A Study of the Receptor-Interaction of Nerve Growth Factor.

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This thesis is submitted in part fulfilment of the requirements of the degree of Doctor of Philosophy at 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.

University of Edinburgh

September 1994

For my parents,

Thaddeus and Philomena

Suaimhneas Dé dá n-anamacha dílse

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ABSTRACT

The interaction of Nerve Growth Factor (NGF) with the high affinity receptor, *trk*, and the low affinity receptor, p75, has been investigated. Peptide fragments of NGF have been chemically synthesised by Solid Phase Peptide Synthesis Techniques. Separate biological assays for each of the receptors have been set up by our collaborators at Parke Davis. These assays test the ability of the synthesised peptide fragments to inhibit NGF binding to the receptor. In this way, potential binding sites of NGF to each of the receptors have been identified.

Work previously carried out in this laboratory led to the identification of a C-terminal region of NGF as a receptor binding domain for the p75 receptor. The synthesis of analogues has highlighted the importance of certain N-terminal residues of this C-terminal fragment, for binding to the low affinity receptor. The identification of a potential secondary binding site to this receptor was made by linking three positively charged residues, believed to form a positively charged complex for binding to the receptor, to the previously identified C-terminal fragment. An eleven carbon chain linker, aminoundecanoic acid, was used for this purpose. It was also found that a dimer of this C-terminal region bound with higher affinity than did the monomer.

Preliminary results have indicated that the C-terminus of NGF is important for binding to the high affinity receptor also. These peptides have been shown to bind competitively with NGF to the high affinity receptor i.e. the question of whether these peptides inhibit NGF binding to the receptor by binding to the receptor themselves or simply by binding to NGF, has been answered. This indicates that these peptides could act as potential agonists for NGF.

ABBREVIATIONS

acetyl CoA	acetylcoenzyme A
AchE	acetylcholinesterase
AcOH	acetic acid
Acm	acetamidomethyl
APP	amyloid precursor protein
aua	aminoundecanoic acid
BDNF	brain derived neurotrophic factor
β/A_4	beta amyloid
BSA	bovine serum albumin
Bum	butoxymethyl
BUPA	*p-t*-butoxyphenylacetic acid
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
C-terminal	carboxy-terminal
DCM	dichloromethane
DIC	diisopropylcarbodiimide
DIEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDT	ethanedithiol
EDTA	ethylenediaminetetracetic acid
FAB	fast atom bombardment
Fmoc	fluorenylmethoxycarbonyl
GdmCl	guanidinium hydrochloride
h	human
HCl	hydrochloric acid
HNGFR	high affinity nerve growth factor receptor
HOBt	1-hydroxybenzotriazole
HOCt	ethyl-1-H-1,2,3-triazole-4-carboxylate
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
LNGFR	low affinity nerve growth factor receptor
m	mouse

mRNA	messenger RNA
n.d.	not detected
NGF	nerve growth factor
NGFR	nerve growth factor receptor
N-terminal	amino-terminal
NTF	neurotrophic factor
NT-3	neurotrophin 3
oxid	oxidised
PC12 cells	phoechromocytoma cells
PBS	phosphate buffered saline
PG	protecting group
phAcm	phenylacetamidomethyl
Pmc	pentamethylchromansulphonyl
PNS	peripheral nervous system
Pre	precursor
R_t	retention time
rhNGF	recombinant human nerve growth factor
SPPS	solid phase peptide synthesis
S-*t*-butyl	S-*t*-butylsulphenyl protection
t	time
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TMSBr	bromotrimethylsilane
TOF	time of flight
trk	tropomysin kinase
Trt	triphenylmethyl
U.V.	ultraviolet
WGA	wheatgerm agglutinin

AMINO ACIDS

Amino Acid	3 Letter Code	1 Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cyclohexylalanine	Cha	_
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Ornithine	Orn	O
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CONTENTS

CHAPTER TWO : DISCUSSION

CHAPTER ONE : INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 Introduction

Alzheimer's Disease (AD) is a form of senile dementia which affects about 10 percent of people in their seventies and 30 percent in their eighties. It is the fourth major cause of death after heart disease, cancer and stroke. The dramatic rise in life expectancy during this century has enabled many more to reach an age at which degenerative diseases of the brain become common. Associated with Alzheimer's Disease is a loss of memory, judgement and emotional stability which usually leads to death in a severely debilitated state between four and twelve years after onset. The total number of sufferers is about 500,000 in the U.K. 400,000 in France and four million in the U.S. The annual cost is estimated at more than $80 billion in the U.S., primarily for custodial care. At the present time there is no cure for this harrowing disease and there is limited treatment for retarding the progression of the disease.

1.1.2 Alzheimer's Disease Pathology

Alzheimer's Disease may only be diagnosed with certainty by close examination of the brain tissue. The characteristic pathological hallmarks are large numbers of amyloid plaques surrounded by neurons containing neurofibrillary tangles[1], vascular damage from extensive plaque deposition[2] and neuronal cell loss[1]. Amyloid is a generic description applied to a heterogeneous class of tissue protein precipitates that have the common feature of β-pleated sheet secondary structure[3]. The main constituent of cerebral amyloid of Alzheimer's Disease has been shown by Glenner and Wong[4] to be a 39-42 amino acid polypeptide,

designated β/A_4 which is derived from a 695-770 amino acid precursor known as amyloid precursor protein (APP). This protein has a single transmembrane domain[5-8] and is encoded by a gene located on chromosome 21. The physiological role of APP is currently unknown. The puzzling thing about the formation of β/A_4 from APP is that normal proteolytic cleavage of APP results in cleavage occurring within the β/A_4 domain[9,10]. Thus, the major processing/secretion pathway for APP cannot be associated with the release of β/A_4. However, it is widely believed that deposition of β/A_4 is the causative agent of Alzheimer's pathology and that the neurofibrillary tangles, cell loss, vascular damage and dementia follow as a direct result of this deposition[11].

There are different forms of Alzheimer's Disease, one of these being what is termed familial Alzheimer's Disease. This form of the disease generally affects people at an earlier age than the sporadic form. In many cases of familial Alzheimer's Disease a mutation in the APP gene at codon 717 has been identified[12]. This mutation has been detected in English, American and Japanese families with early onset Alzheimer's Disease[13]. It is unclear how these mutations cause amyloid deposition but it has been proposed that they may inhibit the breakdown of a COOH-terminal fragment of APP that contains β/A_4[12,13]. Several studies suggest that APP can also be processed by the endosomal-lysosomal pathway. This pathway of APP processing can lead to carboxy-terminal fragments of APP containing the entire β/A_4 sequence[14,15] and may eventually lead to β/A_4 deposition (see Fig.1.1).

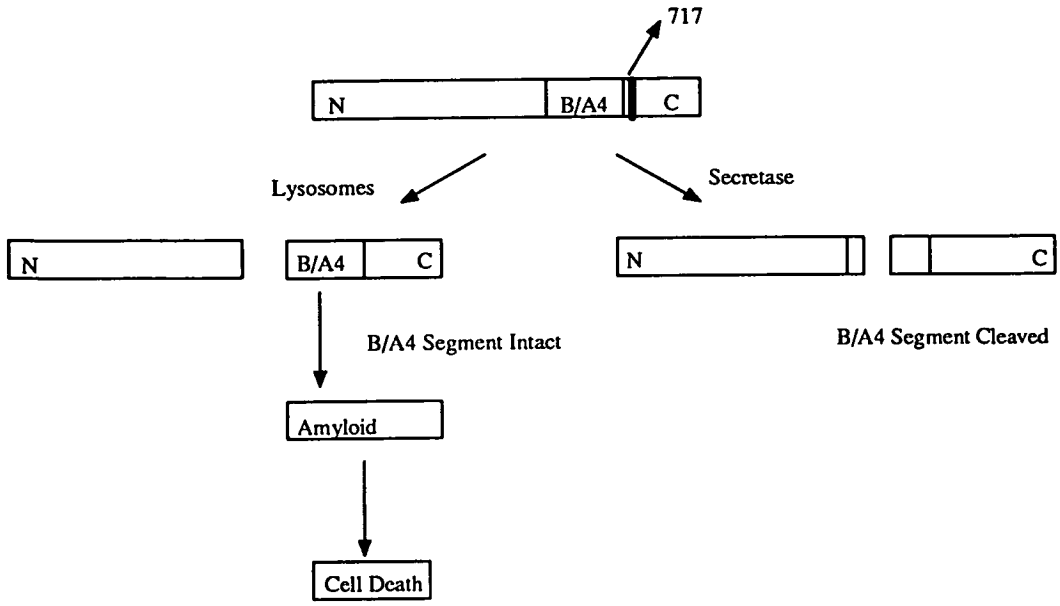


Fig.1.1 Pathways of APP processing

1.1.3 Cholinergic Deficits in Alzheimer's Disease

Many neuronal populations are found to degenerate in the brains of Alzheimer's patients. Those which have attracted most interest are the cholinergic neurons of the basal forebrain. Nerve cells interact with other nerve cells at junctions which are called synapses. Communication across the synapse is carried by small chemical molecules called neurotransmitters. One such neurotransmitter is acetylcholine. Acetylcholine was the first neurotransmitter to be discovered. It is synthesised by the transfer of an acetyl group from acetyl CoA to choline (Fig.1.3). The reaction is catalysed by choline acetyltransferase. The synthesised acetylcholine is stored in synaptic vesicles at the end of the presynaptic axon. On arrival of a nerve impulse, acetylcholine is released into the synapse (Fig.1.2). Diffusion across the synapse to the postsynaptic membrane occurs, and the acetylcholine molecules combine with specific receptor molecules. Excess acetylcholine is broken down to acetic acid and choline by the enzyme acetylcholinesterase.

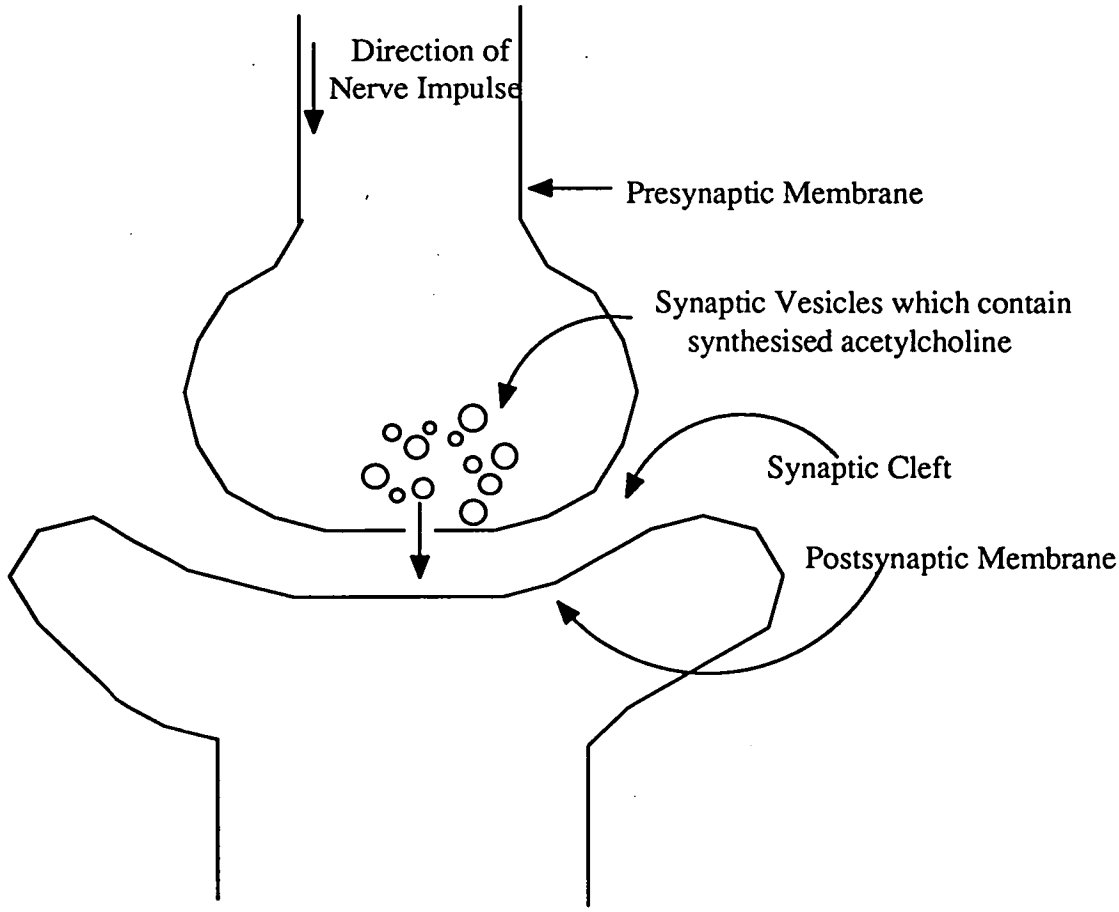


Fig.1.2 Diagrammatic representation of the cholinergic synapse

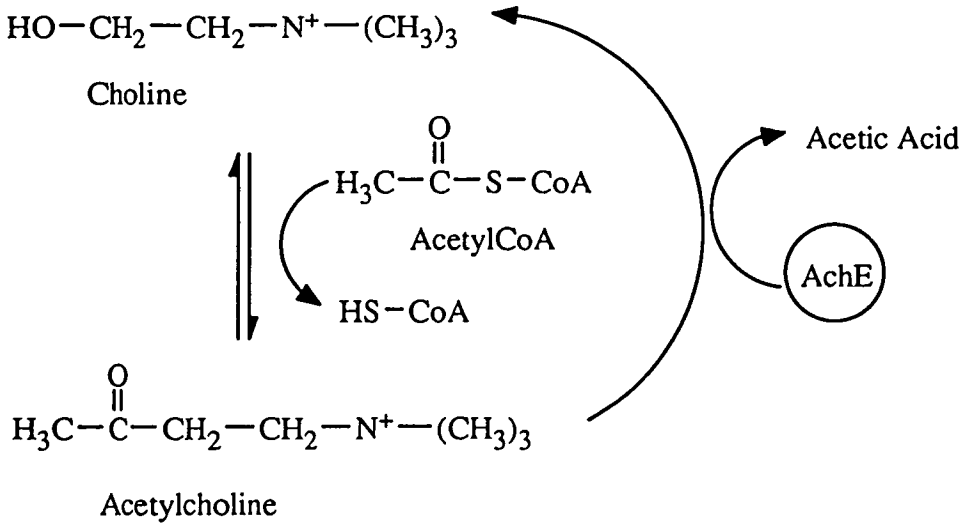
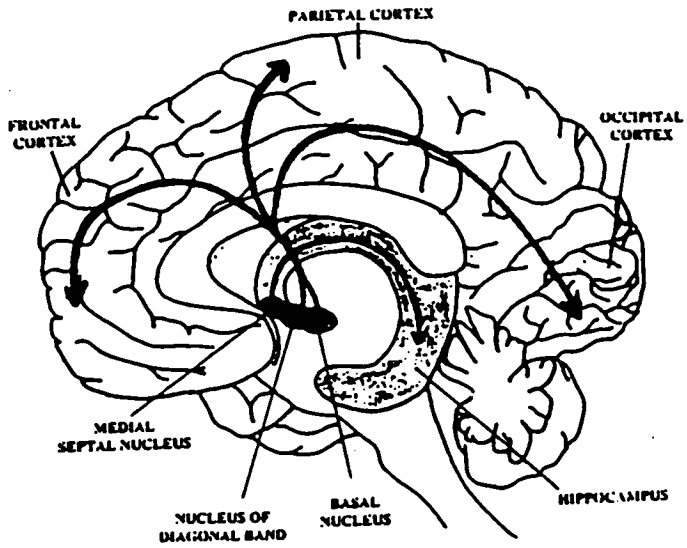


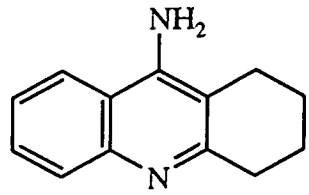
Fig.1.3 Mechanism of synthesis and breakdown of acetylcholine

In the central nervous system, cholinergic neurons of the basal forebrain extend to regions such as the cortex and the hippocampus (Fig.1.4a). In Alzheimer's Disease the activity of choline acetyltransferase (a marker for cholinergic neurons) is considerably reduced in these areas. The finding that neurons of the basal forebrain undergo selective degeneration in Alzheimer patients has led to the belief that the reduction of choline acetyltransferase activity in the cortex and hippocampus is a direct result of degeneration of these cholinergic neurons of the basal forebrain[16,17] (See Fig.1.4a). Levels of post-mortem neocortical choline acetyl transferase have been shown to correlate with severity of mental dysfunction[18]. This is believed to be an important factor in Alzheimer's Disease and as a result, many current drug strategies are based on increasing cholinergic function in the central nervous system. At present the only drug on the market for treating Alzheimer's Disease on this basis is Tacrine (Fig.1.4b). Tacrine is an acetylcholinesterase inhibitor[19]. Inhibition of this enzyme obviously increases the level of acetylcholine in the brain. Other drugs with similar effects are also being studied but none is without unpleasant side effects and as yet none of these drugs has amounted to a cure. In fact, it is found that the effects diminish with time and after a year or two the relentless decline of Alzheimer's Disease continues.

More recently, investigations in to the treatment of Alzheimer's Disease has led to the targeting of cholinergic neurons in a different way. Instead of compensating for the loss of cholinergic neurons by replacing the subsequent loss of acetylcholine levels, scientific investigation into keeping the neurons alive in the first place is being carried out. One obvious target for this purpose is the use of neurotrophic factors[20], proteins which promote the maintenance, growth and functional performances of neuronal populations.



(arrows indicate areas into which the neurons extend)



b. Tacrine

(1)

Fig.1.4 a. Cholinergic nerve tracts of the basal forebrain

b. Structure of Tacrine, used in the treatment of Alzheimer's Disease.

1.2 NERVE GROWTH FACTOR

1.2.1 NGF - A Member of the Neurotrophin Family

Nerve Growth Factor (NGF) was originally discovered in the early 1950's by Rita Levi-Montalcini of Washington University School of Medicine[21]. It was found that NGF was required in the peripheral nervous system for the development and maintenance of sympathetic nerve cells that use catecholamine neurotransmitters such as norepinephrine and dopamine. The search for NGF action in the central nervous system was initially concentrated on catecholamine-producing neurons in the brain. No apparent effects of NGF on catecholaminergic neurons in the brain could be found and it was therefore assumed that NGF did not act in the central nervous system at all. However, in the mid-1980's evidence came to light that NGF did have a role in the central nervous system. It was discovered that NGF increased the choline acetyltransferase activity in the basal forebrain, hippocampus and neocortex, of neonatal rats[22].

NGF is a member of a group of proteins called neurotrophic factors. Neurotrophic factors are proteins that are capable of stimulating survival and growth of nerve cells. Several protein families have neurotrophic factor members. For example, fibroblast growth factors[23], polypeptides involved in various biological activities in vivo and in vitro, and epidermal growth factor[24] which stimulate the growth of epidermal and epithelial cells. NGF belongs to the protein family called neurotrophins. The other known neurotrophins are brain-derived neurotrophic factor (BDNF)[25,26], neurotrophin-3 (NT-3)[27], neurotrophin-4 (NT-4)[28] and neurotrophin-5 (NT-5)[29]. The individual neurotrophins are highly conserved among mammalian species and share approximately 50% of the amino acids with other members of this group (Fig.1.5). NGF is the best characterised member of the group.

```
          1                    26                    50
mNGF      SSTHPVFHMGEFSVCDSVSVWV--GDKTTATDIKGKEVTV LAEVNINNSVFRQYFFETKCRASNPV
hNGF      SSSHPIFHRGEFSVCDSVSVWV--GDKTTATDIKGKEVMV LGEVNINNSVFKQYFFETKCRDPNPV
BDNF      --HSDPARRGELSVCDSISEWVTAADKKTAVDMSGGTVTV LEKVPVSKGQLKQYFYETKCNPMGYT
NT-3      -YAEHKSHRGEYSVCDSESLWVT--DKSSAIDIRGHQVTV LGEIKTGNSPVKQYFYETRCKEARPV

                    79                    100
mNGF      ESGCRGIDSKHWNSYCTTTHTFVKALTTDEKQ-AAWRFIRIDTACVCVLSRKATR
hNGF      DSGCRGIDSKHWNSYCTTTHTFVKALTMDGKQ-AAWRFIRIDTACVCVLSRKAVRRA
BDNF      KEGCRGIDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR
NT-3      KNGCRGIDDKHWNSQCKTSQTYVRALTSENNKLVGWRWIRIDTSCVCALSRKIGRT
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Fig.1.5 Sequence alignment of NGF and related sequences

NGF protein has been purified and sequenced from the submandibular gland of the male mouse[30,31]. It is synthesised as a 305 amino acid protein prepro-NGF. Prepro-NGF is a complex of three types of subunit, α, β, and γ.[32] The γ subunit is a trypsin-like protease[33], the α subunit is a protease inhibitor which is thought to protect NGF from proteloysis[34]. The biologically active NGF is represented by the β subunit. Proteolytic cleavage of the complex releases the mature β-NGF protein which is a basic 118 amino acid protein (120 in human NGF) and forms a non covalent dimer[35].

The crystal structure of β-mouse NGF has been determined[36]. This shows it consists of seven β-strands forming three anti-parallel pairs. The residues which contribute mainly to this structure are well conserved among the neurotrophins giving reason to believe that the other neurotrophins share the revealed NGF structure.

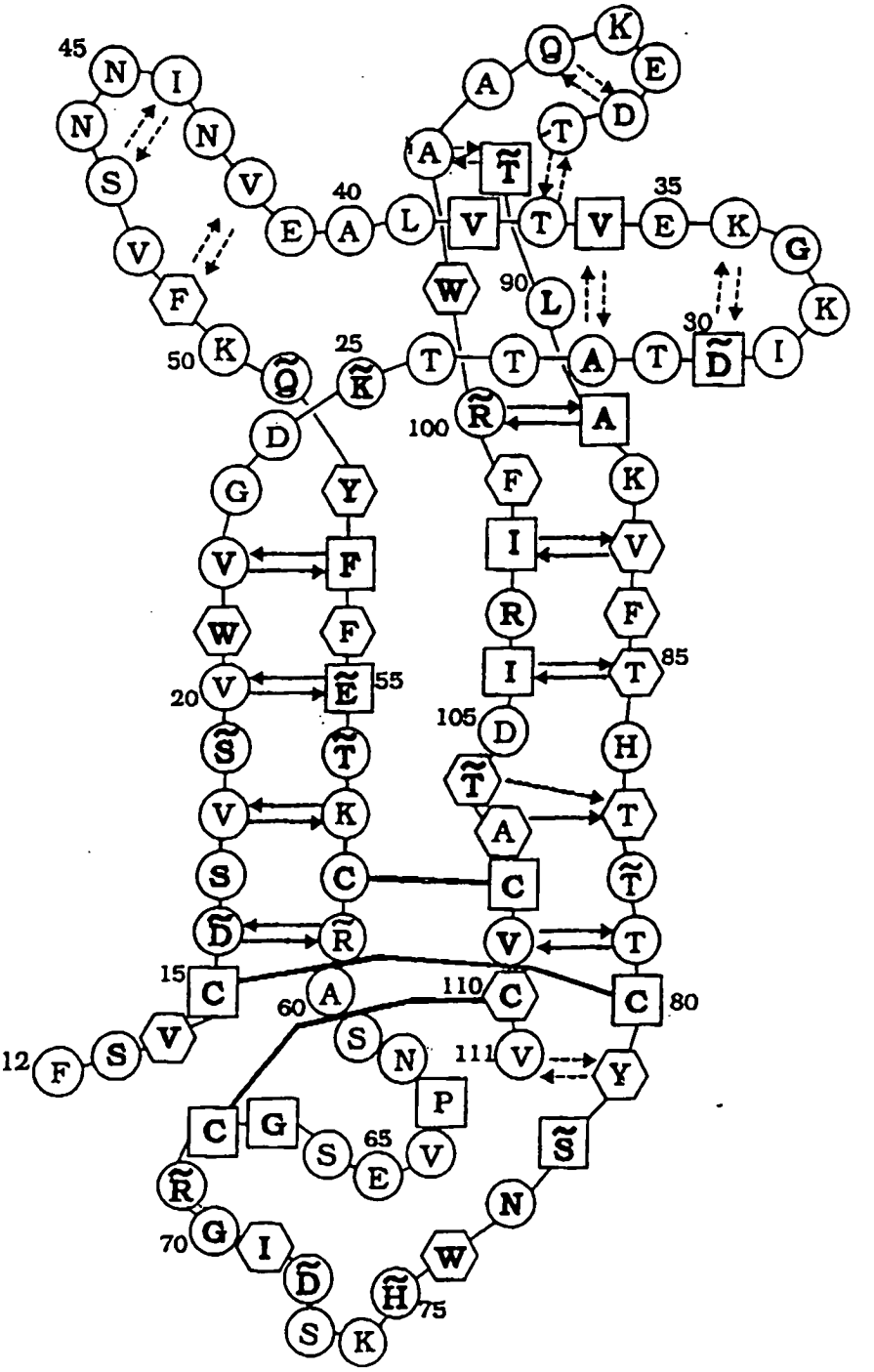


Fig.1.6 Deductions from the crystal structure of mNGF

1.2.2 NGF Receptors p75 and *Trk*

There are two known receptors which bind NGF. The first one to be discovered was the low affinity NGF receptor (LNGFR)[37]. This receptor binds NGF with nanomolar affinity. It is a cysteine-rich glycoprotein which contains approximately four hundred amino acids in a single peptide chain. It has a single membrane-spanning domain separating a slightly longer extracellular domain from a shorter cytoplasmic domain[38]. It has been named p75 NGFR based on the approximate molecular weight of the human receptor of 75000[39]. The extracellular domain contains four cysteine-rich loop regions (Fig.1.7). Each of these loops contains six cysteine residues and it is generally assumed that these cysteines form three disulphide bridges within the loop. These cysteine-rich regions are highly conserved among the species and it is now known that these loops determine the NGF binding domain[40]. Each of the loops is required and they are arranged in the correct order to create the binding site of NGF[41,42]. The short cytoplasmic domain does not contain distinctive motifs but it has been suggested that the conserved C-terminal of p75 may have a G protein linked signal transduction mechanism. However, this has yet to be determined[43]. It should be noted that p75 binds all the neurotrophins with similar affinity[44-46].

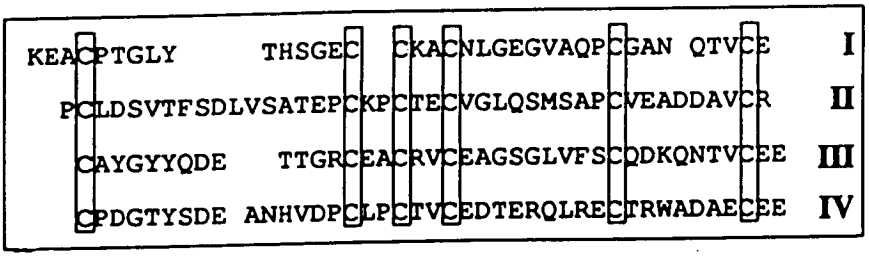


Fig.1.7 Four internal repeats in p75

The identification of a second NGF receptor, which binds NGF with picomolar affinity, was made by Martin-Zanca *et al* when they noticed that the distribution of the product of the proto-oncogene *trk* was restricted to targets of NGF action in neural crest derived sensory neurons[47]. Meakin and Shooter subsequently

reported that high affinity NGF receptors could be immunoprecipitated with anti-phosphotyrosine antibodies suggesting that the high affinity NGF receptor (HNGFR) contains tyrosine kinase activity[48]. This proved to be true when it was found that the tyrosine kinase activity and autophosphorylation of *trk* was stimulated on binding of NGF[49,50]. This tyrosine kinase receptor is a part of a family of tyrosine kinases, the other members of which have been named as *trk* B[51] and *trk* C[52]. These proteins, which have a molecular weight of approximately 140,000, have been found to bind preferentially to the neurotrophins BDNF and NT-3 respectively but not to NGF[53-57]. The kinase domains of *trk*, *trk* B and *trk* C share approximately 85% sequence homology while there is approximately 50% homology among the extracellular domains. The extracelluar domains contain three leucine-rich repeats of 24 amino acids containing 8 leucines which are bounded by two clusters of cysteine residues. There are also two immunoglobulin-like C 2 repeats in the extracellular domain (Fig.1.8). These features give rise to the possibility of potential protein-protein interactions or cell adhesion characteristics of the *trk* receptors.

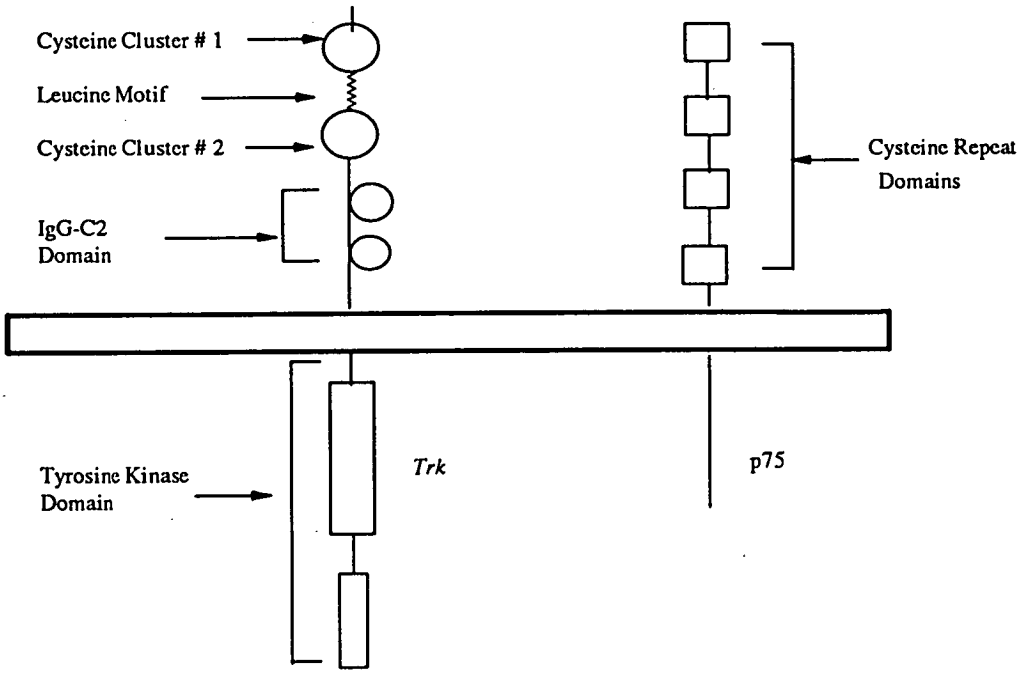


Fig.1.8 Structure of NGF receptors.

1.2.3 The Molecular Nature of the High Affinity Binding Receptor

As stated earlier, NGF plays an important role in the development and maintenance of neurons in both the peripheral and the central nervous systems. In the peripheral nervous system it is noted for its ability to stimulate the growth of sympathetic and sensory neurons. The role of NGF in the central nervous system when investigated by Mobley [22] revealed that intracerebroventricular injections of NGF increased choline acetyltransferase activity in the basal forebrain, hippocampus and neocortex of neonatal rats. The hippocampus and cortex are terminal regions for the fibres of basal forebrain cholinergic neurons. This was supported by the later observation that NGF binding sites in the basal forebrain correspond to the location of cholinergic neurons[58-60].

NGF is produced by the target tissues of the cholinergic basal forebrain neurons in the central nervous system. Binding of the growth factor to receptors on the nerve terminals occurs, followed by rapid internalisation of the NGF:receptor complex[61]. NGF is then retrogradely transported to the perikaryon (cell body) in the septum[62]. This results in the delivery of biologically active NGF to the cell body but the mechanism of signal transduction is as yet unknown.

A number of questions are still unanswered about the mechanism of action of NGF. Firstly, there is much debate about the molecular nature of the receptor which binds NGF with high affinity. A number of cell lines expressing either one or both of the NGF receptors have been developed, in order to study the effects of NGF binding to each of the receptors. The most widely used cell line is a noradrenergic clonal line of rat adrenal pheochromocytoma, PC12 cells[63]. PC12 cells do not require NGF for their growth and survival, however in the presence of this factor the cells respond by shifting from a chromaffin-like cell to one with neurites that resemble a sympathetic neuron[64]. These cells express both p75 and *trk* receptors. Biological responses to NGF are only observed in the presence of high affinity binding. No biological

responses mediated solely by the low affinity receptor have been observed. It has been suggested that co-expression of *trk* and p75 is required for the formation of high affinity NGF binding sites[65]. The proposal is that these proteins form a heterodimer which binds NGF (Fig.1.9a). This model was primarily based on binding studies in which the formation of high affinity NGF binding sites in heterologous COS cells required coexpression of p75 and *trk*. Additionally, high affinity binding was found to require both cytoplasmic and extracellular NGF binding domains of p75[66]. On the contrary, it has also been reported that a percentage of *trk* receptors can bind NGF with high affinity in the physiological picomolar range[67]. Also, coexpression of equal numbers of p75 and *trk* receptors did not appear to affect the dissociation constant or the relative percentage of low and high NGF binding sites displayed by the *trk* receptors. Cross-linking studies in cells expressing both receptors have revealed the existence of both p75 and *trk* homodimers (Fig.1.9b) but p75-*trk* heterodimers were not found[68]. Other studies have also contradicted the idea of a heterodimeric receptor. For example, it has been reported by Weskamp and Reichardt[69] that polyclonal antibodies which were capable of inhibiting NGF binding to p75 receptors in PC12 cells did not eliminate the high affinity receptors or their responsiveness to NGF. Ibanez *et al*[70] have reported that a mutated form of NGF which binds to *trk* but not to p75 receptors, retains much of it's biological activity. Meakin and Shooter[71] reported that three antibodies directed against different epitopes on the low affinity NGF receptor did not recognise the high affinity receptor subtype. Presumably if p75 was associated with the high affinity receptor, these antibodies would recognise the high affinity receptor also. Studies carried out using tyrosine kinase deficient mutants of *trk* showed that coexpression of these mutants with wild type *trk* receptors resulted in a significant inhibition of NGF mediated signal transduction. This indicated that the mutants behave as suppressors presumably by forming inactive wild type-mutant *trk* dimers.[68]

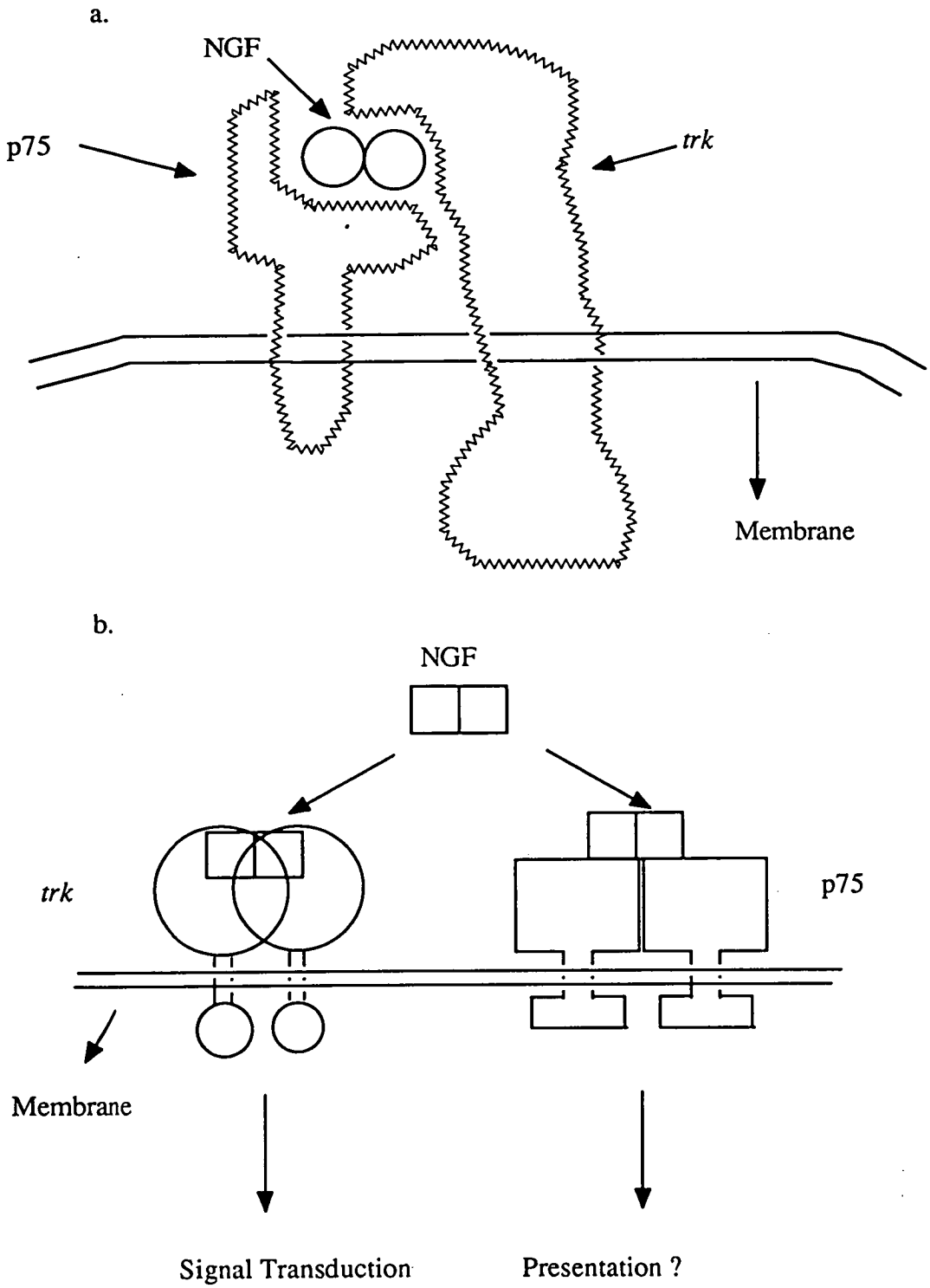


Fig.1.9 Proposed mechanisms for high affinity NGF binding.

a. Heterodimer between p75 and *trk* binds NGF.

b. Homodimers of p75 and *trk* bind NGF separately.

1.2.4 Function of the NGF Receptors p75 And *Trk*

The interaction of NGF with the receptors initiates signal transduction events that are key to the mechanism of action of NGF. It is not clear whether signal transduction occurs only at the level of the nerve terminal membrane or if it also occurs during the transport of the NGF:receptor complex. The high affinity receptor, *trk*, is a tyrosine kinase which has been demonstrated to undergo autophosphorylation on binding of NGF[49,50]. It has been shown that the kinase activity may be blocked by 5'-S-methyladenosine. This inhibitor also blocks NGF-induced differentiation of PC12 cells but does not affect NGF binding to its receptors[72]. These findings confirmed previous suggestions that activation of the NGF receptor complex results in tyrosine phosphorylation[48]. In addition, it was found that a mutant PC12 cell which lacks significant levels of *trk* and is non responsive to NGF, restores its biological response to NGF when a *trk* cDNA is introduced[73]. There can be no doubt, therefore, of the importance of this receptor for signal transduction when bound by NGF.

The function of the low affinity receptor, p75, is less clear however. As explained earlier, this receptor may have a domain resembling mastoparan which raises the possibility that the receptor may interact with a G protein as part of a signal transduction mechanism[43]. However, this suggestion has been made based on the predicted tertiary structure analysis of p75 and it has yet to be determined. Other functions have been suggested for p75. It may act as a presentation receptor which concentrates NGF[74]. There is also some evidence to indicate that it may be involved in the retrograde transport of NGF[75], or it may aid in discriminating among neurotrophic factors[76]. This low affinity receptor belongs to a family of diverse cell surface proteins including two tumour necrosis factor receptors, the Fas antigen, the T cell antigens OX40 and mu4-1BB and the B cell antigen CD40. This family of proteins has a high degree of homology in the cysteine-rich repeats in the

extracellular domain. Rabizadeh *et al*[77] have reported that expression of p75 induced neural cell death when p75 was unbound but binding by NGF or monoclonal antibody inhibited cell death induced by p75. It is possible, therefore, that the function of NGF binding to p75 is to inhibit this process known as apoptosis. Interestingly, another member of this receptor family, the Fas antigen, has been shown to induce apoptosis in murine cells which were transformed with the Fas antigen cDNA[78].

It is clear, therefore, that two separate receptors exist for NGF in responsive cells. The role of the *trk* receptor in neurotrophin function is more clearly defined and understood than that of the p75 receptor. For a review of neurotrophin receptors the reader is referred to reference 20.

1.2.5 NGF and Alzheimer's Disease

Several studies have been carried out to investigate the effect of NGF treatment on cholinergic neurons in the central nervous system. NGF receptors p75 and *trk* are selectively expressed by forebrain cholinergic neurons[79]. Early studies revealed that NGF injected into the hippocampus and cortex was taken up by cholinergic afferents and transported back to the cell bodies in the basal forebrain[80,81]. In support of the hypothesis that NGF acts selectively on cholinergic neurons as a target derived signal, NGF protein levels as well as mRNA coding for NGF are highest in hippocampus and cortex and hippocampal target cells of the cholinergic neurons express NGF mRNA[82-86].

The effect of NGF treatment on the maintenance of cholinergic neurons has been investigated in animals by severing cholinergic projections from the basal forebrain and examining the effect of NGF treatment on these lesioned neurons. It has been found that infusion of NGF can rescue the cholinergic neurons, stimulate axonal growth and improve cholinergic function[87-89]. These ascending cholinergic

projections undergo severe degeneration in Alzheimer's Disease[90,91] and there is strong evidence to suggest that the degeneration of the neurons is directly related to the memory and cognitive dysfunction suffered by these patients[92]. Therefore, it has been proposed that NGF treatment may be beneficial to Alzheimer patients.

Nerve Growth Factor is a large protein which will not cross the blood-brain barrier. This is a problem which will have to be overcome in order to be able to use NGF as a therapeutic agent in Alzheimer's Disease. Several methods of NGF administration have been proposed[93]. The first method involves intracerebral NGF infusion. This method has been tried clinically both in Parkinsons and Alzheimer patients[94]. The invasiveness of this procedure however makes treatment of this nature undesirable. A second method proposed is based on the work by Powell *et al*[95] who demonstrated the release of active NGF from a biocompatible ethylene-vinyl acetate copolymer polymer matrix. When PC12 cells were exposed to polymer-conditioned medium, neurite outgrowth was observed, indicating the bioactivity of NGF administered in this way. It is possible that such a procedure could be used to provide a local source of NGF in the brain for a limited period of time. A third method proposed involves coupling NGF to a carrier to enable it to cross the blood brain barrier. This has been demonstrated by coupling NGF to an antibody to the transferrin receptor. When the conjugate was injected peripherally it successfully crossed the blood brain barrier and retained full biological activity of NGF[96]. Cell grafting techniques have also been proposed as a method of NGF administration to the central nervous system. This would involve transplanting to intracerebral sites, cells capable of NGF synthesis. This technique has been demonstrated to be quite successful in animals[97,98]. Risk of tumour formation using this technique may be circumvented by using primary cell lines[99]. Alternatively, it has also been suggested that it may be possible to transfer to the brain only the genes necessary to produce NGF[100].

An alternative method to using NGF protein for therapeutic treatment of Alzheimer's Disease would be to design a molecule which can act as an NGF agonist in the brain. There is increasing knowledge of NGF receptors and with the knowledge of the crystal structure of NGF it may be possible to identify the regions of NGF required for binding and activation of receptors. These smaller peptides could then be administered using one of the appropriate techniques outlined above or it could be possible to design a smaller organic molecule capable of crossing the blood brain barrier, based on the knowledge of the NGF receptor binding site.

1.2.6 Aim of the Project

The biological significance of NGF and its potential use for the treatment of Alzheimer's Disease is clear. The difficulties of using NGF for treatment have been outlined. The knowledge of NGF interaction with each of the receptors could provide valuable information for the design of drug treatment for Alzheimer's Disease. Knowledge of the amino acid sequence required for binding to p75 and *trk* is limited. Previous work carried out in this laboratory has provided a good insight into the regions of NGF important for binding to p75. It is the aim of this project to develop the binding potential of peptide fragments to p75 and to investigate the regions of NGF important for *trk* binding. Competitive binding assays have been developed by our collaborators at Parke Davis for each of the NGF receptors. The peptide fragments of NGF were to be synthesised by Solid Phase Peptide Synthesis techniques.

1.3 SOLID PHASE PEPTIDE SYNTHESIS

1.3.1 Introduction

During the first half of this century, the synthesis of peptides posed a major challenge for organic chemists. The knowledge of biologically active peptides was ever increasing. The isolation of these naturally occurring peptides often presents a very difficult problem. Therefore, the need for chemical synthesis of these peptides and their analogues became increasingly important for pharmacological studies.

The methods for peptide synthesis were in need of reform. Classical solution chemistry could not meet the ever increasing demand for peptide synthesis. Yields for the formation of peptide bonds were often quite low resulting in poor overall yields for the entire synthesis of a peptide. In the early 1960's a revolution in this area of chemistry was brought about by the development by R. B. Merrifield[101] of Solid Phase Peptide Synthesis (SPPS). The idea behind this technique is quite simple. If an amino acid (A) is anchored to a solid support, it may be allowed to react with a large excess of amino acid (B) until the reaction has gone almost to 100% completion. The excess of B may then be washed away leaving the dipeptide formed and attached to the solid support. This procedure may then be repeated for each amino acid in the sequence until finally the peptide is complete. The design of the solid support is such that it may be easily cleaved to yield a peptide which has been synthesised in extremely high yield. Also, there was great potential for automation of this system and indeed this has been successfully achieved which greatly decreases the length of time involved in the synthesis.

Initially this technique was met with a lot of sceptism from organic chemists who felt instinctively that such high quantitative yields were rare in this area of chemistry. However, the Merrifield technique has stood the test of time and it is now

used world-wide as a very successful technique for the chemical synthesis of peptides.

1.3.2 The Solid Support

The original Merrifield resin was a chloromethylated styrene-divinylbenzene copolymer (2) Fig1.10. Despite much research into other forms of solid support, the original Merrifield resin has stood the test of time and consequently remains the preferred solid support resin for most syntheses. Developments have been made, however, in the coupling of the peptide chain to the solid support. Many 'linkers' have been produced which make cleavage of the peptide from the solid support considerably easier. One such linker was developed by Wang[102] (3). An ester linkage may be formed between the Wang linker and the C-terminus of the peptide. The *p*-alkoxy substituent of the benzyl alcohol increases the sensitivity of the ester linkage to acid. This enables the solid support to be cleaved by trifluoroacetic acid once the synthesis is complete (See Fig.1.10b). Other solid supports which had been used previously including the original Merrifield resin required much more severe conditions for cleavage such as the use of hydrofluoric acid.

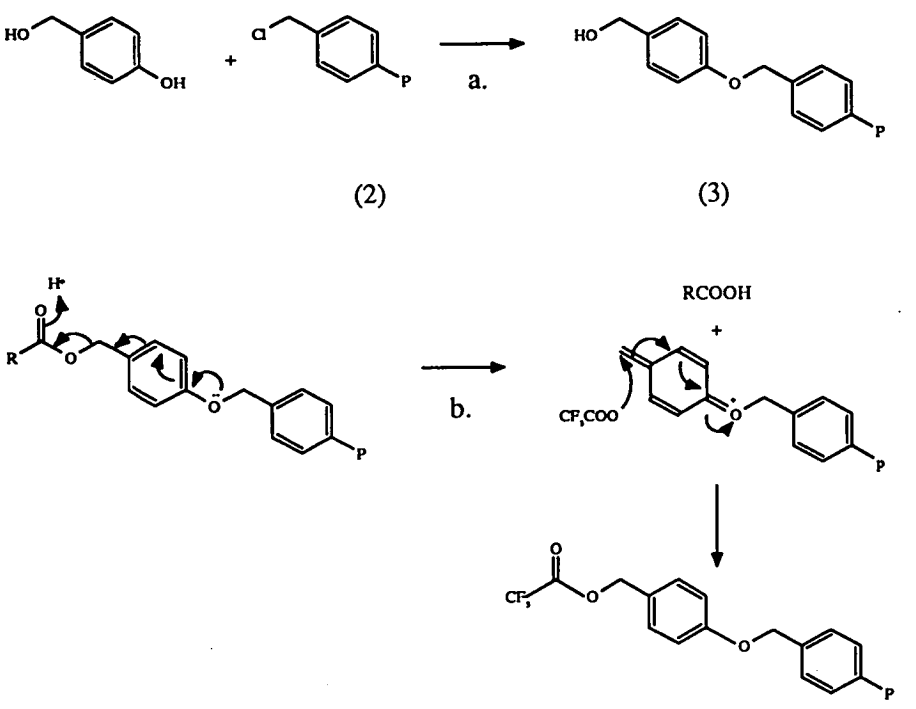


Fig.1.10 a. The synthesis of the Wang linker.

b. Cleavage of the Wang linker.

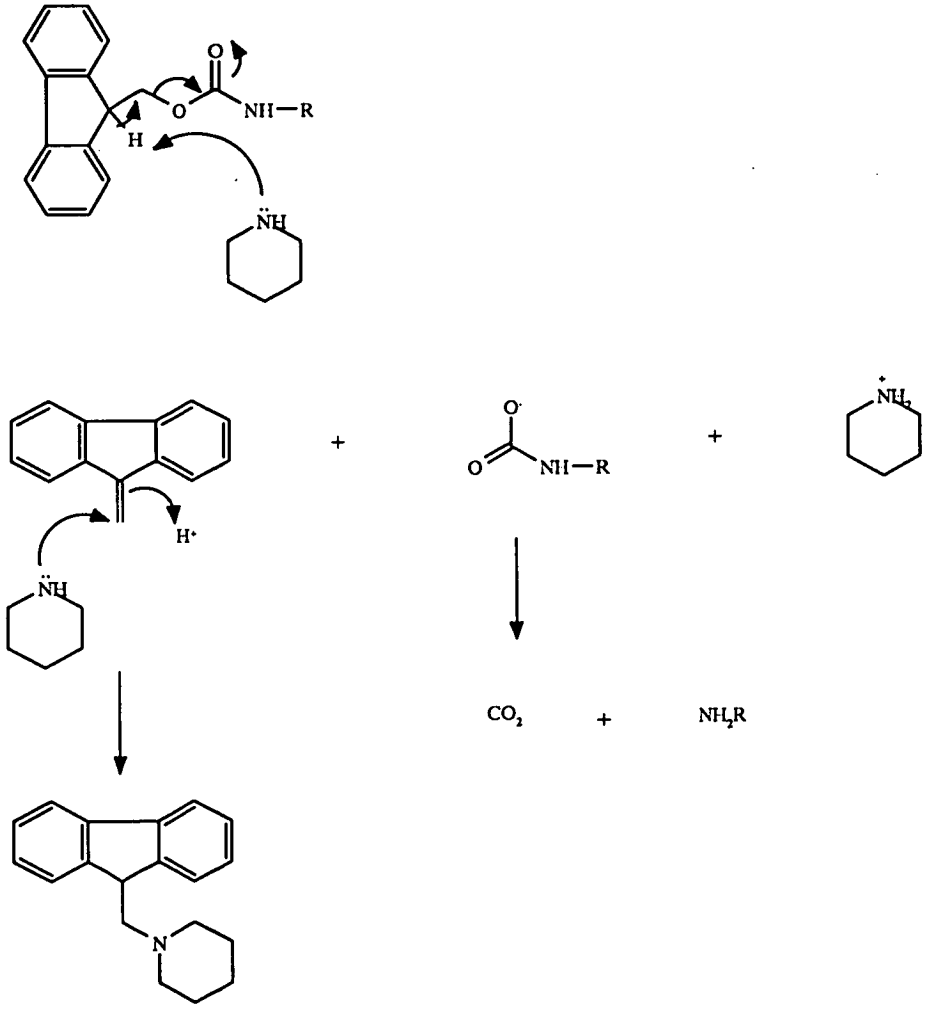
1.3.3 Protecting Groups

1.3.3.1 N-α Protecting Groups

In order to form one particular peptide bond between two amino acids it is necessary that the amino group of one amino acid and the carboxy group of the second amino acid be protected to prevent all but one possible combination of the two amino acids in forming the bond. A number of different α-amino protecting groups have been developed. One such group which is widely used is the fluorenylmethyloxycarbonyl (Fmoc) group (Fig.1.11). This group is base labile and may be easily removed by washing with a solution of 20% piperidine in dimethylformamide (DMF). Therefore, if an Fmoc protected amino acid is attached to the solid support, the Fmoc group may be easily removed to yield a free amine

group. This in turn may then be treated with the next Fmoc protected amino acid in the sequence to form the correct peptide bond.

The advantages of the Fmoc protecting group are as follows. Firstly, the removal of this group in base doesn't affect the peptide linkage to the solid support. Secondly, the adduct formed with piperidine (4) is detectable by U.V. at 302nm. Therefore the formation of the peptide bond may be monitored for each coupling reaction. The amount of Fmoc-piperidine adduct detected correlates directly with the amount of Fmoc protected amino acid which reacted with the resin bound free amine in forming the peptide bond.



(4)

Fig.1.11 Cleavage of the Fmoc protecting group by piperidine.

1.3.3.2 Side Chain Protecting Groups

Many of the common amino acids contain side chain functional groups which could be sensitive to the reaction conditions applied in SPPS. These side chain groups must therefore be protected during the synthesis. Many protecting groups have been developed for this purpose, most of which are easily removed in acids such as trifluoroacetic acid and therefore may be cleaved at the same time as the peptide is being cleaved from the resin. The natural amino acids may be divided in to three groups.

1. Ala, Asn, Gln, Gly, Ile, Leu, Met, Phe, Pro, Trp, Val

Each of these are without functional side chains and therefore do not require protection, although Asn, Gln and Trp may require protection sometimes.

2. Amino acids with OH, COOH, or NH_2 side chain functional groups such as

Thr, Ser, Tyr, Asp, Glu and Lys.

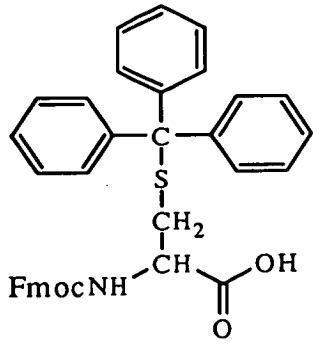
The OH and COOH groups are protected as the *t*-butyl ether or *t*-butyl ester. The NH_2 group of Lys is protected with *t*-butyloxycarbonyl. Each of these protecting groups is removable by treatment with acid.

3. Arg, His and Cys require special protection.

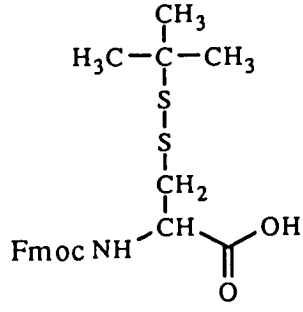
Arginine contains an extremely basic guanadino group. Presently the preferred choice of protecting group for this is the pentamethylchromansulphonyl group[103]. The most commonly used protecting group for histidine is the triphenylmethyl group (Trt). Each of these protecting groups may be cleaved in acid also. The protection of cysteine is discussed in the following section.

1.3.3.3 Protecting Groups for Cysteine

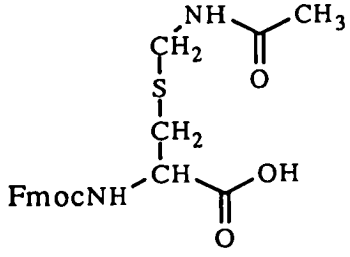
Cysteine requires protection during SPPS because of the nucleophilicity of the sulphhydryl group. This group could potentially compete with amino groups in the acetylation step to form thiol esters. A number of different cysteine protecting groups have been reported in the literature. The main ones used in the work carried out for this dissertation are listed below. Firstly, there is the triphenylmethyl group[104] (5) Fig.1.12. This group is cleaved by acid at the same time as the peptide is being cleaved from the resin, to produce thiol peptides. The S-*t*-butylsulphenyl group (6) is a semi-permanent protecting group. It is stable to acidic and basic conditions. Deprotection is carried out after the peptide has been cleaved from the resin. Reduction of the disulphide bond by phosphines[105] is the commonly used method for deprotection of this group.



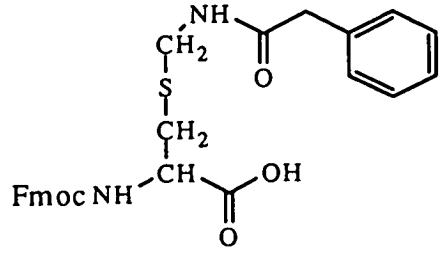
S-trityl (5)



S-*t*-butyl (6)



Acm (7)



phAcm (8)

Fig.1.12 Protecting groups used for cysteine.

The acetamidomethyl (Acm) (7) group is also a semipermanent protecting group. It is also generally stable to the acidic and basic conditions of SPPS. It may be cleaved by a range of electrophilic reagents such as silver triflate[106], iodine[107] and mercury (II) ions[108]. The advantage of this protecting group over the S-*t*-butylsulphenyl group is its ability to reduce the hydrophobicity of the peptide. By increasing the solubility of the crude peptide, this protecting group aids the purification procedure. The final cysteine protecting group employed is an Acm derivative named phenylacetamidomethyl (phAcm) (8). The additional advantage of this protecting group is that it may be cleaved enzymatically under very mild conditions[109].

Cleavage of these protecting groups under the conditions mentioned results in the formation of free thiol groups on the cysteines. The exception to this is the cleavage of the Acm protecting group with iodine. This reaction yields disulphides directly. In all other cases, formation of intramolecular disulphide bonds may be achieved by air oxidation[110] or by reaction in 5-10% dimethylsulphoxide (DMSO) in trifluoroacetic acid[111].

1.3.4 Peptide Bond Formation

In order to form a peptide bond between two amino acids efficiently, chemical activation of the acid function of one is desirable. Activation of the acid function makes it more susceptible to nucleophilic attack by the free amino group. Two commonly used activation procedures are;

(i) to form a symmetrical anhydride of the acid function,
(ii) to form an activated ester of the acid function.

A very good coupling reagent for the formation of symmetrical anhydrides of Fmoc protected amino acids is diisopropylcarbodiimide (DIC) (9) Fig.1.13. DIC reacts with amino acids to form an O-acylurea (10). This may then be reacted upon by a second equivalent of amino acid forming the symmetrical anhydride (13) with release of diisopropylurea. The advantages of this coupling reagent are that it is readily available and that the procedure of activation and coupling is relatively fast. The advantage over using mixed anhydrides is that the two reactive carbonyl groups in symmetrical anhydrides are identical, the same acylated product being obtained through attack at either carbonyl centre, which has the disadvantage that half of the amino acid remains unreacted and is wasted.

Formation of an activated ester of the acid function may be carried out by reaction with 1-hydroxybenzotriazole (HOBt)[112] or ethyl-1-hydroxy-1H-1,2,3-triazole-4-carboxylate (HOCt)[113]. The adduct formed on reaction of DIC with one equivalent of amino acid (10) may be reacted with either of these triazoles to generate an activated HOBt (11) or HOCt (12) ester.

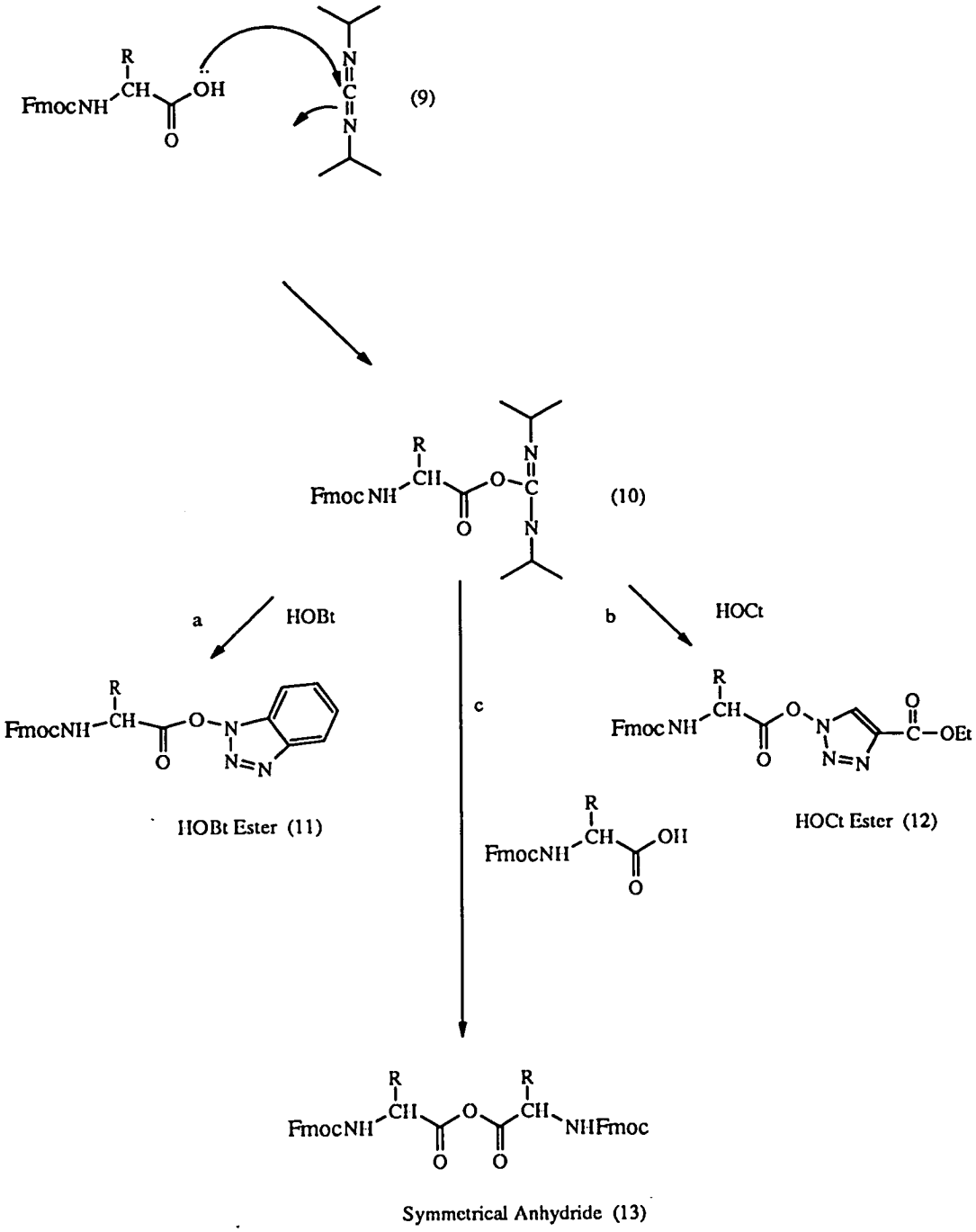
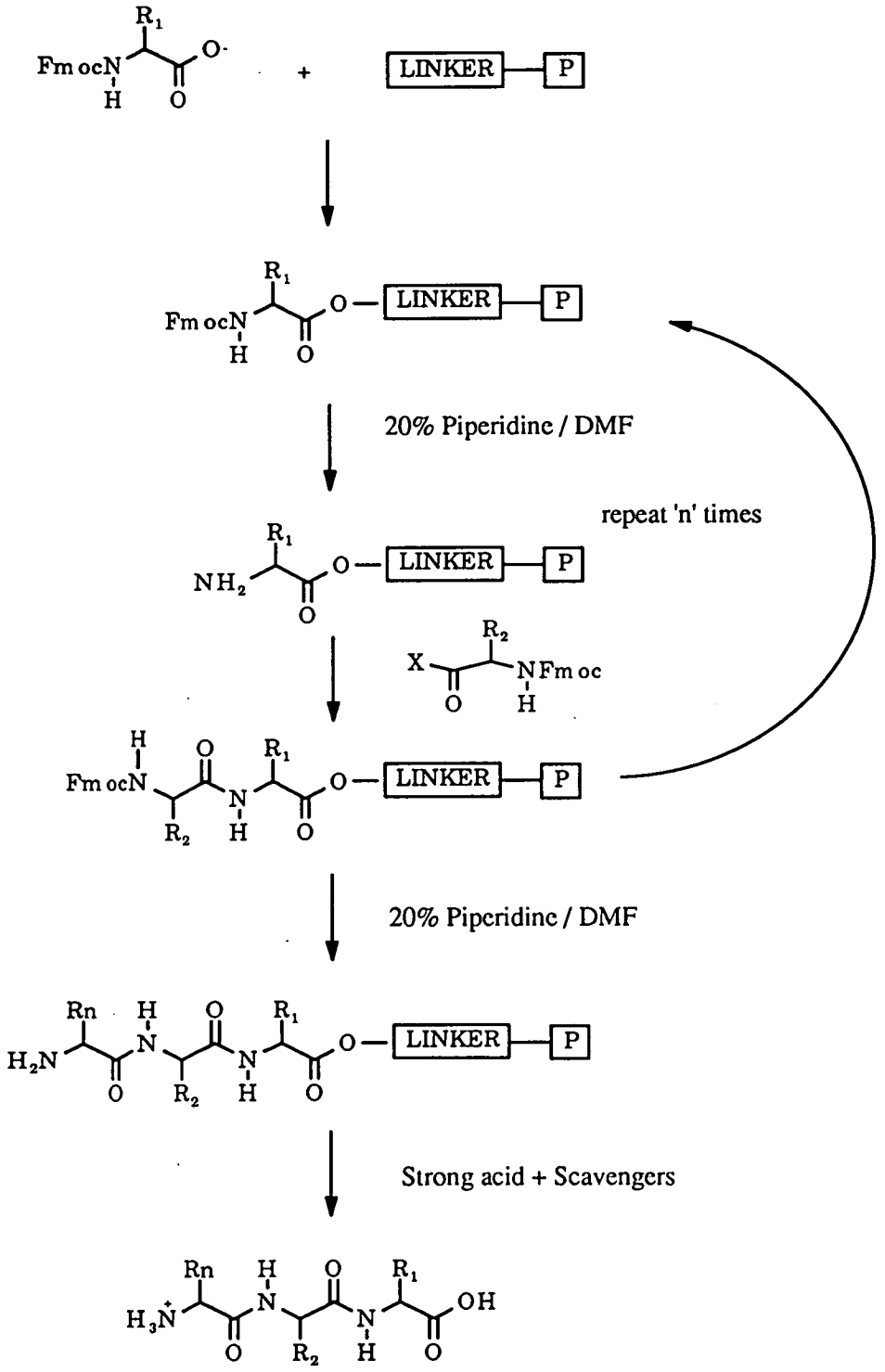


Fig.1.13 Coupling methods used in SPPS.



P = polymer

X = carbonyl activating group.

Fig.1.14 The synthesis cycle for Fmoc SPPS

1.3.5 Problems in Solid Phase Peptide Synthesis

1.3.5.1 Racemisation

Of the twenty common amino acids, nineteen have a chiral α-carbon. Two amino acids, isoleucine and threonine, have second chiral centres in their side chains. In nature, the α-carbon atoms of amino acids mostly possess the same relative (L) configuration. In SPPS, partial conversion to the D configuration of the activated amino acid may occur. The mechanism of racemisation is believed to be due to the formation of oxazolones (Fig.1.15).

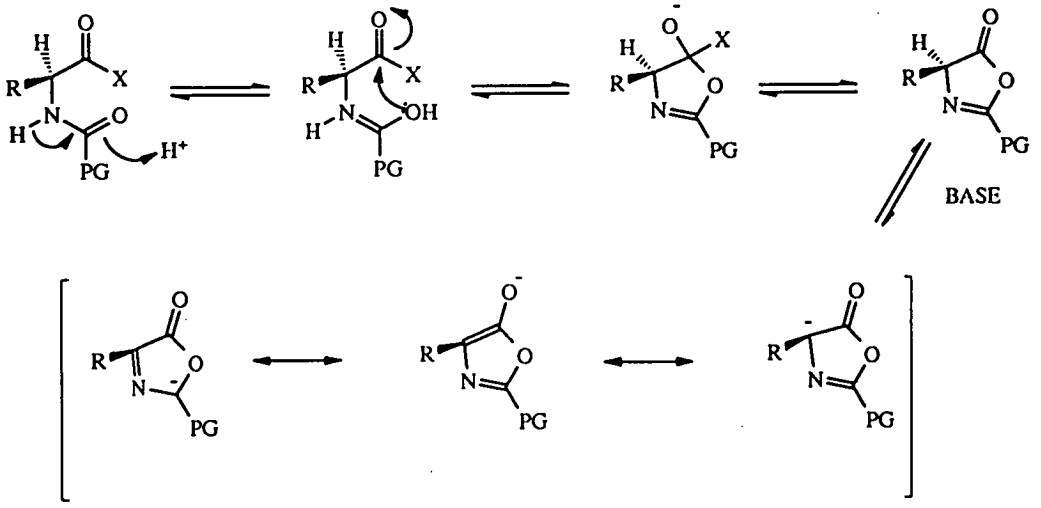
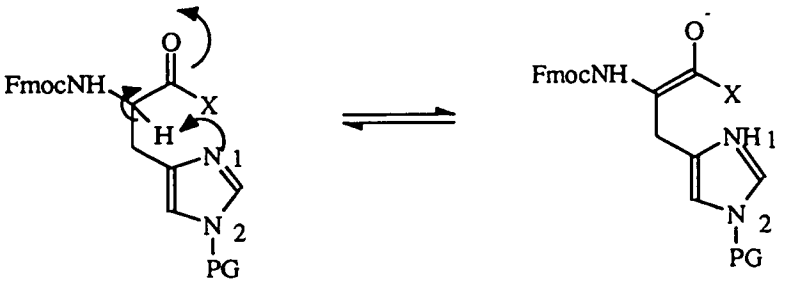


Fig.1.15 Mechanism of racemisation of activated amino acids in SPPS

Reprotonation may occur on either face to give the L or D configuration. The oxazole, racemised or not, can react with the free amino group on the resin in the same way as the original activated amino acid can. This provides a mechanism for incorporation of racemic amino acid residues during peptide synthesis. The presence of base is a requirement for this mechanism and in normal coupling reactions base

would not be present. Therefore racemisation is rarely a problem in this way. However, when the first amino acid is being loaded on to the resin, a catalytic amount of the base 4-dimethylaminopyridine (DMAP), is usually required. If the level of DMAP is too high in the coupling mixture, racemisation of the amino acid may occur.

Histidine creates a problem with racemisation due to the basicity of the nitrogen (N 1) in the side chain ring.



PG = Protecting Group

Fig.1.16 Mechanism of histidine racemisation.

Racemisation may be suppressed through electronic and steric effects of the protecting group on N 2. This may be chosen to so as to reduce the basicity of N 1. eg. the Trt protecting group. The most effective way of suppressing this racemisation is of course to protect N 1. The protecting group tert-butoxymethyl (Bum) is used in this way but this material is a lot more expensive than His(trt) and difficult to purchase in pure form.

1.3.5.2 Deletion Sequences

Deletion sequences may arise from incomplete coupling reactions. If all the amino groups on the resin do not become acylated by the activated amino acid these groups will then be available for reaction in the next coupling cycle, having missed one amino acid in the sequence. To combat this, the free unacylated amino termini may be acetylated by reaction with acetic anhydride. Alternatively deletion peptides may arise from the incomplete deprotection of the Fmoc group in one particular cycle. This prevents the incoming activated amino acids from coupling with the amino terminal, thus giving rise to a deletion sequence being co-synthesised.

CHAPTER TWO DISCUSSION

2.1 Introduction

Much work has been carried out in recent years on the study of the structure-function relationships of Nerve Growth Factor. At present there is limited knowledge of the areas of NGF required for receptor binding and subsequent biological activity. The technique of site directed mutagenesis has been widely used to investigate the importance of specific residues for NGF binding and biological responses[114-118]. Chemical modification of reactive amino acid side chains has also been used in these investigations. For example, N-bromosuccinimide was used to convert $Trp^{21,76,99}$ (all evolutionarily conserved residues) to oxindole derivatives[119,120]. This initially indicated that these residues were not required for biological activity. However, a subsequent study using the same procedure contradicted this[121]. Frazier *et al* reported that iodination or nitration of the two tyrosine residues ($Tyr^{52,79}$) indicated that these residues are exposed on the surface of the molecule but that they are not required for biological activity[119]. Modification of the lysine residues with dimethylsuberimidate[122] or by acetylation or succinylation[123] was reported to be possible without loss of NGF activity. However, modification of the arginine residues with cyclohexanedione resulted in inactivation but kinetic analysis suggests that the loss in biological activity is due to gross structural change rather than modification of specific sites[123].

With site directed mutagenesis studies, changes to the NGF primary sequence have been made. These mutants were then tested for subsequent changes in binding and biological activity of NGF. Ibanez *et al* have reported that three lysine residues ($Lys^{32,34,95}$) form a positively charged interface involved in binding to p75[115]. They have also reported findings that discontinuous stretches of amino acid residues group

together on one side of the NGF dimer to form a continuous surface responsible for binding to and activation of *trk*[118]. A series of mouse NGF deletion mutants were prepared by Drinkwater *et al*[117], using in vitro mutagenesis. They tested the importance of the N-terminal and C-terminal domains and also the surface exposed reverse turn region 59-66, for NGF activity. They found that the C-terminal deleted mutant showed no measurable biological activity compared to the full length NGF suggesting that this region plays a crucial role in mediating receptor recognition and perhaps ligand binding. Mobley *et al* reported that the amino terminal eight residues in murine NGF are susceptible to proteolytic cleavage and were therefore considered not to be involved in interactions with the NGF receptors[124]. Full-length and amino-truncated NGF were considered equal in biological activity based on initial experiments performed with mNGF. When recombinant hNGF became available, Kahle *et al*[116] reported that full length recombinant human NGF (rhNGF 1-118) was more potent than the amino terminal-truncated rhNGF (rhNGF 10-118) with regard to survival of sensory and sympathetic neurons, as well as neurite outgrowth from PC12 cells. They found that *trk* bound full length rhNGF with over 300-fold higher affinity than 10-118 rhNGF whereas only a slight difference in affinity of binding to p75 was observed. This indicated that for hNGF binding to *trk*, the N-terminal region was important.

More recently the structure-function relationships of NGF have been investigated using an alternative and independent technique involving anti-NGF monoclonal antibodies[125]. This is considered a useful strategy since antigenic sites like the receptor binding sites are most likely to be present on the surface of the protein. Three well-characterised monoclonal antibodies were used to probe sites on the surface of NGF involved in binding to p75 and *trk*, and their effects on NGF binding to the receptors were examined. It was found that one of these antibodies (27/21) inhibited biological activity and *trk* binding and phosphorylation by NGF but

did not inhibit p75 binding. This indicated that there are different binding regions for each receptor. It was proposed that the antigenic region that may be involved in binding to *trk* encompasses the N-terminus, the loop region 60-80 and the C-terminus of NGF.

In this study the structure activity relationship of Nerve Growth Factor has been investigated by the synthesis of peptide fragments of NGF and the subsequent testing of the ability of these peptides to inhibit NGF binding to each of the receptors. This method has the advantage that the purity of the peptide fragments may be assured. Chemical modifications and analogues of the fragments may also easily be produced. Previous work carried out on this project in this laboratory, led to the identification of a peptide fragment in the C-terminal region which inhibited NGF binding to the p75 receptor in micromolar concentrations. This provided the starting point of the work carried out here.

2.2 p75 Receptor Binding Assay

The assay used to test the ability of the NGF peptides to bind to the p75 receptor was set up by our collaborators at Parke Davis. This assay used full-length recombinant human p75, produced from Chinese Hamster Ovary (CHO) cell lines. The receptor was biotinylated and then anchored to streptavidin-coated microtitre plates. The high affinity of biotin for the protein streptavidin aided this method of receptor immobilisation. Incubation for two hours, of ^{125}I-NGF (100pM) with a 10μM solution of peptide in dimethylsulphoxide (DMSO), was carried out. After a careful wash cycle the binding ability of the test peptide was determined by comparison of the measured counts per minute for ^{125}I-NGF in the presence and absence of the test peptide. If the test peptide competed with ^{125}I-NGF for binding to the receptor, the counts per minute would decrease as increased quantities of unbound ^{125}I-NGF would be removed during the final wash cycle.

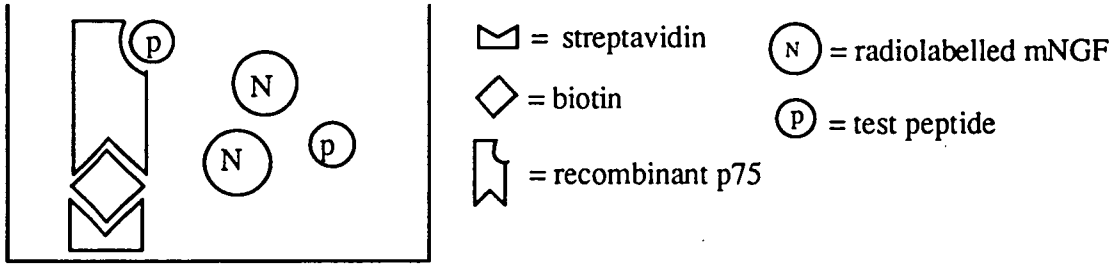


Fig.2.1 Diagrammatic representation of the p75 assay

2.3 *Trk* Receptor Binding Assay

The assay used to test the ability of the NGF peptides to bind to the *trk* receptor was set up by our collaborators at Parke Davis. For this assay, recombinant human *trk* receptor produced from Chinese Hamster Ovary (CHO) cells, was used. The receptor was attached to microtitre plates using wheatgerm agglutinin (WGA) SPA beads. Incubation for one hour at room temperature, of ^{125}I-mNGF (100pM) and a 100μM solution of peptide in binding buffer, was carried out. The binding buffer consisted of phosphate buffered saline (PBS) with 1mg/ml glucose and 1mg/ml bovine serum albumin (BSA). ^{125}I-mNGF was obtained from Amersham. As with the p75 assay, inhibition of ^{125}I-NGF by the test peptide results in a decrease in counts per minute of the receptor-bound ^{125}I-NGF.

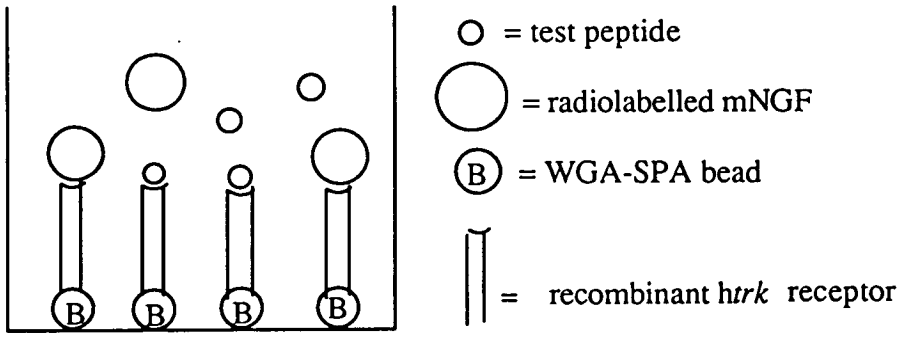


Fig.2.2 Diagramatic representation of the *trk* assay

2.4 Synthesis of Analogues of a C-terminal Active Fragment of NGF

Previous work carried out in this laboratory led to the identification of a C-terminal fragment of NGF required for binding to the p75 receptor[126,127]. This region, which is highly conserved, contains 100-114 NGF.

R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}

It was found that an artificial disulfide bond formed between Cys^{108} and Cys^{110} gave a compound which bound with higher affinity than the reduced form[126,127]. The initial work carried out in this study involved the investigation of the importance of two of the N-terminal residues of this peptide for binding to the p75 receptor. Arg^{100} was replaced by lysine and ornithine, Phe^{101} was replaced by cyclohexylalanine and tyrosine. These substitutions investigated the importance of the functional side chains of these residues , for binding to the p75 receptor.

The synthesis of this sequence proved to be difficult. It was found that using standard double couple cycles with symmetrical anhydride and HOBt ester led to a drop in the synthesis over the entire sequence to approximately 35% coupling. This meant that several deletion sequences would also be present in the crude material and purification of the desired peptide would be extremely difficult. It was therefore decided that standard coupling procedures were insufficient for this sequence. For the synthesis of these analogues, different coupling procedures were investigated in an effort to improve the overall yield of purified peptide obtainable from the synthesis.

The first analogue to be synthesised replaced Arg^{100} with lysine. This was synthesised using standard coupling cycles. A resultant drop in the overall synthesis to 35% coupling was observed, as stated above. By examination of the deprotection

profile (Fig.2.3), areas of poor coupling could be assessed. This information was used in deciding the coupling procedures to be used in subsequent syntheses. It was decided that this analogue would have to be synthesised again.

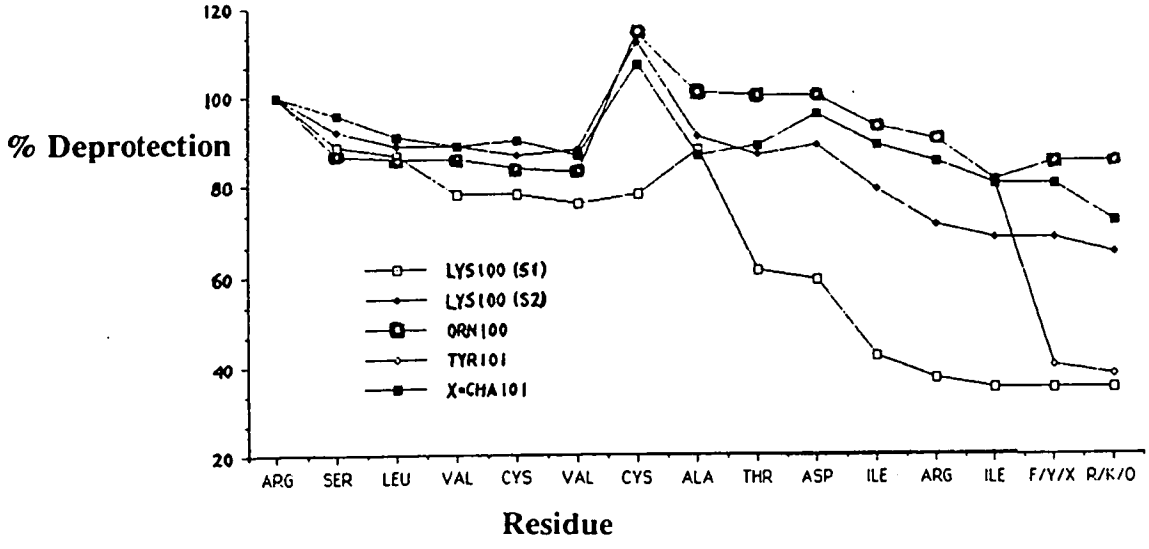


Fig.2.3 Chain assembly for 100-114 NGF analogues

From the deprotection profile of the first analogue (Lys100 S1), areas of poor coupling appeared to be Ala^{107}-Thr^{108} and Asp^{105}-Ile^{106}. Half of the resin was removed from the synthesis before the deprotection of Phe^{101}, but this has been taken into account when calculating the points on the graph for deprotection of Phe^{101} and Arg^{100}.

The second analogue to be synthesised replaced Arg^{100} with ornithine. For this synthesis it was decided that triple coupling cycles would be used with extended coupling times for Ala^{107}-Thr^{108} and Asp^{105}-Ile^{106}, as these were the areas of poor coupling in the previous synthesis. Extended coupling was also used for the coupling of Ile^{102}-Phe^{101}. This resulted in a much improved overall coupling efficiency. The

synthesis ended at approximately 85% coupling compared to 35% coupling observed in the previous synthesis.

The third analogue to be synthesised replaced Phe^{101} with tyrosine. For this synthesis, it was decided to use double couple cycles , as for the first synthesis, but with extended coupling times throughout the sequence. Half of the resin was removed from the synthesis before deprotection of Ile^{102} and stored for the synthesis of the next analogue. Up to this point in the synthesis the coupling had gone as well as it did in the previous synthesis where triple coupling cycles had been used. However, on coupling of Tyr^{101} a 50% drop in coupling was observed (Fig.2.3).

The synthesis of the fourth analogue with Phe^{101} replaced by cyclohexylalanine was carried out using the remaining 50% of the resin removed from the synthesis of the third analogue. In order to prevent the drop in coupling efficiency observed in the previous synthesis for Tyr^{101}, cyclohexylalanine was coupled with triple coupling cycles with extended coupling times.

Finally the synthesis of the first analogue was repeated (Lys100 S2), this time using double couple cycles with extended coupling times. This synthesis finished at 65% coupling compared to 35% coupling for the first synthesis.

Purification of these analogues proved to be quite difficult on account of the hydrophobic nature of the sequence. However, it was noted in each case that cleavage of the S-*t*-butyl protecting groups on cysteine and subsequent oxidation of the crude peptide, greatly improved the solubility. This indicated that the hydrophobic nature of this cysteine protecting group may have been the cause of much of the solubility problems incurred. Each of the analogues was tested on the p75 assay. A comparison between the analogues and the unchanged sequence 100-114, was made. The results obtained are summarised in Table 1.

	Amino Acid Sequence	p75 Assay % Inhibition @ 10μM
100-114 NGF	**R.F.I.R.I.D.T.A.C.V.C.V.L.S.R (oxid)**	**36**
100-114 Lys^{100}	**K.F.I.R.I.D.T.A.C.V.C.V.L.S.R (oxid)**	**12**
100-114 Orn^{100}	**O.F.I.R.I.D.T.A.C.V.C.V.L.S.R (oxid)**	**12**
100-114 Cha^{101}	**R.Cha.I.R.I.D.T.A.C.V.C.V.L.S.R (oxid)**	**30**
100-114 Tyr^{101}	**R.Y.I.R.I.D.T.A.C.V.C.V.L.S.R (oxid)**	**10**

Table 1

From these results it is clear that the substitution of Arg^{100} results in a great depletion of binding of this sequence. Substitution of Phe^{101} by tyrosine also surprisingly appears to destroy the binding ability of the peptide, whereas the substitution by cyclohexylalanine surprisingly does not. This indicates that the aromaticity of the side chain group in Phe^{101} does not appear to be important for stability of binding. It is clear, however, that none of these analogues bind with higher affinity than the native sequence of 100-114 NGF.

2.5 Investigation of a Secondary Binding Site for p75.

A paper published by Ibanez *et al*[115] describes a proposal that Lys^{32}, Lys^{34} and Lys^{95} of NGF form a positively charged interface involved in binding to p75. The mutant proteins with these lysine residues replaced by alanine residues showed loss of binding to the p75 receptor but retained binding to *trk* and biological activity. This demonstrated a functional dissociation between the two NGF receptors. Therefore, it was decided that these lysine residues may form a secondary binding site for p75 along with the C-terminal fragment 100-114 NGF previously identified

here. It was thought that a peptide combining both of these regions may bind more efficiently to the p75 receptor, than the fragment 100-114 NGF, by itself. A peptide was therefore designed which linked the C-terminal fragment 100-114 NGF to these lysine residues using a straight chain carbon linker, aminoundecanoic acid (aua). The flexibility of this linker would hopefully allow the lysine residues to form the required positive charged complex for binding to p75 and also allow 100-114 to stretch to its receptor binding site. Acm protecting groups were used for the cysteine residues as it was expected that this protecting group would improve the solubility of the crude peptide.

The first synthesis of this peptide (compound 8) was carried out using triple coupling cycles (one symmetrical anhydride followed by two HOBt activated ester) with extended coupling times. The aminoundecanoic acid linkers were coupled manually by sonication. The overall synthesis appeared to go quite well ending at approximately 60% coupling. Cleavage of the peptide from the resin indicated, however, that deletion sequences were also present in the crude material (Fig.2.4a). Purification of the required peptide sequence (with Cys still protected) from the deletion sequences was achieved by preparative hplc (Fig.2.4b). However, low yields were obtained.

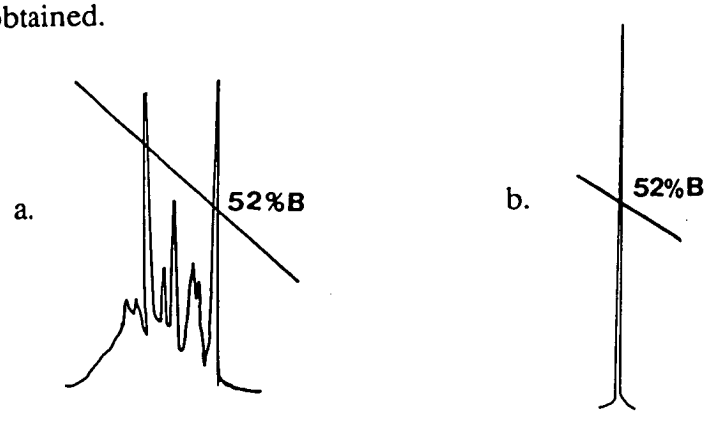


Fig. 2.4 a. Hplc trace of crude peptide (compound 8).

b. Purified peptide.

A second synthesis of this peptide was carried out (compound 8/2). It was decided that a new coupling reagent which was developed in this laboratory[113], ethyl-Hhydroxy-1H-1,2,3-triazole-4-carboxylate, would be used as it had been shown that this coupling reagent improved the overall efficiency of the synthesis of the sequence 100-114 NGF[113]. A new capping solution was also used in this synthesis. The new capping solution of 0.5M acetic anhydride, 0.125M disopropylethylamine (DIEA), 0.2% HOBt in DMF compared to the old capping solution of 0.5M acetic anhydride, 0.5M pyridine in DMF, was believed to be more efficient[128]. The deprotection profile for each synthesis indicated that the second synthesis was as successful as the first. However, comparison of the hplc traces of the crude peptides obtained from each synthesis indicates that a much higher percentage of the required peptide compared to deletion sequences was present in the crude material of the second synthesis (Fig.2.5). The cleavage conditions for both were the same and the cleavage time allowed was three hours. It was clear, therefore, that using the new coupling reagent and capping solution had improved the overall efficiency of the synthesis. Purification of the crude peptide from the second synthesis proved to be considerably easier as a result.

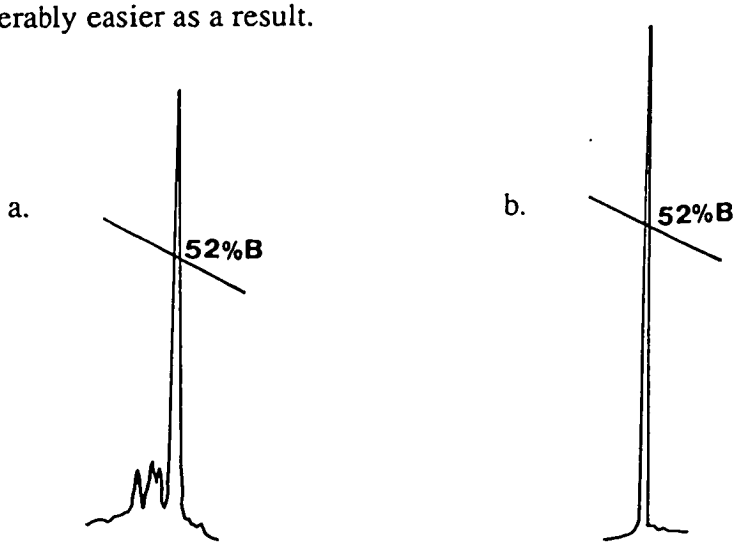


Fig.2.5 a. Hplc trace of crude peptide (cysteine protected compound 8/2).

b. Purified peptide.

To form the disulphide between Cys^{108} and Cys^{110}, deprotection of the cysteine protecting groups had first to be achieved. Several methods of Acm deprotection have been published[106-108]. The method which worked best with this peptide appeared to be cleavage using silver trifluoromethanesulphonate[106]. Cleavage of the Acm groups was confirmed by a positive Ellman's test (section 3.2.6.1). Oxidation was carried out in a solution of 5% DMSO/TFA. The oxidation was monitored by analytical hplc (Fig.2.6). In one hour the oxidation had gone almost to completion. The reaction was stopped and the peptide was purified by preparative hplc.

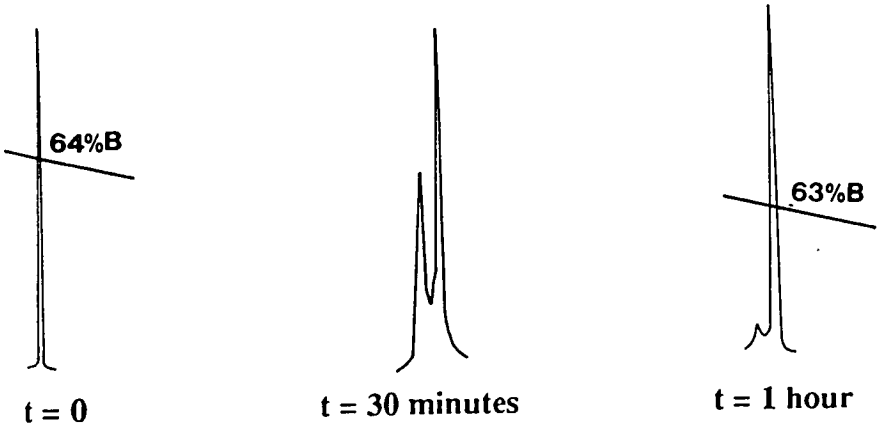


Fig.2.6 Oxidation of compound 8/2 in 5% DMSO/TFA

Both the reduced (with cysteines still protected) and oxidised forms of this peptide were tested on the p75 assay. In addition, a peptide containing only the lysine extension part of this peptide was also synthesised, i.e. the three lysine residues linked to Arg^{100} but with Phe^{101} to Arg^{114} omitted. This was tested for the ability of the lysine residues alone to bind to the p75 receptor. The results obtained in the p75 assay are summarised in Table 2.

Amino Acid Sequence	p75 Assay % Inhibition @ 10μM
K^{32}.G.K^{34}.aua.K^{95}.aua.R^{100}.F.I.R.I.D. T.A.C(Acm).V.C(Acm).V.L.S.R^{114}	49
K^{32}.G.K^{34}.aua.K^{95}.aua.R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R (oxid)	60, 82
K^{32}.G.K^{34}.aua.K^{95}.aua.R^{100}	11
100-114 NGF R.F.I.R.I.D.T.A.C.V.C.V.L.S.R (oxid)	19

Table 2

From these results it was immediately apparent that there was a problem with the p75 assay as the percentage inhibition value for the sequence 100-114 NGF was only about half that of the usual value obtained (36% inhibition @ 10μM). However, it was also clear that the lysine chain attached to the peptide 100-114 greatly increased the inhibition of NGF (60%, 82% inhibition compared with 19% inhibition) with the oxidised from of this linker peptide showing greater inhibition than the cysteine protected form. Interestingly, the lysine residues alone, without the sequence 100-114 attached, did not show much inhibition. The following photograph illustrates how these lysine residues and the C-terminal fragment 100-112 (113-120 not determined in the crystal structure) relate to the 3-D structure of the NGF protein itself. This photograph shows the configuration of the murine protein as found by X-ray crystallography. The plate was prepared from the Brookhaven Protein Databank model of murine NGF and manipulated using the SYBIL programme on an Evans and Sutherland workstation. It is interesting to note the close proximity of the three lysine residues to Arg^{100} and Arg^{103}. It seems likely that these residues form part of a positively charged unit involved in binding to the acidic p75 receptor.

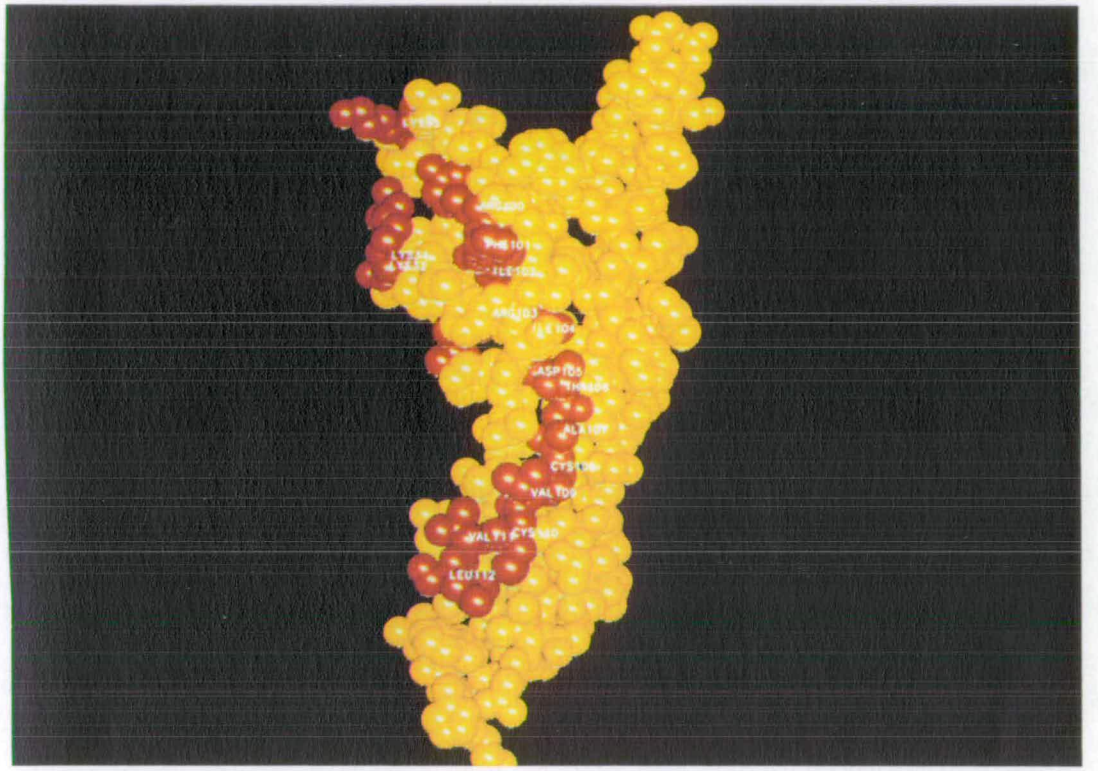


Plate 1

The protein structure of mNGF showing in red, the basic residues $Lys^{95,32,34}$ and the region 100-112 NGF. (Residues 113-120 not resolved in the crystal structure).

2.6.4 Initial *Trk* Assay Results of the NGF Peptides.

The results of the selection of peptides described above are summarised in Table 3.

Amino Acid Sequence		*Trk* Assay % Inhibition (at 100μM)
59-67 (h)	R.D.P.N.P.V.D.S.G	21
46-54 (h)	N.S.V.F.K.Q.Y.F.F	11
34-42 (m)	K.E.V.T.V.L.A.E.V	8
50-60 (h)	K.Q.Y.F.F.E.T.K.C.R.D	10
81-107 (h)	T.T.T.H.T.F.V.K.A.L.T.M.D.G.K.Q.A.A. W.R.F.I.R.I.D.T.A	0
25-40 (h)	K.T.T.A.T.D.I.K.G.K.E.V.M.V.L.G	10.7
1-8.aua.100-114 (h)	S.S.S.H.P.I.F.H.aua.R.F.I.R.I.D.T.A. C.V.C.V.L.S.R (oxid)	83.4

(h) = human sequence, (m) = mouse sequence.

Table 3

The four peptides, which were purified and tested to confirm the epitope scan results (59-67, 46-54, 34-42 and 50-60), appeared to contradict the results. However, it should be noted that for the epitope scan, peptides of mouse sequence were used. This may be a contributing factor to the low inhibition values observed for these human sequence peptides. Alternatively, it may be that none of these peptides contains the entire sequence required for binding and a longer sequence from this region is required. The peptides containing the two loop regions (81-107 and 25-40) also showed very little inhibition in the *trk* assay. However, a very interesting result was obtained for the peptide involving the N-terminal region of NGF. This peptide

showed 83.4% inhibition of NGF binding, at a concentration of 100μM. Initially it was thought that this result indicated good activity due to the N-terminal eight residues. At the same time, our collaborators at Parke Davis synthesised a number of peptides from this N-terminal region. These peptides and their results on the *trk* assay are summarised in Table 4.

	Amino Acid Sequence	*Trk* Assay % Inhibition (at 100μM)
1-7 (h)	**Ac.S.S.S.H.P.I.F.NH_2**	**19**
1-8 (m)	**S.S.T.H.P.V.F.H**	**0**
2-8 (h)	**Ac.S.S.H.P.I.F.H.NH_2**	**4**
2-16 (h)	**Ac.S.S.H.P.I.F.H.R.G.E.F.S.V.A.D.NH_2**	**5.1**
3-9 (h)	**Ac.S.H.P.I.F.H.R.NH_2**	**35**

Ac = acetylated peptide

Table 4

It was clear from these results that the N-terminal region of NGF did not appear to have great inhibitory effects on NGF binding to the *trk* receptor. This led us to investigate the ability of the peptide 100-114 to inhibit NGF binding to the *trk* receptor.

2.7 Investigation of the C-terminal Fragment 100-114 For Binding to *Trk*.

The sequence 100-114 NGF was synthesised (compound 16) using double couple HOCt coupling cycles. The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives. Bromotrimethylsilane (TMSBr) was used in the cleavage solution when the peptide was being cleaved from the resin. Thus the cysteine

protecting groups were cleaved at the same time as the peptide was being cleaved from the resin (see section 2.8.2 pg. 58). The hplc trace of the crude peptide indicated a very good synthesis (Fig.2.9a). The peptide was oxidised in 5% DMSO/TFA and purification by preparative hplc led to the desired compound in very pure form (Fig.2.9b). This peptide was the tested on then *trk* assay. The results obtained are summarised in Table 5.

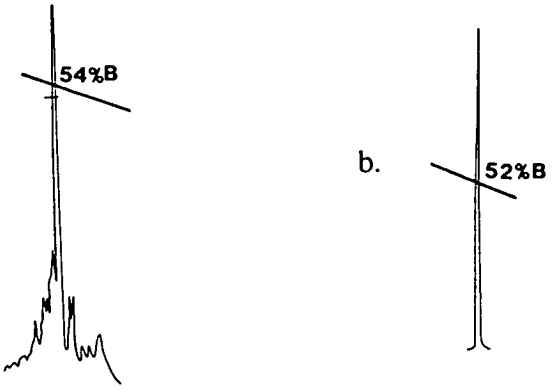


Fig.2.9 a. Hplc trace of crude 100-114 NGF.

b. Purified peptide after oxidation in 5% DMSO/TFA.

	Trk Assay
Amino Acid Sequence	**% Inhibition @ Xμ M**
100-114 NGF R.F.I.R.I.D.T.A.C.V.C.V.L.S.R	**100 @ 200μM**
(oxidised)	**100 @ 100μM**
	92 @ 50μM
	85 @ 10μM

Table 5

These results indicate that the peptide 100-114 NGF has an IC_{50} value, i.e. the concentration required for 50% inhibition of NGF binding, of less than 10μM. Thus it has been found that this C-terminal fragment appears to be important for NGF binding to both p75 and *trk* receptors. The next question concerned how this

peptide inhibited NGF binding. It was not known if the peptide bound to the receptors and thus inhibited NGF binding or if the peptide simply bound to NGF and somehow interfered with the receptor binding region of NGF. It was important to attempt to resolve this question in order to be able to predict whether or not this peptide could act as a potential agonist for NGF.

2.8 Investigation of the Method of Inhibition of *Trk* Binding by Test Peptides

In order to study the interaction of 100-114 NGF with the *trk* receptor it was decided that a tyrosine residue which could be radioiodinated should be attached to the N-terminus of 100-114 NGF. With the radiolabelled peptide, it should be possible to test whether the peptide bound to NGF or to the *trk* receptor.

2.8.1 Synthesis of Tyr^{99}.100-114 NGF

The peptide Tyr^{99}.100-114 NGF was synthesised manually using the apparatus shown in appendix 1. The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives. Cleavage of the peptide from the resin in 90%TFA/2%phenol/4%EDT/4%H_2O was monitored by analytical hplc. Cleavage of up to six hours did not appear to improve the hplc profile of the crude peptide (Fig.2.10). The crude material was found to be very hydrophobic. It was thought that this was probably due to the cysteine protecting groups. An attempt to cleave the S-*t*-butyl groups did not appear to improve the hplc profile either (Fig.2.11). It was clear that this peptide would not be easily purified and

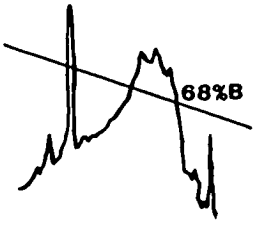


Fig.2.10 Hplc trace of crude peptide.

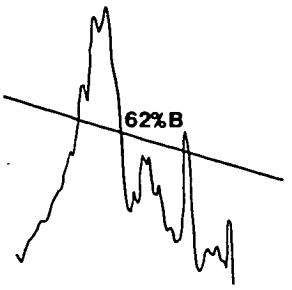


Fig.2.11 Crude peptide after cleavage of S-*t*-butyl groups.

therefore it was decided that the peptide should be synthesised again with incorporation of Acm protecting groups for the cysteine residues. It was predicted that this would improve the solubility of the crude peptide and thus hopefully aid the purification of the peptide. A second synthesis was carried out, this time on the peptide synthesiser, using double couple HOCt coupling cycles. After coupling of Arg^{100}, a small sample of resin was removed from the synthesis and the peptide was cleaved using the same conditions as before. An analytical hplc recorded on the crude material thus obtained showed a greatly improved profile than that of the crude material from the first synthesis (Fig.2.12a). Coupling of the tyrosine residue to the remaining resin-peptide was carried out by overnight sonication with the HOBt ester of Fmoc-tyrosine. The peptide was cleaved from the resin and an analytical hplc was recorded of the crude peptide (shown in Fig.2.12b). It was clear from comparison of Fig.2.12a and b that the coupling of the tyrosine residue to the peptide had in some way affected the cleavage of the peptide from the resin. Once again, purification would be difficult. It was decided that another synthesis would be carried out, this time using the compound *p*-hydroxyphenylacetic acid in replace of the tyrosine residue.

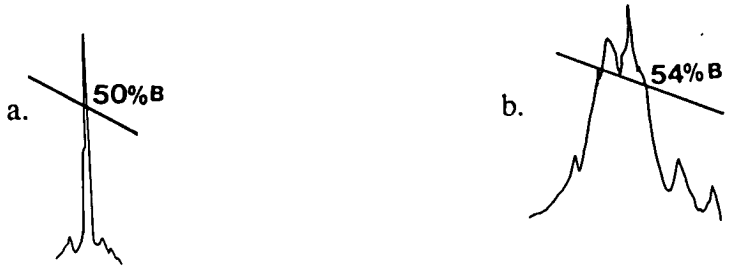


Fig.2.12 a. Hplc trace of crude 100-114 NGF before coupling Tyr^{99}.

b. Crude peptide after coupling Tyr^{99}.

2.8.2 Synthesis of *p*-Hydroxyphenylacetic acid-100-114 NGF

The synthesis of this peptide was first carried out using double couple HOCt coupling cycles and incorporating the cysteine residues as the Acm derivatives. Once again, a small amount of resin was removed from the synthesis after coupling of Arg^{100}. The peptide was cleaved from the resin and the hplc profile of the crude material obtained may be seen in Fig.2.13a. Once again the success of the synthesis of the sequence as far as Arg^{100} was evident. To the remaining resin-peptide *p-t*-butoxyphenylacetic acid (BUPA) was coupled by forming the symmetrical anhydride. The hplc profile of the crude material obtained on cleavage of this peptide from the resin may be seen in Fig.2.13b. By comparison of the hplc profiles in Fig.2.13a and b, it appeared that the coupling of *p*-hydroxyphenylacetic acid to the N-terminus of 100-114 was a success.

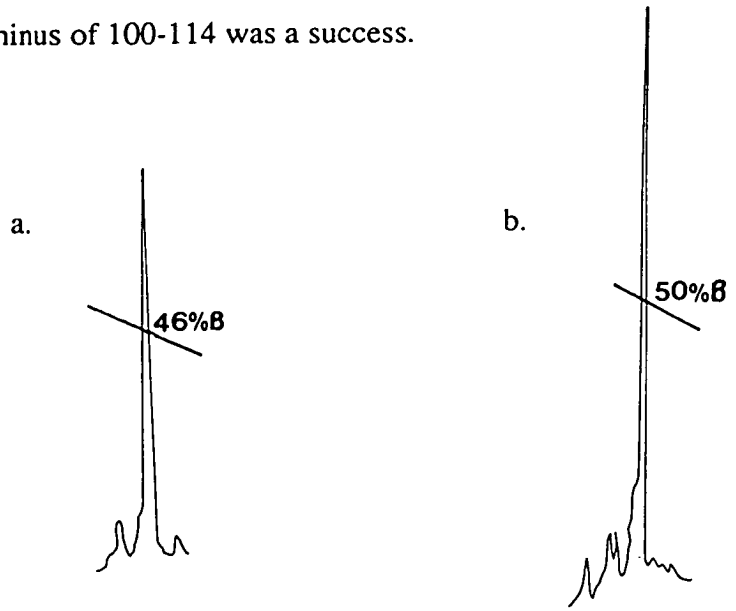


Fig. 2.13 a. Hplc trace of crude 100-114 NGF before coupling BUPA.

b. Crude 100-114NGF after coupling and deprotection of BUPA.

This peptide was purified by preparative hplc. However, an attempt to cleave the Acm protecting groups from the cysteine residues using silvertrifluoromethane

sulphonate resulted in very low yields (approximately 4%). It was apparent that in order to obtain enough material for radiolabelling and subsequent *trk* receptor interaction studies, an alternative method would have to be applied. By comparing the results of the various attempts to synthesise either this peptide or the N-terminal tyrosine peptide, it was decided that using *p*-hydroxyphenylacetic acid in place of tyrosine appeared to have improved the possibility of obtaining a good overall yield of the required peptide. However, the Acm protecting groups used in the synthesis of the *p*-hydroxyphenylacetic acid peptide considerably reduced the overall yields of the oxidised peptide to be obtained as cleavage of these protecting groups proved to be difficult. It was therefore decided that another synthesis of this peptide should be carried out, once again using *p*-hydroxyphenylacetic acid instead of tyrosine for the N-terminal residue but with incorporation of the cysteine residues as the S-*t*-butylsulphenyl derivatives. It was hoped that this cysteine protecting group would be more easily cleaved than the Acm protecting group. The synthesis of this peptide (compound 15) was carried out using double couple HOCt coupling cycles. The Fmoc deprotection profile shown in Fig.2.14, indicated that the synthesis went extremely well.

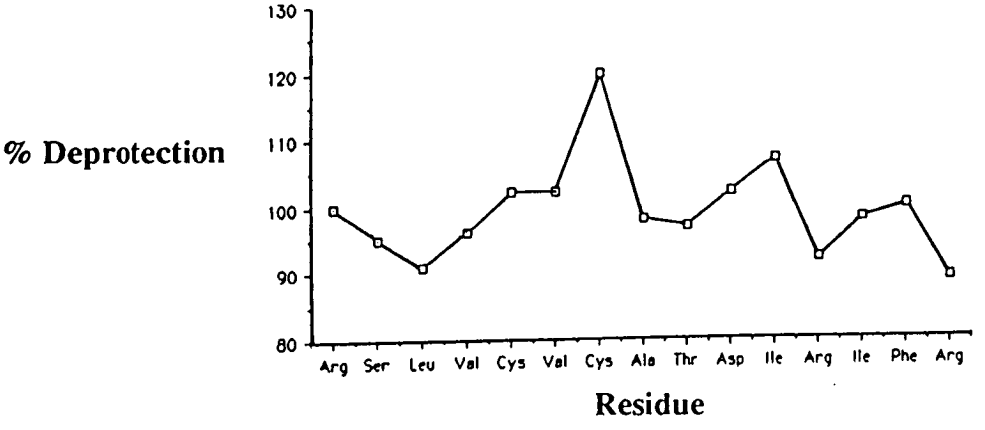


Fig.2.14 Chain assembly for compound 15.

After coupling of Arg^{100}, a small amount of resin-peptide was removed from the synthesis and cleaved in 90%TFA/2%phenol/4%EDT/4%H_2O. The hplc trace of the crude material obtained is shown in Fig.2.15. It was immediately apparent that the hplc profile did not reflect the efficient coupling achieved during the synthesis. It was considered that the nature of the bulky hydrophobic cysteine protecting groups contributed to the lack of resolution observed by analytical hplc. Alternative cleavage conditions were considered. It was decided that it may be advantageous to deprotect the cysteine residues at the same time as the peptide was being cleaved from the resin. This procedure would decrease the hydrophobicity of the crude peptide and thus aid purification. It was thought that the absence of the bulky S-*t*-butyl groups in the crude peptide may improve the resolution of the crude peptide on hplc.

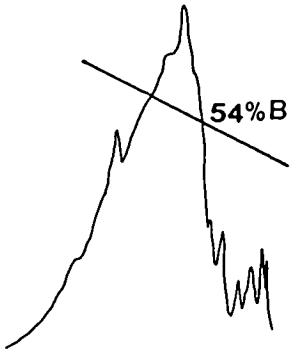


Fig. 2.15 Hplc trace of crude 100-114 NGF before coupling BUPA.

Deprotection of S-*t*-butyl groups at the same time as cleavage of the peptide from the resin has been reported to be achieved by using bromotrimethylsilane (TMSBr) with TFA[130]. A small amount of resin-peptide was cleaved using the reported cleavage solution of 7.5mlTFA/500µlEDT/100µlm-cresol/1.17ml thioanisole/1.3mlTMSBr. The reaction was carried out at 0^0C under N_2. The hplc profile of the crude material thus obtained is shown in Fig.2.16a. A positive Ellman's

test indicated that the cysteine protecting groups had been cleaved. The comparison of Fig.2.15 and Fig.2.16a indicated therefore that the presence of the bulky, hydrophobic S-*t*-butyl protecting groups in the crude peptide contributed mainly to the lack of resolution observed in Fig.2.15. To the remaining peptide-resin, BUPA was coupled by symmetrical anhydride activation. Cleavage of the resin-peptide using TMSBr in TFA was carried out. The analytical hplc profile of the crude peptide thus obtained may be seen in Fig.2.16b. There was clearly a marked improvement in the resolution of this material compared to that obtained by the usual TFA cleavage procedure used up to this point. Oxidation of the peptide in 5% DMSO/TFA and subsequent purification by preparative hplc yielded the required peptide (compound 15). The hplc trace of the final purified product is shown in Fig.2.16c. This peptide was then sent to Parke Davis where the radiolabelling and subsequent *trk* receptor interactions were examined.

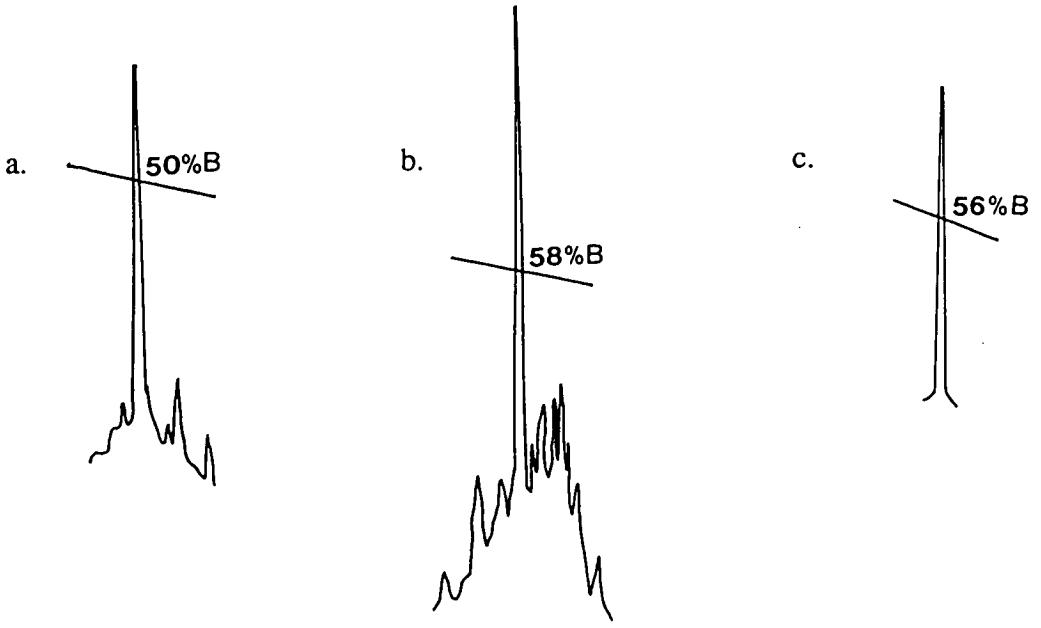


Fig.2.16 a. Crude 100-114 from cleavage in TFA with TMSBr.

b. Crude peptide after coupling BUPA, cleaved in TMSBr with TFA

c. Purified peptide (compound 15).

2.8.3 Cleavage of Tyr^{99}.100-114 with Bromotrimethylsilane in TFA.

The use of bromotrimethylsilane in the cleavage solution for compound 15 prompted an investigation into the use of this cleavage solution for the peptide previously synthesised with tyrosine coupled to the N-terminus of 100-114. As explained above, purification of this peptide was not pursued due to the poor resolution observed in the hplc trace for the crude material obtained from the TFA cleavage solution. However, it was unclear whether the lack of resolution was due to the S-*t*-butyl cysteine protecting groups or if the tyrosine residue in some way affected the cleavage of the peptide from the resin. In order to resolve this question, peptide-resin from the first synthesis of Tyr^{99}.100-114 NGF (with S-*t*-butyl protecting groups on the cysteine residues) was cleaved at 0^{0}C under N_2 using the reported TMSBr cleavage solution of 7.5ml TFA/500µl EDT/100µl m-cresol/1.17ml thioanisole/1.3mlTMSBr. The hplc of the crude peptide obtained is shown in Fig.2.17a. A positive Ellman's test indicated that deprotection of the cysteine residues had occurred. By comparing this hplc trace with Fig.2.11, it is clear that the lack of resolution seen in Fig.2.11 is due to the cysteine protecting groups and not the tyrosine residue. This peptide (compound 11) was purified (Fig.2.17b) and analysis by mass spectrometry and amino acid analysis verified it to be the *title* compound.

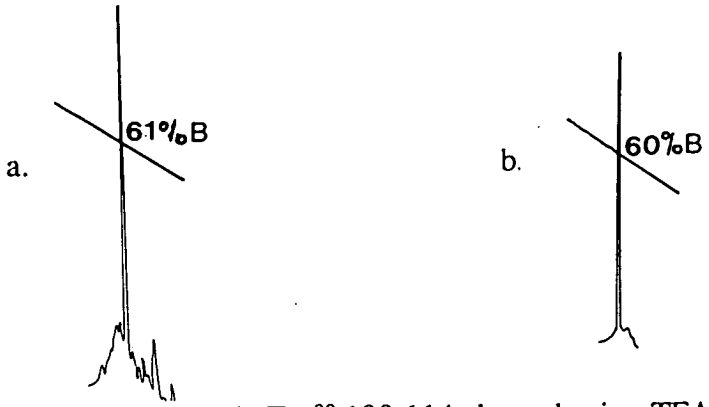


Fig.2.17 a. Hplc trace of crude Tyr^{99}.100-114 cleaved using TFA with TMSBr.

b. Purified peptide.

2.8.4 Results of *p*-Hydroxyphenylacetic acid-100-114 in the *Trk* Receptor Assay.

Before the peptide was radioiodinated it was tested in the *trk* assay for its ability to inhibit NGF binding. It was necessary to establish that the *p*-hydroxyphenylacetic acid group did not interfere with 100-114 inhibition of NGF binding. It was found that this peptide showed 100% inhibition of NGF binding at 100μM concentration indicating that the inhibition of NGF binding by 100-114 was not decreased by the *p*-hydroxyphenylacetic acid residue. Subsequent radiolabelling was carried out and it was confirmed that the peptide 100-114 did not bind to NGF in the *trk* assay[131]. Inhibition of NGF was achieved by the binding of the peptide to the *trk* receptor confirming that this peptide represents at least part of the binding site of NGF to the *trk* receptor. This indicates that 100-114 NGF may act as a potential agonist for NGF binding to the receptor.

2.9 Further NGF Receptor Binding Site Studies.

2.9.1 General

Previous work was carried out in this laboratory involving the investigation of a secondary binding site region in NGF for the p75 receptor[127]. The region 50-70 was linked to 95-120 by five glycine residues and an attempt to selectively form each of the disulphides Cys^{58}-Cys^{108} and Cys^{68}-Cys^{110} was made. This region 50-70 was chosen in particular because of the possibility of forming two of the three disulphide bonds which are present in NGF. However, attempts to form both disulphides were not successful. It was decided that the idea of synthesising a peptide containing the native disulphides should be reconsidered. It was thought that the function of the disulphides could possibly be to bring closer together the residues needed for

interaction with the receptors. Considering 100-114 NGF was found to be required for binding to both the p75 and the *trk* receptors, the discontinuous peptide linking together residues 50 to 70 and 100 to 114, was suggested. The carbon chain linker aminoundecanoic acid was to be used to join the two peptide fragments. The advantage of this linker over using a glycine chain linker was that it was hoped that aminoundecanoic acid would form a less rigid system and would allow the peptide fragments to come together to form the required disulphide bonds. It was decided that both the mouse and human sequences of this peptide would be synthesised as this would include four changes in the sequence 50 to 70 NGF (K^{50} to R, D^{60} to A, P^{61} to S, D^{65} to E). The peptides were to be tested on both assays.

2.9.2 Assembly of 50-70.aua.100-114 hNGF (compound 19).

The peptide was assembled on Wang resin using double couple HOCt cycles. Fmoc-aminoundecanoic acid was coupled manually as the HOBt ester, by overnight sonication. The cysteine protecting groups were chosen so as to allow selective formation of each of the disulphides. $Cys^{58,108}$ were incorporated as the S-*t*-butylsulphenyl derivatives. $Cys^{68,110}$ were incorporated as the phenylacetamidomethyl derivatives. The deprotection profile indicated that the synthesis went well, ending at approximately 60% coupling.

2.9.3 Deprotection and Cleavage from the Resin.

Cleavage of the peptide from the resin was carried out at 0^0C under N_2 in 7.5mlTFA/500µlEDT/100µlm-cresol/1.17mlthioanisole/1.3mlTMSBr. The hplc profile of the crude material obtained after three and five hours is shown in Fig.2.18a. Cleavage for longer than 5 hours was not considered necessary. Cleavage

under these conditions would also have resulted in the deprotection of the S-*t*-butyl protecting groups on $Cys^{58,108}$. Purification of the reduced peptide by preparative hplc was carried out. The hplc profile of the pure reduced material is shown in Fig.2.18b.

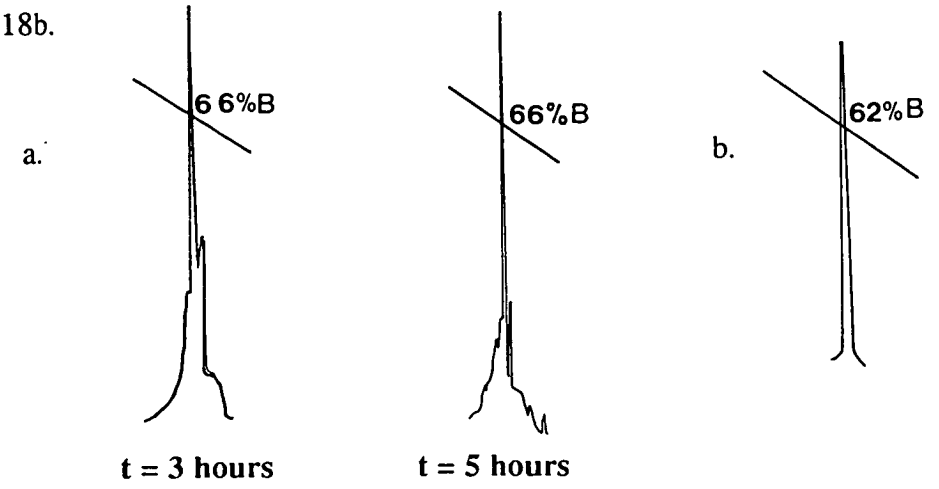


Fig. 2.18 a. Hplc trace of crude compound 19 cleaved in TFA with TMSBr.
b. Purified reduced peptide.

2.9.4 Analysis of the Reduced Peptide

The reduced form of compound 19 was analysed by TOF mass spectroscopy. A single peak at 4660.6 was observed (Fig.2.19). This molecular weight agreed with that expected for the peptide with $Cys^{58,108}$ deprotected and $Cys^{68,110}$ still protected by phenylacetamidomethyl groups. A positive Ellman's test also indicated the presence of free thiol groups which was in agreement with this analysis.

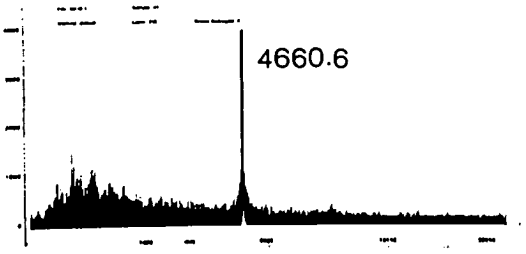


Fig.2.19 TOF mass spectrum of reduced compound 19.

2.9.5 Oxidation of Cys^{58}-Cys^{108} with DMSO.

Formation of the first disulphide bond was carried out by dissolving the reduced material in 5% DMSO/TFA. After one hour oxidation, the retention time of the peptide on analytical hplc had changed from 21 minutes for the reduced peptide to 19 minutes for the oxidised peptide (Fig.2.20a). Subsequent purification by preparative hplc was carried out. The hplc trace of the final purified material obtained is shown in Fig.2.20b. Analysis of this material proved it not to be the required peptide with just one disulphide formed. During the DMSO oxidation of Cys^{58} to Cys^{108} the phAcm protecting groups on Cys^{68} and Cys^{110} were unexpectedly cleaved also. This was evident from the mass spectroscopy results (Fig.2.21). A negative Ellman's test indicated that all four cysteine residues were oxidised. Selective oxidation of each of the disulphides was therefore not achieved and it could not be ascertained if the correct disulphides had formed.

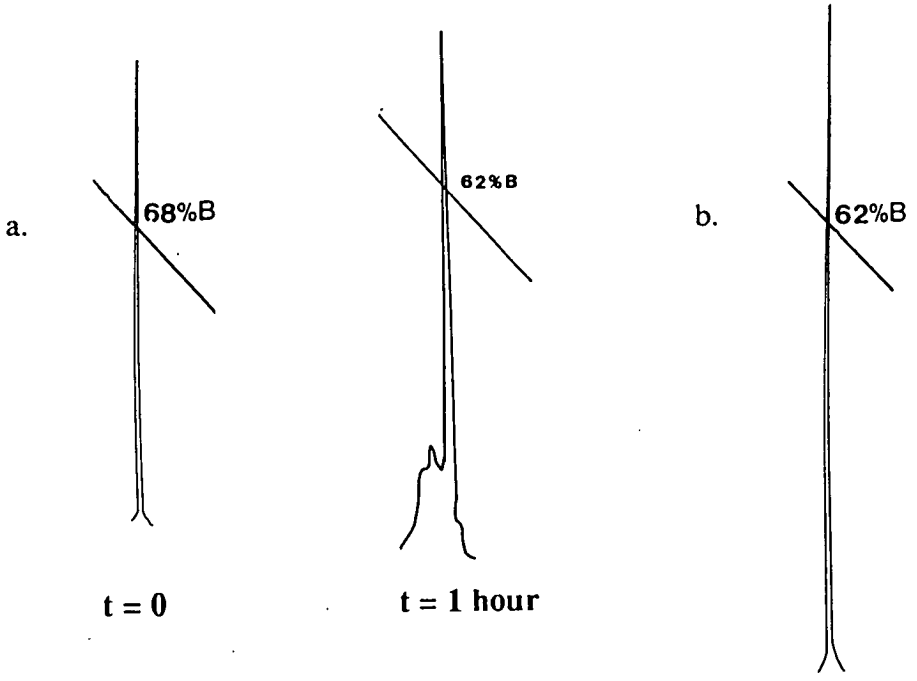


Fig. 2.20 a. Oxidation of Cys^{58}-Cys^{108} in 5% DMSO/TFA.

b. Purified peptide.

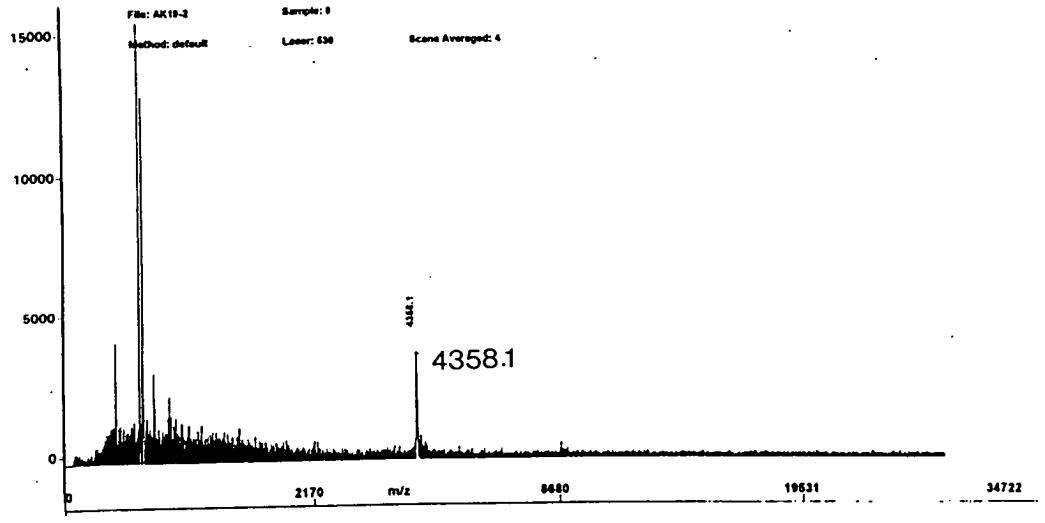


Fig.2.21 TOF mass spectrum of compound 19 oxidised in 5% DMSO/TFA

Cleavage of the phAcm protecting groups in this way was unexpected. However, Akaji *et al* have reported[132] that Acm protecting groups on cysteine may be cleaved to form cystine directly with diphenylsulphoxide, in the presence of methyltrichlorosilane. It is possible, therefore, that following the cleavage of this peptide in bromotrimethylsilane, a certain amount of silyl salts were still present with the peptide during the DMSO oxidation. Perhaps there was enough contamination for the catalysis of the cleavage of the phAcm groups, to take place. As the peptide had been purified by preparative hplc after cleavage from the resin, it was not to be expected that any silyl salts would still be present.

2.9.6 Glutathione Oxidation of Cys^{58}-Cys^{108}.

The conditions of the oxidation of Cys^{58}-Cys^{108} in DMSO/TFA proved to be too harsh for the stability of the phAcm protecting groups on $Cys^{68,110}$. Therefore, it was decided that more gentle oxidation conditions such as oxidation with glutathione should be applied. Glutathione is present *in vivo* in the reduced, GSH, and oxidised, GSSG, forms. It is thought to act as an electron acceptor-donor couple in disulphide exchange reactions. The formation of a disulphide using the reduced and oxidised forms of glutathione is represented in Fig.2.22.

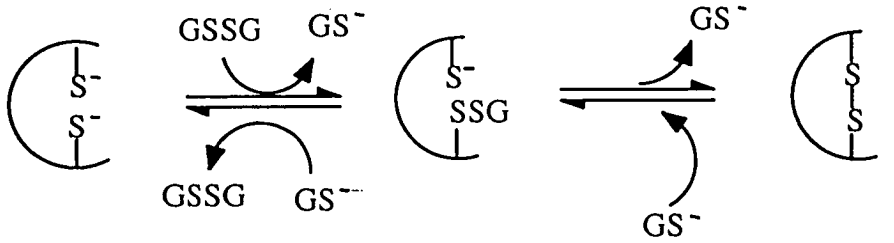


Fig.2.22 Formation of a disulphide with glutathione.

This is an equilibrium process which results in the formation of the most dynamically stable disulphide. Following the procedure described by Ramage *et al*[133] for the formation of a disulphide in the peptide endothelin using glutathione, the oxidation of Cys^{58}-Cys^{108} was carried out by dissolving the reduced peptide (10mg, $1x10^{-5}$M) in buffer at pH 8.0 containing 0.11mmole oxidised and 1.1mmole reduced glutathione. The reaction was stirred overnight at 4^0C and the oxidation was monitored by analytical hplc (Fig.2.23) which indicated that the reaction appeared to go to completion in 24 hours. Purification by preparative hplc gave the pure, disulphide linked Cys^{58}-Cys^{108} peptide (2mg). A negative Ellman's test verified that the disulphide had been formed. Analysis by TOF mass spectroscopy verified that the phAcm protecting groups on $Cys^{68,110}$ were unaffected by the oxidation. The

glutathione oxidation was carried out on a remaining 45mg of reduced peptide. This gave 12mg in total of Cys^{58}-Cys^{108} disulphide formed peptide.

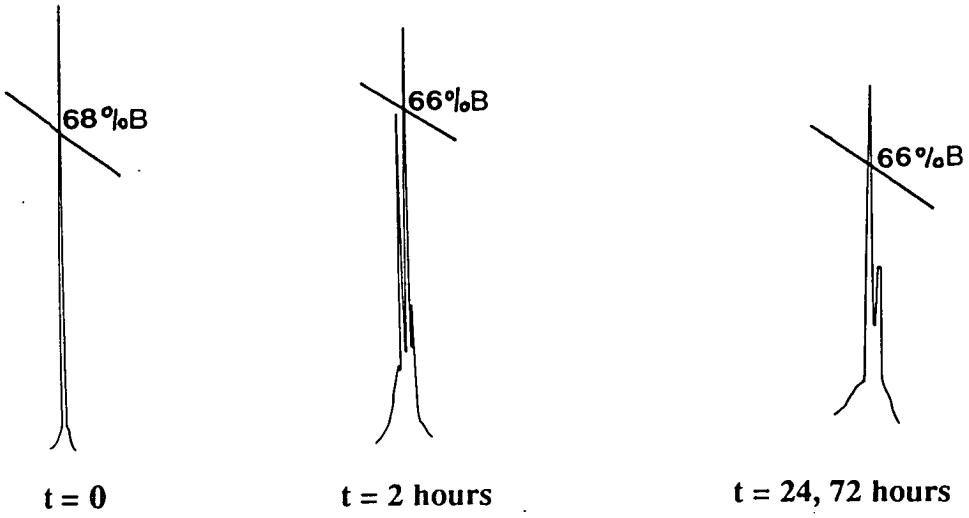


Fig.2.23 Glutathione oxidation of Cys^{58}-Cys^{108} monitored by analytical hplc.

2.9.7 Attempted Formation of Cys^{68}-Cys^{110} Disulphide.

The protecting group used for $Cys^{68,110}$, phenylacetamidomethyl, has been reported to be cleaved enzymatically by penicillin acylase[109]. This procedure was tried on a small scale. However, on addition of the resin bound enzyme to the peptide dissolved in buffer, the peptide was adsorbed by the resin and therefore it was not possible to follow the cleavage of the protecting groups by analytical hplc. It was not possible to ascertain if deprotection had taken place. Cleavage of the phAcm groups had earlier been unexpectedly achieved by DMSO/TFA. Therefore it was decided that this procedure could be tried again. The cleavage and oxidation in 5% DMSO/TFA was followed by analytical hplc. Fig.2.24 shows the change in hplc profile observed over a two hour period.

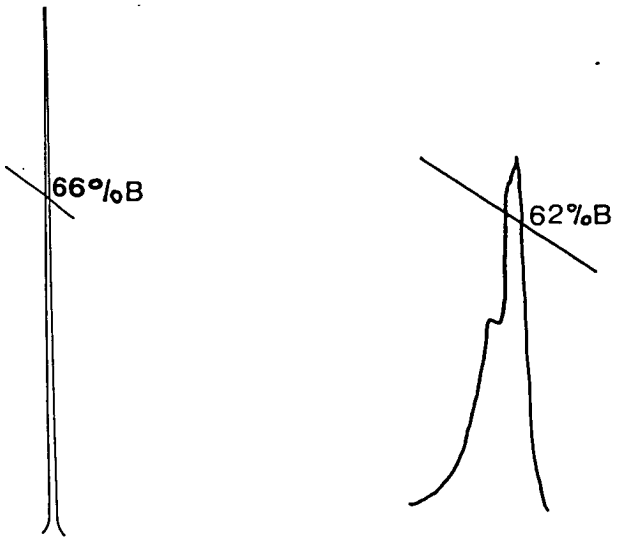


Fig.2.24 Attempted cleavage of phAcm groups and formation of Cys^{68}-Cys^{110} disulphide with 5% DMSO/TFA.

It was clear that a reaction had occurred but the product gave a rather broad peak compared to the pure starting material. The nature of the broad peak indicated that dimerisation had possibly occurred. Given the small amount of material that was left to work with at this stage it was decided that the peptide with one disulphide formed should be sent to Parke Davis for testing and no further attempt at forming the second disulphide was made.

2.9.8 Synthesis of 50-70.aua.100-114 mNGF (compound 21).

The synthesis and purification of this peptide was carried out exactly as for the human sequence peptide (compound 19). As with compound 19, the DMSO oxidation of Cys^{58} to Cys^{108} resulted in cleavage of the phAcm protecting groups from $Cys^{68,110}$ and both disulphides were formed simultaneously. Purification was achieved by preparative hplc.

2.9.9 Results of Human and Mouse 50-70.aua.100-114 NGF in the p75 Assay.

The following results were obtained when these peptides were tested in the p75 assay:

Amino Acid Sequence	p75 assay % Inhibition @ 10μM
50-70.aua.100-114 hNGF (oxid)	**10**
50-70.aua.100-114 mNGF (oxid)	**72**
50-70.aua.100-114 hNGF ($Cys^{58,108}$ oxidised, $Cys^{68,110}$ phAcm protected)	**10**
100-114 NGF (oxid)	**34**

Table 6

The results in Table 6 show that a possible secondary binding site may be present in the mouse sequence 50-70 NGF. This peptide showed 72% inhibition of NGF at 10μM compared to 34% inhibition for 100-114 NGF. The human sequence did not indicate any such inhibition. However, it is not clear whether this may be due to incorrect disulphide formation for this peptide. Perhaps the correct disulphides were formed when the DMSO oxidation was carried out on the mouse sequence peptide. The peptide with just one disulphide formed shows a decrease in binding compared to 100-114, also. It may be that formation of the second correct disulphide would recover the binding observed for the mouse sequence. The following photograph illustrates how these two binding regions relate to the 3-D structure of the NGF protein. It is possible that the function of the disulphides is to bring closer together the residues required for binding to the receptor.

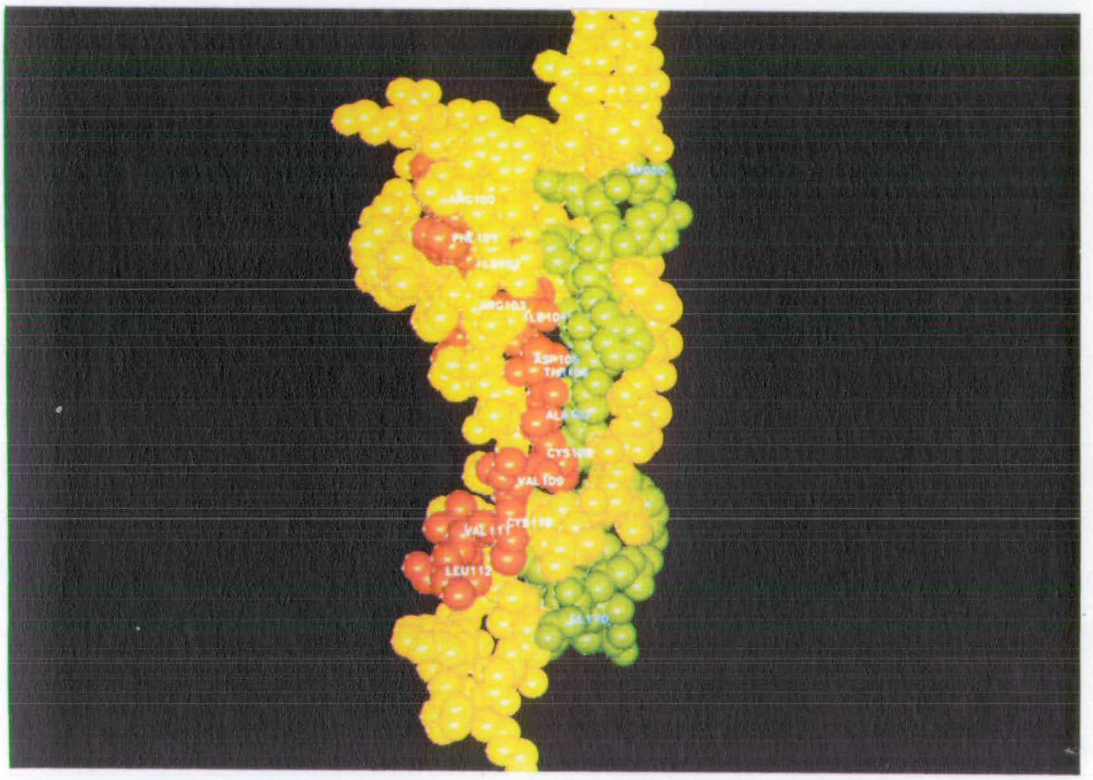


Plate 2

The protein mNGF showing the region 100-112 (red) and 50-70 (green)

2.10 Investigation of the Importance of the N-terminal Region of NGF for p75 Receptor Interaction.

It was mentioned earlier that the N-terminal region of NGF was investigated for interaction with the *trk* receptor. The peptide 1-8.aua.100-114 NGF was synthesised and subsequent testing indicated that the N-terminal region of NGF did not interact with the *trk* receptor. The same peptide was also tested in the p75 assay to see if enhancement of binding by the N-terminal region of NGF could be achieved for this receptor. The following results were obtained:

Amino Acid Sequence	**p75 Assay % Inhibition @ 10μM**
1-8.aua.100-114 hNGF (oxidised)	**63**
1-8.aua.100-114 mNGF (oxidised)	**34**
100-114 (oxidised)	**36**

Table 7

From the results in Table 7 it was clear that the N-terminal eight residues in hNGF appeared to enhance the binding of 100-114 to the p75 receptor. The mouse sequence of this peptide was also synthesised and tested. There are two changes in the N-terminus of NGF between the human (h) and mouse (m) sequences, Ser^3 (h) to Thr (m) and Ile^6 (h) to Val (m). Interestingly, the result obtained for the mouse sequence indicated that the N-terminal region is not important for NGF binding to the p75 receptor, as reported by Mobley[124].

2.11 Further Investigation of 100-114 NGF Binding to *Trk*.

Previous studies had identified 100-114 NGF as the minimum sequence from the C-terminal region of NGF, required for binding to the p75 receptor[126,127]. It was found here that this peptide bound to the *trk* receptor also but it was not known if this sequence represented the optimum binding sequence from the C-terminal region. It was therefore decided that extended sequences from the C-terminal region would be synthesised in an effort to enhance binding to the *trk* receptor. In addition, investigations of the functional importance of the artificial disulphide Cys^{108}-Cys^{110} for *trk* binding, was studied. The reduced form of 100-114 NGF was synthesised, as well as a peptide which replaced Cys-Val-Cys with a proposed β-turn mimic cis-1-amino-4-carboxy-cyclopentane[134] (compound 20). There is a precedent for making this substitution; work carried out in this laboratory showed that replacing Cys-Val-Cys in a peptide sequence from gp120, GC1[135], by this proposed β-turn mimic, resulted in a peptide which retained the full biological activity of GC1[136]. This was an interesting observation as Cys-Val-Cys has been shown to fulfil the requirements of an ideal γ-turn conformation[137]. Making this substitution in 100-114, it was hoped would give some indication of the necessity of the disulphide for interaction of 100-114, with the receptors. The peptides synthesised are listed in Table 7.

	Amino Acid Sequence	Cmpd. no
100-114 NGF	**R.F.I.R.I.D.T.A.C.V.C.V.L.S.R (reduced)**	**16**
111-120 mNGF	**V.L.S.R.K.A.T.R.R.G**	**17**
95-120 mNGF	**K^{95}.Q.A.A.W.R.F.I.R.I.D.T.A.C.V.C.V. L.S.R.K.A.T.R.R.G^{120} (reduced)**	**18**
95-120 mNGF	**Dimer through cysteine residues**	**18/2**
100-107.Turn.111-114	**R.F.I.R.I.D.T.A.Turn.V.L.S.R**	**20**

(Turn = proposed β-turn mimic)

Table 7

Thus, extension of the fragment 100-114 NGF as far as residue 95 on the N-terminus and 120 on the C-terminus was achieved with the peptide 95-120 NGF. This peptide was first synthesised in the reduced form. An attempt at air oxidation of Cys^{108}-Cys^{110} in ammonium acetate (pH 8.0) resulted in dimerisation of the peptide instead of the expected intramolecular disulphide formation. It is believed that NGF may bind to the receptors as a dimeric form so it was decided that this dimer should be tested for enhanced binding, in both receptor assays.

The C-terminal residues 112-120 NGF are poorly defined in the crystal structure of NGF. This is possibly due to the flexibility and solvent accessibility of this region. Drinkwater *et al*[117] reported that the seven carboxy-terminal amino acids in mNGF, 112-118, are required for biological activity. It was therefore decided that a peptide containing the C-terminal residues should be synthesised and tested in the *trk* assay. This peptide 111-120 was chosen so as to avoid the cysteine residues and related purification problems, and yet to include the seven carboxy-terminal residues. It was thought that perhaps the flexibility of this region may indicate a receptor binding region. In addition, the peptide 50-70.aua.100-114 synthesised initially for testing on the p75 assay was also tested on the *trk* assay.

The peptides were tested on both receptor assays. The results obtained are summarised in Table 8 below.

Amino Acid Sequence	***Trk* Assay % Inhibition @ 100μM**	**p75 Assay %Inhibition @ 10μM**
100-114 NGF (reduced)	**0**	**38**
111-120 mNGF	**0**	**-**
95-120 mNGF (reduced)	**24**	**-**
95-120 mNGF (dimer through cysteine residues)	**31**	**77 (IC_{50} = 0.32μM)**
100-107.Turn.111-114	**100**	**-17**
50-70.aua.100-114 hNGF	**100**	**10**

(Turn = proposed β-turn mimic)

Table 8

As may be seen from the results obtained on the *trk* assay, extending the sequence 100-114 NGF appeared to result in a depletion of receptor binding. Interestingly, the reduced form of 100-114 did not show any inhibition of NGF binding to *trk*. This is consistent with the result that replacement of Cys-Val-Cys with the proposed β-turn mimic retained the inhibition previously observed for 100-114 in the oxidised form. This indicates that the cysteine residues are not important for binding of 100-114 NGF to *trk* but instead, the structural constraint implied by the disulphide is what is important. Replacement of this structural constraint by the proposed β-turn mimic, retained the binding ability of the peptide. On the contrary, replacement of the disulphide appears to have completely destroyed the ability of the

peptide to bind to the p75 receptor. This indicates that there is a possible interaction between the cysteine residues and the p75 receptor. On removal of these residues, binding to the receptor is destroyed.

While the peptide 95-120 did not appear to inhibit NGF binding to the *trk* receptor, a very interesting result was obtained for the dimer of this peptide, in the p75 assay. This dimer peptide gave an IC_{50} value of 0.32μM in the p75 assay. This result represents the best result obtained to date for interaction of an NGF fragment with the p75 receptor. It is possible that the high density of positively charged residues required for binding to the p75 receptor may never be obtained from a monomer fragment of NGF. If the dimer succeeds in presenting a higher density of charged residues it perhaps better mimics the binding of NGF which possibly binds in the dimeric form.

2.12 Summary and Conclusions.

The initial aim of this study was to identify regions in the NGF protein which appeared to be involved in receptor-interaction. Interaction with both NGF receptors, p75 and *trk* was studied. Previous work carried out in this laboratory had led to the isolation of a C-terminal fragment of NGF required for binding to the p75 receptor[126,127]. This fragment was 100-114 NGF.

$$R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}$$

Substitutions of various residues in the sequence in previous work and in this study did not lead to a reduction in the IC_{50} value for this sequence.

A secondary binding site in NGF for the p75 receptor was investigated. The lysine residues Lys^{95}, Lys^{32} and Lys^{34} appeared to enhance binding to the p75 receptor when combined with the fragment 100-114 NGF. Computer analysis clearly shows the close proximity of these lysine residues with Arg^{100} and Arg^{103} in the

native protein. Formation of the disulphide between Cys^{108} and Cys^{110} could possibly also bring Arg^{114} in close proximity of this region. Together these basic residues may form a complex which interacts with the highly acidic p75 receptor. The lysine residues alone did not appear to have any binding interaction with the receptor indicating that the combination of both regions is required for higher affinity binding to p75.

Replacing the disulphide in 100-114 NGF by a proposed β-turn mimic, completely destroyed the binding of this peptide to the p75 receptor indicating that the cysteine residues may also play an active part by interacting directly with the cysteine-rich p75 receptor. Alternatively, the change in conformation implied by this β-turn mimic may have sufficiently altered the spatial orientation of the amino acids contained in the sequence which make direct contact with the receptor.

Other regions of NGF investigated for secondary binding sites to p75 included the N-terminal region and the region 50-70 NGF. The N-terminus of hNGF appeared to give a 2-fold increase in the binding affinity to p75. This region was not clearly defined in the crystal structure, possibly due to its flexibility. This may therefore indicate a region flexible enough to interact with the receptor. On the contrary, the region 50-70 NGF is well characterised in the crystal structure. This region is involved in two of the three disulphides formed in native NGF. Following the hypothesis that the function of the disulphides is to bring closer together the residues required for direct receptor interaction, the peptide 50-70.aua.100-114 was synthesised and an attempt to form two of the native disulphides was made. It was not clear which way the disulphides formed in the final product but certainly for the mouse sequence peptide a two-fold increase in percentage inhibition of NGF to the p75 receptor was noted in comparison to that obtained for the peptide 100-114 NGF. This increase was not found for the human sequence peptide indicating that incorrect disulphides possibly had formed in this peptide, or that the amino acid changes

between the human and mouse sequences for the 50-70 region are very significant. Further investigation would be required in order to clarify this point.

Interestingly, it was found that the lowest IC_{50} value obtained for peptide interaction with the p75 receptor was with a dimeric form of the C-terminal fragment 95-120 NGF. This peptide was dimerised through the two cysteine residues $Cys^{108,110}$ and gave an IC_{50} value of 0.32μM which represents a tenfold increase compared to that obtained for the monomer of 95-120 NGF. Possibly the increase in charge density obtained with the dimer is the reason for greater binding to the acidic p75 receptor. It is thought that NGF binds as a dimer to the receptor so it is possible that a dimeric form of the active site better mimics the binding of NGF to the receptor and thereby shows greater inhibition of NGF binding.

The investigation of NGF interaction with the *trk* receptor highlighted the importance of the C-terminal fragment 100-114 for *trk* receptor binding also. This surprising result prompted the question of how this peptide succeeded in inhibiting NGF binding to both receptors. Attachment of a radiolabelled residue to the N-terminus of the 100-114 NGF sequence allowed receptor-interaction of the peptide to be studied. It was thought possible that the peptide could in some way interact with NGF and alter the conformation of the binding site and thereby inhibit binding of NGF. However, it was found that the radiolabelled peptide did not bind to NGF in the *trk* assay. Binding to the *trk* receptor was observed. This indicates that the sequence 100-114 NGF represents at least part of the binding site of NGF for each receptor and therefore may possibly be developed as an agonist for NGF.

Although the sequence 100-114 NGF was found to bind to both NGF receptors, a difference in the functional importance of structure of the sequence for binding to both receptors was observed. The proposed β-turn mimic substituted for Cys.Val.Cys, completely destroyed binding of the sequence to p75 but not to *trk*. This implied that the cysteine residues may be involved in direct interaction with the

cysteine-rich p75 receptor. Substitution of these cysteine residues may therefore severely inhibit the direct interaction of the peptide with the p75 receptor. This substitution did not affect interaction with the *trk* receptor indicating that the relative orientation of the residues when the 1,3 disulphide is replaced by the proposed β-turn mimic, is sufficiently similar to be able to uphold the required conformation for binding to the *trk* receptor. This hypothesis is consistent with the finding that the reduced form of 100-114 NGF (which obviously lacks the structure implied by the disulphide) does not bind to the *trk* receptor but appears to bind with similar affinity as the oxidised form to the p75 receptor. In addition, the dimeric form of 95-120 shows a ten-fold increase in binding to the p75 receptor but a decrease in binding compared to the oxidised 100-114 NGF peptide binding to the *trk* receptor, is observed. This dimeric form of 95-120 would not have the same structural constraints as the oxidised form of 100-114, further implicating the importance of relative spatial orientation of the residues for *trk* receptor interaction.

Attempts to further enhance the binding of 100-114 NGF to *trk* by extending the sequence as far as Lys^{95} on the N-terminus and Gly^{120} on the C-terminus did not produce any significant results. Once again, this could possibly be due to the linear form of these peptides. Perhaps even further enhancement of binding may be achieved by imposing a structural constraint in the sequence 95-120 such as formation of the disulphide Cys^{108}-Cys^{110} or by replacement of the disulphide with the above mentioned β-turn mimic.

Preliminary results suggest that combination of the human sequence 50-70 NGF with 100-114 shows better inhibition of binding to the *trk* receptor than to the p75 receptor. Unfortunately, mouse sequence 50-70.aua.100-114 was not tested in the *trk* assay so a direct comparison of the relative importance of the mouse and human sequence of 50-70 NGF for each receptor cannot be made. .

The results presented here indicate for the first time a region of NGF which is involved in binding to both p75 and *trk* receptors for NGF. A possible secondary binding site for p75 has also been identified. Different structural constraints implied in the C-terminal active fragment have highlighted separate requirements for binding of this region to each receptor. The translation of these results into the construction of compounds with agonistic activity for the neurotrophin NGF is a possibility. It is hoped that such compounds could then be developed for pharmacological interest in the treatment of neurological disease. A complete list of results obtained for all the peptides synthesised in this study and in previous studies[126,127], is given in Appendix 2.

CHAPTER THREE : EXPERIMENTAL

3.1 NOTES

All Fmoc-amino acids were purchased from either Novabiochem, Bachem or Raylo with exception to Fmoc-Cys(phAcm) which was synthesised in this laboratory by Angus Brown[138] and Kirstie Urquhart[134] and Fmoc-aminoundecanoic acid which was synthesised by Jenny Henry[139]. The β-turn mimic Fmoc-1-amino-4-carboxy-cyclopentane described for compound (20) was synthesised by Kirstie Urquhart[134]. Fmoc-*t*-butoxyphenylacetic acid used in compound (15) was synthesised by David Pallin[140]. The peptides were synthesised on an ABI 430A peptide synthesiser. Peptide synthesis grade dimethylformamide (DMF) and 1,4-dioxan were obtained from Rathburn Chemicals. Peptide synthesis grade trifluoroacetic acid (TFA) was obtained from Applied Biosystems. Ultraviolet spectra were recorded on a Varian Cary 210 double beam spectrophotometer. All other instrumentation used is described in the relevant sections in the text.

3.2 SOLID PHASE PEPTIDE SYNTHESIS

3.2.1 Coupling of the First Amino Acid to the *p*-Alkoxy Benzyl Alcohol (Wang) Resin.

Fmoc-L-amino acid (1.6mmoles) was dissolved in DMF (5mls). To this solution diisopropylcarbodiimide (0.8 mmoles) was added and the solution was allowed to stir for 15 minutes. The symmetrical anhydride of the Fmoc amino acid thus formed was then added to 0.5g of Wang resin (0.8 mmole/g), pre-swollen in DMF (5ml). 4-dimethylaminopyridine (DMAP, 10mg) was also added and the mixture was sonicated for 2 hours. The resin was then filtered and washed well with DMF, followed by washing with 1,4-dioxan.

3.2.2 Determination of the Loading of Amino Acid on the Wang Resin.

Approximately 10mg of resin loaded with Fmoc-amino acid was washed with diethyl ether. After filtration the resin was allowed to air-dry under suction for approximately 30 minutes. To an accurately weighed sample (approximately 3mg) of resin-amino acid, a solution of 20% piperidine/DMF (10ml) was added. This was sonicated for 30 minutes. The U.V. absorbance of the piperidine-Fmoc adduct formed was detected at 302nm. The loading of the resin was calculated by a computer programme using the Beer-Lambert law after calibration with known quantities of Fmoc-Gly-OH. The procedure was carried out in duplicate and an average value of the product resin functionality was used to determine the weight of resin-amino acid required to carry out the synthesis on a 0.25mmole scale.

3.2.3 Peptide Assembly on Wang Resin.

The weight of resin-amino acid required to carry out the synthesis on a 0.25mmole scale was determined as outlined in section 3.2.2. The correct weight of resin loaded with the first amino acid was then placed in a clean reaction vessel and the synthesis was carried out using a 430A Applied Biosystems peptide synthesiser. The Fmoc-L-amino acids (0.5mmole) were weighed into cartridges and loaded into the machine. The amino acids were activated for coupling using one or more of the three methods listed below.

3.2.3.1 Formation of Symmetrical Anhydride

Fmoc-amino acid (1mmole) was dissolved in DMF (4ml) and a solution of 0.25M DIC in 1,4-dioxan (2ml) was added. A further 2ml of 1,4-dioxan was added and the solution was allowed to stand for 10 minutes in the activator vessel before being added to the reaction vessel. The standard coupling time allowed was 30 minutes. If extended coupling was used the coupling time allowed was one hour.

3.2.3.2 Formation of HOBt Ester

Fmoc-amino acid (0.5mmole) was dissolved in a solution of 0.25M HOBt in DMF (2ml). This was diluted with DMF (2ml) and transferred to the activator vessel. A solution of 0.25M DIC in 1,4-dioxan (2ml) was added along with a further 2ml of 1,4-dioxan. This was allowed to stand for 15 minutes before being transferred to the reaction vessel. The standard coupling time allowed was 30 minutes. If extended coupling was used the coupling time allowed was 90 minutes.

3.2.3.3 Formation of HOCt Ester.

Two methods were used for the formation of the HOCt ester. The first method involved weighing HOCt (0.5mmole) into cartridges similar to those used for the amino acids. These were then loaded into the machine in the same way as the amino acid cartridges. DMF (2ml) was used to dissolve the HOCt. The Fmoc-amino acid (0.5mmole) was dissolved in DMF (2ml) also and both were transferred to the activator vessel. A solution of 0.25M DIC in 1,4-dioxan (2ml) was added along with a further 2ml of 1,4-dioxan. The solution was allowed to stand for 15 minutes before being transferred to the reaction vessel.

The second method of forming the HOCt ester was exactly the same as for the HOBt ester. The coupling times allowed were also the same as for the HOBt ester.

3.2.3.4 Capping the Resin

After coupling the Fmoc-amino acid the N-α protected resin was washed four times with 50:50 DMF/1,4-dioxan (10mls). Two different capping solutions have been used:

Capping solution A: 0.5M pyridine, 0.5M acetic anhydride in DMF;

Capping solution B: 0.5M acetic anhydride, 0.125M DIEA and 0.2% HOBt in DMF;

In either case the solution (10ml) was transferred to the reaction vessel and the resin was capped for 10 minutes. Finally the resin was washed five times with 50:50 DMF/1,4-dioxan (10mls). For most of the peptides described herein capping solution B was used unless otherwise stated.

3.2.3.5 Deprotection of Fmoc from the Coupled Amino Acid.

A solution of 20% piperidine/DMF (10mls) was transferred to the reaction vessel and deprotection of Fmoc took place over 3 minutes. The resin was washed with 50:50 DMF/1,4-dioxan and a second batch of deprotection solution was added to the reaction vessel. Deprotection for 1 minute was allowed. A sample of each deprotection mixture was analysed by an ABI 758A absorbance detector, with on-line integration via a spectra-physics SP4400 ChromJet integrator/printer. U.V. detection was carried out at 302nm. By comparing the integration peaks for the Fmoc deprotection of each coupled amino acid, the percentage of amino acid coupled could be determined. Finally the resin was washed five times with 50:50 DMF/1,4-dioxan.

3.2.4 Acidolytic Cleavage of the Peptide from the Resin

After peptide assembly the resin-peptide was washed with diethyl ether (50ml) or DCM (50ml) and filtered. The resin-peptide was allowed to air dry under suction for at least one hour before cleavage. The standard cleavage solutions used were as follows:

Cleavage solution A:

0.75g phenol, 0.25ml EDT, 0.5ml thioanisole, 0.5ml H_2O, 8ml TFA

Cleavage solution B:

400µl EDT, 400µl H_2O, 200mg phenol, 9.0ml TFA

Cleavage solution C (carried out under N_2 at 0^oC):

7.5ml TFA, 500µl EDT, 100µl m-cresol, 1.17ml thioanisole, 1.3ml TMSBr

The cleavage mixture used was made up and then added to the dry peptide-resin and stirred using a magnetic stirrer. The cleavage solution used and the cleavage time allowed were determined by carrying out small scale trial cleavages.

Analysis was carried out by analytical hplc of aliquots of the reaction mixture at regular intervals. The conditions used for each individual peptide is described in the text. After the allowed cleavage time the resin was filtered and the cleavage solution was evaporated under high vacuum to yield an oily mixture. The peptide was then triturated with diethyl ether. The diethyl ether was filtered off and the peptide was dissolved in an acetic acid and water solution and freeze dried overnight.

3.2.5 Purification of Peptides by Reversed Phase Hplc

The crude peptide obtained was first analysed by analytical hplc. The hplc machines used were:

1) ABI model 151A separation system;
2) Gilson 305 controller pump with ABI 785A programmable absorbance detector.

The solvent system used to elute the peptides was:

Solvent A: H_20/0.1% TFA;

Solvent B: CH_3CN/0.1% TFA.

The columns used are described below,

Aquapore RP300 reversed phase silica (300 A pore size, 7µm spherical silica)

column 1: 220 x 4.6mm C_{18}

column 2: 100 x 4.6mm C_{18}

column 3: 200 x 4.6mm C_8

Vydac reversed phase silica column (300 A pore size, 5µm spherical silica)

column 4: Vydac 218TP54 C_{18}

column 5: Vydac 208TP54 C_8

For each peptide the analytical gradient used was 5% solvent B to 95% solvent B over 30 minutes at a flow rate of 1ml/minute. The eluent was monitored at

214nm. Unless otherwise stated in the text the samples were injected into a 2.0ml loop.

The peptides were purified by preparative hplc. One of the following columns were used:

column 6: Aquapore RP300 C_{18} 110 x 10mm

column 7: Aquapore RP300 C_{18} 200 x 10mm

column 8: Vydac 218TP1022 200 x 25mm

column 9: Aquapore RP300 C_8 250 x 10mm

column 10: Aquapore RP300 C_8 110 x 10mm

The particular column used depended on the quantity of peptide to be purified and on the peptide length. The solvent gradient was chosen to give best resolution of the required peptide from the deletion sequences also present in the crude material. The conditions used for the purification of each peptide are stated in the text. The fractions collected from each prep run were then combined and freeze-dried. The purity of the peptide was checked by recording an analytical hplc under the same conditions as used for the crude peptide.

3.2.6 Analysis of Purified Peptides

The peptide obtained by the procedure outlined in section 3.2.5 was analysed by amino acid analysis, fast atom bombardment (FAB) and time of flight (TOF) mass spectroscopy. For amino acid analysis, the peptide was hydrolysed in HCl for 24 hours at 110^{0}C. Analysis was carried out on an LKB 4151 amino acid analyser. FAB mass spectra were recorded on a Kratos MS50TC machine. TOF mass spectra were recorded on a Perspective Biosystems LaserTec Benchtop II laser desorption (337nm, 400μJ N_2 laser, 2800V) time of flight system from Vestec mass spectrometry products.

3.2.6.1 Ellman Assay for Thiols

The Ellman assay detects the presence of free thiols in a protein or peptide. Approximately 0.1mg of peptide is dissolved in a solution (2ml) of 6M GdmCl with 0.1M phosphate buffer (pH 7.3) and 1mM EDTA. A solution (100µl) of 3mM dithionitrobenzoic acid (in 0.1M phosphate buffer, pH 7.3) is added. A positive test indicating the presence of free thiols is indicated by the presence of a yellow colour compared to a standard solution. A negative test gives a clear solution.

3.3 EXPERIMENTAL

Synthesis of 100-114 hNGF with Orn replacing Arg^{100} (2)

O^{100}F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}

Fmoc-Arg-resin (570mg, 0.25mmole/g) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled using triple coupling cycles (symmetrical anhydride coupling followed by double HOBt coupling) for each amino acid. Standard coupling times were used except for the HOBt coupling of Ala-Thr, Asp-Ile and Ile-Phe, where extended coupling times were used. Capping solution A was used (section 3.2.3.4). The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives.

The peptide was cleaved from the resin for 3 hours using cleavage solution A (section 3.2.4). The cysteine protecting groups were cleaved by dissolving the peptide (50mg) in a solution of 95% TFE/H_2O (10mls). Tributylphosphine (100µl) was added and the solution was allowed to stir for 3 hours. The TFE solution was then evaporated *in vacuo*. The peptide was triturated with diethyl ether and dried *in vacuo*. An analytical hplc was recorded (column 3, 100µl loop) and this showed a large peak at 44% solvent B. Oxidation of this material was carried out by dissolving the peptide (30mg) in a solution of 10% DMSO/TFA (5ml) and stirring for 1.5 hours. The solution was then evaporated *in vacuo*. The peptide was triturated with diethyl ether and dried *in vacuo* (yield 13mg). Purification was carried out by preparative hplc (column 9) using a gradient of 10-25% solvent B over 10 minutes followed by 25-45% solvent B over 20 minutes. After lyophilisation, 5mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.17, $Thr_1$1.35, $Ser_1$1.14, $Ala_1$1.17, $Val_2$1.73, $Ile_2$1.89, $Leu_1$1.31, $Phe_1$0.86, $Orn_1$0.85, $Arg_2$2.04, Cys_2n.d.; m/z (FAB) 1710 [M^+], HRMS $C_{74}H_{124}N_{22}S_2O_{20}$ requires 1707.90378 found 1707.90377; hplc (column 1, 5.0ml loop) R_t=17.5minutes.

Synthesis of 100-114 hNGF with Tyr replacing Phe^{101} (3)

R.Y.I.R.I.D.T.A.C.V.C.V.L.S.R

Fmoc-Arg-resin (570mg, 0.25mmole) prepared for compound (2) was placed in a reaction vessel and the synthesis was carried out using double couple cycles (symmetrical anhydride followed by HOBt) with extended coupling times. Capping solution A was used (section 3.2.3.4). After coupling Ile^{102} half of the resin was removed and stored for the synthesis of compound (4). The cysteine residues were incorporated as S-*t*-butylsulphenyl derivatives. The Fmoc deprotection peak for Tyr^{101} (section 3.2.3.5) showed that there was a drop of approximately 50% in the coupling of this residue.

The peptide was cleaved from the resin for 3 hours using cleavage solution A (section 3.2.4). The cysteine protecting groups were cleaved using the same procedure as for compound (2). The crude dithiol peptide (25mg) was dissolved in 10% DMSO/TFA (5ml) and oxidation was allowed to proceed for one hour. The DMSO/TFA solution was reduced to approximately 0.5ml by evaporating *in vacuo*. The peptide was triturated with diethyl ether. An analytical hplc was recorded (column 1, 2.0ml loop). This showed the presence of two main peaks at 49% and 45% solvent B. Purification of the peptide by preparative hplc was carried out (column 7) using a gradient of 10-40% solvent B over 30 minutes. After lyophilisation 1.0mg of the *title* compound was obtained. The procedure was repeated on a further 47.3mg of crude dithiol peptide to yield 2.5mg of the *title* compound; amino acid analysis: $Asp_1$1.05, $Thr_1$1.09, $Ser_1$1.15, $Ala_1$1.18, $Val_2$2.04, $Ile_2$2.02, $Leu_1$1.18, $Tyr_1$0.81, $Arg_3$2.96, Cys_2n.d.; m/z (FAB) 1767.3 [M^+], HRMS $C_{75}H_{128}N_{24}O_{21}S_2$ requires 1767.93621 found 1767.92163; hplc (column 4, 2.0ml loop) R_t=14minutes.

Synthesis of 100-114 hNGF with Cyclohexylalanine replacing Phe^{101} (4)

R.Cha.I.R.I.D.T.A.C.V.C.V.L.S.R Cha = Cyclohexylalanine

The peptide-resin which was removed from the reaction vessel after coupling of Ile^{102} in compound (3) was used for this synthesis. To this peptide-resin Cha^{101} and Arg^{100} were coupled with triple extended coupling cycles (symmetrical anhydride followed by double HOBt) to prevent the 50% drop in coupling observed with Tyr^{101} in compound (3).

The peptide-resin was cleaved and the cysteine protecting groups were removed, as for compound (3). An analytical hplc was recorded (column 1, 2.0ml loop). This showed the presence of one main peak at 50% solvent B. The crude dithiol peptide (35mg) was dissolved in 10% DMSO/TFA (5ml) and oxidation was allowed to proceed for 1.5 hours. The solution was then reduced to approximately 0.5ml by evaporation *in vacuo*. The peptide was triturated with diethyl ether and air-dried (yield 22mg). An analytical hplc recorded under the same conditions as for the reduced peptide showed that the main peak eluted at 49% solvent B. The peptide was purified by preparative hplc (column 7) using a gradient of 10-40% B over 30 minutes. After lyophilisation, 4.5mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.17, $Thr_1$1.12, $Ser_1$1.10, $Ala_1$1.16, $Val_2$2.08, $Ile_2$2.16, $Leu_1$1.22, $Arg_3$3.16, Cys_2n.d., Cha_1n.d.; m/z (FAB) 1756.9 [M^+], HRMS $C_{75}H_{134}N_{24}O_{20}S_2$ requires 1755.97252 found 1755.97243; hplc (column 4, 5.0ml loop) R_t=18minutes.

Synthesis of 100-114 hNGF with Lys replacing Arg^{100} (5)

K.F.I.R.I.D.T.A.C.V.C.V.L.S.R

Fmoc-Arg-resin (340mg, 0.15mmole/g) prepared for compound (2) was placed in a reaction vessel and the peptide was assembled using double couple cycles (symmetrical anhydride followed by HOBt) with extended coupling times. Capping solution A was used (section 3.2.3.4). The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives.

The peptide was cleaved from the resin for 3 hours using a cleavage solution of 62.5%TFA/7.5%phenol/20%H_2O/10%anisole. The cysteine protecting groups were cleaved from the crude peptide as for compound (2). The cleavage was monitored by analytical hplc (column 1, 2.0ml loop) and after 3 hours the main peptide peak had moved from 60% solvent B to 51% solvent B indicating the cleavage of the hydrophobic protecting groups had gone to completion. Oxidation of the crude dithiol peptide (56.3mg) was carried out in 10% DMSO/TFA (10ml) for one hour. The oxidation was monitored by analytical hplc using the same conditions as above. The oxidised product eluted at 50% solvent B. The DMSO/TFA solution was reduced to approximately 0.5ml by evaporation *in vacuo* and a solution of 20% AcOH was added. The insoluble particles were filtered off and purification by preparative hplc was carried out (column 7) using a gradient of 10-40% solvent B over 30 minutes. After lyophilisation, 7.5mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.11, $Thr_1$1.04, $Ser_1$1.09, $Ala_1$1.14, $Val_2$2.06, $Ile_2$1.94, $Leu_1$1.14, $Phe_1$0.99, $Lys_1$1.01, $Arg_2$1.81, Cys_2n.d.; m/z (FAB) 1721.7 [M^+], HRMS $C_{75}H_{128}N_{22}O_{20}S_2$ requires 1721.91942 found 1721.91936; hplc (column 4, 2.0ml loop) R_t=15minutes.

Synthesis of Lys.Gly.Lys.aua.Lys.aua.100-114 hNGF (8)

$K^{32}.G.K^{34}.aua.K^{95}.aua.R^{100}.F.I.R.I.D.T.A.C(Acm).V.C(Acm).V.L.S.R^{114}$

Fmoc-Arg-resin (595mg, 0.25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled as far as Arg^{100} using triple couple cycles (one symmetrical anhydride followed by two HOBt cycles). Extended coupling times were used. Capping solution A was used (section 3.2.3.4). The cysteine residues were incorporated as the Acm derivatives. After coupling Arg^{100} the percentage loading of the resin was checked manually using the procedure outlined in section 3.2.2. The resin loading at this point in the synthesis was found to be 0.162 mmole/g. Fmoc-aminoundecanoic acid (348mg, 0.825mmole) was dissolved in DMF (20mls). DIC (0.825mmole) and HOBt (0.825mmole) were added. To the HOBt ester of Fmoc-aminoundecanoic acid thus formed, the peptide-resin (1.0194, 0.165mmole) was added. The resin was sonicated overnight. This resulted in 68% coupling of Fmoc-aminoundecanoic acid. Using the same procedure, a second overnight coupling was carried out. This increased the coupling percentage to 91%. The resin was capped for 15 minutes. The resin was washed with DMF before deprotection of Fmoc with 20% piperidine/DMF (10mls) was carried out. This was followed by a further washing of the resin-peptide with DMF. Fmoc-Lys (936mg, 2mmole) was dissolved in 10mls DMF and DIC (1mmole) was added. The symmetrical anhydride was allowed to form by stirring for 15 minutes before adding the peptide-resin. After 2 hours the percentage coupling was found to be 86%. A second symmetrical anhydride coupling for 1 hour increased the percentage coupling to 98%. The resin was then capped and Fmoc deprotected. Fmoc-aminoundecanoic acid was coupled to Lys^{95} using the same procedure as for coupling to Arg^{100}. After two overnight HOBt couplings the percentage coupling was found to be 84%. A third overnight coupling did not improve on this. The resin was therefore capped and Fmoc deprotected. The coupling of Fmoc-Lys^{34} to aminoundecanoic acid was

carried out using double symmetrical anhydride coupling. The resin was then capped before being returned to the reaction vessel of the peptide synthesiser. After Fmoc deprotection, Gly^{33} was coupled by symmetrical anhydride activation of Fmoc-Gly (2mmole). Extended coupling time was used. Finally Lys^{32} was coupled using double couple symmetrical anhydride activation with extended coupling times.

Cleavage of the peptide from the resin was carried out for 3 hours using cleavage solution B (section 3.2.4). Purification of the cysteine protected peptide (80mg) was carried out by preparative hplc (column 8) using a gradient of 10-35% B over 8 minutes followed by 35-65% over 25 minutes. After lyophilisation, 4.0mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.15, $Thr_1$1.06, $Ser_1$1.16, $Gly_1$1.05, $Ala_1$1.07, $Val_2$2.13, $Ile_2$1.9, $Leu_1$1.1, $Phe_1$0.97, $Lys_3$2.77, $Arg_3$2.97, Cys_2n.d.; m/z (FAB) 2700.4 $[M^+]$, HRMS $C_{123}H_{221}N_{35}O_{28}S_2$ requires 2702.64974 found 2702.64977; hplc (column 1, 5.0ml loop) R_t=18minutes.

Synthesis of oxidised form of (8) (8/2)

K^{32}.G.K^{34}.aua.K^{95}.aua.R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}

Fmoc-Arg-resin (520mg, 0.25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled as far as Arg^{100} using double couple HOCt cycles. The cysteine residues were incorporated as the Acm derivatives. Capping solution B was used (section 3.2.3.4). After coupling Arg^{100}, 50mg of peptide-resin were removed and cleaved for three hours using cleavage solution B (section 3.2.4). An analytical hplc (column 1, 2.0ml loop) of the crude peptide was recorded. This showed a large single peak at 46%B indicating the success of the synthesis thus far. The couplings of the aminoundecanoic acid and the lysine residues were carried out exactly as for (8). After replacing the peptide-resin in the reaction vessel of the peptide synthesiser, Gly^{33} was coupled as in (8). Finally Lys^{32} was coupled using double couple HOCt coupling cycles.

The resin-peptide was cleaved for 3 hours using cleavage solution B (section3.2.4). An analytical hplc (column 4, 2.0ml loop) of the crude peptide showed one main peak at 52% B. Purification of the cysteine protected peptide (290mg) was carried out as for (8). After lyophilisation 81.3mg was obtained. To deprotect the cysteine residues, the peptide (30mg, $1.11x10^{-5}$ moles) was cooled in ice and silver trifluoromethane sulphonate (57mg, $2.22x10^{-4}$ moles, 20 equivalents) was added. This was then dissolved in TFA (4ml). The reaction was stirred in the dark at 0^0C for 1.5 hours. The TFA was evaporated off *in vacuo* and the peptide was precipitated by addition of diethyl ether. The silver salt of the peptide thus formed was dissolved in 50% acetic acid (5mls) and dithiothreitol (171mg, $1.11x10^{-3}$ moles, 100 equivalents) was added. The reaction was stirred at room temperature, in the dark, overnight. The yellow precipitate thus formed was separated by centrifugation (4,500 rpm for 30 minutes). The supernatant liquid was purified by preparative hplc (column 7) using a gradient of 10-35% over 10 minutes followed by 35-50% over 15 minutes. After lyophilisation, 13.8mg of reduced peptide was obtained. A positive Ellman's test carried out (section 3.2.6.1) indicated the presence of free thiol groups. Oxidation of the peptide (12.7mg) was carried in 5% DMSO/TFA (10mls). The oxidation was monitored by analytical hplc (column 1, 2.0ml loop). After one hour the peak at 55% B corresponding to the reduced peptide was replaced by an earlier eluting peak at 52% B. The DMSO/TFA solution was reduced to approximately 2ml by evaporation *in vacuo*. This was diluted to a volume of 9mls using 40% solventB/solventA (section 3.2.5) and purification was carried out by preparative hplc (column 7) using a gradient of 10-40%B over 30minutes. After lyophilisation, 6.0mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.06, $Thr_1$1.00, $Ser_1$0.98, $Gly_1$1.00, $Ala_1$1.06, $Val_2$2.17, $Ile_2$1.91, $Leu_1$1.02, $Phe_1$1.04, $Lys_3$3.17, $Arg_3$3.08, Cys_2n.d.; m/z (TOF) 2556.32 [M^+], HRMS $C_{117}H_{209}N_{33}O_{26}S_2$ requires 2559.57923, found 2559.56781; hplc (column 4, 5.0ml loop) R_t=16minutes.

Synthesis of Lys.Gly.Lys.aua.Lys.aua.Arg (9)

$K^{32}.G.K^{34}.aua.Lys^{95}.aua.Arg^{100}$

Fmoc-Arg-resin (516mg, 0.25mmole) was prepared as explained in section 3.2.1. The peptide was assembled manually, up to the coupling of Gly^{33}, using the same procedure as for the corresponding part of peptide (8/2). The coupling of Fmoc-aminoundecanoic acid was carried out by forming the HOBt ester (1mmole) and sonicating overnight. A second overnight coupling was not required. The resin peptide was then placed in a reaction vessel and the remaining residues were coupled on the machine. Gly^{33} was coupled by symmetrical anhydride activation (2mmole) with extended coupling time. Lys^{32} was coupled using double couple cycles (symmetrical anhydride and HOBt activation) with extended coupling times.

The peptide-resin was cleaved for 2.5 hours using cleavage solution B (section 3.2.4). An analytical hplc (column 1, 5.0ml loop) showed one main peak eluting at 53%B. The peptide (80mg) was purified by preparative hplc (column 8) using a gradient of 10-90% B over 30 minutes. After lyophilisation 10mg of the *title* compound was obtained; amino acid analysis: $Gly_1$1.12, $Lys_3$2.9, $Arg_1$0,96; m/z (FAB) 983.3 $[M^+]$ $C_{48}H_{95}N_{13}O_8$ requires 983.38; hplc (column 2, 5.0ml loop) R_t=16minutes.

Synthesis of 81-107 hNGF (10)

T.T.T.H.T.F.V.K.A.L.T.M.D.G.K.Q.A.A.W.R.F.I.R.I.D.T.A

Fmoc-Ala-resin (463mg, 0.25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled using double couple HOCt cycles with standard coupling times.

The peptide was cleaved from the resin (70mg) for 3 hours using cleavage solution B (section 3.2.4)(crude yield 23mg). The peptide was purified by

preparative hplc (column 6) using a gradient of 10-60% solvent B over 25 minutes. After lyophilisation, 11mg of the *title* compound was obtained; amino acid analysis: $Asp_2$1.82, $Thr_6$5.85, $Glu_1$1.25, $Gly_1$1.06, $Ala_4$4.36, $Val_1$1.06, $Met_1$1.04, $Ile_2$2.10, $Leu_1$1.05, $Phe_2$2.09, $His_1$1.02, $Lys_2$2.12, $Arg_2$2.11, Trp_1n.d.; m/z (FAB) 3084.3 [M^+], HRMS $C_{138}H_{219}N_{39}O_{39}S_1$ requires 3080.62129; found 3080.61849; hplc (column 4, 5.0ml loop) R_t=19.5minutes.

Synthesis of 99-114 with Tyr replacing Trp^{99} (11)

Y.R.F.I.R.I.D.T.A.C.V.C.V.L.S.R

This peptide was synthesised manually using the apparatus shown in Appendix 1. Fmoc-Arg-resin (550mg, 0.25mmole) prepared as outlined in section3.2.4 was placed in the bubbler. The synthesis was carried out by the following order of steps.

1. DMF wash, 10 x 1 minute
2. 20% piperidine/DMF, 1 x 5 minute
3. DMF wash, 10 x 1 minute
4. Kaiser test
5. Couple Fmoc-amino acid
6. DMF wash, 10 x 1 minute
7. Kaiser test
8. If Kaiser test negative return to step 2 and continue. If Kaiser test positive return to step 5.

Each amino acid was coupled (step 5) for one hour as symmetrical anhydride (2mmole) followed by two hours HOBt (1mmole) coupling. The manual monitoring of coupling efficiency was carried out by a Kaiser test. The procedure for the Kaiser test is as follows:

Kaiser Test:

A small sample of washed peptide-resin was removed from the bubbler, placed in a vial and the following solutions were added.

a. 0.28M ninhydrin in ethanol (75μl),

b. 2 x 10^{-4} M potassium cyanide / pyridine (100μl),

c. 76% w/w phenol / ethanol (75μl).

Each of these solutions were obtained from ABI (peptide synthesis reagents).
The vial was heated at 100°C for 7 minutes. A positive test indicating the presence of free amine groups was shown by a blue colour.

The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives. The peptide was cleaved from the resin for three hours using cleavage solution C (section 3.2.4). Purification of the crude dithiol peptide (30mg) was carried out by preparative hplc (column 8) using a gradient of 10-60% over 28 minutes. After lyophilisation, 5.0mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.27, $Thr_1$1.27, $Ser_1$1.06, $Ala_1$1.18, $Val_2$2.00, $Ile_2$2.08, $Leu_1$1.13, $Tyr_1$1.01, $Arg_3$3.18, Cys_2n.d.; m/z (TOF) 1914.3 [M^+], HRMS $C_{84}H_{139}N_{25}O_{22}S_2$ requires 1915.00462, found 1915.00995; hplc (column 4, 5.0ml loop) R_t=19minutes.

Synthesis of 1-8.aua.100-114 hNGF (13)

S^1.S.S.H.P.I.F.H^8.aua.R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}

The synthesis of the first half of this peptide as far as His^8 was carried out exactly as for (8/2). After coupling of Fmoc-aminoundecanoic acid, the resin-peptide was returned to the reaction vessel of the peptide synthesiser and the remaining residues were coupled using double couple cycles with HOCt, except for the two His residues which were coupled using double couple HOBt cycles. The cysteine residues were protected as the Acm derivatives.

The peptide was cleaved from the resin (500mg) for three hours using cleavage solution B (section 3.2.4) (crude yield 300mg). The cysteine protected peptide was purified by preparative hplc (column 8) using a gradient of 10-50%B over 25minutes. The cysteine protecting groups were cleaved from the purified peptide (30mg) using the same procedure as for (8/2). After centrifugation the supernatant liquid of reduced peptide was dissolved in 50% acetic acid and purified by preparative hplc (column 6) using a gradient of 10-50%B over 25minutes. After lyophilisation 14mg of reduced peptide was obtained. Oxidation of this material was carried out in 5% DMSO/TFA for 1 hour. The oxidation was monitored by analytical hplc (column 1, 5.0ml loop). After one hour the peak corresponding to the reduced peptide, eluting at 63%B, had dissapeared and a peak corresponding to the oxidised peptide eluted at 62%B. The DMSO/TFA solution was reduced to approximately 1ml by evaporation *in vacuo*. The peptide solution was diluted to a volume of 6mls with 30% acetic acid and purification was carried out by preparative hplc (column 6) using a gradient of 10-50%B over 25minutes. After lyophilisation, 7mg of the *title* compound was obtained; amino acid analysis, $Asp_1$1.20, $Thr_1$1.11, $Ser_4$3.71, $Ala_1$1.11, $Val_2$2.21, $Ile_3$3.22, $Leu_1$1.15, $Phe_2$2.08, $His_2$2.00, $Arg_3$3.35, Cys_2n.d.; m/z (FAB) 2829 [M^+], HRMS $C_{127}H_{205}N_{37}O_{32}S_2$ requires 2826.52083, found 2826.51047; hplc (column 4, 2.0ml loop) R_t=16.5minutes.

Synthesis of 25-40 hNGF (14)

K.T.T.A.T.D.I.K.G.K.E.V.M.V.L.G

Fmoc-Gly-resin (421mg, 0.25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled using double couple cycles with HOCt. The peptide was cleaved from the resin (50mg) for three hours using cleavage solution B (section 3.2.4) (crude yield 29mg). Purification was carried out by preparative hplc (column 6) using a gradient of 10-40%B over 25minutes. After lyophilisation, 13mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.00, $Thr_3$2.78, $Glu_1$1.12, $Gly_2$2.17, $Ala_1$1.07, $Val_2$2.01, $Met_1$0.85, $Ile_1$1.06, $Leu_1$1.01, $Lys_3$2.87; m/z (FAB) 1692.0 $[M^+]$, HRMS $C_{173}H_{131}N_{19}O_{24}S_1$ requires 1690.94134, found 1690.94893; hplc (column 4, 5.0ml loop) R_t=16.5minutes.

Synthesis of 100-114 hNGF with N-terminal *p*-hydroxyphenylacetic acid (15)

p-hydroxyphenylacetic acid.R.F.I.R.I.D.T.A.C.V.C.V.L.S.R

Fmoc-Arg-resin (550mg, 0.25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled as far as Arg^{100} using double couple cycles with HOCt. The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives. A small sample of the resin was removed and the peptide was cleaved for three hours using cleavage solution C (section 3.2.4). An analytical hplc (column 4, 2.0ml loop) recorded on the crude peptide showed a large single peak indicating the success of the synthesis thus far. To the remaining resin-peptide *p*-*t*-butoxyphenylacetic acid was coupled by symmetrical anhydride (1mmole) coupling.

The peptide was cleaved from the resin (500mg) for three hours using cleavage solution C (section 3.2.4) (crude yield 190mg). Purification of the reduced

peptide was carried out by preparative hplc (column 8, 2.0ml loop) using a gradient of 10-60% B over 30 minutes. The reduced peptide (27mg) was dissolved in 5% DMSO/TFA (14mls) and oxidation was allowed to proceed for one hour. The TFA/DMSO solution was reduced to approximately 2mls by evaporation *in vacuo*. The resulting peptide solution was diluted to 12mls with 30% acetic acid and purification was carried out by preparative hplc (aquapore C_{18}, 5.0ml loop) using a gradient of 10-50% B over 28 minutes. After lyophilisation, 8mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.26, $Thr_1$1.07, $Ser_1$1.08, $Ala_1$1.10, $Val_2$1.51, $Ile_2$2.44, $Leu_1$0.84, $Phe_1$1.17, $Arg_3$3.19, Cys_2n.d.; m/z (TOF) 1883.2 [M^+], (FAB) 1884.0 [M^+], HRMS $C_{83}H_{135}N_{24}O_{22}S_2$ requires 1885.97807, found 1885.97502; hplc (column 4, 2.0ml loop) R_t=16minutes.

Synthesis of 100-114 hNGF (16)

R.F.I.R.I.D.T.A.C.V.C.V.L.S.R

Fmoc-Arg-resin (550mg, 0.25mmole) prepared as outlined in section 3.2.1, was placed in a reaction vessel and the peptide was assembled using double couple cycles with HOCt. The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives.

The peptide was cleaved from the resin (400mg) for three hours using cleavage solution C (section 3.2.4) (crude yield 190mg). Purification of the reduced peptide was carried out by preparative hplc (column 8) using a gradient of 10-60% B over 28 minutes. Oxidation of the purified peptide (15mg) was carried out in 5% DMSO/TFA (10mls) for one hour. This was repeated on a further 12mg of reduced peptide. The DMSO/TFA solution in each case was reduced to approximately 1.5ml by evaporation *in vacuo*. The resulting peptide solutions were diluted to 10mls using 20% acetic acid and purification was carried out by preparative hplc (column 6) using a gradient of 10-55% B over 25 minutes. After lyophilisation, 12mg of the *title*

compound was obtained; amino acid analysis: $Asp_1$1.27, $Thr_1$1.11, $Ser_1$1.13, $Ala_1$1.08, $Val_2$1.54, $Ile_2$2.36, $Leu_1$0.98, $Phe_1$1.20, $Arg_3$2.94, Cys_2n.d.; m/z (TOF) 1751.6 [M^+], (FAB) 1751.9 [M^+], HRMS $C_{75}H_{128}N_{24}O_{20}S_2$ requires 1751.94129, found 1751.93876; hplc (column 4, 2.0ml loop) R_t=14.5 minutes.

Synthesis of 111-120 mNGF (17)

V.L.S.R.K.A.T.R.R.G

Fmoc-Gly-resin (400mg, 0,25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled using double couple cycles with HOCt. After coupling Val^{111} half of the resin was removed and the remainder was used for the synthesis of (18) below.

The peptide was cleaved from the resin (200mg) for three hours using cleavage solution B (section 3.2.4). Purification of the crude peptide (30mg) was carried out by preparative hplc (column 6) using a gradient of 0-20% B over 10 minutes. After lyophilisation, 20mg of the *title* compound was obtained; amino acid analysis: $Thr_1$1.02, $Ser_1$0.71, $Gly_1$1.14, $Ala_1$1.09, $Val_1$0.96, $Leu_1$0.96, $Lys_1$1.03, $Arg_3$2.83; m/z (TOF) 1140.1 [M^+], (FAB) 1143.4, HRMS $C_{47}H_{90}N_{20}O_{13}$ requires 1143.70745, found 1143.71043; hplc (column 4, 2.0ml loop) R_t=10 minutes.

Synthesis of 95-120 mNGF (18)

K.Q.A.A.W.R.F.I.R.I.D.T.A.C.V.C.V.L.S.R.K.A.T.R.R.G

The remaining resin from synthesis (17) was used to assemble this peptide. The coupling of each residue from Cys^{110} to Lys^{95} were carried out using double couple cycles with HOCt. The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives.

The peptide was cleaved from the resin (200mg) for three hours using cleavage solution C (section 3.2.4) (crude yield 95mg). Purification of the reduced peptide was carried out by preparative hplc (column 8) using a gradient of 10-50% B over 28 minutes. After lyophilisation, 32.5mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.06, $Thr_2$1.92, $Ser_1$0.97, $Gln_1$1.09, $Gly_1$1.08, $Ala_4$4.07, $Val_2$2.07, $Ile_2$2.12, $Leu_1$0.99, $Phe_1$1.05, $Lys_2$2.02, $Arg_5$5.46, Cys_2n.d.; m/z (TOF) 3004.8 [M^+], HRMS $C_{130}H_{222}N_{45}O_{33}S_2$ requires 3007.66300, found 3007.67497; hplc (column 4, 5.0ml loop) R_t=20minutes.

Synthesis of 95-120 mNGF dimer (18/2)

15mg of 95-120 mNGF reduced, prepared as explained in (18) was dissolved in a 50mmolar solution of ammonium acetate (150mls). The pH was adjusted to 8.5 using ammonia. The formation of the dimer was monitored by analytical hplc (column 4, 2.0ml loop) over 72 hours. During this time the peak which corresponded to the reduced material (eluting at 52% B) decreased, while the peak corresponding to the dimer formed (eluting at 50%B) increased. The reaction was stopped and the ammonium acetate was removed by freeze-drying. Purification was carried out by preparative hplc (column 7) using a gradient of 10-40% over 22 minutes. After lyophilisation, 5.0mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.06, $Thr_2$1.92, $Ser_1$0.97, $Gln_1$1.08, $Gly_1$1.08, $Ala_4$4.07, $Val_2$2.07, $Ile_2$2.12, $Leu_1$0.99, $Phe_1$1.05, $Lys_2$2.02, $Arg_5$5.46, Cys_2n.d.; m/z (TOF) 6009.8 [M^+], $C_{260}H_{440}N_{90}O_{66}S_4$ requires 6015.22, hplc (column 4, 2.0ml loop) R_t=15minutes.

Synthesis of 50-70.aua.100-114 hNGF (19)

K^{50}.Q.Y.F.F.E.T.K.C.R.D.P.N.P.V.D.S.G.C.R.G^{70}.aua.R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}

Fmoc-Arg-resin (520mg, 0.25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled as far as Gly^{70} using the same procedure as for (8/2). Following the manual coupling of Fmoc-aminoundecanoic acid the resin-peptide was returned to the peptide synthesiser and the remaining residues were coupled using double couple HOCt cycles. $Cys^{58,108}$ were incorporated as the S-*t*-butylsulphenyl derivatives. $Cys^{68,110}$ were incorporated as the phenylacetamidomethyl derivatives.

The peptide was cleaved from the resin (500mg) for 5 hours using cleavage solution C (section3.2.4) (crude yield 300mg). An analytical hplc recorded (column 4, 2.0ml loop) showed the main peak to elute at 62% B. Purification of the reduced peptide ($Cys^{58,108}$ unprotected, $Cys^{68,110}$ protected) was carried out by preparative hplc (column 9, 5.0ml loop) using a gradient of 10-30% B over 6 minutes followed by 30-36% B over 12 minutes. After lyophilisation, 38mg of reduced peptide was obtained; m/z (TOF) 4660.6 [M^+], $C_{209}H_{327}N_{58}O_{55}S_4$ requires 4660.58; hplc (column 5, 5.0ml loop) R_t=21minutes.

An attempted oxidation of Cys^{58} to Cys^{108} was carried out by dissolving 15mg of the reduced peptide in 5% DMSO/TFA (20mls). The oxidation was followed by analytical hplc (column 4, 5.0ml loop). After 60 minutes the retention time of the peptide had changed from 21 minutes for the reduced peptide to 19 minutes for the oxidised peptide. The DMSO/TFA solution was then reduced to approximately 4ml by evaporation *in vacuo*. The resulting peptide solution was diluted to 12mls with 30% acetic acid and purification was carried out by preparative hplc (column 9) using a gradient of 10-40% B over 18 minutes. After lyophilisation, 4mg of pure peptide was obtained. On analysis it was found that the mass of the

product obtained indicated that the protecting groups on Cys^{68} and Cys^{110} were unexpectedly cleaved during the oxidation of Cys^{58} to Cys^{108}. Both disulphides had therefore been formed at the same time; amino acid analysis: $Asn/Asp_4$4.34, $Thr_2$1.92, $Ser_2$1.95, $Glu/Gln_2$2.33, $Pro_2$2.26, $Gly_2$1.99, $Ala_1$1.05, $Val_3$3.02, $Ile_2$2.07, $Leu_1$1.04, $Tyr_1$0.78, $Phe_3$2.84, $Lys_2$1.92, $Arg_5$4.99, Cys_2n.d.; m/z (TOF) 4358.1 [M^+], (FAB) 4363 [M^+], HRMS $C_{191}H_{304}N_{56}O_{53}S_4$ requires 4361.18426 found 4361.14491; hplc (column 4, 5.0ml loop) R_t=19.0minutes; Ellman's test was negative.

Selective oxidation of Cys^{58}-Cys^{108} in 50-70.aua.100-114 NGF (19/2)

The remaining 500mg of peptide-resin was cleaved as outlined above. Purification of the reduced peptide ($Cys^{58,108}$ deprotected, $Cys^{68,110}$ still protected) was carried out by preparative hplc using the same conditions as mentioned above. After lyophilisation 35mg of the reduced compound was obtained.

The reduced peptide (45mg, combined from both syntheses) was dissolved in 6M GdmCl (900ml) to make a solution of $1x10^{-5}$M concentration. Glutathione reduced (304mg, 1.1mM) and oxidised (60mg, 0.11mM) were added. The reaction was allowed to stir overnight at 4^0C. The oxidation was monitored by analytical hplc (column 4, 5.0ml loop). After 24 hours the retention time of the peptide had changed from 20.5 minutes to 20 minutes. The solution was dialysed overnight at 4^0C, using Spectrapor membrane tubing, molecular weight cut off 2000. This reduced the concentration of GdmCl in solution. Purification by preparative hplc (column 10) to remove the remaining GdmCl was carried out. After lyophilisation, 10mg of the *title* compound was obtained; amino acid analysis: $Asn/Asp_4$4.34, $Thr_2$1.92, $Ser_2$1.95, $Glu/Gln_2$2.33, $Pro_2$2.26, $Gly_2$1.99, $Ala_1$1.05, $Val_3$3.02, $Ile_2$2.07, $Leu_1$1.04, $Tyr_1$0.78, $Phe_3$2.84, $Lys_2$1.92, $Arg_5$4.99, Cys_4n.d.; m/z (TOF) 4658 [M^+], (FAB) 4655 [M^+],

HRMS $C_{209}H_{325}N_{58}O_{55}S_4$ requires 4658.34456 found 4658.32495; hplc (column 4, 5.0ml loop) R_t=20minutes.

Synthesis of 100-114 hNGF with cis-1-amino-4-carboxy-cyclopentane replacing Cys-Val-Cys (20)

R.F.I.R.I.D.T.A.β-bend mimic.V.L.S.R

Fmoc-Arg-resin (507mg, 0.25mmole) prepared as outlined in section 3.2.1 was placed in a reaction vessel and the peptide was assembled using double couple cycles with HOCt. Fmoc-1-amino-4-carboxy-cyclopentane was coupled manually by preparing the HOBt ester (1mmole) and sonicating overnight with the peptide-resin.

The peptide was cleaved from the resin (200mg) for 3 hours in 90%TFA/5%H_2O/5%EDT (10mls) (crude yield 85mg). The peptide (25mg) was purified by preparative hplc (column 8) using a gradient of 10-60% over 28 minutes. After lyophilisation, 17.5mg of the *title* compound was obtained; amino acid analysis; $Asp_1$1.11, $Thr_1$0.97, $Ser_1$0.99, $Ala_1$0.99, $Val_1$0.90, $Ile_2$1.96, $Leu_1$1.15, $Phe_1$0.99, $Arg_3$2.90; m/z (TOF) 1558.7 [M^+], (FAB) 1558.0 [M^+], HRMS $C_{70}H_{120}N_{22}O_{18}$ requires 1557.92292 found 1557.92714; hplc (column 5, 5.0ml loop) R_t=20 minutes.

Synthesis of 50-70.aua.100-114 mNGF (22)

R^{50}.Q.Y.F.F.E.T.K.C.R.A.S.N.P.V.E.S.G.C.R.G^{70}.aua.R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}

Fmoc-Arg-resin (550mg, 0.25mmole) was placed in a reaction vessel and the peptide was assembled using double couple cycles (symmetrical anhydride coupling followed by HOBt coupling). Extended coupling times were used from Cys^{110} to Arg^{100}. After coupling Val^{111}, half of the resin was removed and stored for the

symthesis of compound (21). Fmoc-aminoundecanoic acid was coupled manually as for (8/2). $Cys^{110,68}$ were incorporated as the phenylacetamidomethyl derivatives. $Cys^{108,58}$ were incorporated as the S-*t*-butylsulphenyl derivatives.

The peptide was cleaved from the resin (500mg) for 5 hours using cleavage solution C (section 3.2.4) (crude yield 210mg). The reduced peptide (120mg, $Cys^{108,58}$ unprotected, $Cys^{110,68}$ still protected) was purified by preparative hplc (column 9) using a gradient of 10-30% over 6 minutes followed by 30-40% over 20 minutes. After lyophilisation, 20.5mg of pure, reduced peptide was obtained.

Cleavage of the protecting groups on $Cys^{110,68}$ and the formation of both disulphides was carried out by dissolving the reduced peptide (10mg) in 5% DMSO/TFA (10mls). The reaction was allowed to stir for one hour. The oxidation was monitored by analytical hplc (column 4, 2.0ml loop). As the oxidation proceeded the retention time of the peptide changed from 17 minutes to 16 minutes. The DMSO/TFA solution was reduced to 2ml by evaporation *in vacuo*. The resulting peptide solution was diluted to 10mls using 30% acetic acid. Purification of the peptide was carried out by preparative hplc (column 7) using a gradient of 10-60% B over 30 minutes. After lyophilisation, 3.5mg of the *title* compound was obtained; amino acid analysis: $Asn/Asp_2$2.52, $Thr_2$2.22, $Ser_3$3.15, $Glu/Gln_4$3.97, $Pro_1$1.21, $Gly_2$2.19, $Ala_2$1.69, $Val_3$2.74, $Ile_2$1.49, $Leu_1$0.93, $Tyr_1$0.76, $Phe_3$2.95, $Lys_1$1.22, $Arg_6$6.62, Cys_4n.d.; m/z (TOF) 4350.4 $[M^+]$, (FAB) 4348 $[M^+]$, HRMS $C_{189}H_{304}N_{58}O_{52}S_4$ requires 4349.19549 found 4349.19481; hplc (column 4, 5.0ml loop) R_t=19 minutes.

Synthesis of 1-8.aua.100-114 mNGF (21)

$S^{1}.S.T.H.P.V.F.H^{8}.aua.R^{100}.F.I.R.I.D.T.A.C.V.C.V.L.S.R^{114}$

The second half of the resin which was removed from the synthesis of compound (22) was placed in a reaction vessel and the peptide was assembled as far as His^{8} using the same procedure as for compound (22). Coupling of His^{8} to Ser^{1} was carried out using double couple cycles (symmetrical anhydride followed by HOBt coupling) with standard coupling times. The cysteine residues were incorporated as the S-*t*-butylsulphenyl derivatives.

The peptide was cleaved from the resin (300mg) for 3 hours using 90%TFA/5%H_2O/5%EDT (crude yield 210mg). The cysteine protecting groups were cleaved from the crude peptide (80mg) for 5 hours using the same procedure as for (2). The cleavage was monitored by analytical hplc (column 4, 2.0ml loop). The retention time of the peptide changed from 18.5 minutes to 17 minutes as the deprotection proceeded (yield 75mg). The crude dithiol peptide (15mg) was dissolved in 5% DMSO/TFA (10mls) and oxidation was allowed to proceed for one hour. The DMSO/TFA solution was then reduced to approximately 2mls by evaporation *in vacuo*. The resulting peptide solution was diluted to 10mls with 30% acetic acid. Purification by preparative hplc (column 7) using a gradient of 10-60% over 28 minutes. After lyophilisation, 4mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.16, $Thr_2$2.06, $Ser_3$2.74, $Pro_1$1.16, $Ala_1$1.45, $Val_3$2.9, $Ile_2$2.5, $Leu_1$1.36, $Phe_2$2.25, $His_2$2.11, $Arg_3$3.15, Cys_2n.d. m/z (TOF) 2828.4 [M^+], (FAB) 2827 [M^+], HRMS $C_{127}H_{205}N_{37}O_{32}S_2$ requires 2827.51829, found 2827.53432; hplc (column 4, 5.0ml loop) R_t=19minutes.

Purification of 59-67 hNGF (23)

R.D.P.N.P.V.D.S.G

This peptide was synthesised and cleaved from the resin by Alan Cuthbertson[126]. An analytical hplc (column 4, 5.0ml loop) was recorded of the crude material available (178mg). This showed the main peak eluting at 65% B. Purification of the crude peptide (80mg) was carried out by preparative hplc (column 6) using a gradient of 10-36% B over 14 minutes. After lyophilisation, 6.0mg of the *title* compound was obtained; amino acid analysis: $Asn/Asp_3$2.96, $Ser_1$0.82, $Pro_2$2.14, $Gly_1$1.03, $Val_1$1.04, $Arg_1$0.99; m/z (FAB) 957 [M^+], HRMS $C_{38}H_{61}N_{13}O_{16}$ requires 956.44375 found 956.45032; hplc (column 4, 5.0ml loop) R_t=20minutes.

Purification of 46-54 hNGF (24)

N.S.V.F.K.Q.Y.F.F

This peptide was synthesised and cleaved from the resin by Alan Cuthbertson[126]. An analytical hplc (column 4, 5.0ml loop) was recorded of the crude material available (114mg). This showed the main peak eluting at 63% B. Purification of the crude peptide (20mg) was carried out by preparative hplc (column 6) using a gradient of 10-45% B over 15 minutes. After lyophilisation, 10mg of the *title* compound was obtained; amino acid analysis: $Asn_1$0.89, $Ser_1$0.87, $Gln_1$1.20, $Val_1$1.08, $Tyr_1$0.93, $Phe_3$2.98, $Lys_1$1.01; m/z (FAB) 1178 [M^+]; HRMS $C_{59}H_{78}N_{12}O_{14}$ requires 1179.58387 found 1179.58217; hplc (column 4, 5.0ml loop) R_t=19minutes.

Purification of 34-42 mNGF (25)

K.E.V.T.V.L.A.E.V

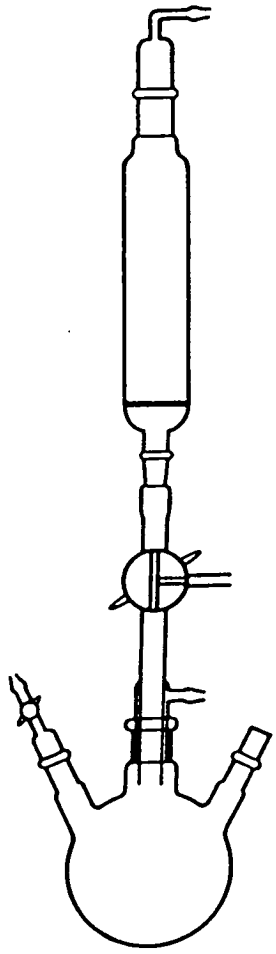
This peptide was synthesised and cleaved from the resin by David Tumelty[127]. An analytical hplc (column 1, 2.0ml loop) was recorded of the crude peptide available (180mg). This showed a single peak eluting at 40% B with a smaller peak eluting at 45% B. Purification of the crude peptide (50mg) by preparative hplc (column 6) was carried out using a gradient of 10-25% B over 15 minutes. After lyophilisation, 22mg of the *title* compound was obtained; amino acid analysis: $Thr_1$1.07, $Glu_2$2.13, $Ala_1$0.91, $Val_3$2.77, $Leu_1$1.00, $Lys_1$1.09; m/z (FAB) 987 [M^+], HRMS $C_{44}H_{78}N_{10}O_{15}$ requires 987.57264 found 987.57279; hplc (column 1, 2.0ml loop) R_t=11minutes.

Purification of 50-60 hNGF (26)

K.Q.Y.F.F.E.T.K.C.R.D

This peptide was synthesised and cleaved from the resin by David Tumelty[127]. An analytical hplc (column 2, 2.0ml loop) was recorded of the crude peptide available (400mg). The main peak eluted at 43% B. Purification of the crude peptide (30mg) was carried out by preparative hplc (column 6) using a gradient of 10-35% B over 25 minutes. After lyophilisation, 10mg of the *title* compound was obtained; amino acid analysis: $Asp_1$1.03, $Thr_1$1.05, $Glu/Gln_2$2.16, $Tyr_1$1.01, $Phe_2$2.11, $Lys_2$2.09, $Arg_1$1.09; m/z (FAB) 1463.7 [M^+], HRMS $C_{66}H_{97}N_{17}O_{19}$ requires 1464.69456 found 1464.70087 ; hplc (column 2, 2.0ml loop) R_t=13minutes.

APPENDIX 1



The nitrogen bubbler apparatus for the manual synthesis of peptides. The resin-peptide is contained within the cylindrical reaction vessel and is agitated with nitrogen. Reagents are added manually from the top and removed under suction through the bottom of the reaction vessel. The lower reservoir collects waste solvents and reagents.

APPENDIX 2

Amino Acid Sequence		p75 assay % Inhibition @10μM	*Trk* assay % Inhibition @100μM
hNGF$^{25-40}$	**KTTATDIKGKEVMVLG**	**20,0**	**10.7**
mNGF$^{34-42}$	KEVTVLAEV	4	8
hNGF$^{46-54}$	**NSVFKQYFF**	**18**	**11**
hNGF$^{50-60}$	**KQYFFETKCRD**	**15**	**10**
hNGF$^{59-67}$	**RDPNPVDSG**	**64**	**21**
mNGF$^{59-67}$	RASNPVESG	-16	2
hNGF$^{81-107}$	**TTTHTFVKALTMDGKQAAWRFIRIDTA**	**38,45**	**0**
mNGF$^{95-120}$	KQAAWRFIRIDTAC(H)VC(H)VLSRK ATRRG	61	24,0
mNGF$^{95-120}$	dimer through disulphides	77 (IC_{50} 0.32μM)	31,10
hNGF$^{1-8,100-114}$	**SSSHPIFHauaRFIRIDTACVCVLSR (oxid)**	**63,52**	**83.4**
mNGF$^{1-8,100-114}$	SSTHPVFHauaRFIRIDTACVCVLSR (oxid)	34	-
NGF$^{32-34,95,100-114}$	**KGKauaKauaRFIRIDTACVCVLSR (oxid)**	**60,82**	**30**
NGF$^{32-34,95,100-114}$	**KGKauaKauaRFIRIDTAC(Acm)V C(Acm)VLSR**	**49**	**0**
NGF$^{32-34,95,100}$	**KGKauaKauaR**	**11**	**0.4**
hNGF$^{50-70,100-114}$	**KQYFFETKCRDPNPVDSGCRGauaR FIRIDTACVCVLSR (oxid)**	**10**	**100**
mNGF$^{50-70,100-114}$	RQYFFETKCRASNPVESGCRGauaR FIRIDTACVCVLSR (oxid)	72	-
hNGF$^{50-70,100-114}$	**KQYFFETKCRDPNPVDSGC(phAcm)R GauaRFIRIDTACVC(phAcm)VLS (oxid)**	**10**	-
NGF$^{100-114}$	**RFIRIDTACVCVLSR (oxid)**	**36**	**100**
NGF$^{100-114}$; Cha^{101}	**RChaIRIDTACVCVLSR (oxid)**	**30**	**100**
NGF$^{100-114}$; Tyr^{101}	**RYIRIDTACVCVLSR (oxid)**	**10**	**58**
NGF$^{100-114}$; Orn^{100}	**OFIRIDTACVCVLSR (oxid)**	**12**	**61**
NGF$^{100-114}$; Lys^{100}	**KFIRIDTACVCVLSR (oxid)**	**12**	-
***p*-hydroxyphenylacetic acid-NGF$^{100-114}$**		**34**	**100**
NGF$^{100-107}$.turn.$^{111-114}$	**RFIRIDTAturnVLSR**	**-17**	**100**
mNGF$^{111-120}$	VLSRKATRRG	32	0

(bold = human or conserved mouse and human sequence; other = mouse sequence)

The following list of peptides were synthesised by Alan Cuthbertson[126] and David Tumelty[127] and tested on the p75 assay. All sequences are conserved human and mouse except those specifically marked as hNGF.

	Amino Acid Sequence	p75 assay % Inhibition @ XμM (or IC_{50})
NGF^{10-17}	GEFSVCDS	0@100μM
NGF^{15-23}	CDSVSVWVG	0@100μM
NGF^{14-28}	VCDSVSVWVGDKTTA-NH_2	19@100μM
NGF^{14-28}	Ac-VCDSVSVWVGDKTTA-NH_2	14@100μM
NGF^{14-28}	VSDSVSVWVGDKTTA-NH_2	25@100μM
NGF^{14-28}	Ac-VSDSVSVWVGDKTTA-NH_2	15@100μM
NGF^{19-22}	SVWV	14@100μM
$hNGF^{19-22}$	Ac-SVWV	11@100μM
NGF^{21-36}	WVGDKTTATDIKGKEV	6@100μM
$hNGF^{25-54}$	KTTATDIKGKEVMVLGEVNINNSVFKQYFF	21@100μM
$hNGF^{34-42}$	KEVMVLGEV	2@100μM
NGF^{41-46}	EVNINN	16@100μM
$hNGF^{46-54}$	NSVFKQYFF	0@100μM
$hNGF^{50-60}$	KQYFFETKCRD	16@100μM
$hNGF^{50-54}$	KQYFF	0@100μM
$hNGF^{59-67}$	RDPNPVDSG	0@100μM
NGF^{66-72}	SGCRGID	15@100μM
NGF^{74-91}	KHWNSYCTTTHTFVKALT	0@100μM
$hNGF^{81-120}$	TTTHTFVKALTMDGKQAAWRFIRIDTACVC VLSRKAVRRA (oxid)	96@100μM
$hNGF^{81-120}$	reduced	90@100μM
$hNGF^{95-120}$	KQAAWRIFIRIDTACVCVLSRKAVRRA	88@100μM (IC_{50} 15μM)
$hNGF^{95-120}$	Cys^{108} S-*t*-butyl, Cys^{110} Acm	95@100μM
$hNGF^{95-120}$	Cys^{110}Acm	10@100μM
$hNGF^{95-120}$	oxidised	88@100μM
NGF^{95-116}	KQAAWRFIRIDTACVCVLSRKA (oxid)	92@100μM
NGF^{95-116}	reduced	(IC_{50} 20μM)
NGF^{95-105}	KQAAWRFIRID	18@100μM

	Amino Acid Sequence	% Inhibition @ XμM
NGF^{97-116}	AAWRFIRIDTACVCVLSRKA	95@100μM (IC_{50}10μM)
$NGF^{100-116}$	RFIRIDTACVCVLSRKA(oxid)	90@100μM (IC_{50} 8μM)
$NGF^{100-116}$	reduced	30@100μM
$BDNF^{100-116}$	RFIRIDTSCVCTLTIKR	(IC_{50}5μM)
$NT\text{-}3^{100-116}$	RWIRIDTSCVCALSRKI	(IC_{50}13μM)
$NGF^{100-116}$	$Gly^{111-113}$, $Cys^{108,110}$Acm	24@30μM
$NGF^{100-116}$	$Gly^{111-113}$, $Cys^{108,110}$oxid	27@30μM
$NGF^{100-116}$	$Gly^{103,104-106,109,111-113}$	6@30μM
$NGF^{100-115}$	RFIRIDTACVCVLSRK (oxid)	84@30μM (IC_{50}12μM)
$NGF^{100-115}$	RFIRIDTACVCVLSRK-NH_2 (oxid)	(IC_{50}3μM)
$NGF^{100-115}$	RFIRIDTACVCVLSRK-NH-heptyl (oxid)	68@100μM
$NGF^{100-115}$	RFIRIDTACCVLSRK(oxid)	24@100μM
$NGF^{100-115}$	RFIRIDTACCVLSRK(reduced)	28@100μM
$NGF^{100-114}$	RFIRIDTACVCVLSR (oxid)	(IC_{50}3μM)
$NGF^{100-114}$	RFIRIDTAPenVPenVLSR (oxid)	-24@10μM
$NGF^{100-114}$	RFRIDTAPenVPenVLSR di-Acm protected	-26@10μM
$NGF^{100-114}$	(cyclised by lactam bridge between Asp^{105} side chain and N-terminus) $Cys^{108,110}$Acm	4@10μM
$NGF^{100-114}$	D-Leu^{102} (oxid)	38@10μM
$NGF^{100-114}$	D-Ile^{104} (oxid)	15@10μM
$NGF^{100-114}$	D-Val^{111} (oxid)	21@10μM
$NGF^{100-114}$	D-$Val^{109,111}$ (oxid)	(IC_{50}12.5)
$NGF^{100-114}$	Aib^{109} (oxid)	23@10μM
$NGF^{100-114}$	Aib^{108} (oxid)	70@10μM
$NGF^{100-114}$	Aib^{111} (oxid)	27@10μM
$NGF^{100-114}$	Nle^{113} (oxid)	(IC_{50}1.2)
$NGF^{100-114}$	$Nle^{106,113}$ $Cys^{108,110}$Acm	44@10μM
$NGF^{100-113}$	RFIRIDTACVCVLS (oxid)	23@30μM
$NGF^{100-110}$	RFIRIDTACVC (oxid)	30@100μM
$NGF^{100-110}$	reduced	0@100μM
$NGF^{101-115}$	FIRIDTACVCVLSRK (oxid)	31@30μM
$NGF^{103-116}$	RIDTACVCVLSRKA (oxid)	15@100μM

	Amino Acid Sequence	% Inhibition @ XμM
$NGF^{106-116}$	TACVCVLSRKA (oxid)	20@100μM
$NGF^{106-116}$	reduced	18@100μM
$NGF^{111-116}$	VLSRKA	13@100μM
$hNGF^{50-70,95-120}$	KQYFFETKCRDPNPVDSGCRGGGGGQAA WRFIRIDTACVCVLSRKAVRRA ($Cys^{58,108}$ S-*t*-butyl, $Cys^{68,110}$Acm)	IC_{50}2μM
$hNGF^{50-70,95-120}$	$Cys^{58,108}$ oxid, $Cys^{68,110}$Acm	27@30μM

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COURSES ATTENDED

Organic Research Seminars, various speakers, 1991-94

The Royal Society of Edinburgh Symposium on Protein Engineering, various speakers, Edinburgh, 1992

The Peptide and Protein Group of the Biochemical Society and the Royal Society of Chemistry 1992 Gregynog meeting, various speakers, Wales, 1992

Medicinal Chemistry, Professor R.Baker, Merk Sharp and Dohme, 1992-93

Advances in Organic chemistry, various speakers, University of Edinburgh, 1992-93

Chemical Development in the Pharmaceutical Industry, various speakers, SmithKline Beecham, 1993-94

NMR spectroscopy, Dr. I.H.Sadler, Edinburgh University, 1993