or keyhole limpet haemocyanin) in order to increase their immunogenicity.[84] Conventional methods of accomplishing this include ligation through the N-terminus, the C-terminus and also through certain amino acid side chains.

(i) Coupling through the peptide N-terminus: Glutaraldehyde[85] is the reagent which is most commonly used to couple the N-terminus or in some cases, the N^{ε} of lysine to a carboxyl group of a protein.

(ii) Coupling through the peptide C-terminus: The water soluble reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride has been extensively used in C-terminal activation.[86] This reagent allows coupling reactions to be performed in aqueous systems which is often essential in maintaining the conformational integrity of biological molecules. Ligation occurs primarily on lysine side chains and on the N-terminus of the carrier protein.

Ubiquitin, a protein which has been implicated in an array of cellular processes[87] has been coupled *via* an acyl azide to various nucleophiles and proteins.[88] This was accomplished by transpeptidation of the C-terminal Gly-Gly sequence with ethyl glycylglycinate followed by hydrazinolysis and subsequently azide formation.

(iii) Coupling through the peptide side chains: This form of conjugation is primarily achieved by coupling through the side chains of tyrosine and cysteine. Tyrosine is coupled using bis diazobenzidine[85] and cysteine with N-succinimidyl-3-(2-pyridyldithio)propionate[89] or maleimidobenzoic acid N-hydroxysuccinimidyl ester.[90]

Chapter 2.
DISCUSSION

2.1. Solid phase synthesis of peptide amides.

2.1.1. Strategy in the synthesis of peptide amides.

In the synthesis of peptide amides by the solid phase method, it is expedient to functionalise the solid support using a linker so as to produce the amide upon cleavage of the peptide-resin link. It is also necessary that the peptide amide be liberated under mild conditions so as to minimise the occurrence of side reactions. In order to satisfy these requirements, the Fmoc methodology should be used and the linker must therefore be compatible with this orthogonal approach. Since the Fmoc N^{α} protection is removed using an organic base, the linker can be cleaved under acidolytic conditions. Due to the stability of the amide bond which would undoubtedly link the peptide to the linker, features must be incorporated into the linker that facilitate the release of the peptide amide under the required conditions. In an S_N1 process, by which the acidolytic cleavage of the peptide-resin link should occur, an intermediate carbenium ion will be generated in the rate determining step. Since carbenium ions are stabilised by electron donation through both inductive and resonance effects, these features then should be requisite in the design of an amide linker. Since benzyl carbenium ions are stabilised effectively by resonance, it would be desirable to have this type of system as an intrinsic feature of the handle.

A number of benzhydryl derivatives were studied by Pless[91] in 1976 (Figure 2.1). He quantified the relative stabilities of these systems by measuring their pK_{R+} values. This value is an indication of the stability of the carbenium ion generated from the corresponding alcohol of the aromatic system. Of the systems that he studied, he

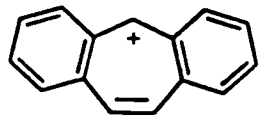
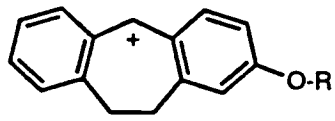
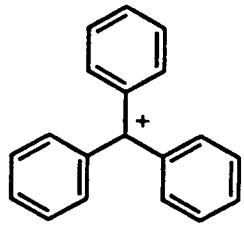
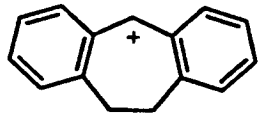
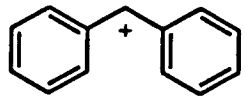
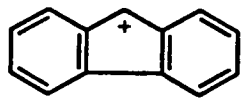
R+		pK_{R+}
	(23)	-3.7
	(24)	-5
	(25)	-6.6
	(26)	-8.0
	(27)	-13.3
	(28)	-14.0

Figure 2.1 Relative stabilities of some aromatic carbenium ions.

$$R+ + H_2O \underset{}{\overset{K_{R+}}{\rightleftharpoons}} R\text{-}OH + H+$$

Equation 1.

reported that the dibenzocycloheptatrienyl ion (23) was the most stable, followed successively by the trityl cation (25), the dibenzocycloheptadienyl cation (26), the benzhydryl cation (27) and lastly the fluorenyl cation (28). It can be seen from equation 1 that the more negative the pK_{R+}, the more the equilibrium will lie to the left in the given equation, i.e. the system will exist in its ionized form. The relative stabilities of the corresponding carbenium ion of these carbinols is a consequence of their aromaticity and the ability of the positive charge to delocalise through the aromatic system.

In the design of an amide linker which would release the completed peptide under mildly acidic conditions, dibenzocycloheptadiene was chosen as the parent system. The two carbon bridge of this system prevents free rotation of the two benzene rings and thus allows both to stabilise the positive charge which would be generated in the 5 position. It was decided also to incorporate an alkoxy substituent onto one aromatic ring *para* to the position of the potential carbenium ion. In this position, the oxygen is able to donate electrons by resonance and further stabilise the incipient carbenium ion upon acidolysis, thus facilitating cleavage of the carbon-nitrogen bond. The mechanism given in figure 2.2, shows how the oxygen in the 2 position stabilises the developing carbenium ion and thus facilitates acidolysis. Protonation of the amide carbonyl occurs in a rapid step, followed by breakage of the carbon-nitrogen bond in the rate determining step. Since the formation of the carbenium ion occurs in the rate determining step, the more stable this cation is, the faster it will be formed. The carbenium ion is then quenched by the counter ion of the acid and this step again may be rate limiting.

It was thought that the alkoxy substituent would have a similar stabilising effect on the carbenium ion as the extra double bond in the dibenzocycloheptatriene system.

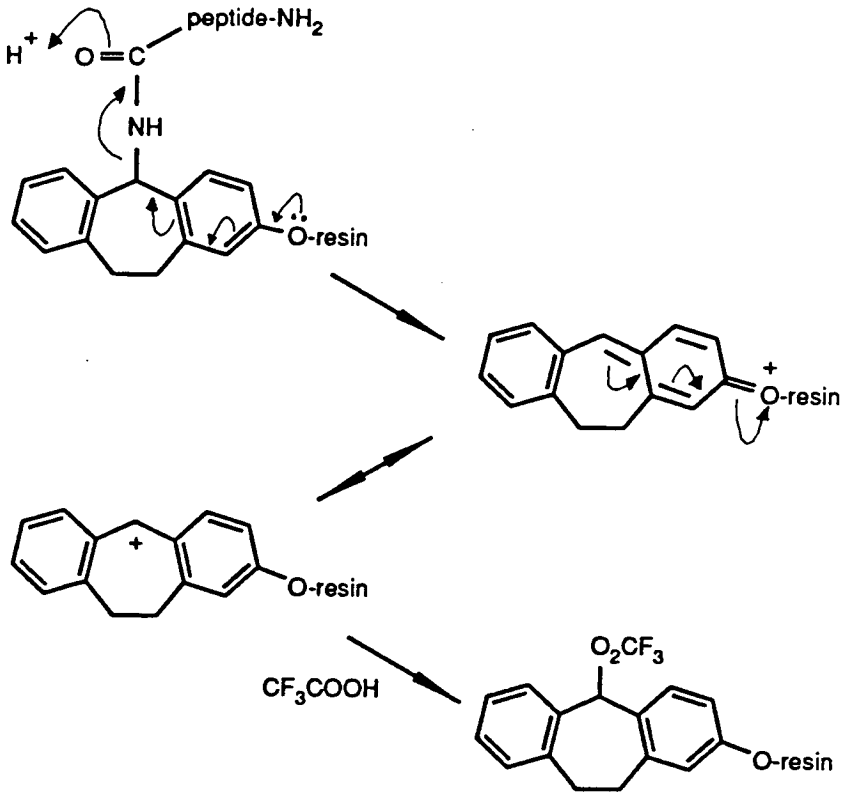
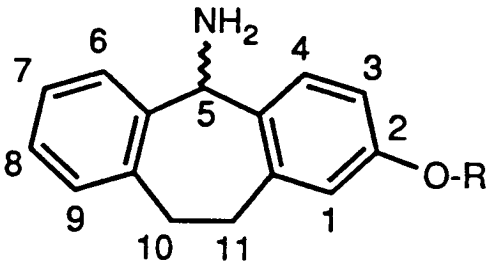


Figure 2.2 Mechanism of acidolytic cleavage with proposed linker.

Having the alkoxyl group would therefore simplify the synthesis while probably giving a cation of similar stability. This conjecture was in part substantiated by the work of Deno.[92] He demonstrated that the pK_{R+} of 4-methoxytrityl was -3.40 compared with -6.63 for the unsubstituted trityl. In a very approximate correlation of this data to the dibenzocycloheptadienyl cation, this would give the alkoxy substituted system (24) a pK_{R+} of approximately -5, bringing it close to the value of -3.7 obtained for the dibenzocycloheptatriene system. With these factors in mind, the 2-alkoxy-5-aminodibenzocycloheptadiene system (29) was proposed as an amide

linker. The amine in the 5 position would form an amide bond with the first amino acid of the desired peptide while the 2 position would be functionalised so as to provide the linkage to the solid support. This linkage would either be an ether or amide bond and would be stable under the conditions employed for the cleavage of the peptide-resin link.

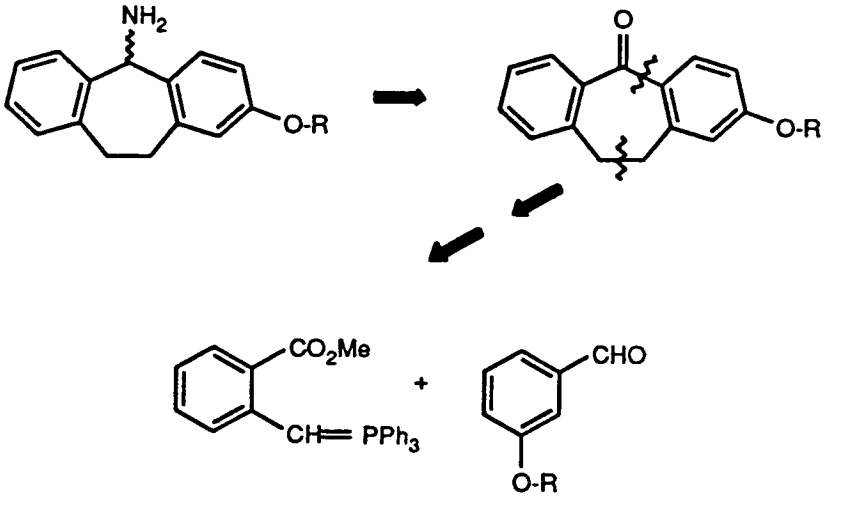


(29)

2.1.2. Synthesis of a novel amide linker.

It was decided that the most facile route to the substituted tricyclic dibenzocycloheptadiene system was through the joining of two substituted benzene derivatives. Dibenzocycloheptadiene and triene systems are commercially available however these are mostly unsubstituted and derivatising the aromatic rings would likely have proved to be synthetically demanding. The two carbon bridge between the two aromatic rings (which would eventually comprise the 10 and 11 positions of the dibenzocycloheptadiene system) could be constructed in an unsaturated form using a Wittig or analogous reaction. After saturation of the bridge, the dibenzocycloheptadiene system could possibly be formed *via* an acid catalysed cyclisation proceeding through an acylium ion. A ring closure occurring through this mechanism would leave a ketone at position 5 which could then be converted to an amine, thus allowing attachment of an amino acid through an amide bond. Position 5

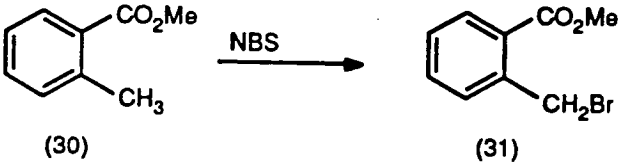
would also be the site of potential carbenium ion formation resulting from lysis of the carbon-nitrogen bond upon release of the resin bound peptide. The dibenzocycloheptadiene system would be substituted with an alkoxyl group in position 2 and this would eventually provide the linkage to the polymer support.



Scheme 4. Retrosynthesis of proposed amide linker.

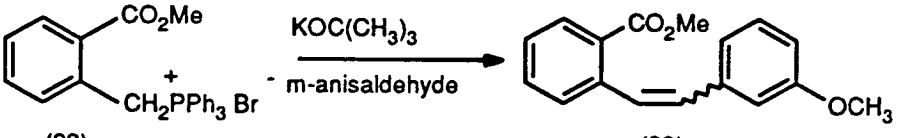
For the Wittig approach to the joining of the two ring systems, the starting material was chosen as methyl 2-methylbenzoate (30). This compound was α-brominated in a light initiated free radical reaction. This reaction was complete in under 2 hours using a slight excess of N-bromosuccinimide as the halogen radical source. A small amount of dibromo compound was observed by t.l.c., however the crude mixture obtained after separation of the succinimide by-product and concentration was used without further purification. The methyl 2-bromomethylbenzoate (31) obtained was then stirred overnight along with triphenylphosphine in toluene to generate the bromo-phosphonium salt (32) as a precipitate. This compound was then recrystallised to obtain 2-(methoxycarbonyl

)benzyltriphenylphosphonium bromide (32) in 73% overall yield from methyl 2-methylbenzoate.



(30) (31)

This compound was then used in a standard Wittig reaction for the formation of the two-carbon bridge. This involved conversion of the phosphonium salt to its corresponding ylide *in situ* , followed by reaction with the requisite aldehyde.



(32) (33)

Several bases were used in this reaction before the conditions were optimised for maximum yield of the desired olefin. These included diazabicyclooctane, lithium methoxide and n-butyllithium. n-Butyllithium was found to give a low yield of product due to a competing side reaction. This involved the S_N2 attack of the butyl anion on the methyl ester giving a significant amount of the butyl ketone (34). The use of potassium *tert* - butoxide as a base at -78°C gave efficient proton abstraction with no apparent side reactions. Condensation of the ylide with *m* -anisaldehyde afforded the expected stilbene derivative (33) in 85% yield. It was noted that a significant amount of transesterification occurred, producing ester (35) subsequent to the addition of *t* -butoxide, if the reaction mixture was not cooled effectively. To counteract this problem, the phosphorane was generated at -78°C for 1.5 hours and the reaction mixture stirred at this temperature for a further 0.5 hours subsequent to addition of the aldehyde.

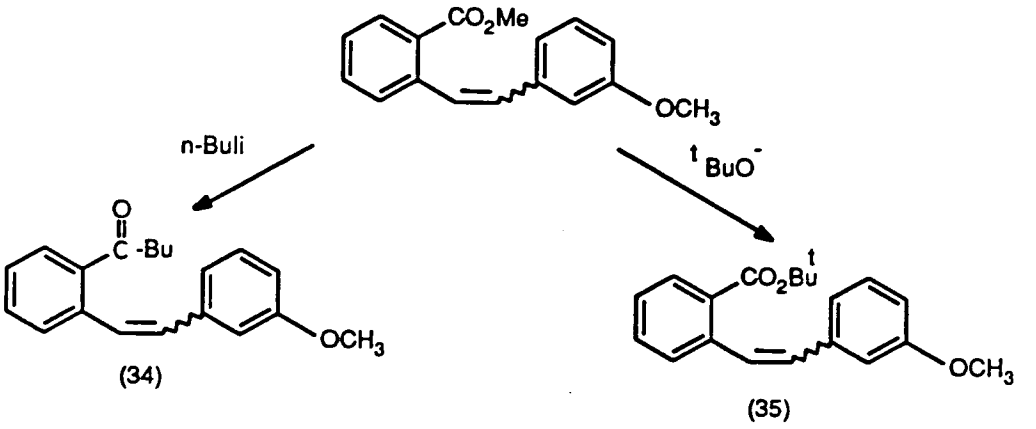
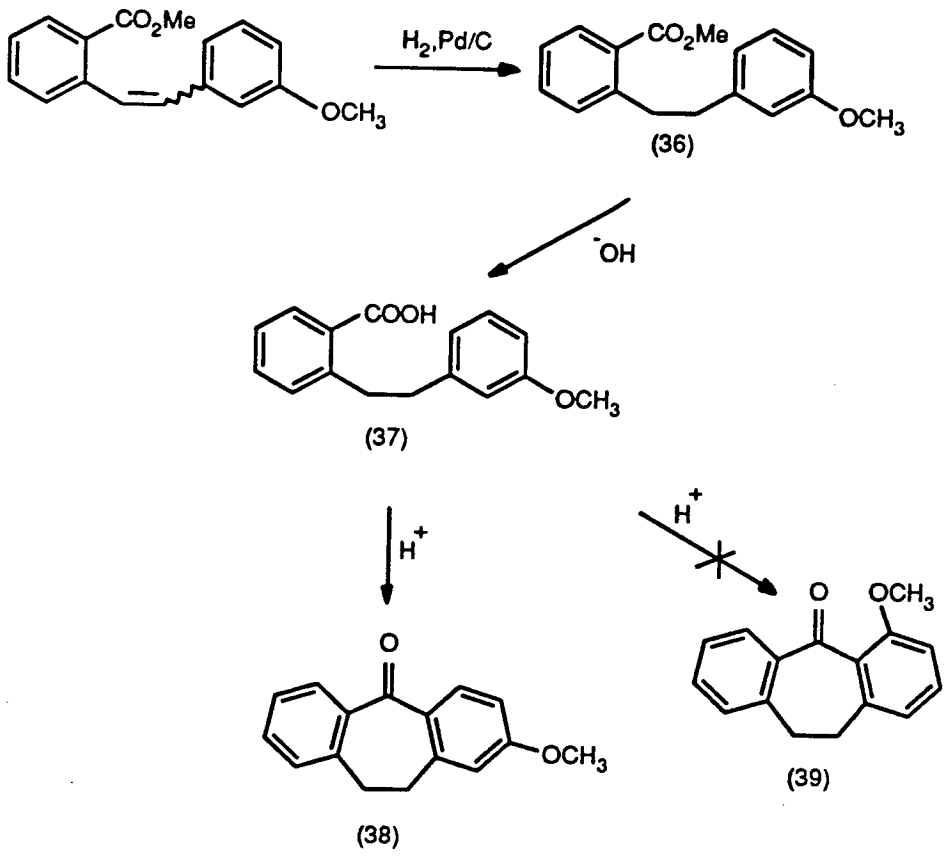


Figure 2.3 Side reactions encountered with Wittig reaction.

Although this reaction gave a high yield of the required compound, isolation proved to be onerous due to the presence of triphenylphosphine oxide. This was eventually separated from the desired material using dry flash chromatography. A more facile synthesis of this compound may involve the use of a Horner-Emmons reaction which would give a water soluble by-product and thus could easily be separated by extraction. The stilbene (33) was then hydrogenated in the presence of palladium on charcoal in aqueous methanol to give the saturated derivative methyl 2-(3'-methoxyphenylethyl)benzoate (36) in quantitative yield. The methyl ester of this compound was then hydrolysed with sodium hydroxide in aqueous methanol to afford the corresponding carboxylic acid 2-(3'-methoxyphenylethyl)benzoic acid (37). This was obtained in a yield of 97%. This benzoic acid derivative was then treated with polyphosphoric acid at 120°C for two hours in order to effect cyclisation and generate the tricyclic system. 2-Methoxydibenzocycloheptadien-5-one (38) was obtained in 74% yield after purification by flash chromatography and recrystallisation.



Scheme 5. Synthesis of tricyclic system showing two possible isomers.

In the acid catalysed ring closure, it is possible for two isomers to be formed. Due to free rotation of the methoxyl substituted aromatic ring, the cyclisation can occur either *ortho* or *para* to the methoxyl group. It was observed however that only one isomer was obtained and this corresponded to the *para* cyclisation. The position of the methoxyl group was confirmed by inspection of the aromatic splitting pattern and coupling constants in a 360 MHz ^{1}H n.m.r spectrum of ketone (38) (figure 2.4). These were found to correspond to dibenzocycloheptadien-5-one substituted in the 2 position (2 isomer) (38) and not in the 4 position (4 isomer) (39).

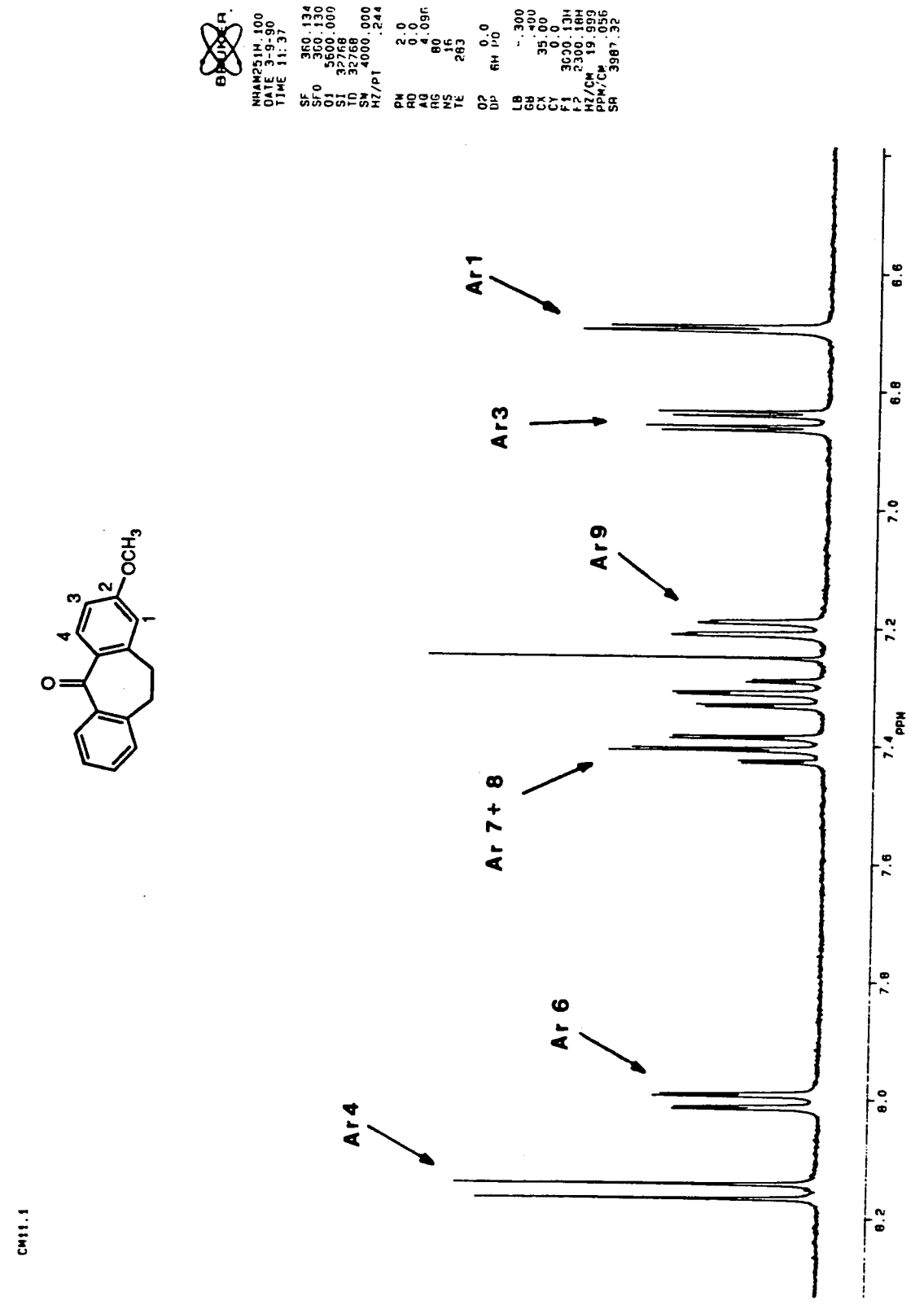
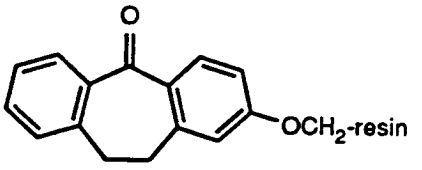


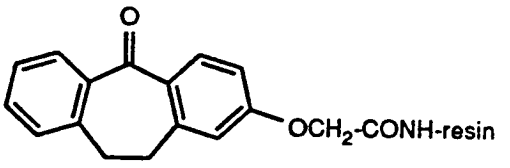
Figure 2.4 360 MHz ^{1}H n.m.r. spectrum of ketone (38).

For the 2 isomer, the aromatic proton in position 1 (Ar1) would have only a *meta* coupling. The aromatic proton in position 3 (Ar3) would have both an *ortho* and a *meta* coupling while the aromatic proton in position 4 (Ar4) would have only an *ortho* coupling. Ar1 and Ar3 would be shielded by the methoxyl while Ar4 should be deshielded by the carbonyl in position 5. For the 4 isomer, Ar1 would have a *ortho* and a *meta* coupling, Ar2 would have two *ortho* couplings and Ar3 would have an *ortho* and a *meta* coupling. Ar4 was observed as a doublet with a typical *ortho* coupling of 8.8 Hz. Ar1 was observed as a fine doublet with a typical *meta* coupling of 2.6 Hz. Ar3 was observed as a doublet split finely into a quartet. This splitting pattern was indicative of an *ortho* and a *meta* coupling and was confirmed by the observed coupling constants of 8.8 and 2.6 Hz. The splitting pattern, coupling constants and chemical shifts for these and all other aromatic protons were consistent with those expected, therefore this confirmed unequivocally that the isomer obtained was substituted in the 2 position. The selectivity in the cyclisation reaction is most likely due to unfavourable steric interactions between the methoxyl and the acylium ion, thus precluding formation of the 4 isomer.

Since the methoxy ketone (38) was obtained in good yield, it was decided to functionalise the 2 position so as to allow loading of the linker onto the solid support. The two standard methods of doing this are through an ether linkage (40) or through an amide bond (41).

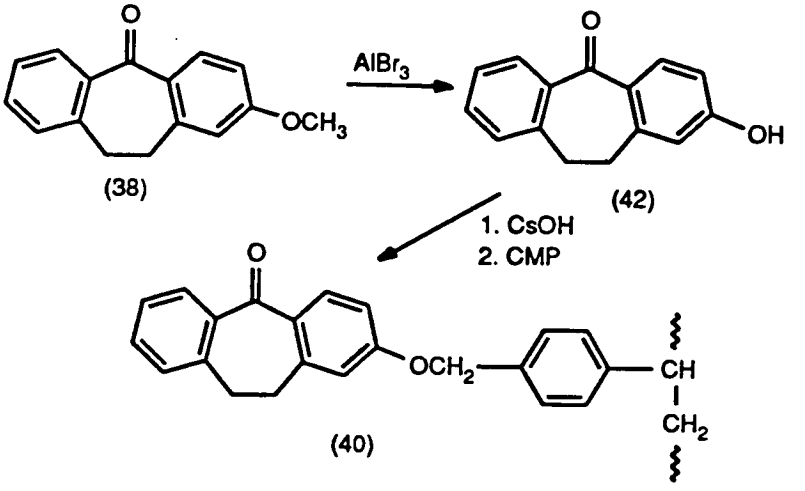


(40)

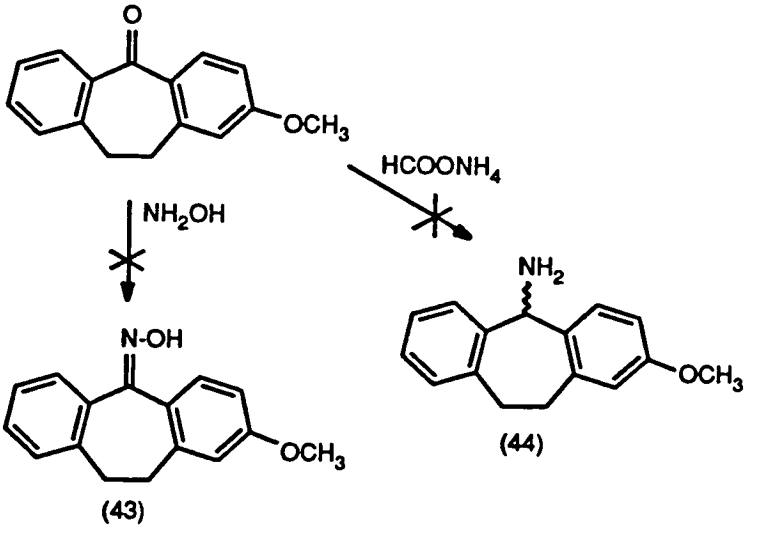


(41)

In order to couple the linker through an ether linkage, it would be desirable to use a Williamson type reaction. The methoxyl would thus be converted to a hydroxyl, the salt of this formed and then reacted with a halogenated solid support. In order to accomplish this, ketone (38) was converted to phenol derivative (42) by treatment with aluminium bromide in benzene whilst heating under reflux for several hours. 2-Hydroxydibenzocycloheptadien-5-one (42) was obtained in 94% yield after work-up and purification. This compound was then attached to polystyrene resin crosslinked with divinylbenzene. This was accomplished by stirring the caesium salt of phenol (42) with chloromethylpolystyrene(1%-divinylbenzene) (CMP) in DMF for several days. After isolation of keto resin (40), the loading was shown to be essentially complete through the use of chlorine analysis.



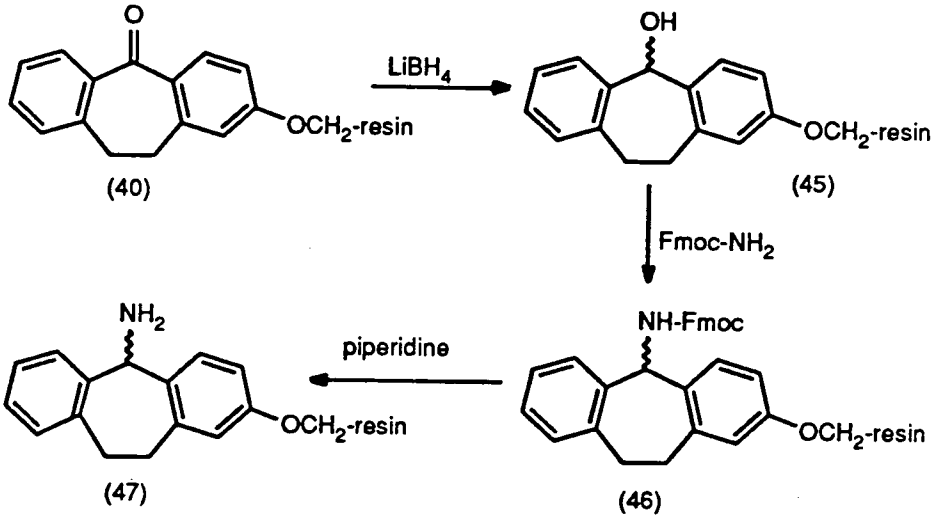
With the dibenzocycloheptadien-5-one system loaded onto the solid support, the 5 position was now ready to be derivatised in order to allow attachment of the first amino acid *via* an amide bond. It had originally been intended to functionalise the 5 position prior to loading onto the resin however this proved to be impractical by the methods used. It was proposed to convert ketone (38) directly to an amine. This was attempted firstly through oxime formation (43) and then by a Leuckart reaction (44).



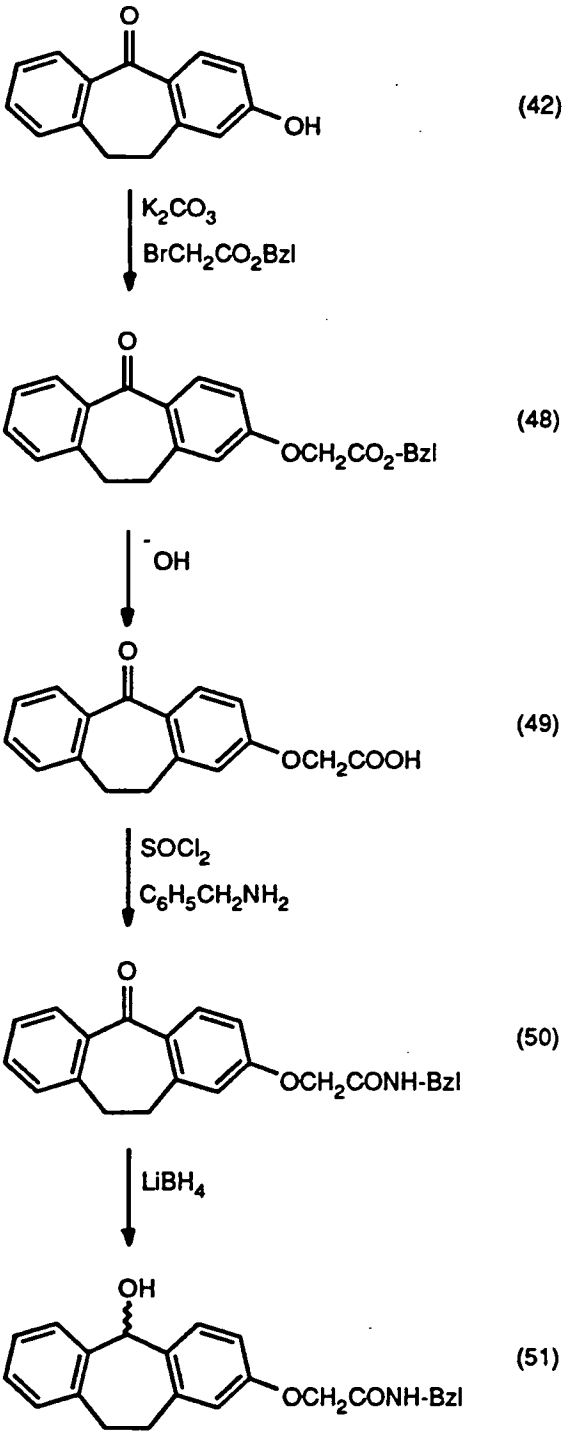
Neither of these methods were useful in generating the desired compound and in both cases the starting material was recovered almost quantitatively. It is conceivable that this was attributable to the vinylogous nature of the 2-alkoxy dibenzocycloheptadien-5-one system. The electron donating effect of the oxygen in the 2 position is propagated through the aromatic system thus making the carbonyl very electron rich. This electron dense ketone is therefore less susceptible to nucleophilic attack. As this approach had failed, it was decided to load ketone (38) directly onto the resin before further functionalisation. This would allow a large excess of reagents to be used since they could be easily washed away. In using forcing conditions, the desired reaction could theoretically be driven further towards completion.

In order to functionalise the 5 position in the desired manner, the ketone was reduced to an alcohol. Keto resin (40) was treated with lithium borohydride in THF for 1 hour. This reaction was judged to be complete by the disappearance of the carbonyl absorbance at 1640 cm^{-1} in the infrared spectrum. 2-Copoly (styrene-1%-divinylbenzene)methoxydibenzocycloheptadien-5-ol (45) was obtained after filtration, washing and drying under vacuum. The nitrogen was introduced into the 5 position in protected form by reaction of the carbinol resin (45) with 5 equivalents of 9-fluorenylmethylcarbamate at 60°C in an acid catalysed reaction (These reactions could have been performed on ketone (38) whilst in solution however purification would have undoubtedly have proved onerous due to the large excess of reagents used). This reaction was shown to have proceeded in quantitative yield through the use of a UV monitoring method.[93] This involves removal of the Fmoc group using piperidine in DMF. The UV absorbance of the piperidine adduct of dibenzofulvene is measured at 300 nm and then used to calculate the number of moles of Fmoc originally on the resin (This procedure has become routine and the data is now processed by computer). The protected amino resin

2-copoly(styrene-1%-divinylbenzene)methoxy-5-(9'-fluorenylmethoxycarbonylamino) dibenzocycloheptadiene (46) was then treated with 20% piperidine in DMF with sonication to generate the free amino resin (47) quantitatively. The success of this reaction was confirmed by disappearance of the urethane carbonyl peak in the infrared spectrum.

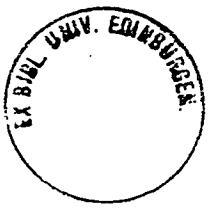


As mentioned previously, the linker can also be coupled to the resin *via* an amide bond. This would involve derivatising the dibenzocycloheptadiene system with a carboxylic acid and then coupling this to aminomethylpolystyrene. In order to test this methodology, a series of model compounds were synthesised (scheme 6). Firstly keto phenol (42) was alkylated by a Williamson reaction using benzyl-2-bromoacetate. This was achieved by stirring these two compounds in a 1:1 ratio with excess potassium carbonate using acetone as the solvent. 2-(Benzyloxycarbonylmethoxy)dibenzocycloheptadien-5-one (48) was obtained in quantitative yield after isolation and crystallisation. This compound was then hydrolysed to give its corresponding carboxylic acid (49) along with benzyl alcohol



Scheme 6. Synthesis of model compound for amide linkage.

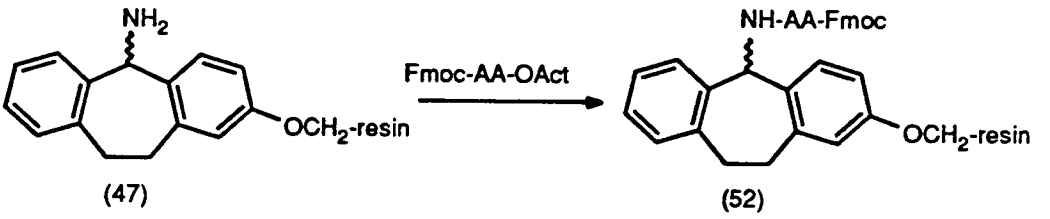
using sodium hydroxide in aqueous methanol. The reaction was complete as judged by t.l.c. after heating under reflux for 1 hour and the carboxylic acid 2-(carboxymethyloxy)dibenzocycloheptadien-5-one (49) was isolated in a high yield of 97%. This was then converted to its benzylamide derivative (50) through activation as its acid chloride and then treatment with benzylamine. This tranformation was accomplished by stirring acid (49) with 40 equivalents of thionyl chloride followed by addition of 2 equivalents of benzylamine after removal of the excess $SOCl_2$ and then stirring overnight at room temperature. Amide (50) was obtained in quantitative yield after work up and recrystallisation. The structure was confirmed as expected by elemental combustion analysis and by the presence of the required NH and carbonyl stretching frequencies in the infrared spectrum. This compound was synthesised as a model in order to test the viability of joining the linker to a solid support *via* an amide bond. In order to introduce the nitrogen in the 5 position, the ketone must first be reduced to its corresponding carbinol. The amide bond providing the link to the resin must therefore be stable to conditions used for reduction. This assumption was corroborated through the conversion of 2-(benzylaminocarbonylmethoxy) dibenzocycloheptadien-5-one (50) to its analogous carbinol (51). The keto group was successfully reduced using $LiBH_4$ in THF with no evidence of concomitant reduction of the amide to the alcohol. The amide was shown by n.m.r. spectroscopy to be unaffected as there was no diminution in the benzyl aromatic resonances or in the benzylic methylene signal. 2-(Benzylaminocarbonylmethoxy)dibenzocyclohepta dien-5-ol (51) was obtained in poor yield, however this was attributable to the difficulty of performing the reaction on a small scale. The crude product obtained upon work up was shown to be essentially homogeneous by t.l.c., also indicating that the amide bond was unaffected by the reduction conditions. This series of model reactions demonstrate that attaching the linker to the solid support through an amide bond is viable.



2.1.3. Application of the amide linker in SPPS.

With the synthesis of solid support (47) completed, this was now available for use in the synthesis of peptide amides. In order for peptide synthesis to be undertaken, it was first necessary to load the C-terminal amino acid residue onto the resin, which involves the facile formation of an amide bond. This is in contrast to the loading of resins to be used in the synthesis of C-terminal free acids, where because the peptide-resin link consists of an ester linkage, N,N-dimethylaminopyridine is required to actuate condensation of the first protected amino acid with the resin. In a general method, Fmoc amino acids were loaded using standard symmetrical anhydride methodology. This involved sonication of 0.5 mmol of resin (47) with 2.0 mmol of preformed symmetrical anhydride (PSA) for 2 hours. The activated species was formed by agitation of 4.0 mmol of amino acid with 2.0 mmol of DIC in DMF for 15 minutes. This method was found to give very high efficiency in loading with coupling yields in the range 95 to 100%. The loading was assessed using the UV monitoring method[93] as previously mentioned. As this resin was intended for use on an automated peptide synthesiser, it was found to be possible to incorporate the loading of the first amino acid through use of a programmed cycle on the machine. This would simplify the synthesis of the peptide amide as all manipulations are carried out by the machine, thus obviating manual operations. Use of the automated method typically involved 0.5 mmol of Fmoc protected amino resin (46) which was then washed several times with DMF in order to pre-swell the resin. A capping cycle was incorporated to block any unreacted free amine therefore preventing the formation of a deletion peptide deficient in the C-terminal residue. After washing of the capped resin, the Fmoc protection was removed by 4 successive treatments with 20% piperidine in DMF. This was again followed by thorough washing with DMF to remove all traces of piperidine. The free amino resin (47) was then loaded with the

first amino acid in a double couple cycle. This is the same cycle as was involved in chain elongation and entails an initial coupling of 2.0 mmol of an Fmoc amino acid as its preformed symmetrical anhydride, followed by a second coupling of 1.0 mmol of the amino acid as its HOBt ester. The loaded resin (52) was then washed with DMF and capped with acetic anhydride in pyridine as before. One disadvantage of the automated loading method is that the loading cannot be accurately measured unless the synthesis is interrupted and a sample of the resin is removed. This is not a major drawback however as the loading reaction was shown by the UV monitoring method in most cases to proceed quantitatively.



(47) (52)

Once the solid support has been loaded with the first amino acid either outwith the machine or as a programmed cycle on the synthesiser, it is ready for stepwise elongation to generate the desired peptide. This was accomplished by repetitive cycles with the required amino acids incorporating deprotection of the N^{α} amine, activation, coupling and capping procedures. All amino acids were double coupled with the exception of glycine which was single coupled with a four fold excess. This precaution is taken with glycine to preclude Gly-Gly formation which may occur as a consequence of the greater reactivity of the amino group of this residue compared with other amino acids. With all amino acids that were double coupled, symmetrical anhydride followed by HOBt activation was used, with the exception of asparagine, glutamine and arginine. These amino acids are incorporated as their HOBt active esters to prevent dehydration of the amide side chains with asparagine and glutamine

and to minimize the cost with arginine. As a consequence of its high cost, arginine is incorporated as an HOBt ester as with this method only half as much amino acid derivative is used as with the symmetrical anhydride method. In the case where pyroglutamic acid was incorporated as the N-terminal amino acid, it was used in ready activated form as a pentachlorophenyl ester and due to poor solubility in DMF, it was coupled in N-methylpyrrolidone.

2.1.3.1. Monitoring the progress of SPPS.

One significant advantage of SPPS using Fmoc N^{α} protection is the chromophoric nature of the fluorenyl system. This chromophore has a characteristic absorbtion maximum at 300 nm in the ultraviolet spectrum and thus enables the monitoring of the fluorenyl species during the course of the synthesis. In order to do this, the filtrate from deprotection and coupling reactions is passed through a UV flow cell and absorption *versus* time is recorded (figure 2.5). When this is done in conjunction with integration of the peak obtained, it is useful in giving a semi-quantitative evaluation of the efficiency of the various steps in SPPS.

This method has been applied with a high degree of success in the monitoring of N^{α} deprotection. The Fmoc group, upon deprotection, affords a piperidine adduct of dibenzofulvene which is then monitored at 313 nm. This is useful in the estimation of coupling efficiency if one assumes that deprotection is quantitative in all cases due to the large excess of base used. Thus by comparing the peak integration arising from the deprotection of the coupled amino acid with that of the previous residue, a relative value can be obtained for the coupling yield.

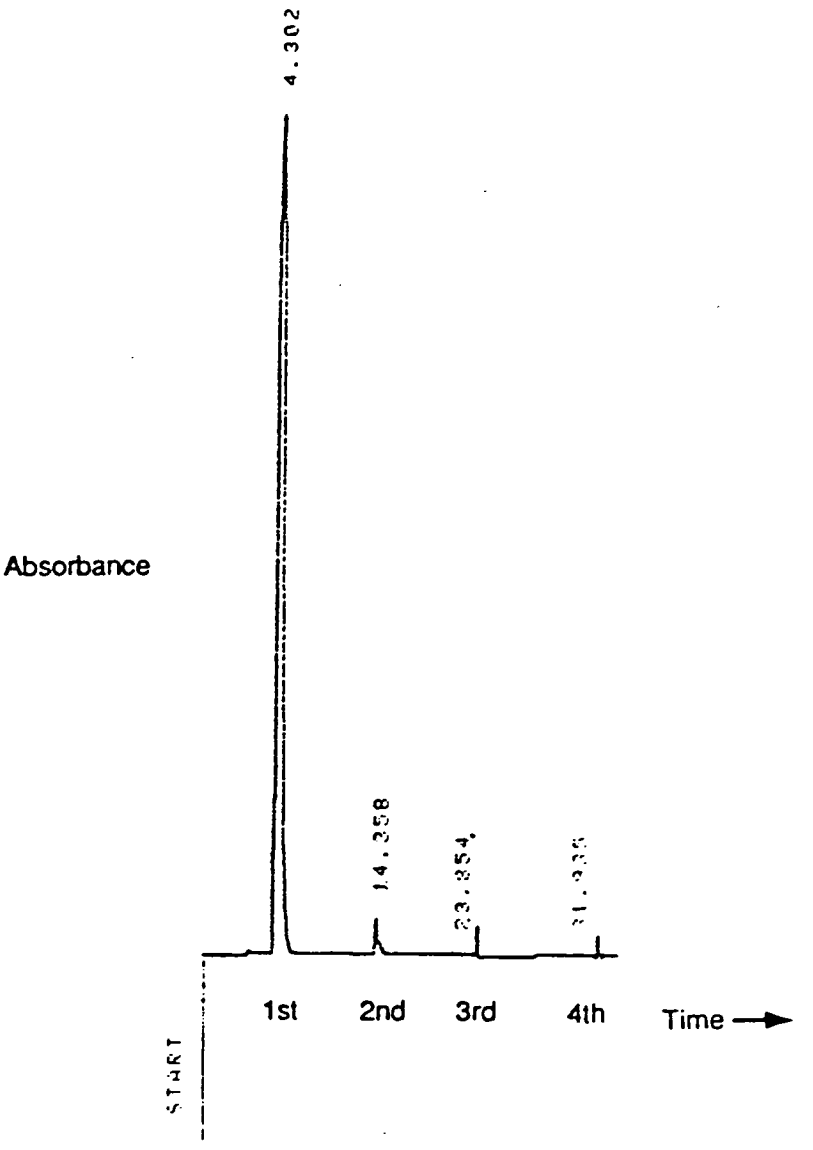


Figure 2.5 Monitoring of Fmoc deprotection at 313 nm.

Through the use of this UV monitoring method, the rate of deprotection can also be observed. Each residue is given four successive deprotections and the effluent from each one is passed through the flow cell. If peaks are observed in the second, third or fourth deprotections, then this is an indication that the deblocking reaction is proceeding at a slower than normal rate. This may also be an indication that the coupling reaction of the next amino acid will also proceed slowly and thus the coupling conditions can be altered accordingly. Through use of this monitoring method, an overall picture of the synthesis is obtained and in the event of a disastrous

synthesis, it can be repeated with some knowledge of where it went wrong. This monitoring method was used to follow the progress of the synthesis of the C-terminal amides made using solid support (47).

2.1.3.2. C-terminal peptide amides synthesised.

Several peptide amides were synthesised using solid support (46). These included little and big gastrin, bombesin, substance P and also fragments of ubiquitin[48], Cro protein[48], interleukin 1β [94] and nerve growth factor.[95]

Little gastrin (55) was synthesised using 0.5 mmol of solid support (46) however 0.25 mmol of resin was removed subsequent to the coupling of the penultimate amino acid (this was used in the synthesis of big gastrin at a later stage). Pyroglutamic acid was then coupled to complete the synthesis of little gastrin. Through the use of deprotection monitoring, the relative efficiencies of each coupling were assessed and it was noted that each one proceeded in high yield with the exception of a small number of residues where a slight drop was observed. Deprotection of the residues between Glu_9 and Gly_2 occurred at a slow rate with relatively large 2nd, 3rd and 4th deprotection peaks being obtained. This was probably due to the steric interactions of the bulky *t* -butyl side chain protection used in the preceeding five consecutive glutamic acids.

The synthesis of big gastrin (56) was carried out as a continuation of little gastrin using the 0.25 mmol of resin previously retained. Deprotection monitoring showed generally efficient couplings throughout the synthesis however a slight drop was noted for the coupling of Gly_3 onto Pro_4. Unlike the little gastrin synthesis, the deprotection reaction did not proceed slowly. This was likely to be a consequence of the greater excess of piperidine as half of the resin had been removed.

SUBSTANCE P (53)

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

BOMBESIN (54)

Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

LITTLE GASTRIN (55)

Glp-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

BIG GASTRIN (56)

Glp-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu-Val-Ala-Asp-Pro-Ser-Lys-Lys-Gln
-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

Figure 2.6 C-terminal amides synthesised using solid support (46).

The synthesis of bombesin (54) was accomplished using 0.5 mmol of resin (46). Monitoring of the deprotection reaction showed efficient coupling and deprotection at each stage.

The synthesis of substance P (53) was found to be without problem if the correct strategy for side chain protection was used. A number of syntheses were attempted using varying methods of protection on the side chain of glutamine. These included use of the trityl group (TRT), the dibenzocycloheptatrienyl group (DBSE) and the dimethoxybenzhydryl group (MBH).

The use of MBH for glutamine protection in the synthesis of substance P[96] was found through monitoring of the deprotection reaction to give poor results. Coupling of Gln_6 onto Phe_7 went only in about 75% yield and the following coupling of Gln_5 onto Gln_6 occurred in a very poor yield of less than 50%. Subsequent couplings were found to proceed almost quantitatively.

The synthesis of substance P using the DBSE group[96] proceeded better, however this was still not satisfactory. Coupling of Gln_6 went in approximately 80% yield and that of Gln_5 occurred in roughly 70% yield. Subsequent couplings tailed slowly off so that the final deprotection peak was approximately one third of the initial peak.

The synthesis using trityl to protect the side shain of glutamine was found to proceed satisfactorily as only minor decreases in coupling efficiency were noted to occur during chain elongation. These results can not be explained on a steric basis alone as they appear to show an inverse correlation to the size of the side chain protecting group. One possible explanation could be that there is a hindrance in the coupling reaction as a result of van der Waals stacking interactions between the side chain of phenylalanine and the aromatic side chain protecting group of glutamine. These interactions could be precluded in the trityl case as this group exists in a

propeller-like conformation and therefore stacking would be sterically impossible.

2.1.3.3. Cleavage of the peptide-resin link.

The rationale behind the use of the alkoxydibenzocycloheptadiene system as a handle for the synthesis of peptide amides lies in its ability to stabilise a positive charge and thus facilitate the acidolytic release of the resin bound peptide. In order to determine the exact minimum conditions for release of the peptide, Fmoc-glycine loaded resin (57) was submitted to varying concentrations of trifluoroacetic acid in DCM (figure 2.7) . Using 1% TFA in DCM, the amino acid amide was found to be quantitatively released from the resin after 2 hours of acid treatment. The rate of cleavage was found to be greatest in the first 30 minutes of acid treatment with 58% of the glycine amide being released after this time. The rate of cleavage slowed appreciably in the next 30 minutes with only a further 17% of the amide being released. The remainder of the glycine amide was released after a further 1 hour under these conditions. By doubling the concentration of trifluoroacetic acid to 2% in DCM, the rate of cleavage during the first 10 minutes was almost tripled with 56% of the glycine amide being released from the resin. Acidolysis of Fmoc-Gly-resin in 3% TFA/DCM resulted in a rate 3.6 times that at 1%. Cleavage in 4% TFA/DCM exhibited a rate 4.3 times as fast as at 1% and twice as fast as the rate in 2% TFA/DCM. Since the rate increased by approximately 3 times from 1 to 2% TFA/DCM, this would indicate that the order of the reaction is somewhere between first and second order at these concentrations, with respect to the trifluoroacetic acid concentration. As the acid concentration is increased to 4%, the rate is approximately 4 times the rate at 1% TFA/DCM and thus the order of the reaction is roughly one at this concentration. Thus the order of the reaction would appear to be changing from between first and second order to closer to first order kinetics as the concentration of the acid is increased and becomes in greater excess.

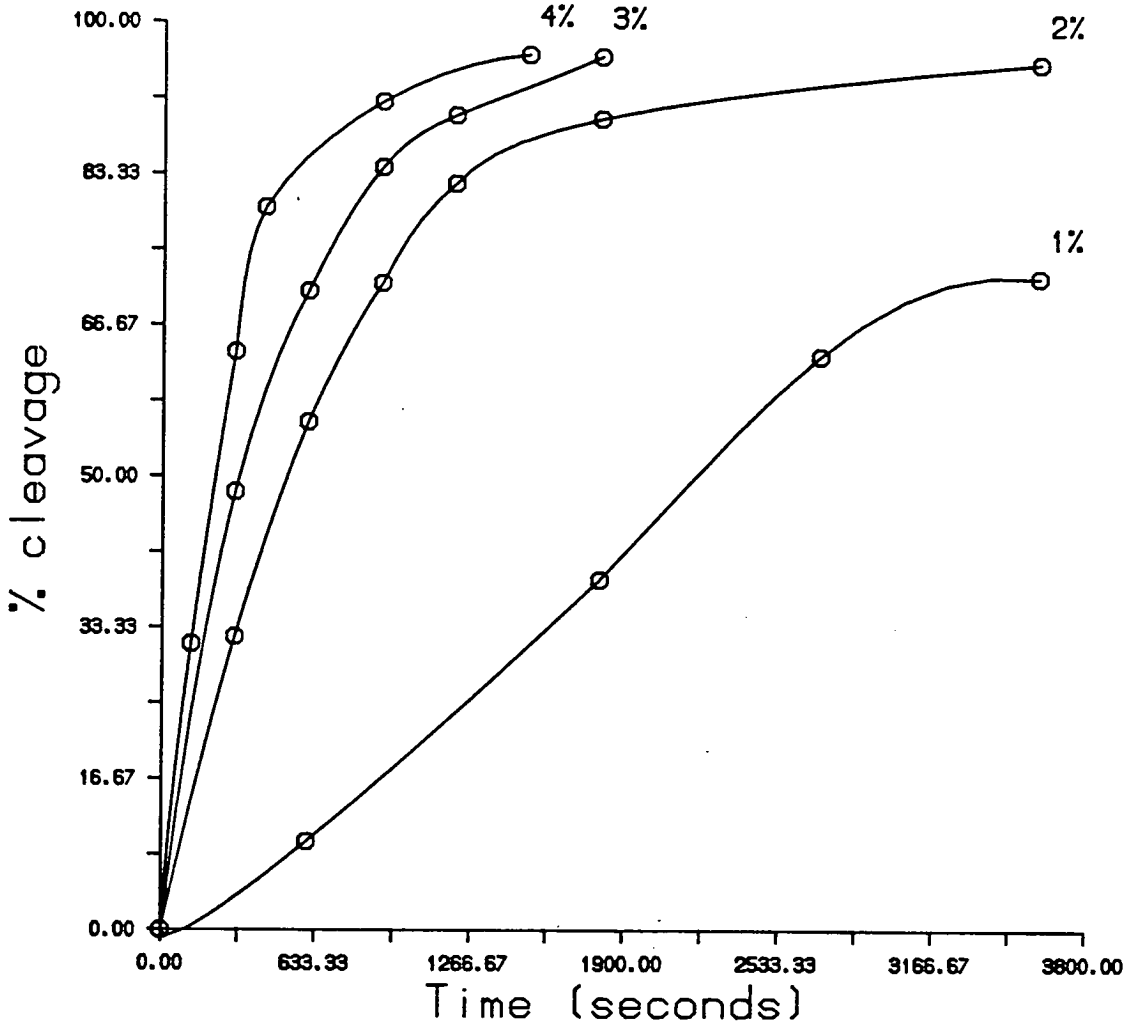


Figure 2.7 Plot of cleavage of Fmoc-Gly-NH_2 from solid support (47) against time at different acid concentrations.

The results obtained for the release of peptide amides from the dibenzocycloheptadiene functionalised solid support indicated that the target of producing a linker which allowed mild cleavage of the peptide-resin link had been achieved. This linker would be compatible with other side chain blocking groups which are removed under mild conditions. Current methodology used in Fmoc chemistry however, dictates the use of 50% trifluoroacetic acid to remove *t* -butyl based side chain protecting groups and thus the resin bound peptide would have to be treated with at least this concentration of acid to liberate the fully deblocked peptide. Therefore it may be possible to achieve a differential acidolysis and obtain a fully or selectively partially protected peptide amide after peptide synthesis on solid support (47).

2.1.3.4. Cleavage, purification and characterisation of peptide amides.

Each peptide amide synthesised using solid support (46) was deprotected and released from the resin by treatment with TFA and the appropriate combination of scavengers. These peptides were subsequently purified using a variety of chromatographic techniques and then characterised mainly by amino acid analysis and fast atom bombardment mass spectrometry (FAB MS).

Little gastrin was deprotected and removed from the solid support by treatment with TFA along with water, thioanisole, ethyl methyl sulphide and N-acetyltryptophan. Thioanisole was added as a carbenium ion scavenger, ethyl methyl sulphide was added to prevent oxidation of methionine and N-acetyltryptophan was added to prevent oxidation and alkylation of tryptophan residues. Little gastrin was initially deprotected for 2 hours however it was found that after this time some *t* -butyl protecting groups remained intact. This was detected by the use of FAB MS as peaks were noted in the spectrum corresponding to the

molecular weight of the peptide with one, two and three blocking groups intact. It was likely that these were on the five consecutive glutamic acids in the sequence of little gastrin as this region is sterically crowded and so may be partially inaccessible to the acid. Treatment of the isolated peptide for a further 2 hours in TFA resulted in completely deprotected little gastrin. At this point, the crude peptide was examined by reverse phase HPLC and a major peak constituting approximately 80% of the total material was observed. The crude material was then purified firstly by gel permeation chromatography. This removed all traces of scavengers and left the crude peptide in a more soluble form. This material was then applied to an anion exchange column and eluted with a buffer gradient of increasing ionic strength. The material obtained from this column was shown to be in a good state of purity however it was purified to homogeneity by semi-preparative reverse phase HPLC. This material was found to give a single peak on HPLC after being isolated in 16% yield. Analysis by FAB MS and amino acid analysis confirmed the presence of the correct product.

Big gastrin was released from the solid support and deprotected by use of similar conditions to those for little gastrin. Analysis of the crude peptide by HPLC showed the presence of a major peak which constituted approximately 70% of the total peak area. Big gastrin was purified through the use of gel permeation chromatography, ion exchange chromatography and semi-preparative HPLC and was shown to be homogeneous by analytical HPLC (figure 2.8). Characterisation of this material by FAB MS and amino acid analysis verified the presence of big gastrin.

The presence of the C-terminal amide in each of these peptides was confirmed by two methods. Accurate mass FAB MS showed the exact mass of the molecular ion to be within 1 part per million. The biological activity of the two peptide amides was also assessed. This was done through the use of radioimmunoassay to measure the antibody binding to the N and C termini of each peptide.[97]

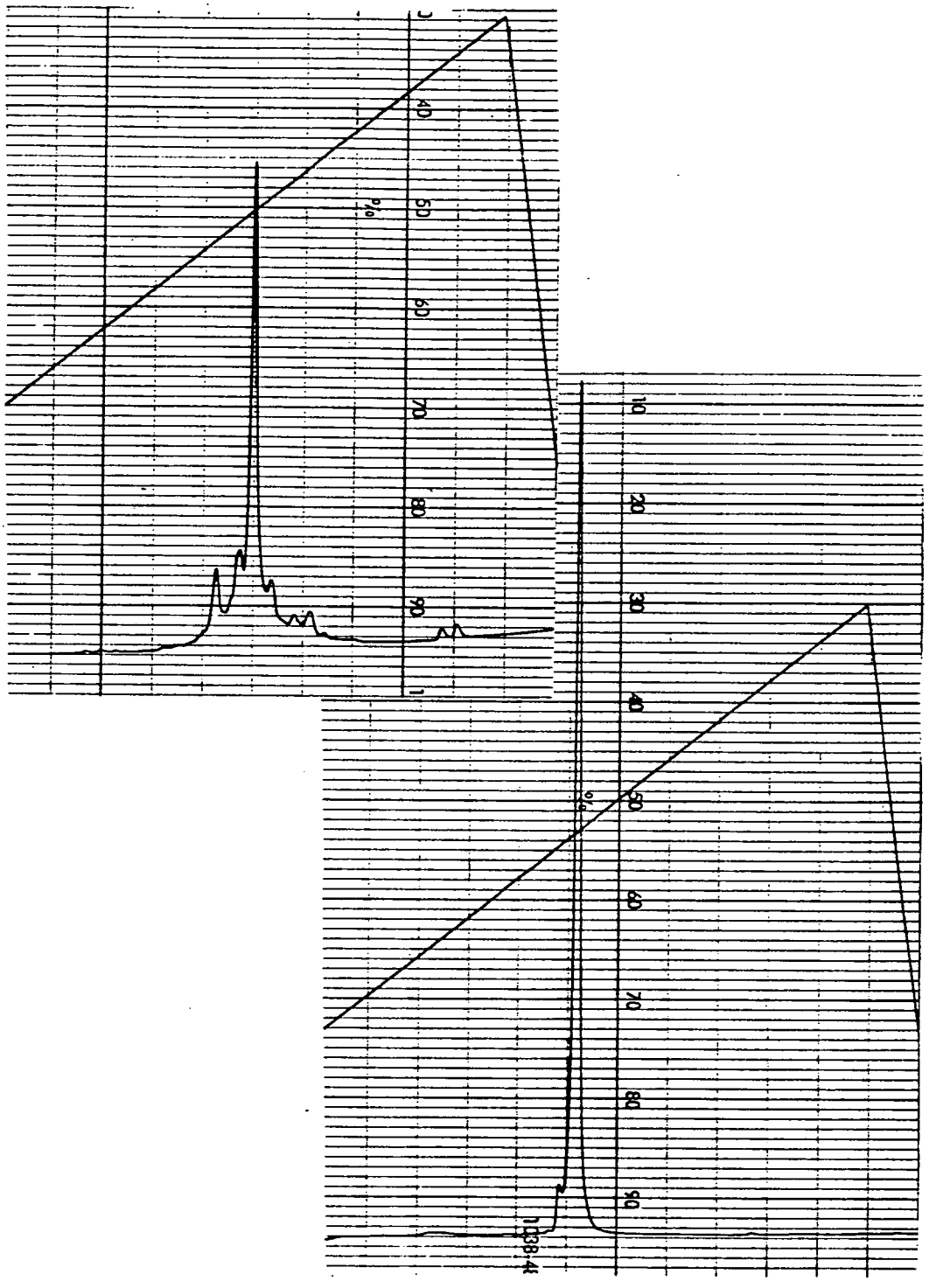


Figure 2.8 Analytical HPLC of crude big gastrin (top) and purified big gastrin (bottom).

Since the C-terminal amide is necessary for binding of the antibody then this will be a direct indication of the presence of this moiety. Little and big gastrin were found to have relative immunochemical potencies of 0.7 and 0.4 respectively at the C-terminus compared to the natural material. These values although slightly low, show that significant antibody binding has occurred and thus confirm the presence of the C-terminal amide. The lower value obtained for big gastrin is likely to be due to its larger size and thus its greater tendency to retain solvent molecules. This would lead to inaccurate weighing of the peptide and would be reflected in the potency obtained.

Substance P was released from the solid support and the side chain protection removed by treatment with TFA, water, thioanisole and ethyl methyl sulphide for 3 hours. The scavengers were removed from the crude peptide by passing it down a gel permeation column. The crude peptide was then purified to homogeneity through the use of cation exchange chromatography and semi-preparative HPLC. Cation exchange chromatography proved essential in this case as a significant amount of material was separated which was shown to be deficient in arginine. Since arginine is a charged amino acid, this technique was especially conducive to the separation of these two peptides. The material obtained from cation exchange was finally purified by semi-preparative HPLC and a 22% yield of the desired peptide was obtained. The authenticity of synthetic substance P was shown through characterisation with accurate mass FAB MS and amino acid analysis. This was further corroborated by showing that the synthetic material had an identical retention time to genuine substance P on analytical HPLC. Substance P was further characterised by 2 dimensional ^{1}H n.m.r. at 600 MHz. A complete assignment of the spin systems of each amino acid was made and no spurious peaks were present (figure 2.9). The methionine C-terminal amide protons were observed at 7.10 and 7.49 ppm in Substance P.

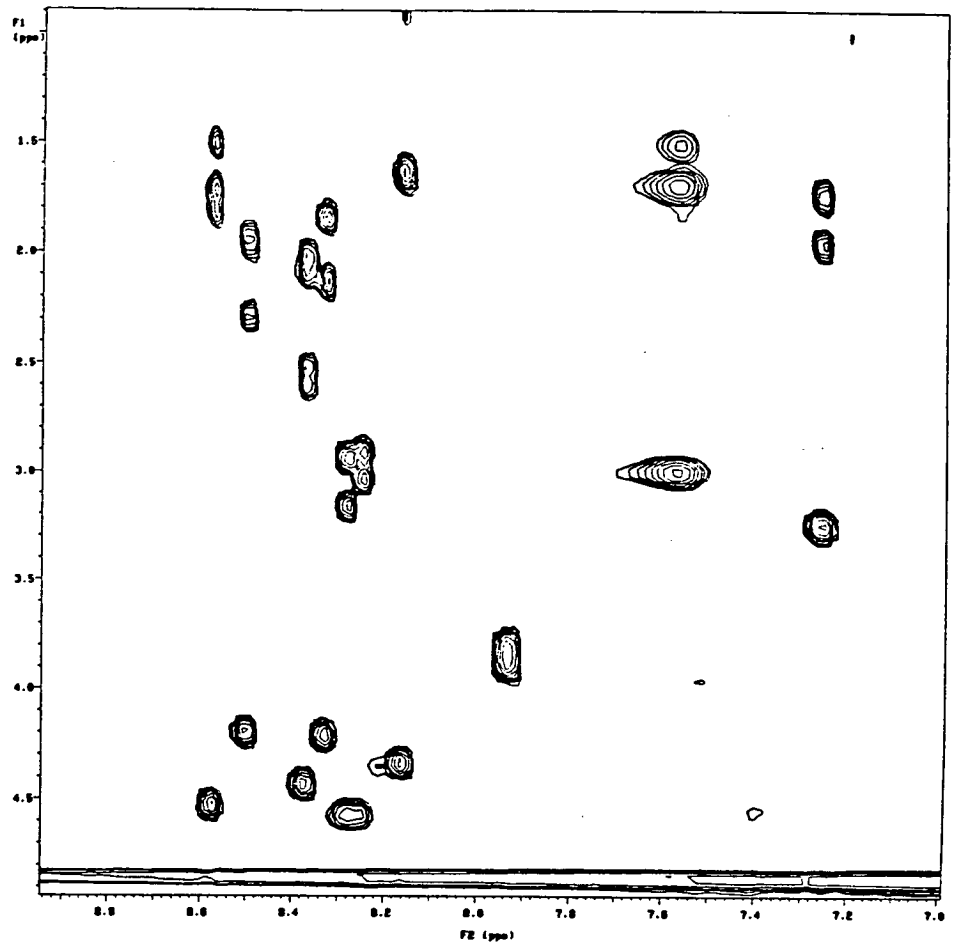


Figure 2.9 TOCSY spectrum of substance P showing α-NH correlations.

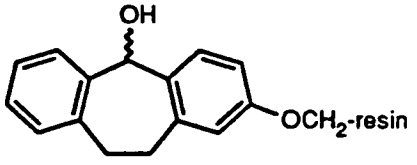
Bombesin was side chain deprotected and removed from the solid support by treatment with TFA, water, thioanisole, ethyl methyl sulphide and N-acetyltryptophan. The crude material obtained was purified using gel permeation chromatography and semi-preparative HPLC. The purified material was obtained in 21% yield and was shown to be homogeneous by analytical reverse phase HPLC. The purified synthetic bombesin was found to co-elute with the genuine material on analytical HPLC and was further characterised by FAB MS and amino acid analysis. A complete assignment of all resonances within the molecule was accomplished by 2

dimensional proton n.m.r. spectroscopy (TOCSY) at 600 MHz and no extra peaks were found. The methionine C-terminal amide protons in bombesin were observed at 7.17 and 7.55 ppm.

The synthesis of these peptides demonstrates the utility of solid support (46) for the facile generation of peptide amides under mild conditions. These peptide amides were all present in high proportion in the crude cleavage mixtures and were also isolated in good yield after several purification steps. The merit of this amide linker was particularly exemplified in the synthesis of the 34 amino acid peptide big gastrin which was synthesised and purified with few problems and was obtained in excellent yield.

2.2. Solid phase synthesis of protected peptides.

As a consequence of the results obtained for the release of peptide amides from the dibenzocycloheptadiene linker, it was envisaged that this linker could be adapted for use in the synthesis of protected peptides with a C-terminal free acid. This linker would require a hydroxyl group at the 5 position of the dibenzocycloheptadiene system instead of an amino function. Therefore in this case an ester bond would constitute the peptide-resin link in contrast to the amide linkage used previously. The ester bond would be very much more acid sensitive than the corresponding amide bond and thus would have application in the synthesis of peptides with fully protected side chains.



(45)

Carbinol resin (45) which had already been synthesised as the immediate precursor of amide linker (46), was chosen for the synthesis of protected peptide acids. This resin was loaded with Fmoc-glycine by reaction with 4 equivalents of its preformed symmetrical anhydride along with a catalytic amount of N,N-dimethylaminopyridine (DMAP). The catalyst is required in this case as the 5 position hydroxyl is not sufficiently nucleophilic to react with the symmetrical anhydride. Solid support (45) was found to be 60% loaded with Fmoc-glycine after 3.5 hours of reaction.

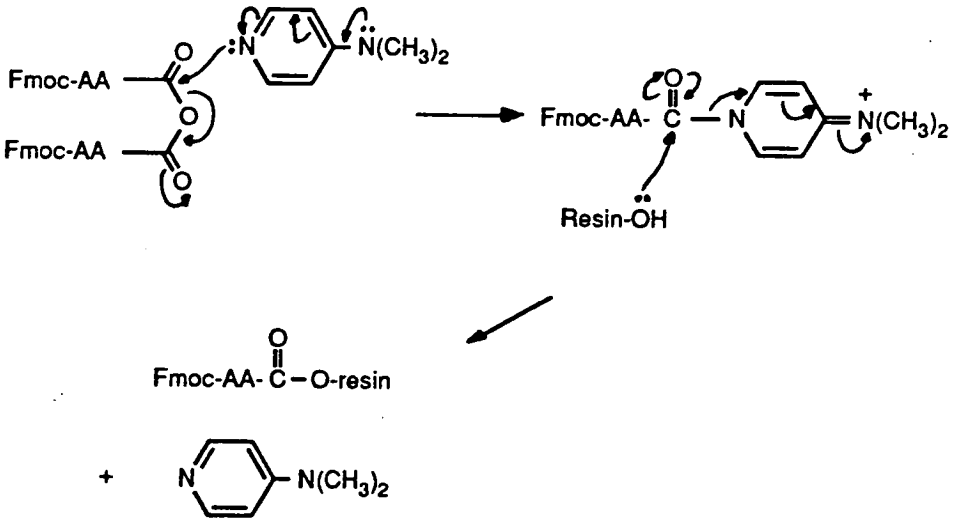


Figure 2.10 Mechanism of catalytic action of DMAP.

Treatment of the glycine loaded resin (58) with 10% acetic acid in DCM resulted in the amino acid being released quantitatively in less than 5 minutes. Release of the peptide from the solid support with this strength of acid would leave side chain protecting groups such as *t* -butyl and Boc intact and therefore this linker would be amenable to the synthesis of protected peptides to be used later in fragment condensation. As a result of the extreme acid sensitivity of the peptide-resin link one drawback however, is the limitation on the use of HOBt as a coupling reagent during

peptide synthesis with resin (45). HOBt is itself a mild acid and thus would effect premature release of the resin bound peptide during peptide synthesis, resulting in a undesirable low yield of peptide. As a measure of the stability of the peptide-resin link, the resin bound peptide H-Thr(*t* -Bu)-Ile-Phe-Ala-Gly (59) was synthesised using loaded resin (58). Through the use of HOBt active ester acylation and quantitative UV measurement of the deprotection effluent, it was found that the amount of resin bound material at the end of the synthesis was only half of that present at the beginning. The loss of this material was due to the acidic properties of HOBt, however this could be circumvented by buffering the reaction mixture with an appropriate base or avoiding the use of HOBt esters as activated species.

The preliminary results obtained using the dibenzocycloheptadiene system as a linker in the solid phase synthesis of fully protected peptides indicate that the peptide-resin link is extremely acid sensitive and thus would have application in producing peptides to be used for fragment condensation.

2.3. Solid phase synthesis of peptide hydrazides.

2.3.1. Introduction.

Due to the preliminary success of using the dibenzocycloheptadiene system as an amide linker, it remained to further develop this system as a means of generally derivatising the C-termini of peptides generated by the solid phase method. It was envisaged that from one particular intermediate compound, a number of different linkers could be synthesised and used in solid phase peptide synthesis. These would be used in the synthesis of C-terminal peptide amides, fully protected peptide free acids, C-terminal secondary amides and C-terminal hydrazides. Since initial experiments attempted in the synthesis of secondary amides had been unsuccessful,

the use of the dibenzocycloheptadiene system was extended firstly to the synthesis of C-terminal hydrazides on a solid support. Peptide hydrazides are useful in the coupling of peptide or protein fragments as they can be easily converted to their corresponding azides, species which are highly reactive and lead to an efficient acylation reaction with minimal racemisation. They may also prove to be of use in the conjugation of peptides to proteins and proteins to other proteins.

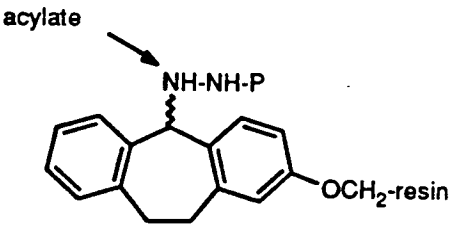
As with the synthesis of peptide amides, the most facile route to the production of C-terminal hydrazides is through the generation of these compounds upon cleavage of the peptide-resin link. This method obviates the use of hydrazine which is a powerful nucleophile (exhibits the α effect) and thus can cause deleterious reactions upon contact with the peptide. It was also desirable to generate the peptide hydrazide under the mildest conditions possible thus introducing scope for leaving the side chain protecting groups intact. Although desirable, this feature would not be essential as a minimal protection strategy could be adopted in the condensation of fragments activated by the azide method. Once generated, the peptide hydrazide can be readily converted to the acyl azide, through use of the optimised conditions developed by Honzl and Rudinger[82] involving the use of butyl nitrite and hydrogen chloride, which would then be coupled to the amino terminus of another peptide.

The rate of acidolysis facilitated by the methoxy dibenzocycloheptadiene linker suggests the use of this type of system for the generation of peptide hydrazides while the utility of the hydrazide linker can be assessed through the synthesis of model peptides.

2.3.2. Synthesis of the hydrazide linker.

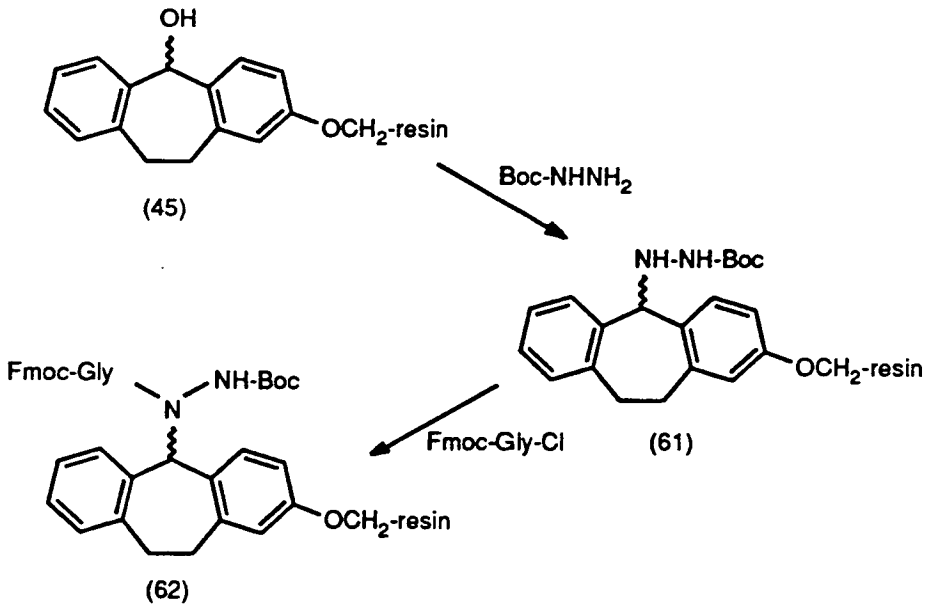
In order to functionalise the dibenzocycloheptadiene system so as to produce peptide hydrazides upon cleavage of the peptide- resin link, it would be necessary to

introduce a hydrazine derivative at the 5 position of the tricyclic system. This hydrazine derivative would have to incorporate a temporary blocking group (P) on one nitrogen as both nitrogens of hydrazine are nucleophilic and would be susceptible to acylation (60).



(60)

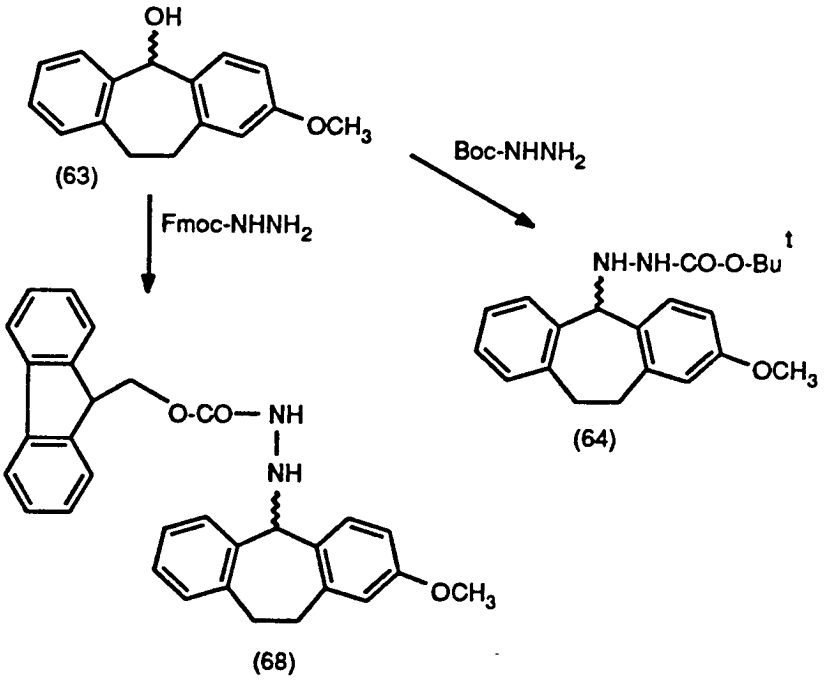
A reaction analogous to the one used for introduction of the amine into the C-terminal amide linker (46) was envisaged for functionalising carbinol resin (45) with a masked hydrazide. Carbinol resin (45) was reacted with *t* -butyloxycarbonylhydrazine (Boc hydrazide) in the presence of a catalytic amount of benzenesulphonic acid to generate hydrazide resin (61). This reaction was intially attempted in DMF and was shown to have been successful by the presence of urethane NH and carbonyl absobtions at 3410 and 1710 cm^{-1} respectively. The reaction was shown to be only partially complete after combustion analysis of the nitrogen content. It was found to go to completion however, if the reactants were heated under reflux in DCM overnight.



Nitrogen analysis of resin (61) in this case gave the expected value for 100% loading of Boc hydrazide. In order to elongate a peptide from this resin, it was first necessary to acylate the free nitrogen of the hydrazide linker (61) using an activated amino acid. Due to the steric bulk of the Boc protection on the second nitrogen of the hydrazide, difficulty in loading the first amino acid was envisaged. To overcome this, Fmoc-glycine was introduced, activated as its preformed acid chloride. It was found that reaction of Fmoc-glycyl chloride in DCM with hydrazide resin (61) in the presence of pyridine gave 50% loading of the amino acid. When the acylation reaction was performed in DCM while being heated under reflux, the loading was found to be quantitative after 5 hours. This loaded resin (62) was now ready to be used in the synthesis of peptide hydrazides.

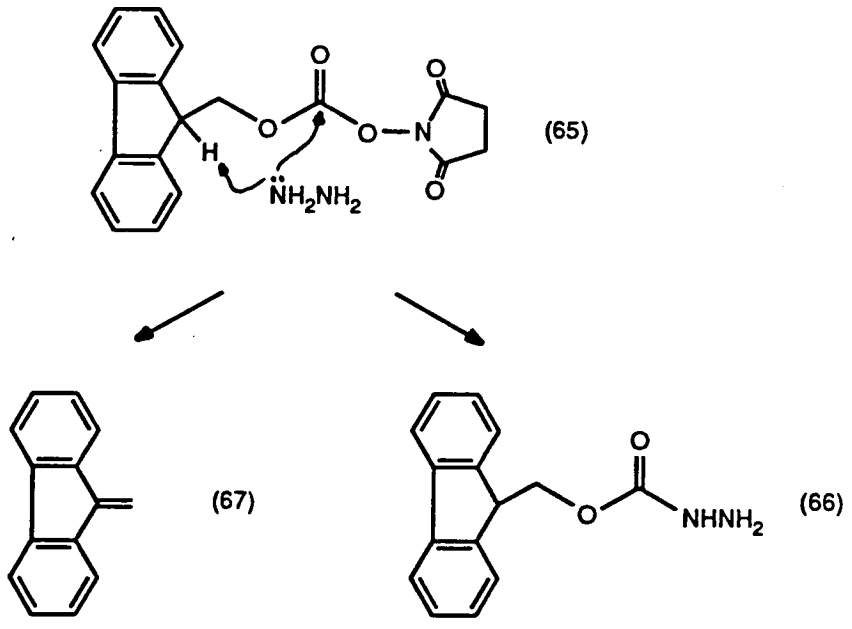
At this point, it was not known which of two possible isomers had been formed during the reaction of the carbinol resin with Boc hydrazide. It was conceivable that either nitrogen of the protected hydrazine could react with the 5 position of the dibenzocycloheptadiene ring. This would be of no consequence in the generation of

the protected hydrazide as acidolytic cleavage of the peptide from both isomers would give the correct peptide hydrazide in each case. However, proper characterisation of the intermediates was believed to be essential in order to approach the problem of synthesising peptide hydrazides in a systematic fashion. In order to achieve this, two model compounds were synthesised and characterised to further elucidate the mechanism of the reaction between Boc hydrazide and carbinol resin (45).

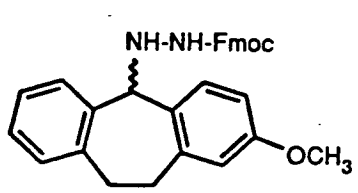


Boc hydrazide was reacted with methoxy carbinol (63), which was produced by $LiBH_4$ reduction of ketone (38), using the same conditions as with the analogous solid phase reaction. Hydrazine derivative (64) was obtained in an excellent yield of 99% after isolation by flash chromatography and was shown to have similar infrared absorbances to the hydrazide resin (61) at 3400 and 1710 cm^{-1}. It was intended to grow crystals of this compound in order to elucidate its structure by X-ray analysis

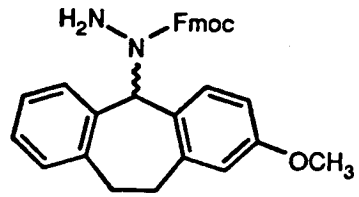
however it did not prove to be conducive to crystallisation. The analogous Fmoc protected hydrazine derivative was envisaged as being a crystalline compound and therefore its synthesis was attempted. In order to accomplish this, the requisite Fmoc protected hydrazine derivative first had to be synthesised. This was achieved by reacting N-(9-fluorenylmethoxycarbonyloxy)succinimide (65) with excess hydrazine hydrate. Fmoc hydrazine (66) was obtained in a yield of 61%. The low yield was probably due to a competing side reaction through β elimination of the starting material. Since hydrazine is a strong base, it can abstract the acidic proton of the fluorenyl system and thus generate the corresponding olefin, dibenzofulvene (67).



The Fmoc protected hydrazine (66) was then reacted with carbinol (63), with acid catalysis, to afford hydrazine derivative (68) in 80% yield. This compound failed to produce crystals of sufficient quality for X-ray analysis however its structure was solved by n.m.r. The two possible isomers which might have been produced during the formation of this compound are (68a) and (68b).



(68a)



(68b)

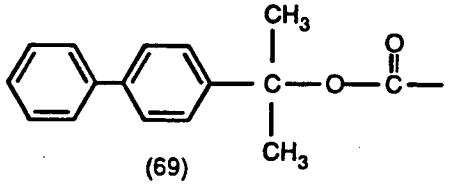
Isomer (68a) would have one proton on each of the two hydrazine nitrogens and these would be expected to have markedly different chemical shifts. Isomer (68b) would have two equivalent protons on one nitrogen of the hydrazine. ^{1}H n.m.r. at 80 MHz of hydrazine derivative (68) showed the presence of two non-equivalent protons which both disappeared upon addition of deuterium oxide to the n.m.r. sample. This supported the structure as isomer (68a).

2.3.3. Cleavage of the peptide-resin link.

Initial results showed that in 1% trifluoroacetic acid in DCM, Fmoc-glycylhydrazide was released from the loaded solid support (62) in 64% yield after 90 minutes. Treatment of the resin bound amino acid with 100% trifluoroacetic acid however, resulted in instantaneous quantitative release of the protected amino acid from the resin.

Although the peptide-resin link is labile to 1% TFA/DCM, a substantial amount of time is required for complete release of the peptide hydrazide. These conditions would not however, be suitable for the synthesis of maximally protected peptide fragments as partial loss of side chain protecting groups would undoubtedly result. Use of the methoxy dibenzocycloheptatriene system would introduce greater acid lability to the peptide-resin link and would allow the synthesis of maximally

protected fragments. In order to accomplish this however, a more acid sensitive group would have to be used for the protection of the second nitrogen of the hydrazine derivative. One such blocking group that could be used is biphenyl isopropyloxycarbonyl (69) or Bpoc.[98]



(69)

Another possible protecting group would be the *p* -toluenesulphonyl or tosyl group. This would be stable to mild acid however it may eliminate during the conversion of the protected hydrazide to the azide and thus would prove useful.

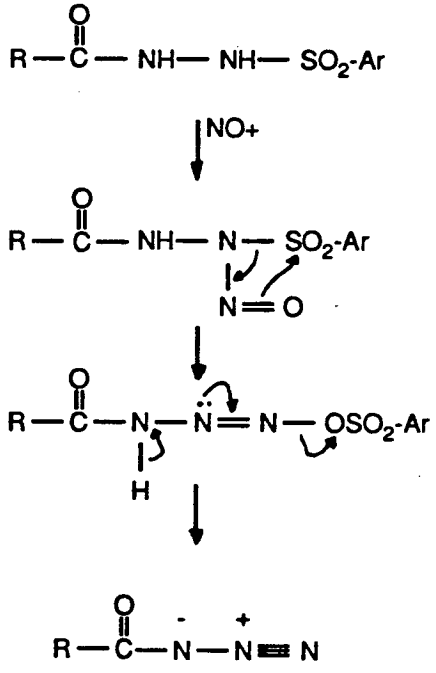


Figure 2.11 Proposed mechanism for azide generation with tosyl protection.

In the synthesis of minimally protected fragments, hydrazide linker (61) would prove very useful as most side chain protection could be removed. There would be a need however, for a lysine blocking group that was stable under the conditions used for side chain and N^{α} protecting group removal i.e. it would be stable to piperidine and to trifluoroacetic acid. This would enable the protection of lysine while leaving other side chains free, thus enhancing the solubility of peptide fragments in aqueous and organic systems.

2.3.4. Synthesis of C-terminal peptide hydrazides.

Using solid support (61) loaded with Fmoc-glycine, peptide synthesis was accomplished using single coupling and HOBt activation in the synthesis of the pentapeptide Ubiquitin 43-47 (70).

H-Leu-Ile-Phe-Ala-Gly-NH-NH_2 (70)

Deprotection and capping cycles were as delineated in the solid phase synthesis of peptide amides. The completed peptide was released from the solid support and the hydrazide protection (Boc) removed by treatment with trifluoroacetic acid, water and anisole for 1 hour. In a refinement of the protocol for isolation of the crude peptide after removal from the solid support, an extraction procedure was used as an alternative to precipitation with diethyl ether. For this method, chloroform was added to a concentrate of the cleavage mixture, followed by 10% acetic acid in H_2O. The biphasic system was then stirred for 0.5 hours and then the aqueous layer was separated and lyophilised. This procedure was effective in removing the scavengers and in leaving the crude peptide in a more soluble form. The crude material obtained was then purified to homogeneity through the use of semi-preparative HPLC. This afforded the peptide hydrazide in 25% yield and its structure was confirmed by

accurate mass FAB MS to within 1 part per million. Amino acid analysis also confirmed the amino acid composition. The crude mixture obtained from the resin was examined for the presence of a peptide with a free acid at the C-terminus. This could arise from the incomplete reaction of Boc hydrazide with the carbinol resin (45). The quantity of free acid peptide could be observed through the use of reverse phase analytical HPLC however it was found to be a minuscule amount. This was accomplished by comparing the retention time of the authentic free acid peptide with that obtained from the crude peptide hydrazide.

As the synthesis of this initial peptide hydrazide proved successful, the methodology was further exemplified in the synthesis of the 10 residue peptide ubiquitin 67-76 (71).

H-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-NH-NH_2 (71)

The synthesis of this peptide was achieved using the standard methodology of preformed symmetrical anhydride/HOBt activation. The completed peptide was then released from the solid support and the side chain and hydrazide protection removed by treatment with trifluoroacetic acid, H_2O, anisole and thioanisole for 3 hours at room temperature. The conditions used were those previously optimised for synthetic ubiquitin.[99] The crude peptide was isolated using the extraction procedure previously outlined for ubiquitin 43-47 (70).

The crude mixture obtained was then examined by analytical HPLC and found to have two major components (A and B) in the ratio of approximately 3:2 (figure 2.12). These were then separated by semi-preparative HPLC and then analysed using a variety of techniques.

Both components had identical amino acid analyses and this showed the amino acid composition for both to be as expected for the desired hydrazide peptide. Each component gave the requisite exact mass of 1147.75390 for $C_{51}H_{95}N_{20}O_{10}$ and the primary sequence of both was confirmed by Edman degradation.

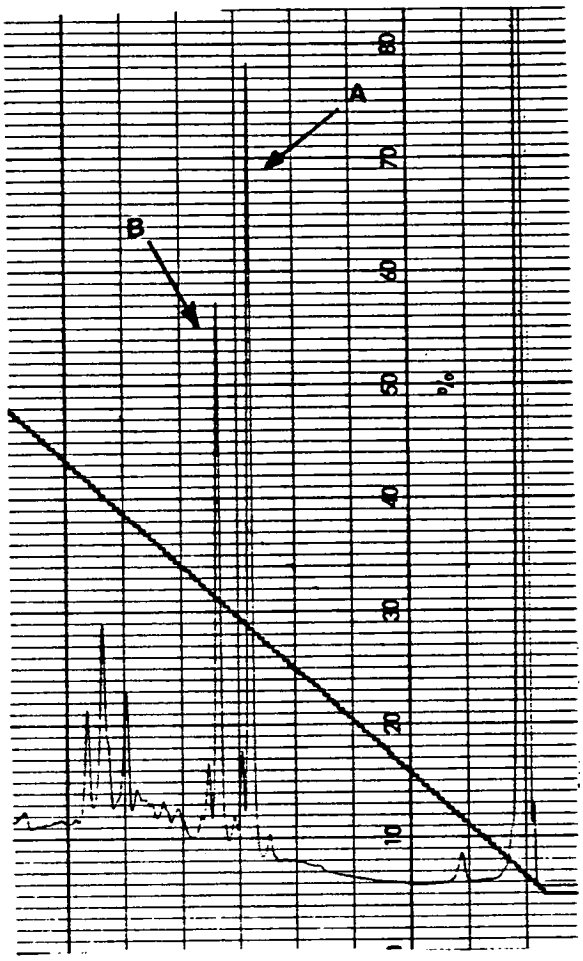


Figure 2.12 Analytical HPLC of crude decapeptide hydrazide (71).

As these two components were indistinguishable by these analytical techniques, it was assumed that they must be isomers of each other. Both compounds were examined by proton n.m.r. at 600 MHz and found to differ markedly in one region of the spectrum. The resonances of all the amino acid spin systems for both components were assigned by the 2 dimensional technique, total correlation spectroscopy (TOCSY). Component A (R_t 11.2 min.) was found to have unusual values for the γ

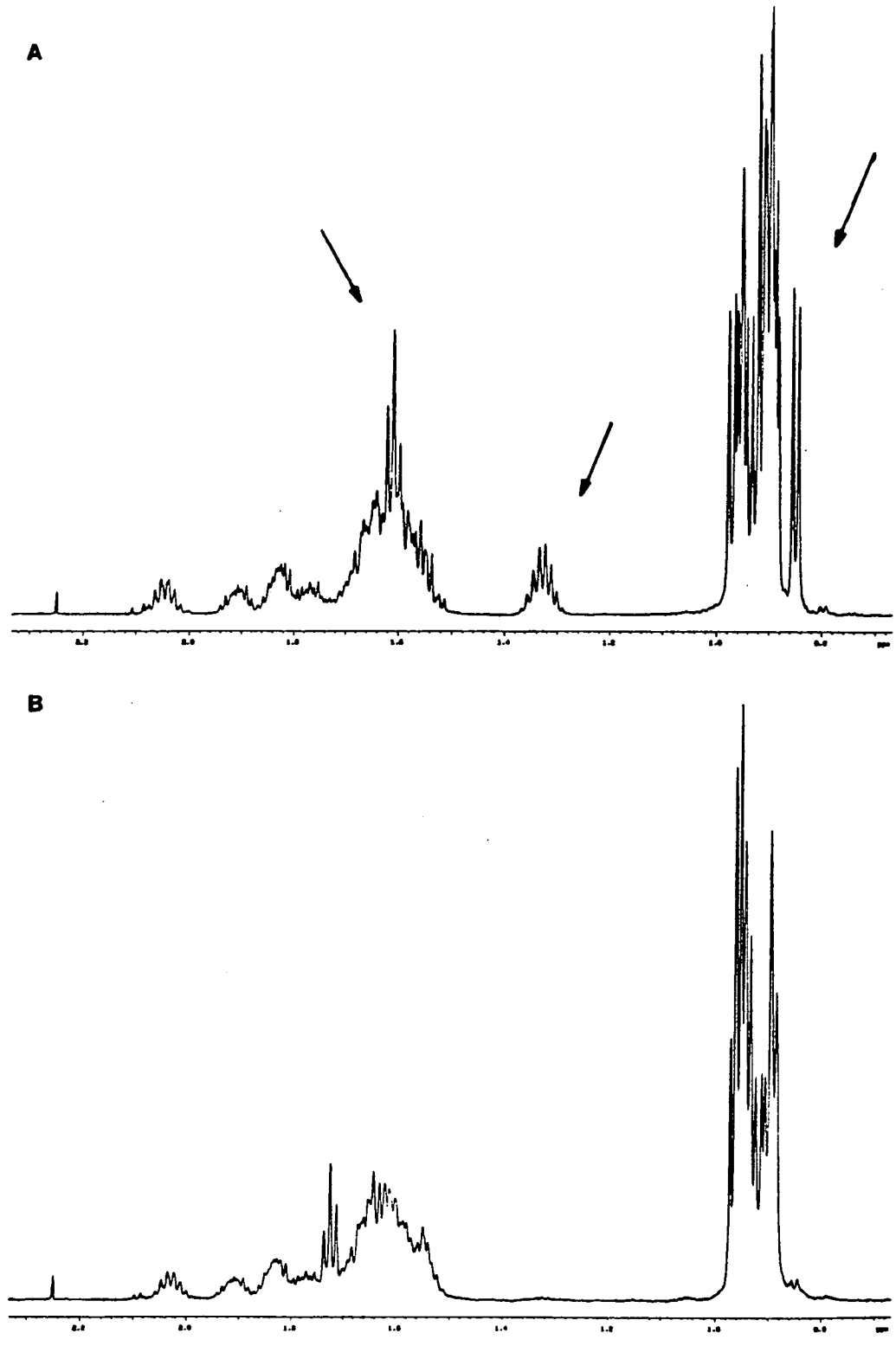


Figure 2.13 1D ^{1}H n.m.r. spectrum of components A & B, arrows indicate unusual leucine resonances.

hydrogens of leucines 67 and 69 i.e. the two leucines on either side of histidine 68. Component B (R_t 12.2 min.) was found to have normal values for the γ protons of these two leucines. The methyl region (δ protons) of component A were also significantly different from those of component B. In addition to this, the values for the 2H and 4H of the imidazole ring of histidine differed by about 0.1 ppm between the two compounds. All other resonances for the two peptides were comparable. These results indicate a difference in the histidine residue in component A causing a different enviroment for the two leucines on either side of it.

Enzymatic hydrolysis of the two peptides using leucine aminopeptidase apparently showed the presence of an unnatural feature in component A. Amino acid analysis of the enzymatic hydrolysis mixture of this component showed the almost complete absence of histidine and valine and a lower amount of leucine than expected. Analysis of the hydrolysate for component B indicated the presence of all amino acids however leucine and arginine were slightly higher than expected. These results appear to indicate the presence of an unnatural characteristic in peptide A as it was not fully recognised by the enzyme. The optical rotations for the two peptides were comparable with the values -49.4 and -56.7 being obtained respectively. ROESY n.m.r spectroscopy, in which through-space interactions of hydrogens can be detected (i.e. when two hydrogens are close in space, a signal is produced) showed that in component A the α CH of leucine 67 was closer in space to the α NH of histidine 68 than in component B.

Although no concrete predication can be made at present about the difference between components A and B, several theories can be postulated. One suggestion is that racemisation of histidine has occurred since this residue is prone to this. The fact that component A is not fully recognised by the enzyme suggests the possibility of a D amino acid being present. The presence of the unusual γ resonances for leucines 67

and 69 in peptide A indicates that they are in a different enviroment for those in peptide B. This could be explained by the presence of D-histidine as the different stereochemistry of this amino acid would place the leucines in a different enviroment and possibly result in different chemical shifts for the γ and δ protons of these residues. Although the D isomer was present in greater proportion than the L isomer (if complete racemisation is the correct explanation) then this could have resulted from a faster rate of acylation of leucine 67 with the D histidine than the L histidine.

One other possible explanation for the formation of two isomers during synthesis is the presence of a *cis* amide bond in one component. Since peptide bonds are planar due to their partial double bond character, it is possible for them to exist in *trans* (Z) or *cis* (E) form. The majority of peptide bonds exist in the *trans* form which is of lower energy, however *cis* amide bonds do occur infrequently especially with proline residues. The existence of a *cis* amide bond between leucine 67 and histidine 68 could explain the difference between the two peptides. The *cis* amide bond may not have been recognised by the enzyme as it is an unusual occurrence in nature and thus it may be present in component A. The *cis* amide bond would also orient the histidine differently in relation to leucines 67 and 69 and could explain the unusual resonances observed in the proton n.m.r. of component A. Components A and B would be identical except for the presence of this bond and thus would have identical amino acid analyses and mass spectra. The presence of the *cis* amide bond in component A may also be indicated by the strong signal obtained for the leucine α CH to histidine α NH correlation in the ROESY spectrum.

An n.m.r. investigation of a 17 residue peptide amide by Peggion et al.[100] showed the presence of a *cis* amide bond between the first two amino acids where the first three amino acids were Ile-Lys-Ile. The n.m.r. investigation of this peptide was carried out in the presence of sodium dodecyl sulphate micelles as a mimic for the

hydrophobic nature of a biological membrane. The *cis* amide bond however was not observed in the absence of the micelles. Similarity can be seen between the conditions for observance of the *cis* amide in this peptide and those in the ubiquitin fragment. A certain amount of homology can be seen between the first three amino acids in both peptides i.e. Leu-His-Leu is similar to Ile-Lys-Ile in that a basic amino acid is sandwiched between two bulky hydrophobic residues. Although components A and B were not studied in the presence of micelles, the sequence is in itself very hydrophobic and thus hydrophobic interactions may stabilise an otherwise unfavourable situation such as a *cis* amide bond. In addition to this, steric constraints may favour the existence of the E rather than the Z form. One piece of evidence pointing against the presence of a *cis* amide bond was discovered again through the use of proton n.m.r. Variable temperature 1 dimensional experiments between 30 and 70°C of components A and B showed no interconversion of the two isomers which would probably be expected as energy is put into the system. These experiments did however show the appearance of two sets of histidine resonances in component A as the temperature was increased and which coalesced upon cooling of the sample.

Due to the lack of further evidence, it was not possible to conclude the exact nature of the two isomers which were formed during the synthesis. Further n.m.r. experiments in different solvents may give greater corroboration to one of these two conjectures or give rise to a different and more appropriate explanation for the unusual occurrence observed.

Despite the occurrence of these two isomeric peptides, it was confirmed by FAB MS that both were the required peptide hydrazide. Studies in this laboratory indicate that the occurrence of these two isomers is a consequence of the peptide sequence and not of the presence of the C-terminal hydrazide.[101]

The synthesis of these two peptides exemplifies the use of hydrazide resin (61) in

the solid phase synthesis of C-terminal peptide hydrazides. Once the hydrazide has been generated, it simply remains to convert this to the acyl azide in order to actuate the condensation of the fragment with another peptide or protein.

Chapter 3.
EXPERIMENTAL

3.1. Notes.

All amino acid derivatives were purchased from Novabiochem and were of the L-configuration. Melting points were determined on an electrically heated Buchi 510 melting point apparatus in open capillary tubes and are uncorrected. Optical rotations were measured in a 10 cm cell using a AA1000 polarimeter. Thin layer chromatography (t.l.c.) was performed on commercially available plastic sheets precoated with silica gel 60 Gf-254 (Merck). Solvent systems are as quoted in the text. Detection of compounds was carried out using an appropriate combination of UV absorption at 254 nm, iodine vapour, potassium permanganate and ninhydrin sprays. High pressure liquid chromatography (HPLC) was carried out mainly on an Applied Biosystems system ie. 2 x 1406A solvent delivery systems, a 1480A injector/mixer, and a 1783A detector/programmer. Analytical and semi-preparative separations were accomplished using the appropriate conditions as indicated in the text. Amino acid analysis was performed on an LKB 4151 amino acid analyser subsequent to Carius tube hydrolysis with constant boiling hydrochloric acid at 110°C for 18 to 36 hours. Peptide sequencing was performed by Welmet on an Applied Biosystems 477A protein sequencer. Infrared spectra were recorded between 4000 and 600 cm^{-1} using a Perkin Elmer 781 spectrophotometer either in solution (CH_2Cl_2) or as a KBr disc. Polystyrene was used as the standard (1603 cm^{-1}). Ultraviolet spectra were obtained using a Varian Cary 210 spectrophotometer and samples were dissolved in the appropriate solvent. High and low resolution fast atom bombardment (FAB) spectra were obtained on a Kratos MS50TC machine. Proton n.m.r. spectra were obtained from a Bruker WP80 (80 MHz), a WP200 (200 MHz), a WP360 (360

MHz) or a Varian VXR 600 (600 MHz) machine in the solvent indicated using tetramethylsilane as the external standard ($\delta = 0.000$). Carbon-13 n.m.r. spectra were recorded on a Bruker WP200 (50.3 MHz) or a WP360 (90.6 MHz) machine in the indicated solvent relative to TMS set at zero. Elemental analyses were performed on a Carlo Erba elemental analyser model 1106. All solvents used were either distilled before use or were of analytical grade (as supplied). The following solvents were dried prior to use using the drying agent indicated in parentheses: Benzene (sodium wire), dichloromethane (calcium hydride), toluene (sodium wire) and tetrahydrofuran (sodium with benzophenone indicator).

3.2. Experimental.

Methyl 2-bromomethylbenzoate. (31)

Methyl 2-methylbenzoate (5.0 g, 22.0 mmol) was dissolved in CCl_4 (50 ml) and to this was added N-bromosuccinimide (6.5 g, 37.0 mmol) and dibenzoylperoxide (0.05 g, 0.21 mmol). The solution was then heated under reflux and irradiated for two hours using a 500 W lamp. The succinimide precipitate was then filtered off and the filtrate concentrated *in vacuo* to give the *required compound* as a yellow oil. This was then used without further purification. (crude yield 9.8 g): $\nu_{max.}$ (CH_2Cl_2) 1720 (C=O), 610 cm^{-1} (C-Br); δ_H (80 MHz, $CDCl_3$) 8.2 - 7.2 (4H, m, aromatic CH's), 4.9 (2H, s, -$C\underline{H}_2$Br), 3.85 (3H, s, -$CO_2C\underline{H}_3$); m/z (EI) 228, 149.

2-(Methoxycarbonyl)benzyltriphenylphosphonium bromide. (32)

The crude product obtained from bromination (31) (9.78 g, 33.0 mmol) was dissolved in toluene (50 ml) together with triphenylphosphine (8.66 g, 33.0 mmol) and the mixture was stirred at room temperature overnight. The resulting precipitate was filtered off, washed with toluene and recrystallised from DCM/toluene to give the *required compound* as a white crystalline solid (11.8 g, 73%): m.p. 230-235°C; t.l.c (10% MeOH/$CHCl_3$) Rf 0.26; Found: C, 66.0; H, 4.9. Calc. for $C_{27}H_{24}O_2PBr$: C, 66.0; H, 4.9%; $\nu_{max.}$ (CH_2Cl_2) 1715 (C=O), 1440 (CH_2), 1110 cm^{-1} (C-O); $\lambda_{max.}$ (EtOH) 275, 268 nm (ε 2550 dm^3 mol^{-1} cm^{-1}), 226 nm (ε $3.79x10^4$ dm^3 mol^{-1} cm^{-1}); δ_H (80 MHz, $CDCl_3$) 7.85-7.25 (19H, m, aromatic CH's), 5.9 & 5.75 (2H, d, J 15 Hz, benzylic $C\underline{H}_2$), 3.4 (3H, s, -$CO_2C\underline{H}_3$); δ_C (50 MHz, $CDCl_3$) 165(-$\underline{C}O_2CH_3$), 134-116 (aromatic C's), 51 (-$CO_2\underline{C}H_3$), 27-28 (-$\underline{C}H_2PPh_3$); m/z (FAB) 411, 379, 315, 277, 262.

2-Methoxycarbonyl-3'-methoxystilbene. (33)

2-(Methoxycarbonyl)benzyltriphenylphosphonium bromide (10 g, 20.0 mmol) was dissolved in THF (75 ml) under an atmosphere of dry nitrogen. The reaction mixture was then cooled to -78°C in a CO_2/acetone bath and potassium tertiary butoxide (2.28 g, 20.0 mmol) was added, after which the solution turned deep yellow in colour. The temperature was then maintained at -78°C for 1.5 h. while stirring, after which time *m* -anisaldehyde (2.88 g, 21.0 mmol) in THF (25 ml) was slowly added. The solution was then stirred at -78°C for a further 0.5 h. and then allowed to warm to room temperature. After stirring overnight, the solvent was then removed *in vacuo* , the triphenylphosphine oxide precipitated with ether and filtered off, and the sample concentrated to leave the *required product* as a yellow oil. The crude product was then purified using flash chromatography (25% ether/n-hexane) to give stilbene (33) as a mixture of *cis* and *trans* isomers (4.56 g, 85%) : t.l.c. (25% ether/n-hexane) Rf 0.25; Found: C, 76.0; H, 5.9. Calc. for $C_{17}H_{16}O_3$: C, 76.1; H, 6.0 %; $\nu_{max.}$ (CH_2Cl_2) 1720 (C=O), 1600 & 1580 (aromatic C=C), 1135 & 1080 cm^{-1} (C-O); δ_H (200 MHz, $CDCl_3$) 8.05-6.6(10H, m, aromatic CH's + -C<u>H</u>=C<u>H</u>-), 3.9 (3H, s, -$CO_2C\underline{H}_3$), 3.6 (3H, s, -$OC\underline{H}_3$); δ_C (50 MHz, $CDCl_3$) 167 (-$\underline{C}O_2CH_3$), 160 (aromatic $\underline{C}$-OCH_3), 139-112 (aromatic C's), 122 (*cis* -<u>C</u>H=<u>C</u>H-), 119 (*trans* -<u>C</u>H=<u>C</u>H-), 55 (-$O\underline{C}H_3$), 52 (-$CO_2\underline{C}H_3$); m/z (EI) 268, 237, 209, 194, 165.

Methyl 2-(3'-methoxyphenylethyl)benzoate. (36)

The above compound (33) (2.5 g, 9.3 mmol) was dissolved in methanol (25 ml) and hydrogenated in the presence of 10% palladium on charcoal (0.25 g). After 4 h. the catalyst was filtered off through celite and the solution was concentrated *in vacuo* to leave a colourless oil. This was then purified using flash chromatography (25% ether/n-hexane) to give the *title compound* (2.4 g, 90%): t.l.c. (25% ether/n-hexane) Rf 0.26; Found: C, 75.7; H, 6.8; Calc. for $C_{17}H_{18}O_3$: C, 75.5; H, 6.7%; $\nu_{max.}$

(CH_2Cl_2) 1720 (C=O), 1600 & 1580 (aromatic C=C), 1490 cm^{-1} (CH_2); $\lambda_{max.}$ (EtOH) 280 nm (ε $3.16x10^3$ dm^3 mol^{-1} cm^{-1}) 273 nm (ε $2.89x10^3$ dm^3 mol^{-1} cm^{-1}); δ_H (80 MHz, $CDCl_3$) 8.0-6.7 (8H, m, aromatic CH's), 3.9 (3H, s, $-CO_2C\underline{H}_3$), 3.8 (3H, s, $-OC\underline{H}_3$), 3.4-2.8 (4H, m, $-C\underline{H}_2x2$); δ_C (50 MHz, $CDCl_3$) 167 ($-\underline{C}O_2CH_3$), 159 (aromatic $\underline{C}$-OCH_3) 143-111 (aromatic C's), 55 ($-CO_2\underline{C}H_3$) , 51 ($-O\underline{C}H_3$), 38, 36 ($-\underline{C}H_2x2$); m/z (EI) 270, 238, 149, 121.

2-(3'-Methoxyphenylethyl)benzoic acid. (37)

Methyl-2-(3'-methoxyphenylethyl)benzoate (36) (2.5 g, 9.2 mmol) was dissolved in MeOH/H_2O (4:1, 50 ml) along with 2 M NaOH (5 ml, 10.0 mmol) and heated under reflux for 2 h. The methanol was then removed *in vacuo* and the remaining aqueous solution washed with ether (2x25 ml). The solution was then acidified to pH1 with 2 M HCl and extracted with ethyl acetate (2x50 ml). The organic layer was then dried over $MgSO_4$ and concentrated *in vacuo* to leave the *required compound* as a white solid (2.3 g, 97%): m.p. 118-120 °C (lit.,[102] 120-121.5 °C); t.l.c. (25% ether/n-hexane) Rf 0.10; Found: C, 75.3; H, 6.4; Calc. for $C_{16}H_{16}O_3$: C, 75.0; H, 6.3%; $\nu_{max.}$ (CH_2Cl_2) 3500 (OH), 1690 (C=O), 1605 (aromatic C=C), 1490 cm^{-1}; $\lambda_{max.}$ (MeOH) 286 nm (ε $3.85x10^3$ dm^3 mol^{-1} cm^{-1}); δ_H (80 MHz, $CDCl_3$) 10.75 (1H, s, $-COO\underline{H}$), 8.2-6.7 (8H, m, aromatic CH's), 3.75 (3H, s, $-OC\underline{H}_3$), 3.5-2.75 (4H, m, $-C\underline{H}_2x2$); δ_C (50 MHz, $CDCl_3$) 173 ($-\underline{C}OOH$), 159 (aromatic $\underline{C}$-OCH_3), 144-111 (aromatic C's), 55 ($-O\underline{C}H_3$), 38 & 37 ($-\underline{C}H_2x2$); m/z (EI) 256, 238, 121.

2-Methoxydibenzocycloheptadien-5-one. (38)

This compound was prepared by the method of Gregori *et al* .[102]

The above compound (37) (1.0 g, 3.9 mmol) and polyphosphoric acid (11 g) were mixed and heated to 120°C while stirring mechanically. The mixture was maintained at 120 °C for 2 h. with stirring and then allowed to cool. Distilled water (50 ml) was

added and the aqueous solution was extracted with ether (2x50 ml). The organic layer was then washed with 10% Na_2CO_3 (2x50 ml), dried over $MgSO_4$ and concentrated *in vacuo* to leave a bright yellow oil. The oil was then purified using dry flash chromatography (25% ether/n-hexane) and recrystallised from ether/n-hexane to give the *title compound* as a pale yellow crystalline solid (0.69 g, 74%): m.p. 74.0-75.0°C (lit.,[102] 74.5-76.0°C); t.l.c. (25% ether/n-hexane) Rf 0.21; Found: C, 80.8; H, 6.0; Calc. for $C_{16}H_{14}O_2$: C, 80.6; H, 5.9%; $\nu_{max.}$ (CH_2Cl_2) 1640 (C=O), 1600 (aromatic C=C), 1115 cm^{-1} (C-O); $\lambda_{max.}$ (MeOH) 300 nm (ε 1.49x10^4 dm^3 mol^{-1} cm^{-1}); δ_H (360 MHz, $CDCl_3$) 8.17-8.14 (1H, d, ^3J 8.8 Hz, Ar4), 8.01-7.99 (1H, q, ^3J 7.8 Hz, ^4J 1.4 Hz, Ar6), 7.43-7.29 (2H, m, Ar7 + Ar 8), 7.21-7.19 (1H, q, ^3J 7.5 Hz, ^4J 1.0 Hz, Ar9), 6.87-6.83 (1H, q, ^3J 8.8 Hz, ^4J 2.6 Hz, Ar3), 6.70-6.69 (1H, d, ^4J 2.6 Hz, Ar1), 3.85 (3H, s, -OC$\underline{H}_3$), 3.17 (4H, s, -C$\underline{H}_2$x2); δ_C (50 MHz, $CDCl_3$) 193 (C=O), 162 (aromatic C2), 145-112 (aromatic C's), 55 (-OC$\underline{H}_3$), 35 & 34 (-C$\underline{H}_2$x2); m/z (EI) 238, 210, 195, 165.

2-Methoxydibenzocycloheptadien-5-ol (63)

Ketone (38) (0.5 g, 2.1 mmol) was dissolved in THF (15 ml) and $LiBH_4$ (0.18 g, 8.4 mmol) added. The reaction mixture was then heated under reflux in an atmosphere of nitrogen for 1.5 h. The solution was then cooled to 0°C in an ice/salt bath and methanol (10 ml) followed by acetone (10 ml) were added very slowly with stirring. The mixture was then concentrated *in vacuo* , the residue was taken up in ethyl acetate (50 ml) and washed with water (3x25 ml). The organic layer was then dried over $MgSO_4$ and concentrated *in vacuo* to leave a clear oil. This was then triturated with n-hexane and cooled to give the *title compound* as a white crystalline solid (0.38 g, 75%): m.p. 78-80°C; t.l.c. (MeOH/$CHCl_3$/AcOH 10:90:0.05) Rf 0.64; Found: C, 80.1; H, 6.7; calc. for $C_{16}H_{16}O_2$: C, 80.0; H, 6.7%; $\nu_{max.}$ (KBr disc) 3350 (OH), 2940-2840 (CH_2), 1615 (C=C), 1255 & 1050 cm^{-1} (C-O); $\lambda_{max.}$ (EtOH) 275 nm (ε =

3033 $dm^3\ mol^{-1}\ cm^{-1}$); δ_H (80 MHz, $CDCl_3$) 7.45-6.62 (7H, m, aromatic CH's), 5.77 (1H, s, C<u>H</u>), 3.77 (3H, s, -OC<u>H</u>$_3$), 3.67-2.83 (4H, m, -C<u>H</u>$_2$x2), 2.44 (1H, d, -O<u>H</u>); δ_C (50 MHz, $CDCl_3$) 159 (aromatic C2), 141-111 (aromatic C's), 77 (-<u>C</u>H), 55 (-O<u>C</u>H$_3$), 33 & 32 (-<u>C</u>H$_2$x2); m/z (EI) 240, 223, 209, 179, 163.

<u>2-Hydroxydibenzocycloheptadien-5-one.</u> (42)

This compound was prepared by the general method of Pfeiffer.[103]

2-Methoxydibenzocycloheptadien-5-one (38) (2.0 g, 4.2 mmol) was dissolved in benzene (20 ml) and anhydrous aluminium bromide (6.0 g, 22.0 mmol) in benzene (20 ml) was added. The reaction mixture was then heated under reflux in an atmosphere of nitrogen for 4 h. The solution was then cooled to 5°C in an ice bath and 2 M HCl (20 ml) was added slowly with mixing. The aqueous and organic layers were then separated and the aqueous layer extracted with ether (2x25 ml). The ether and benzene solutions were then combined and extracted with 2 M NaOH (2x20 ml). The aqueous layer was then acidified to pH 1 with 2 M HCl (40 ml) and extracted with ether (3x50 ml). The ethereal layer was then dried over $MgSO_4$ and concentrated *in vacuo* to leave a brown oil. This was then triturated with ether and recrystallised from ether/n-hexane to give the *title compound* as a white crystalline solid (1.76 g, 94%): m.p. 141.0-141.5°C; t.l.c. (1:1 ether/n- hexane) Rf 0.096; Found: C, 80.5; H, 5.4; Calc. for $C_{15}H_{12}O_2$: C, 80.3; H, 5.4%; $\nu_{max.}$ (CH_2Cl_2) 3560 (OH), 1640 (C=O), 1600 (C=C), 1190 cm^{-1} (C-O); $\lambda_{max.}$ (MeOH) 305 nm (ε $1.28x10^4\ dm^3\ mol^{-1}\ cm^{-1}$); δ_H (80 MHz, $CDCl_3$) 8.14-6.64 (7H, m, aromatic CH's), 6.4-6.1 (1H, s, -O<u>H</u>), 3.12 (-C<u>H</u>$_2$x2); δ_C (50 MHz, $(CD_3)_2CO$) 192 (<u>C</u>=O), 161 (aromatic C2), 145-113 (aromatic C's), 35 & 34 (-<u>C</u>H$_2$x2); m/z (EI) 224, 196, 176, 165.

2-(Benzyloxycarbonylmethoxy)dibenzocycloheptadien-5-one. (48)

2-Hydroxydibenzocycloheptadien-5-one (3.0 g, 13.4 mmol) was added to acetone (100 ml) along with K_2CO_3 (18.5 g, 134 mmol) and benzyl-2-bromoacetate (3.09 g, 13.5 mmol). The mixture was then stirred at room temperature overnight. The excess K_2CO_3 was then removed by filtration and the filtrate was concentrated *in vacuo* . The residue was then taken up in ethyl acetate (50 ml) and washed with saturated Na_2CO_3 (3x25ml) followed by water (1x25 ml). The organic layer was then dried over $MgSO_4$ and the solvent was removed *in vacuo* to leave a clear oil. This was then triturated with ether to give the *title compound* as a white solid (4.78 g, 96%): m.p. 81.5-82.0°C; t.l.c. (1:1 hexane/ether) Rf = 0.21; Found: C, 77.7; H, 5.4; Calc. for $C_{24}H_{20}O_4$: C, 77.4; H, 5.4%; $\nu_{max.}$ (CH_2Cl_2) 1760 (C=O ester), 1640 (C=O ketone), 1600 & 1490 (C=C), 1190 & 1120 cm^{-1} (C-O); $\lambda_{max.}$ (MeOH) 295 nm (ε = 1.57x10^4 dm^3 mol^{-1} cm^{-1}); δ_H (80 MHz, $CDCl_3$) 8.16-6.66 (12H, m, aromatic CH's), 5.22 (2H, s, benzyl $C\underline{H}_2$), 4.69 (2H, s, -$OC\underline{H}_2$), 3.11 (4H, s, -$C\underline{H}_2$x2); δ_C (90 MHz, $CDCl_3$) 193 (C=O), 168 (aromatic C2), 161-112 (aromatic C's), 67 & 65 (-$O\underline{C}H_2$ & benzyl $\underline{C}H_2$), 35 & 34 (-$\underline{C}H_2$x2); m/z (EI) 372, 237, 224, 207, 178.

2-(Carboxymethyloxy)dibenzocycloheptadien-5-one. (49)

Ketone (48) (3.3 g, 8.8 mmol) was dissolved in MeOH (40 ml) along with 2 M NaOH (8.8 ml, 17.6 mmol) and heated under reflux for 1 h. The methanol was then removed *in vacuo* and the solution was acidified to pH1 with 2 M HCl. The aqueous layer was then extracted with ethyl acetate (2x50 ml). The organic layer was then dried over $MgSO_4$ and removed *in vacuo* to leave the *title compound* as a white solid (2.41 g, 97%): m.p. 164-165°C; t.l.c. (n-butanol/AcOH/H_2O 7:2:1) Rf 0.66; Found: C, 72.2; H, 5.0; Calc. for $C_{17}H_{14}O_4$: C, 72.3; H, 5.0%; $\nu_{max.}$ (KBr disc) 2920 (CH_2), 1730 (C=O acid), 1645 (C=O ketone), 1600 (C=C), 1115 cm^{-1} (C-O); $\lambda_{max.}$ (MeOH) 295 nm (ε 1.23x10^4 dm^3 mol^{-1} cm^{-1}); δ_H (80 MHz, D_6 DMSO) 8.10-6.85 (7H, m,

aromatic CH's), 4.8 (2H, s, -OC$\underline{H}_2$), 3.15 (4H, s, -C$\underline{H}_2$x2); δ_C (50 MHz, D_6 DMSO) 192 (C=O ketone), 170 (C=O acid), 161 (aromatic C2), 145-113 (aromatic C's), 65 (-O$\underline{C}H_2$), 35 & 34 (-$\underline{C}H_2$x2); m/z (FAB) 283.

2-(Benzylaminocarbonylmethoxy)dibenzocycloheptadien-5-one. (50)

Carboxylic acid (49) (1.0 g, 3.5 mmol) was dissolved in DCM (10 ml) along with $SOCl_2$ (10 ml, 137 mmol) under nitrogen. The reaction mixture was stirred overnight at room temperature and then concentrated under high vacuum. THF (20 ml) was then added to the residue along with benzylamine (0.77 g, 7.2 mmol) and the mixture was stirred overnight at room temperature under nitrogen. The solvent was then removed *in vacuo* and the resulting solid filtered off. This was then washed with ether and recrystallised from MeOH to give the *title compound* as a white solid (1.3 g, 99%): m.p. 146-147°C; t.l.c. (MeOH/$CHCl_3$/AcOH 10:90:0.05) Rf 0.65; Found: C, 77.4; H, 5.7; N, 3.9; Calc.for $C_{24}H_{21}NO_3$: C, 77.6; H, 5.7; N, 3.8%, $\nu_{max.}$ (KBr disc) 3300 (NH), 2940 (CH_2), 1660 (C=O amide I), 1635 (C=O ketone), 1560 (C=O amide II), 1290 cm^{-1} (C-O); $\lambda_{max.}$ (MeOH) 290 nm (ε $1.49x10^4$ dm^3 mol^{-1} cm^{-1}); δ_H (80 MHz, $CDCl_3$) 8.16-6.67 (13H, m, aromatic CH's & N$\underline{H}$), 4.57 & 4.49 (4H, d+s, benzyl C$\underline{H}_2$ + -OC$\underline{H}_2$), 3.13 (4H, s, -C$\underline{H}_2$x2); δ_C (50 MHz, $CDCl_3$) 193 (C=O ketone), 167 (C=O amide), 160 (aromatic C2), 145-113 (aromatic C's), 67 (-O$\underline{C}H_2$), 43 (benzyl $\underline{C}H_2$), 35 & 34 (-$\underline{C}H_2$x2); m/z (FAB) 371, 256, 217.

2-(Benzylaminocarbonylmethoxy)dibenzocycloheptadien-5-ol. (51)

Amide (50) (0.10 g, 0.27 mmol) was dissolved in THF (15 ml) and $LiBH_4$ (0.023 g, 1.1 mmol) was added in one portion. The reaction mixture was then heated under reflux under nitrogen for 1h. and then was cooled to 0°C with an ice/salt bath. MeOH (5 ml) followed by acetone (5 ml) were added very slowly with stirring. The solvents were then removed *in vacuo* and the residue was taken up in ethyl acetate (35 ml).

This was then washed with water (3x25 ml) and then was dried over $MgSO_4$. The organic layer was then concentrated *in vacuo* to leave a clear oil which upon standing crystallised. This was then filtered off and washed with n-hexane to give the *title compound* as a white solid (0.05 g, 50%): m.p. 128-129°C; t.l.c. (MeOH/$CHCl_3$/AcOH 10:90:0.05) Rf 0.5; $\nu_{max.}$ (KBr disc) 3420 (OH), 2940 (CH_2), 1640 (C=O amide I), 1550 (C=O amide II), 1250 & 1075 cm^{-1} (C-O); δ_H (80 MHz, $CDCl_3$) 7.48-6.58 (13H, m, aromatic CH's + N<u>H</u>), 5.84 (1H, s, C<u>H</u>), 4.52 & 4.42 (4H, s+d, -OC<u>H</u>$_2$ + benzyl C<u>H</u>$_2$), 3.50-2.81 (5H, m, -C<u>H</u>$_2$x2 + O<u>H</u>); δ_C (50 MHz, $CDCl_3$) 168 (C=O amide), 156 (aromatic C2), 141-111 (aromatic C's), 76 (-<u>C</u>H), 67 (-O<u>C</u>H$_2$), 43 (benzyl <u>C</u>H$_2$), 33 & 32 (-<u>C</u>H$_2$x2); m/z (EI) 373, 356, 296, 225, 149.

<u>2-Copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadien-5-one.</u> (40)

2-Hydroxydibenzocycloheptadien-5-one (5.0 g, 22.3 mmol) was dissolved in *t* -BuOH/H_2O (1:1, 50 ml) and caesium hydroxide (3.75 g, 22.0 mmol) was added. The solution was then stirred for 10 min. and the *t* -BuOH was removed *in vacuo* . The caesium salt was then dried by azeotropic distillation with pyridine (2x100 ml) and DMF (3x100 ml). The salt was then dissolved in DMF (50 ml) and added to chloromethylpolystyrene (CMP) (1.106 mmol/g; 5.38 g, 6.0 mmol) previously swollen in DMF (25 ml). The reaction mixture was then stirred mechanically at 60°C for 4 days. After this time, the resin was filtered off, washed with copious quantities of DMF, *i* -PrOH, H_2O, DMF and finally *i* -PrOH, and dried *in vacuo* to give the *title compound* as an off-white solid (excess starting material was recovered for reuse) (6.48 g, 92%): $\nu_{max.}$ (KBr disc) 3100- 3000 (aromatic CH stretch), 3000-2840 (CH_2 stretch), 1640 (C=O), 1610-1560, 1490 (C=C), 1450 cm^{-1} (CH_2); Cl analysis, Found: less than 0.3%; Expected for CMP: 4.2%

2-Copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadien-5-ol. (45)

THF (100 ml) was added to the above compound (40) (0.91 mmol/g; 5.0 g, 4.5 mmol) along with $LiBH_4$ (0.75 g, 34.4 mmol) and the mixture was heated at reflux for 1 h. under nitrogen while stirring mechanically. The reaction mixture was then cooled in an ice/ salt bath and MeOH (10 ml) and acetone (10 ml) were added slowly with mixing. The resin was then filtered off, washed with copious amounts of MeOH, 0.001 M HCl, and MeOH, and dried *in vacuo* to give the *title compound* as white solid (4.95 g): $\nu_{max.}$ (KBr disc) 3560 & 3450 (OH), 3100-2980 (aromatic CH), 2980-2840 (CH_2), 1600,1585 & 1490 (C=C), 1450 cm^{-1} (CH_2).

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(9'-fluorenylmethoxycarbonyl amino)dibenzocycloheptadiene. (46)

DMF (100 ml) was added to the above compound (45) (0.91 mmol/g; 5.0 g, 4.5 mmol) along with 9-fluorenylmethylcarbamate (prepared by the method of Carpino[104]) (4.0 g, 16.7 mmol) and benzenesulphonic acid (0.15 g, 0.95 mmol) and the reaction mixture was stirred mechanically for 6 h. under an atmosphere of nitrogen. The resin was then filtered off, washed with copious amounts of DMF & DCM, and dried *in vacuo* to leave the *title compound* as an off-white solid (5.42 g, functionality of resin 0.65 mmol/g by UV monitoring method[93]): $\nu_{max.}$ (KBr disc) 3420 (urethane NH), 3100-2980 (aromatic CH), 2980-2840 (CH_2), 1750-1680 (urethane C=O), 1600,1585 & 1485 (C=C), 1445 cm^{-1} (CH_2); $\lambda_{max.}$ (20% piperidine/DMF) 300 & 290 nm.

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-aminodibenzocycloheptadiene. (47)

20% piperidine/DMF (20 ml) was added to protected amino resin (0.65 mmol/g; 1.0 g, 0.65 mmol) and the mixture was sonicated for 0.5 h. The resin was then filtered

off and washed with copious amounts of DMF followed by DCM and then dried *in vacuo* to give the *title compound* as a off-white solid (0.8 g): $\nu_{max.}$ (KBr disc) 3020 (CH), 2920 (CH_2), 1600 & 1490 (C=C), 1450 (CH_2), 1110 cm^{-1} (C-O).

9-Fluorenylmethoxycarbonylhydrazine. (66)

N-(9-Fluorenylmethoxycarbonyloxy)succinimide (5.0 g, 14.8 mmol) was dissolved in 1,4-dioxan (60 ml) and hydrazine hydrate (11.1 g, 220 mmol) was added slowly with stirring. The reaction mixture was then stirred overnight and then diluted with DCM (450 ml). This was then washed with water (3x150 ml) and dried over $MgSO_4$. The solvent was then removed *in vacuo* and the residue was then purified using dry flash chromatography ($CHCl_3$) to give the *title compound* as a white crystalline solid (2.3 g, 61 %): t.l.c. (MeOH/$CHCl_3$/AcOH 10:90:0.05) Rf 0.41; Found: C, 70.2; H, 5.5; N, 10.9; Calc. for $C_{15}H_{14}N_2O_2$; C, 70.8; H, 5.5; N, 11.0%; $\nu_{max.}$ (KBr disc) 3320 (NH), 1695 (C=O), 1645 (C=C), 1290 & 1195 cm^{-1} (C-O); $\lambda_{max.}$ (EtOH) 300 nm (ε $6.66x10^3$ dm^3 mol^{-1} cm^{-1}); δ_H (200 MHz, D_6 DMSO) 8.36 (1H, s, -CO-N<u>H</u>), 7.90-7.29 (8H, m, aromatic CH's), 4.32-4.18 (3H, m, C<u>H</u> & C<u>H_2</u>), 4.09 (2H, s, N<u>H_2</u>); δ_C (90 MHz, D_6 DMSO) 158 (C=O), 144-120 (aromatic C's); 66 (-<u>C</u>H_2), 47 (-<u>C</u>H); m/z (FAB) 255, 179, 165, 149, 126; *hrms* found: 255.11333; $C_{15}H_{15}N_2O_2$ requires: 255.11334.

N-*t* -Butyloxycarbonyl-N'-2-methoxydibenzocycloheptadien-5- ylhydrazine. (64)

Carbinol (63) (0.10 g, 0.42 mmol) was heated under reflux in DCM (10 ml) along with *t* -butyloxycarbonylhydrazine (0.17 g, 1.3 mmol) and benzenesulphonic acid (0.015 g, 0.095 mmol) overnight. The solvent was then removed *in vacuo* and the residue purified by flash chromatography (0.5% MeOH/$CHCl_3$) to give the *title compound* as a off-white foam (0.15 g, 99%): t.l.c. (MeOH/$CHCl_3$/AcOH 10:90:0.05) Rf 0.72; Found: C, 71.2; H, 7.2; N, 7.5; Calc. for $C_{21}H_{26}N_2O_3$: C, 71.2; H, 7.4; N,

7.9%; $\nu_{max.}$ (KBr disc) 3400 (NH), 2930 (CH_2), 1710 (C=O), 1610 & 1500 (C=C), 1160 cm^{-1} (C-O); $\lambda_{max.}$ (EtOH) 274 nm (ε 2.07x10^3 dm^3 mol^{-1} cm^{-1}; δ_H (200 MHz, $CDCl_3$) 7.31-6.65 (7H, m, aromatic CH's), 5.86 (1H, s, -CO-NH), 5.13 (1H, s, -CH), 3.85-3.66 (5H, s+m, $-OCH_3$ + $-CH_2$), 2.89-2.75 (2H, s, $-CH_2$), 1.47 (9H, s, $-CH_3$x3); δ_C (90 MHz, $CDCl_3$) 159 (aromatic C2), 157 (C=O), 143-111 (aromatic C's), 80 ($-C-(CH_3)_3$), 70 (-CH), 55 ($-OCH_3$), 33 & 32 ($-CH_2$x2), 28 ($-CH_3$x3); m/z (FAB) 353, 297, 223; *hrms* found: 353.18651; $C_{21}H_{25}N_2O_3$ requires: 353.18654.

N-9'-Fluorenylmethoxycarbonyl-N'-2-methoxydibenzocycloheptadien -5-ylhydrazine. (68)

Carbinol (63) (0.10 g, 0.42 mmol) was dissolved in DCM (10 ml) along with 9-fluorenylmethoxycarbonylhydrazine (0.32 g, 1.3 mmol) and benzenesulphonic acid (0.015 g, 0.095 mmol) and heated under reflux overnight. The reaction mixture was then concentrated *in vacuo* and purified by flash chromatography (1% MeOH/$CHCl_3$) to give the *title compound* as a white solid (0.16 g, 80%): t.l.c. (MeOH/$CHCl_3$/AcOH 10:90:0.05) Rf 0.76; Found: C, 78.0; H, 5.8; N, 5.8; Calc. for $C_{31}H_{28}N_2O_3$: C, 78.1; H, 5.9; N, 5.9%; $\nu_{max.}$ (KBr disc) 3300 (NH), 2940 (CH_2), 1690 (C=O), 1615 (C=C), 1270 & 1170 cm^{-1} (C-O); $\lambda_{max.}$ (EtOH) 295 nm (ε 6.43x10^3 dm^3 mol^{-1} cm^{-1}); δ_H (200 MHz, $CDCl_3$) 7.80-6.66 (15H, m, aromatic CH's), 6.12 (1H, s, -CO-NH), 5.09 (1H, s, -CH), 4.50-4.21 (3H, m, fluorenyl -CH & $-CH_2$), 3.78-3.47 (5H, s+m, $-OCH_3$ & CH_2), 2.83-2.81 (2H, m, $-CH_2$), 1.6 (1H, s, -NH-NH-CO); δ_C (90 MHz, $CDCl_3$) 159 (aromatic C2), 157 (C=O), 144-111 (aromatic C's), 71 (-CH-NH), 67 (fluorenyl CH_2), 55 ($-OCH_3$), 47 (fluorenyl CH), 33 & 32 ($-CH_2$x2); m/z (FAB) 476, 325, 223; *hrms* found: 477.21777; $C_{31}H_{29}N_2O_3$ requires: 477.21780 (< 1 ppm).

N-*t* -Butyloxycarbonyl-N'-2-copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadien-5-ylhydrazine. (61)

Carbinol resin (45) (0.90 mmol/g; 1.0 g, 0.90 mmol) was swollen in DCM (70 ml) and *t* -butyloxycarbonylhydrazine (0.84 g, 6.4 mmol) and benzenesulphonic acid (0.07 g, 0.44 mmol) were added. The reaction mixture was then heated at reflux overnight and then allowed to cool to room temperature. The resin was then filtered off and washed with copious amounts of DMF, DCM, and ether to leave the *title compound* as an off-white solid (0.80 g): $\nu_{max.}$ (KBr disc) 3410 (NH), 2920 (CH_2), 1710 (C=O), 1600 & 1490 (C=C), 1450 (CH_2), 1150 cm^{-1} (C-O); Found: N, 2.35; Expected for 100% loading: N, 2.29%.

N-*t* -Butyloxycarbonyl-N'-(N$^{\alpha}$-9'-fluorenylmethoxycarbonylglycyl) -N'-2-copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadien -5-ylhydrazine. (62)

To Fmoc-Gly-OH (2.0 g, 6.7 mmol) was added DCM (5 ml) and $SOCl_2$ (5 ml, 68 mmol). This was heated under reflux for 2 h. and then cooled to room temperature. The mixture was then concentrated *in vacuo* and then concentrated a further three times following dissolution in DCM (3x15 ml). The residue was then dissolved in DCM (15 ml) and added to resin (61) which had been pre-swollen in DCM (5 ml) and pyridine (2 ml). The reaction mixture was then heated under reflux for 5 h. under nitrogen. The resin was then isolated by filtration and washed with copious quantities of DMF, DCM, and finally ether to give the *title compound* as a pale yellow solid (0.98 g). The loading was found to be 0.84 mmol/g by a UV monitoring method[93]: $\nu_{max.}$ (KBr disc) 3420 (NH), 3030 (CH stretch), 2920 (CH_2 stretch), 1730-1670 (C=O x3), 1600 & 1490 (C=C), 1450 cm^{-1} (CH_2); Found: N, 2.69; Expected for 100% loading: N, 2.80%; $\lambda_{max.}$ (20% piperidine/DMF) 290 & 300 nm.

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(N^{α}-9'-fluorenylmethoxycarbonylglycyloxy)dibenzocycloheptadiene. (58)

DMF (5 ml) was added to Fmoc-Gly-OH (0.64 g, 2.2 mmol) along with DIC (0.17 ml, 1.1 mmol). The mixture was then stirred at room temperature for 15 min. and then added to resin (45) (0.90 mmol/g; 0.30 g, 0.27 mmol) along with N,N-dimethylaminopyridine (5 mg, 0.04 mmol). The reaction mixture was then sonicated for 3.5 h. followed by filtration of the resin. The resin was then thoroughly washed with large amounts of DMF, DCM, and ether to leave the *title compound* as an off-white solid (0.23 g, loading of the functionalised resin 0.49 mmol/g by UV monitoring[93]): $\nu_{max.}$ (KBr disc) 2950 (CH_2), 1720 (C=O x2), 1600 & 1490 (C=C), 1440 cm^{-1} (CH_2); $\lambda_{max.}$ (20% piperidine/DMF) 300 & 290 nm.

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(N^{α}-9'-fluorenylmethoxycarbonylglycylamino)dibenzocycloheptadiene. (57)

Resin (46) (0.65 mmol/g; 0.20 g, 0.13 mmol) was treated with 20% piperidine/DMF (20 ml) for 0.5 h. and was then washed with DMF (5x50 ml). To this was added a mixture of Fmoc-Gly-OH (0.30 g, 1.0 mmol), DIC (0.078 ml, 0.5 mmol) and DMF (5 ml) which had been stirred for 0.5 h. at room temperature. The reaction mixture was then sonicated for 5 h. followed by filtration of the resin. The resin was then washed with copious amounts of DMF, DCM, and ether to give the *title compound* as a light brown solid (0.18 g, functionality 0.67 mmol/g by UV method[93]): $\nu_{max.}$ (KBr disc) 3440 (NH), 1750-1690 cm^{-1} (C=O x2).

3.3. Solid Phase Peptide Synthesis.

The following peptides were prepared using an Applied Biosystems 430A automated peptide synthesiser. All solvents used were peptide synthesis grade and commercially supplied by Applied Biosystems and Rathburn Chemicals.

The solid support (46) was deprotected using 20% piperidine/DMF and the C-terminal residue coupled to the free amine *via* DIC/symmetrical anhydride/ HOBt methodology (Note. This could be performed either outwith the machine or as a programmed cycle on the synthesiser; both methods gave quantitative loading).

The progress of the synthesis was monitored by passing the deprotection effluent through a UV flowcell (313 nm) and thus allowed a semi quantitative analysis of the deprotection and acylation cycles.

Deprotection, activation, coupling and capping were accomplished using programmed cycles on the 430A synthesiser and are summarised below:

(1) Washing and swelling of the resin: DMF (2x, total time = 6 min.). (2) Removal of the N^{α} protecting group: 20% piperidine/DMF (9.0 ml) (4x, 5 + 3 + 3 + 1 min.). (3) Washing: DMF (13x, total time = 31 min.). (4) Activation: 1st coupling - Fmoc-AA-OH (2.0 mmol) in DMF (6.0 ml) + DIC (0.5 M in DMF; 2.0 ml) (15 min.), 2nd coupling - Fmoc-AA-OH (1.0 mmol) in DMF (4.0 ml) + HOBt (0.5 M in DMF; 2.0 ml) + DIC (0.5 M in DMF; 2.0 ml) (30 min.) (5) Coupling: activated species transferred to the 'reaction vessel' (90 min.) (6) Washing: DMF (5x, 6 min. total). (7) Capping: acetic anhydride (0.5 M in DMF; 1.0 ml) + pyridine (0.5 M in DMF; 1.0 ml) (2x, 2.5 + 3.7 min.). (8) Washing: DMF (5x, 5 min.).

Substance P. (53)

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

The synthesis of peptide amide (53) was achieved using resin (46) (0.73 mmol/g; 0.5 mmol) as the solid support, Fmoc N^{α} protected amino acids and symmetrical anhydride/HOBt activation. The guanidine function of arginine was protected with the Pmc group, the N^{ε} of lysine was protected with Boc and the side chain of glutamine with the trityl group.

The completed peptide amide was released from the solid support and all side chain protection removed by treatment with TFA/ water/thioanisole/ethyl methyl sulphide (95:5:2.5:2, 100 ml) with stirring at room temperature for 3 h. The resin was then filtered off, the filtrate was concentrated *in vacuo* and the peptide was precipitated with ether. All solvents and aqueous buffers in the subsequent purification steps were removed by lyophilisation. The crude peptide (1 g) was then filtered, washed with ether, dissolved in 50% AcOH/H_2O and applied to a column of Sephadex G-15 (85 x 1.4 cm) eluting with 30% AcOH/H_2O at 38 ml/h. The product eluted as a single peak at 150 - 250 ml (0.343 g). This was then applied to a column of CM Sephadex resin (40 x 2.6 cm) and eluted with a linear gradient of ammonium acetate (0.025-0.5 M, pH 8.0) at 30 ml/h., followed by isocratic elution (0.5 M, pH 8.0). The product eluted as a single peak (0.087 g) (Gel permeation and ion exchange chromatography were monitored by UV at 254 and 280 nm). This was then further purified by semi-preparative HPLC on an aquapore C_{18} reverse phase column using a linear gradient of H_2O/CH_3CN (0.1% TFA) as the eluant at a flow rate of 5 ml/min. and UV monitoring at 225 nm (0.037 g, 22%): m/z (FAB) 1349 (MH^+), 1002, 854, 751; *hrms* found: 1347.73588; $C_{63}H_{99}N_{18}O_{13}S$ requires 1347.73592 (< 1 ppm); amino acid

analysis Glu_2 2.20, Pro_2 1.95, Gly_1 0.95, Met_1 0.83, Leu_1 0.93, Phe_2 1.90, Lys_1 1.06, Arg_1 1.01; HPLC (aquapore C_{18}, A=H_2O/B=acetonitrile (0.1% TFA), 10-50% B over 30 min.) R_t 16.4 min; $[\alpha]_D^{20}$ -57.8 (C = 0.5, H_2O).

δ_H (600 MHz, 90% H_2O/D_2O)

A.A	α-NH	α-CH	β H's	γ H's	others
Arg		4.43	1.95	1.73	ε 3.23 εNH 7.26
Gln	8.50	4.19	1.95	2.28	6.88,7.51
Gln	8.33	4.21	1.83	2.12	6.88,7.51
Gly	7.94	3.82			
Leu	8.16	4.33	1.63	1.63	0.92,0.97
Lys	8.58	4.52	1.81	1.50	δ 1.69 ε 2.98 NH 7.57
Met	8.37	4.42	2.05	2.52 2.60	δ 2.09 NH 7.10,7.49
Phe	8.28	4.57	2.93 3.16		7.18-7.40
Phe	8.25	4.57	2.90 3.03		7.18-7.40
Pro		4.47	1.92 2.06	2.36	3.62,3.80
Pro		4.38	1.92 2.06	2.30	3.68,3.88

Bombesin. (54)

Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

The synthesis of peptide amide (54) was accomplished using resin (46) (0.73 mmol/g; 0.5 mmol) as the solid support, Fmoc N^{α} protected amino acids and symmetrical anhydride/HOBt activation. The guanidine function of arginine was protected with the Pmc group, the imidazole function of histidine with the trityl group and the carboxamide of asparagine and glutamine with the Mbh group.

The completed peptide amide was released from the solid support with concomitant side chain deprotection by stirring with TFA/H_2O/thioanisole/EMS/N-acetyl tryptophan (95:5:5:2:0.5, 110 ml) for 4 h. at room temperature. The resin was then removed by filtration, the filtrate was concentrated *in vacuo* and the peptide was precipitated with ether. All solvents in subsequent purification steps were removed by lyophilization. The crude peptide (0.9 g) was then filtered off, washed with ether, dissolved in 90% AcOH/H_2O and applied to a column of Sephadex G-15 (100 cm x 2.6 cm i.d.) eluting with 30% AcOH/H_2O at 28.5 ml/h. with UV monitoring at 229 and 280 nm. The product (0.65 g) eluted between 152 & 285 ml. An aliquot (0.115 g) was finally purified by semi-preparative HPLC on a aquapore C_{18} reverse phase column with a linear gradient of H_2O/CH_3CN (0.1% TFA) at a flow rate of 5 ml/min. with UV monitoring at 232 nm (0.030 g, 21%): m/z (FAB) 1619 (MH^+), 1265, 1095, 966; *hrms* found: 1619.82282; $C_{71}H_{111}N_{24}O_{18}S$ requires 1619.82283 (< 1 ppm); amino acid analysis Asp_1 1.01, Glu_3 3.04, Gly_2 1.94, Ala_1 0.99, Val_1 1.02, Met_1 1.02, Leu_2 1.99, His_1 1.06, Trp_1 0.81, Arg_1 0.95; HPLC (aquapore C_{18} A=H_2O B=CH_3CN (0.1% TFA), 10-50% B over 30 min.) R_t 16.4 min; $[\alpha]_D^{20}$ -29.9 (C = 0.8, H_2O).

δ_H (600 MHz, 90% H_2O/D_2O)

A.A	α-NH	α-CH	β H's	γ H's	others
Ala	8.05	4.34	1.35		
Arg	8.56	4.40	1.80	1.60	ε 3.15 εNH 7.19
Asn	8.39	4.67	2.77	6.88,7.38	
Gly	8.48	4.02			
Gly	8.44	3.97			
Gln	8.56	4.38	2.06	2.40	6.94,7.57
Gln	8.56	4.38	2.06	2.40	6.94,7.57
Glp	8.48	4.25	1.93	2.17	
His	8.29	4.71	3.13 3.27		7.26 8.48
Leu	8.39	4.46	1.67	1.67	0.92
Leu	8.34	4.37	1.65	1.65	0.94
Met	8.40	4.51	2.06	2.58	ε 2.17 NH 7.17,7.55
Trp	8.05	4.72	3.24 3.37		7.28, 7.62 7.15, 7.24 7.52 NH 10.17
Val	7.99	4.07	2.13	1.00	

Little Gastrin. (55)

Glp-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

The synthesis of peptide amide (55) was accomplished using resin (46) (0.73 mmol/g; 0.5 mmol) as the solid support, Fmoc N^{α} amino acids and symmetrical anhydride/HOBt activation. Pyroglutamic acid was incorporated by activation as its pentachlorophenyl ester. The carboxylic acid functions of glutamic and aspartic acid were protected their *t* -butyl esters and the hydroxyl function of tyrosine as its *t* -butyl ether.

The synthesis was interrupted prior to coupling of the last amino acid and 0.25 mmol of resin removed to be used later in the synthesis of big gastrin (56). The peptide amide, once fully assembled, was removed from the solid support with simultaneous side chain deblocking by stirring with TFA/H_2O/thioanisole/EMS/N-acetyltryptophan (95:5:5:5:1, 110ml) for 4 h. at room temperature. The resin was then removed by filtration, and the filtrate concentrated *in vacuo* to leave a brown oil. From this, the peptide was precipitated using ether and isolated by filtration to give a crude mixture (1.0 g) (All solvents and aqueous buffers in the following purification steps were removed by lyophilization). This was then dissolved in 0.4% NH_4HCO_3 and applied to a column of Sephadex G-25 (100 cm x 2.6 cm) and eluted with 0.4% NH_4HCO_3 at a pump speed of 21 ml/h. with UV monitoring at 229 and 280 nm. The product (0.32 g) eluted as a single peak between 40 and 70 ml. This was then applied to a column of DEAE DE-25 (40 cm x 2.6 cm) and eluted with a linear gradient of $AcO^-NH_4^+$ (0.1 - 1.0 M, pH 6.5) at a flow rate of 28 ml/h. with UV monitoring at 229 and 280 nm. The product (0.21 g) eluted between 849 and 1000 ml. This product was finally purified by semi-preparative HPLC on an aquapore C_8 reverse phase column using a linear gradient of H_2O/CH_3CN (0.1% TFA) with UV monitoring at 280 nm and a flow rate of 5 ml/min. (0.084 g, 16%): m/z (FAB) 2097 (M+); *hrms* found:

2098.85729; $C_{97}H_{125}N_{20}O_{31}S$ requires: 2098.85732 (< 1 ppm);amino acid analysis Asp_1 0.86, Glu_6 5.94, Pro_1 0.72, Gly_2 1.99, Ala_1 1.10, Met_1 1.00, Leu_1 0.93, Tyr_1 1.09, Phe_1 1.09, Trp_2 1.72; Biological activity (relative immunochemical potency measured by radioimmunoassay) C-terminus 0.7, N-terminus 0.7, Intact 1.4; HPLC (aquapore C_8 A=H_2O B=CH_3CN (0.1% TFA) 10 - 60% B over 25 min.) R_t = 20.0 min; $[\alpha]_D^{20}$ -43.8 (C = 0.6, 1% NH_4HCO_3).

Big Gastrin. (56)

Glp-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu-Val-Ala-Asp-Pro-Ser-Lys-Lys-Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

The synthesis of Big Gastrin was achieved using solid support (46) (0.73 mmol/g; 0.25 mmol), Fmoc N^{α} protected amino acids and symmetrical anhydride/HOBt activation. Pyroglutamic acid was incorporated by activation as its pentachlorophenyl ester. The carboxylic acid functions of aspartic and glutamic acids were protected as their *t* -butyl esters and the hydroxyl function of serine and tyrosine were protected as their *t* -butyl ethers. The N^{ε} of lysine was protected with Boc and the imidazole function of histidine was protected with trityl.

The completed peptide amide was detached from the solid support and all side chain protecting groups removed by treatment with TFA/H_2O/thioanisole/EMS/ N-acetyltryptophan (95:5:5:5:1, 110 ml) for 4h. at room temperature. The resin was then removed from the cleavage mixture by filtration and the solution was concentrated *in vacuo* to leave a brown oil. The peptide was then precipitated by treatment with ether and was isolated by filtration to give a crude product (0.8 g) (All solvents and aqueous buffers in subsequent purification steps were removed by lyophilization and dialysis respectively). An aliquot of this (0.4g) was dissolved in 1% NH_4HCO_3 and applied to a column of Sephadex G-25 (100 cm x 2.6 cm) and

eluted with 0.4% NH_4HCO_3 at a flow rate of 33 ml/h. and with UV monitoring at 229 and 280 nm. The product (0.257 g) eluted as a single peak between 176 and 253 ml. This was then further purified by semi-preparative HPLC on an aquapore C_{18} reverse phase column. A linear gradient of H_2O/CH_3CN (0.1% TFA) was used to elute the product with a flow rate of 5 ml/min. and UV monitoring at 280 nm. This material (0.12 g) was then finally purified by ion-exchange chromatography on DEAE DE-25 resin eluting with a linear gradient of $AcO^-NH_4^+$ (0.05 - 0.5 M, pH 6.8) at a flow rate of 33 ml/h. and UV monitoring at 229 and 280 nm. The product eluted as a single peak between 869 and 1584 ml (0.052 g, 11%): m/z (FAB) *hrms* found: 3848.81011; $C_{176}H_{252}N_{43}O_{53}S$ requires: 3848.80985 (<1ppm); Amino acid analysis Asp_2 2.04, Ser_1 0.95, Glu_8 8.92, Pro_5 4.65, Gly_4 3.91, Ala_2 1.95, Val_1 0.97, Met_1 1.00, Leu_3 3.00, Tyr_1 0.99, Phe_1 0.98, His_1 1.00, Trp_2 1.77, Lys_2 2.29; $\lambda_{max.}$ (0.1 M KOH) 280 nm (ε = 1.093 x 10^4 dm^3 mol^{-1} cm^{-1}, lit.,[105] ε = 1.226 x 10^4) Biological activity (relative immunochemical potency measured by radioimmunoassay) C-terminus 0.4, N-terminus 0.3. HPLC (aquapore C_{18} A=H_2O, B=CH_3CN (0.1% TFA) 20 - 70% B over 30 min.) R_t = 15.0 min; $[\alpha]_D^{20}$ -81.7 (C = 0.5, 1% NH_4HCO_3).

Ubiquitin 43-47 hydrazide. (70)

H-Leu-Ile-Phe-Ala-Gly-NH-NH_2

The synthesis of Ubiquitin 43-47 hydrazide was accomplished using loaded resin (62) (0.84 mmol/g; 0.16 g, 0.13 mmol), Fmoc N^{α} protected amino acids and HOBt activation with single coupling. The cycles employed in the synthesis were as previously mentioned except as follows: (4) Activation: Fmoc-AA-OH (0.5 mmol) in HOBt (0.5 M in DMF; 2.0 ml) + DIC (0.5 M in DMF; 2.0 ml) (30 min.); (5) Coupling: activated species transferred to 'reaction vessel' (45 min.).

The fully assembled peptide hydrazide was released from the solid support by

treatment with TFA/H_2O/anisole (10:0.5:0.5, 11 ml) for 1h. with stirring at room temperature. The resin was then removed by filtration and the filtrate was concentrated *in vacuo* . The residue was then dissolved in $CHCl_3$ (10 ml) and then added slowly to 10% AcOH/H_2O (20 ml) with stirring. The aqueous layer was then lyophilised and subsequently purified by semi-preparative HPLC on an aquapore C_{18} reverse phase column with a linear gradient of H_2O/CH_3CN (0.1% TFA) at a flow rate of 5 ml/min. and with UV monitoring at 225 nm (0.017 g, 25%): m/z (FAB) 534 (MH^+), 445, 404, 374, 199; *hrms* found: 534.34034; $C_{26}H_{44}N_7O_5$ requires: 534.34037 (< 1ppm); amino acid analysis: Gly_1 1.05, Ala_1 1.02, Ile_1 0.98, Leu_1 0.97, Phe_1 0.98; HPLC (aquapore C_{18} A=H_2O B=CH_3CN (0.1% TFA) 10-40% B over 20 min.) R_t 12.8 min; $[\alpha]_D^{20}$ -40.9 (C = 0.35, H_2O).

Ubiquitin 67-76 hydrazide. (71)

H-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-NH-NH_2

The synthesis of peptide hydrazide (71) was accomplished using loaded resin (62) (0.84 mmol/g; 0.60 g, 0.5 mmol) as the solid phase, Fmoc N^{α} protected amino acids and symmetrical anhydride/ HOBt activation. The guanidine function of arginine was protected with the Pmc group and the imidazole function of histidine with trityl.

The completed peptide hydrazide was detached from the solid support with concomitant side chain deprotection by stirring with TFA/H_2O/anisole/thioanisole (85:5:5:5, 100 ml) for 3 h. at room temperature. The resin was then removed by filtration and the filtrate was concentrated *in vacuo* . $CHCl_3$ (50 ml) was then added to the residue which was then extracted with 10% AcOH/H_2O (3x100 ml). The aqueous solutions were then combined and lyophilised to give a crude mixture (0.4 g). An aliquot (0.1 g) of this was then purified by semi-preparative HPLC on an aquapore C_{18} reverse phase column with a linear gradient of H_2O/CH_3CN (0.1%

TFA) at a flow rate of 5 ml/min. and with UV monitoring at 225 nm. Two major products were isolated (A 0.021 g, B 0.005 g).

The following data was obtained for compound A: m/z (FAB) 1147.8 (MH^+), *hrms* found: 1147.75390; $C_{51}H_{95}N_{20}O_{10}$ requires: 1147.75395 (< 1 ppm); amino acid analysis 1. (acid hydrolysis): Gly_2 2.20, Val_1 0.98, Leu_4 3.95, His_1 0.98, Arg_2 1.92; 2. (enzymatic hydrolysis[106]): Gly_2 2.00, Val_1 0, Leu_4 2.04, His_1 0, Arg_2 2.60; sequencing: Edman degradation of peptide hydrazide confirmed the sequence as expected; $[\alpha]_D^{21}$ -49.4 (C = 0.2, H_2O); HPLC (aquapore C_{18} A=H_2O B=CH_3CN (0.1% TFA) 5-50% B over 20 min.) R_t 11.2 min;

δ_H (600 MHz, 90% H_2O/D_2O)

A.A	α-NH	α-CH	β H's	γ H's	others
Arg	8.47	4.35	1.85	1.67	δ 3.24 δNH 7.23
Arg	8.40	4.37	1.79	1.62	δ 3.20 δNH 7.23
Gly	8.48	4.00			
Gly	8.34	3.97			
His	9.02	4.85	3.18 3.30		7.38 8.72
Leu 67		4.00	1.60	1.32	0.89
Leu 69	8.54	4.35	1.55	1.33	0.84 0.91
Leu 71	8.42	4.40	1.61	1.61	0.89 0.94
Leu 73	8.33	4.37	1.63	1.63	0.90 0.95
Val	8.32	4.08	2.05	0.93 0.97	

The following data was obtained for compound B:

m/z (FAB) 1147.8 (MH^+), *hrms* found: 1147.75390; $C_{51}H_{95}N_{20}O_{10}$ requires: 1147.75395 (< 1 ppm); amino acid analysis: 1.(acid hydrolysis): Gly_2 2.05, Val_1 0.99, Leu_4 3.97, His_1 1.02, Arg_2 1.99; 2. (enzymatic hydrolysis[106]): Gly_2 2.00, Val_1 1.16, Leu_4 6.12, His_1 1.02, Arg_2 3.26; sequencing: Edman degradation of peptide hydrazide confirmed the sequence as expected; $[\alpha]_D^{21}$ -56.7 (C = 0.2, H_2O); HPLC (aquapore C_{18} A=H_2O B=CH_3CN (0.1% TFA) 5-50% B over 20 min.) R_t 12.2 min;

δ_H (600 MHz, 90% H_2O/D_2O)

A.A	α-NH	α-CH	β H's	γ H's	others
Arg	8.42	4.38	1.78	1.62	δ 3.22 δ NH 7.31
Arg	8.47	4.38	1.86	1.66	δ 3.25 δ NH 7.31
Gly	8.48	4.01			
Gly	8.33	3.95			
His	8.89	4.85	3.20 3.27		7.32 8.63
Leu 67		4.03	1.72	1.63	0.96 0.96
Leu 69	8.47	4.38	1.56	1.56	0.88 0.92
Leu 71	8.38	4.39	1.59	1.59	0.87 0.94
Leu 73	8.33	4.39	1.62	1.62	0.92 0.92
Val	8.34	4.08	2.02	0.92 0.92	

H-Thr(*t* -Bu)-Ile-Phe-Ala-Gly-O-Resin. (59)

The synthesis of resin bound peptide (59) was achieved using resin (58) (0.49 mmol/g; 0.19 g, 0.093 mmol) as the solid support, Fmoc N^{α} protected amino acids and HOBt activation with single coupling. The cycles used in the synthesis were as mentioned previously except: (4) Activation: Fmoc-AA-OH (1.0 mmol) in HOBt (0.5 M in DMF; 2.0 ml) + DIC (0.5 M in DMF; 2.0 ml) (30 min.). Through the use of UV monitoring of Fmoc deprotection, the final deprotection (threonine) was 40% of the area of the initial deprotection (glycine).

Determination of conditions for release of peptide derivative from the solid support.

A sample of loaded resin (2-3 mg) i.e. Fmoc-Gly attached to the functionalised solid support, was accurately weighed and placed in a 10 ml volumetric flask. The requisite concentration of an organic acid/DCM solution was then added to the volumetric flask up to the mark and the timer started. The flask was then placed in an ultrasonic bath for the required amount of time after which the solution was filtered through a cotton plug (to remove the resin) into a UV cuvette. The UV absorbance at 302 nm was then recorded and used to calculate the percentage of amino acid derivative which had been released from the solid phase (in order to do this, the extinction coefficient of Fmoc-Gly-OH at 302 nm was used and assumed not to vary in each case). The cleavage conditions for each functionalised resin are tabulated below:

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(N^{α}-9'-fluorenylmethoxycarbonylglycyloxy)dibenzocycloheptadiene. (58)

ACID/CONC	TIME FOR 100% RELEASE
TFA/DCM 0.5%	< 5 min.
AcOH/DCM 10%	< 5 min.

N-*t* -Butyloxycarbonyl-N'-(N^{α}-9'-fluorenylmethoxycarbonylglycyl) -N'-2-copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadien -5-ylhydrazine. (62)

1% TFA/DCM

TIME(min.)	% CLEAVED
2.0	9.9
15.0	31.3
30.0	46.2
90.0	63.5
180.0	66.3

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(Nᵅ-9'-fluorenylmethoxycarbonylglycylamino)dibenzocycloheptadiene. (57)

1% TFA/DCM

TIME(min.)	% CLEAVED
10.0	19.5
30.0	57.8
45.0	68.8
60.0	75.2
90.0	79.2
120.0	92.2

2% TFA/DCM

TIME	% CLEAVED
5.0	32.3
10.0	56.0
15.0	71.3
20.0	82.2
30.0	89.4
60.0	95.5

3% TFA/DCM

TIME(min.)	% CLEAVED
5.0	48.3
10.0	70.4
15.0	84.0
20.0	89.7
30.0	96.2

4% TFA/DCM

TIME	% CLEAVED
2.0	31.5
5.0	63.7
7.0	79.6
10.0	83.1
15.0	91.2
30.0	96.4

Chapter 4.
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Advances in Organic Chemistry 1988, 1989 (various lecturers, University of Edinburgh).

Introduction to Management (various speakers, Dept. of Business Studies, University of Edinburgh).

R.S.C. Peptide and Protein Group Meeting, Nottingham (various speakers).

Innovation and Perspectives in Solid Phase Synthesis, Oxford (various speakers).

11th annual Irvine Review Lecture-Asymmetry, St. Andrews (various speakers).