INVESTIGATION OF RECEPTOR BINDING RELATIONSHIPS IN NERVE GROWTH FACTOR

by

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DECLARATION

This thesis is submitted in part fulfilment of the requirements for 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 at any other, University.

To my late father,
William J. Tumelty
(1939-1983)

my mother, Maureen,
and to Caroline.

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ABSTRACT

The synthesis of a series of peptides spanning the entire sequence of the protein nerve growth factor (NGF) has been carried out using solid-phase peptide synthesis (SPPS). A twenty-two residue peptide sequence from the carboxyl-terminal region of NGF was found to inhibit the binding of native NGF to its known receptor, in a cell-free assay. This fragment may define an area in the native NGF molecule which is important for receptor interactions.

The optimisation of this initial fragment found that the inhibitory effect was retained when the fragment was reduced to a fifteen residue, disulphide-linked peptide. Further attempts at optimising this smaller fragment have been carried out by substituting unnatural amino-acids into the sequence, as well as altering the conformation around the disulphide bond.

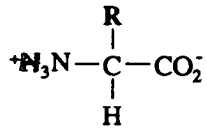
Several of the smaller NGF peptides have been investigated by high-field nuclear magnetic resonance (N.M.R.). The complete assignment of several analogues of the fifteen residue, disulphide-linked peptide has been achieved. Analysis of the 'through-space' interactions indicated that these peptides adopt only random conformations in solution.

As a prelude to a total NGF synthesis, the assembly of the well-characterised protein, hen egg white lysozyme (HL) has been attempted in order to test the current methods for approaching the synthesis of large, multi-cysteine peptides. Some evidence that the peptide chain was successfully assembled has been presented and the difficulties encountered in attempts to purify and fold the protein are discussed.

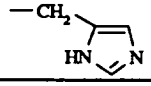
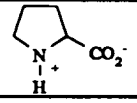
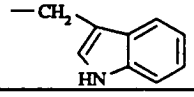
ABBREVIATIONS

Ac	acetyl	**HFIP**	1,1,1,3,3,3-hexafluoro-2-propanol
ACh	acetylcholine	**HL**	hen egg white lysozyme
AChE	acetylcholinesterase	**HPLC**	high-performance liquid chromatography
Acm	acetamidomethyl	**HOBt**	1-hydroxybenzotriazole
AcOH	acetic acid	***hrms***	high-resolution mass spectrometry
AD	Alzheimer's disease	**IC_{50}**	concentration producing 50% inhibition
AE	active ester	**IR**	infrared
BDNF	brain-derived neurotrophic factor	**LHRH**	luteinising hormone releasing hormone
Boc	*tert*-butyloxycarbonyl	**MS**	mass spectroscopy
BSA	bovine serum albumin	**NGF**	nerve growth factor
Bum	*tert*-butoxycarbonyl	**N.M.R.**	nuclear magnetic resonance
Bu^t	*tert*-butyl	**NOESY**	nuclear Överhauser enhancement spectroscopy
Bzl	benzyl	**NTB**	2-nitro-5-thiobenzoate
CAT	choline acetyltransferase	**NT-3 (or 4)**	neurotrophin-3 (or -4)
CHO	Chinese hamster ovary	**NTF**	neurotrophic factor
CNS	central nervous system	**$p140^{trk}$**	protein receptor product of *trk* gene
COSY	correlated spectroscopy	**$p75^{NGFR}$**	low-affinity NGF receptor
DBU	1,8-diazabicyclo-[5.4.0.]-undec-7-ene	**PG**	protecting group
DCC	1,3-dicyclohexylcarbodiimide	**Pmc**	2,2,5,7,8-pentamethylchroman-6-sulphonyl
DCM	dichloromethane	**PNS**	peripheral nervous system
DIC	1,3-diisopropylcarbodiimide	**ROESY**	rotating frame NOESY
DKP	2,5-diketopiperazine	**SA**	symmetrical anhydride
DMAP	4-dimethylaminopyridine	**SBu^t**	*tert*-butylsulphenyl
DMF	N,N-dimethylformamide	**SPPS**	solid-phase peptide synthesis
DMSO	dimethylsulphoxide	**TBNS**	2,4,6-trinitrobenzenesulphonic acid
DPPA	diphenylphosphoryl azide	**TBP**	tri-n-butylphosphine
DTNB	5,5'-dithiobis(5-nitrobenzoic acid)	**TEA**	triethylamine
DTT	dithiothreitol	**TFA**	trifluoroacetic acid
EDT	1,2-ethanedithiol	**TFE**	2,2,2-trifluoroethanol
EMS	ethyl methyl sulphide	**TFMSA**	trifluoromethanesulphonic acid
FAB	fast-atom bombardment	**$(CF_3CO_2)_3Tl$**	thallium (III) trifluoroacetate
FID	free induction decay	**TOCSY**	total correlation spectroscopy
Fmoc	9-fluorenylmethoxycarbonyl	***trk***	tropomyosin receptor kinase
HBr	hydrogen bromide	**Trt**	triphenylmethyl
HF	hydrogen fluoride	**UV**	ultraviolet

CODES FOR AMINO ACIDS



All amino acids used were of the L-configuration unless otherwise stated

AMINO ACID	SIDE-CHAIN (R)	3-LETTER CODE	1-LETTER CODE
Alanine	CH_3	Ala	A
Arginine	$(CH_2)_3NHC(NH)NH_2$	Arg	R
Asparagine	CH_2CONH_2	Asn	N
Aspartic acid	CH_2COOH	Asp	D
Cysteine	CH_2SH	Cys	C
Glutamic acid	$(CH_2)_2COOH$	Glu	E
Glutamine	$(CH_2)_2CONH_2$	Gln	Q
Glycine	H	Gly	G
Histidine		His	H
Isoleucine	$CH(CH_3)C_2H_5$	Ile	I
Leucine	$CH_2CH(CH_3)_2$	Leu	L
Lysine	$(CH_2)_4NH_2$	Lys	K
Methionine	$(CH_2)_2SCH_3$	Met	M
Phenylalanine	$CH_2C_6H_5$	Phe	F
Proline		Pro	P
Serine	CH_2OH	Ser	S
Threonine	$CH(OH)CH_3$	Thr	T
Tryptophan		Trp	W
Tyrosine	$CH_2C_6H_4OH$	Tyr	Y
Valine	$CH(CH_3)_2$	Val	V

GLOSSARY OF BIOLOGICAL TERMS

axons	the long process arising from a nerve cell or neuron, which transmits an impulse or action potential away from the cell body
bacteriophage	a virus containing DNA (sometimes RNA) which infects bacteria
central nervous system (CNS)	main ganglia of nervous system with associated nerve cords, consisting usually of a brain and a dorsal or ventral nerve cord
cholinergic	nerve fibre secreting acetylcholine at its ends when nerve impulses arrive there
Down's syndrome	congenital condition caused by presence of extra copy of chromosome 21, characterised by mental retardation, mongoloid facial features and reduced life expectancy
endocytosis	process by which a cell engulfs material by the infolding of the plasma membrane
ganglia	mass of nervous tissue, encapsulated in connective tissue and containing many synapses and cell bodies. They generally act as integrative centres for areas of high sensory input/motor output
gene cloning (also recombinant DNA technology)	process of isolating a gene DNA sequence, inserting it into a plasmid (a piece of circular DNA) and the subsequent large scale growth of the plasmid in a suitable bacterial host. The appropriate gene (or its protein product) may then be recovered from bulk bacterial cultures
G-proteins	guanyl-nucleotide-binding proteins which act as intermediaries between receptors and secondary messengers (e.g. adenylate cyclase) in signal transduction mechanisms
hippocampus	tract of nervous tissue running back from olfactory lobe to posterior end of the cerebrum
kinase	an enzyme which catalyses the phosphorylation of its substrate by ATP. Act as molecular 'switches' in signal transduction
lysosomes	single-membraned organelle containing hydrolytic enzymes, generally involved in degradative processes
microtubules	minute tubules which occur in groups in cells and involved in cell integrity and orientating the movements of cytoplasm
monoclonal antibody	antibody produced by a cell clone thus comprising only a single species of immunoglobulin molecule
muscarinic receptors	receptor in cholinergic synapses, sensitive to plant poison muscarine
neurons	principal cell types of nervous system, specialised for transmission of information
oncogenes	genetic locus originally identified in RNA tumour viruses, which is capable of the transformation of the host cell. Implicated as the cause of certain cancers
peripheral N.S.	rest of nervous system not including CNS. Consists of nerves that serve as relay channels between receptors/effectors and the integrative parts of the CNS
proto-oncogenes	gene required for normal function in an organism, but which has mutated to become an oncogene
tyrosine kinase	kinase present in intracellular domains of several receptors e.g. EGF, insulin. Binding of ligand stimulates autophosphorylation onto certain tyrosine residues, which activates the kinase to phosphorylate tyrosine residues on its target proteins to initiate signal transduction

CONTENTS

CHAPTER ONE : INTRODUCTION

1.1. ALZHEIMER'S DISEASE

1.1.1. General

Neurological disorders have amongst them some of the most distressing of all human illnesses, such as Alzheimer's Disease (AD) and Huntingdon's Chorea. Typically, the signs and symptoms appear slowly over a period of years, usually in middle or late life, and lead inexorably to dementia (the severe impairment of cognitive function) and eventually death, in a gravely debilitated state, within five to ten years after onset. In the case of AD, the dementia is often heralded by impairments in recent memory. From this point, the higher cognitive functions deteriorate; sufferers lose the ability to read, write or use language properly and their emotional state is commonly affected. Eventually, sufferers will unfortunately die of some complication that afflicts bedridden patients.

The magnitude of AD occurrence was not appreciated until the early 1970's. In the U.S.A. (for which figures are available), it is estimated that 100,000 people die each year of AD-related illnesses. The cost, apart from the human suffering, is reckoned to be $80 billion/annum, primarily in custodial care. The incidence is linked to aging, with a ten or twenty-fold increase between the ages of 60-80; the prevalence may exceed 50% in those aged 95 or more. As the geriatric segment of the population, in the Western World in general, is predicted to grow over the next fifty years or so, it can be expected that the incidence of AD will similarly increase.

1.1.2. Diagnosis and Study of AD

There are certain substantial obstacles which hinder the study of potential AD therapies. These are summarised below in Table 1.1.

Table 1.1 Hurdles to the investigation of AD
a.) No diagnostic tests exist to detect the early onset of the disease
b.) Aetiology unknown, but AD possibly has multiple causes
c.) Not known if AD is a homogeneous or heterogeneous disease[1]
d.) No truly effective therapeutics exist at present
e.) No suitable animal models exist for testing therapeutic strategies

AD is currently diagnosed by the exclusion of other possible causes e.g. drug intoxication, vitamin B_{12} deficiency, for the observed clinical manifestations. Neuropsychological tests may be used to assess the degree of dementia. While diagnosis of the condition is problematical, post-mortem examination of the brain tissue of AD sufferers has clearly shown that substantial changes to the brain's architecture, which typify the disease, have occurred during the course of the disease. The most common changes are;

1. A large number of neuritic plaques appear: these consist of abnormal neurites, associated with a core of an insoluble protein fragment called β-amyloid. In addition, altered glial cells (involved in neuron function and protection) are present in the plaques[2].

2. Neurofibrillary tangles, dense bundles of abnormal fibres, can be observed in the cytoplasm of certain neurons. These fibres are composed of a naturally-occurring neuronal protein called tau. These tangles (as well as the plaques) are found mainly in the hippocampus and neocortex regions[3] (ref. Fig. 1.2).

3. There is extensive degeneration of specific neuronal systems, which use acetylcholine (cholinergic neurons) and noradrenaline (adrenergic neurons) as their transmitters.

Some of these observations have helped in the formulation of models which try to account for the observed biochemical and pathological changes that occur during the course of the disease. Below are listed possible factors involved in the aetiology and pathogenesis of AD;

a.) Genetic defects e.g. AD is linked to Down's syndrome.
b.) Abnormal proteins e.g. β-amyloid.
c.) Infectious agents e.g. prions.
d.) Toxins e.g. aluminium.
e.) Changes in cerebral blood flow e.g. 'hardening of the arteries' observed in AD.
f.) Neurotransmitter deficits e.g. cholinergic hypothesis.

These factors are considered in more detail below.

1.1.3. Possible Models to Explain the Cause(s) and Effects of AD

Each of the above factors has been considered as a basis for working models to aid in the understanding of how AD may be caused, and how the disease causes its effects in sufferers. Each model is supported by some observational or experimental evidence, although each is contradicted by other evidence and none seems to, so far, account for all of the symptoms and pathological findings.

The genetic model for AD has received much attention recently[4]. While AD has generally been considered to be a sporadic disorder, increased risk has been reported in the relatives of AD patients[5]. Individuals with Down's syndrome, who reach the age of forty or more, almost always develop AD-type symptoms. These individuals have three copies of chromosome 21, which also contains the gene coding for the amyloid precursor protein (APP)[2]. The β-amyloid fragment, which is present in the neuritic plaques, is erroneously formed from this APP by abnormal enzymic processing[6]. The resultant amyloid fragment, which is released from APP, becomes deposited in the brain cortex and also around blood vessels. The theory has been forwarded that APP is over-produced in Down's syndrome, leading to the adventitious processing of the protein by inappropriate enzymes (in an attempt to reduce the APP level), which then release the β-amyloid protein. Recently, it has been found that mutations in the APP gene are linked with early-onset, familial AD (FAD)[7,8]. These mutations cause a Val to Ile, Gly or Phe change in APP, which may be the site at which the incorrect processing occurs. Although this mechanism may be involved in FAD, it is unclear if this would encompass all AD cases, and whether or not the amyloid deposition is a primary event in the initiation of the illness. Nevertheless, this remains a very active area of research in the search for AD causation and treatments.

There is some support for the theory that AD may be caused by an infectious agent, perhaps a virus. This derives from certain clinical and neuropathological similarities to Creutzfeldt-Jakob disease. This is a rare brain disorder, usually affecting individuals between 55-75 years old. It leads to a progressive dementia, disturbances in movement and causes death within one to two years of onset. The infective agent was considered to be a 'slow' virus, in that several years were required between exposure to the agent and the onset of symptoms: this was discovered as experimental animals could be infected by brain extracts of infected tissue. However, it has been shown that the agent is a curious protein particle called a

prion[9], which forms rod-like aggregates. Attempts to transmit AD in a similar fashion have been unsuccessful, although it could be argued that other predisposing conditions may be required for such an infection to occur.

The presence of elevated aluminium (Al) levels in the brain of AD patients as a causative factor, has often been put forward: aluminium is present in aluminosilicates in the neuritic plaques and it has been suggested that Al is involved in the early stages of plaque formation. While this continues to be a controversial area, again whether Al is implicated in the onset of AD or simply the result of, for example, an increased affinity of the neurofibrillary tangles for Al, remains to be seen. Similarly, the decreased cerebral blood-flow associated with AD may simply reflect the reduction in the neurons responsible for controlling blood delivery, rather than being a causative factor itself.

1.1.4. The Cholinergic Hypothesis for AD

The most widely accepted hypothesis for the cognitive deficits seen in AD involves the cholinergic system, although this is unlikely to account for some of the other deficits caused by the disease. The cholinergic neurons, which degenerate in the brains of AD sufferers, are those forming the ascending cholinergic pathways of the basal forebrain. The cell bodies of these neurons are located in the septal area and basal nucleus areas of the brain. The axons of these neurons innervate the hippocampus and parts of the neocortex[10] (Fig. 1.2).

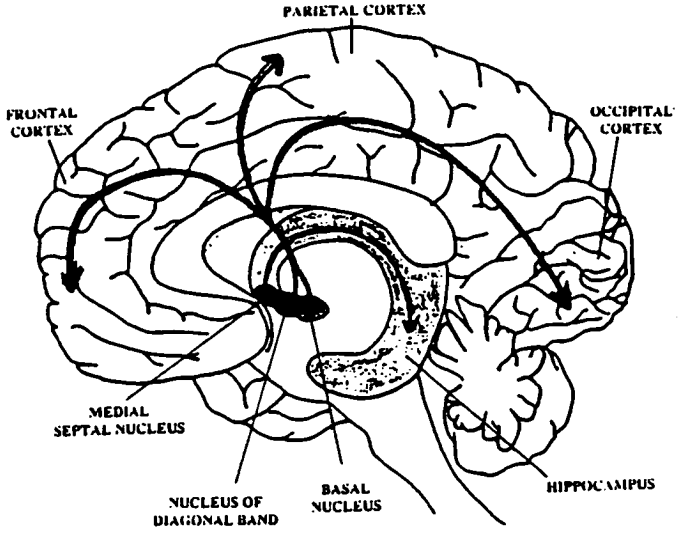


Fig. 1.2 Major cholinergic pathways implicated in AD
(arrows indicate areas into which the neurons extend)

The loss of these neurons is evidenced by the associated decrease in the levels of certain cholinergic marker enzymes e.g. choline acetyltransferase (CAT) and acetylcholinesterase (AChE). Figure 1.3 shows how the cholinergic neurons use these enzymes, and the neurotransmitter acetylcholine (ACh), to carry out their normal functions. As well as the above, there is reduced acetylcholine synthesis in the target areas and the loss of the previously mentioned cholinergic cell bodies. The decrease in general cholinergic factors correlates with the increase in the number of plaques and with the degree of dementia[3]. It is the loss of these specific neurons which is generally regarded as the principal reason for the memory deficits characteristic of AD[11]. In support of this view, is the fact that many researchers have previously shown that disrupting cholinergic transmission affects proper memory functioning[12].

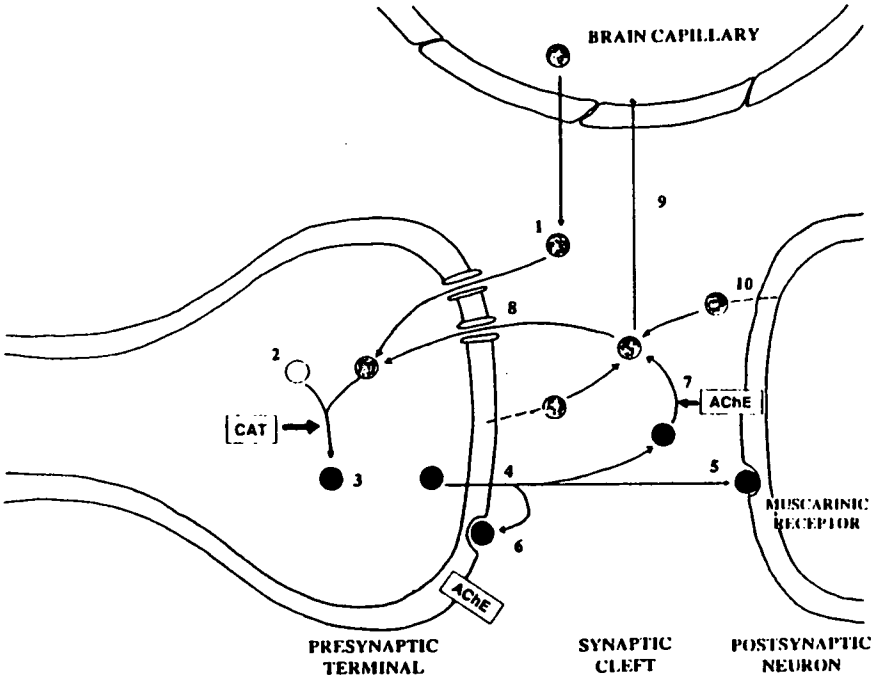


Fig. 1.3 Function and metabolism of acetylcholine in the cholinergic system
Choline (1, grey) passes into the synaptic cleft from blood vessels and is taken into the presynaptic cholinergic terminal via transport systems. Here, acetylcholine (ACh, 3, black) is synthesised from choline and acetyl-coenzyme A (2, white) by the enzyme CAT. ACh is stored in vesicles and released into the synaptic cleft (4) upon neuron depolarisation (caused by an incoming nerve impulse). ACh crosses the cleft to activate mainly muscarinic receptors (5) and hence propagate the signal. ACh release is also self-modulating, by affecting receptors on the presynaptic terminal (6). The action of ACh is rapidly terminated by hydrolysis by AChE, which is located on both pre- and post-synaptic membranes (7). The choline released from this hydrolysis (7) re-enters the pre-synaptic terminal (8) or enters blood vessels (9). The breakdown of phosphatidylcholine (lecithin), a cell membrane constituent, also contributes to choline supply (10).

These findings led to the hypothesis that drugs to stimulate the cholinergic system might revive at least some of the lost mental faculties of AD patients. The simplest approach would be to use replacement drugs which may increase nerve communication by elevating the acetylcholine levels. Unfortunately, cholinergic precursors e.g. choline, lecithin (ref. Fig. 1.3), do not readily cross the blood-brain barrier thus would not be useful as drugs. Alternative approaches have been considered:

a.) Inhibiting cholinesterase: this may raise the levels of acetylcholine in cholinergic neurons and hopefully enhance cholinergic function. Several pharmaceutical companies have produced acetylcholinesterase inhibitors e.g. Tacrine (Parke-Davis), Physostigmine derivatives [Merck, Sharp and Dohme, (MSD)], (Fig. 1.4).

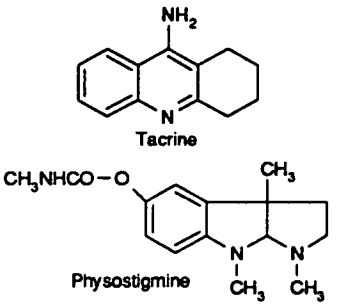


Fig. 1.4 AChE inhibitors

b.) Increasing acetylcholine release from intraneuronal stores: some compounds e.g. 4-aminopyridine appear to alter ACh metabolism to elevate normal levels.

c.) Using acetylcholine mimics: these may interact with ACh (muscarinic) receptors in the post-synaptic membrane and hence perhaps emulate signal transmission[13]. However, three subtypes exist; the M_1 muscarinic receptor sub-type is the one involved in cholinergic function and would make an ideal target for drug development, but the M_2 sub-type is involved in cardiovascular function, while M_3 is associated with the digestive tract, thus finding a M_1-specific drug may prove to be difficult. Some muscarinic drug agonists are currently in clinical trials e.g. a thiopilocarpine derivative (Sandoz), and others are being examined e.g. quinuclidine (MSD), (Fig. 1.5).

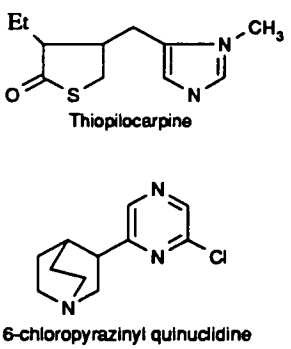


Fig. 1.5 Muscarinic receptor agonists

Unfortunately, all of these strategies have failed to produce significant improvements in AD patients; furthermore such therapies may only limit the symptoms rather than prevent the disease. A better therapy may be one which promotes the survival of

these cholinergic neurons that degenerate during AD. Such a treatment may be able to prevent the onset of AD or stop further deterioration of mildly afflicted patients.

1.1.5. Neurotrophic Factors and AD

In recent years, evidence has accumulated which shows that the survival (during development), and maintenance of specific groups of neurons is influenced by certain proteins, termed neurotrophic factors (NTFs). The best studied of these is nerve growth factor (NGF), which was known to affect the survival and function of peripheral sympathetic and sensory neurons[14,15]. Other NTFs have subsequently been discovered which act on different neuronal groups. The discovery of these proteins led to the concept that a specific group of neurons may have an absolute requirement for a certain NTFs.

Surprisingly, in the early 1980's, NGF was shown to act as a neurotrophic factor in the central nervous system (CNS), as well as the peripheral nervous system[3]. Significantly, NGF was seen to be required by the exact same neurons which are involved in memory functioning and which degenerate in AD. In 1983, Hefti[16] suggested that the lack of NGF may be a direct cause of the pathogenesis of AD. Since then, no evidence to support this view has been uncovered, and it appears that NGF levels are not, in fact, reduced in AD[17]. Nevertheless, NGF and related proteins may potentially represent a new avenue for research into the aetiology and treatment of AD and other neurological disorders, as will be outlined in the following section.

1.1.6. Possible Role for NGF in the Treatment of AD

Several studies have shown that NGF can help in the survival of injured neurons, and even help in the regeneration of damaged axons. Neurons in the medial septal nucleus (and part of the diagonal band nucleus) project into the hippocampus (ref. Fig. 1.2) in a dense bundle of fibres, through a region called the fimbria/fornix: about half of the neurons sending axons through this narrow region are cholinergic. When this pathway is surgically transected in experimental animals e.g. adult rats, many of the septal/diagonal band neurons gradually degenerate and die[18]. An explanation for this could be that the neurons became deprived of their supply of a NTF (e.g. NGF) supplied by the target areas of the hippocampus, which the axons of these neurons innervate. It was hoped that the administration of exogenous NGF to

these axotomised neurons might prevent their degeneration; also NGF might also allow these neurons to regenerate their transected axons and connect back to the hippocampus[18]. Several studies have indeed shown that direct infusion of NGF into the brains of axotomised rats will prevent the loss of these cholinergic neurons[19]. More recently, it has also be demonstrated that the continuous infusion of murine or recombinant human NGF into similarly axotomised monkeys can also protect these neurons from degeneration[20-22]. It may be the case, therefore, that treating AD patients with NGF could prevent the deterioration of these same cholinergic neurons, and hence ameliorate the memory loss associated with the disease[23].

If such a treatment is to be successful, a major problem would be in how to administer the NGF protein to the required areas of the brain. Proteins do not normally cross the blood-brain barrier (BBB), thus alternative strategies to possibly increase the normal concentrations of NGF available have been suggested;

1. Direct injection/infusion of exogenous NGF (perhaps via a small pump mechanism)[23].
2. Transplantation of a cell-line which secretes genetically recombinant human NGF[18,24].
3. Augmentation of the endogenous NGF levels[25].

Methods 1 and 2 will obviously provide problems due to the invasiveness of the procedures, although they have had some success in animal models. The evolving understanding of the regulation of NGF synthesis may eventually help with the realisation of a strategy like method 3.

Perhaps a more realistic approach may be to try to develop a drug which could act as an NGF agonist. A small molecule could more easily cross the BBB, although it would presumably have to be directed to the appropriate areas of the brain to promote the survival of the cholinergic neurons there, without influencing those in the PNS. To design such a drug will require a full understanding of the structure of NGF and on how it produces its biological effects. At the commencement of the work described here, only limited information had available on both of these matters.

1.2. NERVE GROWTH FACTOR (NGF)

1.2.1. The Structure of NGF

NGF was first discovered as a factor from mouse sarcoma cells that caused extensive growth in chick embryo neurons[26,27]. Since then, NGF activity has been found in many tissues, although few mammalian sources are rich enough to allow the purification and biochemical characterisation of their NGF. The major exception to this is the mouse submaxillary gland was found to contain unusually large quantities of NGF[30], the isolation of which has permitted the complete structural elucidation of the molecule from that source. NGF from several other species e.g. human[28], chicken[29], rat[29], has also been produced by recombinant DNA technology, which has enabled their subsequent characterisation. It appears that most NGF molecules from different sources have significant homology with the murine structure, and may have similar three-dimensional structures.

Murine NGF (mNGF) actually exists as a complex of three types of subunit: α, β and γ[31]. The β-subunit represents the active NGF molecule (which exists as a dimer). There is considerable homology between the α and γ forms; γ is a trypsin-like protease and may be involved in the processing of the mNGF complex, thus releasing the active β form. The α-subunit is thought to protect NGF from proteolysis and may regulate its activity. β-NGF is present in the complex as a noncovalently-linked dimer with a M.W. of 26.5 kDal. This dimer is quite stable and remains intact at biologically relevant concentrations[32]. Strong denaturants can separate the dimer into two identical chains, each 118 residues long (120 in human NGF). Both monomers and the dimer are biologically active[33]. The primary sequence for the murine chain was elucidated in 1971[34] and since then, many NGFs from various species have been predicted by molecular cloning[35] (Fig. 1.6). There is a high degree of sequence homology between all NGFs so far discovered; all contain three intrachain disulphides, at conserved positions, and reduction of these bonds causes the complete loss of biological activity[36]. Murine β-NGF is transcribed from a single gene copy, localised on chromosome 3 (1 in humans). From this the β-NGF is synthesised as a large precursor (prepro-NGF), which is processed to form a further precursor (33.8 kDal). This itself is then processed into the mature 13.2 kDal protein. The processing occurs at both ends, with cleavage between two basic residues in each case. The γ-subunit is thought to be responsible for these actions[37].

For many years, only limited progress had been made in elucidating the secondary and tertiary structure of NGF. Spectroscopic analysis had shown that the protein did contain a high amount of antiparallel β-sheet (between 53-67%), with a low amount of α-helix also apparent (0-11%)[38]. Although crystals of murine NGF had been available since the mid-1970's[39], no detailed three-dimensional structure could be obtained until late-1991, when a crystal structure, accurate to 2.3Å resolution, was disclosed[40]. This structure described in section 2.11.

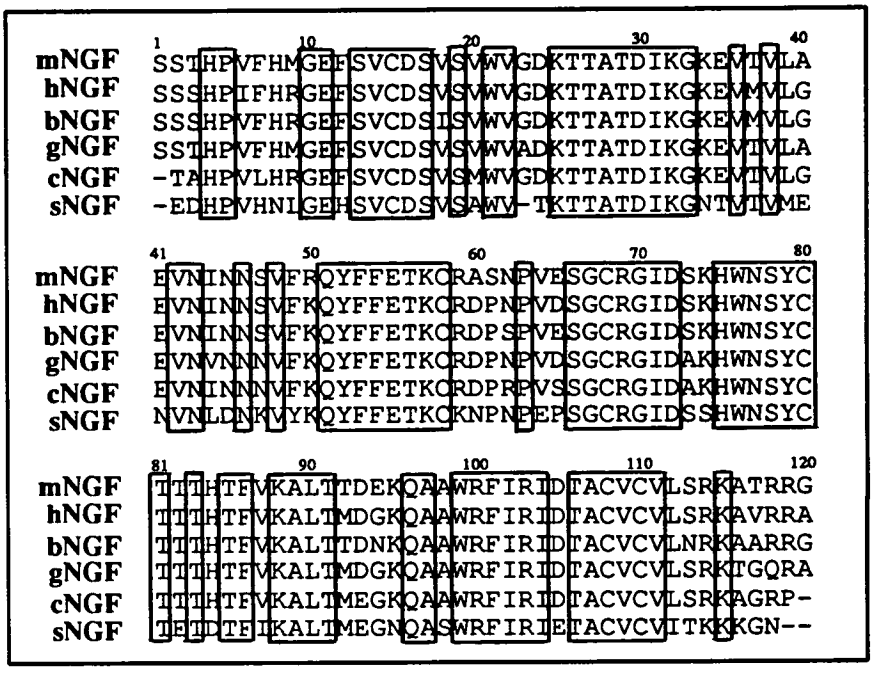
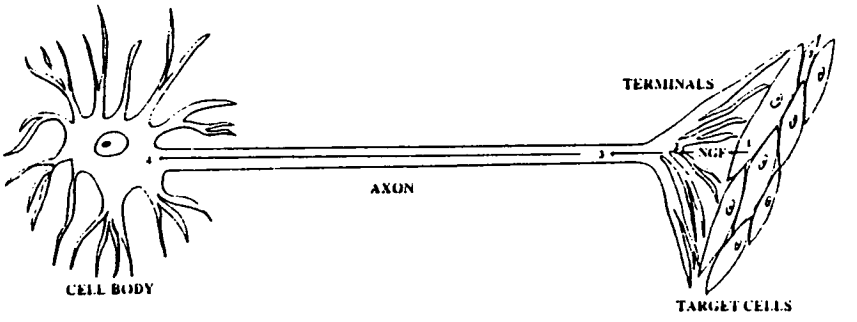


Fig. 1.6 Sequence homology between NGFs from various species
m=murine ; h=human ; b=bovine ; g=guinea pig ; c=chicken ; s=snake
(Boxed areas indicate conserved regions)

1.2.2. The Biological Functions of NGF

NGF is produced in the hippocampus of the basal forebrain, taken up by the (previously mentioned) cholinergic neurons and transported back to the septal cell bodies. This is illustrated in Fig. 1.7. The NGF molecule first binds to a cell surface receptor in the nerve terminals and this NGF:receptor complex is internalised by endocytosis[41]. The complex is transported retrogradely in vesicles along microtubules to the cell body[42]. The NGF, delivered to the cell body, is biologically active and intact, although here it is rapidly degraded by lysosomes[43]. Former reports that NGF bound to nuclear receptors[44] have now been refuted[45]. Thus

somewhere along the pathway taken by the complex inside the neurons, intracellular signals responsible for the function of NGF, must be generated. It seems likely that the actual binding of NGF to its receptor must initiate the signal transduction effects that are the key to the mechanism; however, whether the signal transduction is restricted to the nerve terminal membrane, or also occurs during the transport of the NGF:receptor complex[46] is not yet clear.



1. NGF is released from target cells
2. NGF binds to receptors and is internalised at nerve terminals
3. NGF:receptor complex is retrogradely transported to the cell body e.g. of forebrain cholinergic neurons
4. Signal transduction occurs on route to cell body, triggering trophic actions of NGF

Fig. 1.7 Retrograde transport in cholinergic neurons

Certain biological changes have been observed on the initial binding of NGF to the receptor. These are varied and numerous, but fall into two main classes;

1. Early responses, which occur within seconds to hours, for example,
   (a.) regulation of the uptake of nutrients and precursors, such as amino acids and sugars[47],
   (b.) the induction of protooncogenes c-*myc* and c-*fos*[48],
   (c.) the phosphorylation of ribosomal proteins S6 (increases protein synthesis) and tyrosine hydroxylase (increases catecholamine synthesis)[49].

2. Delayed responses, which occur within hours to days, for example,
   (a.) the initiation of neurite outgrowth[50],
   (b.) the activation of enzymes required for neuronal function, such as tyrosine hydroxylase[51] and ornithine decarboxylase[52].

These changes act to promote the survival and maintenance of the cholinergic neurons in the CNS. There is considerable evidence that NGF is also important in

the survival of the cholinergic cell bodies of the basal forebrain during development[18].

1.2.3. NGF Receptors

The first step in the transduction of the extracellular message of NGF into the required intracellular responses, occurs by its initial association with its receptor[15]. For many years it was observed that two forms of this receptor appeared to exist: a low-affinity form (which had a K_d for NGF of 10^{-9}M) and a lesser-populated high-affinity form (K_d for NGF of 10^{-11}M). The primary structure of the low-affinity form, named $p75^{NGFR}$, has been elucidated by gene cloning[53,54]. This showed the receptor to be 427 a.a.'s long, consisting of a twenty-eight a.a. signal sequence; an extracellular domain containing four, forty-a.a. repeats, followed by a region rich in serine and threonine residues; connected to this is a single transmembrane domain, made up of mainly hydrophobic a.a.'s, then a 155 a.a. cytoplasmic domain[55]. The M.W. of the fully processed receptor protein is approximately 70-80 kDal (hence named p75). The difference between this and the predicted size of the receptor protein (399 a.a.) is due to the extensive O- and N-linked glycosylation in the extracellular domains. Fig. 1.8 illustrates the proposed structure of the $p75^{NGFR}$.

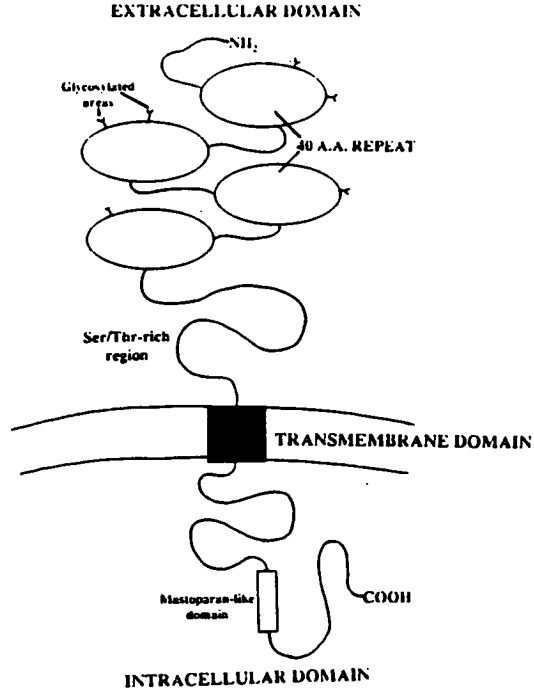


Fig. 1.8 Representation of proposed structure of $p75^{NGFR}$.

The predicted human, rat and chicken sequences show that while there is 92% homology between the rat and human forms, the chicken sequence is less well conserved, although importantly, almost every cysteine residue and the transmembrane region are conserved between the three species. Of the twenty-eight cysteine residues, twenty-four are found within the first 160 residues. Aligning these shows that they are contained within four, forty residue repeats (Fig. 1.9), which may be evolutionarily related. Similar cysteine-rich repeats have been found in the extracellular domains of several proteins e.g. tumour necrosis factor receptor[56]. The cysteine-rich sequences have recently been shown to contain the NGF binding domain(s)[57].

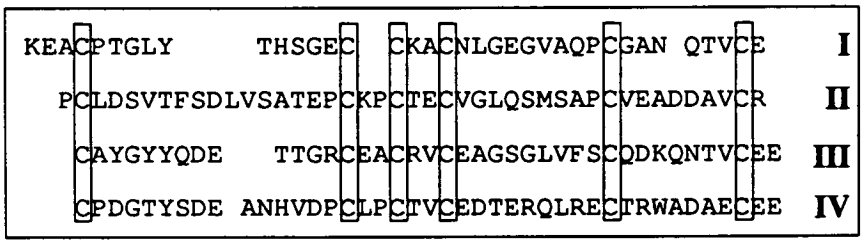


Fig. 1.9 Four internal repeats in p75^{NGFR}
(spaced to allow maximum alignment)

This extracellular region also contains many negatively charged residues (aspartic and glutamic acids), giving a net negative charge of -24. It has been suggested that the folding of the extracellular domain may arrange these charges so that they provide a binding pocket for a corresponding basic (hence positively charged) region on the NGF molecule itself[53].

The deduced a.a. sequence of p75^{NGFR}, whilst having some structural features in common with known receptors, nevertheless lacks significant similarities with any other known receptor or protein. The intracellular domain also bears no similarity to other known growth factors, oncogenes or tyrosine kinases. In fact the p75^{NGFR} appears to have no tyrosine kinase activity[58,59]. This was taken to suggest that the mechanism of action of NGF must differ substantially from that of other peptide growth factors, such as platelet-derived growth factor, epidermal growth factor (EGF) and insulin, which all have tyrosine kinase activity in their receptors. The intracellular domain does however contain a single mastoparan-like domain (a consensus sequence for the binding of G-proteins[60]), which might provide a possible signal transduction route.

In the mid-1980's, it was observed that a second protein species, as well as the $p75^{NGFR}$, could be obtained, bound to radiolabelled NGF, from cells which expressed both high- and low-affinity receptors[61]. This protein had an approximate M.W. of 140 kDal. Further studies showed that both it and the $p75^{NGFR}$ were required to elicit the nerve's responses to NGF. At the time, it was thought that the $p75^{NGFR}$ was part of this larger receptor, along with a smaller 'accessory' protein, perhaps a 60 kDal tyrosine kinase protein; together these would constitute the high affinity form. In early 1991, it was shown that the 140 kDal protein itself contained phosphotyrosine[62], implying that this may participate in a tyrosine kinase signalling pathway. Subsequently, it has been shown that the 140 kDal protein was the product of the *trk* protooncogene, named $p140^{trk}$, and this was required to provide the high-affinity binding observed for NGF[63-65].

The *trk* protein had been identified some years earlier, when the *trk* oncogene was found in a human colon carcinoma[66], and subsequent gene cloning enabled the protein sequence to be predicted. This sequence indicated a tyrosine kinase-like domain fused to a tropomyosin sequence (thus *trk* - tropomyosin receptor kinase). There appear also to be several forms of the *trk* protein[67], all of which have a M.W. of 140-145 kDal, as well as containing an extracellular domain, a single transmembrane domain and an intracellular tyrosine kinase domain. The extracellular domain has cysteine- and leucine-rich regions, as well as several regions characteristic of cell adhesion molecules. When the *trk* proteins were found to be localised in tissues of neural origin[68], the search for its natural ligand was similarly focussed on nervous tissues, which led ultimately to the identification of NGF as its ligand.

What is not clear, at present, is how the $p75^{NGFR}$ and the $p140^{trk}$ receptor are involved in generating the high-affinity binding required to cause the biological effects of NGF. Two theories have been proposed to explain the observed experimental results, and define the component(s) of the high-affinity receptor;

1. The $p140^{trk}$ protein alone mediates all of NGFs biological actions[65].
2. Both $p75^{NGFR}$ and the $p140^{trk}$ receptor are required for high-affinity binding[64].

The second theory suggests that the two receptors interact in some way, perhaps to form a heterodimer, and thus generate the high-affinity binding site[64] (Fig. 1.10). By analogy to some other receptor tyrosine kinases, whose dimerisation is thought to

be required for phosphorylation and activation e.g. EGF receptor, such dimer formation may be required here for the signal transduction of NGF. Conversely, a dimer of the p140^{trk} protein may be the active species[65]. The overall picture of what actually constitutes the high-affinity binding species, and the role that both receptors play in it, is still not complete and represents a very active area of on-going research. Recent evidence has suggested that the p75^{NGFR} may play only a modulating role in the high-affinity binding of NGF[69].

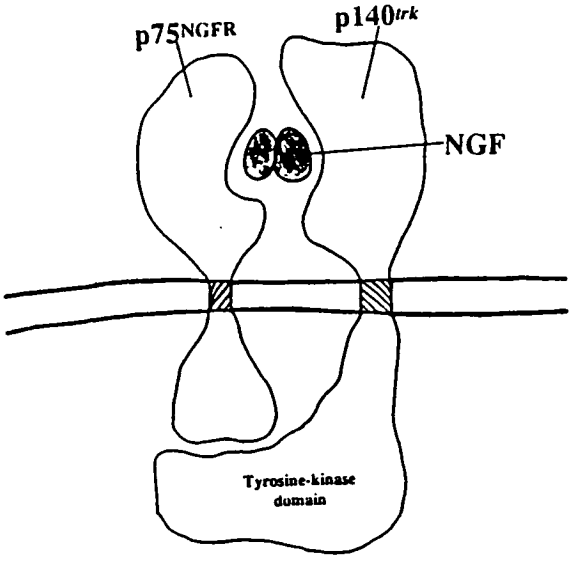


Fig. 1.10 'Two-protein' model of the NGF high-affinity receptor
Both receptors interact with the NGF dimer. The p140^{trk} may additionally interact with the intracellular domain of the p75^{NGFR}.

1.2.4. Other Neurotrophins

NGF is actually part of a family of related neurotrophins, whose other members are now beginning to be characterised. The best defined are brain-derived neurotrophic factor (BDNF)[70] and neurotrophin-3 (NT-3)[71], although recently neurotrophin-4 (NT-4)[72] and neurotrophin-5 (NT-5) have been discovered. As yet the physiological roles of these molecules *in vivo* are unknown, but *in vitro*, they appear to promote the survival of similar neuronal populations as NGF[73]. All of these proteins are structurally related (Fig. 1.11), with about 50% of their sequences conserved between the three molecules. All six cysteines are conserved in similar positions to those in NGF, so perhaps the same disulphide formation also exists. Four variable domains can be seen, which presumably contain residues defining the neuronal specificities of the proteins.

NGF, BDNF, NT-3 and NT-4 all bind to the $p75^{NGFR}$ with roughly equal affinities[74]. They have also been found to bind to other members of the *trk* receptor family. The $p140^{trk}$ receptor, involved in the high-affinity binding of NGF, is coded for by the *trkA* gene, thus has been renamed $p140^{trkA}$; *trkB* and *trkC* genes have also been found and these code for the slightly-higher M.W. receptor proteins, $p145^{trkB}$[75] and $p145^{trkC}$[76]. All of the *trk* receptors share considerable homology, especially in their tyrosine kinase domains.

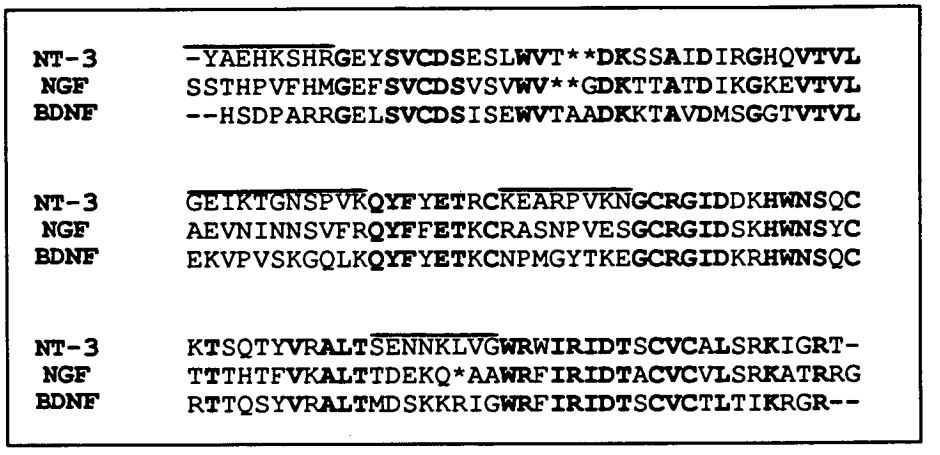


Fig. 1.11 Sequence comparisons of mouse NT-3, NGF and BDNF.

Bold letters show identical a.a.'s at conserved positions; asterisks indicate gaps which allow optimal alignment. The four main variable regions are indicated by lines above the sequences.

It was originally postulated that the $p75^{NGFR}$, along with one of these *trk* proteins, may form a specific high-affinity receptor for an individual neurotrophin e.g. BDNF was, at first, found to bind to $p145^{trkB}$, thus $p75^{NGFR}$ plus $p145^{trkB}$ may form an analogous high-affinity receptor to that proposed for NGF. However, current evidence indicates a more complex picture: it has been shown that $p140^{trkA}$ will bind NT-3 as well as NGF[77], $p145^{trkB}$ will bind BDNF and NT-3, but not NGF[78] and $p145^{trkC}$ binds only NT-3[76]. The structural determinants in these neurotrophins, and in the *trk* receptors, which account for these subtle differences have yet to be characterised.

1.3. THE CHEMICAL SYNTHESIS OF PEPTIDES

1.3.1. General

Amino acids can be covalently linked by the formation of an amide bond between their respective α–amino and α–carboxyl functions. The resultant molecules are termed peptides when the polyamide chain formed is less than 100 a.a. units, thus distinguished from proteins which are generally of much higher molecular weights. The amide or peptide bond (Fig. 1.12) is formed in Nature by a specific enzyme complex, peptidyl-transferase, in the ribosome, elongation of the peptide or protein occurring in the N-to-C-terminal direction. Whilst we are unable to directly mimic this process *in vitro*, peptides (and maybe proteins) are nevertheless accessible by chemical synthesis due to the ingenuity of many synthetic organic chemists throughout this century.

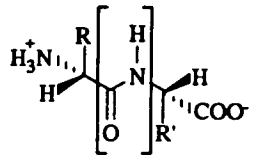


Fig. 1.12 The peptide bond (in a dipeptide)

The interest in peptides and in advancing peptide chemistry has been spurred largely by the discovery of a myriad of naturally-occurring, complex biologically active peptides over the last twenty to thirty years, especially neuropeptide hormones and neurotransmitters[79]. In fact, peptides play key structural and functional roles in many areas of biochemistry, pharmacology and neurobiology[80-83] as demonstrated by the vast literature in these fields. The chemical synthesis of peptides thus has important applications in many areas of research, some of which are outlined below (Table 1.13)

Table 1.13 Some important applications of Synthetic Peptides
Structure determination
Epitope mapping of proteins
Structure-function studies
Protease substrates/inhibitors
Synthetic vaccine production
Peptide hormone agonists/antagonists
Investigation of protein folding

The peptide bond was first recognised as a feature of peptide/protein structure early this century[84,85]. It has about 40% double bond character due to resonance stabilisation - this fact has two important consequences:

a.) -NH- (imide) has no significant tendency to ionise or protonate,

b.) -C-N- bond is relatively rigid and cannot freely rotate hence peptide bonds usually have a *trans*-conformation.

These factors have significant consequences in defining peptide and protein structure. The first small steps to chemically forming this bond were taken by Curtius[86] (benzoylglycyl-glycine) in 1881 and later, around the turn of the century, by Fischer[87] (ethoxycarbonylglycyl-glycine ethyl ester). From here, the field has gradually evolved, punctuated by advances in methodology, which have led ultimately to the development of peptide synthesis on solid supports[88-90] and even the approach to protein synthesis[91,92]. Despite the undoubted complexity of many peptide and protein systems, the underlying basis behind the chemical synthesis of such molecules is the formation of the peptide bond. Before considering the various chemical means of forming these bonds, the subject of protecting groups (PGs) will be discussed as this is most vital to the success of any of the chemical strategies.

1.3.2. N^{α}-Protecting Groups in Peptide Synthesis

Consider the formation of the theoretical dipeptide AB (Fig. 1.14). We require that the carboxyl of A couples with the amine of B, via the activation of the carboxyl of a.a. A by chemical means (see section 1.3.3). If we simply mix activated A with B, we will form some of the required dipeptide AB but also some cyclic A–B and many other undesired products. Additionally, due to the functionality of some a.a. side-chains e.g. Asp, Glu, Lys, we may also get branching and other side-reactions. The solution to these problems has been through the extensive use of PGs which allow only the desired reactions to occur.

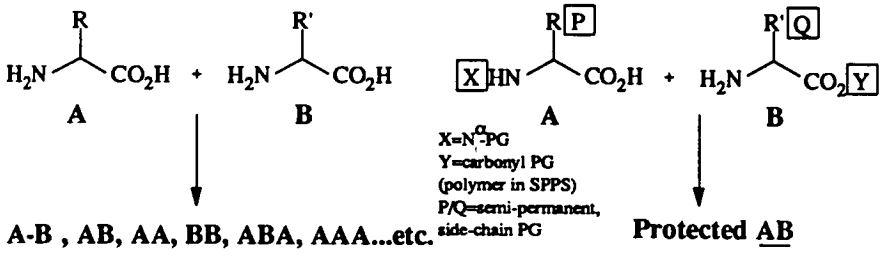


Fig. 1.14 The use of PGs enables the formation of dipeptide AB

The foundation of chemical synthesis of peptides is the N^{α}-PG. This can be selectively removed to allow the coupling of subsequent a.a.'s whereas the other functionalities i.e. C-terminus and side-chains, have 'permanent' PGs which remain intact during chain elongation and are removed at the end of the assembly.

Ideally a useful α-amino PG:

a.) should be introduced into the a.a. in high yield,
b.) should have a chemical procedure for its introduction that will not cause racemisation,
c.) should preserve the chiral integrity of the a.a. during coupling,
d.) should be capable of being removed without damaging the peptide or other PGs.

Most of the currently used groups are based on urethane-type structures, the majority of which may be easily removed by acid or base under mild conditions. The urethane sub-unit appears to especially prevent the formation of azlactones, which have been shown to be involved in racemisation (conversion of normal L-a.a. to D-isomer, see section 1.4.4). Paradoxically, the first urethane PG used by Fischer[93], ethyloxycarbonyl, was unsuccessful as it was difficult to remove and caused side-reactions. In 1932, the benzyloxycarbonyl (Cbz or Z)[94] group was introduced. This could be removed by catalytic hydrogenation or by acidolysis (Fig. 1.15)[95,96].

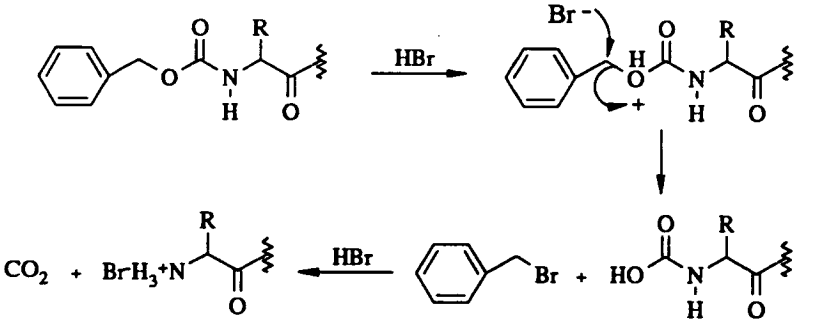


Fig. 1.15 Acidolysis of Z group with HBr/glacial acetic acid

The basis of its deprotection by strong acids was the formation of a stable carbocation. Developing this strategy, groups with greater acid-lability were introduced, the most important being the N^{α}-*tert*-butyloxycarbonyl (Boc)[97,98] group, which can be removed by HCl/acetic acid or trifluoroacetic acid (TFA) [S_N1 mechanism], and the even more labile biphenylisopropyloxycarbonyl (Bpoc)[99] group. Many other acid-labile PGs have been suggested and reviewed[80].

Urethane protection founded on deprotection by base has gradually assumed greater importance over the past decade. Originally, it had been shown that urethanes containing suitably activated hydrogen atoms e.g. arylsulphonylethoxycarbonyl derivatives could be cleaved by base-catalysed β-elimination[100], although strong alkaline conditions were required. Alkyl derivatives[101] of similar compounds found greater application (Fig. 1.16).

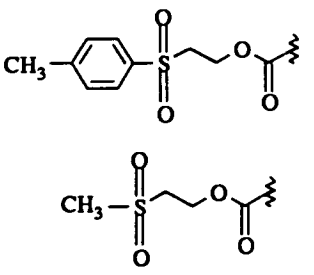


Fig. 1.16 Two base-labile N^{α}-protecting groups

In the early 1970's, Carpino showed that 9-fluorenylmethoxycarbonyl (Fmoc)[102-104] derivatives could be deprotected, again using weak bases e.g. piperidine. The fluorene ring system renders the lone hydrogen on the β-carbon especially acidic. If removed by a base, the carbanion intermediate which is formed collapses to a dibenzofulvene species[105] (Fig. 1.17).

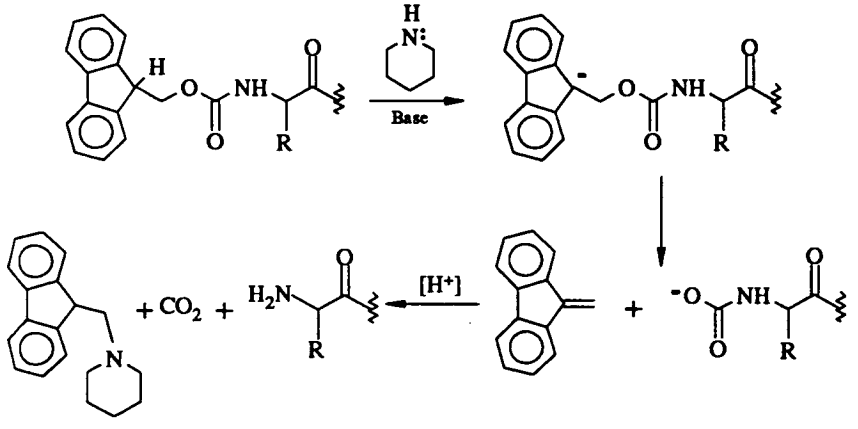


Fig. 1.17 Base deprotection of the Fmoc group

This species is additionally scavenged by the excess base used, resulting in an adduct whose UV detection may be used in monitoring systems[106] (see section 2.2.3). This group is now widely used and has become the basis of an orthogonal scheme for Solid-Phase Peptide Synthesis (SPPS)[107-109]. Other base-labile groups have been suggested, some with certain special properties e.g. 2,2-[bis-(4-nitrophenyl)]-ethoxycarbonyl (Bnpeoc)[110] group, whose deprotection with base leads to a colour change.

While other PGs and methods of deprotection have been suggested and may have uses in special cases, the use of Boc (acid-labile) and Fmoc (base-labile) are most prevalent. The choice of a.a. side-chain protection is dependent on the type of N^{α}-

protection used and will be considered later in the context of SPPS. With suitably protected a.a. residues, their chemical coupling to form peptide chains can be undertaken.

1.3.3. Formation of the Peptide Bond

1.3.3.1. General Methods

Under normal circumstances, carboxylic acids will not react with amines i.e. to form a peptide bond. thus it is usually necessary to augment the electrophilic character of the carboxyl group of the acylating molecule. This can be achieved by replacing its hydroxyl function with a variety of electron-withdrawing groups, whose inductive effect can greatly enhance the carbonyl's susceptibility to nucleophilic attack (Fig. 1.18).

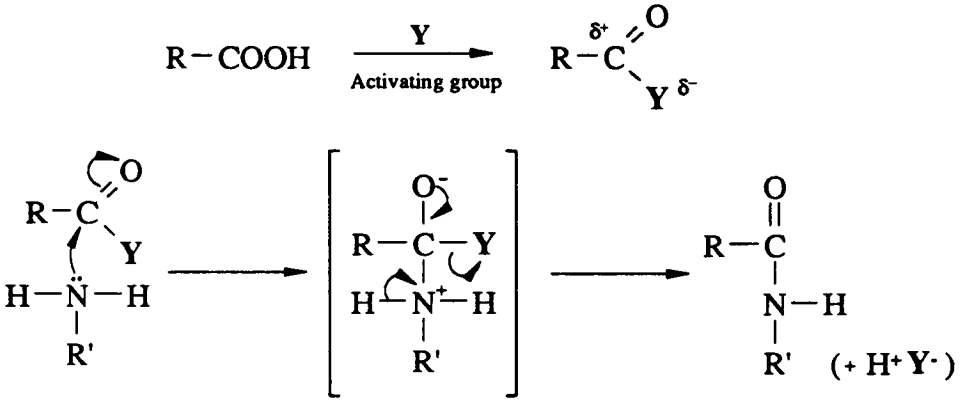


Fig. 1.18 Generalised activation and coupling reactions in Peptide Synthesis

The original method of Fischer[111] used acid chlorides. In combination with his urethane protection at that time, this led to the formation of N-carboxyanhydrides[112] with the subsequent loss of his N^{α}-protection, as well as the formation of oxazolones when using acyl-protected a.a.'s. Thus, although acid chlorides are very reactive, they are not routinely used in peptide synthesis. Recently however, Fmoc-a.a. chlorides have been formed and used in the synthesis of short peptide segments[113]. Stable derivatives bearing the *tert*-butyl moiety were not able to be formed, hence this limited the approach. This problem appears to have been overcome by the use of Fmoc-a.a. fluorides[114], which have subsequently enabled both solution and solid-phase syntheses to be carried out.

The azide method[115], discovered by Curtius (Fig. 1.19) a contemporary of Fischer, is still a major coupling method today, used mainly for segment condensation[116].

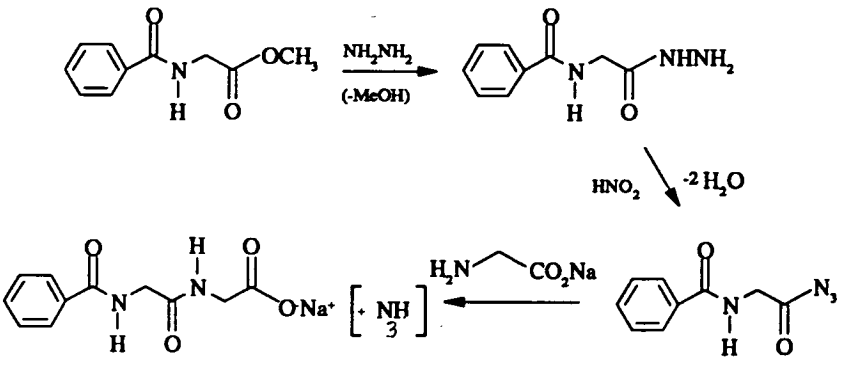


Fig. 1.19 Curtius's original synthesis of benzoylglycyl glycine via azide method

Improvements in strategy[117] and the activation of the hydrazide to the acid azide via alkyl nitrites[118], has led to reagents which may directly form the azide from the free acid e.g. diphenylphosphoryl azide (DPPA)[119] (Fig. 1.20). The major drawback with azides is however their propensity for rearrangement to isocyanates (Curtius rearrangement) (Fig. 1.21).

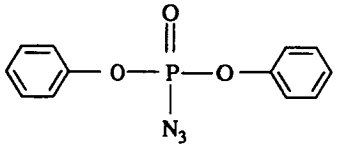


Fig. 1.20 DPPA

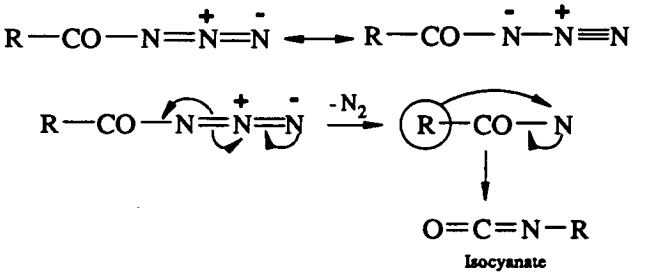


Fig. 1.21 Curtius rearrangement

The isocyanates formed may react with the free amine function to yield ureas. To overcome this problem, azide couplings have to be carried out at low temperatures (at or below 0°C) and with high concentrations of reactants to speed an otherwise slow reaction. The yields obtained are generally low/moderate (30-70%) but an advantage is the low level of racemisation said to be associated with this coupling procedure.

The acid chloride and azide methods were the only two coupling methods available until the early 1950's when mixed anhydrides were introduced[120,121]. The use of these reagents has been reviewed[122]. This procedure was developed and refined, exemplified by the use of isobutylcarbonic acid mixed anhydrides, which provided steric and electronic effects to ensure that nucleophilic attack occurred at the correct

carbonyl (Fig. 1.22). Despite the attractive feature of using only one equivalent of a.a. per coupling (c.f. symmetrical anhydrides), their use has almost exclusively been confined to solution-phase synthesis. Mixed anhydrides formed from substituted phosphonic chlorides e.g. diphenylphosphinyl (DPP) chloride, in the presence of base (Fig. 1.23) have also been suggested and may be employed in SPPS[123].

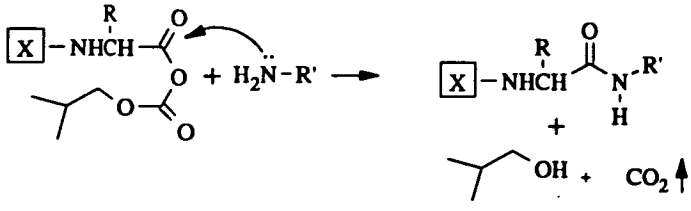


Fig. 1.22 Mixed anhydride coupling

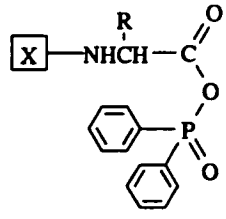


Fig. 1.23 A Dpp anhydride

The above methods of peptide bond formation have found extensive use in solution phase synthesis, although they are not usually applied to SPPS. The two major methods which follow have been applied to both.

1.3.3.2. Symmetrical Anhydrides

With symmetrical anhydrides (SA), acylation of a.a.'s can occur by attack at either carbonyl group. The disadvantage of losing half of the reacting material is largely offset by their high reactivity. Formation of S.A. by the use of carbodiimides was conceived in 1955[124], with dicyclohexylcarbodiimide (DCC) being suggested by Sheehan and Hess[125] that same year. The mechanism of its formation and its use in coupling is shown in Figs.1.24/5.

The dicyclohexylurea (DCU), which forms, is insoluble in most of the solvents used for synthesis e.g. dichloromethane (DCM), dimethylformamide (DMF), thus other carbodiimides which form soluble ureas e.g. diisopropylcarbodiimide (DIC)[126,127] are often used for SPPS to prevent the build-up of insoluble material. The O-acylurea formed in the reaction provides activation leading to:

a.) direct attack by an amine to form a peptide bond.
b.) attack by another carboxyl function to form the S.A.
c.) bond formation via a reactive species, which may be formed by the addition of auxiliary nucleophiles, such as 1-hydroxybenzotriazole (HOBt)[128].

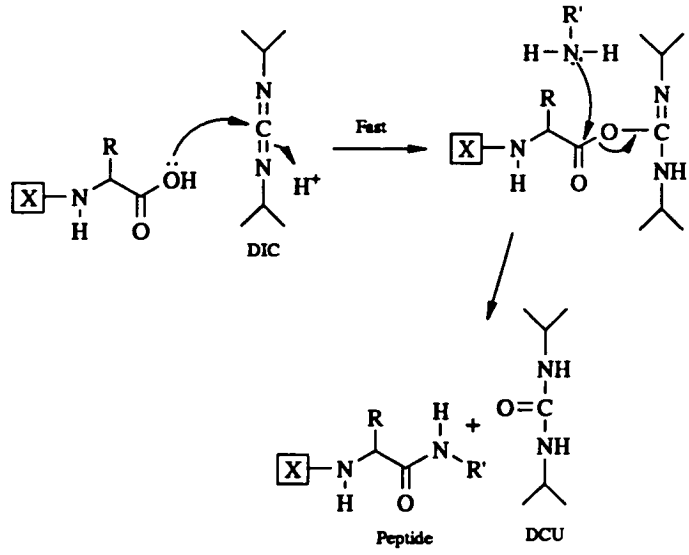


Fig. 1.24 Activation and coupling by carbodiimides

HOBt addition significantly reduces the side-reactions associated with the coupling method, principally the formation of N-acylureas and some racemisation, by reducing the concentration of O-acyl ureas. Thus for *in situ* activation, where all of the components are simply mixed together in the reaction vessel, multiple pathways probably exist (Fig. 1.25). The reactive species formed by HOBt is typical of another class of compounds used for peptide bond formation: active esters.

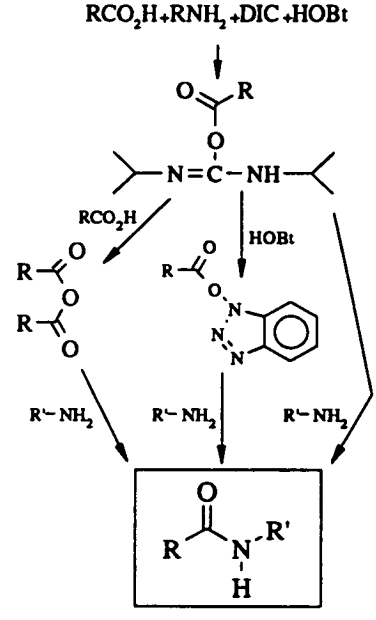
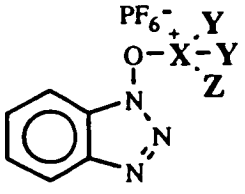


Fig. 1.25 (right) Probable multiple pathways in carbodiimide-mediated couplings.

1.3.3.3. Active esters

Aminolysis of esters has been widely studied as a method of peptide bond formation. Here the alcohol component provides the inductive effect on the carbonyl, leaving it more electrophilic. The most widely used alcoholic function is still HOBt[129], which may form an active ester (AE) *in situ* when added to carbodiimide-mediated couplings, or be preformed outside the reaction vessel. In any case, these esters are unstable and are not usually isolated. Whilst the formation of AE by DIC/HOBt is

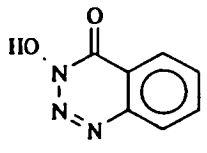
well established, many new coupling reagents for the *in situ* formation of HOBt active esters have been suggested and are claimed to produce faster couplings. These are generally based on the hexafluorophosphate or tetrafluoroborate salts of carbonium or phosphonium species (Fig. 1.26) e.g. BOP[130,131], PyBOP[132], HBTU[133,134],BBC[135]; all require a tertiary base for activation.



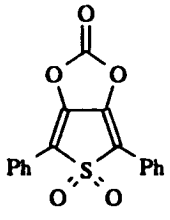
X	Y	Z	Abbrev.
C^+	N⌐	None	BBC
C^+	$N^{CH_3}_{CH_3}$	None	HBTU
P^+	N⌐	N⌐	PyBOP
P^+	$N^{CH_3}_{CH_3}$	$N^{CH_3}_{CH_3}$	BOP

Fig. 1.26 Reagents for *in situ* Active Ester formation

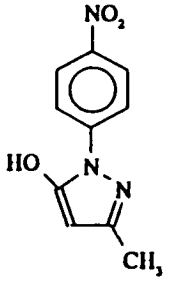
Other types of AE can be obtained in stable, crystalline forms and have gained popularity as they can more easily be used in automatic synthesizers. Representative of these compounds are nitrophenyl esters[136], superseded by trichlorophenyl esters[137,138] and more recently pentafluorophenyl esters[139-141]. All of these react slowly in the absence of HOBt as an additive. Lately, other AE, which may enable the spectroscopic determination of the acylation reaction, have been used e.g. ODhbt[142-144], OTDO[145,146] and Npp[147] esters (Fig. 1.27).



ODhbt
3-hydroxy-2,3-dihydro-4-oxo-benzotriazine



OTDO
2,5-diphenyl-2,3-dihydro-3-oxo-4-hydroxythiophene dioxide



Npp
3-methyl-1-(p-nitrophenyl)-2-pyrazolin-5-one

Fig. 1.27 Alcoholic functions useful for AE formation

It is clear, therefore, that many different types of reagent are available to the peptide chemist for the formation of the amide bond. The choice is sometimes dictated by the particular protection group strategy that is chosen but more often by what can be accommodated in an automatic synthesizer. For reasons which are not entirely clear, often one type of activation method will succeed in a particular coupling reaction

where another fails, thus it is prudent to consider all the above mentioned methods as potentially useful.

Although great advances had been made in peptide chemistry in the first half of the century, it remained a specialised and difficult area. The need to couple a.a.'s, wash, recrystallise, etc. in the solution phase, made the assembly of even quite small peptides a major feat. In the early 1960's, an advance was made which has greatly changed the nature of peptide synthesis and made it into the major field that it is today: the assembly of peptides on solid supports.

1.4. SOLID-PHASE PEPTIDE SYNTHESIS (SPPS)

1.4.1. General: Boc and Fmoc Strategies

The concept of SPPS is straightforward but nevertheless elegant in how it overcomes the problems associated with solution synthesis, especially in the manipulation and solvation of the large, protected peptides formed. A generalised scheme is outlined in Fig. 1.28. An N^{α}-protected a.a. (usually bearing a compatible PG on the side-chain) destined to become the C-terminus of the peptide, is covalently anchored to an insoluble polymeric support, often via a linker. The blocking group is then selectively removed and the peptide chain lengthened by the addition of another N^{α}-protected a.a., using one of the previously mentioned methods, to yield a protected dipeptide. After each of the deprotection and coupling steps, the growing peptide/resin can be thoroughly washed with suitable solvents, simple filtration removing the excess reagents. Subsequent cycles of washing, deprotection, washing, coupling and so on, can extend the peptide chain to the desired length. The coupling cycles may be stepwise with individual a.a.'s or with protected segments (see below). The overriding advantage compared to solution work, is that the substantial excesses of protected a.a.'s used to drive each coupling reaction to completion, can simply be removed by washing/filtration. Additionally, the fact that the growing chain is immobilised largely avoids solubility problems, lengthy purification of intermediate stages and reduces the mechanical loss of material. When the desired peptide length is reached, the peptide is released from the support, usually by mild or strong acid. Strategies are chosen so that the side-chain protection, which has remained intact during chain assembly, can be simultaneously removed to yield the free peptide.

Peptides with acid or amide functions at their C-termini may be formed depending on the nature of the linker used.

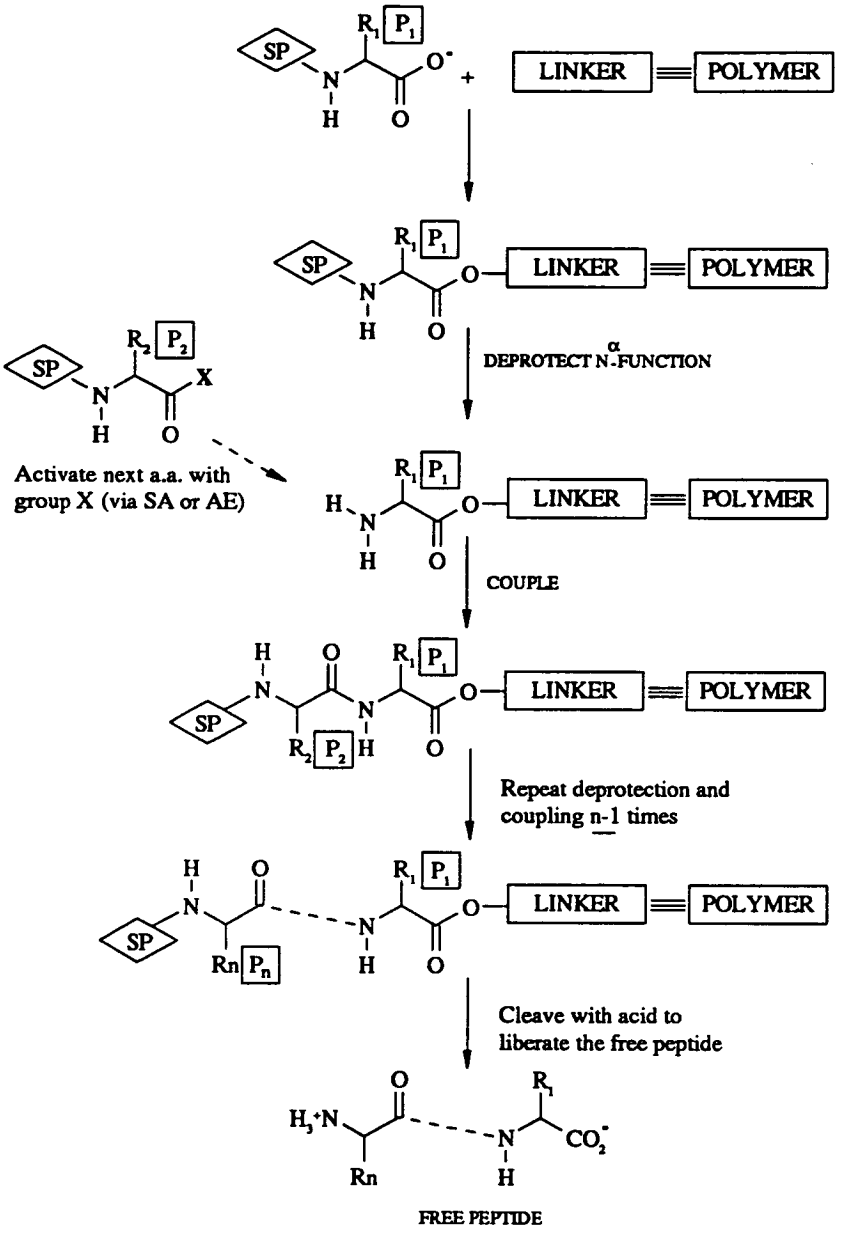


Fig. 1.28 Generalised scheme for SPPS

Such a scheme was first put forward by Merrifield[88,148] in 1963. The solid support that he chose is still widely used today: a copolymer of polystyrene with 1% m-

divinylbenzene[149,150], which provides cross-links, enabling the resin to swell in DCM and DMF. His initial use of the benzyloxycarbonyl (Z) group required HBr/AcOH for deprotection, then triethylamine (TEA) as a base to liberate the free amine before coupling could occur. Unfortunately, this strong acid treatment also removed some of the peptide chains from the resin, requiring nitration of resin to overcome this. In an improved method[151], the Z group was replaced by the Boc group, which could be more easily removed by HCl/AcOH or TFA/DCM. These residues were activated for coupling using DCC. The final cleavage of the peptide from the resin and removal of the side-chain protection called for the use of very strong acids. At first HBr/TFA was used but this has been superseded by anhydrous hydrogen fluoride (HF)[152,153] and trifluoromethanesulphonic acid (TFMSA)[154].

The concern about the detrimental effects of the peptide's repeated exposure to acidolysis[155], with many reports of acid-catalysed side-reactions, led to investigations into orthogonal approaches. Here, instead of graded acid-lability being the foundation to link the protecting strategy of resin/peptide bond, side-chain and N^{α}-blocking groups, instead PGs which theoretically can be removed in any order and in the presence of all other protecting classes, are envisaged. The most successful realisation of this approach was suggested by two research groups in 1978[107,109], using the Fmoc group for N^{α}-blocking (base-labile) and having side-chain functionalities protected by PGs which were labile to mild acid such as TFA, as well as TFA-labile resin linkers. The many improvements made to this basic scheme, primarily in side-chain protection, have been reviewed[156-158]. The Fmoc group is removed quite rapidly by nucleophilic bases e.g. 20% piperidine in DMF, with subsequent coupling by the previously stated methods (SA and AE are generally used). A brief comparison of both Boc and Fmoc strategies is shown in Table 1.29a and b. As the Fmoc strategy was used in this work, only this will be considered in the following sections.

	N^{α}-Boc	**N^{α}-Fmoc**
N^{α} lability	TFA (S_N1)	Piperidine (β-elimination)
Side-chain PGs	Benzyl-based	*tert*-Butyl-based
Side-chain lability	HF, RSO_3H	TFA
Solubilties	High	Moderate

Fig. 1.29a Comparison of Boc and Fmoc strategies

A.A. residue	N^{α}-Boc	N^{α}-Fmoc
Asp, Glu	OBzl ; OcHxl	O-*tert*-butyl
Lys	2-Cl-Z	Boc
Arg	Tos ; Mts	Mtr ; Pmc
His	Tos ; Dnp ; Bom	Trt ; Boc ; Bum
Ser, Thr	Bzl	*tert*-butyl
Tyr	Br-Z	*tert*-butyl
Cys	pMeBzl ; MeOBzl	Acm ; SBu^t ; Trt
Met	None ; Sulphoxide	None
Trp	Formyl	None ; Boc
Asn, Gln	None ; Xan	None ; Mbh ; Trt
Ala, Gly, Ile, Leu, Phe, Pro, Val	None	None

OcHxl, O-cyclohexyl; Tos, 4-toluenesulphonyl; Mts, mesitylene-2-sulphonyl; Dnp, 2,4-dinitrophenol; Bom, benzyloxymethyl; Xan, 9-xanthenyl; Mtr, 4-methoxy-2,3,6-trimethyl-benzenesulphonyl; Pmc, 2,2,5,7,8,-pentamethyl-chroman-6-sulphonyl; Bum, *tert*-butoxymethyl; Acm, acetamidomethyl; SBu^t, *tert*-butylsuphenyl; Trt, triphenylmethyl; Mbh, 4,4'-dimethoxybenzhydryl

Fig. 1.29b Comparison of PGs used in each strategy

1.4.2. Side-Chain PGs

PGs are mandatory for many of the functional side-chains of certain a.a.'s to:

a.) prevent them from reacting during the course of chain assembly e.g. they could be acylated, especially as excess a.a.'s are used during coupling.

b.) prevent modifications to the a.a. during synthesis.

c.) prevent specific side-reactions associated with certain of these side-chains.

The Fmoc strategy enables the design of mild acid-labile protection for the side-chains, thus most have been devised so that they may be removed in TFA solutions in reasonable time (2-4 hr). Several a.a.'s are protected as their *tert*-butyl esters (Asp, Glu)[159] or ethers (Ser, Thr, Tyr)[159]. These may be prepared by the initial attachment of the Bu^t group via isobutylene[160], then the Fmoc added using 9-fluorenylmethylsuccinimidyl carbonate (FmocONSu)[161,162]. The ε-amino function of lysine is primarily blocked by the Boc group, introduced by Boc-azide to a copper-complexed lysine residue[163]. The aliphatic a.a.'s (Ala, Val, Leu, Ile, Phe) are unreactive thus do not require protection. Of the rest, Asn and Gln, Arg and His deserve closer consideration (Cys and Trp protection is discussed later).

1.4.2.1. Asparagine and Glutamine

These are often left unprotected. However, the Fmoc derivatives have poor solubilities and the amide side-chain can undergo dehydration during DIC activation, resulting in nitrile formation[164,165] (Fig. 1.30). This may largely be prevented in carbodiimide couplings by the addition of HOBt[166,167], probably due to the acylation reaction occurring more rapidly. Various PGs have been suggested to try to overcome the solubility problems e.g. 4,4'-dimethoxybenzhydryl (Mbh/Dod)[168], trityl (Trt)[169] and dibenzosuberyl (Dbs)[170].

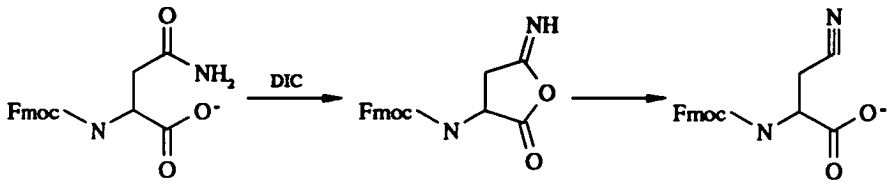


Fig. 1.30 Intramolecular dehydration of Asn during DIC activation

1.4.2.2. Arginine

The guanidino group of Arg is a strong nucleophile and may be acylated during SPPS, hence chosen PGs must be strongly electron-withdrawing to reduce its basicity. The most common side-reactions are, when activated, δ–lactam formation[171,172] and acylation of the guanidino function (during coupling) followed by base-catalysed ornithine formation (during the following deprotection of the Fmoc group)[173]. Many groups have been forwarded to suppress these reactions, the most successful being based on aryl sulphonyl derivatives. The best of these is the 4-methoxy-2,3,6-trimethylbenzene-sulphonyl (Mtr)[174] group; its Fmoc derivative however couples slowly and requires extended TFA/thioanisole treatment for its removal[175]. Leading on from these derivatives, the much more labile 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) group[176-179] (Fig. 1.31) has solved many of the problems associated with Mtr and has rapidly become the choice group for Arg protection, especially useful for multiple arginine-containing peptides.

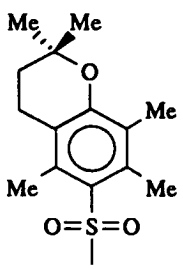


Fig. 1.31 Pmc group

1.4.2.3. Histidine

Two major side-reactions occur when using histidine:

a.) acylation of the imidazole side-chain if left unprotected during coupling[180], and

b.) racemisation due to intramolecular base catalysis by the π-nitrogen[181] (Fig. 1.32).

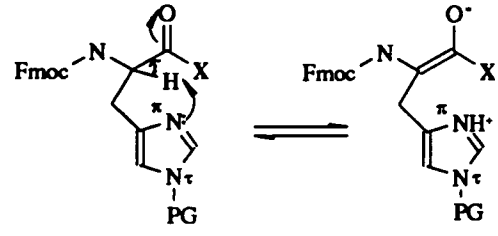


Fig. 1.32 Racemisation of activated His derivatives

Racemisation can be suppressed by blocking the π-nitrogen, although most PGs are derivatised onto the τ-nitrogen. Effective protection of the latter can still operate through electronic and steric effects to reduce the basicity of the more critical π-nitrogen e.g. via Trt[182]. Groups which actually protect this π-nitrogen are the most effective e.g. *tert*-butoxymethyl (Bum)[183], but are expensive and require several steps to synthesise.

After the chain assembly has been completed, these side-chain PGs must be removed, as well as the peptide being released from the resin. This may be achieved most conveniently by acidolysis.

1.4.3. Removal of the Protecting Groups (PGs)

Fmoc SPPS is designed such that relatively weak acids can be used in the deprotection of the side-chains (Fig. 1.33) and release of the peptide from the resin (Fig. 1.34). The ease of side-chain removal is largely governed by the stability of the carbocation formed

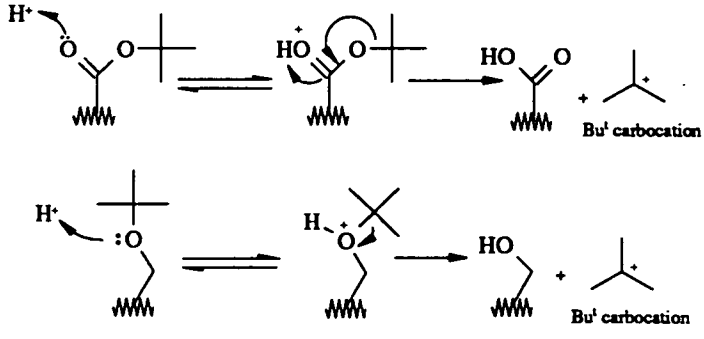


Fig. 1.33 Generalised acidolysis of *tert*-butyl esters and ethers

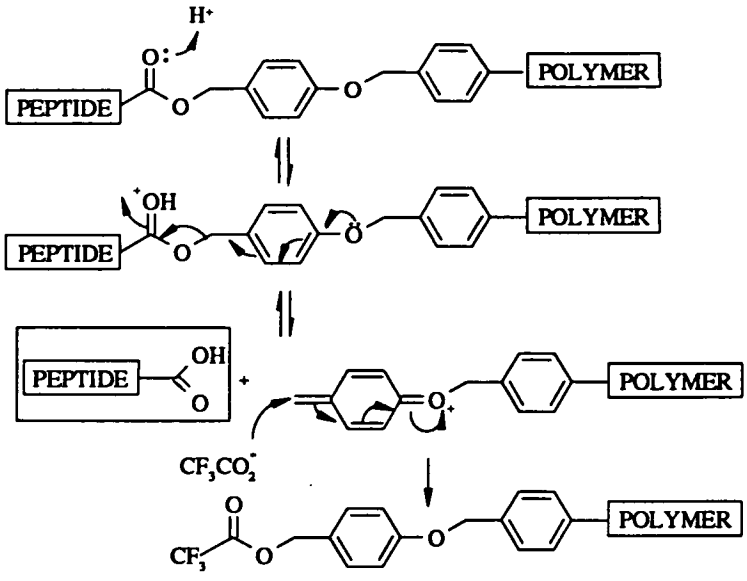


Fig. 1.34 Cleavage of a peptide from the Wang linker using TFA

PGs may thus be designed so that all are removable simultaneously or some can be made to be acid-stable for the purposes of post-synthesis modifications to certain a.a.'s (especially cysteine). The task of removing so many different PGs is problematical and the various stable carbocations formed can attack areas of high electron-density e.g. the indole group of Trp, the rings of Phe and Tyr, the sulphur atoms in Cys and Met, causing modifications if left unchecked. These deleterious side-reactions can however be suppressed to a great extent by the addition of scavengers to the cleavage mixture. When added in excess, these sacrificial compounds are selectively attacked by the released carbocations in preference to the a.a. side-chains, hence minimising these unwanted modifications. The role of scavengers and the classes used will be considered later.

1.4.4. Problems in SPPS

Although many of the original problems in SPPS have been solved by improved strategies, PGs, protocols etc., various others[80,184] are still a cause for concern and require brief consideration.

1.4.4.1. Racemisation

All a.a.'s (except Gly) have a chiral α-carbon. In Nature, a.a.'s mostly exist as the L-isomer but partial conversion to the D-isomer may occur during chain assembly and much of SPPS strategy has been designed to counter racemisation by the control of

coupling conditions, PG strategies, etc. - control in DIC/HOBt couplings and His protection have previously been mentioned. The mechanism of racemisation may be due to the formation of 4,5-dihydro-oxazole-5-ones (azlactones)[185] in activated a.a.'s (Fig. 1.35). In the presence of base, abstraction of the α–proton from the chiral centre gives a resonance-stabilised carbanion. Reprotonation can occur on either face to give either the L- or D-forms. To begin with, it was thought that urethane-type N^{α}-PG's e.g. Z, Boc, Fmoc, did not form azlactones.

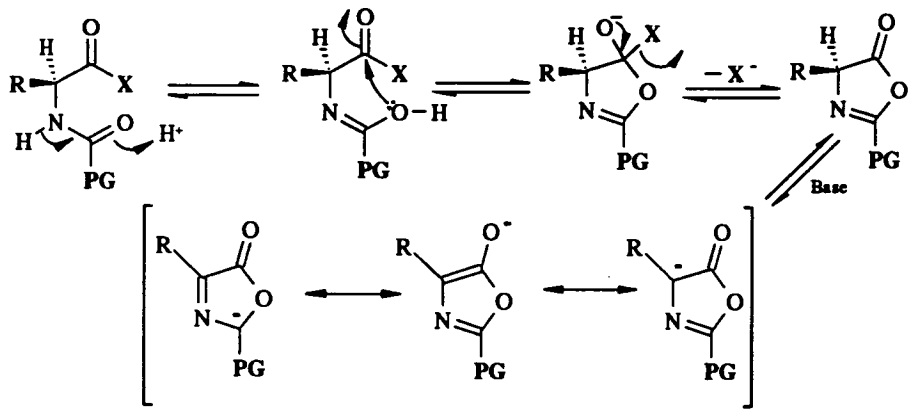


Fig. 1.35 5(4H)Oxazolone formation leading to loss of chirality

However, as such species have now been isolated[186], it may be that electron-donation from these groups can suppress anion formation thus affording protection against racemisation. In the absence of base therefore, amide bond formation usually proceeds with very low levels of racemisation[187]. One potential problem is loading the first a.a. onto the resin. This often requires the creation of an ester bond, catalysed by a base, usually 4-dimethylaminopyridine (DMAP)[188]. When loading with DIC/DMAP, the levels of the base must be kept low (0.1 equiv. or less)[189] and reaction times short to avoid racemisation[190]. Higher concentrations of DMAP may also cause partial removal of the Fmoc group[191].

1.4.4.2. Problems with chain assembly

When a dipeptide on the resin is deprotected, the freed amino group may attack the peptide-resin ester bond. The resulting cyclic dipeptide (a 2,5-diketo-piperazine, DKP) is released from the resin[166] (Fig. 1.36). This side-reaction is sequence-dependent (facilitated by Gly, Pro or D-a.a. residues) but may lessened by increasing the level of piperidine in the deprotection mixture in susceptible sequences[192].

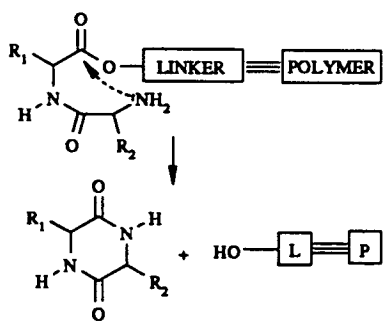


Fig. 1.36 DKP formation

Difficulties with sequences of 'Asn/Gln-X' or 'Asp-X' where X=Gly, Ala, Ser or Thr, are well-known. Base-catalysed imide formation of Asn/Gln-X, especially where X=Gly[193], results in either chain termination or the formation of α- or β-aspartyl (or α/γ-glutamyl) peptides[193]. These reactions may occur when coupling using powerful activation methods. They may however largely be overcome by the choice of an appropriate PG. The situation for 'Asp-X' sequences is even more serious. Attack of the backbone nitrogen onto the side-chain carboxyl, promoted by base e.g. piperidine, results results in the formation of aspartimides[194,195] (Fig. 1.37), with the subsequent loss of any PG that is present on the side-chain. This imide can open to give either the correct α- or incorrect β-aspartyl peptides. This reaction is further considered later in section 2.2.2.

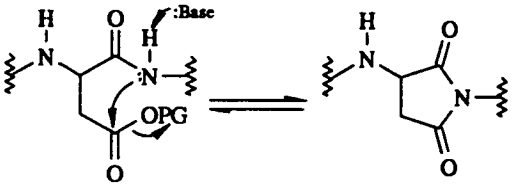


Fig. 1.37 Aspartimide formation

1.4.4.3. Deletions

Not all coupling reactions can be driven to completion, for a variety of reasons e.g. unavailability of the amine on the resin, solvation factors, steric factors. To combat this, covalent blocking of the free, unreacted amino termini of unacylated peptides is carried out, often via acetylation using acetic anhydride. The capped deletion peptides can usually be separated from the required sequence as they often have different physical characteristics. Apart from this 'deliberate' chain termination, some side reactions are known which act to prematurely halt or interrupt chain elongation:

i.) cyclisation of N-terminal Glu residues (when side-chains are left unprotected) to pyroglutamine. This can be catalysed by weak acids, e.g. a.a.'s or HOBt[196],

ii.) solvent or resin-bound aldehydes may form Schiff's bases with deprotected amine groups, thus preventing acylation[197], and

iii.) in some long sequences, it has been reported that the Fmoc group may not be completely removed in some cases[198], even in the presence of high concentrations of piperidine hence may lead to deletions.

Additionally, the sequence Asp-Pro is often acid-labile and can be cleaved by weak acids in the work-up[199].

Overall, although most problems with SPPS have been more or less solved, some or all of the above may combine in certain circumstances to thwart even the best planned and executed synthesis.

1.5. CYSTEINE AND DISULPHIDES IN PEPTIDES AND SPPS

1.5.1. General

The thiol group of cysteine is probably the most reactive of any a.a. side-chain. At slightly alkaline pH, it ionises to form the strongly nucleophilic thiolate anion. It can react with a wide variety of species e.g. alkyl halides and metal ions, to give stable compounds. Complexes with metals ions like mercury and silver are common and can be used to titrate the number of thiols present in a peptide/protein[200]. The sulphur atom may exist in a number of oxidation states and thiols are easily oxidised by air, a reaction catalysed by the presence of trace amounts of certain metal ions, e.g. Cu^{2+}, Fe^{3+}. A disulphide is the usual product of such oxidation processes (Fig. 1.38).

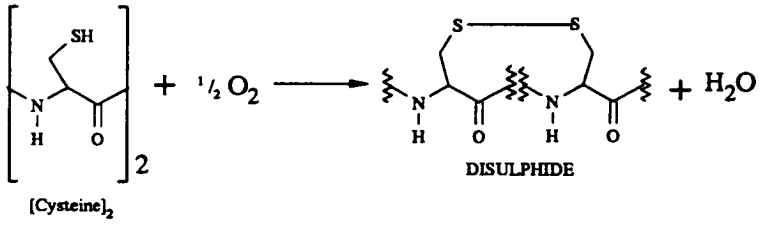


Fig. 1.38 Cysteine may form a disulphide in the presence of an appropriate electron acceptor

Disulphide bonds often occur between cysteine residues in peptides and proteins, being introduced *in vivo* as a post-translational modification. The bond is greatly affected by the particular conformation of the protein backbone: cysteines must have their αC-atoms within 4-9Å. for a disulphide to form between them[201]. Disulphides appear almost exclusively in extracellular proteins, where their role is probably in providing additional stability. Moreover, in some proteins e.g. insulin, disulphides act to stabilise a relatively unstable folded conformation. Disulphides can be converted back to their original thiols groups under strongly reducing conditions and at alkaline pH, where thiol/disulphide interchange is greatest[202].

1.5.2. Cysteine Protecting Groups

In SPPS, protection of Cys is obviously required to prevent:

a.) oxidation to thiols during assembly.
b.) acylation (as thiol function is nucleophilic).

The fact that some peptides/proteins require the presence of a disulphide bond whereas others need only the free thiol, adds another dimension to cysteine protection strategies. Many groups and oxidation methods have been suggested, although few have found common usage in Fmoc SPPS. The following Table 1.39 summarises most of the Cys PG's used. Methods of preparation (for a general review of these, see ref. 203) and removal procedures are included. Codes for the removal strategies are given below. Some of these procedures lead directly to the formation of disulphide bonds from protected species. The PG's and various methods for forming disulphides as they relate to this work will be discussed in more detail later

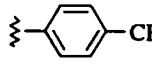
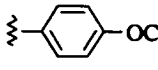
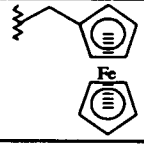
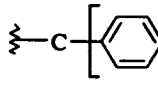
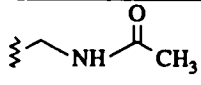
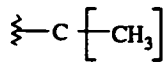
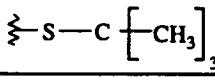
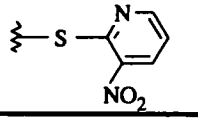
Protecting Group	STRUCTURE	PREPARATION	REMOVAL	Prep. Ref.	Examples of Use
pMe-Bzl		pMeBzlBr	HF S/S/TFA Tl^{3+}	204	80
pMeOBzl		pMeOBzlCl	HF. D/TFA S/S/TFA Ag/Hg/Tl	205	80 217
Fcm		Fcm.OH + TFA	TFA	206	206
Trt		Trt.OH + $BF_3.OEt_2$	TFA I_2 Ag/Hg/Tl	207	212 216
Acm		Acm.OH + TFA	Ag/Hg/Tl S/S/TFA I_2	208	212 214 215
Bu^t		Isobutylene	TFMSA Hg/Tl S/S/TFA	209	212 215
SBu^t		Bu^t-SH	RSH Phosphines	210	213-215
Npys		Npys-Cl	RSH Phosphines	211	218 219

Table 1.39 Various cysteine PGs useful in the Fmoc strategy

Key to Table

Fcm	Ferrocenylmethyl.
Npys	3-nitro-2-pyridinesulphenyl.
S/S/TFA	Various silyl chloride/sulphoxide/TFA systems[220,221].
D/TFA	DMSO/TFA system[222].
Ag/Hg/Tl or Hg/Tl	Silver (Ag^{+})[223,224], Mercury (Hg^{2+})[208,209], and Thallium (Tl^{3+})[225] salts/various solvents.
I_2	Iodine in various solvents[216].
RSH	Various reducing agents e.g. tri-n-butyl-phosphine[213], β-mercaptoethanol[214], (for Npys, this may be the thiol group of another cysteine residue)[219].

1.6. PROTEIN SYNTHESIS : LYSOZYME

1.6.1. General : Protein Synthesis

Stepwise SPPS has been successfully applied to the preparation of some small proteins in the region of 50-100 a.a.'s. e.g. HIV-1 protease (99 residues - using Boc strategy)[226] and ubiquitin (76 a.a.'s - Fmoc strategy)[227]. The fact that both synthetic proteins were able to be crystallised and the X-ray structures determined[228,229], indicates that products of the highest purity had been obtained. To realise such syntheses requires much initial investigation into optimising coupling steps, protection strategies, deprotection and cleavage protocols and extensive purification. Additionally, actually determining whether or not synthesis of the target protein has been achieved, necessitates using a battery of techniques e.g. sequencing, mass spectrometry, isoelectric focussing etc., few of which can distinguish whether or not minor changes to the synthetic protein had occurred, for instance, a side-chain PG may still be present or may have modified another residue. Despite these hurdles, the assembly of even larger, more complex proteins has been attempted. Several workers have used a convergent strategy, in which protected segments of the protein are first assembled either in solution[230] or by SPPS (then released as the protected segment[231]). These are then subsequently coupled in the solution phase or on a solid-phase support[232]. Whilst theoretically this should result in a purer final product (if the intermediate stages can be purified), in practice the frequent poor solubility of these protected fragments, and problems of C-terminal racemisation during coupling, often limit this approach. More successful may be couplings using

minimal side-chain protection[233] or perhaps using enzymes[91]. Examples of large peptides/proteins made by chemical methods are shown in Table 1.40.

Protein	No. A.A.	Strategy	Comments	Ref.
Interleukin-3	140	SW/Boc	Purification problems unresolved	234
Bovine Pancreatic RNase	124	Frag/Boc	Four disulphides. Full enzymic activity claimed	235
Apocytochrome C	104	Frag/Boc	Coupled three frag. with C-terminal Gly-thiocarboxyls	236
BP Trypsin Inhibitor	58	Frag/Boc	Three disulphides. Solid-phase frag. syn., frag. linked in soln.	237
Human TGFα	53	SW/Fmoc	Three disulphides	238
Human βCG	74	SW/Fmoc	9.5% yield. Continuous flow methodology used	239
RET	79	SW/Fmoc	Continuous flow methodology used	240

(Notes; SW = stepwise ; Frag. = fragment)

Table 1.40 Examples of chemical syntheses of proteins

Multi-cysteine proteins with more than 100 residues have provided targets which presently set the upper limits for chemical synthesis. Perhaps prompted by the display that the reduced forms of the proteins can refold into biologically active species[241,242], several attempts have been made to chemically produce the enzymes Bovine Pancreatic Ribonuclease[235,243] and Hen Egg White Lysozyme (HL). Although synthesis of the ribonuclease appears to have been accomplished[235], two attempts at lysozyme analogues have not produced successful outcomes[244,245].

1.6.2. Structure of Lysozyme

HL is one of the most widely studied enzymes. It was the first to have its structure determined by X-ray crystallography[246]. This structure, in fact, along with those of complexes with various small saccharides, enabled a theory of its catalysis to be forwarded[247]. The residues Glu35 and Asp52 appear to be directly involved but several others, like the Trp's at 62,63 and 108, are important in substrate binding. This mechanism appears as the standard example of enzyme catalysis in most biochemistry texts[248]. Lysozyme derives its name from its capacity to destroy certain bacteria by cleaving the polysaccharide components of their cell walls, specifically hydrolysing the β(1-4) glycosidic link between N-acetylglucosamine and N-acetylmuraminic acid.

The enzyme is relatively small, 129 a.a.'s long (14.6 kDal.) with four disulphides, linking 6/127, 30/115, 64/80 and 76/94[249], and is not greatly conserved between species (some have only two or three disulphides). The structure has become the model for extensive work on protein folding, with the classic hydrophobic core and disulphides whose interchanging during the folding process has enabled intermediate stages to be trapped[250,251]. The study of the renaturation of reduced, denatured lysozyme using differing concentrations of oxidised/reduced glutathione (see section 2.7.6.1) to yield up to 85% of the original activity, has given us a general method for disulphide formation in peptides/proteins[242]. It may also provide a way to fold a chemically synthesised lysozyme. The enzymic activity regained can easily be quantified by a sensitive spectroscopic assay, where the regenerated protein can clarify a suspension of *Micrococcus lysodeikticus* cells[252], the transmittance increase being directly proportional to the enzyme activity present.

How the initial stages of an attempt at a total HL synthesis were carried out, and their potential importance in aiding work towards the total synthesis of a protein like NGF, will be discussed in detail later (see section 2.10).

CHAPTER TWO : DISCUSSION

2.1. RATIONALE AND APPROACH TO THE INVESTIGATION

2.1.1. General

It is clear that NGF exerts its biological effects through binding to various cell surface receptors[253-255], in common with all other growth factors. What was not apparent at the commencement of this study, was which areas on the NGF molecule were actually responsible for triggering these effects. Knowledge of which a.a.'s are important for binding to the NGF receptor, and essential for this biological activity, should provide clues to NGF's mechanism of action. Perhaps more importantly, agonists or antagonists could be discovered from such facts. These would be potentially useful in clinical/research studies as mentioned earlier. For example, agonists which imitated NGF's actions could be useful as potential drugs for treating neuropathies such as AD, by enabling the damaged cholinergic neurons in the CNS to survive or perhaps even regenerate. This could ameliorate the chronic memory losses associated with such conditions. Alternatively, as one study has suggested that NGF may actually potentiate the toxic effects of amyloid (the major constituent of the neural plaques seen in AD patients)[256], it may turn out that antagonists may also be helpful. No major systematic study of the protein has been undertaken and little conclusive structure-activity relationship (SAR) data has appeared previously in the literature.

The few studies that had been reported relied on altering murine NGF by the specific chemical modification of certain residues. The results are summarised in Table 2.1.

Residue(s)	Chemical modification	Effect on receptor binding/activity	Ref.
$Lys^{25,32,34,57,74,88,95,115}$	Dimethylsuberimidate	None/None	257
$Lys^{25,32,34,57,74,88,95,115}$	Acetylation	None/None	258
$Lys^{25,32,34,57,74,88,95,115}$	Succinylation	None/None	258
$Arg^{50,59,69,100,103,114,118}$	1,2-cyclohexanedione	Inactivates*	258
$Tyr^{52,79}$	Nitration/Iodination	None/None	259
Trp^{21}	N-bromosuccinimide	Large reduction/LR	259,260
$Trp^{21,99}$	N-bromosuccinimide	Small reduction/SR	259,260
Disulphides	Reduction	Inactivates	261

*evidence suggests that this is due to structural changes rather than specific modifications.

Table 2.1 Effects of chemical modifications on mouse NGF

These may be difficult to interpret as certain modified residues, which are not themselves necessary for binding or biological activity, may nevertheless disrupt the secondary/tertiary[262] structure hence will alter NGF's properties. More sophisticated approaches had to be utilised if sensible results were to be obtained and progress made.

2.1.2. Method of Investigation

It was decided to probe the biologically relevant areas in NGF by synthesising fragments from across the entire protein and testing these in several assays against authentic NGF, in the hope that potential 'active-site' sequences would be discovered which would give receptor binding/biological activity similar to the native protein. At the commencement of this study, the relationship between receptor binding (to the $p75^{NGFR}$) and the biological effects caused by this binding, were still unclear. Hence assays were to be carried out which would determine the degree of binding to $p75^{NGFR}$ alone: it was thought that searching for agonist activity (i.e. receptor binding with the elicitation of a biological response) would be too complex at the early stages of our work. There is some precedence for this approach; it has been shown that, at relatively high concentrations (μM or mM), peptide fragments containing an active region blocked the biological activity of the protein fibronectin[263] and mimicked the activity of basic fibroblast growth factor[264]. In fact shortly after our study had commenced, a group published results on NGF based on a similar plan[265].

The potential value of using synthetic peptides can also be assessed by considering earlier published work in the NGF field. It was claimed that a fragment from a tryptic digest of murine NGF showed activity (as judged by neurite outgrowth from a chick sensory ganglia assay) which was about one hundred times more effective than intact NGF on a molar basis[266]. This fragment was found to consist of residues 10-25/75-88 linked by the disulphide between 15-80, which is present in the native protein. Later this same research group also alleged that the sequence 58-59/60-69/104-114, again joined by disulphides, could partially prevent native NGF from binding to an antibody (raised to NGF) in a competitive assay[267]. To clarify the situation, the earlier peptide was chemically synthesised and shown to have none of the said activity[268]. The original assertion has since been further refuted, being found to be due to trace amounts of incompletely digested NGF[269]. Thus the dangers of obtaining peptides from digests are clear, as is the value of synthetic peptides whose purity can be assured.

The next step was to decide exactly which sequences should be made. Several avenues had previously been investigated. Some work had been carried out on raising antibodies (ABs) to hydrophilic regions of NGF[35]. Highly hydrophilic areas (determined via Hopp and Woods plots[270]) often lie on the surface of proteins (hence represent antigenic epitopes) and may be involved in protein:receptor interactions. Interestingly, none of the NGF peptides (residues 23-35, 58-67, 87-97 and 111-120 in rat; 23-35, 55-70 and 109-118 in chicken)[35] used in that study to raise the ABs, mimicked NGF activity nor did the ABs themselves block NGF binding. Alternatively, other investigators had begun to look at the effect of site-directed mutagenesis on the protein[271]. Here, molecular biology techniques were used to change individual a.a.'s in the protein to see what effects (if any) these had on activity or receptor binding. The limitation of this approach may be in initially choosing the appropriate target residues to alter and that, like the chemical modifications, a change in an a.a. which is not essential for binding may still cause destabilisation of the protein structure, often meaning that the mutant protein cannot even be isolated. Having considered what had already been done, we decided to adopt a more general and fundamental approach.

As previously stated, NGF is highly conserved over all of the species so far sequenced. It has been shown that the N-terminal octapeptide is not required for activity[272], thus comparing the remainder of the sequences for human[273], bovine[274], mouse[34,275] and chicken[276] NGF's, several areas containing long stretches of completely conserved residues can be identified. Presumably, these should be structurally and/or functionally significant. Accordingly the conserved sequences of five residues or more were considered as initial synthetic targets, as summarised in Fig. 2.2. Several peptides containing non-conserved residues were also synthesised to overlap peptides from these areas as will be discussed later.

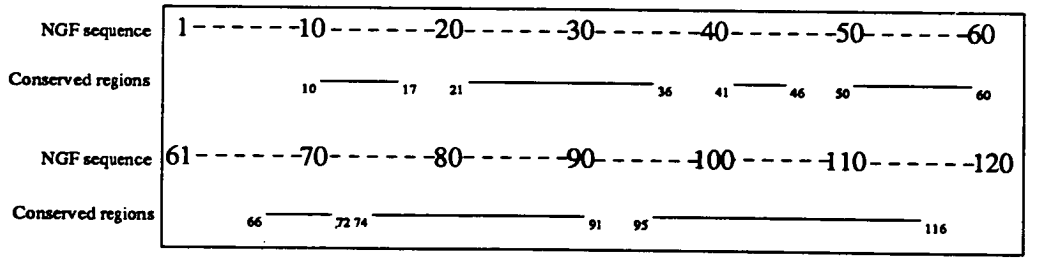


Fig. 2.2 Conserved regions across human, bovine, mouse and chicken NGFs, which were to be synthesised

2.1.3. Potential Agonist/Antagonist Molecules from Peptide Leads

There are few examples of peptide analogues of peptide/protein fragments being used directly as drugs: LHRH has been modified to produce anti-cancer compounds e.g. Zoladex (ICI)[277], and gastrin analogues (e.g. Pentagastrin) are used for the diagnostic stimulation of gastric acid secretion[278]. Increasingly however, synthetic peptides are being used as a method of producing 'leads' in drug discovery programmes. This is witnessed by the explosion in methods for producing multiple peptides in the minimum time as a way of speeding an initial screening process e.g. simultaneous multiple syntheses on polystyrene pins[279], on beads[280], on cover-slips[281], and even presented on the surface of recombinant bacteriophage[282]. Central to these schemes is, of course, the hope that some linear sequence can be found which will give receptor binding and there are many successful examples of this approach.

One important difference, between a native protein and a peptide binder, which must be borne in mind, is how they interact with their receptor. Peptides below around forty residues tend to have a large number of conformers in dynamic equilibrium when in solution. However, these peptides tend to adopt more defined conformations when they bind to their receptors, which themselves often have pre-defined structures: their fit to the receptor is thus 'induced' so that they form complementary conformations to those of their respective receptors. On the other hand, proteins tend to have inherently stable three-dimensional structures. In the interaction with its receptor therefore, the native protein has the advantage, over a peptide, of having a sufficiently large area to enable intramolecular, noncovalent interactions to occur and hence to overcome the unfavourable conformational entropy associated with adopting a defined three-dimensional structure. A consequence of this is that a linear fragment (from a native protein) which binds to the receptor, is unlikely to have any innate structure itself (with some exceptions[283]). Thus if any structural information is to be obtained, it will be necessary to make analogues of this peptide binder which may lock the molecule into its biologically active conformation, and, in restricting the population of conformers in solution, may enable its structural elucidation by, for example, nuclear magnetic resonance (N.M.R.). Several illustrations of this approach have been reported[284,285].

Before a conformationally restricted analogue of a peptide can be made, it is important to first delineate which side-chains may be crucial for activity. This can

be done by making progressively shorter analogues of the original parent peptide until the minimum sequence, that still retains the degree of receptor binding or biological activity, is reached. From here, the side-chains can be varied to determine the significance of charge, steric size, hydrophobicity, chirality etc., at each (or at a specific) position. The arrangement of side-chains involved in receptor binding can be envisioned on the basis of these results and conformationally restrained analogues could be made to test these theories. Such analogues could consist of macrocyclisations of the peptide chain (N-to-C-terminus) or between side-chains (e.g. Asp/Glu to Lys) or further by the introduction of alkyl groups to restrict the movement of the peptide backbone. Often these constrictions may improve the degree of receptor binding due to the smaller decrease in entropy associated with its binding compared to the unstrained parent peptide. An outline for the approach to discovering agonist/antagonist molecules from peptide fragments is shown in Fig. 2.3.

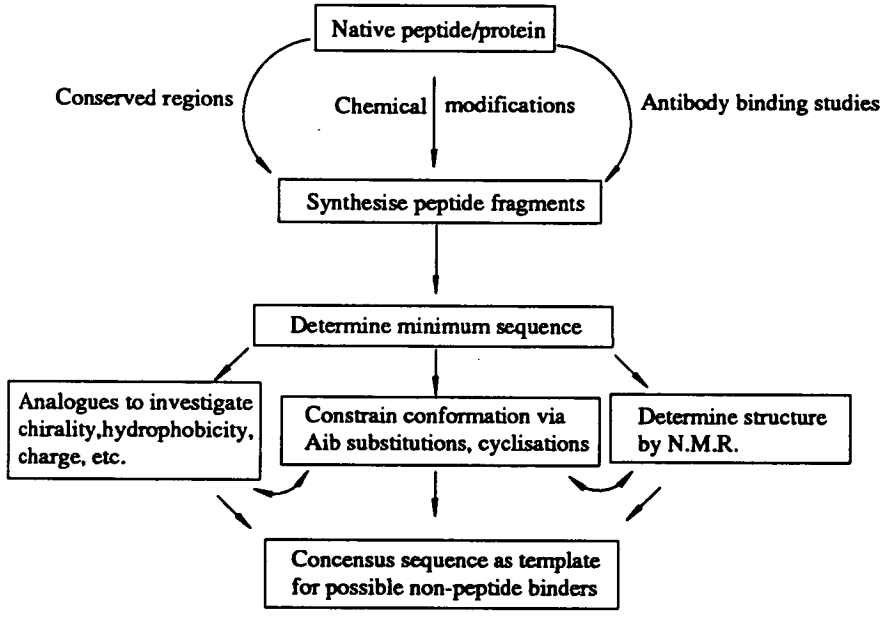


Fig. 2.3 Generalised protein 'active-sequence' investigation strategy

2.2. THE CHEMICAL SYNTHESIS OF NGF FRAGMENTS

2.2.1. Types of Resin used in the Syntheses

The standard resin used for most of the syntheses was the Wang resin[286,287]. This is a 4-(hydroxymethyl)phenoxymethyl derivative of a copolymer of styrene cross-linked with 1% DVB (Fig. 2.4). The C-terminal Fmoc-a.a. was coupled to this, with

SA activation, to form an ester bond in a reaction catalysed by a small amount of DMAP (< 0.1 equiv.) (Fig. 2.4). Alternatively, a type of resin developed in this laboratory enabled the formation of peptide amides upon TFA cleavage.

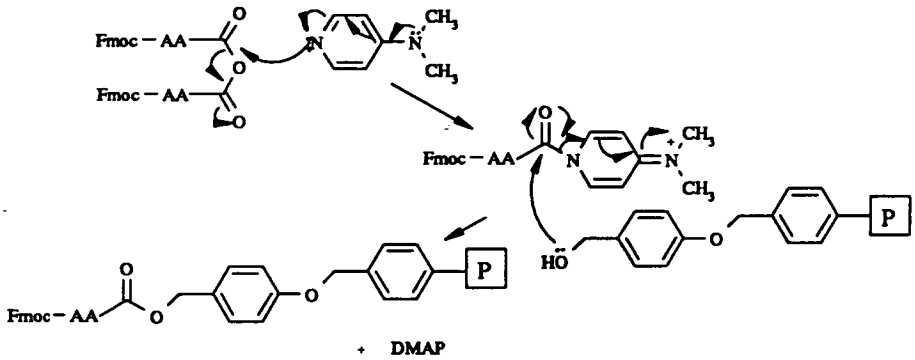


Fig. 2.4 Catalytic action of DMAP in loading the Wang resin

The 2-copoly(styrene-1%-divinylbenzene)methoxy-5-aminodibenzocycloheptadiene resin[288] (referred to as the dibenzosuberyl resin) (Fig. 2.5) could be derivatised using the standard SA/AE couplings to form an amide linkage. An intermediate in the preparation of this resin was modified to enable the formation of a heptylamide peptide (NGF 100-115-NH-heptyl). The heptadiene-5-one (1) had previously been prepared[289] (Fig. 2.6). This was reacted with heptylamine in the presence of titanium (IV) chloride/DCM to yield the imine (2). Monitoring the reaction by IR showed the gradual disappearance of the ketone band at 1640 cm^{-1} over a 2.5 h. period. corporation of the heptylamine was confirmed by nitrogen analysis. The imine structure on the resin was assigned on the basis of previously reported analogous reactions[290].

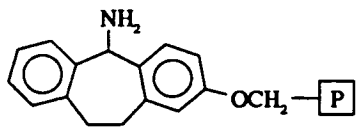


Fig. 2.5 Dibenzosuberyl resin

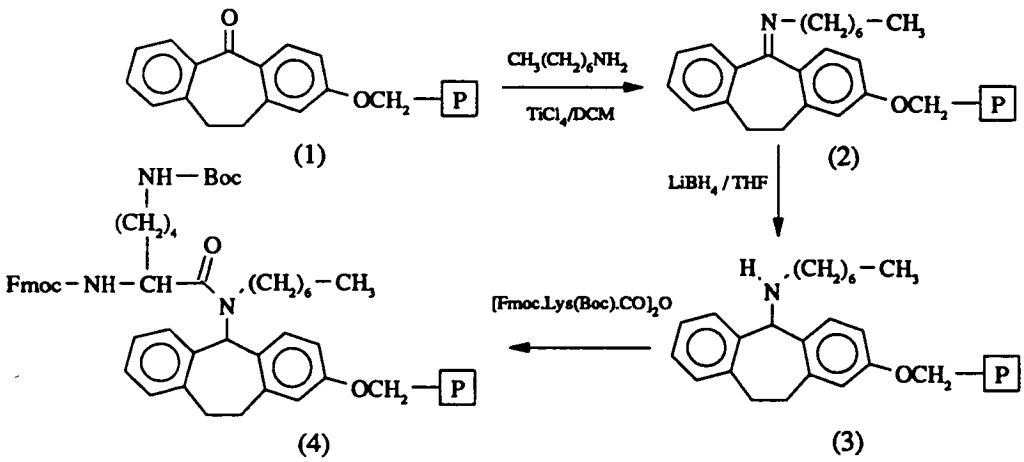


Fig. 2.6 Scheme for synthesis of the heptylamine resin

The imine/resin was reduced to the secondary amine (3), characterised by the appearance of a broad band in the IR at around 3400 cm^{-1}. The coupling of the first residue, Fmoc-Lys(Boc)-OH, required extended reaction times and large excesses of SA. Presumably, steric factors made this reaction unfavourable: previously, only glycine had been coupled to the resin using acid chloride activation[289]. The derivatised resin (4) was used in the assembly of the required peptide with no further difficulties.

Whichever resin was used, the amount of derivatisation (loading) was assessed by measuring the amount of dibenzofulvene adduct released during a sample 20% piperidine/DMF deprotection under standard conditions[291] (see section 2.2.3). Loadings were generally between 0.5-0.6 mmol/g resin. For longer syntheses (> 25 a.a.'s), a lower loading was used (effected by decreasing the derivatisation reaction time from two to one h.) as recommended by Kent[91], to lessen possible steric effects (which may interfere with coupling reactions) between the growing chains.

2.2.2. Manual and Automatic Methods of Peptide Assembly

The NGF peptides were assembled by one of two methods; manual or automatic, both employing the same principles. The apparatus for manual assembly and its operation are described in Appendix A. Basically, the resin was suspended in DMF inside a cylindrical glass vessel fitted with a fine sintered-glass frit. Mixing of the resin/reagents was by nitrogen, introduced through a three-way tap. Reagents were added via wash-bottles or pipettes and could be drained from the vessel into a lower waste reservoir under slight vacuum. The cycles for Fmoc-deprotection and coupling are given in sections 3.2.3 and 3.2.4. Each coupling was by a single SA, which was usually sufficient; however if the monitoring tests (section 2.2.3) showed that acylation was incomplete, a further AE acylation was carried out, sometimes using *in situ* activation by BOP or HBTU in difficult cases. Changing the coupling method often produced complete acylations, perhaps due to AE being less sterically hindered (although also less reactive) than SA. If a coupling was incomplete after three attempts, the peptide/resin was acetylated with acetic anhydride. Usually the resulting truncated, acetylated peptides could be easily separated from the target peptides.

Automatic assembly was carried out on an Applied Biosystems 430A peptide synthesizer. The cycles of washing, deprotection, coupling and capping were similar

to those used in manual assembly. Here, deprotection was with batches of 20% piperidine/DMF for a total of twelve minutes (5, 3, 3 and 1 min.). It was later found that this could be reduced to four minutes (3 and 1 min.) without any adverse effects. This was prudent as extended exposure to piperidine may lead to the formation of β-aspartyl peptides[195]. This is usually not considered to be a significant problem when using the Asp(OBu^t) residue but the side-reaction was emphasised in the assembly of NGF 100-114 [Asp(OBzl)^{105}, Pro^{109a}-dVal^{109b}, Cys(Acm)^{108,110}]. The terminal Fmoc group had been inadvertently left on the peptide; this was not discovered until after the TFA cleavage (the OBzl group is stable to TFA and also remained on the peptide). An attempt was made to remove the Fmoc group using 20% piperidine/DMF for two minutes. This led to almost 100% conversion to the β-aspartyl form (Fig. 2.7), with loss of the benzyl protecting group (using 15% piperidine/DMF for 1 min. led to 35% conversion). The suppression of this side-reaction remains a challenge in peptide chemistry.

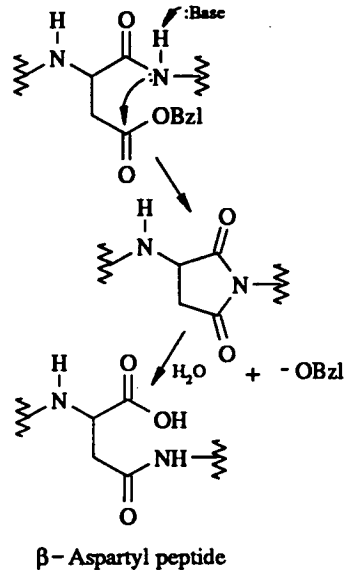


Fig. 2.7 β-Aspartyl formation when using the Asp(OBzl) PG

The coupling reactions used were double-couple cycles, SA then AE. If triple-coupling was required, an additional AE was used. Some residues were incorporated as their AE only, due to previously mentioned problems e.g. Arg(Pmc), His, Asn and Gln. The full cycles are considered in section 3.2. Table 2.8a and 2.8b compare the two methods of assembly.

Method	ADVANTAGES
Manual	Flexible-changes in methodology e.g. different activation methods, increasing coupling times, are easily incorporated.
	Real-time monitoring of the acylation and coupling steps is possible and the operator can intervene as required.
Automatic	Continuous assembly thus faster, especially for longer sequences.
	Controlled amounts of reagents, reaction times etc. are used thus the assembly may be standardised and is usually reproducible.

Fig. 2.8a Comparison of Manual and Automatic assembly methods

Method	LIMITATIONS
Manual	Labour-intensive and generally slow.
	Assembly is not continuous.
	Resin samples must be removed for testing thus reducing the yield, especially in longer syntheses.
	Generally impractical for peptides > 30 residues.
	Requires exposure of operator to chemicals.
Automatic	Inflexible-usually conditions and methods must be assigned at the start of the assembly.
	Monitoring is retrospective i.e. poor couplings are only detected after that residue has been deprotected. This necessitates that double-couple and capping cycles must be always be used whether needed or not.
	All reagents must be completely soluble in the solvents used (DMF or dioxan/DMF).

Fig. 2.8b Comparison of Manual and Automatic assembly methods

2.2.3. Monitoring

In manual assembly, the extent of the acylation reaction could be checked by simple colour tests, which detected free amine functions and hence indicated incomplete couplings. In the Kaiser test[292], a sample of beads would show a blue colouration (Ruhemann's purple) due to a complex between any free amine and the ninhydrin reagent used. A qualitative assessment of the bead/solution colour (aided by experience) determined whether complete acylation had occurred. The test shows positive if the coupling is less than 99% complete. To check, a second test using 2,4,6-trinitrobenzenesulphonic acid (TNBS)[293] was used, a positive test here being indicated by a red colouration on the resin beads. Quantitative ninhydrin tests[294] can also be used here but are laborious to perform.

Automatic assembly using synthesizers has removed much of the routine involved in manual SPPS. With a new generation of synthesizers being evolved that can determine in 'real-time' whether incomplete acylation has occurred during assembly, the realisation of Merrifield's hope of truly 'automated' peptide synthesis may soon be realised. Here, a non-invasive, monitoring method was used which avoided the removal and destruction of resin samples (as in manual assembly) and, more importantly, enabled some 'post-coupling' information to be obtained. When the Fmoc-resin is treated with piperidine during the deprotection step, a dibenzofulvene species is released. The excess piperidine present adds to this olefin to give an

adduct (Fig. 2.9). This adduct has UV absorption maxima at 267, 290 and 301 nm[159].

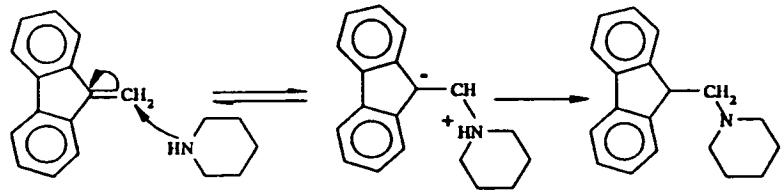


Fig. 2.9 Generation of the chromophoric dibenzofulvene adduct

Monitoring the UV absorbance at 302 nm allows a semi-quantitative determination of the deprotection efficiency and hence the level of incorporation of the preceding residue. By comparison to the integral for the first deprotection of the original derivatised resin, subsequent falls in a.a. incorporation (representing poor acylations) could be observed. This proved useful in deciding whether or not to continue a particular assembly and also in optimising certain syntheses. Often some predictive information was obtained; the integral trace would rise (sometimes even above 100%) one or two residues before a fall in coupling efficiency. This was thought to be due to solvent effects caused by resin desolvation. Poor coupling sequences were also apparent when the peak integral for the second (and third or fourth) deprotection washes began to appear for particular residues (implying a slow deprotection profile for those residues). These signs enabled the next residue to be triple-coupled or checked by manual colour tests. Some reports have suggested that the addition of low concentrations of 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) to the deprotection mixture may increase the speed of Fmoc removal in difficult cases[295]. Examples of monitoring traces are shown in Fig. 2.10 to illustrate some of the above points.

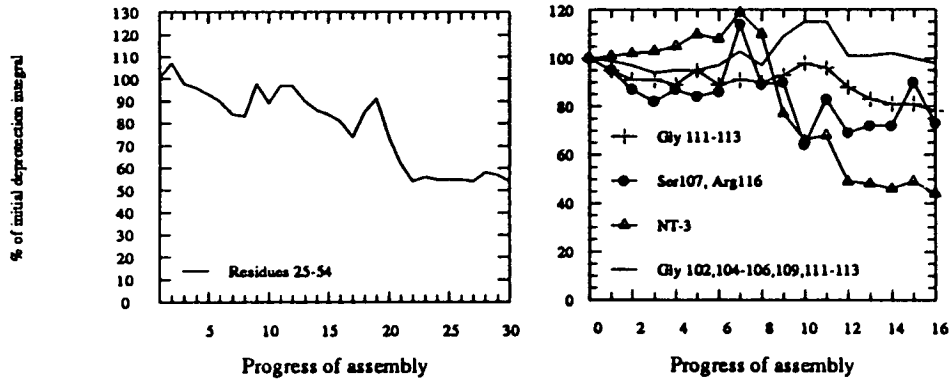


Fig. 2.10 Deprotection profiles of NGF 25-54 assembly and some NGF 100-116 analogues

Fig. 2.10 shows drops in incorporation in the assembly of NGF 25-54, and compares the monitoring traces for four NGF 100-116 analogues (NT-3 100-116 can be

considered to be an NGF analogue). A fall in coupling was predicted for NT-3 by a rise in the trace at residue 109 (point 7 on the trace). For the Ser^{107}, Arg^{116} analogue, although a similar sequence to NT-3, the same fall in apparent incorporation did not occur to the same extent and the trace actually rose again towards the end of the assembly. Such coupling differences were probably due to the steric/hydrophobic effects of using the trityl group on Cys for NT-3 compared to the smaller, more hydrophilic Acm group for the other analogue. Substituting some of the residues in these two peptides for glycine led to near perfect assemblies (as seen in the traces for the Gly analogues).

2.2.4. Peptide Solvation

The solvation of the peptide/resin was seen to be a problem in manual assembly of some of the peptides from the C-terminal region of NGF. Effective solvation of the cross-linked polystyrene resin should leave the peptide chains as accessible as if they were in free solution and, under proper solvating conditions, it has been observed that no decrease in synthetic efficiency may occur up to residue number sixty[296]. Thus the swelling of the peptide/resin should never (theoretically at least) limit a.a. couplings as long as the correct solvating conditions exist. Using polar solvents such as DMF, help the solvation process and aid the solubility of Fmoc-a.a.'s. However, in these syntheses, desolvation was observed as a dramatic decrease in the volume of the peptide/resin bed in the bubbler reaction vessel. This occurred most frequently when the peptide was eight or nine residues long, resulting in slow and incomplete couplings. The fall in incorporation was seen to be sequence-dependent but was overcome somewhat by triple-coupling the affected residues, as observed in later automatic assemblies.

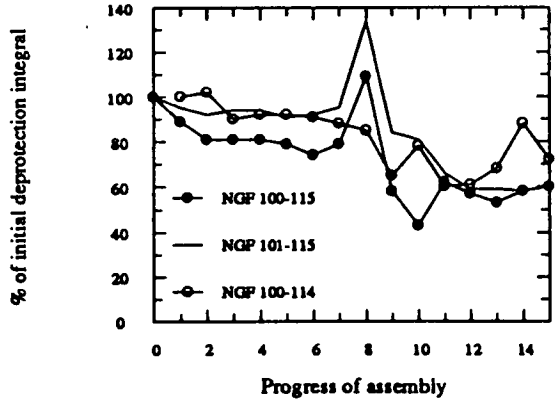


Fig. 2.11 Sequence-dependent falls in incorporation in NGF peptides

Fig. 2.11 shows that triple-coupling Asp[106] and Thr[107] (residue no. 9 and 10) led to improved incorporations of these residues in the assembly of NGF 101-115 and 100-114 compared to 100-115. It has been suggested that such effects may be due to β-sheet formation on the resin[297,298]. When this occurred, the addition of 20% hexafluoro-2-propanol (HFIP) in 50% dioxan/DMF in extended wash cycles improved the solvation as previously observed[299] and aided difficult couplings during the manual assembly of some of the C-terminal peptides.

2.2.5. Acidolysis of the NGF Peptides

The removal of the side-chain PG's and cleavage of the resin-bound peptides was carried out using TFA. This results in the release of highly reactive carbonium ions, derived from these PG's e.g. the Bu^t cation (released from Ser/Thr/Tyr residues). This is held in solution as its trifluoroacetate salt, as opposed to converting to isobutylene and being lost as a gaseous by-product, providing a soluble source of such carbonium ions[300]. These stable cations can cause the modification of sensitive a.a. residues (as mentioned in section 1.4.3) To prevent these side-reactions, various nucleophilic scavengers[301,302], which trap these cations, were added in excess to the TFA solution. These are then selectively attacked in preference to the a.a. side-chains. Examples of the scavengers used are given in Table 2.12.

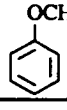
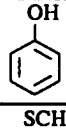
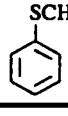
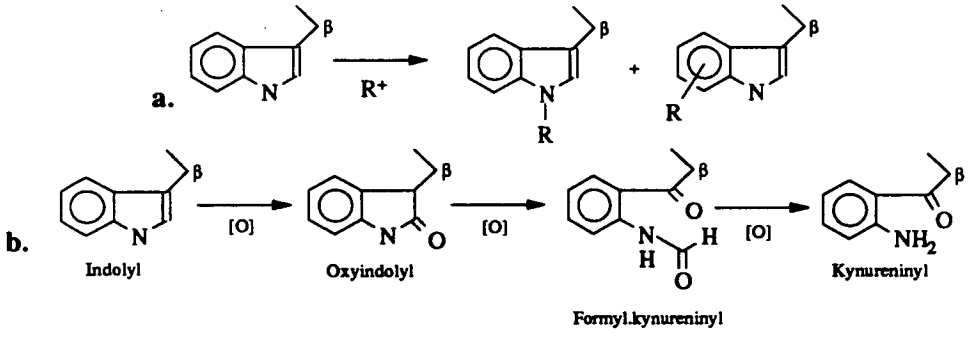
Scavenger	Structure	Comments
Anisole		Scavenges tert-butyl trifluoroacetate (Bu^{t}TFA). Poor scavenger of Bu^t carbonium ions.
Ethyl methyl sulphide (EMS)	CH_3-S-CH_2-CH_3	Prevents Met oxidation. Scavenges tBuTFA.
1,2-ethanedithiol (EDT)	HS-CH_2-CH_2-SH	Best Bu^{t}TFA scavenger. Scavenges Trt. Prevents Trp alkylation by Pmc group. With water, prevents Trp t-butylation.
Water	H-O-H	Good for Bu^t/Boc scavenging. Essential for Pmc scavenging.
Phenol		Scavenges Bu^{t}TFA.
Thioanisole		Accelerates Pmc deprotection. Partially deprotects Cys(Acm) and (SBu^t). Prevents Met oxidation

Table 2.12 Scavengers used in acidolytic cleavages
(after ref. 302)

The optimum cleavage conditions were dependant on which residues were present, their number and sequence, the side-chain PG's used and the a.a. attached to the resin. Small-scale trial reactions using small amounts of resin were carried out, varying the reaction time and combination of scavengers in some cases, to optimise the cleavage conditions for each peptide. A general mixture of 3% water, 3% EDT and 2% (wt./vol.) phenol in TFA was found to be useful in many cases. Similar compositions have previously been reported to be applicable to a wide variety of peptides[303]. Thioanisole, although reported to speed the removal of Pmc from Arg[179], was omitted from the cleavages of NGF peptides containing Cys(Acm) or Cys (SBu^t) as it causes the partial removal of these PG's[214]. It was observed that the inclusion of EDT did not remove the SBu^t group from Cys as had been previously reported[304]. Scavengers were observed to act most efficiently when used from freshly opened bottles.

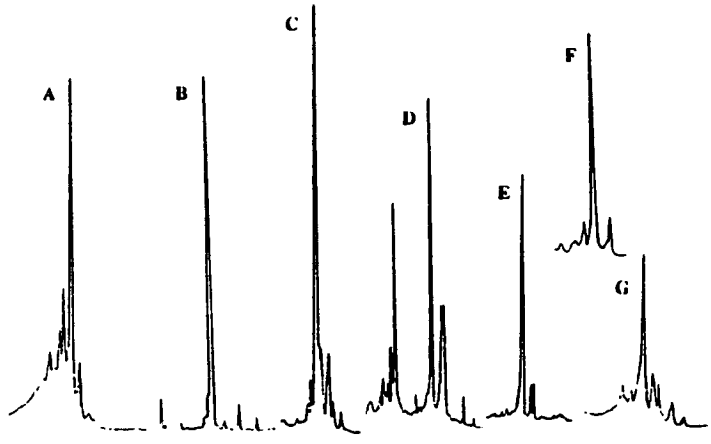
Tryptophan may be alkylated on the indole ring by a various species (e.g. Pmc) in the absence of the correct scavengers[302] (Fig. 2.13a). However, in the presence of EDT and water as scavengers, this side-reaction was not observed in any of the Trp-containing NGF peptides (with the exception of NT-3 100-116). Cleavages were carried out under nitrogen to minimise any possible acid-catalysed oxidation of the Trp residues (Fig. 2.13b).



Figs. 2.13a&b. Alkylation and oxidation of tryptophan

The presence of Trp in the 53 a.a. NGF peptide did cause severe problems in the formation of the disulphide bridges (see section 2.7.7). Fmoc-Trp(Boc)[305] was used in the second HL assembly. On cleavage, a relatively stable N^{in}-carboxy-indole intermediate is said to form from this protecting group. This appeared to provide protection against the tryptophan damage that occurred on extended acidolysis; such damage was observed in the first assembly of HL where Trp was unprotected.

After cleavage for the appropriate length of time, the free peptide was obtained by evaporation of the TFA solution then precipitation of the peptide with excess ice-cold ether. The crude peptide was further dissolved in either aqueous acidic or basic solution, extracted from ether (to remove the scavengers) then lyophilised. HPLC traces of some of the crude NGF peptides are shown in Fig. 2.14.



Peptide	%CH_3CN/min.; Rt	Peptide	%CH_3CN/min.; Rt
A : 25-54	5-65/30 ; 21.5 min.	**E : 100-116** Gly analogue	10-90/30 ; 21.8 min.
B : 100-116 $G^{111-113}$	10-50/30 ; 15.8 min.	**F : 100-115**	10-75/30 ; 15.0 min.
C : 100-116 S^{107}, R^{116}	5-95/30 ; 20.2 min.	**G : 100-114**	5-95/30 ; 22.0 min.
D : NT-3 100-116	10-90/30 ; 12.2 min.		

Fig. 2.14 HPLC traces of some crude NGF peptides

2.2.6. Purification Techniques

2.2.6.1. General

Initially, the products from SPPS will be impure, as would be expected for any technique where multiple reactions were carried out without the isolation and purification of intermediate stages. The crude peptide will generally contain a mixture of some or all of the following:

1. Solvents, scavengers and reagents.
2. Terminated peptides (acetylated or truncated by other means).
3. Partially protected peptides.
4. Modified or rearranged peptides (caused during acidolysis).
5. Deletion peptides (lacking one or more of the required residues).

Whilst 1-3 can usually be separated from the required peptide, examples of 4 and 5 will often have physicochemical properties which are similar to the target sequence. These problems can be expected to become more significant with increasing peptide length. Additionally, purification often becomes disproportionately more difficult as the crude product becomes more impure, generally due to solubility problems. In practice, if a crude peptide is not soluble in largely aqueous systems, purification may not be possible at all. Considering these points, the value of optimising the assembly and cleavage protocols for a peptide cannot be stated highly enough. Crude mixtures can be separated by various methods based on:

a.) Molecular size - gel filtration,
b.) Hydrophobicity - HPLC (various forms),
c.) Charge - ion exchange,
d.) Affinity techniques.

2.2.6.2. Gel Filtration (Size-exclusion chromatography)

This technique fractionates peptide or protein mixtures on the basis of molecular size (strictly hydrodynamic volume). Peptides/proteins partition themselves between the mobile phase (eluent, an acetic acid solution was used here) and the stationary phase, the liquid inside a bead-formed polysaccharide gel matrix. The partitioning depends on the rate of access into the small pores on the beads; large peptides are excluded from the internal volume and thus emerge first from the column, whereas smaller peptides, which can gain access to the pores, emerge later. The fact that components do not bind to the matrix limits the resolution of the technique so it is generally only useful for group separation. Various media are commercially available, differing in structure, degree of cross-linking (hence fractionation range) and robustness. The media used are summarised in Table 2.15.

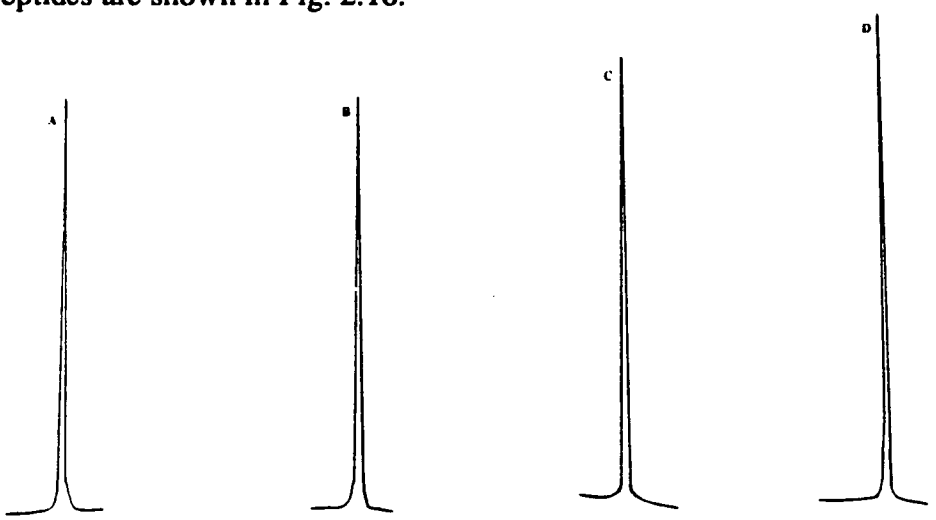
Media	Uses
Sephadex G25	1. to desalt crude peptide mixtures 2. to separate dithiothreitol (DTT) from peptide in Cys side-chain deprotection strategies
Sephadex G50	1. initial purification of NGF 53-mer peptide 2. initial purification of HL
Sephadex G75	1. additional purification of HL

Table 2.15 Uses of Gel Filtration media

2.2.6.3. High Performance Liquid Chromatography (HPLC)

The principle of separation here relies on the hydrophobic interaction between the a.a. side-chains of the component peptides in a crude mixture, and silica-bound octyl- (C8) or octadecyl- (C18) alkyl chains. The crude mixture is applied to a silica column under aqueous conditions whereupon the components reversibly 'bind' to the silica. These can then be selectively and specifically eluted by increasing the percentage of organic modifier (usually acetonitrile) in the aqueous mobile phase; hydrophilic components eluting early, more hydrophobic components being retained until higher proportions of acetonitrile are reached. With small peptides, the processes of partition and adsorption between the mobile and stationary phases produce the separation, whereas for large polypeptides/proteins, an adsorption-displacement model has been postulated[306].

Analytical HPLC enabled the peptides to be analysed. The speed, resolution and low sample requirements made this method especially convenient. Samples were run under various gradients and also isocratically to check for impurities running under the main peak. The use of different counter-ions e.g. ammonium hydrogen carbonate or sodium hydrogen phosphate, in place of 0.1% TFA aided this process. Semi-preparative HPLC was used extensively to purify the NGF peptides. Judicious adjustment of the counter-ions, organic modifier or, more importantly, the gradient of aqueous/organic eluent and flow rate[307], led to the effective isolation of the target peptide from its crude product in every case. HPLC traces of some of the purified NGF peptides are shown in Fig. 2.16.



A : NGF 95-116 ox.	B : NGF 100-115 ox.	C : NGF 100-114 ox.	D : NT-3 100-116 ox.
10-90%/30 min. ; 17.5 min.	10-75%/30 min. ; 15.0 min.	5-95%/30 min. ; 20.0 min.	10-90%/30 min. ; 11.3 min.

Fig. 2.16 Some purified NGF peptides

2.2.6.4. Other purification methods

Ion exchange chromatography : peptides display a net charge at all pH values other than at their isoelectric point, which is characteristic for individual peptides. A counter-ion to this charge can be immobilised on a solid-phase support to act as a stationary phase. Partitioning between this stationary and a mobile phase (a buffer at a particular pH) affects the separation of components depending on the strength of interaction between the component and the support. Altering the ionic strength or pH of the eluent selectively elutes the peptides. This method was envisaged for the purification of the NGF 53-mer and HL, but was not utilised for reasons which will be explained.

Affinity chromatography : other strategies, based on modifications to the Fmoc group of the N-terminal residue, may have future applications in the purification of these larger polypeptides. The 9-COR-Fmoc group[308], where R=Lys (for ion exchange), R=biotin (for affinity chromatography) or R=2-aminoundecanoic acid (for RP-HPLC), has been suggested. Similarly, the Tbfmoc group[309] can be used, with separations carried out on graphite or activated charcoal columns. Both methods rely on increasing the physicochemical differences between the target peptides and terminated (by acetylation) peptides lacking the final derivatised residue.

2.2.7. Chemical Formation of Disulphide Bridges

The formation of disulphide bridges became an integral part of this work. Some examples of the removal of acid-labile and acid-stable Cys PG's, and the methods used in disulphide bond formation, are summarised in Table 2.17.

NGF peptides containing a single cysteine residue (thus not requiring a disulphide bridge) were assembled using the trityl group. This was easily removed by TFA, liberating the trityl cation (which turns the cleavage solution yellow). This cation was prevented from adding back to the thiol group by the presence of excess EDT in the mixture. The trityl group was also used for many peptides which actually required a disulphide. With many of these peptides, a simple air-oxidation strategy was adopted. The purified, reduced peptide was dissolved in water (at 0.5 mg/cm^3 or less) and stirred. The pH was adjusted to around neutrality with concentrated ammonium hydroxide, if required. By HPLC, the reduced peak could be seen to

decrease in height with time (over 1-6 days), to be gradually replaced by an oxidised peak (which almost always eluted slightly earlier).

NGF peptide	Cys PG	Removal	Disulphide formed by..
14-28, 66-79	Trt	EDT/TFA	-
100-110, 100-115	$Trt^{108,110}$	EDT/TFA	Air oxidation
95-116	$Trt^{108,110}$	EDT/TFA	Air ox., catalysed by $K_3Fe(CN)_6$
100-115-NH_2	$Trt^{108,110}$	EDT/TFA	10% DMSO/TFA
100-114	$Trt^{108,110}$	EDT/TFA	20% DMSO/TFA
100-116 Ser^{107},Arg^{116}	$Acm^{108,110}$	AgOTf/TFA, DTT/AcOH	Air oxidation
100-114 Aib^{111} 100-114 Nle^{113}	$Acm^{108,110}$	AgOTf/TFA, DTT/AcOH	10% DMSO/TFA
53-mer	$Acm^{108,110}$	$(CF_3CO_2)_3Tl$/TFA	
100-115-NH_2	$SBut^{108,110}$	TBP/TFE	Air oxidation
53-mer	$SBut^{58,108}$ $Acm^{68,110}$	TBP/TFE	a.) Air oxidation b.) GSSG/GSH
100-114[Pro-dVal][109]	$SBut^{108,110}$	TBP/TFE	15% DMSO/TFA

Table 2.17 Cys PG removal and disulphide formation in NGF peptides

The extent of the reaction was usually between 90-99% and could be followed by Ellman's test (section 2.7.6), in which the colour intensity also decreased over time to a constant value. The reactions could be speeded in some cases by the addition of small amounts of potassium ferricyanide $[K_3Fe(CN)_6]$[212], although this often reduced the final yield of the oxidised product. Separation of the reduced/oxidised peaks was most conveniently achieved by first adjusting the solution to low pH (with 0.1% TFA) then directly pumping this solution onto a semi-preparative HPLC column. The peptide components bound to the column and oxidising agents e.g. ferricyanide or DMSO (see below), were eluted under mainly aqueous conditions before purification was carried out using an acetonitrile gradient in the usual way.

A few NGF peptides (e.g. 100-114) could not be purified from their crude forms due to their insolubility. This led to the use of 10-20% DMSO in TFA as an oxidant. These peptides easily dissolved in the solvent system enabling conversion to the disulphide form within one h. at 25°C[222]. The oxidised products were soluble and were able to be purified by conventional means. This method was also used for some of the more soluble NGF peptides due to its speed and convenience. Test reactions were also carried out using DMSO in aqueous buffer systems[310] but these were observed to take longer (> 3 h. at pH 5.0).

The poor crude products obtained for some of the peptides when using Cys(Trt) and the requirement for selective disulphide formation in the NGF 53-mer (see section 2.7.1), led to the use of Cys(Acm) and Cys(SBu^t) in certain assemblies. The SBu^t group could be removed easily using n-tributylphosphine (TBP) in 95% 2,2,2-trifluoroethanol/water (95% TFE)[311] (Fig. 2.18).

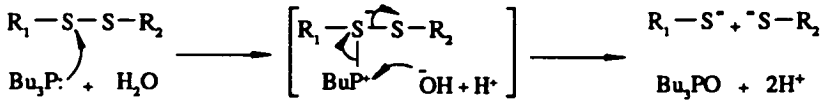


Fig. 2.18 Generalised cleavage of mixed disulphides with TBP

Although this PG made the crude peptides much more hydrophobic, its removal could easily be monitored by HPLC due to the large reduction in HPLC retention time that occurred.

The Acm group improved the assemblies of some NGF peptides, also giving more soluble crude products (compared to SBu^t protected or free thiol peptides). It has conventionally been removed using mercury (II) acetate at pH 4, followed by reduction; some investigators have however reported difficulties in reducing the mercuric/thiol complexes that form in this reaction[245]. In this work, the protecting group was more conveniently removed using silver trifluoromethanesulphonate (AgOTf) in TFA[223] (silver tetrafluoroborate has also been suggested[224]), followed by reduction with dithiothreitol (DTT). The reduced peptide could be separated from the DTT by gel filtration. On one occasion the method was unsuccessful.

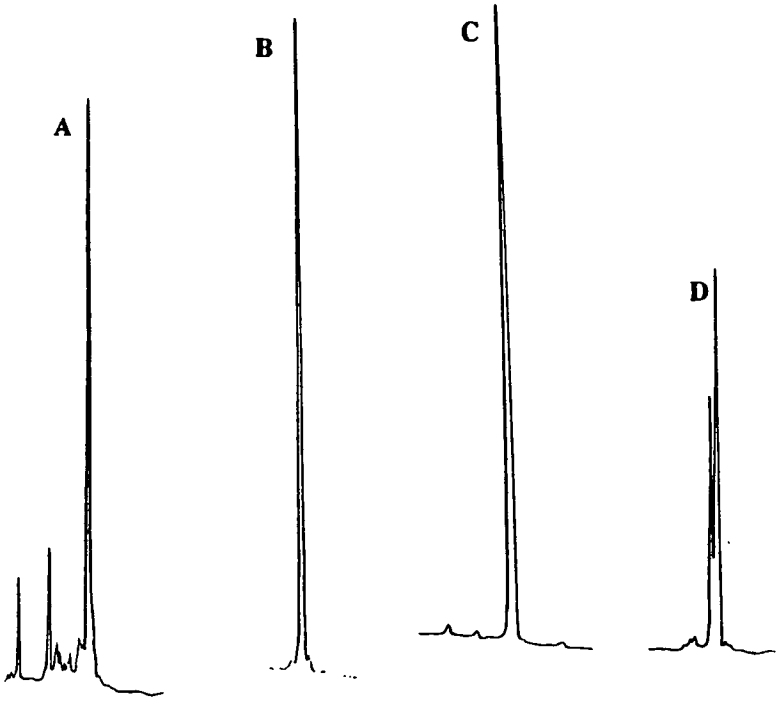
Several methods of forming a disulphide directly from the di-Acm peptide were investigated. Using NGF 100-116 S^{107},R^{116} as a model, the following reactions were carried out;

a.) Iodine (several concentrations) in (i) 80% acetic acid/water or (ii) 80% methanol/water (various reaction times).
b.) DMSO (0.4M)/trimethylchlorosilane (1.0M) in TFA[220] for 15 min.
c.) Diphenylsulphoxide (10 equiv.)/methyltrichlorosilane (100 equiv.) in TFA[312] for 10 min.

Method a.) showed that poor conversion to the oxidised form had occurred, with several additional peaks present under all conditions. Methods b.) and c.) showed the characteristic peak shifts expected; however when the putative oxidised peaks

were isolated and examined by FABMS, as well as the correct molecular ion (m/z 2052), several other unidentified, higher M.W. peaks were observed. These reactions were especially time-dependent, increasing the reaction times gave rise to many unknown side-products (on HPLC).

More successful was the direct formation of the disulphide in NGF 100-114[Pro-dVal][109], whilst the peptide was still attached to the resin. Treatment with TBP in HFIP/DMF for 3 h. removed the SBu^t PGs. The free thiol peptide on the resin was oxidised using either; (i) a $K_3Fe(CN)_6$ solution in HFIP/DCM for 12 h., or (ii) an iodine-saturated solution of HFIP/DCM for 1 h. On cleavage from the resin, it was seen that protocol (i) had given only a low (10%) conversion to the oxidised form whereas (ii) had led to around 65% conversion (Fig. 2.19). These results were not optimised but show that the time of production of such peptides could be reduced in favourable circumstances. An efficient chain assembly is probably vital to such a strategy.



A	Trial cleavage crude after SBu^t protecting groups had been removed from resin-bound peptide. Grad. 5-95% CH_3CN/30 min. ; main peak eluted at 52% CH_3CN.
B	Pure, reduced 100-114 [PdV][109]. Elutes at 52% CH_3CN.
C	Pure, oxidised 100-114 [PdV][109]. Elutes at 51% CH_3CN.
D	Resin-bound crude after iodine oxidation. Major peak coeluted with sample C. Purification isolated peak, which gave correct FABMS.

Fig. 2.19 Traces illustrating resin-bound disulphide formation

One NGF peptide (100-115-NH_2) was assembled using each of the above PGs (Fig. 2.20). In this assembly, Trt gave the poorest crude product, with Acm and SBu^t PGs giving similar traces, although the final oxidised product was obtained in low yield when using Acm. SBu^t was the optimal PG in this particular case, although in other closely related assemblies, both Trt and Acm PGs proved satisfactory. The choice of Cys PG is often overlooked in the optimisation of an assembly but this example illustrates that it may well be vital to successful synthesis of the target peptide. The use of Cys PGs for selective disulphide formation is considered in section 2.7.

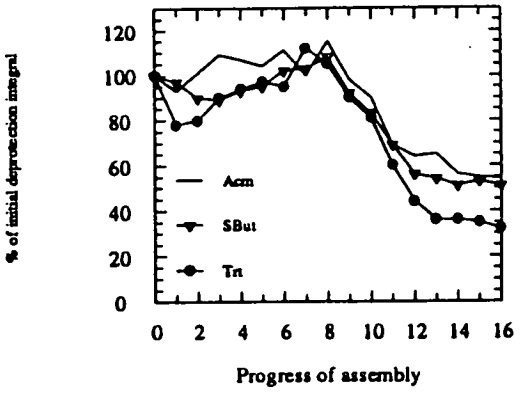


Fig. 2.20 NGF 100-115-NH_2 syntheses using various Cys protecting groups

2.3. BIOLOGICAL TESTING OF THE NGF PEPTIDES

2.3.1. General

The NGF peptides were tested for their ability to bind to the low-affinity NGF receptor ($p75^{NGFR}$). These assays were carried out by staff at Parke-Davis Pharmaceutical Research Co. (Ann Arbor, MI) and Chiron Corporation (Emeryville, CA), following the development of the tests at Chiron. Typically 2-15 mg of the purified NGF fragments were sent for testing. Two cell-free systems were mainly used for testing; the R2 assay and the Biotin assay. In the R2 assay, a human extracellular domain of the $p75^{NGFR}$ was prepared by recombinant methods from a Chinese hamster ovary (CHO) cell line. A monoclonal antibody, R2, which had been raised against the extracellular domain of the $p75^{NGFR}$, was adhered to the wells of microtitre plates. This system was then used to 'capture' the extracellular domain of the receptor, to which the native murine NGF could then bind. The mNGF used in these assays was isolated from mouse submandibular glands as before and radioiodinated by the Iodogen method for use in these assays. The assay could tolerate small amounts of organic solvent, e.g. 10% methanol, without the binding level of authentic NGF being compromised. This system was used to test some of

the first NGF peptides to be made but it did suffer from two problems; (a.) some receptor/AB dissociation occurred during the assay, and (b.) the AB appeared to interact with the NGF/receptor affinity, causing a shallow displacement curve. These difficulties were overcome by the development of the more widely used Biotin assay, which had very low receptor/plate dissociation and gave a well-defined, S-shaped dose-response curve. In the Biotin assay, a full-length recombinant human $p75^{NGFR}$ was used, again produced from CHO cell lines. The receptor was then biotinylated and could be firmly adhered to streptavidin-coated microtitre plates for use in the assays. The basic principles of both assays is illustrated in Fig. 2.21 below.

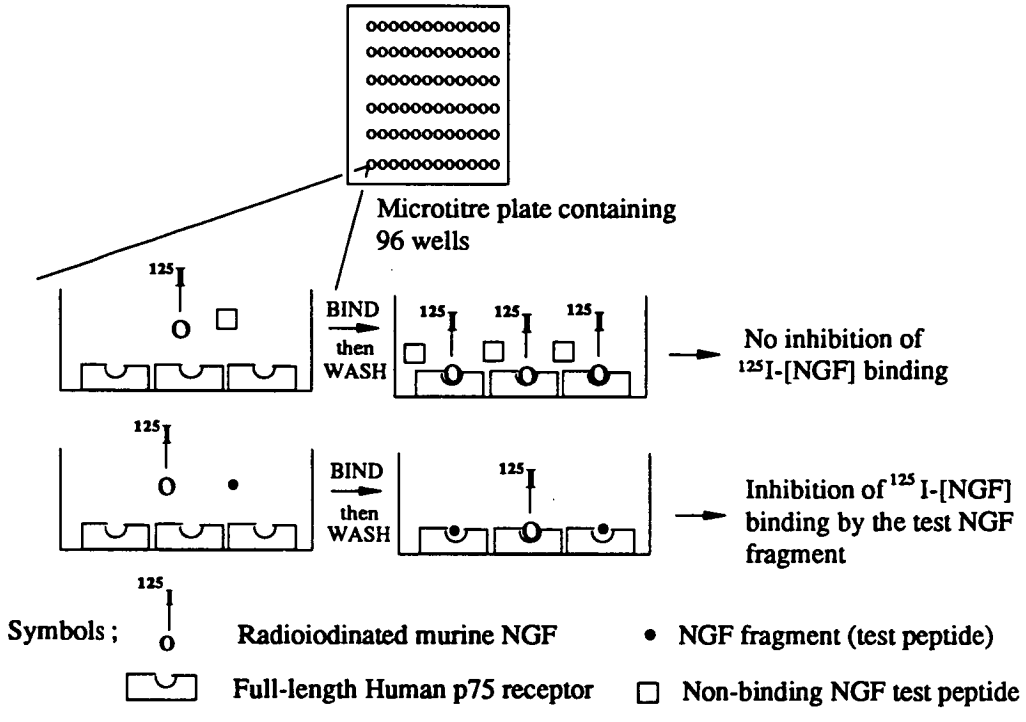


Fig. 2.21 Generalised NGF binding assay

2.3.2. Biotin Assay

The microtitre plates were derivatised by coating for six hours with 50µl/well of streptavidin (20µl/cm^3) in phosphate buffered saline (PBS). The non-specific sites were blocked with 1% bovine serum albumin (BSA) in PBS. The wells were then washed with 0.1% BSA/PBS then 50µl/well of biotinylated $p75^{NGFR}$ (5µg/cm^3) was added and allowed to incubate for two hours. The plates were then ready for testing.

The test peptides were assayed using the following protocol:

1. Wells were rinsed three times with binding buffer (0.25 cm^3, 0.2% BSA/PBS, 0.05% sodium azide (NaN_3).
2. 98µl/well of ^{125}I-NGF, (2 nM) in binding buffer, was added.
3. 2µl/well of the TEST PEPTIDE (in DMSO) was added to final concentrations of 1, 10, 30 and 100µM.
4. The plates were incubated for two hours at room temperature.
5. The reaction was terminated with three washes of buffer (0.1% BSA/PBS, 0.05% NaN_3).
6. The radioactivity in the wells was determined in a gamma-counter.

By comparison with control samples, the decrease (if any) in radioactivity (and hence in ^{125}I-NGF binding) caused by the test peptides was determined. This implies that the test peptides had competed with the radioiodinated NGF for the available binding sites on the immobilised $p75^{NGFR}$, thus decreasing the level of radioactivity. Accordingly, competitive peptides may contain areas important for binding, analogous to those in the native protein. By testing each NGF fragment at several concentrations, a dose-response curve could be plotted. This enabled IC_{50} values, the concentration required to cause a 50% reduction in native NGF binding, to be calculated as illustrated in Fig. 2.22. These proved useful for comparing the binding efficiencies of different fragments, and evaluating changes made to a particular peptide. In these tests, ^{125}I-NGF typically bound to the $p75^{NGFR}$ with an IC_{50} value of 5-10 nM.

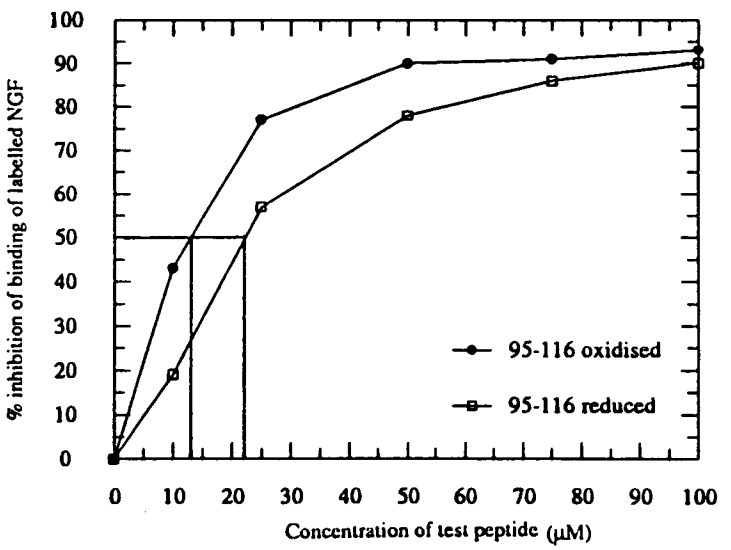


Fig. 2.22 Dose response curves for NGF 95-116 peptides showing the determination of IC_{50} values

2.4. RESULTS OF INITIAL SCREEN ACROSS NGF MOLECULE

The results of the binding assays for NGF fragments from all areas of the native protein are summarised in Table 2.23. IC_{50} values (where they have been determined) are included in brackets in micromoles/litre (μM).

NGF peptide	Sequence	R2 assay % binding @100 μM	BIOTIN assay % binding @100 μM (or IC_{50} value)
10-17	GEPSVC^{SH}DS	0	-
14-28	VC^{SH}DSVSVWVGDKTTA	-	19
Ac-14-28	Ac-VC^{SH}DSVSVWVGDKTTA	-	14
19-22	SVWV	-	14
Ac-19-22	Ac-SVWV	-	11
25-54	KTTATDIKGKEVTVLAEVNINNSVFRQYFF	-	21
34-42	KEVTVLAEV	0	-
46-54	NSVFRQYFF	0	-
50-54	RQYFF	2	-
50-60	KQYFFETKC^{SH}RD	16	-
59-67	RASNPVQSG	0	-
66-79	SGC^{SH}RGIDSKHWNSY	0	-
70-74	GIDSK	5	-
71-79	IDSKHWNSY	0	-
95-116 red.	KQAAWRFIRIDTAC^{SH}VC^{SH}VLSRKA	73	(20μM)
95-116 ox.	KQAAWRFIRIDTAC^{OX}VC^{OX}VLSRKA	-	92% ; (15μM)

Notes : C^{SH} = free thiol cysteine; C^{OX} = disulphide-linked cysteine
Ac = acetyl; ox. = disulphide-linked; red. = free thiol

Table 2.23 Results of initial screen

The results showed that most of the linear NGF peptides did not cause much reduction in the level of murine ^{125}I-NGF's binding to the p75^{NGFR}. In one region, however, at the C-terminus of the native protein, a fragment did show that significant competition had occurred. The peptide 95-116 showed binding in both the R2 and Biotin assays. This sequence contained cysteine residues at positions 108 and 110 (which form disulphides with cysteines residues at 58 and 68 respectively in NGF) The peptide was, at first, tested in its reduced form, having been assembled using Cys(Trt). Later, it became clear from HPLC studies that a disulphide bond

rapidly formed between these residues, even at acidic pH values. Further studies confirmed that in relatively dilute solutions (0.5 mg/cm^3 or less), an intramolecular disulphide readily formed; this could be reversed by DTT reduction. No intermolecular dimer could be made to form, even at high peptide concentrations (up to 20 mg/cm^3). Testing of the purified, oxidised form showed an improved IC_{50} value of 15 μM against 20 μM for the reduced form; presumably, the reduced peptide had formed this disulphide during testing and this lower value reflected the advantage of having a 'preformed' disulphide in the oxidised peptide. All subsequent peptides in this region were tested in their more stable, disulphide-linked forms. The NGF 95-116 ox. peptide was also tested in a similar epidermal growth factor (EGF) assay, but binding was not observed, implying that the peptide can discriminate between the two receptors. The possible consequences of the presence of this disulphide bridge, as they relate to binding, are further considered below. The next stage undertaken was to determine the minimum fragment of the sequence which would still maintain this high level of binding.

2.5. DETERMINATION OF THE MINIMUM BINDING SEQUENCE

2.5.1. Results of Binding Assays

The original NGF 95-116 peptide was progressively shortened at the N- and C-termini to see what effects on binding this would produce. The results of these modifications are summarised in Tables 2.24a and b.

N-TERMINUS Peptide	Sequence	% binding in assay		IC_{50} (μM)
		Biotin @ 30 μM	Biotin @ 100 μM	
95-116 ox.	KQAAWRFIRIDTAC^{ox}VC^{ox}VLSRKA	-	92	15
100-116 S^{107}, R^{116} ox.	RFIRIDTSC^{ox}VC^{ox}VLSRKR	-	-	7
100-115 ox.	RFIRIDTAC^{ox}VC^{ox}VLSRK	84	-	12
101-115 ox.	FIRIDTAC^{ox}VC^{ox}VLSRK	30	-	-
103-116 ox.(313)	RIDTAC^{ox}VC^{ox}VLSRKA	-	15	-
106-116 red.	TAC^{SH}VC^{SH}VLSRKA	-	18	-
106-116 ox.	TAC^{ox}VC^{ox}VLSRKA	-	20	-
111-116	VLSRKA	-	13	-

Fig. 2.24a Optimisation of N-terminus of 95-116

C-TERMINUS Peptide	Sequence	% binding in assay		IC_{50} (μM)
		Biotin @ 30 μM	Biotin @ 100 μM	
95-116 ox.	KQAAWRFIRIDTAC^{ox}VC^{ox}VLSRKA	-	92	15
100-116 S^{107}, R^{116} ox.	RFIRIDTSC^{ox}VC^{ox}VLSRKR	-	-	7
100-115 ox.	RFIRIDTAC^{OX}VC^{ox}VLSRK	84	-	12
100-115-NH_2 (SBu^t) ox.	RFIRIDTAC^{ox}VC^{ox}VLSRK-NH_2	-	-	3
100-115-NH_2 (Trt) ox.	RFIRIDTAC^{ox}VC^{ox}VLSRK-NH_2	-	-	19
100-115-NH-heptyl (SBu^t)^{108,110}	RFIRIDTAC^{SB}VC^{SB}VLSRK-NH-heptyl	39	-	-
100-115-NH-heptyl oxidised	RFIRIDTAC^{ox}VC^{ox}VLSRK-NH-heptyl	68^{+}	-	-
100-114 ox.	RFIRIDTAC^{ox}VC^{ox}VLSR	-	-	3
100-113 ox.$^{(313)}$	RFIRIDTAC^{ox}VC^{ox}VLS	23	-	-
100-110 ox.	RFIRIDTAC^{ox}VC^{ox}	-	30	-
100-110 red.	RFIRIDTAC^{SH}VC^{SH}	-	0	-

(+ data was not able to be fitted to curve to determine IC_{50} value ; Notes ; SB = SBu^t PG on cysteine)

Fig. 2.24b Optimisation of C-terminus of 95-116

2.5.2. Optimisation of the N-terminus

Starting from residue 95, it was found that the sequence could be reduced to residue Arg^{100} without any loss in binding. This was convenient as it omitted Trp^{99}, a fact which facilitated the oxidative disulphide formation in some of the later peptides. Removal of residues 100-102 drastically reduced the level of binding, although little decrease was observed on further reducing the length of this smaller peptide. The high binding was retained in NGF 100-115 but subsequent removal of Arg^{100} to form NGF 101-115 caused a significant fall in binding. Thus it appeared that Arg^{100} was important in the maintenance of binding integrity.

2.5.3. Optimisation of the C-terminus

Beginning with the peptide NGF 100-116 Ser^{107}, Arg^{116} ox., the peptide was shortened by one residue at a time. NGF 100-115 retained the binding and its C-terminus became the subject of several changes. Assemblies of the amide form of this peptide were achieved using both SBu^t and Trt as Cys PGs. Interestingly, the binding values for these peptides were different, although this was probably due to

the SBu^t assembly giving a slightly purer final product. A heptylamide of this peptide was also produced in the hope that increasing the hydrophobicity of the peptide may be a way of increasing binding. The final oxidised peptide gave results that could not be fitted to a curve to determine an IC_{50} value. This was thought to be due to solubility difficulties at the higher concentrations used in some of the tests. Removing residue 115 to give 100-114 ox. had little effect, but removing Arg^{114} considerably reduced the level of binding. These truncated peptides gave considerable solubility problems in their crude forms as previously stated, although the purified, oxidised forms were soluble. Overall, it appeared that the smallest active fragment was the oxidised form of NGF 100-114. This peptide was used as the basic model for further a.a. substitution strategies.

Significantly, the removal of Arg residues from either end of this peptide led to decreased levels of binding. In the original description of the $p75^{NGFR}$ receptor[53], it was observed that the extracellular region contained a large concentration of negatively-charged residues (Asp/Glu). It was suggested that this may represent a complementary binding pocket to particular positively-charged areas on the NGF molecule. It was also further postulated that the Arg residues in NGF were close together in the 3-D structure[53], thus perhaps presenting such a positively-charged surface. It may be that the arginine residues in NGF 100-114 (at 100, 103 and 114) may be so arranged as to mimic such a structure, additionally stabilised by the disulphide bond formed between $Cys^{108,110}$. This point is further developed in section 2.11.

2.6. REPLACEMENT STRATEGIES

2.6.1. General

Before the final optimisation of the NGF 95-116 peptide had been completed, some a.a. replacements were carried out on the 100-116 sequence to investigate the contribution to binding of some of its constituent residues, and to see if some changes might further increase the binding of the parent peptide. The results are summarised in Table 2.25. The NGF, BDNF and NT-3 100-116 peptides all bound to the recombinant receptor with roughly equal affinities, despite having four or five a.a. changes between them. The non-conserved positions were at 99, 107, 111, 113, 114 and 116. The Ser^{107}, Arg^{116} analogue incorporated the change at Ala^{107} (Ser^{107} in

BDNF and NT-3), but again had a similar affinity for the receptor. Thus, it seemed clear that some replacements would be tolerated. Certain drastic changes were incorporated in which glycine replaced (a.) $V^{111}L^{112}S^{113}$ and (b.) all the residues which appeared to tolerate a single substitution as seen in an alanine scan[314] (where each residue is replaced in turn by alanine). This effectively removed these side-chain functionalities and would allow a greater degree of flexibility in the peptide backbone.

Peptide	100-116 ANALOGUE Sequence	% binding in assay		IC_{50} (μM)
		Biotin @ 30 μM	Biotin @ 100 μM	
100-116 ox.[313]	RFIRIDTAC^{ox}VC^{ox}VLSRKA	-	90	8
100-116 red.[313]	RFIRIDTAC^{SH}VC^{SH}VLSRKA	-	30	>100
S^{107},R^{116} diAcm	RFIRIDTSC^{SA}VC^{SA}VLSRKR	7	-	-
S^{107},R^{116} ox.	RFIRIDTSC^{ox}VC^{ox}VLSRKR	-	-	7
$G^{111-113}$ diAcm	RFIRIDTAC^{SA}VC^{SA}GGGRKA	24	-	-
$G^{111-113}$ ox.	RFIRIDTAC^{ox}VC^{ox}GGGRKA	27	-	-
Gly analogue	RFGRGGGAC^{ox}GC^{ox}GGGRKA	6	-	-
" " diSBu^t	RFGRGGGAC^{SB}GC^{SB}GGGRKA	0	-	-
BDNF[313]	RFIRIDTSC^{ox}VC^{ox}TLTIKR	-	-	5
NT-3	RWIRIDTSC^{ox}VC^{ox}ALSRKI	-	-	13

Notes : SA = Acm PG on cysteine

Table 2.25 Binding results for NGF 100-116 analogues

2.6.2. Results of Residue Replacements

The $Gly^{111-113}$ analogue showed a significant loss in binding; although residues 111 and 113 are not greatly conserved between species (Leu^{112} is conserved), the side-chains may make a contribution to receptor binding or the conformation of the peptide. When many residues (even conserved ones) were replaced with Gly, the binding was very low (as expected). This showed that the presence of the Arg residues and the disulphide alone were not sufficient to maintain the level of binding, but that other side-chains must be required to attain the binding conformation.

Different effects due to the presence or absence of the disulphide bridge could also be observed, e.g. the oxidised form of the Ser^{107}, Arg^{116} analogue gave an IC_{50} of 7 μM, whereas its Cys(Acm)-protected form (no disulphide present) gave only 27% binding at 30 μM. This could obviously be due either to the steric effects of having

the PG present or the inability of the analogue to form the usual disulphide, and consequently not obtaining an optimal conformation. The presence of the SBu^t PGs on the other glycine analogue abolished the already very low binding value. Overall, these results show that although the arginine residues are essential and the presence of the disulphide also aids binding, certain other a.a.'s are also needed for optimal binding. Considering this, it was thought unlikely that simple replacement strategies would lead to increases in binding, especially considering the high number of conserved residues already in this region (ten a.a.'s in NGF 100-114 are also conserved in BDNF and NT-3) and the fact that all of the changes here gave either somewhat unchanged or reduced binding. Accordingly, modifications which would alter a.a. chirality and backbone flexibility were conceived with the intention of incorporating these into the NGF 100-114 sequence. As screening at each a.a. position across the whole sequence was impracticable, it was decided to concentrate around the 109-113 region (see section 2.8).

2.7. A DISCONTINUOUS NGF PEPTIDE : NGF 53-MER

2.7.1. General

It is possible that the NGF binding site(s) may consist of residues which are distant from one another in the primary sequence; only in the three-dimensional arrangement of such residues in the folded structure of the native protein would the binding site be properly constituted. As previously mentioned, Johnson[53] had suggested that the arginine residues in the C-terminal region may well be close to one another in NGF and thus form such a binding region complementary to the Asp/Glu-rich areas on the $p75^{NGFR}$. It was argued that residues near to the disulphides should be close to one another; if disulphides analogous to those in NGF could be formed, perhaps certain a.a.'s (especially arginines) could be arranged to give higher levels of binding to those observed in the linear fragments. To be included in the peptide was the sequence 95-116 which (at this stage) had already shown high binding. Considering this, the following 'discontinuous' peptide was suggested; residues 95-120 would be joined to residues 50-70. This would enable the following:

1. The chemical formation of the 'native' disulphides, Cys^{58}-Cys^{108} and Cys^{68}-Cys^{110}.
2. The arginine residues at 59, 69, 100, 103, 114 and 118 to be included.

3. The effect of extending the C-terminus to 120 to be examined as this would be made during the first part of the assembly.
4. Residues 71-93, including Cys^{80}, to be omitted as their inclusion would hinder the overall objectives.

It was decided to join the segments together by a small glycine chain. Residues 70 and 94 are Gly in NGF and an additional five glycines were added between these. It was hoped that this would give additional flexibility and hydrophilicity to the peptide and enable the disulphides to form more readily. This gave a chain length of fifty-three overall. Crucial to the success of the strategy was the choice of Cys PGs, as they had to provide the opportunity for selective disulphide bond formation. The SBu^t protecting-group was chosen for $Cys^{58,108}$ and Acm for $Cys^{68,110}$. The overall strategy is summarised below:

a.) The peptide was assembled stepwise from 120 until residue 95 when about half of the resin was removed.
b.) The assembly was continued with the remaining half of the resin by the addition of seven glycines, then residues 70 through to 50 were added.
c.) The fully, Cys-protected 53-mer was purified.
d.) The SBu^t groups on $Cys^{58,108}$ were removed, the first disulphide formed then the disulphide-linked peptide was purified.
e.) Attempts were made at forming the second disulphide (68-110) from the di-Acm, disulphide-linked peptide using various direct methods.

2.7.2. Assembly of NGF 53-mer

The peptide was assembled on a 0.21 mmol scale, using 0.7 g of a Wang resin, which had been pre-derivatised with Fmoc-Ala to a loading of about 0.30 mmol/g. This lower loading (usually 0.5 mmol/g was used) enabled a greater excess of a.a.'s to be used in the coupling reactions and probably led to an improved overall assembly. Some of the residues were triple-coupled based on difficulties encountered in the NGF 95-116 and 95-120[313] assemblies and most of residues towards the end of the assembly were triple-coupled also. The traces for the first part of the assembly, NGF 95-120 $C^{108}(SBu^t)$, $C^{110}(Acm)$, and the continuation to form NGF 50-70/GGGGGG/95-120 $C^{58,108}(SBu^t)$, $C^{68,110}(Acm)$, are shown in Fig. 2.26. The initial section showed a gradual fall in incorporation, although no major drops occurred. When the assembly of this 95-120 was complete, about half of the DMF-wet resin was removed. The remainder was used to form the 53-mer in a

continuous assembly. Little change in the assembly trace was observed over the sequence Gly^{94}-Lys^{50} and it was thought that these residues had been incorporated at a high level.

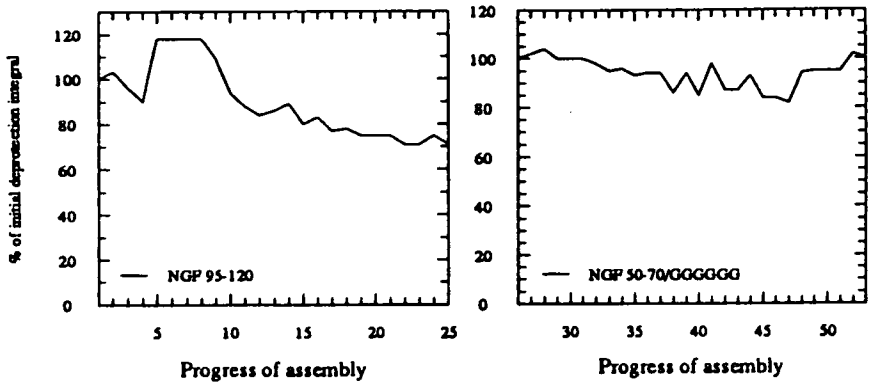


Fig. 2.26 Monitoring traces for residues 95-120 and 50-70-$(Gly)_6$.

2.7.3. Cleavage of NGF 53-mer

Both peptides were cleaved as previously outlined. The presence of EDT did not appear to affect the integrity of the SBu^t PGs over the three hour cleavage period. HPLCs for the crude peptides are shown in Fig. 2.27.

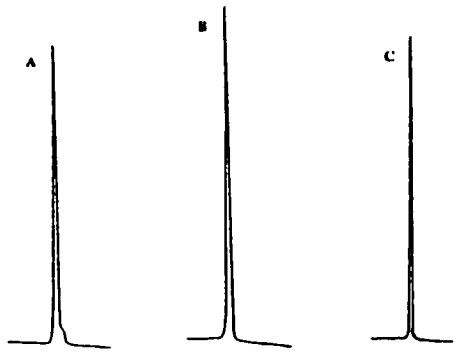
A : 5-95% CH_3CN/30 min. Rt=16.3 min.
B : 5-85% CH_3CN/25 min. Rt=18.8 min.

Fig. 2.27 HPLC traces of (a) crude 95-120, (b) crude 53-mer

2.7.4. Purification of NGF 53-mer

Both of the crude products were quite soluble. Extraction from 10% acetic acid removed most of the scavengers (especially phenol) and initial purification by gel filtration gave a reasonably pure product in both cases. Further purification by semi-preparative HPLC gave a pure, single peak product in each case. At this stage, additional purification by ion-exchange chromatography was considered; however, in view of the further reactions and purification required on the 53-mer, ion exchange was not carried out here but was to be used later if required. A sample of

the 95-120 peptide additionally had its SBu^t group removed from Cys^{108} in the usual manner before purification by semi-preparative HPLC. The HPLC traces of each of these purified peptides are shown in Fig. 2.28.



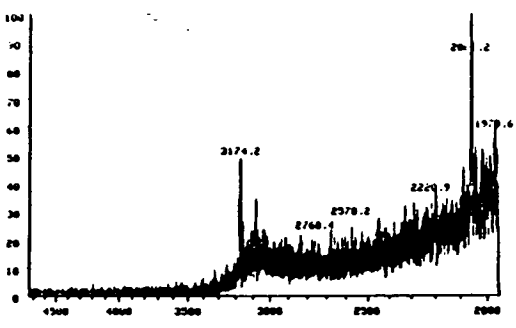
PEPTIDE	All 5-95% CH_3CN/30 min. Rt (min.)
A : 95-120 $C^{108}(SBu^t)$, C^{110}(Acm)	16.3
B : 95-120 C^{110}(Acm)	15.0
C : 53-mer	18.1

Fig. 2.28 HPLC traces of the pure peptides

2.7.5. Analysis of the Peptides

The level of purification of the fully cysteine-protected 53-mer could be seen by considering the amino-acid analysis results for the crude and pure forms. The mean error was calculated by totalling the deviations of the experimentally obtained a.a. values from the actual values and dividing this by the total number of a.a.'s. The crude 53-mer gave a value of 24%, whereas after the two purification steps, this had reduced to 6% for the final pure peptide. The crude 95-120 peptide itself gave satisfactory results, with a mean error of 4%.

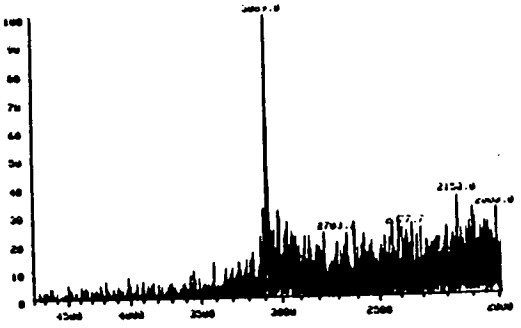
Fast atom bombardment mass spectrometry (FABMS) was used to show the presence of the correct molecular ion. The machine used was accurate to 0.1% (on low resolution). The cysteine-protected 95-120 peptide and Cys^{108}-reduced form both gave molecular ions within this tolerance (3174 and 3089 respectively). The M.W. of the Cys-protected 53-mer was probably close to the limit of detection of the machine. Nevertheless, a molecular ion of 6105 (calculated value was 6109, thus <0.07% error) was observed. These results are illustrated in Fig. 2.29. It was observed that only the pure peptides gave these high molecular ion results; the signals could not be detected with the crude or partially purified (after the gel filtration) peptides. A sample of each of the pure products was sent for testing in the assays.



NGF 95-120 $Cys^{108}(SBu^t)$, $Cys^{110}(Acm)$

Observed *m/z* = 3177.7

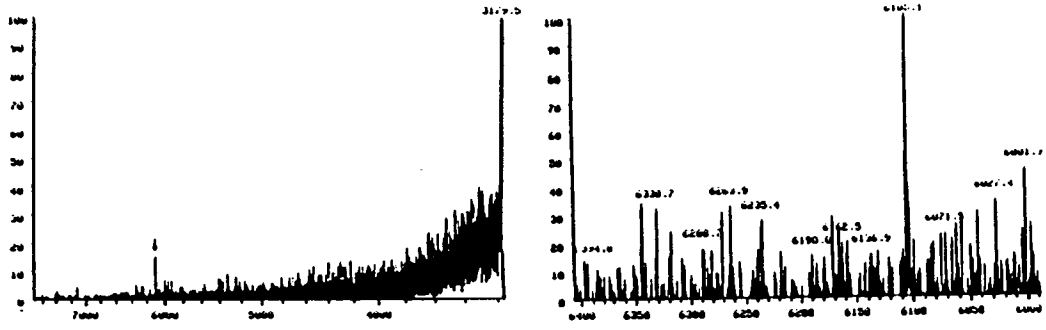
$C_{139}H_{238}N_{46}O_{33}S_3$ requires 3177.9



NGF 95-120 $Cys^{110}(Acm)$

Observed *m/z* = 3089.8

$C_{135}H_{230}N_{46}O_{33}S_2$ requires 3089.7



NGF 53-mer 50-70/GGGGGG/95-120 $Cys^{58,108}(SBu^t)$, $Cys^{68,110}(Acm)$

Observed *m/z* = 6105.1 ; $C_{263}H_{426}N_{84}O_{72}S_6$ requires 6107.4 (< 0.04% error)

Fig. 2.29 FABMS traces for the 95-120 and 53-mer peptides

2.7.6. Selective Disulphide Formation

150 mg of the pure NGF 53-mer, with all of the protecting groups intact, had been produced to enable studies on the selective formation of the disulphide bonds to be carried out. The first bond would be that between $Cys^{58,108}$. Samples of this disulphide-linked peptide were formed by two methods;

1.) Oxidised/reduced glutathione (GSSG/GSH) couple.

2.) Air oxidation.

2.7.6.1. GSSG/GSH oxidation

This system has been used extensively for controlling disulphide formation in peptides[315] and proteins[242]. Reduced glutathione is a tripeptide [γGlu-Cys-Gly], which is thought to act *in vivo* (along with its oxidised form) as an electron acceptor-donor couple in disulphide exchange reactions. The system acts to increase the rate and yield of oxidation by facilitating disulphide interchange. Since the process is reversible, as shown in Fig. 2.30, the equilibria may be forced between the oxidised and reduced states depending on the relative proportions of GSSG and GSH present.

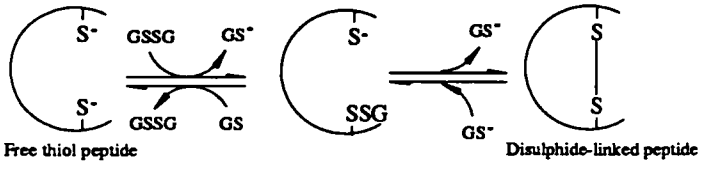


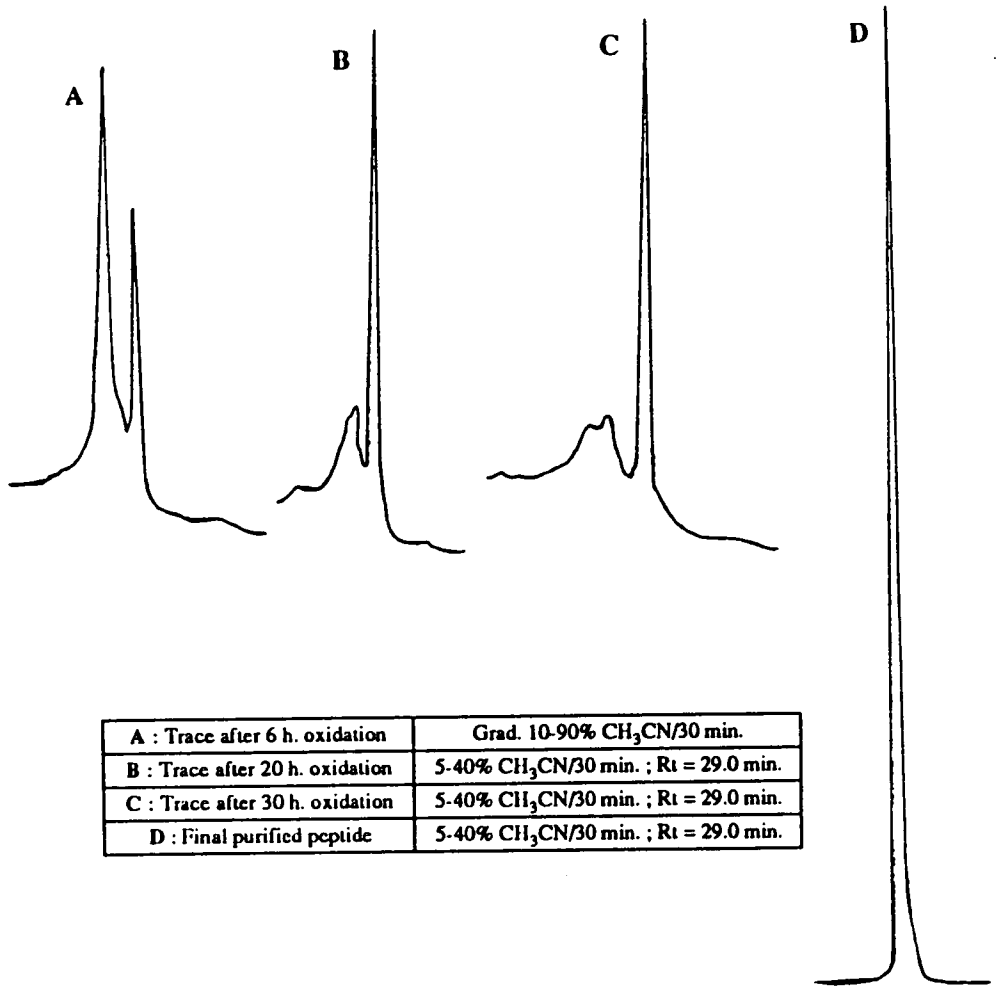
Fig. 2.30 GSSG/GSH couples in disulphide formation

Using a two-fold excess of GSSG over GSH, alkaline pH and moderate ionic strength, free-thiol peptides may be converted to their oxidised forms within 12-24 h. at room temperature.

Firstly, 70 mg of the Cys-protected 53-mer was reacted with TBP/TFE to remove the butylsulphenyl groups from $Cys^{58,108}$. Their removal was confirmed by the large change in retention time observed on analytical HPLC. This reduced peptide (58 mg) was dissolved in buffer at pH 8.0, containing 0.15 mM GSSG and 0.075 mM GSH. Stirring under nitrogen for 20 hours led to the formation of the oxidised product. Purification by semi-preparative HPLC gave the pure, disulphide-linked 53-mer (9.5 mg). Several later-running (by HPLC) peaks were thought to represent mixed peptide/glutathione species. These were combined and reoxidised to give a further portion (5 mg) of the disulphide-linked product.

2.7.6.2. Air oxidation

Another 53 mg of the fully cysteine protected 53-mer had its SBu^t PGs removed as before. The reduced peptide was dissolved in water, the solution adjusted to pH 8.0 then stirred for 24 h. The reaction was followed by HPLC (Fig. 2.31). Purification of the solution gave the disulphide-linked product as before (9 mg).



A : Trace after 6 h. oxidation	Grad. 10-90% CH_3CN/30 min.
B : Trace after 20 h. oxidation	5-40% CH_3CN/30 min. ; Rt = 29.0 min.
C : Trace after 30 h. oxidation	5-40% CH_3CN/30 min. ; Rt = 29.0 min.
D : Final purified peptide	5-40% CH_3CN/30 min. ; Rt = 29.0 min.

Fig. 2.31 Traces showing time-course for air-oxidation of reduced 53-mer

Both reactions gave the required product in about 20% yield and the peptides obtained coeluted with each other on HPLC. Ellman's test[316] (section 3.2.7) measures the nitrobenzoate (NTB) released upon the reaction of the thiol with DTNB and hence detects free thiol functions (Fig. 2.32). This gave a negative result with this peptide, indicating the disulphide had formed, having originally given a positive result with the reduced peptide solution before the oxidation reaction had commenced. A correct FABMS result was obtained for the pure peptide (Fig. 2.33) and a small sample was sent for testing.

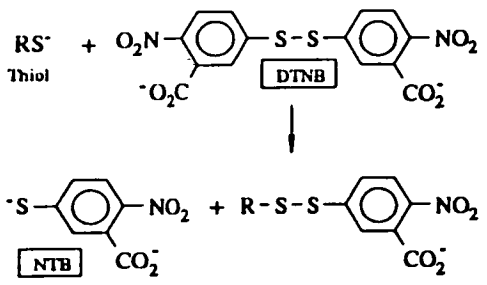


Fig. 2.32 NTB (λ_{max} at 412 nm) allows thiol determination in Ellman's test

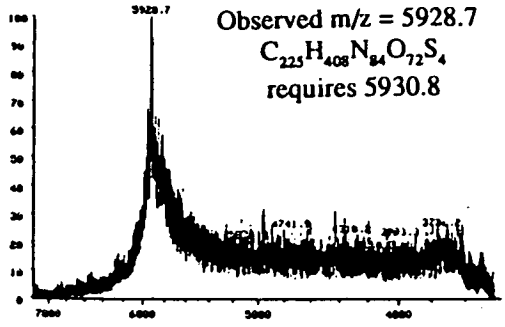


Fig. 2.33 FABMS of 53-mer containing a disulphide between Cys[58,108]

2.7.7. Attempted Formation of the Second Disulphide Bond

18 mg of the disulphide-linked 53-mer, with the Acm groups still on Cys[68,110], remained for attempts to be made at the formation of the second disulphide bond. It was thought that this would be problematical, especially due to the presence of Trp[99], which may be affected by the more severe reaction conditions necessary for direct disulphide formation. Many small-scale trial reactions were carried out using the same conditions as those used for NGF 100-116, S[107], R[116] (see section 2.2.7);

1. Iodine, in various solvents.
2. DMSO/Me_3SiCl/TFA.
3. Ph_2SO/$MeSiCl_3$/TFA.
4. $(CF_3CO_2)_3Tl$/TFA. (1.2 equiv., 0°C for 20-60 min.)[317].

Reactions 2. and 3. suffered from the obvious deleterious effect that they had on Trp[99]. On addition of these reagents in TFA, the peptide solution rapidly became purple (blank solutions remained colourless), indicative of tryptophan damage. This was confirmed by the HPLC traces, which showed that many additional peaks had appeared. Treatment with iodine (reaction 1), under conditions favoured by Kamber for insulin syntheses[216], also showed that many side-reactions had occurred, with little evidence that the required oxidised product had formed.

One of the main difficulties encountered here was in trying to ascertain if the disulphide bond had actually formed from the protected species. Experience with other NGF peptides indicated that differences in retention time on HPLC between di-Acm and disulphide-linked peptides were a good indicator of bond formation, although the differences were often slight. FABMS could show that the target peptides had been made, due to the mass change involved (decrease of 144). However in this case, the mass was so great that this was not feasible for trial experiments. Additionally, the change in conformation caused by disulphide

formation in a small peptide may add to these changes in retention time; here, a disulphide was being introduced into a peptide already containing one, thus it was thought that any change in retention time, incurred in direct bond formation, may well be negligible.

Reaction 4 initially looked more promising, the reaction leading to a slightly earlier-eluting product on HPLC (using a shallow gradient to maximise any difference in retention). However, a reaction time that produced this effect (20 min.), also gave a purple colouration to the solution, indicating tryptophan damage. A larger scale reaction using 5 mg of the 53-mer with 1.2 equiv. of $(CF_3CO_2)_3Tl$ in TFA (0.6 mg in 1 cm^3, reacted at 0°C for 20 min.) gave 1 mg of product after purification by HPLC. On analytical HPLC, the pure main peak had moved only slightly. FABMS of this product showed a diffuse peak around the area which would represent the loss of two Acm groups. However, it was thought that the product of a larger scale reaction could not be sufficiently characterised, nor could the integrity of the tryptophan residue be ensured to a degree that would make the production and testing of the peptide a meaningful exercise.

2.7.8. Results of Testing Peptides from the NGF 53-mer Synthesis

The results obtained from the binding assays on these peptides are shown in Table 2.34. The results for the 95-120 peptides appear somewhat anomalous.

Peptide	Sequence	% binding in assay		IC_{50} (μM)
		Biotin @ 30 μM	Biotin @ 100 μM	
95-120 $C^{108}(SBu^t)$ $C^{110}(Acm)$	KQAAWRFIRIDTAC^{SB}VC^{SA}VLSRKAVRRA	-	95	10-20
95-120 $C^{110}(Acm)$	KQAAWRFIRIDTAC^{SH}VC^{SA}VLSRKAVRRA	-	10	-
95-120 ox.[63]	KQAAWRFIRIDTAC^{OX}VC^{OX}VLSRKAVRRA	-	88	20
NGF 53-mer $C^{58,108}(SBu^t)$ $C^{68,110}(Acm)$	KQYFFETKC^{SB}RDPNPVDSGC^{SA}RGGGGGGG KQAAWRFIRIDTAC^{SB}VC^{SA}VLSRKAVRRA	-	-	2
NGF 53-mer $C^{58,108}$ ox. $C^{68,110}(Acm)$	KQYFFETKC^{OX}RDPNPVDSGC^{SA}RGGGGGGG KQAAWRFIRIDTAC^{OX}VC^{SA}VLSRKAVRRA	27	-	-

Table 2.34 Binding results for 95-120 and 53-mer peptides

The cysteine-protected 95-120 peptide gave the same binding affinity as the oxidised 95-120 peptide[313]. Perhaps in this case, the increased peptide length compensated for the conformational disturbance that the PG's may have caused. However, it is difficult to reconcile this with the results obtained when the SBu^t had been removed (a ten-fold decrease in binding). It may be that due to the testing being carried out in DMSO, dimer formation may have been favoured in this peptide, leading to decreased solubility or hindering the previously observed binding process. The fully cysteine-protected 53-mer gave as high a level of binding as that observed for any previous NGF fragment. Disappointingly, when the first disulphide was formed, this binding was reduced, implying that the region responsible for eliciting the binding process had been masked by the presence of the disulphide bond. It was unfortunate that the second disulphide was not able to be formed reliably as it would have been interesting to see if the binding could be recovered in the two-disulphide peptide.

Considering the overall methods used, it would have been advantageous to omit or substitute the tryptophan[99] residue; the fact that residues 100-114 could have replaced the 95-120 peptide had not been discovered at that time. It may have been easier to assemble a 50-70/100-114 peptide and try to form the correct disulphide in this. On reflection, the presence of the small glycine chain may not have been necessary, although it probably contributed to the success of the assembly. Replacing the Acm groups with the more hydrophobic trimethylacetamidomethyl (Tacm)[223] may have facilitated the analysis of the results of attempts to form the second disulphide bond. On HPLC, a greater change in retention time between the di-Tacm and oxidised product would probably have been observed.

Before the commencement of the 53-mer assembly, other strategies had been considered; forming the residue 59-67 loop by joining two separate peptides together, via disulphide bonds, was considered. Similar work had previously been carried out using NGF peptides, where fragments with the sequences 10-25 and 78-88 had been linked by cysteine residues at 15 and 80 on the respective chains[268]. The idea was rejected, at the time, as being technically too difficult with peptides as large as 50-70 and 95-120. However, the method may be viable if smaller fragments e.g. 50-70 (or a smaller fragment which still contained the cysteine residues) and 100-114 were used; this may enable both parallel and anti-parallel dimers to be formed form both chains using directed disulphide-bond formation[268,318].

2.8. FURTHER ATTEMPTS TO INCREASE THE BINDING OF NGF 100-114

2.8.1. General

Attempts were made to try to increase the level of binding of the NGF 100-114 peptide. The investigation of possible methods for carrying out this process was carried out in three main areas:

1. The chirality of certain a.a.'s was reversed and some conformationally constrained a.a.'s were included.
2. The conformation around the disulphide bond was altered.
3. Various types of macrocyclisation of the peptide were also tried.

Analogues using methods 1 and 2 were confined to the previously examined residue 109-113 region.

2.8.2 Altering Chirality and Conformational Freedom of Some Residues

Reversing the chirality of certain residues is a popular method of enhancing the stability of the resulting analogue to enzymic degradation and may sometimes cause a favourable change in conformation. Also the commercial availability of protected a.a.'s makes such substitutions relatively easy. Here, the valine residues at 109 and 111 were replaced to see what effect this may have on binding.

A widely used method for constraining the conformation of an a.a. unit is to replace the hydrogen at the α-carbon by a methyl group (or various other alkyl groups) to give a dialkylamino acid[319]. When a single methyl is added, the resulting residue is α-aminoisobutyric acid (Aib, α-methylalanine). A Ramachandran plot[320] of ϕ (torsional angle about the N^{α}-C^{α} bond) and ψ (torsional angle around the C^{α}-$C^{carbonyl}$ bond) verses the calculated conformational energy (showing the conformational space allowed to a particular residue), has shown that 50% of the plot is accessible to glycine. Adding a methyl group to form alanine reduces this to about 16%, whereas only a few percent is available to an Aib residue[321]. This available space is particularly favoured by helixes and turns, so including Aib residues may lead to the formation of a type I or III turn conformation[322].

Additionally in this series, norleucine[113] (Nle) replaced serine[113] to see the effect removing the only hydrophilic side-chain in this area, as well as extending the side-chain length. A double-norleucine insertion, where Nle[106] also replaced Thr[106], was also assembled, although the final oxidised form was not achieved in this case.

2.8.3. Results of Amino-Acid Substitutions

The results of these substitutions are shown below in Table 2.35.

Peptide	Sequence	Biotin @ 10 μM	IC_{50} (μM)
100-114 ox.	RFIRIDTAC^{ox}VC^{ox}VLSR	-	3
dVal[111] ox.	RFIRIDTAC^{ox}VC^{ox}**dV**LSR	21%	-
dVal[109,111] ox.	RFIRIDTAC^{ox}**dV**C^{ox}**dV**LSR	-	12.5
Aib[109] ox.	RFIRIDTAC^{ox}**Aib**C^{ox}VLSR	23%	-
Aib[111] ox.	RFIRIDTAC^{ox}VC^{ox}**Aib**LSR	27%	-
Nle[113] ox.	RFIRIDTAC^{ox}VC^{ox}VL**Nle**R	-	1.2
Nle[113] diAcm	RFIRIDTAC^{SA}VC^{SA}VL**Nle**R	29%	-
Nle[106,113] diAcm	RFIRID**Nle**AC^{SA}VC^{SA}VL**Nle**R	44%	-

Table 2.35 Binding results for NGF 100-114 analogues

These peptides were tested at 0.1, 0.3, 1.0, 3.0 and 10 μM concentrations to enable more accurate IC_{50} values to be determined. Unfortunately, all of the peptides failed to give significantly increased binding levels compared to that of the 100-114 parent, thus the percentage binding at 10 μM (the highest concentration tested) is quoted here. The IC_{50} value for the norleucine[113] peptide was slightly lower than the parent peptide, although this perhaps reflected the increased accuracy of calculation allowed by using these lower peptide concentrations. Overall, it is difficult to draw firm conclusions from what was, of necessity, quite a small substitution programme. It was interesting to observe that changing the chirality of both valines appeared to have relatively little effect on binding. The Aib substitutions may have had deleterious effects on the conformation around the disulphide bond, affecting the relative positions of other a.a.'s in the peptide; perhaps these constraints prevented the peptide from forming the optimum binding conformation. The overall pattern caused by such changes may have been more easily observed if the permutations of chirality and conformational constraints for each residue could have been

investigated. Without additional information, the exact role that these substitutions had in changing the binding of the parent molecule remains open to speculation.

2.8.4. Altering the Conformation around the Disulphide Bond

The structure around the -$C^{108}VC^{110}$- disulphide bond area was thought to be that of a γ-turn by analogy with previously published work[323] (Fig. 2.36). The turn has three residues, here -CVC-, and is stabilised by the presence of the disulphide bond. The carbonyl of residue *i* (Cys^{108}) may be hydrogen-bonded to the NH of residue *i*+2 (Cys^{110}). Such a structure may be contributory to the increased binding affinities seen in the oxidised forms of the C-terminal NGF peptides. Indeed, when the cysteine residues were side-chain protected, the degree of receptor binding almost always dropped significantly, although it was unclear whether or not the disulphide itself remained intact during testing. Initially, it was surmised that the disulphide bond may be undergoing exchange with one of the numerous cysteine residues (or disulphide bonds) on the $p75^{NGFR}$, or even with NGF itself, thus causing the observed binding affinity. Some peptide-based affinity labels, possessing a disulphide bond, have been described for the derivatisation of cysteine-containing enzymes[324]: it was conceivable that a similar process may be occurring here. There is however no evidence for such a mechanism, although some simple experiments which would have disproved such a theory had not been undertaken at the initial testing stage.

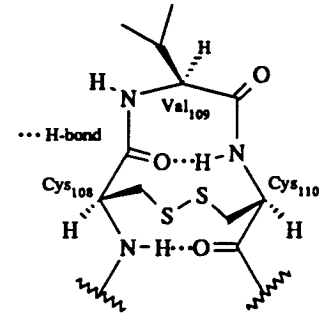


Fig. 2.36 Proposed γ-turn in NGF 100-114

As the spatial arrangement of the residues around the disulphide bond may not have been greatly altered by any of the former substitutions, the addition of another residue between the cysteines was contemplated. It was hoped that this would;

1. by increasing the peptide length, alter the linear distance between the important residues. If the disulphide did not remain intact during testing, this change may change the binding properties of the peptide.

2. enable the incorporation of a β-bend (if the disulphide did remain intact during testing).

β-bends (also called β-turns) are well-known features of protein structure, generally occurring at the protein surface, where the peptide chain reverses its overall direction. The β-bend consists of four residues and may, like the γ-turn, be stabilised by an inter-turn hydrogen-bond[322]. There are many examples of peptides where a β-bend seems likely to be present in the bioactive conformation of a naturally-occurring peptide[325]. For peptides having the general structure -Cys-X-Y-Cys-, and containing a disulphide bond, it appears that the presence of the disulphide forces the 14-membered ring system into a favourable conformation, thus allowing the internal '4→1' hydrogen-bond to form[326,327]. The model peptide 'Ac-L-Cys^{ox}-L-Pro-D-Val-L-Cys^{ox}-NH_2' was shown to adopt a type II β-bend in DMSO[322,328]. Accordingly, the [Pro^{109a}-D-Val^{109b}] unit replaced Val^{109} in an analogue of NGF 100-114 and the oxidised form of the analogue was produced as before (Fig 2.37). The result of the assay is shown in Table 2.38. Although only a single result, it seems that the incorporation of the extra proline residue, and reversing the chirality of the valine^{109}, did not greatly affect the level of binding. This perhaps suggests that the conformation around the disulphide bond is less critical than previously thought. Overall, the change in structure brought about by the β-bend may not have altered the favoured orientations of the side-chains directly involved in receptor binding. It would have been interesting to see the effect caused by increasing the size of the disulphide ring by either placing additional residues between the cysteines or moving the cysteines further apart, towards either end of the 100-114 parent peptide. Such substitutions may have imposed some favourable (or unfavourable) constraints on the molecule and helped to elucidate the exact role of the disulphide bond in the action of these peptides.

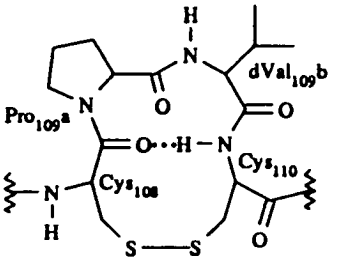


Fig. 2.37 Putative β-bend in NGF 100-114 analogue

Peptide	IC_{50} (μM)
NGF 100-114 ox.	3
NGF 100-114 [Pro^{109a}-D-Val^{109b}] ox.	1

Table 2.38 Assay result for β-bend analogue

2.8.5. Cyclic Analogues of NGF Peptides

The simplest way to introduce a conformational constraint into a peptide is by way of a cyclisation, either through a disulphide bond or by coupling the α-amino

function (or a lysine ε-amino group) with the carboxyl terminus (or an aspartic/glutamic acid side-chain). Although the resulting peptide may still have considerable flexibility, the cyclisation will nevertheless substantially reduce the number of accessible conformations and may improve or alter the biological activity of the peptide. Several examples of cyclic fragments having greater binding affinities and/or biological activities are known e.g. somatostatin[329], α-melanotrophin[330] and fibronectin[331]. Additionally, cyclisation of some of the NGF peptides may have enabled structural investigations by N.M.R.

It was envisaged that cyclic forms of the peptides NGF 100-114 ox. and 100-114 [PdV][109] ox. could be created. Several difficulties existed in these peptides. The presence of Asp^{105} would complicate the cyclisation procedures thus it was protected in these assemblies as the TFA-stable benzyl ester. This was to be removed later by brief treatment with TFMSA. The cysteine residues also required protection; it was hoped that if the cyclisation was successful, it might be possible to form the disulphide thus making a bicyclic peptide. Initially, a head-to-tail cyclisation was attempted using diphenylphosphoryl azide (DPPA) as the condensing agent and using procedures based on previous work[332].

The general procedures were carried out as follows. The peptides were assembled and purified as before. The pure Cys- and Asp-protected peptides were then dissolved in DMF (5 mg/ml) and the solution adjusted to pH 8.0 with TEA. DPPA (3 equiv.) was added at 0°C, the solution maintained at -20°C for 72 h., then at 4°C for 48 h., and finally for 24 h. at room temperature. Evaporation of the solvent, neutralisation with acetic acid, extraction with ether then lyophilisation gave a solid product. The reaction had been followed by HPLC and several additional peaks had appeared. On separation by semi-preparative HPLC, none of the resulting compounds obtained from these peaks gave a FABMS value indicative of cyclisation; the major peak appeared to represent a DPPA-derivatised form. Adjustment of the peptide/DPPA concentrations and reaction times did not lead to the required products either. It was considered that steric factors may have hampered the head-to-tail cyclisation process so a side-chain-to-side-chain approach was tried. The peptide NGF H-100-115-NH_2 had previously been assembled using Cys(Acm) PGs. The peptide/resin was acetylated, the peptide cleaved and the resulting acetylated peptide was purified. The pure peptide thus had a free ε-amino on Lys^{115} and a free γ-carboxyl on Asp^{105}. The DPPA reaction was carried out as before, but again no evidence of cyclisation was found in the products.

As the activation process may not have been optimal, it was proposed to use a resin developed in this laboratory, which would release a C-terminal hydrazide on acid cleavage[228] (Fig. 2.39). This could then be converted to the azide for subsequent head-to-tail cyclisation without the need to protect Asp[105]. Unfortunately, despite several attempts using long coupling times, the amount of derivatisation by Fmoc-Arg(Pmc)-OH that could be obtained was too low to make a further attempt at a NGF 100-114 assembly viable.

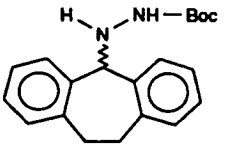


Fig. 2.39 Hydrazide resin

Overall, the cyclisation attempts were unsuccessful. The peptide was probably severely hindered by the presence of the protecting groups on the Asp and Cys residues, making the head-to-tail cyclisation unfavourable. The peptide itself may have had little propensity for a cyclic form, especially considering that the reaction is very sequence-dependent[333]. On reflection, while the bicyclic form was seen as the ultimate goal, an easier initial route may have been to attempt the reactions in peptides lacking the Cys residues e.g. they could have been substituted by Ser, and to determine were the problems lay in the overall cyclisation process.

2.9. NUCLEAR MAGNETIC RESONANCE STUDIES ON NGF FRAGMENTS

2.9.1. General

Some of the smaller NGF fragments were examined by nuclear magnetic resonance (N.M.R.) for evidence of secondary structure. These were;

a.) 100-110 ox. in 90% H_2O/10%D_2O.
b.) 100-115 ox. in 90% H_2O/10%D_2O.
c.) 100-114 ox. in d_6-DMSO.
d.) 100-114 [PdV][109] ox. in 90% H_2O/10%D_2O and d_6-DMSO.

The large number of protons in even these small peptides necessitated the use of various two-dimensional N.M.R. (2-D N.M.R.) experiments to aid in the assignment of each resonance. A general scheme for 2-D N.M.R. is shown in Fig. 2.40.

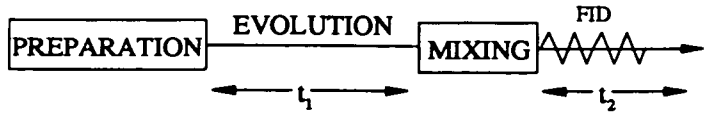


Fig. 2.40 Generalised 2-D N.M.R. pulse sequence

In the preparation period, one or more pulses are applied to the sample to generate a magnetisation, which evolves during time t_1. This is followed by a mixing period during which more pulses are applied. After this, the signal is recorded as a free induction decay (FID) as a function of a second time variable t_2. This process is repeated for a series of values of t_1 to generate a set of FIDs. This data is Fourier transformed twice to give a spectrum which is a function of two frequency variables. The types of experiment that can be carried out, and the information that can be obtained from them, depend on the manipulation of this basic pulse sequence. Table 2.41 summarises the experiments used in this study and the information that they contain.

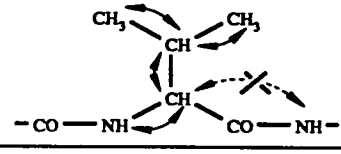
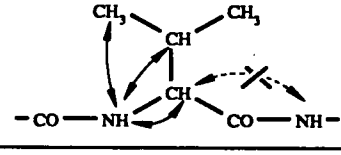
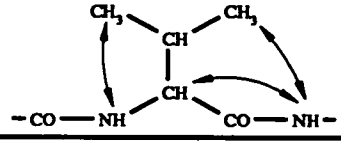
Experiment	Couplings observed between...	Information
COSY		Shows through-bond (geminal and vicinal) couplings
TOCSY		Shows all through-bond couplings
ROESY/NOESY		Shows through-space couplings. Used for sequential assignment. May enable 3-D information to be gained

Table 2.41 Types of 2-D N.M.R. experiment

2.9.2. Interpretation of the 2-D N.M.R. Spectrum of NGF 100-114[PdV][109]

NGF 100-114[PdV][109] ox. can be considered as an example to show how the spectra were interpreted. The procedures used were based on the discussion of Wüthrich[334]. The initial step in determining whether any preferred structure exists, is to assign all of the resonances observed to the respective residues from which they originated. A portion of the TOCSY spectrum is shown in Fig. 2.42. This indicates the connectivities between the amide proton and the other protons (α, β, etc.) for some

residues in the peptide. Most a.a.'s could be identified by their characteristic connectivity patterns; however, some a.a.'s can give the same spin system pattern e.g. AMX system is observed here for Asp, Cys, Phe and Ser. These residues cannot be differentiated by TOCSY experiments alone.

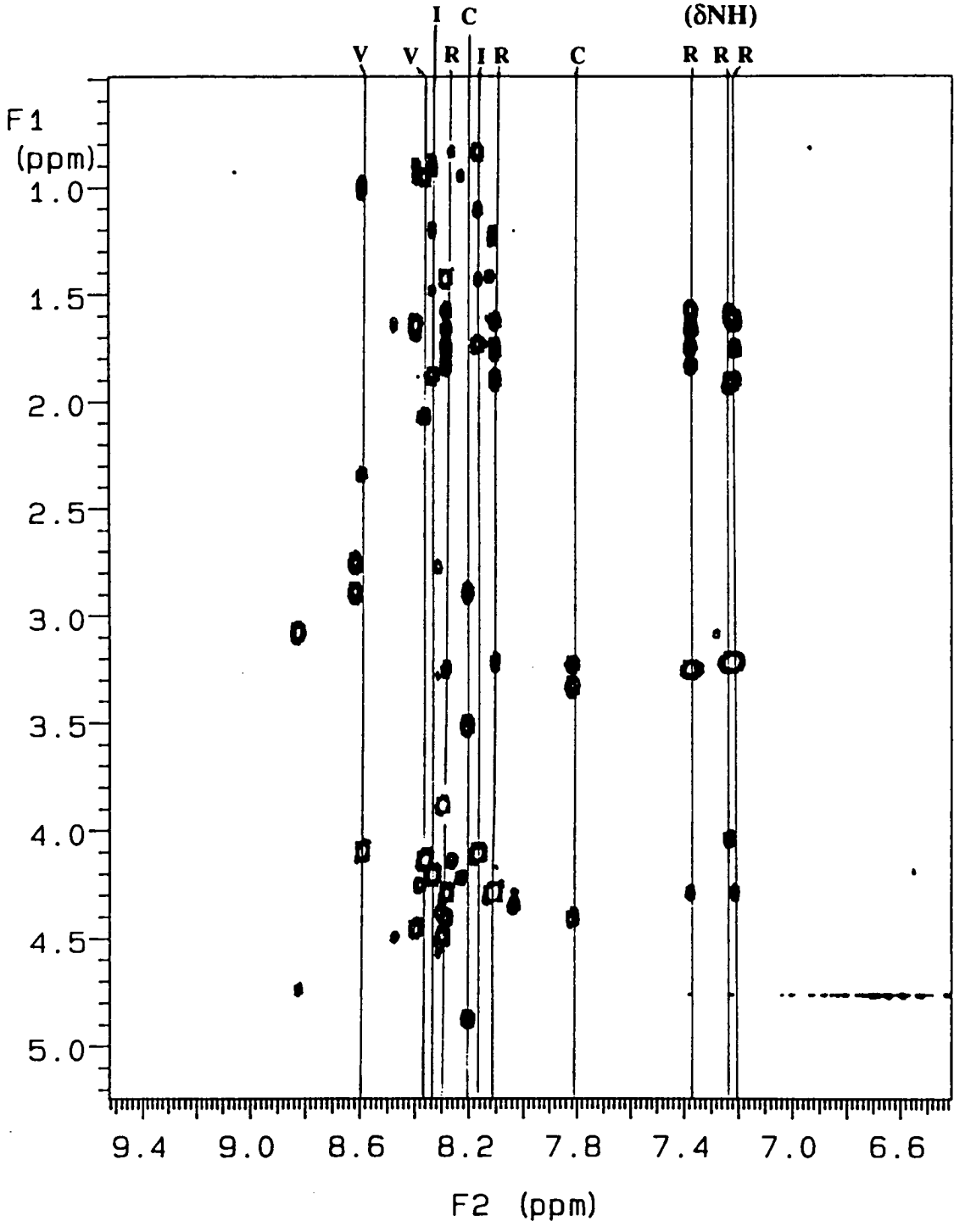


Fig. 2.42 Part of TOCSY spectrum for NGF 100-114[PdV][109] showing identification of some of the multiple spin systems

Furthermore, multiple copies of certain residues were present in this peptide i.e. $Arg^{100,103,114}$, $Ile^{102,104}$, $Cys^{108,110}$ and $Val^{109b,111}$ (labelled in Fig. 2.42); again these were not able to be distinguished, although Arg^{100} could be identified due to the absence of backbone-amide connectivities for this residue. Additionally, on closer examination, the αH peak for Asp^{105} was not present and the αH of Thr^{106} gave a weak signal.

The identification of the above residues was simplified by the through-space information obtained from a ROESY experiment. Usually a ROESY peak will be observed between the αH (or sometimes βH) and the NH proton of the adjacent residue. This is illustrated in Fig. 2.43.

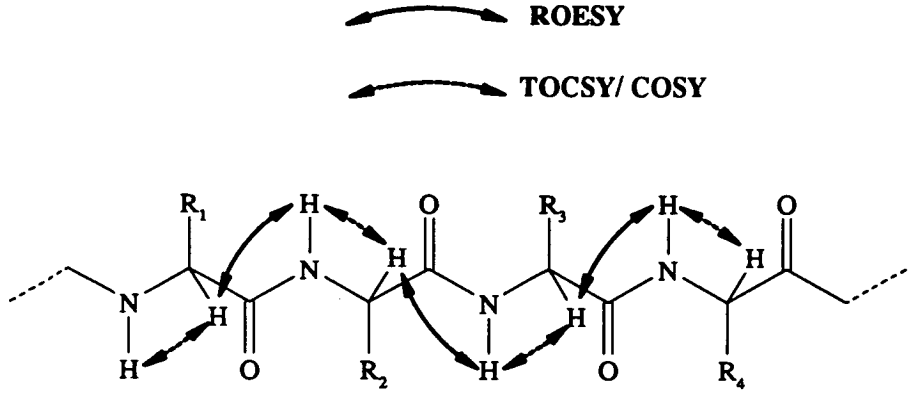


Fig. 2.43 2-D N.M.R. interactions in the peptide backbone

If the TOCSY and ROESY spectra are overlaid, the resulting spectra can be used to 'walk the backbone' of the peptide. Starting from the αH of the N-terminal residue of the peptide, it was often possible to follow the peptide sequence by joining related TOCSY (or COSY) and ROESY cross-peaks with a series of horizontal and vertical lines. This is shown in Fig. 2.44 for the [Pro-D-Val]^{109} analogue. This completed and confirmed the overall assignment.

After this, evidence of structure, exemplified by ROESY peaks between non-sequential residues, was sought. All of the remaining ROESY cross-peaks were found to be either within a residue or between adjacent residues; none were observed between residues distant in the primary structure, indicating the probable lack of a preferred conformation. This was also found to be true for the other peptides which were examined by N.M.R. It therefore appeared that the highest binding peptides existed in a random-coil conformation in the solution phase.

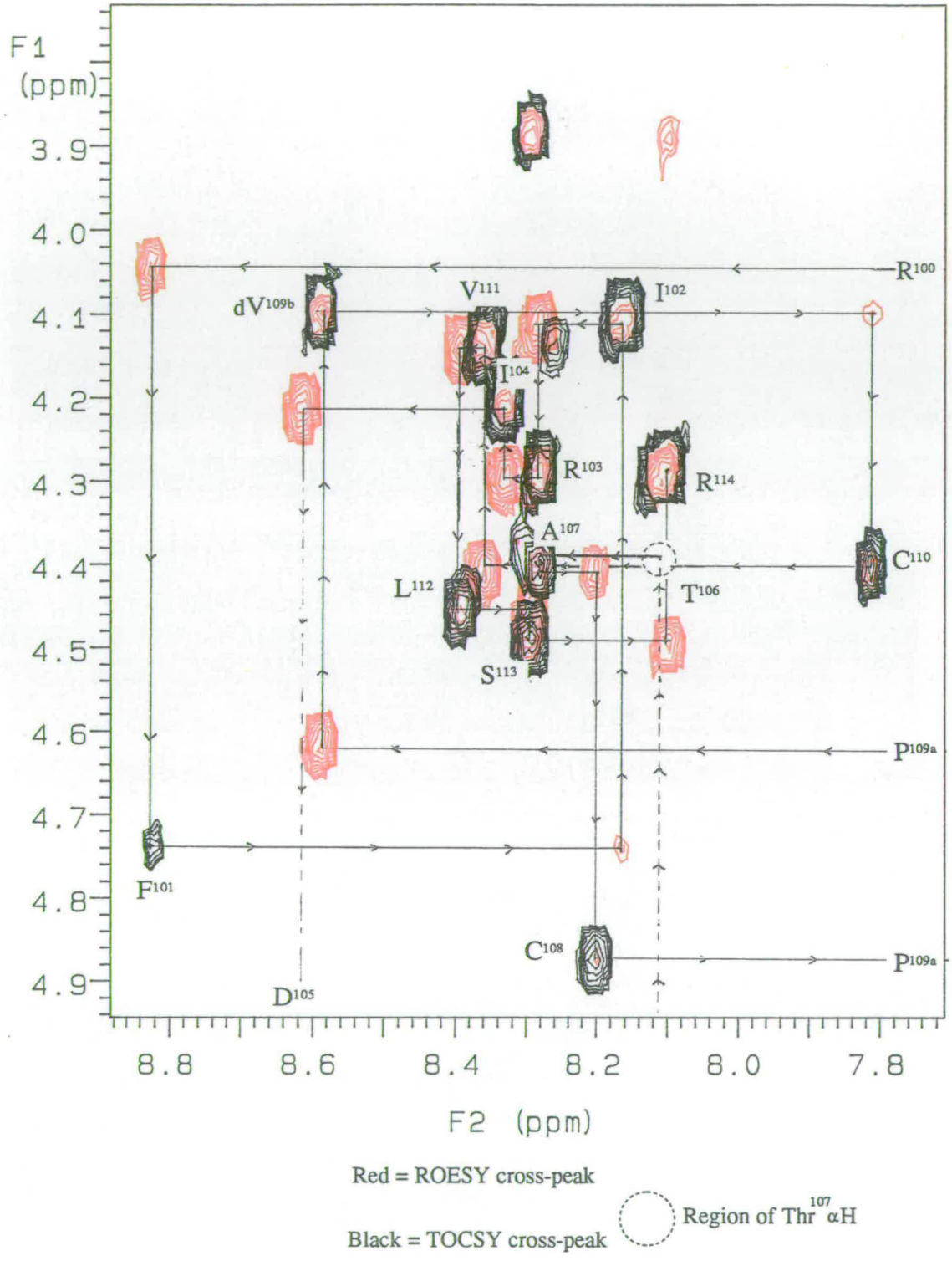


Fig. 2.44 "Walking the backbone" of NGF 100-114 [PdV]^{109} ox.

2.10. PROTEIN SYNTHESIS : LYSOZYME

2.10.1. General

The total synthesis of NGF was considered as an objective relatively early in this study. However, considerable hurdles to the assembly, purification and even analysis of such a large and complex peptide (or more accurately, a small protein) had to be overcome before such a project could be realised. Some of the major problems were;

1. The lack of access to authentic NGF.
2. It was not known if NGF would fold properly to give an active form.
3. The size of the protein lies outside the range and accuracy of most common analytical spectroscopic techniques.

Added to these, of course, were the usual technical difficulties of assembling, deprotecting and purifying the protein, as well as attempting to refold it into an active form. Whilst these obstacles face all protein syntheses to a greater or lesser extent, it was thought that at least some of the hurdles could be addressed by first testing our techniques on a well-characterised protein; the small enzyme, hen egg white lysozyme (HL) was selected for this task. This protein has the following attributes, which may help in the determination of where the major difficulties would arise in an overall strategy;

a.) The authentic material is inexpensive and readily available.
b.) HL is similar to NGF in length and complexity (129 a.a.'s long, four disulphides).
c.) The protein contains six tryptophan and two methionine residues, thus it would represent a good test of the acidolysis and disulphide-forming steps.
d.) The pure, reduced protein has been shown to refold into an active form.

This last point was potentially useful: Saxena[242] had shown that reduced and denatured HL [HL(red.)] can be reactivated using a GSSG/GSH couple. The reactivated protein can also be easily assayed in solution to determine the presence and degree of renaturation. It may be that such a system could be utilised to form an active enzyme from a chemically synthesised, primary structure. The appearance of any enzymic activity would prove to be a good indicator that the synthetic procedures were satisfactory.

2.10.2. Assembly of Synthetic Hen Egg White Lysozyme (SL)

Considerable experience in the assembly of relatively large peptides (e.g. ubiquitin) has been accumulated in this laboratory and the monitoring system used in automatic assembly has been shown to be a reliable indicator of the progress of such assemblies. In view of the time required for the usual double-couple cycles, the expense involved and the fact that some previous assemblies using only single-couple cycles had been successful[335], it was decided that a single, ten-fold excess of HOBt ester would be used for each residue. The resin loading was lower than usual (0.25 mmol/g) as recommended by Kent[91]. The cysteine residues were to be protected by an acid-stable PG to enable the option of purifying a free- or protected-thiol protein. The SBu^t group was chosen in preference to Acm as its removal could be more easily observed and it had previously been reported that Acm-protection caused difficulties in a similar lysozyme assembly[245].

Two assemblies were carried out. In assembly A, a 30 min. coupling time was used. A large fall in incorporation was observed after only twenty residues. A sample of the resin (about 10% was removed for examination, whilst the assembly was continued. The assembly was completed, when the final deprotection value indicated that only around 30% of the original substitution level remained.

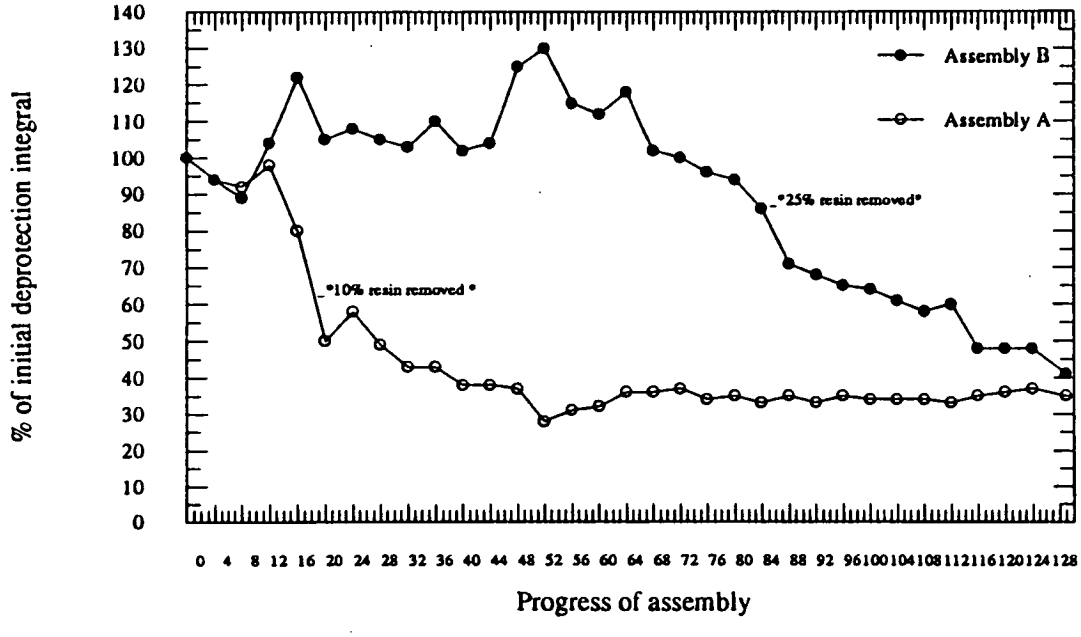


Fig. 2.45 Monitoring profiles for both SL assemblies

In assembly B, several modifications were added which improved the overall assembly; His(Bum) and Trp(Boc) derivatives were used to give additional protection, the HOBt was dried before use[229], and most importantly, the coupling time was extended to 90 min. None of the previously observed falls occurred, indeed the trace stayed at (or above) the same value as the initial deprotection for much of the assembly. This was perhaps due to minor fluctuations in the sample volume (from the deprotection solution) taken for the monitoring tests; this difference would have been amplified due to the very small amount of Fmoc present (only 0.1 mmol scale was used), causing this effect. About one quarter of the peptide/resin was removed towards the end of the assembly to facilitate vortexing. The trace gave a final deprotection value of around 55% (allowing for the resin already removed). The monitoring traces for both syntheses are shown in Fig. 2.45 (above).

2.10.3. Cleavage of SL

In assembly A, a cleavage protocol using a higher than usual level of scavengers (in 85% TFA) for three h., followed by a lower level (in 92% TFA) for a further three h., was tried. This gave a broad peak on HPLC. Extending this cleavage time by two h. led to a purple colouration in the solution and the crude product, indicative of tryptophan damage. The crude protein gave a much poorer trace on HPLC. In assembly B, the cleavage protocol was more conventional, using the necessary scavengers in 90% TFA for six hours. Extending this reaction time did not appear to alter the HPLC trace and no Trp damage was apparent, even after 24 hr: presumably the Boc protecting group on the indole ring had prevented such acidolytic degradation. The crude product of both assemblies gave a reasonable AAA.

2.10.4. Initial Purification: Gel Filtration

The crude material from assembly A was passed down a Sephadex G50 column. Four areas were collected and combined (fractions K, L, M and N) Some of the material eluted at (or near) the void volume, indicating its total exclusion from the resin (fractions K, L and M), although a substantial amount of lower M.W. material had been separated from this fraction N. This fact was shown by the high V_e/V_o (elution/void volume) ratio for this fraction. The AAA for these four fractions showed that while K, L and M had similar compositions to that of native lysozyme (HL), fraction N was quite different. Further crude synthetic protein was passed

down a G75 Superfine column, in the hope that this would give better discrimination between the high M.W. components of the crude sample. HL and reduced, denatured HL [HL(red.)] were also subjected to the same conditions. From the crude material, four peaks (fractions W, X, Y and Z) were again observed in the UV trace. These were combined, lyophilised and the V_e/V_o ratios calculated as before. Fraction W had eluted near to V_o. This was thought to represent noncovalent oligomeric species, which had formed due to the relatively high protein concentrations initially obtained when dissolving the crude protein. Fractions X and Y eluted roughly in the same region as HL, and the HL(red.) eluted earlier than the native protein as previously observed[251].

The crude protein from assembly B was subjected to gel filtration on a Sephadex G75 column. Three fractions were collected here; P, Q and R. Fraction P appeared again to be due to high M.W. oligomeric forms; fraction R had the appearance of lower M.W. peptides; however, fraction Q eluted with approximately the same volume as HL. A sample of combined P+Q had its SBu^t PGs removed in the usual way, then the reduced product was passed down the column. This reduced, synthetic lysozyme [SL(red.)] eluted with the same volume of eluent as that of the HL(red.), and also earlier than the original Cys-protected fractions. HPLC traces of the crude Cys-protected SL and SL(red.) proteins, along with HL(red.) are shown in Fig. 2.46.

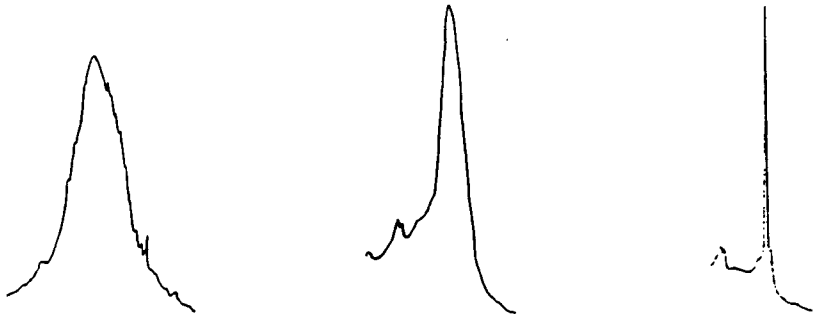


Fig. 2.46 HPLC traces comparing synthetic lysozyme, at various stages, with the reduced, natural protein

2.10.5. Analysis of SL

Ellman's test on the lyophilised SL(red.) and HL(red.) showed that only 3.6 and 4.1 thiol groups/molecule respectively, were present. Comparing this to the value of 7.6 for freshly prepared HL(red.), it is clear that some disulphide formation and folding

had occurred during the gel filtration step. However, the effect on the SL(red.) was not known; either the cysteine deprotection had not been complete, or worse, the required number of cysteine residues was not present at all, or perhaps partial oxidation had also occurred in this case. It would be difficult, of course, to ascertain exactly how many Cys residues should be present in this impure sample, in that existing deletion/truncated sequences of similar size may have adversely affected the calculation of the apparent cysteine content. The deprotection/reduction was thus repeated with TBP/TFE, furthermore this was carried out under denaturing conditions using 6M guanidinium chloride (Gdm.HCl, pH 8.0) in Tris buffer. The SL(red.) was rapidly isolated on a G25 gel column, then lyophilised. The SL(red.) now gave a similar value of 3.5 SH groups/molecule. The HL(red.) value had declined even further to 1.2.

AAA results on the HL(red.) and SL(red.) proteins showed that while HL(red.) had a mean error/residue of 7%, under the same conditions the SL(red.) gave an error of 16%. Both samples were subjected to polyacrylamide gel electrophoresis under denaturing conditions. The resulting gel showed that the SL(red.) lane had a band at the same level as the strong band appearing in the HL(red.) lane (at around the 14.6 kDal standard). At least two other bands of lesser intensity could be observed in the SL(red.) lane, at a level between the 14.6 and 8.2 kDal markers.

Overall, the results implied that at least some portion of the crude samples from both assemblies contained protein material of approx. the same M.W. as that of HL. Significantly, in view of the monitoring traces, the crude material from assembly B contained a greater proportion of high M.W. material compared to assembly A (70% against 50%). The exact nature of this material would be difficult to define without the aid of further purification steps; AAA of the fractions from the G50 column were the same as that of native HL, and even the crude material from assembly A gave largely equivalent results. Some attempts were made at further characterisation. A sample of fraction L, a fully Cys-protected product from assembly A (after the G50 column) was subjected to plasma-desorption mass spectrometry (PDMS), which can determine the mass of high M.W. biological molecules e.g. proteins[336]. Unfortunately, the initial ion-spectrum produced from the sample was too complex to permit the calculation of the mass, obviously reflecting the expected lack of purity of such a sample.

2.10.6. Attempts at Folding SL

At this stage further purification steps were obviously required. The cysteine-protected protein could perhaps have been purified by ion-exchange chromatography or affinity methods. However, a different approach was tried, which also enabled the investigation of the renaturation process in HL. It was clear from the Ellman's test results that the reduced and denatured authentic protein, and perhaps the synthetic protein also, had lost its free thiol groups over a certain time-period whilst in solution, presumably by disulphide formation. It was decided to investigate whether the thiols in the synthetic protein could be formed into stable disulphides (perhaps even the native ones) using the previously stated GSSG/GSH couple. This may have enabled the resulting synthetic protein to be more easily purified by applying techniques and conditions already reported for the purification of the native protein. Additionally, any enzymic activity which appeared even at this relatively crude stage could be assessed.

Using the conditions suggested by Saxena[242], the HL(red.) was able to be regenerated, returning about 56% of the original enzyme activity, as measured in the *M. lysodeikticus* assay of Jollés[252]. The same protocol was to be used for samples of the SL(red.). To begin with, a portion of the high M.W. gel filtration fractions from assembly B, had its SBu^t groups removed under denaturing conditions (as before). The resulting solution was then dialysed against 6M Gdm.HCl (pH 8.0)/β-mercaptoethanol at 4°C for 24 h. This should have completely reduced and denatured the synthetic protein. Over the next seven days, the protein solution was sequentially dialysed against solutions containing decreasing amounts of denaturant: These were one litre each of de-aerated and nitrogen-purged 6M, 4M, 2M and finally 0.5M Gdm.HCl (all at pH 8.0 and at 4°C). It was hoped that the gradual decrease of denaturant (in the absence of undesirable air-oxidation processes) would encourage any of the correct sequence present to form some secondary structure[337]. The final step was to use the glutathione couple to reshuffle the disulphide bonds and perhaps attain a native-like structure in the synthetic protein.

One of the things that was not known was the effect that the presence of other peptide/protein impurities may have on the folding process; also, assuming that some of the correct sequence was present (and full deprotected), it was also not known what proportion (presumably small) this represented and whether or not this could be

detected if the folding process had proved to be completely successful. The dialysis series had clearly altered the synthetic protein, comparing the HPLC traces of the cysteine-protected protein (after the gel column), with the reduced, 'folded' protein after dialysis (Fig. 2.47) shows an obvious narrowing of the peak. Presumably the number of conformers had been decreased somewhat during the dialysis series, leading to a narrower peak on HPLC.

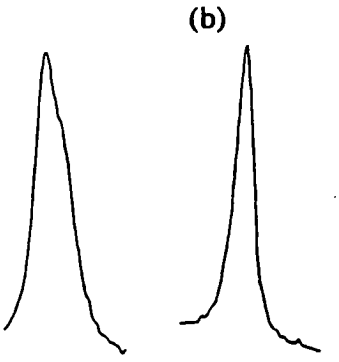


Fig. 2.47 (a) Cysteine-protected SL (b) 'Folded' SL(red.) after dialysis.

Using this dialysis solution directly (after dilution to the appropriate concentration), the glutathione reaction was carried out under conditions which had succeeded in reactivating the authentic reduced and denatured protein. This was not successful; extensive investigations into the effects of varying denaturant levels, temperature and the concentrations of both the synthetic protein and the GSSG/GSH couple, proved fruitless. No enzymic activity could be observed, although this was not really surprising in view of the large number of variables involved. What was discovered from these reactions was that the reduced protein had to be maintained at very high dilutions: the reduced protein could not be increased beyond 1 μM (about 14.5 mg/l) without extensive loss of the protein material through precipitation. This may have significant consequences for any future attempts at reactivating such a synthetic protein on a preparative scale.

In conclusion, it appears that it will be necessary to purify the synthetic protein with the cysteine PGs intact. It may be difficult to work with the fully-reduced crude product considering its tendency to aggregate, even at relatively low concentrations.

			Nº. observed in ...	
Residue	Nº. in entire sequence	Nº. in final 25 residues	Nº. observed in HL(red.)	Nº. observed in SL(red.)
Leu	8.0	3.0	8.0	5.5
Ala	12.0	3.0	12.5	1.3
Phe	3.0	1.0	3.2	1.8
Tyr	3.0	1.0	3.1	2.0
His	1.0	1.0	1.3	0.6
Met	2.0	1.0	2.3	1.3

Table 2.48 Comparison of AAA for natural and synthetic reduced lysozymes.

A comparison of the AAA results for HL(red.) and SL(red.) [Table 2.48] perhaps points to the relative lack of purity of the latter: many of the final 25 residues of the

assembly are low indicating the probable presence of terminated/deletion sequences with comparable molecular sizes (as the protein had been previously subjected to gel filtration). Accordingly, the crude protein is now the subject of purification strategies which will utilise the Tbfmoc/graphite affinity techniques recently developed in this laboratory[309]. This may provide a facile first route to the rapid purification of such large peptides/proteins.

2.11. SUMMARY : OVERVIEW OF NGF STUDIES

During the course of this study, other research programmes led to several important discoveries in the NGF field. It had long been recognised that while NGF bound to the $p75^{NGFR}$, this receptor itself did not cause the signal transduction which led to the biological effects triggered by NGF binding. As previously stated, the demonstration that the protooncogene *trk* encodes a functional NGF receptor[65,339], led to the theory that the *trk* product (a 140 kDal protein, $p140^{trk}$) acted alone as the high-affinity receptor species (and hence elicited the biological effects). Alternatively, it was suggested that $p140^{trk}$, together with the $p75^{NGFR}$, formed a two-protein, high-affinity receptor[64,339]. Furthermore, although NGF, BDNF and NT-3 all bind to the $p75^{NGFR}$ with roughly equal affinity[74], there appear to be distinct high affinity forms of the $p140^{trk}$, namely $p140^{trkA}$, $p140^{trkB}$[78] and $p140^{trkC}$[76] respectively. Thus, it has been postulated that NGF may contain two, spatially-separated binding sites, with which NGF may be able to interact simultaneously with each receptor. It could be surmised that the region which binds to the $p75^{NGFR}$ would be common to all three neurotrophins, and that which binds to their respective *trk* receptors would be composed of a region (or regions) not conserved across the proteins.

In late 1991, a crystal structure of mouse NGF (to 2.3Å resolution) was disclosed[40]. This showed NGF to have a novel structure, consisting of three anti-parallel pairs of β-strands, which formed a flat surface (Fig. 2.49). Two NGF subunits associate through this surface to form the NGF dimer. Interestingly, these β-strands are linked by four, surface-lying loop regions, which contain most of the variable regions present in the NGF-related proteins. It may be that these variable loop regions will ultimately define the discrete high-affinity binding sites observed on NGF and its related molecules.

In mid-1992, Ibáñez and co-workers[339], using site-directed mutagenesis on NGF, had shown that the mutation of residues $Lys^{34,34,95}$ abolished binding to the $p75^{NGFR}$. Importantly, these mutants did retain binding to the $p140^{trkA}$ receptor and stimulated outgrowth from chick sympathetic ganglia (a biological assay for NGF). These residues do not play a structural role in NGF and, in the dimer, the area close to and around this β-loop, between residues Asp^{30}-Lys^{34}, forms a continuous, positively-charged surface[40]. Thus, it was suggested that the positively-charged a.a.'s around this loop constituted the low affinity binding site on NGF[339]. The lysine residues at 32 and 34 are, however, not conserved in BDNF and NT-3, perhaps implying a separate binding region for these proteins. Ibáñez suggested that in BDNF, the three positively-charged residues at 95-97 (KKR), in the near-by variable loop 94-98 of BDNF, may compensate for the lack of these lysine residues.

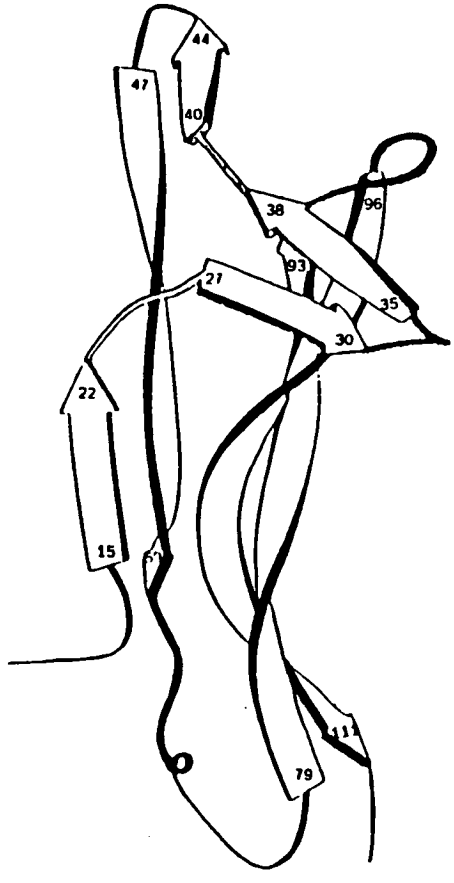


Fig. 2.49 Ribbon cartoon of mNGF showing β-strand structure

(loops regions 31-34, 45-46, 94-95 and 60-78 are in black)

How the highest binding fragments discovered in this study relate to the 3-D structure of the NGF protein itself, is illustrated in the following photographs (Plates 1-4), which show the configuration of the murine protein as found by X-ray crystallography[40]. The plates were prepared from the Brookhaven Protein Databank model of murine NGF and manipulated using the SYBIL programme on an Evans and Sutherland workstation. Residues 1-11 and 112-118 were poorly defined on the original X-ray map and are likely to be flexible and solvent accessible[40]. The key to Plates 1-4 gives a full explanation of the residues highlighted.

KEY TO PLATES 1-4

PLATE 1

Residues 50-70 (magenta) run from the top right side to the bottom right side of the molecule. The positions of Cys^{58} (with its disulphide-bonded Cys^{108}) and Cys^{68} (with its disulphide-bonded Cys^{110}) are labelled.

Residues 95-120 (green) run from top left to bottom left. Residues 113-120 have been added and set to a random-coil conformation. The positively-charged residues in this region are highlighted (yellow) and labelled (from the top; Lys^{95}, Arg^{100}, Lys^{34}, Lys^{32}, Arg^{103}, Arg^{114} and Arg^{118}: Lys^{115} is also labelled).

PLATE 2

This shows the reverse view of Plate 1. The positively-charged area comprising Lys^{95}, Arg^{100}, Lys^{34}, Lys^{32} and Arg^{103}, can clearly be seen jutting out from the top right of the molecule. Note again that residues 113-120 have been added and have no defined position in this structure. The surface-lying, accessible variable loop segment comprising residues 59-66 (magenta) can also be observed in this reverse view. Note also that the position of this model corresponds closely to that of Fig. 2.49 so that it is just possible to follow the backbone and secondary structure elements on Plate 2 by comparison with Fig. 2.49.

PLATE 3

The backbone is shown as a ribbon (magenta) in the same orientation as Plates 1 and 4. The lysine and arginine residues highlighted in Plate 1 are coloured red and blue respectively. The clustering of the disulphides (from top to bottom), Cys^{58}-Cys^{108} (green), Cys^{15}-Cys^{80} (orange) and Cys^{68}-Cys^{110} (yellow) can be observed. The turns of the 59-66 loop region can just be seen behind the disulphides.

PLATE 4

The position of residues 100-114 (113 and 114 have been added) is shown (magenta) in relation to the entire sequence (white).

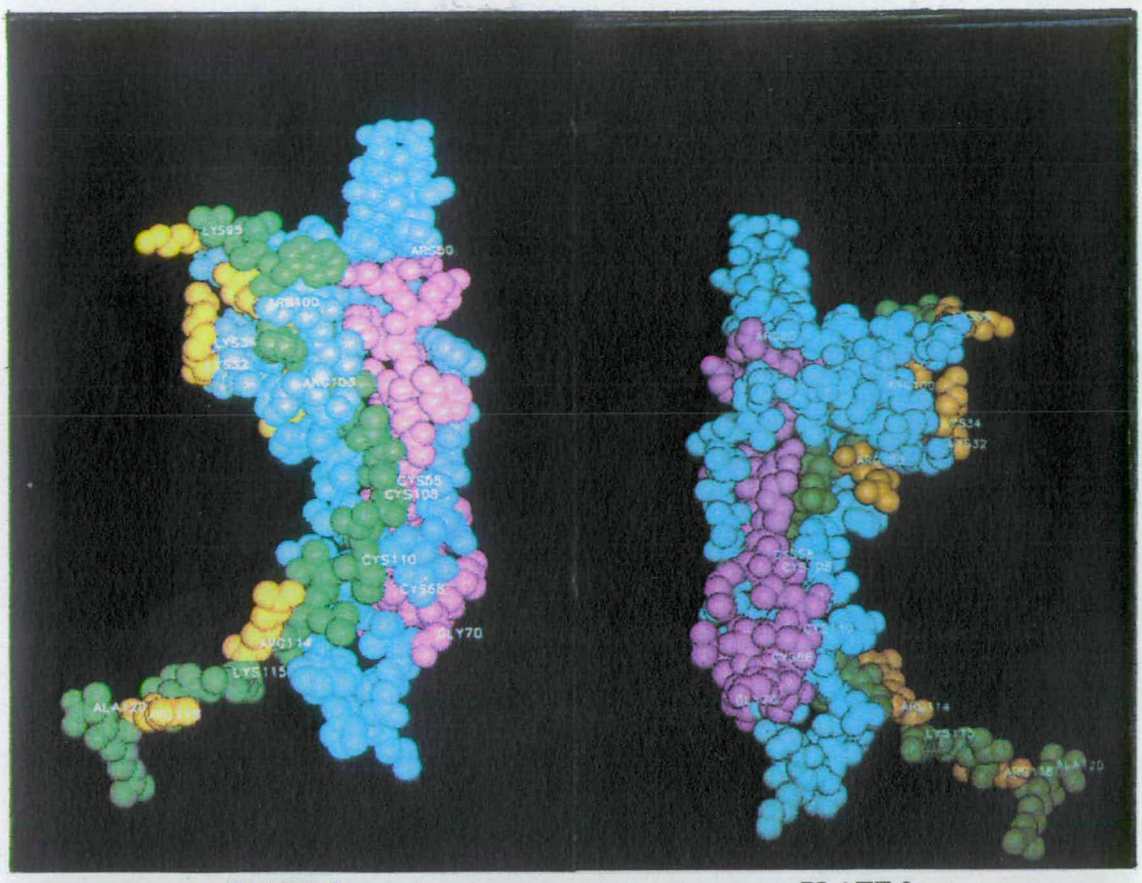


PLATE 1

PLATE 2

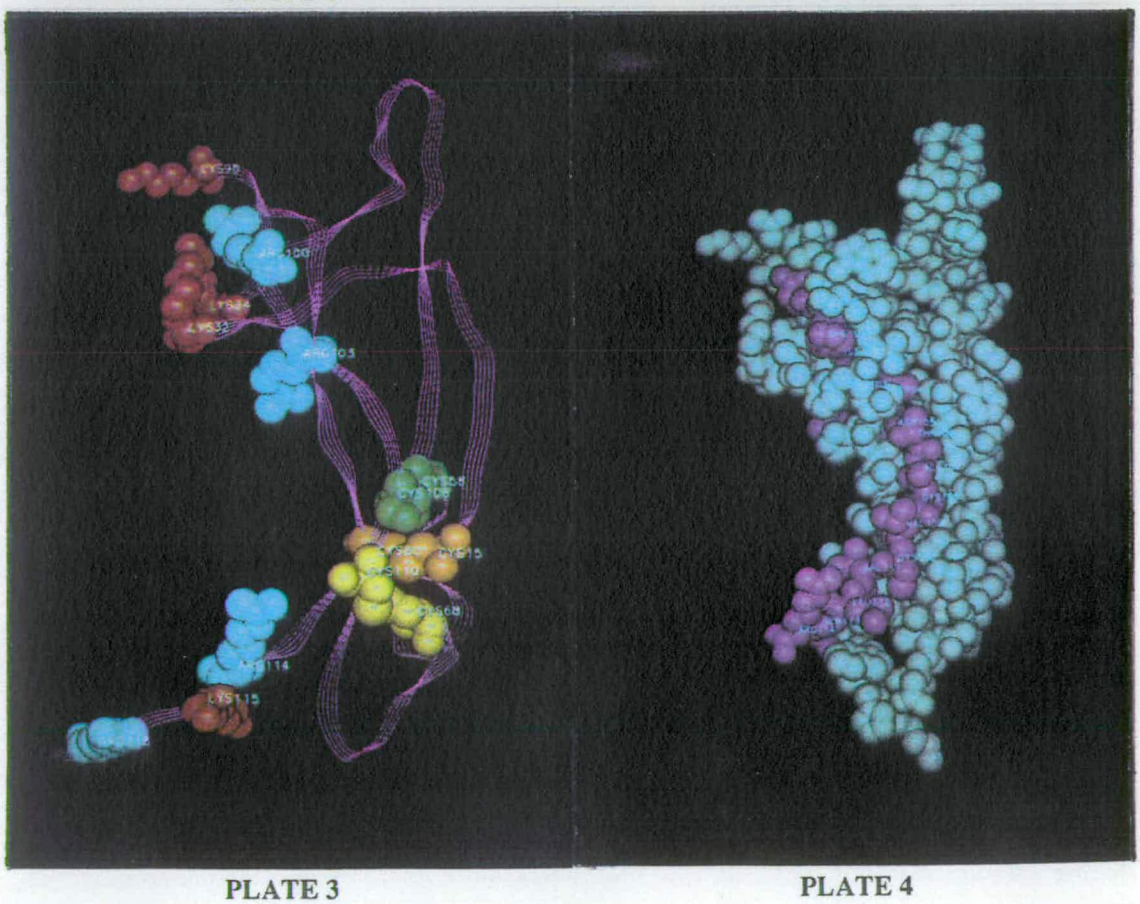


PLATE 3

PLATE 4

These models suggest possible theories for the observed binding of the NGF fragments. The lysine residues (32, 34 and 95), implicated in $p75^{NGFR}$ interaction, are in the immediate vicinity of Arg^{100} and Arg^{103}. It has been suggested that these together form the binding site to the p75 receptor[339]. In the 100-114 ox. fragment, a similar binding region may have been simulated by the $Arg^{100,103}$ residues present, with additional aid by Arg^{104}. The latter may have been manoeuvred into a favourable position by the presence of the disulphide bond in this fragment and together these may have mimicked a positively-charged surface. Significantly, neither Arg^{100} nor Arg^{114} could be removed from this peptide without causing a substantial reduction in the binding affinity. Considering Ibáñez's explanation for BDNF binding[339], it may be that simply a concentration of positively-charged a.a.'s, in the correct conformation (or able to form the required conformation), is necessary to constitute a binding peptide. As it is thought that NGF may bind as a dimer, it may be that a single peptide could never achieve the level of binding observed for NGF, in that the high density of positively-charged residues required could not be reached in a single peptide chain.

From the crystal structure information, it is clear that many of the residues in the 100-114 fragment are intimately involved in the stabilisation of the NGF dimer[40]. The residues Phe^{101}, Ala^{107}, Val^{109} and Val^{111} all contribute to interactions between the two chains constituting the dimer, and, in addition to the polar Thr^{106}, these residues are buried in the dimer interface. Perhaps, at the concentrations used in the assays, the 100-114 peptide itself interacted with the NGF dimer, thereby reducing the apparent level of ^{125}I-NGF binding to the recombinant $p75^{NGFR}$.

The models of the X-ray structure show that the sequences 50-70 and 95-120 (which are present in the NGF 53-mer as the discontinuous fragments) run parallel to one another in the native NGF protein, thus residues 70 and 95 are at opposite ends (and sides) of the molecule. The 53-mer peptide would probably not have mimicked the relative positions of many of the residues in NGF; also to form the correct disulphides would have required the disulphides to form across each other in the 53-mer - this may have been sterically inhibited in a single, continuous chain. However, it would have been interesting to form the variable loop bordered by the two disulphides, if it could have been achieved. In view of the variability and obvious surface-accessibility of this loop (see Plate 2), it may be that this region could be responsible for the interaction with the $p140^{trkA}$ receptor: it is tempting to suppose that the loop is held in place by the disulphides due to its biological importance.

Also, as mentioned, not one of the residues in this loop between 59-66 is conserved across NGF, BDNF and NT-3. Such an area could account for the existence of the different *trk* receptors, each different loop region being responsible for defining which of the proteins bound to which *trk* sub-class[340].

Overall, current ideas perhaps indicate that creating an NGF agonist may be difficult if the 'two-component' ($p75^{NGFR}$ and 140^{trkA} receptors), high-affinity receptor theory is confirmed. Such a peptide would presumably have to span both receptors and provide two binding sites, thus would probably have to be both large and complex. However, there seems to be a growing body of evidence and opinion that suggests that the $p140^{trkA}$ receptor alone may represent the high-affinity receptor and mediate the biological responses of NGF, and that the two receptors may interact only through their signal transduction mechanisms[69]. The interpretation of the results of many of the current receptor experiments (especially from cultured cells) is complex (and sometimes contradictory) and many of the issues involved are still the subject of considerable controversy.

If the $p140^{trkA}$ receptor alone does prove to represent the high-affinity receptor, it should be possible to follow a similar approach to that undertaken here in order to determine which regions may bind to this receptor. Such binding may directly trigger the biological activity and lead to the elucidation of structures required for agonist activity. The basis of this work would, of course, be the creation of a reliable $p140^{trkA}$ receptor assay. Advances towards such an approach, perhaps even utilising many of the NGF fragments described here, are currently being investigated[341].

CHAPTER THREE : EXPERIMENTAL

3.1. NOTES

All a.a.'s were purchased from Novabiochem or Raylo Chemicals and were of the L-configuration unless otherwise stated. The integrity of each was confirmed before use by MP, TLC and optical rotation under conditions stated on the data sheet supplied, and additionally by N.M.R. (200 MHz). TLC was carried out on some peptides using plastic plates, precoated with silica gel 60GF-254 (Merck 5735) in the following systems: n-butanol/pyridine/acetic acid/water, a.) (15:10:3:12) or b.) (1:1:1:1). Visualisation of certain a.a. residues in peptides was achieved using a suitable combination of the following methods: UV absorption at 254 nm, ninhydrin for compounds with free amino groups, Mary's spray [4,4'-bis(dimethylamino)diphenylcarbinol] for acid functions, Pauli's spray for tyrosine- and histidine-containing peptides, Ehrlich's spray for tryptophan and Sakaguchi's test for arginine (see section 3.2.7. for details). Optical rotations were measured on an AA1000 polarimeter (Optical Activity Ltd.) in DMF (for a.a. derivatives) using a 10 cm cell. UV and visible spectra were recorded on a Varian Cary 210 double-beam spectrophotometer in the appropriate solvent. Infrared spectra were recorded between 4000 and 600 cm^{-1} on a Perkin-Elmer 781 spectrophotometer using KBr discs. Polystyrene was used as the standard (1603 cm^{-1}). Amino acid analysis was performed on a LKB 4150 alpha amino acid analyser on a hydrolysate obtained from one of the methods described in section 3.2.9. High and low resolution fast atom bombardment mass spectra (FABMS) were obtained from a Kratos MS50TC machine, using thioglycerol or 3-nitrobenzyl alcohol as a matrix. ^1H N.M.R. spectra were recorded on either a Bruker WP200 (200 MHz) or a Varian VXR5000 (600 MHz) machine in the solvents indicated using tetramethylsilane (TMS) as an internal standard. 2-D experiments were carried out using the 600 MHz machine. Peptide sequencing was carried out on an Applied Biosystems (ABI) 477A sequencer at the WellMet sequencing facility, University of Edinburgh. Polyacrylamide molecular weight gels (for lysozyme) were run using 20% gels on a LKB Pharmacia PhastGel electrophoresis system and developed using Coomassie Blue and silver staining.

All solvents were distilled before use and the following were dried using the reagents given in parentheses: diethyl ether (sodium wire) ; dichloromethane (calcium hydride) ; tetrahydrofuran (sodium). Dimethylformamide, 1,4-dioxan and piperidine

were Peptide Synthesis grade supplied by Rathburn Chemicals, Walkerburn, Scotland. Biochemical grade trifluoroacetic acid (TFA) was obtained from ABI. H2O used was Milli-Q grade. High performance liquid chromatography (HPLC) was carried out using an ABI system, comprising 2 x 1406A solvent delivery systems, a 1480A injector/mixer and a 1783A detector/controller.

ANALYTICAL COLUMNS used were as follows:
ABI Aquapore RP300 reverse-phase silica (300 Å pore size, 7 μm spherical silica)
Column A : 220 x 4.6 mm C18 or C8, as stated in text, flow rate = 1.0 cm^3/min.
Column B : 110 x 4.6 mm C18 or C8, flow rate 1.0 cm^3/min.

Vydac C18 reversed-phase silica (5 μm particle size)
Column C : Column 218TP54, 250 x 4.6 mm, flow rate 1.0 cm^3/min.

PREPARATIVE COLUMNS used were:
ABI (matched to analytical columns)
Column D : 250 x 9.2 mm C18 or C8, flow rate 5.0 cm^3/min.
Column E : 110 x 9.2 mm C18 or C8, flow rate 5.0 cm^3/min.

Vydac C18 reversed-phase silica (10 μm particle size)
Column F : Column 218TP1022, 250 x 22 mm, flow rate 10.0 cm^3/min.

Components were eluted from these columns with a gradient of acetonitrile (far UV grade, Rathburn Chemicals) in double-distilled, deionised water with 0.1% TFA in both solvents. The appropriate gradients are quoted in the text in (time, % acetonitrile) e.g. gradient of 10-30% acetonitrile (CH_3CN) over 30 min., is quoted as (0,10), (30,30). Eluents were detected at either 214 or 229 nm. The following standard analytical gradients are referred to in the text:

Gradient	time, % CH_3CN	Gradient	time, % CH_3CN
A	(0,0), (20,40)	E	(0,5), (30,65)
B	(0,5), (20,55)	F	(0,5), (30,95)
C	(0,5), (25,85)	G	(0,10), (25,75)
D	(0,5), (30,35)	H	(0,10), (30,90)

3.2. SOLID-PHASE PEPTIDE SYNTHESIS

3.2.1. Preparation of Fmoc-Amino Acid Resins

The Fmoc-amino acid residues used were as previously stated, the individual types used in a particular assembly are referred to in the text.
A solution of Fmoc-a.a. (5.0 mmol) and N,N'-diisopropylcarbodiimide (0.39 cm^3, 2.5 mmol) in DMF (10 cm^3) was stirred for 15 min., then p-benzyloxybenzyl alcohol polystyrene resin (cross-linked with 1% divinylbenzene, functionalised to 0.80 mmol/g, 1.0 g, 0.80 mmol) was added. The slurry was swirled and more DMF added to ensure mobility. 4-(N,N'-dimethylamino)-pyridine (DMAP, Aldrich Chemicals, 15 mg, 0.1 mmol) was added in a little DMF and the entire mixture sonicated for 2 hr. The resin was collected by filtration, washed thoroughly with DMF then dioxan and stored at 4°C until required. A sample of this was washed with ether then dried *in vacuo* to give the Fmoc-a.a.-resin as off-white granules. The loading was determined by treating an accurately weighed sample (2-5 mg) with 20% piperidine/DMF (10 cm^3) and sonicating for 20 min. The UV absorbance of the supernatant was measured at 302 nm and the loading calculated using the Beer-Lambert Law (ε_{302} = 15,400). The coupling efficiency was usually in the region of 60-80%, giving a typical loading of 0.5-0.6 mmol/g.

3.2.2. Capping Unreacted Resin/Peptide Sites

The Fmoc-a.a.-resin (0.5 mmol) was treated with acetic anhydride (0.1 g, 1.0 mmol) and pyridine (0.08 g, 1.0 mmol) in DMF (7 cm^3). The mixture was shaken/vortexed for 2.5 min., filtered then the treatment repeated, with shaking for a further 3.5 min. The resin was filtered off and washed thoroughly with DMF. This process was used in automatic assembly after the coupling cycles for each residue were complete, and in manual assembly only when required.

3.2.3. Methods Used in Manual Assembly of Peptides

3.2.3.1. Assembly

The bubbler apparatus used in manual assembly is shown, and its operation described, in Appendix A. Manual assembly of the NGF peptides was carried out on the pre-loaded resin using the following protocol. Volumes of reagents used were approximately 10 cm^3/g of resin:

Operation		Reagent	Time (no. x min.)
1.	Wash	DMF	10 x 1
2.	Deprotect	20% Pip./DMF	2 x 3
3.	Wash	DMF	10 x 1
4.	Test resin	Kaiser/TBNS	1 x 5
5.	Couple	SA or AE	1 x 60 (SA) ; 1 x 90 (AE)
6.	Wash	DMF	10 x 1
7.	Test resin	Kaiser/TBNS	1 x 5
8a	If test is negative, continue cycle at Step 2		
8b	If test is still positive, repeat coupling with AE using an appropriate activation method. Continue coupling (twice more only) until the resin test is negative. Cap the resin if required. Continue at Step 2.		

3.2.3.2. Manual activation of Fmoc-amino-acids

a.) SA : The Fmoc-a.a. derivative (4 equiv.) and DIC (2 equiv.) were stirred in a minimum of DMF (5-10 cm^3) for 15 min. then added to the resin.
b.) AE : The Fmoc-a.a. derivative (2 equiv.), HOBt (Fluka, 2 equiv.) and DIC (2 equiv.) were stirred in a minimum of DMF (5-10 cm^3) for 30 min. then added to the resin.
c.) AE/other : The Fmoc-a.a. derivative (2 equiv.), BOP or HBTU (Novabiochem, 2 equiv.) and diisopropylethylamine (DIPEA, 4 equiv.) were stirred in a minimum of DMF (5-10 cm^3) for 5 min. then added to the resin.
At the end of the assembly, the completed peptide/resin was washed in excess DMF, dioxan then ether, with final drying overnight *in vacuo*.

3.2.3.3. Manual monitoring

i.) Kaiser test : A small sample of washed resin was withdrawn from the reaction vessel, placed in a vial then three drops of each of the following solutions were added,

a.) Ninhydrin in ethanol (5 g in 100 cm^3).
b.) Phenol in ethanol (80 g in 20 cm^3).
c.) 1.0 mM aqueous solution of KCN in water (2 cm^3) added to distilled pyridine (98 cm^3).

The vial was heated at 100°C for 5 min. A positive test (indicating the presence of free amine groups) was shown by a blue colouration.

ii.) TBNS : 5 mg of trinitrobenzenesulphonic acid was dissolved in 10% DIPEA/DMF (0.5 cm^3) then three drops of this solution added to a small sample of washed resin. A positive test indicating the presence of free amine was observed when the beads became strongly orange-coloured.

3.2.4. Methods Used in Automatic Assembly of Peptides

3.2.4.1. General

This was carried out using an Applied Biosystems 430A peptide synthesizer. Deprotection integrals were calculated by UV detection at 302 nm on an ABI 759A absorbance detector, with on-line integration via a Spectra-Physics SP4400 ChromJet integrator/printer. The machine compartmentalises the dissolution of the a.a. derivative (in small plastic cartridges), the activation (in a glass activator vessel) and the coupling steps (in a Teflon reaction vessel containing the resin). Mixing the resin/reagents was by vortexing and draining by positive-nitrogen pressure. Syntheses were on a 0.50 or 0.25 mmol scale using the appropriate amount of pre-derivatised resin. The washing, deprotection, coupling and capping steps used were similar to the manual synthesis protocols and are outlined below. All washes used 50% DMF/dioxan. Coupling was via double-couple cycles, a SA then an AE, except that some residues e.g. Asn, Gln, His and Arg were double-coupled with AE only.

3.2.4.2. Removal of the Fmoc group

The peptide/resin was treated with 20% piperidine/DMF (9 cm^3) in four batches reagent for periods of 5, 3, 3 and 1 min., with washing steps in between each batch. A sample of each deprotection mixture was passed through the on-line UV (with detection at 302 nm) and the peak integrated. The steps were reduced to 3 and 1 min. only in later syntheses, with no adverse effects.

3.2.4.3. Activation of the Fmoc-amino-acids

For a 0.25 mmol scale synthesis ;
SA : The Fmoc-a.a. (0.5 mmol) was dissolved by adding DMF (4 cm^3) and bubbling nitrogen through the solution for 3 min. [extended to 15 min. for Arg(Pmc) and 20 min. for Asn/Gln]. The solution was transferred to the activator where DIC/dioxan (2 cm^3, 0.25M) and dioxan (2 cm^3) were added. This solution was left to stand for

15 min. before transfer to the reaction vessel. The resin/SA solution was then vortexed for 30 min. to couple the a.a. This was later extended to 60 min., which gave better results in some circumstances.

AE : The Fmoc-a.a. (0.5 mmol) was dissolved by adding DMF (2 cm^3) and HOBt/DMF (2 cm^3, 0.25M). This solution was transferred to the activator, where DIC/dioxan (2 cm^3, 0.25M) and dioxan (2 cm^3) were added. The solution was left to stand for 20 min. before transfer to the reaction vessel. The resin/SA solution was then vortexed for 30 min. to couple the a.a. derivative. This was extended to 90 min. in some of the later syntheses.

At completion of the assembly, the peptide/resin was washed with excess dioxan then ether and dried overnight *in vacuo*.

3.2.5. Acidolytic Cleavage

The ether-dried peptide/resin (0.20-1.00 g) was placed in a small round-bottomed flask along with a stirring bar. The appropriate scavengers (specified in the text) were mixed together then TFA added to the required amount (5-20 cm^3). This was added to the resin and stirred (usually under nitrogen) for 2-5 hr. as necessary. Small samples (0.1-0.2 cm^3) were withdrawn at hourly intervals, evaporated to dryness under a stream of nitrogen, partitioned between 10% aqueous acetic acid and ether then the aqueous layer examined by HPLC. Cleavage was halted when two identical traces were obtained. At this point, the solution was concentrated *in vacuo* to an oil (often yellow or brown) and the crude peptide precipitated by the addition of excess ice-cold ether. The crude peptide was collected by filtration and washed with excess ether to remove the scavengers. The crude peptide was usually taken up in a minimum volume of aqueous acetic acid (10-30%) or ammonium hydroxide (10%, if it was more soluble in this and Cys was absent) then extracted twice from large excesses of ether (x 10-20 volumes). The aqueous layer was then lyophilised to give the extracted crude peptide as a white solid.

3.2.6. Hydrolysis of Peptides for Amino Acid Analysis (AAA)

i.) HCl hydrolysis : The peptide (2-4 mg) was treated with constant boiling HCl (2 cm^3) and placed in a sealed, evacuated Carius tube. This was heated at 110°C for 18-48 hr. as required. The hydrolysate was then evaporated to dryness *in vacuo*, taken up in a little H_2O (5 cm^3) and again evaporated to dryness. This process was

repeated twice then the residue dissolved in the appropriate loading buffer (2 cm^3) for subsequent analysis.

ii.) 4-TSA hydrolysis : The peptide (2-4 mg) was treated with 3M aqueous p-toluenesulphonic acid (1 cm^3), which had been saturated with tryptamine, and hydrolysed as above. The resulting hydrolysate was treated with 1M NaOH (2 cm^3) and water (2 cm^3) then analysed.

3.2.7. Detection of Individual Amino Acids in the Peptides

These were used on TLC plates and electrophoresis paper for qualitative assessment of a peptide's composition ;

1.) Ninhydrin : Detected free amine groups. The plate was sprayed with a 0.2% solution of ninhydrin in acetone then heated at 100°C for 5 min.

2.) Pauli's : Detected Tyr and His. The following solutions were required ;

a.) Sulphanilic acid (0.1 g) in 1 cm^3 of conc. HCl, diluted with 20 cm^3 of water, 4°C,

b.) $NaNO_2$ (5% in water at 4°C), and

c.) Na_2CO_3 (10% in water at 4°C).

1 cm^3 of a.) and 1 cm^3 of b.) were mixed and allowed to stand at -20°C for 5 min. 2 cm^3 of c.) was added and the plate lightly sprayed. Tyr-containing peptides stained pink, His-containing peptides stained orange.

3.) Ehrlich's : Detected tryptophan. Plates were sprayed with a solution of 20% p-dimethylaminobenzaldehyde (10% in conc. HCl) in acetone. Trp-containing peptides turned purple after a few minutes.

4.) Sakaguchi's : Detected arginine. Plates were sprayed with 8-hydroxyquinoline (1% in acetone) then air-dried for 10 min. The plate was then sprayed with Br_2/NaOH solution (0.67 cm^3 in a 0.1M NaOH solution). Arginine-containing peptides gave orange-red spots.

5.) Ellman's : This detects free thiol groups and can be used quantitatively or qualitatively.

A sample of the thiol-containing peptide (20-50 pmol) was dissolved in 0.1M Tris-Cl buffer (pH 8.0, 2.5 cm^3), containing 8M urea and 10mM EDTA (denaturing buffer). A fresh solution of 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB, 0.01M in 0.05M

sodium phosphate buffer, pH 7.0] was prepared. With 2.5 cm^3 of denaturing buffer alone in the reference cell, and the peptide/denaturing buffer solution (2.5 cm^3) in the sample cell (of a double-beam spectrophotometer), a small amount of DTNB solution (0.10 cm^3) was added to each and the absorbance read at 412 nm after a few minutes. The value of ε_{412} for the carboxylate-4-nitrothiophenolate used to calculate the thiol content was 14290 $M^{-1}cm^{-1}$.

For qualitative analysis, a small sample of the peptide (< 0.5 mg) was dissolved in the denaturing buffer (0.20 cm^3) and two drops of the DTNB solution added. The immediate appearance of a strong yellow colour indicative a positive test.

3.3. EXPERIMENTAL

Conserved NGF 10-17

H-Gly-Glu-Phe-Ser-Val-Cys^{SH}-Asp-Ser-OH

The initial resin had been derivatised with Fmoc-Ser(Bu^t)-OH (0.60 mmol/g ; 1.00 g ; 0.60 mmol). Cysteine was incorporated as Fmoc-Cys(Trt). The peptide was assembled manually, using single-couple cycles, except Asp(OBu^t) and Cys(Trt) which required an additional AE/HOBt coupling, resulting in 1.77 g of dried peptide/resin. A portion of this (0.82 g) was cleaved for 3 hr. in a solution of 4% water, 4% EDT and 2% anisole in TFA (15 cm^3, nitrogen-purged) to yield the crude peptide (0.21 g) as a white solid. This was relatively insoluble and appeared to polymerise readily. On ether extraction (2 x 70 cm^3) from 10% aqueous acetic acid (21 cm^3), a crystalline white solid (0.17 g) was obtained. A portion of this material (90 mg) was dissolved with difficulty in glacial acetic acid (5 cm^3) then water (10 cm^3) was added. Some insoluble material had to be filtered from this solution (through a 0.45 μm membrane filter). The filtrate was purified in four portions by semi-preparative HPLC [column D(C18), gradient (0,15), (2,15), (27,55)]. The purest fractions, as judged by analytical HPLC , were combined and lyophilised to give the *title compound* as a fluffy, white solid (10 mg).

FABMS m/z 843 ($M+H^+$), *hrms* found 842.31163, $C_{34}H_{50}N_8O_{15}S_1$ requires 842.31161 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient B] Rt 12.4 minutes (in the absence of added acetic acid, a small peak appeared at Rt 13.6 minutes, probably due to dimerisation) ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.03, Ser_2 1.77, Glu_1 1.08, Gly_1 0.98, Val_1 1.01, Phe_1 1.01, Cys_1 N.D. ; Ellman's test was positive.

Conserved Nerve Growth Factor 14-28

H-Val-Cys^{SH}-Asp-Ser-Val-Ser-Val-Trp-Val-Gly-Asp-Lys-Thr-Thr-Ala-OH

The initial resin had been derivatised with Fmoc-Ala-OH (0.58 mmol/g ; 1.20 g ; 0.70 mmol). Cysteine was protected as Cys(Trt), Trp was unprotected. Automatic assembly was carried out on the ABI machine, using the normal double-couple

cycles and monitoring showed that no drops in incorporation had occurred. On completion of assembly, the DMF-wet resin was divided into three portions : one portion (0.61 g) was used in this synthesis, 0.59 g was acetylated to form NGF Ac-14-28-OH and 0.80 g used in unsuccessful dansylation reactions. Some dried peptide/resin (0.30 g) was cleaved for 3 hr. in a mixture of 3% water, 2% EDT and 2% phenol in TFA (10 cm^3), resulting in a crude product (0.13 g). A sample of this material (70 mg) was dissolved in 20% aqueous acetic acid (21 cm^3), filtered and purified in seven portions by semi-preparative HPLC [column D(C18), gradient (0,10), (20,30), (30,30)]. The purest fractions yielded an almost pure, white solid (30 mg). Final purification by semi-preparative HPLC [column E(C18), gradient (0,10), (30,35)] yielded the *title compound* (7.5 mg) as a white powder.

FABMS m/z 1566.8 ($M+H^+$) ; *hrms* found 1565.73950, $C_{67}H_{107}N_{17}O_{24}S_1$ requires 1565.73956 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 12.7 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_2 1.98, Thr_2 2.00, Ser_2 1.65, Gly_1 0.95, Ala_1 0.97, Val_4 3.73, Lys_1 1.21, Cys_1 N.D., Trp_1 N.D.

Conserved Nerve Growth Factor acetylated 14-28

Ac-Val-Cys^{SH}-Asp-Ser-Val-Ser-Val-Trp-Val-Gly-Asp-Lys-Thr-Thr-Ala-OH

Half of the resin from NGF H-14-28-OH was acetylated and dried to give 0.59 g. The cleavage was under the same conditions used for the initial peptide, yielding the crude peptide (0.15 g). Ether extraction of this crude product gave 96 mg. A portion of this (20 mg) was dissolved in 15% aqueous acetic acid (8 cm^3), filtered and purified by semi-preparative HPLC [column D(C18), gradient (0,10), (30,40)] to yield the *title compound* (8 mg) as a white solid after lyophilisation of the appropriate fractions.

FABMS m/z 1608.7 ($M+H^+$), $C_{69}H_{109}N_{17}O_{25}S_1$ requires 1607.8 ; Analytical HPLC [column A(C18), gradient F] Rt 14.6 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_2 1.98, Thr_2 2.00, Ser_2 1.65, Gly_1 0.95, Ala_1 0.97, Val_4 3.73, Lys_1 1.21, Cys_1 N.D., Trp_1 N.D.

Conserved Nerve Growth Factor 19-22

H-Ser-Val-Trp-Val-OH

The initial resin had been derivatised with Fmoc-Val-OH (0.52 mmol/g ; 1.00 g ; 0.52 mmol). Trp was unprotected. The peptide was assembled manually, using single SA couplings. The weight of dried resin obtained was 1.50 g (half of this was used to form NGF Ac-19-22-OH). A portion of the resin (0.30 g) was cleaved for 2 hr. in a solution of 5% water, 2% anisole and 1% EDT in TFA (5 cm^3), to give the crude peptide (77 mg) as a single peak product (by HPLC). A sample of crude material (30 mg) was purified in a single portion by semi-preparative HPLC [column D(C18), gradient (0,10), (15,40)]. Lyophilisation of the purest fractions gave the *title compound* (17.5 mg) as a crystalline, white solid.

FABMS m/z 490 ($M+H^+$), *hrms* found 489.25877, $C_{24}H_{35}N_5O_6$ requires 489.25873 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 13.3 minutes ; Amino acid analysis (4-TSA hydrolysis, 18 hr.) Ser_1 0.98, Trp_1 0.76, Val_2 2.05.

Conserved Nerve Growth Factor acetylated 19-22

Ac-Ser-Val-Trp-Val-OH

Half of the resin from NGF 19-22 was acetylated. The cleavage was under the same conditions used for the initial peptide, yielding the crude peptide (78 mg) as a single product (by HPLC). A portion of this (40 mg) was purified in four runs by semi-preparative HPLC [column D(C18), gradient (0,10), (20,45)] to yield the *title compound* (19 mg) after lyophilisation of the appropriate fractions.

FABMS m/z 532 ($M+H^+$), *hrms* found 531.26941, $C_{26}H_{37}N_5O_7$ requires 531.26931 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 14.7 minutes ; Amino acid analysis (4-TSA hydrolysis, 18 hr.) Ser_1 0.98, Trp_1 0.76, Val_2 2.05.

Mouse Nerve Growth Factor 25-54

H-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-Leu-Ala-Glu-Val-Asn-Ile-Asn-Asn-Ser-Val-Phe-Arg-Gln-Tyr-Phe-Phe-OH

The initial resin had been derivatised with Fmoc-Phe-OH (0.35 mmol/g ; 1.00 g ; 0.35 mmol). Asn and Gln were unprotected. Automatic assembly was carried out on the ABI machine, using the normal double-couple cycles, except in the following cases : Thr^{26} and Val^{42} (1 x SA, 2 x AE/HOBt), $Asn^{43,45}$ (2 x AE/HOBt) and Asn^{46} (3 x AE/HOBt). Monitoring showed that significant falls in incorporation had occurred over the regions Gly^{33}-Gln^{35} and Val^{38}-Ile^{44}. The weight of dried peptide/resin after assembly was 2.60 g. Several different conditions were investigated to optimise the cleavage protocol. Finally, a portion of resin (1.00 g) was cleaved for 4 hr. in a mixture of 4% water, 3% anisole, 2% EDT and 1% thioanisole in TFA (25 cm^3). After ether extraction, the crude peptide was obtained as a white solid (0.51 g). Full analysis on this material was carried out.

A portion of crude material (0.20 g) was dissolved in 15% CH_3CN/H_2O (75 cm^3), with 10% aqueous acetic acid added (25 cm^3) and purified in twenty portions by semi-preparative HPLC [column E(C8), gradient (0,15), (25,40)]. The combined fractions yielded an almost pure, white solid (55 mg). This was repurified in five lots [dissolved in 10% aqueous acetic acid (11 cm^3)] by semi-preparative HPLC [column D(C18), gradient (0,10), (30,50)], to give the *title compound* as a white solid (22 mg) after lyophilisation of the appropriate fractions.

Crude product

FABMS m/z 3530, 3511, 3494, 3434 ($M+H^+$) ; Analytical HPLC [column A(C18), gradient E] Rt 21.5 minutes [major peak (70%)] ; Amino acid analysis (HCl hydrolysis, 24 hr.) Asx_4 6.13, Thr_4 3.89, Ser_1 1.57, Glx_3 5.10, Gly_1 1.01, Ala_2 2.26, Val_4 5.78, Ile_2 2.64, Leu_1 1.41, Tyr_1 1.71, Phe_3 5.43, Lys_3 2.97, Arg_1 1.85.

Title compound

FABMS m/z found 3434.1 ($M+H^+$), $C_{156}H_{246}N_{40}O_{47}$ requires 3433.9 ; Analytical HPLC [column A(C18), gradient F] Rt 14.7 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asx_4 4.20, Thr_4 3.39, Ser_1 1.04, Glx_3 2.88, Gly_1 1.05, Ala_2 1.89, Val_4 3.84, Ile_2 1.99, Leu_1 0.94, Tyr_1 0.92, Phe_3 3.14, Lys_3 2.79, Arg_1 1.12.

Mouse Nerve Growth Factor 34-42

H-Lys-Glu-Val-Thr-Val-Leu-Ala-Glu-Val-OH

The initial resin had been derivatised with Fmoc-Val-OH (0.55 mmol/g ; 1.00 g ; 0.55 mmol) Automatic assembly was carried out on the ABI machine using the normal double-couple cycles (SA and AE). Monitoring showed that no major drops in incorporation efficiency had occurred. The weight of dried resin obtained was 1.28 g. A portion of this (0.60 g) was cleaved for 2 hr. in a solution of 5% water and 5% anisole in TFA (10 cm^3), to yield the crude peptide (0.24 g) as a single peak product (by HPLC). Some of the crude material (50 mg) was dissolved in 5% CH_3CN/H_2O (5 cm^3) and purified in two portions by semi-preparative HPLC [column E(C18), gradient (0,5), (2,5), (17,20)]. Combining the purest fractions, as judged by analytical HPLC , and lyophilisation gave the *title compound* as a white solid (19 mg).

FABMS m/z 988 ($M+H^+$), *hrms* found 986.56474, $C_{44}H_{78}N_{10}O_{15}$ requires 986.56481 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 15.6 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Glu_2 2.37, Thr_1 1.07, Ala_1 1.16, Val_3 3.20, Leu_1 1.11, Lys_1 0.87.

Mouse Nerve Growth Factor 46-54

H-Asn-Ser-Val-Phe-Arg-Gln-Tyr-Phe-Phe-OH

The initial resin used was the remaining half of NGF 50-54. Fmoc-Asn was not side-chain protected. The peptide was assembled manually using single-couple, SA cycles, except Val^{48} (double-coupled with AE/BOP) and Asn^{46} (double-coupled, 1 x AE/BOP, 1 x AE/HBTU). The weight of dried resin after assembly was 2.10 g. A portion of this (1.00 g) was cleaved for 2 hr. in a solution of 5% water, 5% anisole and 5% phenol in TFA (10 cm^3), to yield a rather insoluble crude product (0.50 g). This was subsequently ether extracted (2 x 150 cm^3) from 20% aqueous acetic acid (50 cm^3) and lyophilised to yield the crude product (0.31 g). A portion of this material (60 mg) was dissolved in 5% CH_3CN/H_2O (15 cm^3) then purified in six portions by semi-preparative HPLC [column D(C18), gradient (0,5), (5,5), (20,25)].

Combining the purest fractions, as judged by analytical HPLC , and lyophilisation gave the *title compound* as a white solid (12 mg).

FABMS m/z 1207 (M^+), *hrms* found 1206.58224, $C_{59}H_{78}N_{14}O_{14}$ requires 1206.58219 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 23.9 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asn_1 1.03, Glu_1 1.19, Ser_1 0.91, Val_1 1.01, Tyr_1 1.04, Phe_3 3.05, Arg_1 1.01.

Mouse Nerve Growth Factor 50-54

H-Arg-Gln-Tyr-Phe-Phe-OH

The initial resin had been derivatised with Fmoc-Phe-OH (0.57 mmol/g ; 2.00 g ; 1.14 mmol). The peptide was assembled manually using single-couple, AE/BOP cycles. Gln was unprotected. Half of the DMF-wet resin (about 3 g) was removed and dried to give 1.40 g dried peptide/resin (the remainder was used in assembly of NGF 46-54). A portion of this (0.50 g) was cleaved for 2 hr. in a solution of 5% water, 5% anisole and 5% phenol in TFA (10 cm^3) to yield the crude peptide (0.17 g) as a white solid, which was essentially a single peak on analytical HPLC. A portion of this material (50 mg) was dissolved in 5% CH_3CN/H_2O (5 cm^3) and filtered. Purification was in three portions by semi-preparative HPLC [column D (C18), gradient (0,0), (5,5), (15,25), (25,25)]. The purest fractions, as judged by analytical HPLC , were combined and lyophilised to give the *title compound* as a fluffy, white solid (22 mg).

FABMS m/z 760 ($M+H^+$), *hrms* found 759.99472, $C_{38}H_{49}N_9O_8$ requires 759.99481 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 16.8 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Gln_1 1.16, Tyr_1 0.99, Phe_2 2.01, Arg_1 1.01.

Human NGF 50-60

H-Lys-Gln-Tyr-Phe-Phe-Glu-Thr-Lys-Cys^{SH}-Arg-Asp-OH

The initial resin had been derivatised with Fmoc-Asp(OBu^t)-OH (0.58 mmol/g ; 0.95 g ; 0.55 mmol). Cysteine was incorporated as Fmoc-Cys(Trt), Gln was unprotected. The peptide was assembled manually, using SA cycles, except Arg(Pmc), Cys(Trt)

and Gln (double-coupled), which were by AE/HOBt couplings. Assembly proceeded well until Tyr(Bu^t) was added, when the resin began to aggregate badly. Washes were carried out in 50% DMF/dioxan to try to resolvate the resin. The deprotection steps at Tyr(Bu^t), Gln and Lys(Boc) were extended by three minutes. Lys(Boc) required triple-coupling (SA, AE/HOBt, AE/BOP). The weight of the peptide/resin after chain assembly was 1.77 g. A portion of this (1.50 g) was cleaved for 3 hr. in a solution of 5% water, 5% EDT and 5% anisole in TFA (20 cm^3) to yield the crude peptide (0.64 g) as a white solid. On ether extraction, a crystalline white solid (0.52 g) was obtained. A portion of this material (50 mg) was dissolved in glacial acetic acid (2 cm^3) then water (8 cm^3) was added. Some DMF (1 cm^3) was added and the solution sonicated (for 30 min.) to solubilise the crude mixture. The solution. was filtered and purified in five portions by semi-preparative HPLC [column B(C18), gradient (0,5), (30,35)]. The purest fractions, as judged by analytical HPLC, were combined and lyophilised. A small amount of contaminating material still remained. The purest fractions (20 mg) were dissolved in 10% aqueous acetic acid (4 cm^3) and purified again by semi-preparative HPLC [column B(C18), isocratic at 20% acetonitrile for 30 min.]. Lyophilisation of the appropriate fractions gave the *title compound* as a fluffy, white solid (6 mg).

FABMS m/z 1466 ($M+H^+$), *hrms* found 1463.68671, $C_{66}H_{97}N_{17}O_{19}S_1$ requires 1463.68673 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient A] Rt 13.1 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.01, Thr_1 0.90, Glx_2 2.06, Tyr_1 0.93, Phe_2 1.97, Lys_2 2.01, Arg_2 2.06, Cys_1 N.D. ; Ellman's test was positive.

Mouse Nerve Growth Factor 59-67

H-Arg-Ala-Ser-Asn-Pro-Val-Gln-Ser-Gly-OH

The initial resin had been derivatised with Fmoc-Gly-OH (0.45 mmol/g ; 1.10 g ; 0.5 mmol). Automatic assembly was carried out on the ABI machine using normal double-couple cycles (SA/AE). Monitoring showed that no major drops in a.a. incorporation had occurred. The weight of dried resin obtained was 1.38 g. Some of the resin-bound peptide (0.70 g) was cleaved for 3 hr. in a solution of 5% water, 2.5% thioanisole and 2.5% phenol in TFA (15 cm^3), giving a mixture of crude peptide and scavengers (0.35 g). Ether extraction (2 x 100 cm^3) of this solution

from 10% aqueous acetic acid (15 cm^3) and lyophilisation gave the crude peptide (0.20 g) as almost a single peak (by HPLC). A portion of this crude material (60 mg) was dissolved in 10% aqueous acetic acid (6 cm^3) and purified in three runs by semi-preparative HPLC [column E(C18), gradient (0,2), (2,2), (12,12)]. Combining the purest fractions then lyophilisation gave the *title compound* as a white solid (45 mg).

FABMS m/z 916 (M^+), *hrms* found 915.44090, $C_{36}H_{61}N_{13}O_{15}$ requires 915.44101 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 16.6 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asn_1 0.98, Ser_1 1.62, Glu_1 1.09, Pro_1 0.93, Gly_1 0.97, Ala_1 1.20, Val_1 0.98, Arg_1 0.94.

Mouse Nerve Growth Factor 66-79

H-Ser-Gly-Cys^{SH}-Arg-Gly-Ile-Asp-Ser-Lys-His-Trp-Asn-Ser-Tyr-OH

The initial resin had been derivatised with Fmoc-Tyr(Bu^t)-OH (0.61 mmol/g ; 0.50 g ; 0.31 mmol). Cys and His were incorporated as their trityl derivatives, Asn and Trp were unprotected. The peptide was assembled manually, using double-couple (1 x SA, 1 x AE/HBTU) cycles, except in the following cases : His(Trt), (2 x AE/HBTU) ; Arg(Pmc), (2 x AE/HOBt) ; Ser^{66}, Cys^{68} and $Gly^{67,70}$ were coupled by single SA. The weight of dried resin obtained was 0.96 g. A portion of this (0.50 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% anisole in TFA (15 cm^3), to give 0.22 g (after ether extraction) as almost a single peak product (by HPLC). A sample of crude material (50 mg) was dissolved in 10% aqueous acetic acid (6 cm^3) and purified in five portions by semi-preparative HPLC [column D(C18), gradient (0,2), (25,19), (35,19)]. The purest fractions yielded the *title compound* as a white solid (15 mg) after lyophilisation.

FABMS m/z 1610 ($M+H^+$), *hrms* found 1608.71021, $C_{68}H_{100}N_{22}O_{22}S_1$ requires 1608.71032 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 12.3 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asx_2 1.97, Ser_3 3.06, Gly_2 2.13, $Ile_1$0.99, Tyr_1 1.08, His_1 1.04, Lys_1 0.86, Arg_1 1.03, Cys_1 N.D., Trp_1 N.D.

Conserved Nerve Growth Factor (NGF) 70-74

H-Gly-Ile-Asp-Ser-Lys-OH

The initial resin had been derivatised with Fmoc-Lys(Boc)-OH (0.42 mmol/g ; 0.90 g ; 0.38 mmol). The peptide was assembled manually, using double-couple cycles (SA and AE) resulting in 1.21 g of dried peptide/resin. A portion of this (0.90 g) was cleaved for 2 hr. in a solution of 5% water in TFA (10 cm^3), to yield the crude peptide (0.30 g) as a white solid. On ether extraction (2 x 50 cm^3) from 10% aqueous acetic acid (5 cm^3), a crystalline white solid (0.27 g) was obtained. A portion of this material (40 mg) was dissolved in 10% aqueous acetic acid (4 cm^3) and purified in four portions by semi-preparative HPLC [column C (C18), gradient (0,0), (30,20)]. The purest fractions, as judged by analytical HPLC , were combined and lyophilised to give the *title compound* as a fluffy, white solid (19 mg).

FABMS m/z 519 ($M+H^+$), *hrms* found 519.27786, $C_{21}H_{39}N_6O_9$ requires 519.27783 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient A] Rt 12.5 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.11, Ser_1 0.94, Gly_1 1.07, Ile_1 1.07, Lys_1 0.92.

Conserved NGF 71-79

H-Ile-Asp-Ser-Lys-His-Trp-Asn-Ser-Tyr-OH

The initial resin had been derivatised with Fmoc-Tyr(Bu^t)-OH (0.50 mmol/g ; 1.00 g ; 0.50 mmol). Asn and Trp were unprotected. Histidine was incorporated as Fmoc-His(Trt). The peptide was assembled manually, using single-couple SA cycles, except Asn (1 x AE/HOBt), resulting in 2.06 g of dried peptide/resin. A portion of this (0.83 g) was cleaved for 3 hr. in a solution of 3% water, 3% EDT, 3% anisole, 3% phenol (w/v) and 3% ethyl methyl sulphide (EMS) in TFA (15 cm^3, nitrogen-purged) to yield (after ether extraction) the crude peptide (0.39 g) as a white solid. The material contained two peaks, as detected by analytical HPLC. A portion of the crude peptide (60 mg) was dissolved in 10% aqueous acetic acid (9 cm^3) then acetonitrile (1 cm^3) was added. After sonication and filtration, the filtrate was purified in ten portions by semi-preparative HPLC [column E(C18), gradient (0,2), (30,12), (45,12)]. Lyophilisation of the appropriate fractions yielded the major peak

(10 mg) as a white solid and the minor peak (5 mg) as a brown, sticky oil. The major peak was confirmed as the *title compound* by subsequent analysis.

FABMS m/z 1149 ($M+H^+$), *hrms* found 1148.52501, $C_{52}H_{72}N_{14}O_{16}$ requires 1148.52507 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient D] Rt 19.0 minutes ; Amino acid analysis (4-TSA hydrolysis, 18 hr.) Asx_2 1.85, Ser_2 1.83, Ile_1 1.01, Tyr_1 1.03, His_1 1.01, Trp_1 0.83, Lys_1 1.12 ; The major peak was confirmed by the successful sequencing of the peptide to give correct values for all a.a.'s.

Conserved NGF 95-116 (reduced form)

H-Lys-Gln-Ala-Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{SH}-Val-Cys^{SH}-Val-Leu-Ser-Arg-Lys-Ala-OH

The initial resin had been derivatised with Fmoc-Ala-OH (0.50 mmol/g ; 1.10 g ; 0.55 mmol). Cysteine was incorporated as Fmoc-Cys(Trt), Gln and Trp were unprotected. The peptide was assembled manually, using single-couple SA cycles, except in the following cases : Arg^{103} and Cys^{108} were triple-coupled (2 x AE/BOP, 1 x AE/HOBt :1 x SA, 2 x AE/BOP), Trp^{99} and Thr^{106} were double-coupled (2 x SA), Arg^{100} was double-coupled (2 x AE/BOP) and Lys^{95} Gln^{96} were coupled singly (1 x AE/BOP). Resin desolvation was seen after the coupling of Val^{109}. The weight of dried resin obtained was 3.00 g. A portion of this (0.35 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3, under nitrogen) to yield the crude peptide (0.20 g). On ether extraction, a crystalline white solid (0.17 g) was obtained. A portion of this material (66 mg) was dissolved in glacial acetic acid (3 cm^3) then aqueous 0.1% TFA (27 cm^3) was added. The solution was filtered and purified in six portions by semi-preparative HPLC [column E(C18), gradient (0,5), (30,35)]. The purest fractions, as judged by analytical HPLC , were combined and lyophilised to give the *title compound* as a fluffy, white solid (6 mg).

FABMS m/z 2536.4 ($M+H^+$), *hrms* found 2536.38367, $C_{112}H_{188}N_{35}O_{28}S_2$ requires 2536.38370 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 18.1 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.06, Thr_1 0.93, Ser_1 0.96, Glu_1 1.13, Ala_4 3.98, Val_2 1.99, Ile_2 1.87, Leu_1 1.02, Phe_1 0.95, Lys_2 2.00, Arg_3 2.85, Cys_2 N.D., Trp_1 N.D. ; Ellman's test was positive.

Conserved NGF 95-116 (oxidised form, $Cys^{106,116}$ disulphide-linked)

H-Lys-Gln-Ala-Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-Lys-Ala-OH

A further batch of dried NGF H-95-116-OH resin (1.60 g) was cleaved under the same conditions as above to yield the crude product (0.79 g). A portion of this (0.30 g) was dissolved in a minimum amount of 30% aqueous acetic acid (30 cm^3), passed down a column of G25 Sephadex (Fine, 2.5 x 150 cm) and eluted at 30 cm^3/hr. with the same solvent. UV absorbance was monitored at 254 and 277 nm. Fractions containing peptide were verified with Ehrlich's spray. The purest of these was combined and lyophilised to yield a white solid (0.20 g). This was dissolved in glacial acetic acid (4 cm^3), diluted with aqueous 0.1% TFA (16 cm^3) and purified in three portions by semi-preparative HPLC [column F, gradient (0,5), (45,30)]. Combining the appropriate fractions yielded a further amount of reduced NGF H-95-116-OH (21 mg). A portion of this (15 mg) was dissolved in H_2O (50 cm^3) and stirred continuously. The original reduced peak decreased in height to be replaced by a new, more hydrophilic peak [by analytical HPLC, column A(C18), gradient (0,10), (20,60), Rt changed from 13.6 to 12.4 min.]. After 24 hr., 50% conversion had occurred. Addition of $K_3Fe(CN)_6$ (0.7 cm^3, 2 mg/cm^3 solution in H_2O), led to 80% conversion in a further 2 hr. The entire solution (50 cm^3) was loaded onto a semi-preparative HPLC column through the aqueous pump and the mixture purified [column F, gradient (0,5), (10,5), (40,30)]. Lyophilisation of the appropriate fractions gave the *title compound* as a white solid (4.5 mg).

FABMS m/z 2534.8 ($M+H^+$), *hrms* found 2534.36798, $C_{112}H_{186}N_{35}O_{28}S_2$ requires 2534.36805 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient B] Rt 12.4 min. Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.01, Thr_1 0.97, Ser_1 0.96, Glu_1 1.11, Ala_4 4.05, Val_2 1.99, Ile_2 1.88, Leu_1 1.02, Phe_1 1.09, Lys_2 2.06, Arg_3 3.01, Cys_2 N.D., Trp_1 N.D. ; Ellman's test was negative.

Conserved NGF 100-115, $Cys^{108,110}$ disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-Lys-OH

The initial resin had been derivatised with Fmoc-Lys(Boc)-OH (0.67 mmol/g ; 1.00 g ; 0.67 mmol). Cysteine was incorporated as Fmoc-Cys(Trt). The peptide was assembled automatically, using the normal double-couple cycles. A drop in incorporation occurred over the residues Asp^{105}-Thr^{106}. The weight of dried resin obtained was 2.02 g. All of this was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.73 g) as a white solid. This was purified in nine runs [9 x 80 mg, each portion dissolved in 25% aqueous acetic acid (10 cm^3)] by semi-preparative HPLC [column F, gradient (0,5), (30,40)]. Lyophilisation of the combined fractions gave the reduced peptide (0.13 g) as a white solid.

A sample of this pure, reduced peptide (32 mg) was dissolved in H_2O (32 cm^3), stirred continuously and the reaction followed by analytical HPLC. After 24 hr., the reduced peak showed partial conversion to an oxidised form [column A(C18), gradient G, a change from reduced peak (75%) Rt 22.9 to oxidised peak (25%) Rt 12.7 min. was observed]. To speed the reaction, $K_3Fe(CN)_6$ (0.5 cm^3 of a solution of 2 mg/cm^3 in H_2O) was added. The solution became light yellow and turbid. After a further four hours, the entire solution was filtered to remove a fine, white insoluble precipitate that had formed. The filtrate was loaded onto a semi-preparative HPLC [column F] and the components separated [gradient (0,0), (60,35)]. The appropriate lyophilised fractions gave the *title compound* (6.5 mg) in low yield. A further amount (45 mg) of the pure, reduced peptide was dissolved in H_2O (45 cm^3) and allowed to oxidise over a 48 hr. period. 80% conversion to the oxidised form was seen to occur. The same purification procedure as before [except TFA (45 µl) was added and the gradient was (0,0), (0,5), (60,35)] yielded a further 18.5 mg of the *title compound* as a white solid.

Reduced form

FABMS m/z 1880.3 ($M+H^+$), *hrms* found 1879.02838, $C_{81}H_{142}N_{26}O_{21}S_2$ requires 1879.02842 (< 1 ppm) ; Analytical HPLC [column B(C18), gradient G] Rt 15.0 minutes ; Ellman's test was positive.

Title compound

FABMS m/z 1878.1 ($M+H^+$), *hrms* found 1877.01280, $C_{81}H_{140}N_{26}O_{21}S_2$ requires 1877.01272 (< 1 ppm) ; Analytical HPLC [column B(C18), gradient G] Rt 14.1 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.06, Thr_1 0.92, Ser_1 0.62, Ala_1 1.02, Val_2 1.92, Ile_2 1.89, Leu_1 1.00, Phe_1 0.94, Lys_1 1.00, Arg_3 2.83, Cys_2 N.D. ; Ellman's test was negative.

600 MHz ^{1}H NMR (90% H_2O, 10%D_2O)

δ in ppm

Residue	α-NH	α-CH	β-CH	Others
Arg^{100}	-	4.02	1.90,1.90	γ1.59;δ3.21;NH7.21
Phe^{101}	8.82	4.71	3.03,3.07	Ring7.04,7.12
Ile^{102}	8.35	4.18	1.85	$\gamma CH_2$1.17,1.45;$\gamma CH_3$0.88;δ0.88
Arg^{103}	8.28	4.25	1.72,1.80	γ1.56,1.63;δ3.25;NH7.28
Ile^{104}	8.15	4.08	1.70	$\gamma CH_2$1.08,1.43;$\gamma CH_3$0.80;δ0.80
Asp^{105}	8.64	-	2.80,2.92	
Thr^{106}	8.25	4.35	4.25	γ1.37
Ala^{107}	8.13	4.28	1.21	
Cys^{108}	8.10	4.68	3.10,3.28	
Val^{109}	9.04	4.12	2.22	γ1.00
Cys^{110}	8.33	4.52	3.20,3.50	
Val^{111}	8.11	4.13	2.15	γ0.94
Leu^{112}	8.36	4.40	1.66,1.66	γ1.60;δ0.86,0.93
Ser^{113}	8.24	4.44	3.85,3.85	
Arg^{114}	8.30	4.39	1.76,1.90	γ1.66;δ3.21;NH7.19
Lys^{115}	8.26	4.27	1.75,1.86	γ1.43;δ1.67;ε2.99;εNH_3^+7.54

Conserved NGF 101-115, $Cys^{108,110}$ disulphide-linked

H-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-Lys-OH

The initial resin had been derivatised with Fmoc-Lys(Boc)-OH (0.53 mmol/g ; 0.95 g ; 0.50 mmol). Cysteine was incorporated as Fmoc-Cys(Trt). The peptide was assembled automatically, using the normal double-couple cycles, except Asp^{105} and Thr^{106}, which were triple-coupled. A drop in incorporation occurred over the residues Thr^{106}-Ala^{107}. The weight of dried resin obtained was 2.75 g. All of this was

cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (20 cm^3) to yield the crude peptide (1.05 g) as a white solid. Purification of some of the crude product (0.65 g) was in thirteen runs [13 x 50 mg, each portion dissolved in 10% aqueous acetic acid (5 cm^3)] by semi-preparative HPLC [column F, gradient (0,5), (25,35)]. Lyophilisation of required fractions gave an almost pure product (73 mg) Repurification under the same conditions [except gradient was (0,5), (25,35)] yielded the pure, reduced peptide (31 mg) as a white solid.

Oxidation of this product in H_2O (150 cm^3) led to 80% conversion to the oxidised form [by HPLC; column A(C18), gradient G, a change in Rt from 18.7 to 18.1 minutes was observed]. TFA (0.15 cm^3) was added to the solution, followed by filtration then the filtrate was loaded onto a semi-preparative HPLC [column F] and the components separated [gradient (0,0), (10,0), (55,35)]. The appropriate fractions were lyophilised to give the *title compound* (9 mg) as a white solid.

Reduced form

FABMS m/z 1725.2 ($M+H^+$) ; Analytical HPLC [column A(C18), gradient G] Rt 18.7 minutes ; Ellman's test was positive.

Title compound

FABMS m/z 1722.4, *hrms* found 1720.91159, $C_{75}H_{128}N_{22}O_{20}S_2$ requires 1720.91161 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient G] Rt 18.7 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.98, Thr_1 0.80, Ser_1 0.61, Ala_1 0.97, Val_2 1.95, Ile_2 1.83, Leu_1 1.01, Phe_1 0.93, Lys_1 0.97, Arg_2 1.82, Cys_2 N.D. ; Ellman's test was negative.

Conserved NGF 106-116 (reduced form)

H-Thr-Ala-Cys^{SH}-Val-Cys^{SH}-Val-Leu-Ser-Arg-Lys-Ala-OH

The peptide/resin NGF H-111-116-OH was used in this assembly. Cysteine was incorporated as Fmoc-Cys(Trt). All residues were added using single-couple SA cycles, except Thr^{106} and Cys^{108} (2 x SA). Resin desolvation was apparent after the incorporation of Val^{109}. The weight of dried resin obtained was 1.32 g. A portion of this (0.61 g) was cleaved for 2.75 hr. in a solution of 3% water, 3% EDT and 2% anisole in TFA (15 cm^3) to yield the crude peptide (0.22 g) as a white solid. A portion of this material [40 mg dissolved in 15% aqueous acetic acid (8 cm^3)] was purified in four runs by semi-preparative HPLC [column D(C18), gradient (0,10),

(2,10), (10,20), (25,20)]. The purest fractions were combined and lyophilised to give the *title compound* as a fluffy, white solid (11 mg).

FABMS m/z 1151 ($M+H^+$), *hrms* found 1150.11479, $C_{47}H_{87}N_{15}O_{14}S_2$ requires 1150.11479 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient C] Rt 11.6 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Thr_1 0.97, Ser_1 0.94, Ala_2 2.11, Val_2 1.97, Leu_1 1.00, Lys_1 0.99, Arg_1 1.06, Cys_2 N.D. ; Ellman's test was positive.

Conserved NGF 106-116 (oxidised form, $Cys^{108,110}$ disulphide-linked)

H-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-Lys-Ala-OH

The remainder of the crude material (0.17 g) from NGF H-106-116-OH (reduced form) was purified in two lots by semi-preparative HPLC [column F, gradient (0,10), (30,25)], to yield a further 43 mg of pure reduced product. 40 mg of this was dissolved in H_2O (200 cm^3) and stirred continuously over six days. After this time, analytical HPLC [column C, gradient (0,10), (25,50)] showed a change in Rt from 15.9 to 16.3 minutes, to give a main peak (75% by peak height) and two later running peaks (X and Y), where X = 12%, Rt 16.9 min. ; Y = 15%, Rt 19.4 min. The entire solution was therefore pumped onto a semi-preparative HPLC [column F, gradient (0,0), (10,0), (40,25), (45,25), (55,30), (65,30)]. Lyophilisation of the separated peaks gave the major peak (17.5 mg) and peaks X (5 mg) and Y (3.5 mg). Subsequent analysis showed that the major peak represented the *title compound.* Peaks X and Y appeared to represent dimeric forms (FABMS m/z 2296.0).

FABMS m/z 1148.5 (M^+), *hrms* found 1147.99921, $C_{47}H_{85}N_{15}O_{14}S_2$ requires 1147.99912 (< 1 ppm) ; Analytical HPLC [column C, gradient F] Rt 15.3 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Thr_1 0.97, Ser_1 0.94, Ala_2 2.11, Val_2 1.97, Leu_1 1.00, Lys_1 0.99, Arg_1 1.06, Cys_2 N.D. ; Ellman's test was negative.

Conserved NGF 111-116

H-Val-Leu-Ser-Arg-Lys-Ala-OH

The initial resin had been derivatised with Fmoc-Ala-OH (0.45 mmol/g ; 1.50 g ; 0.68 mmol). The peptide was assembled manually, using single-couple SA cycles. Half of the Fmoc-protected resin was dried down to give 0.58 g (the assembly was continued to form NGF H-106-116-OH using the other half). A portion of this dried resin (0.25 g) was cleaved for 2 hr. in a solution of 5% water and 5% thioanisole in TFA (10 cm^3) to yield the crude peptide (0.11 g) as a white solid. The soluble crude product [45 mg dissolved in 10% aqueous acetic acid (6 cm^3)] was purified in three portions by semi-preparative HPLC [column D(C18), gradient (0,5), (15,25)]. The purest fractions, as judged by analytical HPLC , were combined and lyophilised to give the *title compound* as a dense, crystalline solid (27 mg).

FABMS m/z 673 ($M+H^+$), *hrms* found 672.42831, $C_{29}H_{56}N_{10}O_8$ requires 672.42826 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient C] Rt 9.1 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Ser_1 0.96, Ala_1 1.08, Val_1 0.95, Leu_1 0.98, Lys_1 0.96, Arg_1 1.06.

Conserved NGF 100-115 amide, $Cys^{108,110}$ disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-Lys-NH_2

The initial resin used had the dibenzosuberyl linker to provide an amide on cleavage (0.45 mmol/g ; 0.50 g ; 0.23 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t). The peptide was assembled automatically, using the normal double-couple cycles, except for residues Arg^{100}-Ala^{107}, which were triple-coupled. A drop in incorporation again occurred over the residues Ile^{104}-Ala^{107}. The weight of dried resin obtained was 1.18 g. A portion of this (0.60 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.30 g) as a white solid. A portion of this material (0.10 g) was placed in a silanised flask and was completely dissolved in 95% aqueous TFE (5 cm^3) by stirring under nitrogen. Excess tributylphosphine (TBP, 0.3 cm^3) was added under nitrogen. Stirring was continued under N_2 for 2 hr., after which time the entire solution was evaporated *in vacuo* to a colourless oil and the crude, reduced peptide precipitated by the addition

of excess ether. Filtration gave a solid which was immediately taken up in 10% aqueous acetic acid (25 cm^3), extracted with ether (2 x 150 cm^3) then loaded directly onto a semi-preparative HPLC [column F] and the components separated [gradient (0,20), (10,20), (40,40)]. Lyophilisation of the required fractions gave the pure, reduced peptide (16 mg) as a white solid. This was dissolved in H_2O (32 cm^3) and oxidised over 36 hr. with stirring, to form the pure, oxidised peptide. No further purification was required. Lyophilisation of the entire solution gave the *title compound* (15 mg) as a white solid.

Reduced form

FABMS m/z 1880.8, *hrms* found 1879.05221, $C_{81}H_{144}N_{27}O_{20}S_2$ requires 1879.05216 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 14.6 minutes (c.f. for di-SBu^t peptide, Rt 17.6 min.) ; Ellman's test was positive.

Title compound

FABMS m/z 1877.6 ($M+H^+$), *hrms* found 1877.03655, $C_{81}H_{142}N_{27}O_{20}S_2$ requires 1877.03651 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 14.1 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.80, Thr_1 0.87, Ser_1 0.78, Ala_1 1.00, Val_2 2.11, Ile_2 1.85, Leu_1 1.04, Phe_1 0.94, Lys_1 1.05, Arg_3 3.15, Cys_2 N.D. : Ellman's test was negative.

Conserved NGF 100-115 amide, $Cys^{108,115}$ disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-Lys-NH_2

The initial resin used had the dibenzosuberyl linker to provide an amide on cleavage (0.45 mmol/g ; 0.50 g ; 0.23 mmol). Cysteine was incorporated as Fmoc-Cys(Trt). The peptide was assembled automatically, using the normal double-couple cycles, except for the sequence Arg^{100}-Ala^{107}, in which each residue was triple-coupled. A drop in incorporation occurred over the residues Ile^{104}-Ala^{107}. The weight of dried resin obtained was 1.14 g. A portion of this (0.60 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.23 g) as a white solid. The analytical HPLC trace of this crude was poor. A sample of this crude (90 mg) was purified by semi-preparative HPLC [column F, gradient (0,10), (40,40)] to give the pure, reduced peptide in low yield (4.5 mg) and a deletion product (7 mg), which analysis showed to lack the residues $R^{100}FI^{102}$. The remainder (0.14 g) was stirred in 10% DMSO/TFA (5 cm^3) for 1 hr., evaporated *in*

vacuo to near dryness and a white solid (0.35 g), precipitated by the addition of excess ice-cold ether. Filtration and drying gave the crude, oxidised product. Purification of this was by semi-preparative HPLC [column F, gradient (0,20), (40,40)]. Lyophilisation of appropriate fractions gave the *title compound* (6 mg) as a white solid.

Reduced form

FABMS m/z 1881.0 ($M+H^+$), *hrms* found 1879.05217, $C_{81}H_{144}N_{27}O_{20}S_2$ requires 1879.05216 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 17.4 minutes ; Ellman's test was positive.

Title compound

FABMS m/z 1877.9 ($M+H^+$), *hrms* found 1877.03655, $C_{81}H_{142}N_{27}O_{20}S_2$ requires 1877.03651 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 16.9 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.84, Thr_1 0.86, Ser_1 0.70, Ala_1 1.29, Val_2 2.29, Ile_2 1.87, Leu_1 1.11, Phe_1 0.96, Lys_1 1.16, Arg_3 2.91, Cys_2 N.D. : Ellman's test was negative.

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-heptyliminodibenzocyclo-heptadiene

Resin [Compound 1 (ref. section 2.2.1, p. 45), 0.62 mmol/g, 0.80 g] was swollen in DCM (16 cm^3) along with heptylamine (1.29 cm^3, 1.00 g, 8.70 mmol), cooled in an ice/salt bath and stirred under an atmosphere of dry nitrogen. A solution of 1.0M titanium (IV) chloride in DCM (1.50 cm^3, 1.50 mmol) was added dropwise then the mixture heated under reflux for three hours. After cooling for one hour under N_2, the resin was filtered off, washed with copious amounts of DCM and dried *in vacuo* to yield the *title compound* (Compound 2, 0.88 g) as a pale-yellow solid.

Nitrogen analysis found : N, 0.79% ; expected for 100% loading, 0.81% ; ν_{max} (KBr disc) : 3080-2840 (CH), 1600 cm^{-1} (C=C) [the original band at 1640 cm^{-1} (C=O) had completely disappeared]

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-heptylaminodibenzocyclo-heptadiene

Resin [Compound 2 (ref. p.45), 0.76 g] was stirred in THF (20 cm^3), $LiBH_4$ added (0.10 g, 4.55 mmol) and the mixture heated under reflux for two hours under an atmosphere of dry nitrogen. The mixture was then cooled in an ice/salt bath, methanol (5 cm^3) added then acetone (5 cm^3) was added dropwise. The resin was filtered off and washed extensively with methanol, water, then methanol again. Drying *in vacuo* gave the *title compound* (Compound 3, 0.71 g) as a white solid.
ν_{max} (KBr disc) : 3420 (NH), 3080-2840 (CH), 1600 cm^{-1} (C=C)

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(N^{α}-fluorenylmethoxy-carbonyllysyl(N^{ε}-*tert*-butyloxycarbonyl)heptylaminodibenzocyclo-heptadiene

Fmoc-Lys(Boc)-OH (2.30 g, 5.00 mmol) was dissolved in a minimum amount of DMF/dioxan (1:1, 4.0 cm^3) then DIC (0.4 cm^3, 2.5 mmol) was added. After 20 min. stirring, a portion of resin [Compound 3 (ref. p.45), 0.35 g, 0.25 mmol] was added and the slurry sonicated at 20°c for twenty-two hours. After this time, the resin was washed with excess DMF and a small sample (5 mg) dried with ether then *in vacuo* overnight. The functionality of the resin by UV monitoring was 0.29 mmol/g (total ~0.1 mmol). The loading reaction was repeated for a further sixteen hours using all of the resin to give the *title compound* (Compound 4, 0.36 g) as a pale-yellow solid with a functionality of 0.40 mmol/g (total ~0.14 mmol).

ν_{max} (KBr disc) : 3410 (NH), 3080-2840 (CH), 1730 (C=O), 1640 (C=O), 1600 cm^{-1} (C=C).

Conserved NGF 100-115 heptylamide, $Cys^{108,110}$ (SBu^t)

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{SB}-Val-Cys^{SB}-Val-Leu-Ser-Arg-Lys-NH-$(CH_2)_6CH_3$

The resin [Compound 4 (ref. p.45)] used was to provide a heptylamide on cleavage (0.40 mmol/g ; 0.36 g ; 0.14 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t).

The peptide was assembled automatically, using triple-couple cycles for each residue. The weight of dried resin obtained was 0.62 g. A portion of this (0.31 g) was cleaved for 4 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.11 g) as a white solid. A portion of this material (50 mg) was dissolved in 25% aqueous acetic acid (12 cm^3), filtered and purified by semi-preparative HPLC [column F, gradient (0,20), (45,60)]. Lyophilisation of the purest fractions gave the *title compound* (10 mg) as a fluffy, white solid.

FABMS m/z 2154.7 ($M+H^+$), *hrms* found 2154.23442, $C_{96}H_{174}N_{27}O_{20}S_4$ requires 2154.23440 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 20.1 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.84, Thr_1 0.73, Ser_1 0.76, Ala_1 1.00, Val_2 2.17, Ile_2 2.13, Leu_1 1.08, Phe_1 0.87, Lys_1 1.09, Arg_3 3.10, Cys_2 N.D. ; Ellman's test was negative.

Conserved NGF 100-115 heptylamide, $Cys^{108,110}$ disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-Lys-NH-$(CH_2)_6CH_3$

The remainder of the crude from the above assembly (60 mg) was extracted from 20% aqueous acetic acid (30 cm^3) with ether and lyophilised to yield a more soluble product (45 mg). A portion of this (26 mg) was dissolved in 95% aqueous TFE (4 cm^3), TBP (0.1 cm^3) added then stirring continued under nitrogen for 3 hr. Evaporation *in vacuo* followed by the addition of ether gave the crude, reduced peptide (20 mg). This was purified by semi-preparative HPLC [column F, gradient (0,15), (45,45)] and the purest fractions lyophilised to gave the pure, reduced peptide (5 mg) as a white solid. This was dissolved in H_2O (30 cm^3) and air-oxidised, with stirring, over five days to form the pure, oxidised peptide. No further purification was required. Lyophilisation of the entire solution gave the *title compound* (3.5 mg) as a white solid.

FABMS m/z 1975.5, *hrms* found 1975.14609, $C_{88}H_{156}N_{27}O_{20}S_2$ requires 1975.14614 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 15.0 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.84, Thr_1 0.73, Ser_1 0.76, Ala_1 1.00, Val_2 2.17, Ile_2 2.13, Leu_1 1.08, Phe_1 0.87, Lys_1 1.09, Arg_3 3.10, Cys_2 N.D. ; Ellman's test was negative.

Conserved NGF 100-114, $Cys^{108,114}$ disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Val-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.60 mmol/g ; 1.00 g ; 0.60 mmol). Cysteine was incorporated as Fmoc-Cys(Trt). The peptide was assembled automatically, using the normal double-couple cycles, except Asp^{105} and Thr^{106}, which were triple-coupled. The weight of dried resin obtained was 2.37 g. All of this was cleaved for 3.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (20 cm^3) to yield the crude peptide (0.82 g) as an insoluble white solid. An attempt was made to purify some of the crude product [50 mg, dissolved in 20% aqueous acetic acid (10 cm^3)] by semi-preparative HPLC, which led to poor recovery of the pure, reduced product (2 mg). The remainder of the crude peptide was ether extracted from 20% aqueous acetic acid to yield a more soluble white solid (0.52 g). A sample of this (0.40 g) was dissolved in 20% DMSO/TFA (25 cm^3), with anisole added (0.1 cm^3) and stirred for 2 hr. at RT. The solution was evaporated *in vacuo* to near dryness (some DMSO remained) and a white solid (0.30 g) formed on the addition of excess ice-cold ether. A change in Rt from 24.0 to 23.3 minutes for the major peak was seen on analytical HPLC [column A(C18), gradient E]. Purification of all of the oxidised, crude product was in six runs [6 x 50 mg, each portion dissolved in 20% aqueous acetic acid (10 cm^3)] by semi-preparative HPLC [column F, gradient (0,10), (45,30), (55,30)]. Lyophilisation of the required fractions gave the *title compound* (46 mg) as a white solid.

Reduced form

FABMS m/z 1753.3 ($M+H^+$) ; Analytical HPLC [column A(C18), gradient E] Rt 24.0 minutes ; Ellman's test was positive.

Title compound

FABMS m/z 1751.7 ($M+H^+$), *hrms* found 1749.92656, $C_{75}H_{129}N_{24}O_{20}S_2$ requires 1749.92654 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 20.0 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.12, Thr_1 0.69, Ser_1 0.63, Ala_1 0.98, Val_2 1.95, Ile_2 2.20, Leu_1 1.00, Phe_1 1.11, Arg_3 3.16, Cys_2 N.D. ; Ellman's test was negative.

600 MHz ^{1}H NMR (d_6-DMSO)

δ in ppm

Residue	α-NH	α-CH	β-CH	Others
Arg^{100}	-	3.74	1.68	γ1.49;δ3.05;NH7.64
Phe^{101}	8.54	4.69	2.77,3.00	Ring7.26
Ile^{102}	8.24	4.22	1.72	$\gamma CH_2$1.06,1.41;$\gamma CH_3$0.80;δ0.80
Arg^{103}	8.04	4.20	1.75	γ1.59;δ3.08;NH7.51
Ile^{104}	8.58	4.28	1.68	$\gamma CH_2$1.06,1.49;$\gamma CH_3$0.85;δ0.85
Asp^{105}	8.48	4.65	2.52,2.70	
Thr^{106}	7.58	4.18	3.98	γ1.01
Ala^{107}	7.87	4.32	1.19	
Cys^{108}	8.24	4.55	2.51,2.62	
Val^{109}	8.81	4.01	2.09	γ0.89
Cys^{110}	7.34	4.63	2.83,3.14	
Val^{111}	8.03	4.34	1.95	γ0.84
Leu^{112}	7.93	4.22	1.70	γ1.70;δ0.80
Ser^{113}	7.81	4.28	3.54	
Arg^{114}	8.00	4.18	1.75	γ1.49;δ3.08;NH7.51

Conserved NGF 100-110 (reduced form)

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{SH}-Val-Cys^{SH}-OH

The initial resin had been derivatised with Fmoc-Cys(Trt)-OH (0.32 mmol/g ; 0.80 g ; 0.26 mmol). Cysteine was incorporated as Fmoc-Cys(Trt). Automatic assembly was carried out on the ABI machine, using the normal double-couple cycles and monitoring showed that no drops in incorporation had occurred. The weight of dried resin obtained was 1.61 g. A portion of this (0.78 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3, under nitrogen) to yield an insoluble crude product, which on ether extraction (2 x 150 cm^3) from 20% aqueous acetic acid (50 cm^3), yielded a more soluble crude product (0.18 g). A portion of this material (60 mg) was purified in six runs by semi-preparative HPLC [column D(C18), gradient (0,15), (2,15), (20,25)]. The purest fractions were combined and lyophilised to give the *title compound* as a fluffy, white solid (10 mg).

FABMS m/z 1297 ($M+H^+$), *hrms* found 1295.64790, $C_{55}H_{93}N_{17}O_{15}S_2$ requires 1295.64785 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient B] Rt 14.8 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.04, Thr_1 0.97, Ala_1 1.14, Val_1 1.00, Ile_2 1.85, Phe_1 0.96, Arg_2 1.95, Cys_2 N.D. ; Ellman's test was positive.

Conserved NGF 100-110 (oxidised form, $Cys^{108,110}$ disulphide-linked)

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-OH

A portion of the crude product from NGF 100-110 reduced (0.80 g) was purified in by semi-preparative HPLC [column F, gradient (0,10), (35,30)], with poor recovery of the pure, reduced product (7 mg). The remainder of the resin (0.83 g) was cleaved under the same conditions as above to yield a further amount of crude peptide (0.25g). All of this material [dissolved in 15% aqueous acetic acid (35 cm^3)] was purified in six portions [column F, gradient (0,10), (35,30)] to give the pure, reduced peptide (50 mg). The combined pure, reduced peptides (57 mg) were dissolved in H_2O (250 cm^3) and stirred continuously for six days. After this time, analytical HPLC [column A(C18), gradient (0,10), (25,50)] showed a change in Rt from 22.9 to 22.1 minutes for the main peak. TFA (0.25 cm^3) was added, the entire solution filtered, loaded onto a semi-preparative HPLC [column F] and the components separated [gradient (0,0), (5,0), (55,30)]. The lyophilised main peak fractions gave the *title compound* (26 mg), with two trace hydrophobic impurities appearing as dimeric forms (3 mg, m/z 2588.8 ; 2 mg, m/z 2591.0).

FABMS m/z 1294.6 ($M+H^+$), *hrms* found 1293.631201, $C_{55}H_{91}N_{17}O_{15}S_2$ requires 1293.631195 (< 1 ppm) ; Analytical HPLC [column A(C18), gradient F] Rt 16.7 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.04, Thr_1 0.97, Ala_1 1.14, Val_1 1.00, Ile_2 1.85, Phe_1 0.96, Arg_2 1.95, Cys_2 N.D. ; Ellman's test was positive. N.M.R. results are included below.

600 MHz ^{1}H NMR (90% H_2O, 10%D_2O)

δ in ppm

Residue	α-NH	α-CH	β-CH	Others
Arg^{100}	-	4.01	1.89	γ1.58;δ3.20;NH7.22
Phe^{101}	8.82	4.71	3.05	Ring7.34
Ile^{102}	8.31	4.16	1.86	$\gamma CH_2$1.18,1.45;$\gamma CH_3$0.90;δ0.85
Arg^{103}	8.28	4.24	1.72,1.79	γ1.55,1.62;δ3.23;NH7.26
Ile^{104}	8.16	4.06	1.72	$\gamma CH_2$1.08,1.40;$\gamma CH_3$0.80;δ0.80
Asp^{105}	8.64	-	2.84,2.97	
Thr^{106}	8.26	4.35	4.16	γ1.37
Ala^{107}	8.13	4.29	1.19	
Cys^{108}	8.16	4.51	3.07,3.28	
Val^{109}	8.87	4.07	2.10	γ0.98
Cys^{110}	8.24	-	3.10,3.33	

NGF 100-116 Ser^{107}, Arg^{116}, $Cys^{108,110}$ (Acm)

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ser-Cys^{SA}-Val-Cys^{SA}-Val-Leu-Ser-Arg-Lys-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.51 mmol/g ; 1.00 g ; 0.51 mmol). Cysteine was incorporated as Fmoc-Cys(Acm). The peptide was assembled automatically, using the normal double-couple cycles. The weight of dried resin obtained was 2.77 g. A portion of this (1.50 g) was cleaved for 2 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (20 cm^3) to yield the crude peptide (0.74 g) as an almost single product (by HPLC). The crude peptide was ether extracted from 10% aqueous acetic acid to yield a white solid (0.54 g). Purification of crude product (0.50 g) was in five runs [each portion dissolved in 10% aqueous acetic acid (4 cm^3)] by semi-preparative HPLC [column F, gradient (0,5), (25,25), (35,25)]. Lyophilisation of required fractions gave the *title compound* (57 mg) as a white solid, with a further portion of almost pure product (57 mg, 90% pure by HPLC).

FABMS m/z 2196.0 ($M+H^+$), $C_{93}H_{164}N_{32}O_{25}S_2$ requires 2194.6 ; Analytical HPLC [column A(C18), gradient E] Rt 19.3 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.08, Thr_1 0.84, Ser_2 2.12, Val_2 2.17, Ile_2 2.11, Leu_1 1.10, Phe_1 1.04, Lys_1 1.07, Arg_4 3.74, Cys_2 N.D. ; Ellman's test was negative.

NGF 100-116 Ser^{107}, Arg^{116}, $Cys^{108,110}$ disulphide-linked.

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ser-Cys^{α}-Val-Cys^{α}-Val-Leu-Ser-Arg-Lys-Arg-OH

A sample of the pure, di-Acm protected peptide (50 mg, 0.03 mmol) and silver triflate (AgOTf, 0.15 g, 0.6 mmol) were dissolved in TFA (4 cm^3) and stirred at 0°c for 90 min. in the absence of light. The solution was then evaporated to dryness *in vacuo* and a fine, white solid precipitated on the addition of excess ether. Collection by filtration and drying gave the silver salt of the peptide as a white powder (70 mg). By HPLC [column E, gradient. F], the original peak due to the di-Acm compound, had been replaced by a later-running, broad peak [Rt changed from 16.3 to 17.0 min.]. Dithiothreitol (DTT, 0.45 g, 3.0 mmol) was dissolved in glacial acetic acid (2 cm^3) then H_2O (2 cm^3) added. The silver salt of the peptide (65 mg) was added to this with vigorous stirring, which was continued for 2 hr. in the absence of light. After this time, the milky, yellow suspension that had formed was centrifuged (6000 x g, 30 min.) to pellet an insoluble, yellow precipitate. Analytical HPLC [column A(C18), gradient E] of the filtrate showed that a single peak had formed, with a Rt of 19.2 minutes. The filtrate (4 cm^3) was diluted to 30% aqueous acetic acid [H_2O (2 cm^3) added] then passed down a Sephadex G50 column (Fine, 1.5 x 35 cm), eluting at 15cm^3/hr. with the same solvent. Fractions containing peptide were identified using Sakaguchi's test, then examined by HPLC. The purest, reduced fractions were combined and lyophilised to yield the reduced form of NGF H-100-116-OH, Ser^{107}, Arg^{116} (21 mg) as a white solid [m/z 2053.7 ($M+H^+$)].

A portion of the pure, reduced peptide (15 mg) was dissolved in H_2O (50 cm^3) and allowed to air oxidise, with constant stirring over three days. By HPLC [column A(C18), gradient E], a change in Rt from 17.5 to 17.0 min. was observed. No further purification was required (conversion > 99%). Lyophilisation of the entire solution gave the *title compound* (14 mg) as a fluffy, white solid.

FABMS m/z 2050.7 (M^+), $C_{87}H_{152}N_{30}O_{23}S_2$ requires 2050.5 ; Analytical HPLC [column C, gradient E] Rt 13.1 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.08, Thr_1 0.84, Ser_2 2.12, Val_2 2.17, Ile_2 2.11, Leu_1 1.10, Phe_1 1.04, Lys_1 1.07, Arg_4 3.74, Cys_2 N.D. ; Ellman's test was negative.

NGF 100-116 $Gly^{111-113}$, $Cys^{108,110}$ (Acm)

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ser-Cys^{SA}-Val-Cys^{SA}-Gly-Gly-Gly-Arg-Lys-Ala-OH

The initial resin had been derivatised with Fmoc-Ala-OH (0.58 mmol/g ; 0.50 g ; 0.29 mmol). Cysteine was incorporated as Fmoc-Cys(Acm). The peptide was assembled automatically, using the normal double-couple cycles. No drops in incorporation were observed. The weight of dried resin obtained was 0.89 g. A portion of this (0.70 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.23 g) as essentially a single product (by HPLC). Purification of crude product (0.22 g) was in two runs by semi-preparative HPLC [column F, gradient (0,10), (30,30)]. Lyophilisation of the appropriate fractions gave the *title compound* (100 mg) as a fluffy, white solid.

FABMS m/z 1966.2 ($M+H^+$) ; *hrms* found 1965.02749 $C_{82}H_{142}N_{29}O_{23}S_2$ requires 1965.02755 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 15.8 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.05, Thr_1 0.97, Gly_3 3.28, Ala_2 2.21, Val_1 1.12, Ile_2 1.86, Phe_1 0.88, Lys_1 1.14, Arg_3 2.94, Cys_2 N.D. ; Ellman's test was negative.

NGF 100-116 $Gly^{111-113}$, $Cys^{108,110}$ disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ser-Cys^{Ox}-Val-Cys^{Ox}-Gly-Gly-Gly-Arg-Lys-Ala-OH

The pure, di-Acm peptide was oxidised using essentially the same procedures as used for NGF H-100-116-OH, Ser^{107}, Arg^{116}.
A sample of the di-Acm peptide (50 mg) was treated with AgOTf (0.15 g) in TFA (5 cm^3) to yield the silver salt (73 mg). This was then reduced with DTT (0.45 g) in 50% aqueous acetic acid for 2 hr. Centrifugation, gel filtration of the filtrate (as before) then lyophilisation gave the pure, reduced peptide (20 mg).
A sample of this (15 mg) was dissolved in H_2O (50 cm^3) and allowed to air oxidise, with constant stirring. The oxidation proceeded slowly, HPLC [column C, gradient H] showing a change in Rt from 16.5 to 15.9 min. After ten days, the entire solution was lyophilised to give the *title compound* (14 mg) as a fluffy, white solid.

FABMS m/z 1822.3 ($M+H^+$), $C_{76}H_{129}N_{27}O_{21}S_2$ requires 1821.0 ; Analytical HPLC [column C, gradient H] Rt 15.9 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.05, Thr_1 0.97, Gly_3 3.28, Ala_2 2.21, Val_1 1.12, Ile_2 1.86, Phe_1 0.88, Lys_1 1.14, Arg_3 2.94, Cys_2 N.D. ; Ellman's test was negative.

NGF 100-116 $Gly^{102,104-106,109,111-113}$, $Cys^{108,110}$ (SBu^t)

H-Arg-Phe-Gly-Arg-Gly-Gly-Gly-Ala-Cys^{SB}-Gly-Cys^{SB}-Gly-Gly-Gly-Arg-Lys-Ala-OH

The initial resin had been derivatised with Fmoc-Ala-OH (0.58 mmol/g ; 0.50 g ; 0.29 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t). The peptide was assembled automatically, using the normal double-couple cycles. No drops in incorporation were observed. The weight of dried resin obtained was 0.92 g. A portion of this (0.60 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.2 g) as essentially a single product (by HPLC). Purification of all of the crude product was in two runs by semi-preparative HPLC [column F, gradient (0,10), (30,40)]. Lyophilisation of the appropriate fractions gave the *title compound* (94 mg) as a fluffy, white solid.

FABMS m/z 1744.3 ($M+H^+$), $C_{69}H_{119}N_{27}O_{18}S_2$ requires 1743.1 ; Analytical HPLC [column C, gradient H] Rt 21.8 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Gly_8 7.97, Ala_2 2.03, Phe_1 0.98, Lys_1 1.03, Arg_3 2.92, Cys_2 N.D. ; Ellman's test was negative.

NGF 100-116 $Gly^{102,104-106,109,111-113}$, $Cys^{108,110}$ disulphide-linked

H-Arg-Phe-Gly-Arg-Gly-Gly-Gly-Ala-Cys^{Ox}-Gly-Cys^{Ox}-Gly-Gly-Gly-Arg-Lys-Ala-OH

A sample of pure, Cys-protected peptide from the above synthesis (67 mg) was placed in a silanised flask and was completely dissolved in 95% aqueous TFE (4 cm^3) by stirring under nitrogen. Excess tributylphosphine (TBP, 0.1 cm^3) was added under nitrogen. Stirring was continued under N_2 for 2 hr. Analytical HPLC [column E, gradient. H] showed a change in the Rt of the main peak from 12.9 to 7.5 minutes. After this time the entire solution was evaporated *in vacuo* to a colourless

oil, which was taken up by stirring in 10% aqueous acetic acid (30 cm^3) for 20 minutes. This solution was extracted with ether (3 x 100 cm^3) then lyophilised to yield the reduced peptide (51 mg). Purification of crude product was by semi-preparative HPLC [column F, gradient (0,10), (40,20)]. Lyophilisation of the appropriate fractions gave the pure, reduced peptide (20 mg) [FABMS m/z 1567.9] A sample of this (15 mg) was dissolved in H_2O (50 cm^3) and allowed to air oxidise, with constant stirring, over 36 hr. A change in Rt from 12.0 to 11.3 min. was observed by HPLC [column. E, gradient (0,10), (30,30)] over this period. After complete conversion to the oxidised form, the entire solution was lyophilised to give the *title compound* (8.5 mg) as a fluffy, white solid.

FABMS m/z 1564.5 (M^+), $C_{61}H_{101}N_{27}O_{18}S_2$ requires 1564.8 ; Analytical HPLC [column C, gradient (0,10), (30,30)] Rt 11.3 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Gly_8 7.97, Ala_2 2.03, Phe_1 0.98, Lys_1 1.03, Arg_3 2.92, Cys_2 N.D. Ellman's test was negative.

NT-3 100-116, $Cys^{108,110}$ disulphide-linked

H-Arg-Trp-Ile-Arg-Ile-Asp-Thr-Ser-Cys^{ox}-Val-Cys^{ox}-Ala-Leu-Ser-Arg-Lys-Ile-OH

The initial resin had been derivatised with Fmoc-Ile-OH (0.51 mmol/g ; 0.60 g ; 0.31 mmol). Cysteine was incorporated as Fmoc-Cys(Trt). The peptide was assembled automatically, using the triple-couple cycles for every residue. The weight of dried resin obtained was 1.91 g. A portion of this (0.97 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.45 g) as a white solid. The analytical HPLC trace of this crude showed three major peaks (named X, Y and Z). A portion of the crude (0.24 g) was purified in three runs by semi-preparative HPLC [column F, gradient (0,10), (60,50)], fractions containing each of the three peaks were lyophilised separately and analysed by AAA and HPLC (column C., gradient H). Peak X (Rt 12.0 min., 48 mg) was shown to lack the residues $R^{100}WIR^{103}$, peak Y (Rt 13.1 min., 22 mg) was the pure, reduced peptide and peak Z (Rt 15.8 min., 14 mg) had the correct AAA but the FABMS was +265 (corresponding to a Pmc group). The reduced peptide (20 mg) was dissolved in H_2O (50 cm^3), stirred for 72 hr., then the solution was lyophilised to give the oxidised peptide (18 mg). This was purified further by semi-preparative

HPLC [column F, gradient (0,20), (40,40)] and lyophilisation of appropriate fractions gave the *title compound* (7.5 mg) as a white solid.

Reduced form

FABMS m/z 2021.3 ($M+H^+$), *hrms* found 2020.09480, $C_{97}H_{151}N_{28}O_{23}S_2$ requires 2020.09475 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 12.2 minutes ; Ellman's test was positive.

Title compound

FABMS m/z 2018.7, *hrms* found 2018.07913, $C_{97}H_{149}N_{28}O_{23}S_2$ requires 2018.07910 (< 1 ppm) ; Analytical HPLC [column C, gradient H] Rt 11.3 minutes ; Amino acid analysis (4-TSA, 18 hr.) Asp_1 1.11, Thr_1 0.82, Ser_2 1.47, Ala_1 1.15, Val_1 1.15, Ile_3 3.11, Leu_1 1.13, Lys_1 1.19, Trp_1 0.78, Arg_3 2.97, Cys_2 N.D. : Ellman's test was negative.

Human NGF 95-120 $Cys^{108}(SBu^t)$, $Cys^{110}(Acm)$.

H-Lys-Gln-Ala-Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{SB}-Val-Cys^{SA}-Val-Leu-Ser-Arg-Lys-Ala-Val-Arg-Arg-Ala-OH

The initial resin had been derivatised with Fmoc-Ala-OH (0.30 mmol/g ; 1.50 g ; 0.45 mmol). Cys was incorporated as $Cys^{108}(SBu^t)$ and $Cys^{110}(Acm)$. Gln and Trp were unprotected. The peptide was assembled automatically with the normal cycles except Arg^{100}, Ile^{102}, Thr^{106} and Val^{111}, which were triple-coupled. Monitoring showed no major drops in incorporation. The DMF-wet peptide/resin (6.6 g) was left Fmoc-protected and divided into two portions. One half was dried with ether to give the required resin for the above peptide (1.30 g), the remainder was used in the synthesis of NGF H-50-70-$(Gly)_5$-94-120-OH. A portion of this (1.20 g) was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (15 cm^3) to yield a mixture of crude peptide and scavengers (0.51 g). Part of this product was dissolved in a minimum amount of 10% aqueous acetic acid (25 cm^3), passed down a column of G50 Sephadex (Superfine, 2.5 x 150 cm) and eluted at 30 cm^3/hr. with the same solvent. UV absorbance was monitored at 254 and 277 nm. Fractions containing peptide were verified with Ehrlich's spray. The purest of these was combined and lyophilised to yield a white solid (0.18 g). This was purified in four portions by semi-preparative HPLC [column F, gradient (0,10), (10,10), (30,35), (45,35)].

Combining the appropriate fractions yielded the *title compound* as a fluffy, white solid (66 mg) after lyophilisation.

FABMS m/z 3177.7 ($M+H^+$), $C_{139}H_{238}N_{46}O_{33}S_3$ requires 3177.9 ; Analytical HPLC [column A(C18), gradient F] Rt 16.3 minutes ; Amino acid analysis (HCl hydrolysis, 20 hr.) Asp_1 1.08, Thr_1 1.02, Ser_1 0.98, Ala_5 5.19, Val_3 3.18, Ile_2 1.85, Leu_1 1.10, Phe_1 0.94, Lys_2 1.99, Arg_5 5.22, Cys_2 1.97, Trp_1 N.D. ; Ellman's test was negative.

Human NGF 95-120 Cys^{110}(Acm).

H-Lys-Gln-Ala-Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{SH}-Val-Cys^{SA}-Val-Leu-Ser-Arg-Lys-Ala-Val-Arg-Arg-Ala-OH

A portion of the crude material from the above synthesis (0.16 g) was dissolved in 95% aqueous trifluoroethanol (TFE, 12cm^3) and stirred until complete dissolution had occurred (30 min.). Excess tributylphosphine (TBP, 0.15 cm^3) was added under nitrogen. Stirring was continued under N_2 and the course of the reaction followed by HPLC. After 10 minutes, the Rt of the main peak had changed from 12.8 to 11.6 min. [column A(C18), gradient C]. After one hour, the entire solution was evaporated *in vacuo* to dryness and the reduced peptide was precipitated as a white solid by the addition of ice-cold ether (100 cm^3). Isolation by filtration and further ether washes removed most of the remaining TBP to give the crude reduced product (0.14 g). This was purified in three portions by semi-preparative HPLC [column F, gradient (0,10), (30,35)]. Lyophilisation of the appropriate fractions gave the *title compound* as a fluffy, white solid (24 mg).

FABMS m/z 3089.8 (M^+), $C_{135}H_{230}N_{46}O_{33}S_2$ requires 3089.7 ; Analytical HPLC [column A(C18), gradient E] Rt 24.0 minutes ; Amino acid analysis (HCl hydrolysis, 20 hr.) Asp_1 1.08, Thr_1 1.02, Ser_1 0.98, Ala_5 5.19, Val_3 3.18, Ile_2 1.85, Leu_1 1.10, Phe_1 0.94, Lys_2 1.99, Arg_5 5.22, Cys_2 1.97, Trp_1 N.D. ; Ellman's test was positive.

Human NGF 50-70-$(Gly)_5$-94-120 $Cys^{58,108}(SBu^t)$, $Cys^{68,110}$(Acm).

H-Lys-Gln-Tyr-Phe-Phe-Glu-Thr-Lys-Cys^{5B}-Arg-Asp-Pro-Asn-Pro-Val-Asp-Ser-Gly-Cys^{5A}-Arg-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Lys-Gln-Ala-Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{5B}-Val-Cys^{5A}-Val-Leu-Ser-Arg-Lys-Ala-Val-Arg-Arg-Ala-OH

This peptide was a continuation of half of the resin used in the synthesis of NGF H-95-120-OH, $Cys^{108}(SBu^t)$, Cys^{110}(Acm). Cys was incorporated as $Cys^{58}(SBu^t)$ and Cys^{68}(Acm). Gln and Asn were unprotected. The peptide was assembled automatically with the normal cycles except across the sequences Lys^{50}-Gln-Tyr-Phe-Phe-Glu-Thr^{56} and Pro^{61}-Asn-Pro^{63}, in which each a.a. was triple-coupled. Monitoring showed no major drops in incorporation across the sequence. The resin weight after assembly was 1.80 g. The majority of this (1.70 g) was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (30 cm^3, under nitrogen) to yield a mixture of crude peptide and scavengers. This was ether extracted from 10% aqueous acetic acid to yield the crude peptide (0.93 g). This product was dissolved in a minimum amount of 10% aqueous acetic acid (35 cm^3), passed down a column of G50 Sephadex (Superfine, 2.5 x 150 cm) and eluted at 30 cm^3/hr. with the same solvent. UV absorbance was monitored at 254 and 277 nm. Fractions containing the peptide were verified with Ehrlich's spray. The purest of these was combined and lyophilised to yield a white solid (0.85 g). This was purified in seventeen portions by semi-preparative HPLC [column F, gradient (0,5), (60,40)]. Combining the appropriate fractions yielded the *title compound* as a fluffy, white solid (0.15 g) after lyophilisation, and a further amount (38 mg) of almost pure product (90% by HPLC).

FABMS m/z 6105.1 (error < 0.04%) , $C_{263}H_{426}N_{84}O_{72}S_6$ requires 6107.4 ; Analytical HPLC [column A(C18), gradient C] Rt 18.8 minutes.

Amino acid analysis ;

Crude (HCl hydrolysis, 18 hr.) Asx_4 4.19, Thr_2 2.00, Ser_2 2.45, Glx_3 2.70, Pro_2 1.87, Gly_8 8.00, Ala_5 8.00, Val_4 6.01, Ile_2 2.84, Leu_1 1.64, Tyr_1 0.84, Phe_3 3.20, Lys_4 5.35, Arg_7 9.20, Cys_4 3.64, Trp_1 N.D.

Title compound (HCl hydrolysis, 24 hr.) Asx_4 3.68, Thr_2 1.62, Ser_2 1.72, Glx_3 2.67, Pro_2 4.02*, Gly_8 8.00, Ala_5 5.60, Val_4 4.20, Ile_2 2.02, Leu_1 1.10, Tyr_1 0.77, Phe_3 2.57, Lys_4 3.52, Arg_7 7.17, Cys_4 1.72*, Trp_1 N.D. : *Cys degrades on extended acid

hydrolysis to form a species which coelutes with Pro. : Ellman's test on the *title compound* was negative.

Human NGF 50-70-$(Gly)_5$-94-120 $Cys^{68,110}$(Acm), $Cys^{58,108}$ disulphide-linked.

H-Lys-Gln-Tyr-Phe-Phe-Glu-Thr-Lys-Cys^{Ox}-Arg-Asp-Pro-Asn-Pro-Val-Asp-Ser-Gly-Cys^{SA}-Arg-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Lys-Gln-Ala-Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{Ox}-Val-Cys^{SA}-Val-Leu-Ser-Arg-Lys-Ala-Val-Arg-Arg-Ala-OH

A sample of pure, fully Cys-protected peptide from the above synthesis (70 mg) was placed in a silanised flask and was completely dissolved in 95% aqueous TFE (10 cm^3) by stirring under nitrogen (30 min.). Excess tributylphosphine (TBP, 30 µl) was added under nitrogen. Stirring was continued under N_2 and the course of the reaction followed by HPLC. After 90 minutes, the original peak (on HPLC) had almost completely vanished, to be replaced by a more hydrophilic peak [column A(C18), gradient (0,10), (30,90), the Rt of the main peak had changed from 20.3 to 18.0 min.]. After three hours, the entire solution was evaporated *in vacuo* to a colourless oil, which was taken up by stirring in 15% aqueous acetic acid (100 cm^3) for 20 minutes. This solution was extracted with ether (3 x 300 cm^3) then lyophilised to yield the $Cys^{58,108}$-reduced peptide (58 mg), which was not analysed.

Oxidation of the reduced peptide.

Glutathione oxidation : A portion of the reduced peptide (50 mg) was oxidised in a Tris buffer solution (0.1M, pH 8.0, 100 cm^3), containing EDTA (29 mg, 0.1 mmol.), oxidised glutathione (GSSG, 92 mg, 150 µmol.) and reduced glutathione (GSH, 23 mg, 75 µmol.). After 20 hr., the entire solution was filtered, TFA added (0.1 cm^3) then pumped onto a semi-preparative HPLC [column F] at 10 cm^3/min. The components were separated [gradient (0,0), (20,0), (60,35)] and the fractions lyophilised to yield the *title compound* (9.5 mg) along with some other products, which were identified as mixed disulphide (peptide/glutathione) species. These were reoxidised and purified under the same conditions to yield a further amount of the *title compound* (5 mg).

Air oxidation : Another portion of the fully cysteine-protected peptide (53 mg) was treated with TBP under the above conditions to again give a colourless oil. H_2O (40 cm^3) was added to this with stirring. An additional amount of H_2O (100 cm^3) was added and stirring continued until all of the residual oil had dissolved (15 min.),

leaving a cloudy, white solution. This was extracted with ether (2 x 300 cm^3) to give a clear solution, the pH adjusted to 8.0 by the addition of three drops of 2M NaOH and the solution stirred vigorously. After 24 hr., the entire solution was filtered, TFA added (0.1 cm^3) then pumped onto a semi-preparative HPLC [column F] at 10 cm^3/min. The components were separated [gradient (0,0), (15,0), (40,30)] and lyophilisation of the appropriate fractions yield gave more of the *title compound* (9 mg) as a fluffy, white solid.

FABMS m/z observed 5928.7. For $C_{255}H_{408}N_{84}O_{72}S_4$, calculated average mass = 5930.8, calculated monoisotopic mass = 5926.973. ; Analytical HPLC [column A(C18), gradient E] Rt 21.8 minutes ; Ellman's test on the *title compound* was negative.

NGF 100-114, dVal^{111}, Cys^{108,110} disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-dVal-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.52 mmol/g ; 0.50 g ; 0.25 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t), Val^{111} was replaced by dVal^{111}. The peptide was assembled automatically, using the normal double-couple cycles. A 50% drop in incorporation was observed in R^{103} to I^{104} coupling. The weight of dried resin obtained was 1.27 g. A portion (0.70 g) of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.33 g) as an insoluble white solid, which showed two major peaks on HPLC (X and Y ; column C, gradient F, Rt for X, 19.3 min. ; Rt for Y, 21.3 min.). A portion of the crude (0.25 g) was dissolved in 95% aqueous TFE (20 cm^3) then TBP (0.25 cm^3) was added. HPLC showed that the main peaks had moved to give a Rt of 18.6 (for X) and 19.3 minutes (for Y). Evaporation *in vacuo*, precipitation with ether, collection by filtration and extensive ether washing gave an insoluble crude, reduced product (0.20 g). Purification of half of this material was by semi-preparative HPLC [column F, gradient (0,10), (15,10), (55,40)] and the two peaks lyophilised to give peak X (14 mg) and peak Y (11 mg) as white solids. Subsequent analysis (AAA, FABMS) showed peak X to be a deletion product (m/z 1220 thus corresponds to Ac-I^{104}-R^{114}-OH) and peak Y to be the pure, reduced peptide (m/z 1752). Peak Y (10 mg) was stirred in 10% DMSO/TFA (5 cm^3), with anisole added (0.1 cm^3) for 2 hr. at RT., then evaporated *in vacuo* to

near dryness. The oily residue was taken up in 20% aqueous acetic acid (5 cm^3) and purified by semi-preparative HPLC [column F, gradient (0,5), (10,5), (50,45)]. Lyophilisation of appropriate fractions gave the *title compound* (6 mg) as a fluffy, white solid.

FABMS m/z 1751 ($M+H^+$), *hrms* found 1749.92569, $C_{75}H_{129}N_{24}O_{20}S_2$ requires 1749.92557 (< 1 ppm) ; Analytical HPLC [column C, gradient C] Rt 13.8 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.05, Thr_1 0.95, Ser_1 0.98, Ala_1 0.83, Val_2 2.23, Ile_2 1.77, Leu_1 1.21, Phe_1 0.83, Arg_3 2.67, Cys_2 N.D. ; Ellman's test was negative.

NGF 100-114, dVal^{109,111}, Cys^{108,110} disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-dVal-Cys^{ox}-dVal-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.52 mmol/g ; 0.50 g ; 0.25 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t), Val^{109,111} were replaced by dVal^{109,111}. The peptide was assembled automatically, using the normal double-couple cycles. The weight of dried resin obtained was 1.40 g. A portion (0.70 g) of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.35 g) as a white solid. A portion of the crude (0.15 g) was dissolved in 95% aqueous TFE (20 cm^3) then TBP (0.25 cm^3) was added. Evaporation *in vacuo*, precipitation with ether, collection by filtration and extensive ether washing gave a crude, reduced product, which was directly stirred in 10% DMSO/TFA (10 cm^3), with anisole added (0.1 cm^3) for 1.5 hr. at RT., then evaporated *in vacuo* to near dryness. The oily residue was taken up in 20% aqueous acetic acid (15 cm^3) and purified by semi-preparative HPLC [column F, gradient (0,5), (20,5), (60,40)]. Lyophilisation of appropriate fractions gave the *title compound* (13 mg) as a fluffy, white solid.

FABMS m/z 1751.1 ($M+H^+$), *hrms* found 1749.92565, $C_{75}H_{129}N_{24}O_{20}S_2$ requires 1749.92557 (< 1 ppm) ; Analytical HPLC [column C, gradient B] Rt 13.2 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.92, Thr_1 0.86, Ser_1 1.16, Ala_1 1.09, Val_2 2.13, Ile_2 1.93, Leu_1 1.18, Phe_1 1.07, Arg_3 2.70, Cys_2 N.D. ; Ellman's test was negative.

NGF 100-114, Aib^{109}, Cys^{108,110} disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Aib-Cys^{ox}-Val-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.52 mmol/g ; 0.50 g ; 0.25 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t), Val^{109} was replaced by α-aminoisobutyric acid, Aib^{109}. The peptide was assembled automatically up to Cys^{110} then Fmoc Aib-OH coupled manually as an AE/HOBt for 2 hr. The coupling was confirmed by a Kaiser test. Assembly was continued by machine. The weight of dried resin obtained was 1.5 g. A portion (0.70 g) of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (15 cm^3) to yield the crude peptide (0.36 g) A portion of the crude (0.26 g) was dissolved in 95% aqueous TFE (20 cm^3) then TBP (0.20 cm^3) was added. A peak shift from Rt of 17.8 to 14.7 minutes was observed [by HPLC, column C, gradient H]. Evaporation *in vacuo*, precipitation with ether, collection by filtration and extensive ether washing gave the crude, reduced product (0.21 g). This was purified in two runs by semi-preparative HPLC [column F, gradient (0,5), (30,30)] and lyophilisation of appropriate fractions gave the pure, reduced peptide (86 mg). A portion (60 mg) of this was stirred in 10% DMSO/TFA (10 cm^3) for 1.5 hr. at RT, then evaporated *in vacuo* to near dryness. The oily residue was taken up in 20% aqueous acetic acid (3 cm^3) and purified by semi-preparative HPLC [column E(C18), gradient (0,5), (30,95)]. Lyophilisation of appropriate fractions gave the *title compound* (33 mg) as a fluffy, white solid.

Reduced peptide

FABMS m/z 1740 ($M+H^+$), *hrms* found 1737.92564, $C_{74}H_{129}N_{24}O_{20}S_2$ requires 1737.92557 (< 1 ppm) ; Analytical HPLC [column C, gradient F] Rt 19.8 minutes.

Title compound

FABMS m/z 1736.1 ($M+H^+$), *hrms* found 1735.91000, $C_{74}H_{127}N_{24}O_{20}S_2$ requires 1735.90993 (< 1 ppm) ; Analytical HPLC [column C, gradient F] Rt 19.0 minutes.

Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.10, Thr_1 1.09, Ser_1 1.03, Ala_1 1.19, Val_1 1.08, Ile_2 2.20, Leu_1 1.07, Phe_1 1.12, Arg_3 3.32, Cys_2 N.D., Aib_1 N.D. ; Ellman's test was negative.

NGF 100-114, Aib^{111}, Cys^{108,110} disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-Val-Cys^{ox}-Aib-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.52 mmol/g ; 0.50 g ; 0.25 mmol). Cysteine was incorporated as Fmoc-Cys(Acm), Val^{111} was replaced by α-aminoisobutyric acid, Aib^{111}. The first five residues were coupled manually as AE/HOBt for 1.5 hr. each. The couplings were confirmed by Kaiser tests. The assembly using Fmoc-Cys^{110}(Acm)-Arg^{114}-resin was continued by machine. The weight of dried resin obtained was 1.60 g. A portion (0.80 g) of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.35 g) as a single product by HPLC. All of this crude was purified in five runs by semi-preparative HPLC [column F, gradient (0,10), (25,50)] and lyophilisation of appropriate fractions gave the pure, di-Acm peptide (84 mg). The Acm groups were removed as in NGF H-100-116-OH, Ser^{107}, Arg^{116}, Cys^{108,110} disulphide-linked : A portion of the di-Acm peptide (50 mg, 0.03 mmol) and silver triflate (AgOTf, 80 mg, 0.3 mmol) were dissolved in TFA (5 cm^3) and stirred at 0°c for 1 hr. in the absence of light. The solution was then evaporated to dryness *in vacuo* and a fine, white solid precipitated on the addition of excess ether. This was reduced with excess DTT (0.45 g, 3.0 mmol) in 50% aqueous acetic acid (10 cm^3) with vigorous stirring for 2 hr. Centrifugation as before removed an insoluble, yellow precipitate. The supernatant was directly purified by semi-preparative HPLC [column F, gradient (0,10), (10,10), (30,30), (40,30)] and lyophilisation of the appropriate fractions gave the pure, reduced peptide. This was directly oxidised in 10% DMSO/TFA (10 cm^3) for 1.5 hr. at RT, then evaporated *in vacuo* to near dryness. The oily residue was taken up in 20% aqueous acetic acid (5 cm^3) and purified, again by semi-preparative HPLC [column F, gradient (0,5), (5,5), (40,45)]. Lyophilisation of the purest fractions gave the *title compound* (11 mg) as a fluffy, white solid.

FABMS m/z 1737.2 ($M+H^+$), *hrms* found 1735.91001, $C_{74}H_{127}N_{24}O_{20}S_2$ requires 1735.90993 (< 1 ppm) ; Analytical HPLC [column C, gradient F] Rt 12.7 minutes. Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 1.08, Thr_1 0.91, Ser_1 1.30, Ala_1 1.11, Val_1 1.01, Ile_2 1.95, Leu_1 1.02, Phe_1 0.99, Arg_3 2.78, Cys_2 N.D., Aib_1 N.D. ; Ellman's test was negative.

NGF 100-114, Nle^{113}, Cys^{108,110} (Acm)

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{SA}-Val-Cys^{SA}-Val-Leu-Nle-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0..60 mmol/g ; 1.00 g ; 0.60 mmol). Cysteine was incorporated as Fmoc-Cys(Acm), Ser^{113} was replaced by norleucine, Nle^{113}. The first seven residues were coupled manually as AE/HOBt for 1.5 hr. each. The couplings were confirmed by Kaiser tests. Severe resin desolvation was seen after the coupling of Cys^{108}(Acm). The resin was halved after the addition of Ala^{107} (the other half was used in the assembly of NGF H-100-114-OH, Nle^{106,113}). The assembly using the Fmoc-Ala^{107}-Arg^{114}-resin was continued by machine. The weight of dried resin obtained was 1.41 g. A portion (1.00 g) of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.48 g) as an insoluble crude product. A portion (95 mg) of this was dissolved in 20% aqueous acetic acid (10 cm^3), filtered and purified by semi-preparative HPLC [column F, gradient (0,20), (45,50)]. Lyophilisation of appropriate fractions gave the *title compound* (30 mg) as a fluffy, white solid.

FABMS m/z 1919.6 ($M+H^+$), $C_{84}H_{144}N_{26}O_{21}S_2$ requires 1918.4 ; Analytical HPLC [column C, gradient F] Rt 13.3 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.86, Thr_1 1.03, Ala_1 1.03, Val_2 1.92, Ile_2 1.89, Leu_1 0.89, Phe_1 0.86, Arg_3 2.75, Cys_2 N.D., Nle_1 N.D.

NGF 100-114, Nle^{113}, Cys^{108,110} disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{Ox}-Val-Cys^{Ox}-Val-Leu-Nle-Arg-OH

The Acm groups were removed as in NGF H-100-116-OH, Ser^{107}, Arg^{116}, Cys^{108,110} disulphide-linked : A portion of the di-Acm crude peptide (0.15 g) and silver triflate (AgOTf, 80 mg, 0.3 mmol) were dissolved in TFA (15 cm^3) and stirred at 0°c for 1.5 hr. in the absence of light. The solution was then evaporated to dryness *in vacuo* and a fine, white solid precipitated on the addition of excess ether. This was reduced with excess DTT (0.45 g, 3.0 mmol) in 50% aqueous acetic acid (20 cm^3) with vigorous stirring for 3 hr. Centrifugation as before removed an insoluble, yellow precipitate. The supernatant was directly purified by semi-preparative HPLC [column E(C8), gradient (0,20), (10,20), (40,70)] and lyophilisation of the

appropriate fractions gave the pure, reduced peptide (42 mg). This was oxidised in 10% DMSO/TFA (15 cm^3) for 1.5 hr. at RT, then evaporated *in vacuo* to near dryness. The oily residue was taken up in 20% aqueous acetic acid (10 cm^3) and purified, again by semi-preparative HPLC [column F, gradient (0,10), (15,10), (55,50)]. Lyophilisation of the purest fractions gave the *title compound* (16 mg) as a fluffy, white solid.

FABMS m/z 1777.9, $C_{78}H_{134}N_{24}O_{19}S_2$ requires 1776.4 ; Analytical HPLC [column C, gradient F] Rt 13.0 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.86, Thr_1 1.03, Ala_1 1.03, Val_2 1.92, Ile_2 1.89, Leu_1 0.89, Phe_1 0.86, Arg_3 2.75, Cys_2 N.D., Nle_1 N.D.

NGF 100-114, $Nle^{106,113}$, $Cys^{108,110}$ (Acm)

H-Arg-Phe-Ile-Arg-Ile-Asp-Nle-Ala-Cys^{SA}-Val-Cys^{SA}-Val-Leu-Nle-Arg-OH

The initial resin used was a continuation of NGF H-100-114-OH, Nle^{113} synthesis. Thr^{106} was replaced by Nle^{106}. All subsequent a.a.'s were added manually via double-AE/HOBt couplings for 1.5 hr. each. The couplings were confirmed by Kaiser tests. Problems with resin aggregation occurred and washes with 50% DMF/dioxan were used. The weight of dried resin obtained was 1.50 g. A portion (1.00 g) of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.40 g) as an very poorly soluble crude product. A portion (0.10 g) of this was dissolved in 40% CH_3CN/H_2O (9 cm^3) with glacial acetic acid (1 cm^3) added, filtered and purified by semi-preparative HPLC [column F, gradient (0,20), (30,60)]. Lyophilisation of appropriate fractions gave the *title compound* (23 mg) as a fluffy, white solid.

FABMS m/z 1932.9 ($M+H^+$), $C_{86}H_{150}N_{26}O_{20}S_2$ requires 1932.4 ; Analytical HPLC [column C, gradient F] Rt 14.7 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.95, Ala_1 1.12, Val_2 2.18, Ile_2 1.80, Leu_1 0.93, Phe_1 0.95, Arg_3 3.07, Cys_2 N.D., Nle_2 N.D.

NGF 100-114 [Pro^{109a}-dVal^{109b}], Cys^{108,118} disulphide-linked

H-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{ox}-[Pro-dVal]-Cys^{ox}-Val-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.52 mmol/g ; 0.50 g ; 0.25 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t). Val^{109} was replaced by two residues, referred to as Pro^{109a} and dVal^{109b}. The peptide was assembled automatically, using triple-couple cycles for Pro^{109a} and dVal^{109b}. The weight of dried resin obtained was 0.93 g. A portion of this (0.43 g) was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.23 g) as a white solid, almost a single product by HPLC. A portion of this material (0.15 g) purified in two runs by semi-preparative HPLC [column F, gradient (0,15), (30,45)]. Lyophilisation of the purest fractions gave the pure, di-SBu^t peptide (51 mg) as a fluffy, white solid. 35 mg of this was stirred for 3 hr. in 95% aqueous TFE (4 cm^3) with TBP (0.1 cm^3) added. The solution was evaporated *in vacuo* to an oily residue, which was dissolved in 10% aqueous acetic acid (20 cm^3), ether extracted then lyophilised to give the pure, reduced peptide (27 mg). Most of this compound (25 mg) was dissolved in 15% DMSO/TFA (10 cm^3) and stirred for 1 hr. This was concentrated *in vacuo*, !0% aqueous acetic acid (10 cm^3) added then the solution applied directly to a semi-preparative HPLC column (column F). The components were separated [gradient (0,5), (30,75)] and lyophilisation of the purest fractions gave the *title compound* (13 mg) as a fluffy, white solid.

Reduced peptide

FABMS m/z 1850.4 (M+H$^+$), *hrms* found 1848.99366, $C_{80}H_{138}N_{25}O_{21}S_2$ requires 1848.99398 (< 1 ppm) ; Analytical HPLC [column B(C8), gradient F] Rt 12.8 minutes.

Title compound

FABMS m/z 1847.8 (M+H$^+$), *hrms* found 1846.97838, $C_{80}H_{136}N_{25}O_{21}S_2$ requires 1848.97833 (< 1 ppm) ; Analytical HPLC [column B(C8), gradient F] Rt 12.4 minutes ; [column C, gradient F] Rt 13.5 min. ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.94, Thr_1 0.92, Ser_1 1.09, Pro_1 2.00*, Ala_1 1.09, Val_2 2.10, Ile_2 2.06, Leu_1 1.12, Phe_1 1.06, Arg_3 2.79, Cys_2 N.D*. ; Ellman's test was negative ; *Cys degrades on acid hydrolysis to give a product which coelutes with Pro. N.M.R. results are included below.

600 MHz ^{1}H NMR (90% H_2O, 10%D_2O)

δ in ppm

Residue	α-NH	α-CH	β-CH	Others
Arg^{100}		4.08	1.93,1.93	γ1.62;δ3.21;NH7.24
Phe^{101}	8.85	4.74	3.09,3.12	Ring7.29
Ile^{102}	8.19	4.14	1.73	$\gamma CH_2$1.10,1.43;$\gamma CH_3$0.84;δ0.84
Arg^{103}	8.31	4.32	1.75,1.84	γ1.57,1.65;δ3.25;NH7.38
Ile^{104}	8.36	4.23	1.88	$\gamma CH_2$1.19,1.48;$\gamma CH_3$0.91;δ0.87
Asp^{105}	8.64	-	2.76,2.89	
Thr^{106}	8.14	4.42	4.32	γ1.22
Ala^{107}	8.31	4.43	1.42	
Cys^{108}	8.23	4.88	2.89,3.51	
Pro^{109a}		4.63	2.03,2.23	γ2.03;δ3.69
$dVal^{109b}$	8.62	4.13	2.34	γ0.99
Cys^{110}	7.84	4.43	3.23,3.33	
Val^{111}	8.38	4.16	2.06	γ0.95
Leu^{112}	8.42	4.49	1.65	γ1.65;δ0.89,0.95
Ser^{113}	8.32	4.52	3.89	
Arg^{114}	8.13	4.32	1.75,1.81	γ1.61;δ3.22;NH7.21

Formation of NGF 100-114 [Pro^{109a}-$dVal^{109b}$], $Cys^{108,110}$ disulphide-linked on the resin

A sample of dried resin from the above assembly (90 mg) was swollen with 50% hexafluoroisopropanol (HFIP)/DMF (1 cm^3) then TBP (0.2 cm^3) added and the solution sonicated for 3 hr. at 40°C. The resin was filtered off and washed with excess DMF (x 5), isopropanol (x 3), DMF (x 3), then dioxan (x 3). A sample (20 mg) of this was further dried with ether then cleaved as before (5 cm^3) for 2.5 hr. to give the crude product (5 mg). Analytical HPLC showed that deprotection of the SBu^t groups had occurred (84% by peak integration). The remainder of the resin (ether dried to give 40 mg) was then treated in two ways :

(a) 20 mg was suspended in an iodine-saturated solution of HFIP/DCM (1:3, 1 cm^3) and sonicated for 1 hr. The resin was then collected by filtration, washed with excesses of DCM, DMF and dioxan, then dried with ether.

(b) 20 mg was suspended in a solution of 1M aqueous $K_3Fe(CN)_6$, HFIP and DCM (1:1:8, 1 cm^3) and sonicated for 12 hr. The resin was then collected by filtration, washed with excesses of DMF, H_2O, DMF and dioxan, then dried with ether.

Each resin was then cleaved for 3 hr. with 2% anisole, 2% water and 1% EDT in TFA (5 cm^3) to give 6 mg for (a.) and 4 mg for (b.). Comparison of the analytical HPLC traces of authentic reduced and oxidised NGF 100-114 [Pro^{109a}-$dVal^{109b}$] with those for the crude products for protocols (a.) and (b.), showed that (a.) had given 65% conversion to the oxidised form, whereas (b.) had given only 10% conversion (by peak integrals). Purification of the crude of (a.) by semi-preparative HPLC [column E(C18), gradient. 25% isocratic] gave a small sample of the *title compound* (0.8 mg).

FABMS m/z 1847.5 ($M+H^+$), *hrms* found 1846.97840, $C_{80}H_{136}N_{25}O_{21}S_2$ requires 1848.97833 (< 1 ppm) ; Analytical HPLC [column C, gradient F] Rt 13.5 min.

NGF 100-114, $Asp(OBzl)^{105}$, $Cys^{108,110}$ (SBu^t)

H-Arg-Phe-Ile-Arg-Ile-Asp^{OBzl}-Thr-Ala-Cys^{SB}-Val-Cys^{SB}-Val-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.60 mmol/g ; 0.40 g ; 0.24 mmol). Cysteine was incorporated as Fmoc-Cys(SBu^t), Asp^{105} was added with its side-chain protected as a TFA-stable benzyl ester. Automatic assembly was carried out until Thr^{106} was coupled then assembly was continued manually by single SA couplings, except Arg^{103} and Ile^{104} (2 x AE/HOBt). The weight of dried resin obtained was 0.70 g. All of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.30 g) as a poorly soluble, although a single product (by HPLC), crude product. A portion (0.25 g) of this was dissolved in 20% aqueous acetic acid (25 cm^3), filtered and purified by semi-preparative HPLC [column E(C8), gradient (0,20), (10,20), (40,70)]. Lyophilisation of appropriate fractions gave the *title compound* (70 mg) as a fluffy, white solid.

FABMS m/z 1984.8 ($M+H^+$), $C_{88}H_{146}N_{26}O_{22}S_2$ requires 1984.4 ; Analytical HPLC [column C, gradient F] Rt 14.0 minutes ; Amino acid analysis (HCl hydrolysis, 18

hr.) Asp_1 0.89, Thr_1 0.87, Ser_1 1.00, Ala_1 1.07, Val_2 2.14, Ile_2 2.00, Leu_1 1.11, Phe_1 0.92, Arg_3 3.01, Cys_2 N.D.

NGF 100-114 Asp(OBzl)^{105}, [Pro^{109a}-dVal^{109b}], Cys^{108,110} (Acm)

H-Arg-Phe-Ile-Arg-Ile-Asp^{OBzl}-Thr-Ala-Cys^{5A}-[Pro-dVal]-Cys^{5A}-Val-Leu-Ser-Arg-OH

The initial resin had been derivatised with Fmoc-Arg(Pmc)-OH (0.52 mmol/g ; 0.50 g ; 0.25 mmol). Cysteine was incorporated as Fmoc-Cys(Acm). Asp^{105} was added as Asp(OBzl). Val^{109} was replaced by two residues, referred to as Pro^{109a} and dVal^{109b}. The peptide was assembled automatically using the normal cycles. The weight of dried resin obtained was 0.89 g. All of this was cleaved for 3 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.40 g) as a white solid, a single hydrophobic product by HPLC. This material was purified in five runs [5 x 80 mg in 25% aqueous acetic acid (20 cm^3)] by semi-preparative HPLC [column F, gradient (0,40), (30,70)]. Lyophilisation of the purest fractions gave a pure peptide (0.15 g). Analysis showed that the terminal Fmoc group had not been removed (FABMS m/z 2336.6 $\therefore$+221) due to a machine error. The Fmoc group was removed by dissolving a sample of peptide (93 mg) in 15% piperidine/DMF (5 cm^3), stirring for 1 min. then immediately evaporating the solution to dryness *in vacuo*. The residue was taken up in 50% aqueous acetic acid (10 cm^3) and the components separated by semi-preparative HPLC [column F, gradient (0,20), (20,35), (25,35), (40,50), (50,50)]. Two major products (X and Y) had formed : by HPLC (column C, gradient F), compound X (35%, Rt 15.3 min., FABMS m/z 2006.7) and compound Y (50%, Rt 15.7 min., FABMS m/z 2115.8). Compound X corresponded to the aspartimide-rearranged peptide (Bzl group was lost) whereas compound Y gave the *title compound* (33 mg) as a white solid.

FABMS m/z 2115.8, $C_{95}H_{159}N_{25}O_{21}S_4$ requires 2115.7 ; Analytical HPLC [column C, gradient F] Rt 15.3 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.88, Thr_1 0.98, Ser_1 1.12, Pro_1 0.71, Ala_1 1.08, Val_2 2.11, Ile_2 2.08, Leu_1 1.12, Phe_1 0.97, Arg_3 3.12, Cys_2 N.D.

Conserved NGF acetylated 100-115 amide, $Cys^{108,110}$ (Acm)

Ac-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys^{SA}-Val-Cys^{SA}-Val-Leu-Ser-Arg-Lys-NH_2

The initial resin used had the dibenzosuberyl linker to provide an amide on cleavage (0.45 mmol/g ; 0.50 g ; 0.23 mmol). Cysteine was incorporated as Fmoc-Cys(Acm). The peptide was assembled automatically using the normal double-couple cycles. A large drop in incorporation occurred over the residues Ile^{104}-Asp^{105}. The weight of dried resin obtained was 0.83 g. 0.40 g was replaced in the machine and acetylated. All of this was cleaved for 2.5 hr. in a solution of 3% water, 3% EDT and 2% phenol in TFA (10 cm^3) to yield the crude peptide (0.14 g) as a white solid. The crude was purified in three runs by semi-preparative HPLC [column F, gradient (0,10), (30,30)]. Lyophilisation of appropriate fractions gave the *title compound* (20 mg) as a white solid.

FABMS m/z 2063.5, $C_{89}H_{155}N_{29}O_{23}S_2$ requires 2063.5 ; Analytical HPLC [column C, gradient F] Rt 11.8 minutes ; Amino acid analysis (HCl hydrolysis, 18 hr.) Asp_1 0.97, Thr_1 0.98, Ser_1 1.20, Ala_1 1.08, Val_2 2.22, Ile_2 1.93, Leu_1 1.10, Phe_1 0.92, Lys_1 1.10, Arg_3 2.91, Cys_2 N.D. ; Ellman's test was negative.

Hen Egg White Lysozyme (HL) 1-129

K^{1}-V-F-G-R-C^{6}-E-L-A-A^{10}-A-M-K-R-H-G-L-D-N-Y^{20}-R-G-Y-S-L-G-N-W-V-C^{30}-A-A-K-F-E-S-N-F-N-T^{40}-Q-T-T-N-R-D-T-N-G-S^{50}-T-D-Y-G-I-L-Q-N-I-S^{60}-R-W-W-C^{64}-N-D-G-R-T-P^{70}-G-S-R-N-L-C^{76}-N-I-P-C^{80}-S-A-L-L-S-S-D-I-T-A^{90}-S-N-V-C^{94}-A-K-K-I-V-S^{100}-D-G-D-G-M-N-A-W-V-A^{110}-W-R-N-R-C^{115}-K-G-T-D-V^{120}-Q-A-W-I-R-G-C^{127}-R-L^{129}

Disulphides : 6-127, 30-115, 64-80, 76-94.

Two assemblies were undertaken, A and B ; each will be considered separately.

<u>Assembly A</u>

The resin used here had been derivatised with Fmoc-Leu-OH (0.25 mmol/g ; 0.40 g ; 0.10 mmol). The following derivatives were used : Arg (Pmc); Cys (SBu^{t}); Ser, Thr, Tyr as (Bu^{t}); Asp, Glu as (OBu^{t}); Lys (Boc); Asn, Gln, Trp were unprotected. Each residue was single-coupled with a ten-fold excess of AE/HOBt (1.0 mmol) for 30 min. The continuous assembly took eight days. A large drop in the apparent incorporation was seen over residues Ala^{110}-Arg^{114} (50% fall). 10% of the resin was removed at this stage and investigated whilst the assembly continued. Manual UV testing confirmed the observed fall. A further gradual decline (to 30% of the initial deprotection integral) occurred over the next thirty residues. Transient drops in apparent incorporation were observed on refilling the solvent reservoirs on the synthesizer. No further large falls in coupling efficiency were observed. The resin was dried with ether to give a yellow solid (1.32 g).

Acidolysis of A

Many trial cleavages were carried out using small amounts of resin (< 20 mg), varying scavenger composition, reaction times, etc. On the basis of these, the following protocol was carried out :

A portion of resin (0.50 g) was stirred for 3 hr. (under nitrogen) in a solution of 4% water, 4% EDT, 3% phenol, 2% EMS and 2% anisole in TFA (20 cm^3). This solution was then filtered off the resin, which was washed with a little neat TFA, the combined filtrates were concentrated *in vacuo* and a white solid precipitated by the addition of excess ether. The solid (not weighed) was collected by filtration. It gave a broad band on HPLC [column C, gradient F], eluting between 70-80% CH_3CN. This solid was stirred for a further 3 hr. under N_2 in a solution of 4% water, 2% EDT, 1% phenol and 1% EMS in TFA (20 cm^3). Treatment as before gave the

crude protein as a white solid (0.36 g). A portion of this was cleaved for another 2 hr. under the latter conditions. Upon the usual work-up, the resultant material (70 mg) was a dark purple colour (indicative of tryptophan damage) and HPLC showed that the main peak had broadened considerably. No further work was carried out on this material.

Sephadex G50 gel filtration

The rest of the crude synthetic lysozyme (SL) material (0.27 g) was dissolved, with sonication, in 30% aqueous acetic acid (30 cm^3) and subjected to gel filtration in three runs (3 x 10 cm^3) on a Sephadex G50 column (Superfine, 78 x 2.5 cm), eluting at 30 cm^3/hr. with the same solvent. The crude material eluted over tubes 20-40 ; four main peak areas were collected and lyophilised to give (in order of elution), compound K (33 mg), compound L (44 mg), compound M (42 mg) and compound N (15 mg, collected from first run only). By HPLC (column C, gradient F), compounds K and L looked similar to the crude material (wide peaks, elution over 48-52% CH_3CN), compound M was similar also (v. wide peak, 40-52% CH_3CN) but compound N consisted of scavengers and other low M.Wt. deletions (many small peaks over 35-55%). On analytical C8 columns (A and B), the peaks looked more symmetrical and eluted earlier. The void volume, V_o, of the column was determined by running a Blue Dextran (Pharmacia, M.Wt. > 200 kDal) solution [2 mg/cm^3, in 30% aqueous acetic acid (10 cm^3)] down the column under the same conditions as above. This eluted as a sharp band over tubes 20-22. Finally, natural hen egg white lysozyme (HL) [Sigma, Grade IV, 1.25 mg/cm^3, in 30% aqueous acetic acid (30 cm^3)] was passed down the column. The elution volume, V_e, of each peak was calculated as the volume of eluent required to elute the compound (estimated to the mid-point of each peak on the UV trace at 225 nm). The value of V_e/V_o was calculated for each compound.

Component	BD	HL	K	L	M	N
Ve/Vo	1.00	1.28	1.02	1.15	1.57	1.81

Amino acid analysis

AAA was carried out on the crude SL product before gel filtration : the hydrolysis was in HCl for 24 and 45 hr.

AA	24 hr.	Actual	45 hr.	AA	24 hr.	Actual	45 hr.
Asx	20.1	21.0	19.5	Ile	7.3	6.0	5.0
Thr	5.3	7.0	4.5	Leu	8.1	8.0	5.5
Ser	4.2	10.0	3.5	Tyr	2.6	3.0	2.0
Glx	6.3	5.0	3.8	Phe	2.8	3.0	1.8
Gly	12.9	12.0	11.6	His	1.3	1.0	1.1
Ala	12.3	12.0	10.6	Lys	6.7	6.0	6.4
Cys	6.2	8.0	6.1	Arg	12.1	11.0	11.9
Val	8.5	6.0	7.7	Trp	N.D.	6.0	N.D.
Met	2.4	2.0	2.2	Pro*	N.D.	2.0	N.D.

* The proline peaks were too small to be integrated.

AAA was also carried out for the samples of compounds A-D and for HL after Sephadex G50 gel filtration: hydrolysis was in 4-TSA for 22 hr.

AA	HL	Actual	K	L	M	N
Asx	19.8	21.0	23.6	22.5	20.4	18.1
Thr	7.3	7.0	7.9	6.9	6.4	6.3
Ser	8.8	10.0	11.0	10.4	10.5	4.6
Glx	5.6	5.0	5.4	5.1	5.8	5.8
Pro	1.8	2.0	3.9	5.3	7.0	5.3
Gly	11.3	12.0	12.6	12.8	11.4	15.2
Ala	11.2	12.0	12.1	11.9	13.7	12.3
Cys	6.2	8.0	(0.5)	(0.3)	N.D.	N.D.
Val	5.1	6.0	4.2	3.9	6.1	6.5
Met	2.0	2.0	1.5	1.3	2.1	2.3
Ile	4.6	6.0	5.1	5.4	4.6	4.0
Leu	7.1	8.0	7.2	7.3	7.2	7.6
Tyr	2.7	3.0	3.8	4.3	4.6	4.6
Phe	2.6	3.0	2.4	2.3	2.3	1.8
His	1.0	1.0	0.9	1.2	1.0	1.2
Trp	4.8	6.0	4.7	8.2	5.6	6.7
Lys	5.5	6.0	5.3	5.1	6.8	6.6
Arg	10.5	11.0	11.5	12.1	10.5	15.3
Mean error per residue	10.7%	0.0%	11.4%	11.3%	9.4%	21.1%

The total and mean errors were calculated omitting Cys and Pro values.

Preparation of reduced and denatured, natural lysozyme [HL(red.)].

After Jaenicke (Ref. 337)

HL (0.10 g) and DTT (0.11 g) were dissolved in a solution of 8M urea in 0.05m Tris buffer (pH 8.7, 10 cm^3), containing 1M EDTA. The solution was stirred for 3 hr. under nitrogen, the pH adjusted to around 3 by the addition of glacial acetic acid then applied to a Sephadex G25 gel column (Coarse, 45 x 1.5 cm), eluting with 0.1M aqueous acetic acid at 30 cm^3/hr. Fractions giving a positive Ehrlich's test were examined by HPLC, the purest being combined and lyophilised to give the HL(red.) protein (69 mg) as a fluffy, white solid. This was stored in 5 mg aliquots at -20°C.

Quantitative Ellman's test : observed 7.6 thiol groups/molecule ; required 8.0.

HPLC (column C , gradient F) Rt 15.0 min.

Gel filtration G75

The remainder of the resin (0.80 g) was cleaved under the same conditions as before to yield a crude product (0.39 g). Most of this (0.30 g) was dissolved in 25% aqueous acetic acid (20 cm^3), diluted to 10%, then passed (in three runs) down a Sephadex G75 column (Superfine, 150 x 2.5 cm), eluting with 0.1M acetic acid at 20 cm^3/hr. Four areas were collected, combined and lyophilised to give (the earliest eluting first); compounds W (31 mg), X (37 mg), Y (55 mg) and Z (0.13 g). Blue Dextran (20 mg), HL (20 mg) and HL(red.) (10 mg) were passed in order down the column under the same conditions to enable the value of V_e/V_o to be calculated for each compound.

Component	BD	HL	HL(red.)	W	X	Y	Z
Ve/Vo	1.0	2.7	2.1	1.4	2.0	2.7	3.5

Assembly B

A second assembly of HL was undertaken. The resin used was as before, except that here the resin was first capped with excess benzoyl chloride in DMF (at 0°C for 2 hr.) then warmed up and allowed to react overnight. The resin was collected by filtration and extensively washed with DMF, DCM, DMF and dioxan before use. The Fmoc derivatives used were as before except that His(Bum) and Trp(Boc) replaced those used in assembly A. The HOBt used here was dried by recrystallisation from ethanol before use. The time for each ten-fold excess AE/HOBt coupling was extended to 90 min./residue and each a.a. in the sequences

Cys^{76}-Cys^{80} and Ala^{107}-Cys^{115} was triple-coupled. At residue Thr^{43} (the 87th coupling), approx. one quarter of the peptide/resin (1.7 of 6.9 g wet-weight) was removed from the reaction vessel to facilitate the washing and vortexing steps. The amount of Fmoc present in a sample of this resin was checked by manual UV monitoring giving a value of 90% of the initial deprotection (c.f. 86% for the on-line monitoring). From this level, the apparent incorporation fell to 55% at the end of the assembly (correcting for the resin already removed). The resin was left Fmoc-protected after the final coupling and manual UV indicated a value for this of 50% of the initial Fmoc deprotection. 3.0 g of yellowish resin was obtained on drying with ether.

Cleavage

A sample of resin (0.11 g) had the terminal Fmoc-group removed then was dried down in ether and cleaved in a solution of 3% water, 3% phenol, 2% EDT, 1% anisole and 1% EMS in TFA (10 cm^3), under nitrogen. Samples (~ 1.5 cm^3) were removed at times t = 3, 4, 6 and 8 hr. and overnight (25 hr.). Each sample was concentrated *in vacuo*, precipitated with ether and collected by filtration. Each sample was then examined by HPLC.

A further 0.48 g of resin was stirred for 6 hr. under nitrogen in a TFA (20 cm^3) solution of the same composition as before. The usual work-up gave the crude SL product (0.33 g).

Gel filtration G75

The crude SL was dissolved in 0.1M acetic acid (30 cm^3) with sonication for 1 hr. Addition of some glacial acetic acid (2 cm^3) and resonication was required for complete dissolution. This was then, in three runs (1-3), passed down a Sephadex G75 (Standard, 70 x 3 cm) column, eluting with 0.1M acetic acid at 30 cm^3/hr. Three major fractions were collected and combined ; fraction A, B and C.

Column 1+2 : Fraction P (tubes 9-12, 19 mg), Q (tubes 13-22, 25 mg) and R (tubes 23-27, 28 mg).

Column 3 : Fraction P (tubes 10-13, 24 mg), Q (tubes 14-23, 15 mg) and R (tubes 24-28, 12 mg).

Removal of SBu^t groups

Fractions P and Q (from columns 1+2) [44 mg] were dissolved in 90% TFE (20 cm^3) with stirring. Excess TBP (0.30 cm^3) was added under nitrogen and stirring continued for 3 hr. By HPLC (column C, gradient F), the original wide peak (Rt 20.3) narrowed and changed to Rt 19.0 minutes. On the same gradient, HL(red.) gave a sharp peak with Rt 19.0 min. The solution was concentrated *in vacuo*, the residue dissolved in 5% aqueous acetic acid (10 cm^3) and extracted three times with ether. The aqueous solution was directly applied to the Sephadex G75 column and eluted with 0.1M acetic acid at 30 cm^3/hr. Lyophilisation of the appropriate fractions recovered the SL(red.) protein (40 mg). Samples of the HL(red.) (32 mg columned, 20 mg recovered after lyophilisation) and BD were also passed down the column to allow the V_e/V_o values to be calculated as before. Ellman's test on the recovered HL(red.) gave only 4.1 SH/molecule whilst that of the SL(red.) was 3.6 SH/molecule.

Component	BD	HL	HL(red.)	SL(red.)	P	Q	R
Ve/Vo	1.0	2.9	2.3	2.4	1.2	2.9	3.9

Polyacrylamide Molecular Weight Gels

Samples of HL(red.) and SL(red.) (both from the above G75 column, 0.1 mg of each) were dissolved in a 10 mM Tris-Cl buffer (pH 7.2, 0.10 cm^3), containing EDTA (1.0 mM), 2.5% sodium dodecyl sulphate (SDS), 2.5% β–mercaptoethanol and 0.01% Bromophenol Blue. The solutions were boiled for 5 min. These were then subjected to SDS-PAGE using the Pharmacia PhastGel system. A pre-cast, high-density 20% gel, with SDS added, was used (fractionation range 1-20 kDal). M.W. standards (2.6, 6.4, 8.2, 14.6 and 17.2 kDal) were also run. The protein bands were stained using Coomassie Blue in accordance with the manufacturer's procedures (PhastSystem Separation Technique File No. 111).

***Micrococcus lysodeikticus* assay for HL**

[After Jollés (Ref. 252)]

M. lysodeikticus cells (25 mg) were suspended in 0.067M sodium phosphate buffer, pH 6.2, containing 0.1M NaCl. Thorough mixing gave a broth-like suspension, which had a transmittance of between 20-22% at 650 nm when water was set at 100% transmittance. A standard solution of HL was prepared by dissolving 1.25 mg in 0.1M Tris-acetate buffer (pH 8.1, 45 cm^3) containing 1mM EDTA with 0.1M

acetic acid added (5 cm^3) to give a final pH of 7.7. The assay was run at a constant 37°C, all solutions and the spectrophotometer being maintained at that temperature.

Assay: A sample of the cell suspension (2.5 cm^3) was placed in a quartz cuvette (1 cm pathlength, 3 cm^3 capacity) and the test solution (0.5 cm^3, at various concentrations) was squirted into this to aid mixing. The rise in transmittance was recorded as a function of time over a three minute period. Using the standard HL solution, it was found that a graph of ΔA_{650} against HL concentration gave a straight line, passing through the origin, with a gradient (in arbitrary units) of 0.005 ΔA_{650}/min./mg HL in 1L buffer.

Regeneration of activity from inactive HL(red.)

[After Saxena and Wetlaufer (Ref. 242)]

All solutions were duplicated and maintained at 37°C. HL(red.) (1.20 mg) was dissolved in 0.1M acetic acid (7 cm^3) to give an approx. 10 μM solution. A sample of this (1 cm^3) was added to 0.1M Tris-acetate buffer (pH 8.1, 9 cm^3) containing 1.0 mM EDTA, 6.0 mM GSH and 0.6 mM GSSG. A control containing only 0.1M acetic acid (1cm^3) and the renaturation buffer (9 cm^3) was also set up. These solutions were checked for lysozyme activity by assaying samples (0.5 cm^3) at suitable intervals. A standard native lysozyme solution was also checked each time. The results are summarised below:

Sample	Time (min.)	ΔA_{650}/min.
Natural HL	0	1.14×10^{-1}
HL(red.) in 0.1M acetic acid	0	0
Control	0	0
HL(red.) in regeneration buffer	60	0.43×10^{-1}
HL(red.) in regeneration buffer	100	0.43×10^{-1}
HL(red.) in regeneration buffer	300	0.43×10^{-1}
HL(red.) in Tris buffer only	60	0.09×10^{-1}

Natural HL	ΔA_{650}/min./mg protein = 5.76
Regenerated HL(red.)	ΔA_{650}/min./mg protein = 3.24

Further reduction of SL(red.) under denaturing conditions

A sample of the previously reduced, synthetic lysozyme [SL(red.), 20 mg] was stirred for 3 hr. in a solution containing 6M guanidinium chloride (Gdm.HCl) in 0.1M Tris-Cl (pH 8.2, 2.5 cm^3), propanol (2.3 cm^3) and TBP (0.2 cm^3). The solution was concentrated slightly *in vacuo* then applied to a Sephadex G25 (Coarse, 25 x 1.5 cm) and eluted with 0.1M acetic acid. The appropriate fractions were lyophilised to give the SL(red.) protein (5 mg). Ellman's test on this gave a value of 3.5 SH/molecule. Under the same conditions, HL(red.) gave only 1.2 SH/molecule.

Amino acid analysis on HL(red.) and SL(red.).

Both of the reduced samples were hydrolysed in HCl for 18 hr. then analysed.

AA	HL(red.)	Actual	SL(red.)	AA	HL(red.)	Actual	SL(red.)
Asx	22.4	21.0	23.4	Ile	5.6	6.0	5.0
Thr	7.0	7.0	8.4	Leu	8.0	8.0	5.5
Ser	9.6	10.0	11.5	Tyr	3.1	3.0	2.0
Glx	5.8	5.0	5.6	Phe	3.2	3.0	1.8
Gly	12.2	12.0	11.8	His	1.3	1.0	0.6
Ala	12.5	12.0	10.7	Lys	5.5	6.0	5.2
Cys	(2.4)	8.0	(5.5)	Arg	9.3	11.0	8.9
Val	5.6	6.0	5.4	Trp	N.D.	6.0	N.D.
Met	2.3	2.0	1.3	Pro	(10.0)	2.0	(4.4)

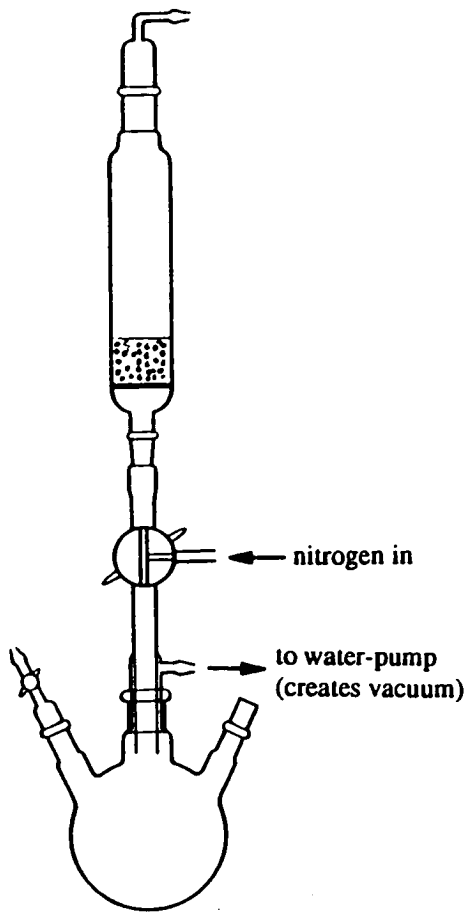
Attempts at renaturation of SL(red.)

Fractions P+Q (from G75 column 3, 39 mg) were stirred for 3 hr. in a solution containing 6M Gdm.HCl in Tris buffer (pH 8.0, 3 cm^3), isopropanol (2.5 cm^3) and TBP (0.5cm^3). After this time, the solution was concentrated slightly *in vacuo*, placed in a dialysis bag (Spectrophor, M.Wt. cut-off 2 kDal) then dialysed for 24 hr. at 4°C against a solution of 6M Gdm.HCl in Tris buffer (pH 8.0, 500 cm^3), containing 1 mM EDTA and 0.3M β-mercaptoethanol, with two changes of this dialysing solution in that time. The protein solution inside the bag was then transferred into a larger bag and the volume adjusted to 100 cm^3 with the following solution, which also formed the next dialysis solution; 6M Gdm.HCl in Tris buffer (pH 8.0, 1000 cm^3) containing 1mM EDTA. This and subsequent dialysis solutions were degassed and purged with nitrogen before use. After 48 hr. and several changes of dialysis solution, the bag was dialysed against a similar solution except

this contained 4M Gdm.HCl. The process was repeated at 2M Gdm.HCl then at 0.5M Gdm.HCl. After 24 hr. in this final solution, the contents of the bag became cloudy. The solution was increased to 200 cm^3, put into two dialysis bags and dialysis continued. No further cloudiness was observed and this final solution was used in the following reactivation studies. The solution was estimated to have a protein concentration of 10^{-5}M, ten times more concentrated than that required for optimum regeneration.

Regeneration studies : Many small-scale studies were carried out using the crude, 'renatured' SL(red.) solution under conditions which had successfully regenerated enzymic activity from HL(red.). The resulting solutions were assayed as before. None gave any significant activity. More studies were carried to test the effect of temperature, denaturant concentration and GSH/GSSG composition. None of these tests gave any activity either. It was however observed that the SL(red.) concentration in all of the reactivation studies, could not be increased beyond 10^{-6}M without causing precipitation of protein from the solution or its adherence to glassware.

APPENDIX A



Merrifield 'bubbler'

Resin is suspended in a suitable solvent mixture e.g. DMF, 50% dioxan/DMF, inside the cylindrical glass reaction vessel. The mixture is agitated by nitrogen, which is introduced through a three-way tap as shown. A glass frit prevents the loss of the resin and enables nitrogen to 'bubble' into the solution. Reagents may be added through the top of the reaction vessel from wash bottles, automatic pipettes or measuring cylinders. Excess reagents and solvents may be drained from the resin into the lower reservoir under slight vacuum (via connection to a water pump). It is best not to allow the resin to become completely drained of solvent to prevent resin aggregation. Resin collecting above the liquid surface can be washed down at each reagent addition. Small samples of resin can be removed by Pasteur pipette to enable monitoring of the resin by Kaiser tests etc.

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COURSES ATTENDED

Organic Research Seminars (various speakers) University of Edinburgh, 1989-92.

Recent Advances in Organic Chemistry (various speakers) University of Edinburgh, 1989-92.

Current Topics in Organic Chemistry (various speakers) University of Edinburgh, 1989-92.

Scottish Protein Group (various speakers) University of Edinburgh, 1992.

Medicinal Chemistry (Prof. R. Baker and colleagues, Merck, Sharp and Dohme) 1990, 1992.

Medicinal Chemistry (Members of ICI Pharmaceuticals, various speakers) 1992.

N.M.R. Spectroscopy (Dr. I.H. Sadler) University of Edinburgh, 1990-92.

Scottish Perkin Meeting (various speakers) University of Strathclyde, 1989.

12th American Peptide Symposium (various speakers) M.I.T., Boston, 1991.