Studies on Interleukin-1-β-pre Subpeptides

Derek Maclean

A thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh September 1991
This thesis is submitted in part fulfillment of the requirements of the degree of Doctor of Philosophy in the University of Edinburgh. Unless otherwise stated the work described is original and has not been previously submitted, in whole or in part, for any degree at this or any other university.
To my parents,
Murdo and Helen,
and to Stephanie
Acknowledgements

I would like to thank Prof. R. Ramage for the provision of research facilities and to gratefully acknowledge his constant advice and encouragement throughout the course of this work.

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Finally, I would like to thank all my friends and colleagues in Edinburgh for making my time here so enjoyable and productive.
Abstract

The synthesis of a series of peptides from the processing region of interleukin-1-β precursor (IL-1-β-pre) has been carried out using automated solid-phase methods. The subpeptide comprising residues 102-138 was found to possess inhibitory activity towards the cleavage of IL-1-β-pre in studies using human monocyte cell lines.

Attempts have been made to define more closely the region of the peptide responsible for this activity. Overlapping dodecapeptides have been prepared which cover this region of the precursor. The highest level of residual biological activity was found to reside in the subpeptide 111-122, comprising six residues on either side of the site of processing. Various derivatives of this peptide have been prepared in order to enhance this activity.

Several of these peptides have been investigated by high-field nuclear magnetic resonance (NMR). Complete assignment of the spectra of most of the dodecapeptides has been achieved. The NMR spectra of an analogue of IL-1-β-pre 102-138 where cysteine-124 has been replaced by alanine (Ala-124-IL-1-β-pre 102-138) have been obtained and partially assigned. Analysis of through-space interactions indicated that these peptides adopt random conformations.

A survey of protecting groups for the side-chain amide functional groups of asparagine and glutamine in solid-phase peptide synthesis has been carried out. Novel dibenzosuberenyl-type derivatives have been prepared and successfully applied to the preparation of a series of peptides. A comparison between these derivatives and commonly used alternatives (unprotected amide, trityl and 4,4'dimethoxybenzhydryl) indicated that the trityl group was the most generally suitable means of incorporating these amino acids.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>Acm</td>
<td>acetamidomethyl</td>
</tr>
<tr>
<td>atm.</td>
<td>atmospheres</td>
</tr>
<tr>
<td>b</td>
<td>broad</td>
</tr>
<tr>
<td>Bnpeoc</td>
<td>bis-(4-nitrophenyl)-ethoxycarbonyl</td>
</tr>
<tr>
<td>Boc</td>
<td>t-butyloxycarbonyl</td>
</tr>
<tr>
<td>Bpoc</td>
<td>biphenyloxycarbonyl</td>
</tr>
<tr>
<td>Bum</td>
<td>butoxymethyl</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CM</td>
<td>carboxymethyl</td>
</tr>
<tr>
<td>COSY</td>
<td>correlated spectroscopy</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>Dbsa</td>
<td>10,11-dihydro-5H-dibenzo-[a,d]-cyclohepten-5-yl</td>
</tr>
<tr>
<td>Dbse</td>
<td>5H-dibenzo-[a,d]-cyclohepten-5-yl</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N'-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DKP</td>
<td>2,5-diketopiperazine</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(N,N-dimethylamino)-pyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>EDT</td>
<td>ethane-1,2-dithiol</td>
</tr>
<tr>
<td>EMS</td>
<td>ethylmethysulphide</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>FAB</td>
<td>fast-atom bombardment</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenlymethoxy carbonyl</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform IR</td>
</tr>
<tr>
<td>HOBr</td>
<td>1-hydroxybenzo-triazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution MS</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-1-β-converting enzyme</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-1r</td>
<td>interleukin-1-receptor</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>IL-1r antagonist</td>
</tr>
<tr>
<td>IR</td>
<td>infra-red</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>Mbh</td>
<td>4,4'-dimethoxybenz-hydryl</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Überhauser effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>NOE spectroscopy</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PG</td>
<td>protecting group</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristyl acetate</td>
</tr>
<tr>
<td>PMc</td>
<td>pentamethylchroman-sulphonyl precursor</td>
</tr>
<tr>
<td>pTSA</td>
<td>p-toluene sulphonic acid</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>resin</td>
<td>resin</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>resin</td>
<td>copoly(styrene/2% divinylbenzene)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROESY</td>
<td>rotating frame NOESY</td>
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<tr>
<td>RP</td>
<td>reversed-phase</td>
</tr>
<tr>
<td>s</td>
<td>strong</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulphate</td>
</tr>
<tr>
<td>sIL-1r</td>
<td>soluble IL-1r</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid-phase peptide-synthesis</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
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<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TOCSY</td>
<td>totally correlated spectroscopy</td>
</tr>
<tr>
<td>trityl</td>
<td>triphenylmethyl</td>
</tr>
<tr>
<td>Trt</td>
<td>trityl</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>w</td>
<td>weak</td>
</tr>
<tr>
<td>X</td>
<td>leaving group</td>
</tr>
<tr>
<td>Z</td>
<td>benzyloxy carbonyl</td>
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Genetically Coded Amino Acids

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<thead>
<tr>
<th>Amino Acid</th>
<th>Side-Chain</th>
<th>3-Letter Code</th>
<th>1-Letter Code</th>
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<tr>
<td>Alanine</td>
<td>CH₃</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Arginine</td>
<td>(CH₂)₃NHC(NH)NH₂</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>Asparagine</td>
<td>CH₂CONH₂</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>CH₂COOH</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Cysteine</td>
<td>CH₂SH</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>(CH₂)₂COOH</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Glutamine</td>
<td>(CH₂)₂CONH₂</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td>Glycine</td>
<td>H</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td>Histidine</td>
<td>CH₂(im)¹</td>
<td>His</td>
<td>H</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>CH(CH₃)C₂H₅</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>Leucine</td>
<td>CH₂CH(CH₃)₂</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Lysine</td>
<td>(CH₂)₄NH₂</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Methionine</td>
<td>(CH₂)₂SCH₃</td>
<td>Met</td>
<td>M</td>
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<td>Phenylalanine</td>
<td>CH₂C₆H₅</td>
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<td></td>
<td>Pro</td>
<td>P</td>
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<tr>
<td>Serine</td>
<td>CH₂OH</td>
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<tr>
<td>Threonine</td>
<td>CH(OH)CH₃</td>
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<td>T</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>CH₂(ind)³</td>
<td>Trp</td>
<td>W</td>
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<tr>
<td>Tyrosine</td>
<td>CH₂C₆H₄OH</td>
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<td>Y</td>
</tr>
<tr>
<td>Valine</td>
<td>CH(CH₃)₂</td>
<td>Val</td>
<td>V</td>
</tr>
</tbody>
</table>

All amino acids are of the L-configuration unless otherwise noted.

Notes:

1. The imidazolyl group of histidine
2. Proline
3. The indole group of tryptophan
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Chapter 1. Introduction

1.1 The Immune System

1.1.1 General

The ability to repel invading organisms is a vital part of the physiological make-up of all members of the animal kingdom. Even the simplest invertebrates such as helminths (flukes and worms) or jellyfish possess elements of innate immunity (Latin *immunitas*, freedom from). They contain cells which are capable of delivering a bombardment of chemical weapons against an intruder. Higher animals have more complex immune systems which display adaptive methods for eliminating invaders. These systems are characterised by their ability to learn and adapt to their environment. Innate responses remain constant over time. The study of the mechanisms of immunity is called immunology, and many good textbooks give detailed accounts of the state of current knowledge (e.g. refs. 1 and 2).

Immunology is moving fast in the light of the discoveries of cell and molecular biology in recent years. Techniques such as gene cloning and expression, and monoclonal antibody production have led to considerable advances in our understanding of the immune system.

The work described in this thesis is an attempt to understand and ultimately control a pivotal step of the immune response. Before this can be discussed in detail some facets of the subject must be introduced more thoroughly.

1.1.2 Innate Immunity

The first lines of defence against infection are the body surfaces. Skin provides an effective physical barrier, and secretions generate an acidic environment in which most bacteria cannot survive. Broken or damaged skin is much more vulnerable.
The entrances to the body's internal surfaces are also closely guarded. Membranes produce mucus which traps foreign bodies, and reflexes such as coughing and vomiting help with the mechanical removal of invaders. Most bodily secretions such as tears, semen and saliva have antimicrobial properties.

Despite these defences, infectious agents do sometimes succeed in entering the body where they can do real damage if left to multiply unchecked. Special cells are found in the tissues, which can bind to foreign particles and destroy them. Macrophages (Greek "big eater") engulf and digest their prey, whereas granulocytes expel chemicals which damage the invader. Complement proteins assist these processes by binding to foreign bodies and opsonising them, in other words enhancing their attractiveness to macrophages and granulocytes. These processes are relatively inefficient, and have been circumvented in many ways by various organisms.

1.1.3 Adaptive Immunity

Innate immunity was for a long time thought to be a primitive method of protection but in fact it is the basis of the adaptive immune response. The cells and molecules of adaptive immunity merely serve to enhance the innate mechanisms.

The adaptation is based on the ability of certain cells to respond to specific markers of foreignness and to selectively reproduce those cells which are best at dealing with the invader. The cells responsible are the small lymphocytes: B-cells which produce antibodies and T-cells which control the intensity and direct the effects of the immune response.

Antibodies, or immunoglobulins, are the key molecules of adaptive immunity. They act in an analogous manner to the complement proteins in innate immunity, opsonizing invaders for disposal by other cells. They are however capable of exercising exquisite sensitivity in choosing the targets to which they bind. Each antibody binds to an antigen (antibody generator) which is part of a foreign body, e.g. a bacterial flagellum. The precise part of the antigen to which the antibody binds is called an epitope. Thus an antigen may possess several epitopes and be bound by several
different antibodies. B-cells carry antibodies on their cell membranes and so can recognise antigens.

Four polypeptide chains:
- light
- heavy
- heavy
- light;

joined by disulphide bridges.

The antigen-binding site is made up of regions of hypervariable sequence towards the N-terminus of each chain.

![Diagram of antibody structure]

**Fig.1.1 the Fundamental Unit of Antibody Structure**

T-cells do not recognise intact antigens: the antigen must be presented by a particular type of macrophage in a form which the T-cell recognises. This is in fact the initial step in adaptive immunity. The T-cell is stimulated by antigen presentation and it in turn sends messages to B-cells and other T-cells to multiply and make ready to attack invaders. The ensuing cascade of activated cells ensures that the invader is overwhelmed before it can multiply. The messages referred to are delivered in the form of soluble proteins - the cytokines or "cell movers"

### 1.1.4 Cytokines and Interleukins

A cytokine is an inducible secreted polypeptide which affects the growth and function of cells. An Interleukin is a cytokine of known primary structure produced by leukocytes and active during the immune response.

By the late 1970s around one hundred cytokines had been described and named on the basis of some observed *in vitro* or *in vivo* activity. With increasingly common use of gene and protein sequencing it became clear
that single molecular species were often responsible for several of these effects. Rationalisation of the field has now reduced the number of cytokines to around twenty, though new ones are constantly being discovered.

Cytokines are now loosely classified into several families. The divisions are largely based on historical rather than well-defined scientific differences. The main cytokine families are the interferons\(^4\), colony stimulating factors\(^5\), tumour necrosis factors\(^6\), and interleukins\(^7\). The definition of an interleukin is so broad that many of the other cytokines, if discovered today, would probably be placed in this group. To date at least eight proteins have been so designated, interleukins 1 to 8 (IL-1 to -8).

Most studies on cytokines have looked at their role in the immune response, but they may have important functions in homeostasis, growth, and development. They are important mediators of local cell communication (autocrine and paracrine) (see fig.1.3), but many may also
act in a systemic, hormone-like (endocrine) manner. One example of such a pleiotropic protein is the "endogenous pyrogen" interleukin-1.

Fig. 1.3 Modes of Hormone Action

1. Autocrine
2. Paracrine
3. Endocrine

1.1.5 Interleukin-1

The wide range of properties of interleukin-1 is indicated by the number of separate names which it previously held (table 1.1). Its activity was first described by Atkins in 1955 as a protein which induced fever when injected into animals (endogenous pyrogen). The other names arose over the next 25 years. The present form, interleukin-1 was introduced in 1979 at the 1st International Lymphokine Workshop held in Switzerland. Cloning of the other factors soon established their common identity.

<table>
<thead>
<tr>
<th>Previous Name</th>
<th>Abbreviation</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Endogenous pyrogen</td>
<td>EP</td>
<td>8</td>
</tr>
<tr>
<td>Lymphocyte Activating Factor</td>
<td>LAF</td>
<td>9</td>
</tr>
<tr>
<td>Leukocyte Endogenous Mediator</td>
<td>LEM</td>
<td>10</td>
</tr>
<tr>
<td>Mononuclear Cell Factor</td>
<td>MCF</td>
<td>11</td>
</tr>
<tr>
<td>B-Cell Activating Factor</td>
<td>BAF</td>
<td>12</td>
</tr>
<tr>
<td>Osteoclast Activating Factor</td>
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<td>13</td>
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<td>Synovial Factor</td>
<td>SF</td>
<td>14</td>
</tr>
<tr>
<td>Epidermal T-Cell Activating Factor</td>
<td>ETAF</td>
<td>15</td>
</tr>
<tr>
<td>Haemopoietin-1</td>
<td>HP-1</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 1.1 Previous Designations of Interleukin-1
There are known to be many cellular sources of interleukin-1 but the major laboratory source is from monocytes (mononuclear phagocytes, of which the macrophage is an example). Interleukin-1 is an inducible protein, i.e. only produced in the presence of certain stimuli. For example, when monocytes are treated with lipopolysaccharide (LPS, a component of bacterial cell walls) IL-1 activity is detectable intracellularly within 30 minutes and outside the cell after approximately one hour\textsuperscript{17}. On the timescale of the immune response this is extremely rapid (see fig.1.4). In fact IL-1 is thought to be the earliest cytokine produced on encounter with antigen and an obligatory mediator of a mature immune response.

![Fig. 1.4 The Primary and Secondary Immune Response](image-url)
Fig. 1.5 Selected Biological Properties of Interleukin-1

**Immune System**
- Proliferation
- IL-2 production
- IL-4
- IL-2r
- Radioprotection

**Central Nervous System**
- Fever induction
- Slow-wave sleep
- Appetite suppression

**Musculo-Skeletal System**
- Bone resorption
- Osteoclast activation
- Bone-marrow radioprotection
- Cartilage resorption
- Collagenase
- Muscle proteolysis

**Vascular Tissue**
- Endothelial cells: proliferation
- Prostaglandin
- Smooth muscle, IL-1

**Other Organs**
- Liver: Acute-phase proteins
- Pancreas: Toxicity to β-islet cells
1.1.6 The IL-1 Gene and Gene Products

Murine IL-1 cDNA was first cloned in 1984\textsuperscript{18}, and the human gene was identified soon after\textsuperscript{19-21}. March \textit{et al} \textsuperscript{22} identified and expressed two different human cDNAs encoding proteins with IL-1 activity which were designated Interleukin-1-\(\alpha\) and \(\beta\) (IL-1-\(\alpha\) and IL-1-\(\beta\)).

![Diagram of IL-1 Genes](image)

\textbf{Fig. 1.6 Interleukin-1 Genes}

IL-1-\(\alpha\) and -\(\beta\) show 26\% homology of amino-acids but 45\% at the nucleic acid level\textsuperscript{22}. The organisation of the gene is identical in both cases, with seven exons and six introns. The two genes may well have arisen from a gene duplication event\textsuperscript{23}. Each cDNA codes for a protein of \textit{circa} 31 kDa molecular weight (269 or 271 amino acids) although IL-1 activity had previously been mainly detected for a 17 kDa protein. It was shown that the 17 kDa species comprised the C-terminal region of the larger molecule in each case\textsuperscript{22}, so it seemed probable that IL-1-\(\alpha\) and -\(\beta\) gene products were precursors which were processed to the active forms.

1.1.7 Receptors for IL-1

The initial report of a specific IL-1 receptor was published in 1985 when Dower \textit{et al} \textsuperscript{24} showed binding of radiolabelled IL-1 to murine T-cells. Further work estimated that there were approximately five hundred receptors per cell which bound IL-1-\(\alpha\), IL-1-\(\beta\) and the precursor to IL-1-\(\alpha\) (IL-1-\(\alpha\)-pre) with identical affinity (\textit{kM} =10\textsuperscript{-10} M) and kinetics but which did not bind IL-1-\(\beta\)-pre\textsuperscript{25}. This receptor has since been identified on several different cell types\textsuperscript{26,27} and cloned from human\textsuperscript{28} and murine\textsuperscript{29} T-cells.
The cDNA codes for a protein of 576 amino acids comprising three domains\textsuperscript{30} (see fig. 1.7). The cytosolic domain of this receptor has no recognisable enzymic activity\textsuperscript{30}. Any signal transduction via secondary messengers will probably involve another protein bound to this domain. Internalisation of IL-1 following receptor binding is also possible. Some intact IL-1 has been detected at the cell nucleus\textsuperscript{33,34} as have IL-1 receptors\textsuperscript{35}.

Notes

i-iii: Three extracellular domains belong to the immunoglobulin superfamily\textsuperscript{30}. Presumably responsible for ligand binding.

iv: Intracellular domain may be linked to an additional effector protein\textsuperscript{31,32}.

G: Glycosylation accounts for the observed variability in M.Wt\textsuperscript{27}.

Fig. 1.7 The 80 kDa IL-1 Receptor

A distinct receptor was described by Matsushima \textit{et al}\textsuperscript{66} of 60 kDa molecular weight, reducible to 48 kDa on deglycosylation\textsuperscript{37}. This receptor has so far only been reported on B-cells. It shows a lower affinity for IL-1-\(\alpha\) than IL-1-\(\beta\), both being less tightly bound than for the 80 kDa receptor.

A third class of IL-1 receptor has been partially purified and characterised\textsuperscript{38}. This has lower affinity for IL-1 than the 80 kDa receptor but binds IL-1-\(\beta\)-pre as well as mature IL-1-\(\beta\).

Like IL-1 itself, the IL-1 receptor is inducible (at least for the 80 kDa form). Its concentration at the cell surface is affected by stimuli such as LPS\textsuperscript{27,39,40}. Unstimulated cells bear very low levels of IL-1r\textsuperscript{24}. This system is characteristic of several cytokines\textsuperscript{41} and has been particularly well studied in the case of interleukin-2.
1.1.8 The Structure of Interleukin-1 Proteins

*In vitro* IL-1-α and β mediate the same responses in all systems studied (though as we saw in section 1.1.7 the precursors do behave differently). As they can share the same receptor a comparison of their structures might be expected to indicate those areas of the molecules which are responsible for receptor binding and hence biological effects.

Analysis of the primary structure of the two precursor molecules revealed no strongly conserved regions of homology and hence little evidence for the position of shared functional domains. The main similarities were in fact found in the N-terminal region of the precursors, in the segment which is removed to give the mature proteins.

The tertiary structure of the two proteins has also been investigated. Priestle *et al.* reported the crystal structure determination of mature IL-1-β in 1988 and later refined their analysis to 2.0Å. Nuclear magnetic resonance (NMR) spectroscopic analysis of a solution of mature IL-1-β was in agreement with the solid-state structure. IL-1-β is a compact globular protein containing twelve β-strands which form a barrel-like structure. The overall folding pattern is very similar to that of soybean trypsin inhibitor, and correlated well with structure predictions based on circular dichroism (CD) and fluorescence measurements.

![Interleukin-1-β Structure](image)

Fig.1.8 Interleukin-1-β Structure
The crystal structure of mature IL-1-α was solved in 1990\textsuperscript{47}. The overall shape of the molecule is identical to IL-1-β, again taking up the β-barrel conformation (the "IL-1-fold"\textsuperscript{47}). The N-termini of the two mature proteins are positioned differently. This may offer an explanation of the differential activity of the precursor molecules. Some functional region of IL-1-β may be hidden by the additional residues in the precursor whereas the homologous region of IL-1-α-pre may be available for receptor binding.

CD studies on IL-1-β-pre have been carried out\textsuperscript{48} and indicate that this protein has some α-helical character. It seems likely that the additional 116 N-terminal residues form a new domain.

Comparison of the tertiary structures reveals little more on the question of functional sequences. Most of the conserved residues lie in the hydrophobic core of the respective molecules where they are not available for interaction with the receptor.

Various other techniques have been applied to the problem of identifying functional areas of IL-1. Point mutations have been introduced at various positions in the IL-1 gene but in most cases both folding and biological activity remained unchanged\textsuperscript{44,49}. Histidine-30 of IL-1-β was shown to be important in stabilising part of the protein structure\textsuperscript{50}, and replacing arginine-127 with glycine reduced bioactivity\textsuperscript{51}. Truncated IL-1 molecules have been tested showing that the loss of more than a few residues from either terminus considerably reduces the function of both IL-1-α and -β\textsuperscript{52-4}. This is probably due to disruption of the IL-1 fold\textsuperscript{55}.

1.1.9 IL-1 Production

Regulation of IL-1 levels is a possible mechanism by which the in vivo immune response is controlled. There are many potential steps leading to IL-1 production at which this control could be exerted (see figure 1.9).
LPS-induced IL-1 production is blocked by actinomycin and cycloheximide indicating de novo RNA synthesis\textsuperscript{17}. Also IL-1-\(\beta\) mRNA is produced more quickly and to a higher level than IL-1-\(\alpha\) mRNA\textsuperscript{17}. Thus some control is exercised at the transcriptional level. Analysis of the IL-1 genes has identified several potential promoter motifs and other regulatory sites\textsuperscript{23}. Possible transcriptional activator\textsuperscript{23} and suppressor\textsuperscript{56} proteins have been identified. It has been reported that transcription of the IL-1 gene may occur without induction of protein synthesis\textsuperscript{57}. This may be a result of post-transcriptional modification affecting the half-life of the resultant mRNA\textsuperscript{58}.

IL-1 must be secreted from the producing cell in order to have biological effect, but unlike most secretory proteins\textsuperscript{60} no form of IL-1 has a recognisable signal sequence. The kinetics of IL-1 secretion are unusual\textsuperscript{61} and there is some evidence that an alternative mechanism of exportation
may operate. Several other proteins have also been identified as non-standard secretory products and many parallels with IL-1 exist in these cases.

A: most secreted proteins are exported (i) via the endoplasmic reticulum. A hydrophobic signal sequence helps passage through the membrane and is cleaved at the same time. Other post-translational modifications such as glycosylation (G) are also carried out at this stage.

B: proteins lacking a signal sequence have been postulated as being exported by two methods: (ii) membrane-bound proteolytic activity releases part of the protein to the supernatant; or (iii) the protein is enclosed in a vacuole. This fuses with the membrane expelling the protein.

Fig 1.10 Secretion of Proteins

The IL-1-β gene has been transfected into a rodent cell-line and engineered such that the precursor protein was constitutively produced. No extracellular IL-1 activity was detected. It would appear that the ability to secrete IL-1 is specific to those cells which normally produce it.

Cell death and lysis have been postulated as alternative pathways for IL-1 release but studies of IL-1-producing cell cultures have failed to identify
other cytosolic proteins which would be expected to escape if such a passive route was used.

The final link in the chain of events leading to IL-1 production is the proteolytic processing of the IL-1 precursor molecules. Studies in this area have largely concentrated on IL-1-β, following the observation that IL-1-α-pre was active and bound to the T-cell (80 kDa) receptor.

The site of IL-1-β-pre cleavage in monocytes was characterised by Cameron et al. (see figure 1.11). Subsequent studies have characterised several products of IL-1-β-pre proteolysis of approximately the same size as mature IL-1-β. None of these gave full IL-1 bioactivity though in general the closer to the correct cleavage the more active the product. It was therefore suggested that there was a specific protease which cleaved IL-1-β-pre to give the fully active protein. However the fact that other proteolytic activities could give considerable IL-1 activity again raises the question of the nature of the secreted form of IL-1. Though the in vitro evidence suggests that secretion and processing events are active and specific for IL-1-β it cannot be ruled out that in vivo cell lysis and non-specific cleavage is a major route for the generation of IL-1 activity, indeed it is possible that incorrectly processed IL-1 is a characteristic of certain disease states.

![Fig. 1.11 Proteolysis of IL-1-β](image)

Recently workers at Merck in the U.S. have succeeded in partially purifying an IL-1-β-converting enzyme (which they have called ICE) which cleaves the IL-1β precursor at the correct point for full biological activity. This enzyme is located in the cytosol of monocyte and monocyte-like cell-lines. The specificity of ICE has been investigated by these workers and
by a team from the Immunex Corporation of Seattle\textsuperscript{72,73}. The results are summarised in fig. 1.12 and show that the enzyme is highly selective with regard to the primary sequence of the substrate.

<table>
<thead>
<tr>
<th>Peptide Substrate</th>
<th>Rate of Cleavage</th>
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<tr>
<td>A Y V H D A P V R S</td>
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<tr>
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<tr>
<td>............E............</td>
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<tr>
<td>............G............</td>
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<tr>
<td>............V............</td>
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<td>............F............</td>
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<tr>
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<tr>
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<tr>
<td>Y V H D A P V R</td>
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<tr>
<td>A Y V H D A P V R S</td>
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</tr>
<tr>
<td>E A Y V H D A P V R S L</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Fig. 1.12 Substrate Specificity of ICE

When the precursor to IL-1-β was denatured by heat, specific cleavage still occurred\textsuperscript{73}. This and the fact that relatively short synthetic peptides are also substrates suggests that the overall tertiary structure is of little importance to the enzyme activity. The sensitivity of the enzyme to peptide length does suggest that elements of local secondary structure may be important. The convertase has not yet been fully purified and very little characterisation data has been published, though it has been reported to be a member of the serine protease family.
1.1.10 Modulators of IL-1 Activity

As IL-1 is such a key mediator of the immune response it would be expected that its level would be carefully regulated in vivo. Control at the various steps of production was discussed in the last section, but there is also a need for post-production control mechanisms. These generally control the effective concentration of IL-1 by altering protein/receptor interactions. Blocking of IL-1 receptor-binding may be achieved by molecules which have affinity for either IL-1 or its receptor.

1.1.10.1 IL-1 Receptor Antagonists

IL-1 antagonist activity has been observed from a number of sources for instance the urine of febrile patients, synovial fluid, virus-infected cultures, or UV-treated biological samples. In most cases the nature of the inhibitory species has not been determined, but in two cases the activity has been characterised. Seckinger et al. isolated a protein from the urine of patients with monocytic leukaemia and showed that it competed with IL-1 for binding sites on a T-cell clone.

More recently Hannum et al. identified three monocyte-derived IL-1-receptor antagonist proteins (IL-1ra or IRAP) which turned out to be differentially glycosylated forms of the same protein. All three species bound equally well to IL-1r-bearing cells with a similar affinity to both forms of mature IL-1. Cloning of the IL-1ra gene indicated that the protein was related to IL-1-α and -β (19 and 26 % amino-acid homology; similar gene organisation) though it possessed a recognisable signal sequence (hence the glycosylation). The protein showed no IL-1 agonist activity in several recognised assays.

It was later shown that this IL-1ra did not bind to B-cells, nor did it show antagonist (or agonist) activity in another assay. This fits well with the results discussed in section 1.1.7 indicating the existence of distinct classes of IL-1 receptors. It also suggests that different parts of the IL-1 molecules may be responsible for different biological effects.

Once again most conserved residues between this IL-1ra and the two forms of IL-1 lay in the hydrophobic core, but Eisenberg et al. showed
that the C-terminal region had a similar hydrophobicity plot to the analogous region of IL-1-β. A synthetic peptide comprising part of this sequence, IL-1-β-233-269 (the C-terminal 33 residues of both IL-1-β and its precursor) prevented thymocyte activation when present in an IL-1 assay at micromolar levels. Other studies with shorter peptides have shown antagonist activity but only when present at high concentrations in unusual model systems. The exact nature of the inhibition in these reports was not investigated.

1.1.10.2 IL-1-Binding Proteins

Uromodulin is a glycoprotein found in the kidney and urine which has been shown to bind IL-1. Its location suggests that this may be a mechanism for the excretion of IL-1. The major source of elimination of administered radiolabelled IL-1 has been shown to be via the urine.

Soluble forms of cytokine receptors have been known for some time, and have been shown to compete with the membrane-bound receptor for ligand, thus exerting inhibition of the ligand's effects. A protein of this type was recently identified which derives from the 80 kDa IL-1r by proteolytic cleavage on the extracellular side of the membrane-spanning domain. This soluble form of the IL-1 receptor (sIL-1r) shows unchanged binding properties to intact IL-1r.

Autoantibodies to IL-1-α have been detected in healthy individuals. It is not clear what effect this has on biological activity. Anti-interferon-1 has been administered together with interferon-1 with no loss of anti-viral activity. It seems possible that such binding proteins protect cytokines from degradation.

Such a role has long been suggested for α2macroglobulin, a serum protein which binds other active proteins. The binding of IL-1 to this protein has been shown to accompany resistance to proteolysis.
1.1.11 Pathological and Physiological Roles of IL-1

Most studies on the biological properties of IL-1 have focussed on its role in adaptive immunity, and this is indeed a key function of the molecule. Analysis of IL-1 genes in various species indicate that the molecule appeared somewhat before lymphocytes and antibodies in evolutionary history$^{95}$, suggesting a more fundamental role for IL-1.

Inflammation is the main evidence of an *in vivo* immune response. Four main things happen to cause this state:

- blood supply to the affected area is increased;
- increased permeability of capillaries allows soluble immune mediators to reach the site;
- leukocytes migrate towards the site of infection;
- local or body temperature rises (fever).

These are all innate processes, and all are known to be IL-1 dependent$^{96}$. They ensure the optimal ability of the body to respond to invasion by foreign organisms.

An ideal immune response would restore the full function and integrity of the site of infection, but inflammation has a fundamentally dualistic nature - the repair process is accompanied by undesirable side-effects including attack on healthy tissue and excess tissue deposition. This is normally of little importance and a small price to pay for the protection of the rest of the body, but several disorders are characterised by inappropriate and damaging immune responses.

Allergies such as hay-fever result from an over-zealous response to a relatively innocuous allergen (pollen granules). A similar, if rather artificial effect is observed in organ transplantation and tissue grafts in general. The recipient displays immunity to the graft to a greater or lesser extent depending on the apparent "foreignness" of the donated tissue, resulting in graft rejection. If substantial numbers of leukocytes are present in the transplanted tissue they can mount an immune response against the recipient. This graft-versus-host reaction can prove fatal.
Notes

1 Recognition of antigen results in the production of immune mediators such as IL-1.
2 T-cell activation results in the production of further soluble immune mediators.
3 Endothelial cells retract allowing effector cells to leave circulation and move towards the site of infection.
4 B-cell activation initiates antibody production.
5 Soluble immune mediators enter the bloodstream with local and hormonal effects

Fig. 1.13 The Site of Inflammation
More common and probably more damaging to society are the autoimmune diseases. This class of disorders are characterised by prolonged and harmful immune response in the absence of detectable infectious agents. Many diseases fit this pattern, e.g. juvenile (type-1) diabetes, pernicious anaemia, thyroiditis and many others. Mechanisms which have been suggested for these illnesses include the breakdown of intercellular communication, the production of abnormal antibodies and the excess production of pro-inflammatory mediators, of which IL-1 is a prime suspect. The role of IL-1 in many such diseases has been reviewed by DiGiovine and Duff. One particularly prevalent and well-studied autoimmune disease is rheumatoid arthritis.

1.1.12 IL-1 and Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a disease characterised by chronic inflammation of the synovial membrane of articulated joints. The resulting damage done to neighbouring cartilage and bone leads to severe curtailment in the use of the joint. Systemic features are also observed, such as fever, muscle wasting and weight loss.

It had long been suspected that IL-1 might be important in RA since many of the cytokine's in vivo and in vivo activities mirrored RA symptoms. Endogenous pyrogen activity was detected in synovial exudates in 1967, and other factors from table 1.1 were identified in the same fluid. The discovery of the common identity of these factors led to attempts to correlate the level of IL-1 with disease activity. Most early studies proved inconclusive because assays depended on measuring IL-1 bioactivity. Interfering factors in complex clinical samples made results impossible to assess quantitatively. Furthermore, IL-1-α and -β behave identically in such analyses.

The advent of immunoassay has permitted quantitative determination of both forms of IL-1, and application to RA has proved notably fruitful. High levels of IL-1-α and -β have been detected in RA synovial fluid, with the concentration of the β form being approximately ten-fold higher.
than that of the α form. This relationship has been independently observed in most studies and also holds for mRNA levels. The reason for the differential production of the two forms is unclear, as are the biological consequences.

Measurement of IL-1-β concentration in the plasma of RA patients revealed significant correlation with Ritchie joint score (a measure of joint movement) and several other indicators of disease state. The level of IL-1-β in individual patients varied dramatically over a period of several weeks, being typically high on admission to hospital (corresponding to a flare-up in stiffness and pain), falling steadily as the symptoms declined, then often rising again. The correlation of serial measurement of IL-1-β with Ritchie score is shown in figure 1.14.

![Figure 1.14 Correlation of IL-1-β and Ritchie Score](image)

The level of IL-1-α detected in these studies was, typically, one order of magnitude less than the level of IL-1-β, and only a low correlation with RA symptoms was observed.

These results do not provide evidence for or against a causative role for IL-1-β in RA, but indicate that elevated IL-1 release is pathogenic independent of its place in a chain of events leading to clinical disease.
1.2 Solid-Phase Peptide Synthesis

1.2.1 General

Peptides were originally defined as being derived from the digestion of proteins. The name "protein" reflects the recognition of the crucial importance of these compounds to biology. Proteins are responsible for the structural integrity, homeostasis, growth and proliferation of all organisms on earth. Even the replication and translation of the genetic message, though carried by nucleic acids, is under the control of proteins.

The terms peptide and protein are not mutually exclusive. Mature interleukin-1-β is by definition both a peptide and a protein. Generally a peptide is of low molecular weight, though again "low" is open to interpretation. One hundred residues is a generally accepted cut-off point. The problem is really one of semantics and not biology.

Solid-phase peptide synthesis (SPPS) is one of several "chemical" methods for making peptides, and there are many other "biological" routes to obtaining these useful molecules.

SPPS was conceived by Merrifield in 1959 and has gone on to become the predominant chemical means of producing peptides. Successful application of this advance could not have taken place without a thorough understanding of the principles of peptide synthesis, a science already almost 60 years old at the conception of SPPS.

1.2.2 Principles of Peptide Synthesis

The smallest peptide comprises two alpha-amino acids joined by an amide bond (1). All proteins contain amino acids linked in this manner.

\[
\text{H}_3\text{N} \quad \text{H} \quad \text{R} \quad \text{H} \quad \text{COO}^- \\
\text{H} \quad \text{N} \quad \text{H} \quad \text{COO}^- \\
\text{R} \quad \text{H} \quad \text{COO}^- \\
\text{R} \quad \text{H} \quad \text{COO}^-
\]

Fig. 1.15 A Dipeptide With the Peptide Bond Indicated
A mixture of two amino acids and a coupling reagent reacts in a random manner to give a complex mixture of products. Many of the amino acid side-chains carry functional groups (e.g. 7 and 8) which may also react, increasing the number of possible products.

\[
\text{H}_3\text{N}^-\text{COO}^- + \text{H}_3\text{N}^-\text{COO}^- \overset{\text{(2)}}{\rightarrow} \overset{\text{coupling reagent}}{\downarrow} \overset{\text{(3)}}{\rightarrow} \overset{\text{(4)}}{\rightarrow} \overset{\text{(5)}}{\rightarrow} \overset{\text{(6)}}{\rightarrow} + \text{other dimers, trimers, and higher polymers}
\]

**Fig 1.16 Ambiguity in Coupling Amino Acids**

Nature manages to assemble proteins from their constituent monomers in an unambiguous manner, but chemists have to protect certain parts of reagent molecules and activate others to achieve the desired result. Fig. 1.18 shows a scheme for preparing a dipeptide using amino acids 7 and 8.

**Fig 1.17 Amino Acids With Side-Chain Functionality**
Fig 1.18 Unambiguous Coupling of Lysine and Glutamic acid

Activation of the carboxyl component is achieved by replacing the hydroxyl group with an electron-withdrawing substituent. These will be discussed in section 1.2.4. There is no good method for activating the amine component.

In order to produce peptides containing three or more amino acids the nature of the protecting groups must be considered in more detail. One of the termini of the dipeptide must be freed whilst leaving all other protecting groups intact. For a number of reasons which are discussed in section 1.2.3 it is usually best to remove the protection on the \( \alpha \)-amino group. Temporary \( \text{N}^\circ \)-protection is dealt with in section 1.2.5 and semi-permanent protection in section 1.2.6.

1.2.3 Strategy of Peptide Synthesis

Three possible routes to a tetrapeptide are shown in figure 1.19. Assuming that the yield for each coupling is 90\%, then the overall yield of ABCD in strategy i) (fragment condensation) is 81\% as opposed to 73\% in either strategy ii) or iii) (stepwise condensations).
Fig. 1.19 Strategies for Tetrapeptide Synthesis

For the octapeptide ABCDEFGH the corresponding yield in a strategy i)-type scheme is 73% and only 48% in the other two cases. It would seem that fragment condensation methods are fundamentally better. However some chemical aspects must be considered.

It can be seen that strategies i) and iii) require the activation of the carboxyl terminus of a peptide fragment. By contrast, the active species in strategy ii) will be a Nα-protected amino acid. This is of crucial importance because racemisation can take place.

Racemisation occurs principally via proton abstraction following 5(4H)oxazolone (14) formation (fig. 1.20) from the activated derivative, and this is suppressed when suitably protected amino acids (section 1.2.5) (rather than peptides) are activated. This is a major reason why strategy ii), C→N stepwise condensation has become the most common method for chain assembly.
1.2.4 Activation and Coupling Methods

Many different methods for activating carboxyl groups have been applied in peptide chemistry\textsuperscript{111}. The general form of an activated amino acid is shown in fig 1.21.

The group $X$ should be electron-withdrawing, rendering the carboxyl carbon more electrophilic. The stronger this polarisation, the faster the coupling will proceed. However, strong activation also favours racemisation by an analogous pathway to fig 1.20. An efficient coupling method should not overactivate the carboxyl group. Amino acid chlorides were used in one of the very first attempts at peptide synthesis\textsuperscript{112}, but successful applications have been extremely limited because the activation they provide is too powerful.
Acid azides (18)\textsuperscript{113} have retained favour as coupling agents for somewhat longer. Their more gentle activation normally gives negligible racemisation, though a troublesome side-reaction (fig 1.22) means that careful precautions must be taken in their usage.

[Chemical equation]

\[ R\overset{\text{N}=\text{N}=\text{N}}{\rightarrow} R'\cdot\text{NH}_2 \]

Fig. 1.22 The Curtius Rearrangement

Various anhydrides have been used with some success. Several reviews of this method have been published\textsuperscript{114}. Examples of mixed anhydrides which have been successfully applied are shown in fig 1.23.

[Table]

<table>
<thead>
<tr>
<th>Ref.</th>
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<th>R</th>
<th>PG·NH</th>
</tr>
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<tbody>
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<tr>
<td>113</td>
<td>(CH\textsubscript{3})\textsubscript{3}CCO\textsuperscript{2-}</td>
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<td>114</td>
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<td>116</td>
<td>(Ph)\textsubscript{2}PO\textsuperscript{2-}</td>
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Fig 1.23 Mixed Anhydrides For Peptide Synthesis

Considerations of regioselectivity, important in the mixed anhydrides become irrelevant if the symmetrical anhydride of each amino acid is used. These give good activation with little racemisation and have found widespread use. They can be conveniently prepared by treatment of protected amino acids with reagents such as dicyclohexyl\textsuperscript{-120} or diisopropylcarbodiimides\textsuperscript{121}.
Esters of amino acids have only one activated carbonyl group and thus also give unambiguous acylation of the amine component. The activation provided in these cases is distinctly less than in anhydrides but this minimises racemisation and some other side-reactions. N-Hydroxysuccinimide (23), pentafluorophenyl (25) and 1-hydroxybenzotriazole (24) derivatives have been particularly widely used.

\[
\text{PG-NH}_2 \underset{\text{O}}{\overset{\text{O}}{\text{O}}} \text{NH-PG}^{(22)}
\]

**Fig 1.24 Symmetrical anhydride of amino acid**

1.2.5 N\textsuperscript{\alpha}-Protecting Groups

The main requirements for N\textsuperscript{\alpha}-protecting groups were introduced in sections 1.2.2 and 1.2.3, viz:

- they must be easily removable in conditions which leave other protecting groups and all peptide bonds intact;
- they must prevent acylation at the protected amine
- they must prevent the activated amino acid from racemisation.
Various \(\text{N}^\alpha\)-protecting groups are discussed in ref 125.

The urethane group (26) was recognised as a good potential \(\text{N}^\alpha\)-protecting group very early in peptide synthesis. These derivatives decrease the nucleophilicity of the \(\alpha\)-nitrogen and hence minimise racemisation via oxazolone formation (see fig. 1.20). Initial cleavage of the urethane ester linkage results in formation of a carboxylic acid (27) which spontaneously decarboxylates to the free amine (fig. 1.26)

![Fig. 1.26 Urethane Cleavage](image)

The first practically applicable urethane \(\text{N}^\alpha\)-protection to be introduced was the benzyloxy carbonyl or Z group (29)\(^{126}\) which can be removed by either hydrogenolysis or acidolysis (fig. 1.27).

![Fig. 1.27 Removal of Z group](image)

Tertiary butyloxy carbonyl (Boc) derivatives (32) were the prototypic highly acid-labile urethane \(\text{N}^\alpha\)-protected amino acids\(^{127}\). The sensitivity to acid arises because of the stable \(t\)-butyl carbocation which is produced (33). 2-(4-Biphenyl)propyl(2)oxycarbonyl (Bpoc) derivatives (34)\(^{128}\) are even more
acid-labile. The Z group is stable under the conditions for cleavage of these groups, thus allowing the use of benzyl-derived side-chain protection with Boc Nα-protection.

Fig. 1.28 Acid-Labile Urethanes

Urethanes are also available which readily lose a proton to give a stabilised anion. These are deprotected under basic conditions via β-elimination. Foremost among this class are the 9-fluorenylmethoxycarbonyl (Fmoc) derivatives (35).
This base-lability offers a further dimension for peptide chemistry since true orthogonality can be achieved in the conditions required for cleavage of the $N\alpha$- and side-chain protecting groups.

1.2.6 Semipermanent Protecting Groups

Up to this point only generic aspects of peptide chemistry have been discussed. No account has been taken of the diverse nature of individual amino acids. The different side-chain functional groups may sometimes affect the choice of coupling method or $N\alpha$-protection, but most generally applied techniques work well with all amino acids.

The specific properties of each amino acid must be taken into account in the choice of semipermanent side-chain protecting groups. The requirements for protection are shown in table 1.2.

Extensive reviews of side-chain protecting groups in peptide synthesis are available\(^{130}\) and a review by Bodanszky and Martinez gives a clear insight into the individual nature of each amino acid\(^{131}\).

1.2.7 The C-terminus: The Solid-Phase Principle

The C-terminal carboxyl group is chemically very similar to side-chain carboxylic acids, and was most often protected in an identical manner though orthogonal protection was available if required\(^{108}\).

In 1963 Merrifield introduced a new C-terminal "protecting group" in the form of a functionalised insoluble polymer, chloromethyl polystyrene crosslinked with a small amount of divinylbenzene\(^{109}\). This resulted in him being awarded the Nobel prize for chemistry in 1984\(^{133}\).

The process was designed to combat some fundamental problems faced by solution-phase methodology, namely the extremely difficult and time-consuming process of purifying intermediates and the poor solubility of large protected peptide fragments.
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<td>Glutamine</td>
<td>Optional</td>
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<tr>
<td>Asparagine</td>
<td></td>
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<tr>
<td>Methionine</td>
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<tr>
<td>Tryptophan</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Alanine</td>
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<td>Valine</td>
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<td>Leucine</td>
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<td>Phenylalanine</td>
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<td>Proline</td>
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Table 1.2. Side-Chain Protection Requirements

Following attachment of the C-terminal amino acid to the polymer, chain assembly takes place towards the N-terminus, with all excess soluble reagents and by-products being removed by filtration and washing of the peptide-"resin". Treatment with acid then gives the desired peptide.

Merrifield's classical SPPS scheme is shown in fig 1.30 and the modified scheme for use with Fmoc-amino acids in fig. 1.31.
Fig 1.30 Merrifield Solid-Phase Peptide Synthesis (SPPS)
Fig. 1.31 Modified SPPS
These protocols confer several practical advantages over solution-phase syntheses:

- purification of intermediates is eliminated
- reactions can be driven to completion by the use of excess reagents
- vessel transfer is eliminated thereby minimising mechanical losses
- automation is possible.

These advantages have led to the development of peptide synthesiser machines which can automatically assemble protected peptide chains from suitably derivatised amino acids, greatly easing syntheses. Merrifield's introduction of SPPS is arguably the biggest single advance in the history of peptide synthesis. Many more and longer peptides could be produced and applied to research into biological processes. This method of peptide synthesis is now by far the major means for producing these important molecules.
Chapter 2. Discussion

2.1 Study Aims

The following observations are a summary of the pathophysiological role of interleukin-1 and the reasons for the considerable academic and industrial interest in this cytokine.

1. Inflammation can become damaging if uncontrolled for some reason, e.g. in rheumatoid arthritis and other autoimmune diseases.

2. IL-1 is the earliest detected mediator of the immune response.

3. Observed IL-1 activities match the damaging effects of inflammation.

4. IL-1 levels show positive correlation with the severity of a number of disease symptoms.

5. IL-1 activity may be controlled by a complex set of mechanisms, at the level of transcription, translation, processing and receptor binding.

Based on these observations we have set out to gain an insight into IL-1 production and regulation, and to try and modulate IL-1 activity with a view towards identifying potential anti-inflammatory actions.

The difference between the two forms of IL-1 has been a topic of much debate for some time. IL-1-α and -β behave identically in all assays which have so far been described, and are produced in response to the same stimuli. It might therefore be expected that only one such protein would be needed to mount a mature immune response, and that the other form would have been lost in evolution.

This pairing system is not unique in cytokine biochemistry. The two tumour necrosis factors TNF-α and -β are structurally related proteins which share the same receptor and apparently identical biological properties. The TNFs have many functional homologies to IL-1 and are also produced very
early in the immune response. It is interesting that these related proteins should have such similar organisation.

Other cytokines also appear to show this pairing homology\(^{35}\). It appears to be a conserved feature of the evolution of this class of proteins, though the driving force remains unclear.

Several mechanisms can be conceived of as to why two IL-1s are better than one. Firstly it may act as a fail-safe. If each IL-1 is independently capable of eliciting an effective immune response then even if one system is eliminated or eluded the other will still protect the body. Secondly the fail-safe could serve to protect the body's own tissues from the damaging effects of inflammation by only mounting an immune response when both signals were received. Thirdly the double system may somehow accelerate the onset of the immune response thus clearing invading organisms more quickly. Some combination of these and other reasons may provide the driving force for the conservation of two IL-1s.

One important difference between IL-1-\(\alpha\) and -\(\beta\) is in the activity of their respective precursor molecules, the precursor to IL-1-\(\alpha\) being an IL-1 agonist and binding to IL-1 receptor whilst IL-1-\(\beta\)-pre is inactive and does not bind the receptor. Thus inhibiting the proteolytic processing of both IL-1s would remove IL-1-\(\beta\) activity but leave IL-1-\(\alpha\) activity. It is probable that both forms of IL-1 are cleaved by separate, specific enzymes as there is little homology between the two proteins around their respective sites of proteolysis.

![Cleavage Site](image)

**Fig. 2.1 The Cleavage Regions of IL-1-\(\alpha\) and -\(\beta\)**
Hence it should be possible to design agents which will selectively prevent the processing of IL-1-β, offering the possibility of studying an immune response in the absence of this protein. This would be a useful step towards an understanding of the *in vivo* immune response.

Our approach to the design of ICE inhibitors (interleukin-1-β converting enzyme) was set out in two stages. Firstly synthetic subpeptides of IL-1-β were to be prepared in an effort to find those parts of the precursor molecule which were involved in interaction with the enzyme. Secondly, chemical modifications would be introduced in the most important of these subpeptides to produce tight-binding inhibitors of the enzyme. The work presented in this thesis relates entirely to the first stage of this programme.

### 2.2 Synthesis of IL-1-β-pre Subpeptides

#### 2.2.1 Initial Target

The initial aim was to make a peptide which contained the cleavage site of IL-1-β-pre and included a similar number of residues on either side of it. The peptide should be of sufficient length such that it may be expected to possess secondary structure similar to that region of the natural precursor, and hence be likely to interact with the processing enzyme.

The sequence of this first peptide and its relationship to mature IL-1-β and IL-1-β-precursor are shown in fig. 2.2.

#### 2.2.2 Peptide Synthesis

The principles of solid-phase peptide synthesis are described in section 1.1. The general strategy employed in the synthesis of all IL-1-β-precursor subpeptides described here was the orthogonal protection scheme (modified Merrifield SPPS) as shown in fig. 1.31.
Precursor to Interleukin-1-β

MAEVPLASE MMAYYSQNED DLFFEADGPK GMKCSQGDL DCPDGGGIGL
RISDHYSKG FRGAAAVVVA MDKLRKMLVP CGQTFGENDL STFFPFIFEE
EPIFFDTWDN EAYVHDAPVR SLNCTLRDSQ GKLVSMPGSY ELKALHLGGQ
DMEGGVVFSM SFVQGEESND KIPVALGLKE KNLYLSFLVLK DDKPTLGQES
VDPKNYPKKK MEKRFVFNKH EINNKLEFES AQFPNWYIST SGAENMPVFL
GGTKGGQDIT DFTMQFVSS

IL-1-β

APVR SLNCTLRDSQ GKLVSMPGSY ELKALHLGGQ
DMEGGVVFSM SFVQGEESND KIPVALGLKE KNLYLSFLVLK DDKPTLGQES
VDPKNYPKKK MEKRFVFNKH EINNKLEFES AQFPNWYIST SGAENMPVFL
GGTKGGQDIT DFTMQFVSS

cleavage site

101 EPIFFDTWDN EAYVHDAPVR SLNCTLRDSQ GKLVSMPGS 138

Fig. 2.2 IL-1-β Proteins and Initial Target

Fmoc-amino acids were coupled in a stepwise manner to a peptide chain anchored to the solid support by the Wang linker \(^{136}\) (fig. 2.3). Electron donation from the \(p\)-oxygen enhances the acid-lability of the peptide-resin link. Acid-labile side-chain protecting groups were based on the tert-buty group, except in the case of arginine (Pmc, 58) \(^{137}\), histidine (trityl) \(^{138}\) and cysteine (Acm, 59) \(^{139}\). In this case the carboxamide side-chains of asparagine and glutamine were left unprotected. Use of the acetamidomethyl (Acm) group for cysteine protection allows the free thiol to remain masked during purification of the peptide, preventing oxidation and dimerisation to cystine. Acm can be removed when desired by mercuric ions at pH \(^{4139}\).
Fig. 2.3 Cleavage of a Peptide From the Wang linker
Fig. 2.4 Activation of Fmoc-Amino Acids
The peptide chain was elongated by one amino acid residue using an automated double-couple cycle. In most cases the first coupling used preformed symmetrical anhydrides (63) which were generated immediately before use by treating the Fmoc- amino acid (60) with diisopropylcarbodiimide, thus forming the symmetrical anhydride (63). The second coupling included 1-hydroxybenzotriazole (24) in the activation step thus forming the active HOBt ester (64). Knorr’s reagent (65) and Castro’s reagent (66) are reported to form the HOBT ester (64) more rapidly, but are more expensive and difficult to prepare. Aminolysis of either activated derivative in fig. 2.4 forms the desired peptide bond.

The use of two coupling methods has certain practical advantages which help drive reactions towards completion. In SPPS most peptide chains grow in cavities within the polymer matrix. Steric effects are enhanced under these conditions, so the highly activated symmetrical anhydride (63), which is rather bulky, may couple poorly compared to the less active but also less hindered HOBT-ester (64).

Certain amino acid derivatives are not suited to this general procedure. Glycine is prone to diacylation and is generally only coupled once. For differing reasons the symmetrical anhydrides of asparagine, glutamine, arginine and histidine derivatives are not used. These amino acids are introduced by means of two couplings with the HOBT-ester.

2.2.3 Monitoring of Chain Assembly

It is very useful to have a measure of the incorporation of each successive amino-acid so that couplings which proceed slowly or incompletely may be identified and dealt with. If couplings do not proceed in a quantitative manner then deletion and termination side-products will be produced. These may differ in their physical properties only slightly from the desired peptide and be very difficult to remove from it following cleavage from the resin support.

The goal of monitoring in SPPS is to achieve fully automated synthesis in
which the completeness of each coupling reaction is tracked and acylating conditions maintained until quantitative peptide bond formation is attained. Only then will the cycle proceed to the next amino acid.

This goal has not yet been successfully attained despite several attempts. One major difficulty is the fact that the desired product is resin-bound and thus difficult to characterise by spectroscopic means. This can be overcome if samples of the peptide-resin are removed and analysed in some way, but the additional time, manipulation and mechanical losses are undesirable and render the process less amenable to automation. Testing in this manner with ninhydrin is still a useful technique in manual and semi-automated syntheses.

Several methods measure the removal of activated amino acid from the reaction solution. This can be difficult to quantify in SPPS because reagents are generally applied in excess. Further, this technique does not give direct information about the efficiency of coupling. Decreases in concentration of the active species may be due to effects other than aminolysis, for example hydrolysis.

Because of the difficulties in monitoring the coupling step we chose to concentrate on deprotection. When Fmoc-peptide-resin is treated with piperidine the N-terminus becomes exposed with the resulting production of dibenzofulvene (37)(see fig. 1.29). Piperidine adds to this olefin as shown in fig.2.5.

![Fig.2.5 Generation of the "Deprotection Chromophore"

44
The resulting adduct (69) has a strong absorbance at 300nm, measurement of which allows calculation of the amount of Fmoc present and hence the degree of incorporation of the last amino acid.

This measurement can be utilised as a monitoring tool in several conceivable ways. Firstly as a batchwise procedure, where the effluent from deprotection can simply be passed through a colorimeter on its way to waste. Secondly as a continuous flow set-up where the deprotection solution is recirculated between the reaction vessel and colorimeter, giving time-dependent monitoring of the reaction. Thirdly as an analytical technique, whereby small amounts of peptide-resin can be removed, treated with piperidine, and the appropriate action taken depending on the amount of Fmoc present.

We have developed a system combining the simplicity of the first method with some of the information content of the second. The peptide-resin batch is treated with four separate volumes of piperidine and the absorbance of the effluent from each treatment is determined. The quantity of reagent and time of reaction are chosen so that for most peptides deprotection will proceed almost quantitatively in the first run. Substantial quantities of chromophore in subsequent runs signify an unusually slow deprotection. Fig. 2.6 shows the application of this monitoring method to the assembly of IL-1-β-pre 102-138.
Fig. 2.6 Successive Amino Acid Incorporation in the Solid-Phase Synthesis of IL-1-β-pre 102-138
Fig. 2.6 illustrates several noteworthy features of this technique. The drop in incorporation is preceded by a rise in apparent incorporation to over 100%. This drop is also preceded and accompanied by an increase in the relative absorbance of the second and third deprotection steps. The method appears to have unexpected predictive properties. Once deprotection has occurred it is too late to rectify a problem which happened at the previous coupling step, but if the next step can be predicted to be difficult then action can be taken to improve incorporation, such as using a larger excess of acylating agent or allowing coupling to proceed for longer.

2.2.4 Acidolysis of Protected Resin-Bound Peptides

Use of base-labile Fmoc Nα-protection means that the resin-peptide linker and all side-chain protecting groups may be chosen to be highly sensitive to acid. This permits relatively gentle cleavage conditions to be used. The general reagent in this case is 95% aqueous TFA, which contrasts with the final HF-based cleavage required for peptides assembled using Boc Nα-protection. This is advantageous in minimising certain side-reactions, e.g. rearrangement of aspartyl peptides (fig. 2.7)\(^{131}\), and is also a less hazardous procedure.

![Fig 2.7 β-Aspartyl Formation](image)

Cationic species are produced on acidolysis, and these can react with
nucleophilic centres to generate undesired products, e.g. the irreversible alkylation of tryptophan by the tertiary butyl cation\textsuperscript{143}.\n
\begin{align*}
\text{(73)} & \quad \text{(74)} \\
\text{H} & \quad \text{H} \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{O}
\end{align*}

Fig. 2.8 Alkylation of Tryptophan

Additional reagents are normally included in the cleavage mixture in order to minimise these side-reactions. Examples of auxiliary nucleophiles which scavenge these reactive species are shown in fig. 2.9. Suitable combinations of these (and many other) compounds are included, depending on the sequence of the particular peptide which has been assembled.

\begin{align*}
\text{OH} & \quad \text{Me} & \quad \text{CH}_3 \\
\text{Me} & \quad \text{S} & \quad \text{SH} & \quad \text{Me} & \quad \text{SH} & \quad \text{Me} & \quad \text{CH}_3 \\
\text{S} & \quad \text{SH} & \quad \text{Me} & \quad \text{SH} & \quad \text{Me} & \quad \text{SH} & \quad \text{Me} & \quad \text{CH}_3
\end{align*}

\begin{align*}
\text{phenol} & \quad \text{thioanisole} & \quad \text{ethane-1,2-dithiol} & \quad \text{ethylmethylsulphide} & \quad \text{3-methylindole}
\end{align*}

Fig. 2.9 Useful Scavengers in Acidolytic Cleavage

Another way of reducing the extent of these side-reactions is to carry out cleavage at reduced temperatures. However this also slows the desired reactions, so acidolysis is normally carried out at room temperature with a suitable scavenger mixture. Reference \textsuperscript{144} gives a practically-oriented review of cleavage procedures.

IL-1-\beta-pre 102-138 contains several residues which are subject to this type
of side-reaction, especially tryptophan, tyrosine and methionine. Because of the protecting groups used, t-butyl cations will be generated on deprotection, along with the particularly troublesome trityl and Pmc cations from histidine and arginine respectively. A suitable scavenger mixture includes water, phenol, thioanisole, EDT, EMS and N-acetyltryptophan.

The cleavage mixture for IL-1-β-pre 102-138 was analysed by HPLC at intervals. Little change in the trace was observed after 2.5 hours and this was taken to be the optimum deprotection time. The crude peptide is obtained by concentrating the deprotection mixture followed by trituration with diethyl ether. Subsequent dissolution of the material so obtained was, on occasion, troublesome. Attempts were made to extract the peptide from the cleavage mixture by treating the concentrate with water/diethyl ether mixtures and separating the phases. However IL-1-β-pre 102-138 has a rather low solubility in water, and increasing the water-soluble organic content of the mixture with methanol or acetonitrile led to inseparable mixtures.

2.2.5 Purification of IL-1-β-pre 102-138

The purification of peptides produced by stepwise solid-phase synthesis is in general a non-trivial problem. The crude peptide will be a mixture of the desired product and some combination of the following contaminants:

i) solvents, scavengers, and reagents;
ii) terminated peptides;
iii) partially protected peptides;
iv) rearranged peptides;
v) products of side-reactions.

The first type of impurity is normally relatively easy to remove from the mixture, being rather different in character to the desired product, but those in types ii) to v) are rather more troublesome. The physicochemical properties of these impurities become relatively more similar to the correct product with increasing chain length, and in general longer peptides are more difficult to purify than shorter ones.
2.2.5a Gel Filtration, or Size-Exclusion Chromatography

This simple group separation exploits differences in the size of components in a mixture. It involves passing a solution through a bed of bead-formed polysaccharide gel. Molecules partition between the mobile phase (eluant) and the stationary liquid phase within the beads of the gel. The partition coefficient depends on the rate of access to the stationary phase via pores in the beads. Large molecules enter the beads less easily than small molecules and are thus eluted more quickly. Various gel-filtration media are commercially available, differing in their structure, degree of cross-linking and mechanical robustness.

The results of Sephadex column chromatography on crude IL-1-β-pre 102-138 are shown in fig. 2.10.

2.2.5b Ion-Exchange Chromatography

Peptides carry a nett charge at all values of pH other than at the iso-electric point (pI), which is a characteristic of each peptide. The counter-ion of the peptide can be immobilised on a solid-support which can then act as the stationary phase for this type of separation. Components of the mixture will then partition between the stationary and mobile phases to an extent depending on the relative strength of interaction with the solid support. Increasing the ionic strength of the eluant or bringing its pH closer to the pI of the desired peptide results in a reduction in the strength of this interaction. A gradient using either or both of these properties will thus result in elution of components in order of pI.

Figs. 2.11a and b show the result of ion-exchange chromatography on the peptidic fraction of the gel filtration eluant.
Fig. 2.10 Gel Filtration Chromatography on IL-1-β-pre 102-138

Fig. 2.11a Anion-Exchange Chromatography on IL-1-β-pre 102-138
2.2.5c High Pressure Liquid Chromatography (HPLC)

Reversed-phase HPLC depends in principle on the hydrophobic interaction of the components in a mixture with alkyl chains (normally C₈ or C₁₈) which are bound to the solid-phase. Application of a gradient of an organic solvent in the aqueous mobile-phase results in elution of peptides of increasingly hydrophobic character. The speed of this technique and the high degree of control over the separation makes this a particularly good tool for the demanding task of separating very similar molecules.

The results of preparative HPLC on the major component after ion-exchange chromatography is shown in fig.2.12.

2.2.6 Analytical Techniques in Peptide Purification

Analysis of the products of each stage of the protocol is crucial to the success of the purification. HPLC in analytical mode was the primary method for studying the products of each step in purification. Speed, resolution and low sample requirements make this especially convenient. Fig. 2.13 shows how the sample became increasingly pure after the application of each separation technique. Electrophoresis (iso-electric focussing) was applied at each stage, and an increasingly sharp band corresponding to a pI of 4.3 was observed.

Other analytical methods are essential for confirming the purity and more importantly the identity of the product. Amino acid analysis and mass spectrometry are particularly useful. Fig. 2.14 shows how the amino acid analysis of acid hydrolysates of the various samples gave results which were closer to the predicted values at each stage of the purification.
Fig. 2.11b Cation-Exchange Chromatography on IL-1-β-pre 102-138

Fig. 2.12 Preparative Reversed-Phase HPLC on IL-1-β-pre 102-138
C-18 Silica Gel Filtration Anion Exchange Cation Exchange HPLC

Fig. 2.13 Analytical HPLC on IL-1-β-pre 102-138

After purification by:

Fig. 2.14 Amino Acid Analysis of IL-1-β-pre 102-138
Fast atom bombardment mass spectrometry (FAB-MS) gave little evidence for the presence of the correct molecular ion until after the peptide had been purified by HPLC, but the peak resulting from the required molecular ion was then the strongest in the spectrum.

2.2.7 Alternative Approaches to the Initial Target

The purification of IL-1-β-pre 102-138 was a time-consuming and difficult task yielding only 3.8 milligrams of the desired product after HPLC, an apparent efficiency of approximately 0.2%. Though this could certainly be optimised, some thought was put in to methods of easing the difficulties which had been encountered.

As the choice of the exact termini of the target peptide was somewhat arbitrary it was assumed that slightly different peptides would have similar biological properties to IL-1-β-pre 102-138 whilst possibly allowing easier purification.

IL-1-β-pre 98-138 differs from 101-138 only by extending the N-terminus of the original peptide by the sequence Phe-Glu-Glu-Glu. It was felt that introduction of this highly charged region would provide a useful "tag" for anion-exchange chromatography, emphasising the difference between prematurely terminated peptides and the desired product, and hence easing purification. Surprisingly however, this peptide had rather low solubility in aqueous solvent necessitating the use of denaturing agents and their subsequent removal. These complications made purification of this peptide even more difficult than IL-1-β-pre 102-138 and was abandoned at a relatively early stage.

Attempts to purify IL-1-β-pre 102-138 with a free cysteine were equally problematical. Aggregation and the resulting insolubility proved unsurmounted problems.

Removing the cysteine altogether and replacing it with alanine (Ala124-IL-1-β-pre 102-138) proved more practical. This change was felt to be
reasonable in light of the fact that IL-1-β-pre 102-138 showed biological activity whilst carrying the ACM group on the thiol side-chain of cysteine, implying that the free thiol was not essential. In this case synthesis proceeded rather better, and the crude peptide was purified in one step by carefully controlled reversed-phse HPLC. The resulting product gave a good analysis in comparison to that obtained by multi-step purification on IL-1-β-pre 102-138.

Fig. 2.15 Analysis of Ala124-IL-1-β-pre 102-138
2.2.8 Mapping the Cleavage Region of IL-1-β-pre

In general, peptides of the size discussed in the last section are expensive and difficult to prepare. It was felt important to define more precisely those areas of the 37-peptide which were responsible for any biological activity.

We set out to map this region of the precursor by preparing overlapping dodecapeptides as shown in fig. 2.16. This can be considered as analogous to epitope mapping of proteins.

![Diagram of peptides]

**Fig. 2.16 Target Peptides From the Cleavage Region**

Fig. 2.17 shows the incorporation of each successive amino acid in the assembly of these six peptides as determined by the absorbance of the appropriate deprotection solution.
The synthesis of these peptides was generally satisfactory. A drop in incorporation of 2-15% was observed over the first three couplings in five cases, though IL-1-β-pre 117-128 suffered a 35% drop on one particularly hindered coupling. Four of the peptides gave essentially complete acylation throughout the remainder of their syntheses, but IL-1-β-pre 117-128 had one more poor coupling (5% drop) and IL-1-β-pre 127-138 had a run of several consecutive poor incorporations. All these peptides were purified in one step by reversed-phase HPLC, and all gave satisfactory amino acid analyses, FAB mass spectra, and analytical HPLC profiles.

2.2.9 Synthesis of Peptide Amides

IL-1-β-pre 111-122 and 117-128 were also prepared as the C-terminal amide/ N-terminal acetyl derivatives. This gives a peptide which should more closely resemble the character of that segment of the protein which it is designed to model.

The Wang linker is not suitable for the preparation of C-terminal amides. Peptide-resin linkages such as those shown in fig. 2.18 are required.
Treatment with acid causes cleavage of the linker-nitrogen bond as indicated in fig. 2.18 to generate a resin-bound stabilised carbocation which is presumably scavenged in an identical manner to other cations produced in acidolysis. Assembly of peptides on resin linker 82 proceeded similarly to assembly on the Wang linker and no additional problems were encountered in the purification of the products thus obtained.

### 2.2.10 Modifications to IL-1-β-pre 111-122

Other derivatives of IL-1-β-pre 111-122 were prepared in order to assist determination of biological properties. The addition of an N-terminal aminoundecanoyl group was expected to enhance the interaction of peptide with any membrane-bound IL-1-β-pre processing activity. Introduction of the fluorescent dansyl group\(^\text{147}\) was designed to aid visualisation of the cell location of these peptide derivatives and any products thereof.
Cleavage of the appropriate peptide-resin derivative prepared as shown in fig. 2.20 gives either 11-aminoundecanoyl-IL-1-β-pre111-122-OH (84), N-dansyl-11-aminoundecanoyl-IL-1-β-pre111-122- OH (85), or dansyl-IL-1-β-pre111-122-OH (86).

The products of step ii) (fig. 2.20) were investigated by analytical HPLC using diode-array detection (fig. 2.21). Information as to the nature of each component of the mixture was derived from the UV spectra thus obtained, and revealed that dansylation had not proceeded to completion. This was in fact a practical advantage as it allowed the isolation of both compounds 84 and 85 from one preparative HPLC treatment.

There are in fact four major components in the mixture at the end of step ii), two pairs of peaks of equal intensity. A similar unexpected pattern was found in the synthesis of acetyl-IL-1-β-pre111-122-NH₂ and IL-1-β-pre 106-117.

In the case of acetyl-IL-1-β-pre 111-122-NH₂ the two products were separated by isocratic HPLC (fig 2.22). Amino-acid analyses of acid hydrolysates of these products indicated that the composition was identical, and FAB-MS of each sample confirmed this (fig 2.23). High field two-dimensional NMR showed significant differences between the two components, as shown in fig 2.24.

Conformational isomerism was suspected, for instance cis-trans forms of the peptide bond adjacent to proline. This initially seemed to fit with the NMR evidence. The most revealing evidence came from amino-acid analysis of an enzyme hydrolysate of the two products (fig 2.25). Histidine and the two adjacent amino acids valine and aspartic acid were present in rather lower amounts in one component than in the other. It would seem that the two peptidic products are epimerically related, differing only in the configuration of the histidine residue. A more thorough analysis of the NMR spectra revealed that this hypothesis fitted rather better than conformational isomerism. A similar analysis was performed on the two forms of synthetic IL-1-β-pre 106-117 and identical conclusions were arrived at.
Fig. 2.20 Dansyl/Aminoundecanoyl IL-1-B-pre 111-122

i: a) Fmoc-aminoundecanoic acid/DCC/HOBt
   b) piperidine
ii,iii: dansyl chloride
Fig. 2.21 Diode-Array Detection of Dansylated IL-1-β-pre 111-122
Fig 2.22 Purification of Acetyl-IL-1-β-pre 111-122-NH₂

Fig 2.23 FAB-MS of Acetyl-IL-1-β-pre 111-122-NH₂
Fig 2.24 2-D NMR of Acetyl-IL-1-β-pre 111-122-NH₂

Fig. 2.25 Enzyme Digestion on Acetyl-IL-1-β-pre 111-122-NH₂
Histidine is known to be particularly sensitive to racemisation. Urethane Nα-protecting groups normally ensure the chiral integrity of most amino acids under normal coupling conditions, but when Boc-L-histidine protected with a benzyl group on the N(τ) position of the imidazole ring was activated using DCC, rapid racemisation occurred\textsuperscript{148}.

Jones \textit{et al} investigated the mechanism of this troublesome side reaction and found that the π-nitrogen was capable of intramolecular proton abstraction of the α proton, as shown in fig.2.26.

Even electron-withdrawing τ-substituted protecting groups such as the phenacyl group did not entirely overcome this reaction.

N(τ)-protected histidine derivatives are, however, generally acceptable provided that rather gentler methods of activation are employed, such as the use of HOBT esters. For most of the histidine-containing peptides prepared in our laboratory the use of N(τ)-trityl-protected histidine offered no problems. The reason for the racemisation which occurred in the above noted cases is not known.

\textbf{Fig. 2.26 Racemisation of Activated Histidine Derivatives}
Better, though more expensive alternatives are now commercially available with protecting groups regiospecifically introduced at the π-position, such as the t-butoxymethyl derivative\textsuperscript{150} (89, fig. 2.27). These have been shown to give racemisation-free coupling even when activated with DCC. Compound 89 is now routinely used in our laboratory for the incorporation of histidine residues.

\[ \text{Fig. 2.27 } \text{N}_{\pi-}-(t\text{Butoxymethyl})-\text{histidine} \]
2.3 Biological Activity of IL-1-β-pre Subpeptides

Studies on the effect of these synthetic peptides on the processing of IL-1-β-pre were carried out by J. Eastgate and G. Duff of the Rheumatic Diseases Unit, Northern General Hospital, Edinburgh\textsuperscript{\textsuperscript{151}}.

The main method for the detection and identification of IL-1 proteins was Western blotting following SDS-polyacrylamide gel electrophoresis. In this technique the components of a mixture are separated on the basis of molecular weight. The gel is then blotted onto a nitrocellulose membrane, transferring a portion of the separated materials. Treating this membrane with labelled antibodies allows visualisation of all components to which the antibody will bind.

IL-1-β-production was investigated in a series of cell lines. One particular monocytic line (THP-1) was shown to produce high levels of both 31 kDa IL-1-β-precursor and the active 17 kDa form when stimulated with PMA, and was used in further studies of the processing event.

The cellular localisation of this proteolytic cleavage was determined by fractionating cell culture samples into supernatant, membrane and lysate. The ability of each fraction to cleave the IL-1-β-precursor was tested. Membrane preparations were found to liberate the largest amount of 17 kDa IL-1-β. The addition of fresh THP-1 cells to a sample of the IL-1-β-precursor also resulted in the production of 17 kDa IL-1-β.

These studies were repeated in the presence of known protease inhibitors, and in the presence of several of the IL-1-β-pre subpeptides whose synthesis was described earlier. The serine protease inhibitors aprotinin and leupeptin decreased the amount of 17 kDa IL-1-β produced from membrane preparations or fresh cell cultures as judged by Western blotting, as did synthetic IL-1-β-pre 102-138 (see fig. 2.30).
Fig. 2.29 The Cellular Location of IL-1-β-pre Processing Activity

1. Decant
2. Sonicate
3. Centrifuge, Decant
4. Add sample to IL-1-β-pre solution
5. SDS-polyacrylamide gel electrophoresis
6. Blot on nitrocellulose wash with labelled anti-IL-1-β serum

C Cell Culture
S Cell Supernatant
L Cell Lysate
M Cell Membranes
F Fresh Cells
P Solution of IL-1-β-pre precursor
A: membranes + IL-1-β-pre
B: membranes + IL-1-β-pre + aprotinin
C: membranes + IL-1-β-pre + IL-1-β-pre 102-138

Fig. 2.30 Western Blot Showing Inhibition of IL-1-β-pre Processing
Acetyl IL-1-β-pre 111-122-NH$_2$ altered the time course of 17kDa IL-1-β production in the same cultures (data not shown), apparently showing partial inhibitory activity only during the early stages of the experiment (1-2 hours). None of the other synthetic peptides showed inhibitory activity.

Fig. 2.30 shows several low molecular weight bands in lane C that are detected by the IL-1-β-pre antiserum. This suggests: i) that IL-1-β-pre 102-138 is recognised by antibodies to IL-1-β-pre; and ii) that the peptide is being broken down in some way during the experiment. More detailed investigation of the nature of these bands would be necessary in order to determine if this peptide is a substrate for specific IL-1-β-pre convertase enzymes.

The time-dependent inhibition shown by Ac-IL-1-β-pre 111-122-NH$_2$ suggests that this peptide is being broken down to inert products. Black et al 73 have shown that this peptide is indeed a substrate for their partially purified ICE activity, being cleaved between the same residues as IL-1-β-pre. The identity of the processing enzyme(s) in our experiments has not been determined. Characterisation of this activity would be extremely useful to establish if more than one specific IL-1-β-pre converting enzyme exists.
2.4 NMR Studies on IL-1-β-pre Subpeptides

A major goal of this project was to characterise the structure of the processing site of the precursor to IL-1-β, and a key hypothesis was that subpeptides from this region of the protein should closely mimic the native conformation of the processing site. Indirect evidence for the validity of this hypothesis came from studies on the intact protein. CD spectra indicate that the N-terminal third of IL-1-β-pre forms a separate domain to the C-terminal two thirds\(^{46}\). The cleavage region thus appears to take the form of the link between the two domains and might be expected to have a distinct structure independent of that of the domains themselves. The noted susceptibility of this region to various proteolytic activities indicates a rather open structure for this part of the precursor. Finally the X-ray structure of active IL-1-β\(^{43}\) indicates that the N-terminal residues do not occupy well-defined positions in the crystal lattice.

X-ray diffraction crystal structure determination remains the most important method for determining the structure of proteins. This technique is not particularly well-suited to the study of peptides because in the solution phase many different conformational states may be populated. This makes crystallisation of peptides rather difficult and limits the practicality of this approach. Nuclear magnetic resonance (NMR) allows analysis of samples in the solution phase and is thus more practical and possibly more relevant than X-ray diffraction studies on peptides. It may be possible to identify several populated conformations and the dynamic relationship between them.

The large number of protons in even relatively small peptides (around 100 for a dodecapeptide) leads to very complicated NMR spectra. To assign the data thus obtained it is necessary to use two-dimensional techniques which both resolve the information and provide additional aids to its assignment.

The general scheme for 2-D NMR spectroscopy is shown in fig. 2.31 along with examples of the sort of information obtained. Systematically varying one of the parameters of the pulse sequence, commonly the time between
two pulses, permits a set of free induction decays (FIDs) to be obtained. Two dimensional Fourier Transform converts this into a matrix $m_{ij}(F_1, F_2)$, $m$ being the magnitude of signal at any data point and $F_x$ being the chemical shift in dimension $x$.

Fig. 2.31 2-D NMR

[Diagram showing the sequence: preparation $\rightarrow$ evolution $\rightarrow$ mixing $\rightarrow$ detection, with the phases corresponding to COSY, TOCSY, ROESY/NOESY]
2.4.1 Interpretation of 2-D NMR Spectra of IL-1-β-pre Subpeptides

The procedure used here for the assignment of 2-D spectra has been tailored from the discussion of Wüthrich\(^{152}\). All IL-1-β-pre subpeptides studied here have been treated in essentially the same manner, illustrated here using the spectra of IL-1-β-pre 111-122.

The COSY spectrum shown in fig. 2.32 was obtained in D\(_2\)O. Amide and other exchangeable protons do not give signals under these conditions. For a peptide of this size it is relatively straightforward to assign most of the signals for each amino acid. As an example, the assignment for proline is shown. Repeating this experiment in H\(_2\)O allows assignment of the amide protons by correlation with the αCH signals.

Another method leading to the same information requires the TOCSY spectrum. Each vertical line in the portion shown in fig. 2.33 joins all the signals of one spin system. The protons of each amino acid form one or more distinct spin system(s). Several amino acids can give the same spin system, for example Ser, Cys, Asp, Asn, His, Phe, Tyr, and Trp all show an AMX system. There are four examples of this class of amino acid in IL-1-β-pre 111-122 (marked with a ■ in fig. 2.33) which cannot be unambiguously distinguished from the TOCSY (or COSY) data. A further complication is the presence of multiple copies of certain amino acids. The A\(_3\)X patterns deriving from Ala 112 and 117, and the A\(_3\)B\(_3\)MX systems of Val 114 and 119 cannot be distinguished from this data alone.

These problems can only be surmounted with any certainty with the aid of the through-space information obtained in the ROESY spectrum. Firstly certain spin systems of ambiguous origin can be assigned to specific amino acids. Observation of the ROESY cross-peaks indicated in fig. 2.34 allows identification of the resonances due to the Tyr and His residues of IL-1-β-pre 111-122.
Fig. 2.32 Part of the COSY Spectrum of IL-1-β-pre 111-122
Showing the Assignment of Pro-118 Signals
Fig. 2.33 Part of the TOCSY Spectrum of IL-1-β-pre 111-122
Showing Identification of the Spin Systems
A rather more powerful technique allows sequence specific assignment of all signals, dealing at a stroke with the problems outlined above. In general, a ROESY cross-peak is observed between an αCH proton and the αNH proton of the adjacent residue on the C-terminal side. Fig 2.35 shows how this relates to the COSY/TOCSY through-bond interactions already obtained.

Fig 2.35 NMR Interactions in the peptide backbone

Fig 2.36 shows the key part of the TOCSY/ROESY spectrum of IL-1-β-pre 111-122. Related cross-peaks can be joined by horizontal or vertical lines as shown allowing sequential assignment, "walking the backbone" of the peptide.

Once the full sequential assignment had been carried out, the remainder of the ROESY spectrum was searched for evidence of long range through-
Fig. 2.36 "Walking the Backbone" of IL-1-β-pre 111-122

Red = ROESY crosspeak
Black = TOCSY crosspeak
space interactions. The only inter-residue cross-peaks were found to be due to protons on neighbouring amino acid residues. No indication of secondary structure was obtained.

Spectra for IL-1-β-pre 101-112 were not obtained due to the low solubility of the peptide, but an identical analysis was successfully performed on each of the remaining dodecapeptides. Full sequence specific assignment of each peptide was obtained, but in no case was evidence of secondary structure found.

The available quantities of pure IL-1-β-pre 102-138 were too low to allow NMR spectra of this peptide to be obtained. However a sufficient amount of its analogue, Ala-124-IL-1-β-pre 102-138 was prepared, and COSY, TOCSY, and NOESY data sets were acquired from a solution of the peptide in 20% CD₃OH/80% H₂O. Identification of the spin systems is shown in fig 2.37, and the results of correlating the TOCSY/NOESY i-i+1 data are shown in fig. 2.38.

There are two main reasons why it is impossible to complete the sequence-specific assignment of this peptide. Firstly several peaks lie under, and are obscured by, the water signal. Secondly, and more importantly, many of the NH or CH signals are degenerate causing ambiguity in identification of related cross-peaks. Uncertainty as to the origin of around half of the observed peaks makes searching for elements of secondary structure unproductive. In fact the high degree of degeneracy is an indicator of a random coil-type structure for the peptide.

Observation of elements of secondary structure in this peptide would provide strong evidence for the conformation of this region of IL-1-β precursor. The indications from our studies are that the peptide takes up a more random structure. This is rather inconclusive: whilst it is possible that this accurately reflects the nature of the cleavage site it may be that the role of the domains in determining the structure of the inter-domain region has been underestimated. With improved knowledge of the structure of IL-1-β and its precursor it should be possible to design better mimics of this region which give a more clear insight into the structure of the processing site.
Fig. 2.37 Part of the TOCSY Spectrum of Ala-124 IL-1-β-pre 102-138
Showing Identification of the Spin Systems

\[ \bullet = \text{AM(PT)}X \text{ spin system} \]
\[ \blacksquare = \text{AMX spin system} \]
Fig. 2.38 Partial Walk Along the Backbone of Ala-124 IL-1-β-pre 102-138
2.5 Side-Chain-Protected Carboxamide Amino Acids

2.5.1. Problems with Carboxamide-Containing Amino Acids

The primary amide side-chains of asparagine and glutamine do not in general compete with the \( \alpha \)-carboxyl group in activation and coupling steps of peptide synthesis. Hence they do not give rise to branched peptides and have most often been incorporated with a free carboxamide group.

There are however several problems which are encountered when this strategy is employed. A number of troublesome side-reactions have been encountered, the most important of which are shown in fig. 2.39.

Fig. 2.39 Side-reactions of Glutamine

The main difference between these amino acids is the reluctance of asparagine to close the ring in which its own \( \alpha \)-amino group participates (fig 2.39ii). The lactam (95) produced when an N-terminal glutamine undergoes this side reaction may ring-open to give a glutamyl rather than glutaminyl derivative. Also, the acylated \( \alpha \)-amine is a poor nucleophile and can cause chain termination.
Imide formation (fig 2.39i) offers the possibility of subsequent ring-opening to either the α- or ω-peptide bond. Similar imide formation can occur even within peptide chains in the presence of base (93; X=NH-peptide). Ring-opening of 94 has the net effect of either hydrolysing the ω-amide to an aspartyl/glutamyl peptide or cleaving the peptide bond. This is particularly troublesome in stepwise syntheses with Fmoc-amino acids where repetitive treatments with base are used to cleave each successive Nα-protecting group.

Dehydration of asparagine/glutamine (fig 2.39iii) results in the incorporation of β-cyanoalanine or γ-cyanobutyryne (97) respectively. This product can be the major component of the synthesis if free side-chain Gln/Asn derivatives are overactivated at the coupling stage.

Side-reactions i) and iii) only occur to a significant extent during the activation of asparagine or glutamine, and parallels can be drawn between this side-reaction and the more general problem of racemisation (section 1.2.3). The key intermediate is rather similar in each case. Appropriate choice of coupling conditions can reduce all these problems. In fact it is found that the activation conditions for asparagine and glutamine must be rather gentler than that necessary to minimise the racemisation of most amino acids. Treatment of Boc-asparagine with DIC gives 30-50% dehydration, but no detectable racemisation. Addition of HOBt was thought to eliminate this problem, but recent reports have cast doubt on this assumption.

Peptide and amino acid derivatives with free carboxamide side-chains often have troublesome physical properties. They tend to have rather low solubility in the solvents used for peptide synthesis, an observation which has been attributed to their propensity towards intermolecular hydrogen-bonding, leading to aggregation.

The use of side-chain-protected asparagine and glutamine derivatives will disrupt these interactions and will also prevent the aforementioned side-reactions. Amides are generally much more resistant to acidolysis than the corresponding esters. For instance Nβ-tert-butylasparagine is stable to TFA/anisole at room temperature whereas Nβ-tert-butylaspartate is rapidly
cleaved under the same conditions. It is necessary to use side-chain protecting-groups which will be easily cleaved on acidolytic deprotection. Several protected amide derivatives which have been used for this purpose are shown in fig. 2.40. The order of acid lability shown derives largely from the stability of the resulting carbocation. These have been derived experimentally for a series of the parent species as shown in fig. 2.41.

We reasoned that the dibenzosubereryl group (104) might provide useful amide protection, and set out to prepare the appropriate amino acid derivatives.

Fig. 2.40 Asn/Gln Side-Chain Protecting Groups
\[ R-\text{OH} + H^+ \rightleftharpoons R^+ + H_2O \]

\[ K_{R^+} = \frac{[R-\text{OH}][H^+]}{[R^+][H_2O]} \]

\[
\begin{align*}
\text{(102)} & \quad \text{pK}_{R^+} = 4.75 \\
\text{(103)} & \quad \text{pK}_{R^+} = 1.6 \\
\text{(104)} & \quad \text{pK}_{R^+} = -3.7 \\
\text{(105)} & \quad \text{pK}_{R^+} = -6.6 \\
\text{(106)} & \quad \text{pK}_{R^+} = -8.0 \\
\text{(107)} & \quad \text{pK}_{R^+} = -13.3 \\
\text{(108)} & \quad \text{pK}_{R^+} = -14.0
\end{align*}
\]

**Fig. 2.41** pK\textsubscript{R+} Values for Selected Carbocations\textsuperscript{157}
2.5.2 Synthesis of Dibenzosuberenyl Derivatives

König and Geiger\textsuperscript{154} prepared the dimethoxybenzhydryl-protected derivatives of asparagine and glutamine by treatment with dimethoxybenzhydrol in glacial acetic acid with a catalytic amount of conc. sulphuric acid. We applied the same procedure to the preparation of Fmoc-Gln/Asn-(Dbse)-OH (109/110) and found that the work-up was eased by the precipitation of the desired product from the reaction mixture following overnight stirring. Compounds of this type were found to have a rather low solubility in all solvents, only dissolving appreciably in highly polar solvents such as DMSO or DMF.

The dihydro-derivatives (Fmoc-Gln/Asn-(Dbsa)-OH, 111/112) and Bnpeoc-derivatives\textsuperscript{155} (113/114) were also prepared by the same general method.

2.5.3 Synthesis of Trityl Derivatives

Sieber and Riniker\textsuperscript{156} attempted to prepare Fmoc-Gln/Asn-(Trt)-OH derivatives by an analogous procedure to that described above, but obtained the desired product in only low yield after a laborious work-up. They found better results by side-chain tritylation of Z-Gln/Asn-OH, then removing the Z group by hydrogenolysis and reprotecting with Fmoc. We repeated their preparation of Fmoc-Asn-(Trt)-OH and obtained identical results. These compounds are now commercially available.

2.5.4. Properties of Side-Chain-Protected Asn/Gln Derivatives

The acidolytic cleavage of each amide protecting group was studied by time-dependent TLC or HPLC, and the results are shown in fig 2.43. The dibenzosuberenyl group showed a similar degree of acid-lability to the trityl group, and was removed much faster than dimethoxybenzhydryl or dibenzosuberyl.
Fig 2.42 Dibenzosubereryl-type Asn/Gln Derivatives

<table>
<thead>
<tr>
<th>Protection Group</th>
<th>t½ (mins.)</th>
<th>% Deprotection (t= 10 mins.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dbsa (112)</td>
<td>10-15</td>
<td>60</td>
</tr>
<tr>
<td>Mbh (100)</td>
<td>5-10</td>
<td>90</td>
</tr>
<tr>
<td>Trt (101)</td>
<td>&lt;5</td>
<td>100</td>
</tr>
<tr>
<td>Dbse (110)</td>
<td>&lt;5</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 2.43 Acidolysis of Glutamine Derivatives

Acidolytic cleavage would be expected to be prolonged, and the differences
in deprotection times magnified, when these compounds are applied to SPPS, as steric effects due to the polymeric support and the other residues of the peptide would hinder the reaction.

Solubility is another key property which must be considered. It is important that the amino acid derivative should dissolve readily in suitable solvents. In automated SPPS an insoluble reagent could cause blockages in filters or piping with disastrous effects on the synthesis in progress.

The solubilities of the relevant derivatives in two common solvents which are used for peptide synthesis are shown in fig. 2.44.

<table>
<thead>
<tr>
<th>Protected Derivative</th>
<th>Solubility (g/ml) in:</th>
<th>Solubility (g/ml) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMF</td>
<td>DMF/dioxan (1:1)</td>
</tr>
<tr>
<td>Fmoc-Asn-(Trt)-OH</td>
<td>&gt;0.2</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Fmoc-Gln-(Trt)-OH</td>
<td>&gt;0.2</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Fmoc-Asn-(Dbse)-OH</td>
<td>0.08</td>
<td>insol.</td>
</tr>
<tr>
<td>Fmoc-Gln-(Dbse)-OH</td>
<td>0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Bnpeoc-Asn-(Dbse)-OH</td>
<td>&gt;0.2</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Bnpeoc-Gln-(Dbse)-OH</td>
<td>&gt;0.2</td>
<td>&gt;0.2</td>
</tr>
</tbody>
</table>

Fig. 2.44 Solubility of Protected Asn/Gln Derivatives

The minimum solubility for a suitable reagent for SPPS is around 0.075 g/ml. Fmoc-Asn-(Dbse)-OH in DMF barely meets this criterion, and must be used with great care in an automated system. Fmoc-Gln-(Dbse)-OH proved to be much better, though the rate of dissolution was rather slow and the
resulting solution was rather viscous, causing some difficulty in transfer steps.

Bnpeoc/Dbse and Fmoc/Trt derivatives posed no solubility problems in either of the solvents tried, and would, at least in this respect offer suitable protecting group combinations for Asn/Gln.

2.5.5 Application of Dbse Derivatives to Peptide Synthesis

One side-reaction which has been noted in the use of side-chain-protected asparagine and glutamine arises from the stable cation which is produced on acidolysis. As discussed in section 2.2.4, this can result in alkylation of sensitive amino acid residues. The indole ring of tryptophan has been noted as being particularly sensitive to this modification.

Sieber and Riniker\textsuperscript{158} prepared the decapeptide shown in fig. 2.45 and showed that alkylation of tryptophan occurred in case ii) (82\%) whereas the correct product was obtained in 92\% analytical yield in case i).

\begin{align*}
\text{Fmoc-Lys-Gln(R)-His-Asn(R)-Pro-Lys-Tyr-Gln(R)-Trp-Asn(R)-O R} \\
\text{R=(i) trityl or R=(ii) 2,4,6-trimethoxybenzyl}
\end{align*}

\textit{Fig. 2.45}

The hexapeptide Phe-Pro-Asn-Trp-Tyr-Leu comprises residues 233-238 of the precursor to IL-1-\(\beta\). As well as having potential IL-1 activity this is a useful test peptide for the new protecting group.

Assembly of the tripeptide Trp-Tyr-Leu-O R proceeded as normal. Activation of Fmoc-Asn-(Dbse)-OH was achieved by adding the solid amino acid derivative in small portions to a stirred solution of DIC and HOBt in DMF. Thus activation and dissolution were combined in one step. The solution thus obtained can simply be taken up by the peptide synthesiser and chain assembly continued as normal.
This procedure overcomes the problem of the low solubility of Fmoc-Asn-(Dbse)-OH in DMF. Though it requires the presence of the operator (hence not automated) it is not necessary to disrupt the coupling cycle. Asn-(Dbse) was successfully incorporated as determined at the subsequent deprotection step. The remaining residues were coupled without further difficulty.

Acidolytic cleavage of the assembled peptide-resin gave a crude product which was purified by semi-preparative HPLC to give pure peptide in 39% overall yield from the initial coupling. The predicted amino-acid analysis and molecular ion (FAB-MS) were obtained.

### 2.5.6 Comparative Studies on Asn/Gln Protection

Four other peptides have been used to study the effect of altering the carboxamide protection on the chain-assembly process. These test peptides were chosen because of known difficulties in their preparation.

Substance P is an undecapeptide amide which is involved in the transmission of the sensation of pain from sensory neurons. This had previously been prepared in this laboratory\(^{146}\) and was known to be a difficult synthesis, having several successive poor couplings. Fig 2.46 allows comparison of the effect of different amide protecting groups on the synthesis of this peptide.

All the protected glutamine derivatives show the same type of behaviour, dropping at the same point in each case. Coupling of the first glutamine residue results in a large drop and incorporation falls even further on the second glutamine. After this point all remaining couplings were more successful. The differences between the behaviour of Fmoc-Gln-(Trt)-OH and the corresponding Dbse, Dbsa, and Mbh derivatives are quantitative rather than qualitative, trityl-protection clearly giving the best overall incorporation.
The peptide containing two unprotected glutamine side-chains behaved rather differently. Coupling of the first glutamine proceeded in quantitative yield, and the second also coupled relatively well compared to protected derivatives. However from this point onwards, several couplings did not go to completion. Incorporation of the N-terminal arginine(Pmc), essentially quantitative in all glutamine-protected syntheses, proceeded to less than 40% in the glutamine-unprotected case.

Kent\textsuperscript{159} has discussed the phenomenon of difficult sequences in peptide synthesis and has attributed this to aggregation of protected peptide chains. Evidence supporting this hypothesis has been found in NMR studies of peptide-resins from known difficult sequences, revealing a marked decrease in the mobility of the polystyrene backbone compared to the resins of known facile peptides. Further, FTIR studies of the same resins has indicated the presence of $\beta$-sheet-type hydrogen bonding\textsuperscript{160}.
Milton et al. have applied Chou and Fasman conformational parameters to predict the structure of resin-bound peptides and have found positive correlation between the predicted degree of β-sheet formation and incomplete acylation. They found that where the cumulative average $P_c*$ was lowest (corresponding to the greatest likelihood of β-sheet formation) couplings were most likely to fail.

Using their parameters, the prediction for the assembly of substance P is shown in fig 2.47. The prediction appears good in the case of assembly using unprotected glutamine, at least for the first eight or nine residues, only breaking down where the first side-chain-protected amino acids are incorporated. This might be expected since the Chou and Fasman parameters are derived from the structures of proteins which do not carry these groups. However, an even better correlation is found between the predicted degree of β-sheet formation and incorporation in the glutamine-protected cases. The reason for this is unclear.

![Graph showing prediction for assemble of Substance P](image)

Note: Low $P_c*$ = high predicted likelihood of β-sheet formation

Fig. 2.47 Substance P: β-sheet Prediction
In the case of IL-1-β-pre 102-138 (fig. 2.48) less pronounced effects are predicted, but the region where incorporation begins to fall (see fig. 2.6) does indeed correlate with the most strongly favoured β-sheet region.

![Graph with amino acid sequence and Pc]

**Fig 2.48 IL-1-β-pre 102-138 ; β-sheet Prediction**

Of rather more direct interest here is the difference between the glutamine derivatives. Why is trityl so much better than all the others? The answer could lie in the peptide-polymer rather than peptide-peptide interaction (as in the β-sheet interactions described above).

The planar aromatic rings of the various protecting groups would be expected to interact with the phenyl groups of the polystyrene matrix via Van der Waals stacking forces. Trityl's bulkiness discourages stacking interactions. Dibenzosubereryl is rigid and almost planar, hence imposing strict demands on the orientation of stacking phenyl groups. Dibenzosuberyl is more flexible, and dimethoxybenzhydryl even more so, able to interact with phenyl groups at many orientations.
Thus incorporation correlates in a negative manner with ability to participate in stacking arrangements with the polymer. Such interaction would hinder coupling in two ways. Firstly an activated protected amino acid would diffuse less freely within the gel bead, and secondly a peptide carrying such a protected residue would effectively introduce additional non-covalent cross-links to the matrix, reducing gel permeability.

The behaviour in the case of IL-1-β-pre 101-112 was rather different. Fig 2.49 shows that incorporation of Fmoc-Asn-(Trt)-OH was very poor indeed, whereas all other asparagine derivatives gave near quantitative acylation. The same pattern is observed again when the corresponding glutamine derivatives are used in the assembly of IL-1-β-pre 121-132 (fig 2.50).

![Graph showing incorporation of IL-1-β-pre 101-112](image)

**Fig. 2.49** IL-1-β-pre 101-112 : Incorporation
The common factor in these two cases is the presence of the side-chain-carboxamide derivative as the third residue to be coupled to the resin-linker. This position is particularly troublesome in peptide chemistry because the resin-dipeptide is uniquely well set up for intramolecular cyclisation.

![Graph showing incorporation of IL-1-β-pre 121-132](image)

**Fig. 2.50 IL-1-β-pre 121-132: Incorporation**

The dipeptide is lost from the resin and chain termination occurs. Evidence
for this mechanism operating in the cases of the IL-1-β-pre subpeptides mentioned above was obtained from amino acid analysis of an acid hydrolysate of the resin-bound product. If simple capping had occurred after the poor incorporation of the trityl-protected amino acid then a large amount of the first two amino acids should be detected. If, however, diketopiperazone (DKP) formation had occurred, then these amino acids should be present in approximately the correct proportion, as was indeed found.

In fact the product obtained on acidolytic cleavage of 109-Gln-(Trt)-IL-1-β-pre 101-112 was of superior quality to that derived from the other four preparations, though the yield was greatly reduced.

Why does the coupling of trityl derivatives encourage DKP formation? The steric bulk of this group would again seem to offer an explanation. The reactive carboxyl group of Fmoc-Gln-(Trt)-OH may be sterically hindered, thus lowering the rate of activation and, especially, coupling. If the rate of coupling is lowered and the rate of DKP formation remains constant (all other conditions being equal) then more of the side-reaction will take place.

The rate of cyclisation to the diketopiperazone depends on the particular dipeptide involved\(^{162}\). The necessary formation of a \(\text{cis}\) peptide bond is greatly affected by the steric and chiral features of the constituent amino acids. Proline- and glycine-containing peptides are particularly disposed towards this side-reaction. The dipeptide IL-1-β-pre 111-112, H-Glu-(O-\(^{13}\)Bu)-Ala-O\(\text{R}\) would not be expected to be particularly sensitive to DKP formation. Indeed with any glutamine protecting group other than trityl this is the case, and acylation proceeds much more quickly. IL-1-β-pre 131-132, H-Gln-(PG)-Lys-(Boc)-O\(\text{R}\) is even more sterically hindered, hence even less DKP would be expected even allowing for a slow acylation. This is in fact what is observed.

Modifications to the coupling strategy could lessen this problem. Use of a faster or less hindered activating agent should accelerate coupling and thus reduce the relative amount of DKP formation.

We applied Knorr’s reagent\(^{140}\) to the activation of Fmoc-Asn-(Trt)-OH in the synthesis of IL-1-β-pre 101-112. This reagent is reported to accelerate
production of the HOBt ester. We found that the level of asparagine incorporation was raised to 60%, still considerably poorer than when any of the other derivatives when used with standard coupling methods.

As has already been noted, this side-reaction has no damaging effect on the quality of the final peptide, indeed it may even give better results. Nevertheless it may result in a large decrease in yield and should be borne in mind.

The conclusion of this section must be that trityl side-chain-protected carboxamide derivatives are the most suitable reagents developed to date for the incorporation of asparagine and glutamine in SPPS, except possibly when these residues occur as the third residue from the C-terminus of the desired peptide. They gain over unprotected derivatives in terms of solubility, prevention of side-reactions and prevention of inter-chain aggregation. They are however more expensive and difficult to produce, and may be slower acylating agents.

Many improvements on the dibenzosubereryl-type protecting group can be envisaged. Removing one or both benzyl groups would result in enhanced acid-lability whilst reducing the bulk of the side-chain (e.g. 119, and see fig. 2.41). Brief attempts by us to prepare $N_\alpha$-cycloheptatriene derivatives met with no success. The precise degree of acid sensitivity could be tailored by the introduction of substituents. Bulky substituents such as t-butyl on the dibenzosubereryl moiety (118) may improve solubility and decrease peptide-polymer interactions.

\[ \text{Fig.2.52 Other Dibenzosubereryl-Type Protecting Groups} \]
2.6 Concluding Remarks

The results presented in this thesis offer some encouragement in the search for ICE inhibitors, though the goal is clearly still some way off.

Biological testing in this system has proved to be an extremely demanding task, and the results have often been difficult to interpret. The current literature holds much promise of a clearer understanding of the mysteries which presently envelop interleukin-1, and it is to be hoped that this will guide and simplify future work in this area. The ever-growing evidence of the role of IL-1 in disease states and the intellectual challenge of unravelling its role in homeostasis make this a field of great interest and potential. The excitement currently surrounding cyclosporin and related immunosuppressants offer a tantalising parallel to the IL-1 story.

Structural studies of the subpeptides prepared in this study have shown that, in solution at least, they exist largely in random-coil-type conformations. Small linear peptides such as those described herein are unlikely to adopt a defined shape, since solvation and inter-molecular aggregation serve to disrupt all but the strongest elements of intramolecular structure. In this respect the results of NMR analysis of these dodecapeptides are to be expected.

The results of structural studies on the larger peptide, ala-124-IL-1-β-pre 102-138 are rather more disappointing. It had been hoped that a self-contained subdomain might be present within these residues which could adopt a definite structure even in the absence of the large N- and C-terminii of IL-1-β-pre. This would appear not to be the case.

Molecular modelling studies of IL-1-β have been initiated in this department and clearer insights into the next generation of peptides to be studied are already being obtained.
Studies on protected side-chain carboxamide groups have, it is hoped, made a small but useful contribution to the developing methodology of solid-phase peptide synthesis. The novel dibenzosubereryl-type protecting groups described offer considerable potential, although trityl derivatives remain the most suitable thus far reported. The electronic properties of the dibenzosubereryl group which make it a useful protecting group also invite applications in general synthetic organic chemistry. The first stages of investigations in this area are presently being undertaken, and initial results have been extremely promising.

SPPS has reached a stage of maturity such that peptides can be rapidly and successfully prepared by non-specialists, resulting in a great multiplication of the applications of these compounds. The lingering presence of nagging problems such as the incorporation of Asn/Gln residues and the constant challenge of producing bigger and better molecules ensure that the role of the peptide chemist is far from obsolete.
Chapter 3 Experimental

3.1 Notes

Melting points were taken in open capillaries on a Büchi 510 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out using plastic sheets precoated with silica gel 60GF-254 (Merck 5735) in the following solvent systems: A) chloroform/methanol/acetic acid (9:1:0.5); B) butanol/acetic acid/water (3:1:1); C) cyclohexane/diethyl ether (1:1). Visualisation of the components was achieved by a suitable combination of the following methods: UV absorption at 254 nm; acidic potassium permanganate; ninhydrin for compounds with free amino groups; Mary’s reagent (4,4’-bis(dimethylamino)diphenylcarbinol) for acid functions. Optical rotations were measured on an AA1000 polarimeter (Optical Activity Ltd.) using a 10 cm cell in the solvent indicated. Mass spectra were recorded on a Kratos MS50TC machine. Ultra-violet spectra were recorded on a Cary 210 spectrophotometer. Infra-red spectra were recorded on a Perkin-Elmer 781 spectrophotometer in the solution indicated or as a KBr disc. $^1$H NMR spectra were recorded on either Bruker WP80 (80 MHz), WP200 (200 MHz), WH360 (360 MHz), or Varian VXR5000 (600 MHz) machines in the solvent indicated using tetramethylsilane (TMS) as external standard ($\delta_H=0.00$). $^{13}$C NMR spectra were recorded on a Bruker WP200 machine operating at 50.1 MHz in the solvent indicated using tetramethylsilane as external standard ($\delta_C=0.00$). All solvents were distilled before use, and the following were dried using the reagents given in parentheses when required: diethyl ether (sodium wire); dichloromethane (calcium hydride); tetrahydrofuran (sodium); methanol (magnesium-iodine). Dimethylformamide, 1.4-dioxan and piperidine were Peptide Synthesis grade supplied by Rathburn Chemicals, Walkerburn, Scotland. High performance liquid chromatography was carried out using an Applied Biosystems system comprising 2x1406A solvent delivery system, a 1480A injector/mixer, and a 1783A detector/controller. Analytical columns were Applied Biosystems Aquapore RP300 C18 reversed-phase silica (300Å pore size, 7μm spherical silica, 4.6mm internal diameter). Columns were eluted with a gradient of acetonitrile (far UV grade: Rathburn Chemicals) in water,
with 0.1% TFA (far UV grade: Applied Biosystems) in both solvents, at a flow rate of 1 cm³/minute. Some combinations of column length and gradient are shown below, others are specified in the text.

<table>
<thead>
<tr>
<th>Column</th>
<th>Gradient; (time in mins., %acetonitrile)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>22 cm</td>
</tr>
<tr>
<td>B</td>
<td>11 cm</td>
</tr>
<tr>
<td>C</td>
<td>11 cm</td>
</tr>
<tr>
<td>D</td>
<td>11 cm</td>
</tr>
<tr>
<td>E</td>
<td>11 cm</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0, 10), (30, 90), (31, 10)</td>
</tr>
<tr>
<td></td>
<td>(0, 10), (25, 90), (26, 10)</td>
</tr>
<tr>
<td></td>
<td>(0, 10), (30, 90), (31, 10)</td>
</tr>
<tr>
<td></td>
<td>(0, 15), (15, 30), (16, 15)</td>
</tr>
<tr>
<td></td>
<td>(0, 14), (20, 14)</td>
</tr>
</tbody>
</table>

The eluant was monitored at 226 nm unless otherwise noted. Preparative HPLC was carried out on the same equipment using columns of 9.2 mm internal diameter unless otherwise noted. The conditions used are specified in the text. Simultaneous multiple wavelength detection was carried out using a LKB Pharmacia Diode-Array Detector and data processor. Amino acid analyses were carried out using an LKB 4150 alpha amino acid analyser on a hydrolysate obtained by one of the methods described in section 3.2.9. Peptide titration curves and iso-electric-focussing were carried out using LKB Pharmacia PhastGel electrophoresis equipment and developed using silver staining. Sequencing of peptides was carried out on an Applied Biosystems 477A sequencer at the WellMet sequencing facility, University of Edinburgh.

### 3.2 Solid-Phase Peptide Synthesis

#### 3.2.1 Preparation of Fmoc-Amino Acid Resins

A solution of Fmoc-amino acid (9.5 mmol, NovaBiochem or Raylo Chemicals unless otherwise noted) and N,N'-diisopropylcarbodiimide (DIC; 0.744 cm³; 4.75 mmol) in dimethylformamide (DMF; 30 cm³) was allowed to stand for 15 minutes prior to addition to a mixture of p-alkoxybenzyl alcohol polystyrene resin (crosslinked with 2% divinylbenzene)(0.79 mmol g⁻¹ w.r.t. hydroxyl group; 2.0 g, 1.58 mmol) and 4-(N,N-dimethylamino)-pyridine (DMAP; Aldrich Chemicals; 30 mg) in DMF (15 cm³). The mixture was
sonicated for 2 h then filtered and the resin washed thoroughly with DMF then diethyl ether. Drying in vacuo yielded Fmoc-amino acid-resin as off-white granules.

The loading of the Fmoc-amino acid-resin was determined by treating a weighed sample (2-4 mg) with piperidine (2.0 cm$^3$) and DMF (8.0 cm$^3$). After sonicating for 20 minutes the UV absorbance of the supernatant was measured at 300 nm and the loading calculated using the Lambert Beer law. A typical preparation gave 75% coupling for a loading of ca. 0.6 mmol g$^{-1}$.

3.2.2 Capping of Unreacted Resin Sites

Fmoc-peptide-resin (0.5 mmol) was treated with acetic anhydride (0.10 g, 1.0 mmol) and pyridine (0.080 g, 1.0 mmol) in DMF (7 cm$^3$). The mixture was shaken for 2.5 minutes then filtered and treated with a further identical portion of acetic anhydride and pyridine in DMF. After shaking for a further 3.5 minutes the mixture was filtered and the resin washed thoroughly with DMF.

3.2.3 Removal of Fmoc Group

Fmoc-peptide-resin (0.5 mmol) was treated with four portions of 20% piperidine in DMF (9.0 cm$^3$) and shaken for 5, 3, 3, and 1 minute respectively. The mixture was filtered prior to each addition and the UV absorbance of the filtrate measured at 300 nm on each occasion. After the last treatment the peptide-resin was washed thoroughly with DMF.

3.2.4 Activation of Fmoc-Amino Acid

i) Fmoc-amino acid (1.0 mmol) was dissolved in DMF (8.0 cm$^3$) and treated with 0.5 M DIC in DMF (2.0 cm$^3$; 1.0 mmol). The solution was allowed to stand for 15 minutes.
ii) Fmoc-amino acid (1.0 mmol) was dissolved in DMF (4.0 cm$^3$) and treated with 0.5 M DIC in DMF (2.0 cm$^3$; 1.0 mmol) and 0.5 M 1-hydroxybenzotriazole (HOBt; Fluka) in DMF (2.0 cm$^3$; 1.0 mmol). The solution was allowed to stand for 15 minutes.

Initially most Fmoc-amino acids were coupled using procedure i), then recoupled using procedure ii). The following were coupled using procedure ii) twice: Arg, Gln, Asn, His.

3.2.5 Coupling of Activated Amino Acid to Peptide-Resin

Activated amino acid solution (from 3.2.4 i or ii) was added to peptide-resin (0.5 mmol). The mixture was shaken for 30 minutes then filtered and the resin washed thoroughly with DMF.

3.2.6 Automated Chain Assembly

All peptides were assembled using an Applied Biosystems 430A peptide synthesiser. The initial resin for the synthesis of peptides with free C-terminii was that described in step 3.2.1. If a C-terminal amide was desired then 5-(Fmoc-amino)-dibenzosuberyl-2-resin was used. Syntheses were normally carried out on an initial scale of 0.5 mmol. Steps 3.2.2 to 3.2.5 inclusive ("capping", "deprotection", "activation"(i and/or ii), and "coupling") comprise the coupling cycle used to elongate the resin-bound peptide by one residue. The timing and logistics of the coupling cycle are shown in fig. 3.1.

Once all amino acids have been coupled, one further round of capping and deprotection (steps 3.2.1 and 3.2.2) gives the free N-terminus. If desired the peptide can then be acetylated by once more repeating the capping step.
Fig. 3.1
Automated Solid-Phase Peptide Synthesis
3.2.7 Fmoc-Amino Acids

The following Fmoc-amino acids were always incorporated with no further protection:

Glycine; Alanine; Valine; Leucine; Isoleucine; Phenylalanine; Tryptophan; Proline; Methionine.

The following were always incorporated with the side-chain protection shown:

Glutamic acid; Aspartic acid; Tyrosine; Serine; Threonine: (OtBu), Arginine: (pentamethylchromansulphonyl; Pmc¹³⁷), Lysine (Boc).

The particular derivative used to incorporate the following amino acids will be specified for each peptide.

Glutamine; Asparagine; Histidine; Cysteine.

3.2.8 Acidolytic Deprotection

Peptide-resin (0.5 g) was treated with 95% aqueous TFA (10 cm³) containing an appropriate scavenger mixture (specified in the text). Aliquots of the mixture were analysed at regular intervals by HPLC and the optimal cleavage time determined (1-4 h). The mixture was filtered and the filtrate concentrated in vacuo to ca. 1 cm³ of oily liquid. Addition of ice-cold diethyl ether (20 cm³) gave the peptide as a white to off-white solid (0.1-0.4 g).

3.2.9 Hydrolysis of Peptides for Amino Acid Analysis

i) Acid hydrolysis: Peptide (1-3 mg) was treated with 3 M aqueous p-toluenesulphonic acid (pTSA; 1.0 cm³) and placed in a sealed, evacuated Carius tube. For peptides containing tryptophan a tryptamine-saturated pTSA solution was used. After heating to 110°C for 18-36h the hydrolysate was treated with 1 M sodium hydroxide (2.0 cm³) and water (2.0 cm³).
ii) **Proteolytic digestion:** Peptide (2-3 mg) was treated with 5% formic acid (0.25 cm$^3$) in an Eppendorf tube and pepsin in 5% formic acid (Sigma) (20 μl; 1 mg/cm$^3$) added. The tube was incubated at 40°C for 24 h, then lyophilised. 0.2 M Ammonium hydrogen carbonate solution (0.25 cm$^3$) was added to the residue, and the pH adjusted to 7.75 ±0.25 with dilute ammonia or acetic acid. 0.025 M Manganese chloride (50 μl) and leucine aminopeptidase M (Sigma) (40 U/cm$^3$; 15 μl) were added, and the tubes incubated at 40°C for a further 48 h. The samples were repeatedly lyophilised to remove salt, then taken up in citrate buffer (pH 2.2; 2.0 cm$^3$).
3.3 Experimental

Interleukin-1-β-Precursor 102-138 (120)

H-Pro-Ile-Phe-Phe-Asp-Thr-Trp-Asp-Asn-Glu-Ala-Tyr-Val-His-Asp-Ala-Pro-
Val-Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys-Ser-Leu-Val-
Met-Ser-Gly-OH

The initial resin was Fmoc-Gly-\(\text{R}\) (0.6 mmol g\(^{-1}\); 0.85 g, 0.5 mmol). The side-chain amide groups of asparagine and glutamine were left unprotected. Histidine was protected with a trityl group on the \(\tau\)-nitrogen of the imidazole ring (His-(Trt)). The thiol group of cysteine was protected by an acetamidomethyl group (Cys-(Acm)). Approximately one third of the peptide-resin was removed after 21 couplings because of excessive swelling of the peptide-resin. The weight of peptide-resin after chain assembly was 2.05g. Resin-bound-peptide (0.4 g) was cleaved for 2 h with a solution of 2.5% thioanisole, 2.5% anisole, 2.5% ethylmethylsulphide (EMS), 2.5% N-acetyltryptophan, and 5% water in TFA (10 cm\(^3\)), giving a mixture of crude peptide and scavengers (0.75 g). This mixture was dissolved in the minimum volume of 30% aqueous acetic acid and passed down a column of G-25 Sephadex (2.5x150 cm) eluted with 30% aqueous acetic acid at 25 cm\(^3\)/hour. UV absorbance of the eluant was monitored at 254 and 277 nm. Appropriate fractions were combined and lyophilised to yield crude peptide (0.19 g). A portion (0.10 g) was dissolved in the minimum volume of 0.05 M aqueous ammonium hydrogen carbonate solution and the pH adjusted to 8.0 with 0.2M aqueous ammonia. The resulting solution was passed down a DEAE-cellulose column (1.5x30 cm) and eluted with 0.05 M aqueous ammonium hydrogen carbonate solution (pH 8.0). The UV absorbance of the eluant was monitored at 254 and 277 nm. After a small amount of unbound material was eluted a linear gradient of increasing concentration of ammonium hydrogen carbonate was applied to a final concentration of 0.3M. The total volume of the eluant was 600 cm\(^3\) and the pH was maintained at 8.0 throughout. Appropriate fractions were combined and lyophilised. Those fractions corresponding to the main absorbance peak showed the cleanest analytical HPLC trace and amino acid analyses closest to the desired values. A portion of the major product was dissolved in the minimum volume of 0.025 M aqueous ammonium acetate solution and the pH adjusted to 3.5 with 0.1 M aqueous acetic acid.
This solution was passed down a column of CM-Sephadex (1x20 cm) and eluted with 0.025 M aqueous ammonium acetate solution (pH 3.5) at 15 cm³/hour. The UV absorbance of the eluant was monitored at 254 and 277 nm. After a small amount of unbound material had been washed off a linear gradient of increasing ammonium acetate concentration was applied, the final concentration being 0.3 M. The total volume of the eluant was 600 cm³ and the pH at the end of the procedure was 4.5. Appropriate fractions were combined and lyophilised. The remainder of the major product from DEAE column chromatography was dissolved in the minimum amount of 20% aqueous acetonitrile and purified in six portions by preparative HPLC (22 cm column; flow 3.5 cm³/minute; isocratic 29% acetonitrile). Lyophilisation of the appropriate fractions yielded the *title compound* (3.8 mg).

FAB-MS m/z 4313.0, C₁₈₀H₂₈₇N₆₅O₇₅S₂ requires 4312.9; Analytical HPLC (22 cm column; gradient ((time, %acetonitrile), (0, 10), (3, 30), (25, 55), (26, 10))) Rt 12.6 minutes.

**Amino Acid Analysis (acid hydrolysate; 24 h) after purification by:**

<table>
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<tr>
<th>Gel Filtration</th>
<th>Anion: Exchange</th>
<th>Cation: HPLC</th>
<th>Prep. Ideal</th>
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<td>0.86 1</td>
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<tr>
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<td>0.96</td>
<td>0.96 1</td>
</tr>
<tr>
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<td>3.25</td>
<td>2.95 3</td>
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<tr>
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<td>0.95</td>
<td>0.92 1</td>
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<tr>
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<td>2.04</td>
<td>2.06</td>
<td>1.95 2</td>
</tr>
<tr>
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<td>0.93</td>
<td>0.89 1</td>
</tr>
<tr>
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<td>1.74 1</td>
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<tr>
<td>Arg 2.51</td>
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<td>2.37</td>
<td>2.07 2</td>
</tr>
<tr>
<td>Total Error</td>
<td>8.15</td>
<td>5.14</td>
<td>4.56 3.01</td>
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<tr>
<td>Mean Error</td>
<td>22.6%</td>
<td>14.3%</td>
<td>12.6% 8.4%</td>
</tr>
</tbody>
</table>

107
Ala-124-Interleukin-1-β-Precursor 102-138 (121)

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

The initial resin was Fmoc-Gly-[R] (0.6 mmol g⁻¹; 0.85 g, 0.5 mmol). The side-chain amide groups of asparagine and glutamine were left unprotected. Histidine was protected with a butoxymethyl group on the π-nitrogen of the imidazole ring (His-(Bum)). The weight of peptide-resin after chain assembly was 3.03 g. Resin-bound peptide was cleaved for 1.5 h with a solution of 5% ethanedithiol, 2.5% anisole, 2.5% ethylmethylsulphide, 2.5% phenol, and 5% water in TFA (10 cm³), giving crude peptide (0.27 g) as a white solid. A portion (0.16 g) of this material was ground in acetic acid (2 cm³) and water (2 cm³) added. This mixture was sonicated for 20 minutes at 35°C and a further portion of water (2 cm³) added. A small amount of insoluble material was removed by filtration. The residue was washed with a further portion of water (2 cm³). The combined washings and filtrate were purified in three portions by preparative HPLC (28x2.5 cm diam. column; flow 7 cm³/minute; gradient [(time, %acetonitrile), (0, 0), (5, 20), (35, 60), (38, 90), (39, 0)]). Fractions were analysed by HPLC and combined and lyophilised as appropriate. The title compound was obtained as the major product (20.6 mg).

FAB-MS m/z 4209.4; C₁₈₆H₂₉₃N₅₁O₅₉S requires 4209.7; HPLC (gradient A) Rt 15.1 minutes; Amino acid analysis Asx₆5.82, Thr₁1.03, Ser₄4.00, Glu₃3.67, Pro₂1.95, Gly₁1.20, Ala₃3.25, Val₂2.61, Met₁0.98, Ile₁0.78, Leu₂2.83, Tyr₀1.94, Phe₂2.02, His₁0.82, Lys₁1.12, Arg₂1.36, Trp₁1.11.

600 MHz ¹H NMR (30%CD₃OH, 65%H₂O, 5%D₂O)

<table>
<thead>
<tr>
<th>Residue</th>
<th>α-CH</th>
<th>α-NH</th>
<th>β-CH</th>
<th>others</th>
</tr>
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<td>N/A</td>
<td>2.02,2.33 γ2.10;δ3.68,3.82</td>
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<tr>
<td>103-Ile</td>
<td>4.21</td>
<td>8.43</td>
<td>1.75</td>
<td>γCH₂:1.15,1.44; γCH₃:0.89; δCH₃:0.78</td>
</tr>
<tr>
<td>104-Phe</td>
<td>8.27</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

108
| 105-Phe | 106-Asp | 4.62 | 8.35 | 2.77,2.90 |
| 107-Thr | 108-Trp | 4.26 | 7.94 | 4.20 | γ1.14 |
| 109-Asp | | indole 2H:7.34; 4H:7.69; 5H:7.24; 6H:7.32; 7H:7.57; NH:10.21 |
| 110-Asn | 111-Glu | 4.31 | 8.19 | 2.05,2.14 | γ2.50 |
| 112-Ala | 113-Tyr | 4.24 | 8.12 | 1.62 |
| 114-Val | 115-His | 4.60 | 7.87 | 2.97,3.13 | 2H:7.40; 4H:7.17 |
| 116-Asp | 117-Ala | 4.04 | 7.79 | 2.06 | γ0.87,0.95 |
| 118-Pro | 119-Val | 4.51 | 8.17 | 2.12 | γ0.97,1.03 |
| 120-Arg | 121-Ser | 4.13 | 8.40 | 1.84,1.93 | γ1.71; δ3.32; NH:7.28 |
| 122-Leu | 123-Asn | 4.45 | 8.08 | 1.72 | γ1.67; δ0.93,0.96 |
| 124-Ala | 125-Thr | 4.31 | 8.08 | 4.31 | γ1.27 |
| 126-Leu | 127-Arg | 4.39 | 8.08 | 1.72 | γ1.67; δ0.93,0.96 |
| 128-Asp | 129-Ser | 4.31 | 8.08 | 1.72 | γ1.67; δ0.93,0.96 |
| 130-Gln | 131-Gln | 4.40 | 8.33 | 1.86,1.94 | γ1.54; δ1.76; ε3.07; NH:7.62 |
| 132-Lys | 133-Ser | 4.44 | 8.28 | 1.61 | γ1.61; δ0.96,1.01 |
| 134-Leu | 135-Val | 4.44 | 8.28 | 1.61 | γ1.61; δ0.96,1.01 |
| 136-Met | 137-Ser | 4.17 | 8.03 | 2.15 | γ1.00,1.03 |
| 138-Gly | | 4.63 | 8.41 | 2.08,2.20 | γ2.63,2.71 |
| 139-Ser | | 3.94 | 8.41 | |
Interleukin-1-β-Precursor 101-112 (122)

H-Glu-Pro-Ile-Phe-Phe-Asp-Thr-Trp-Asp-Asn-Glu-Ala-OH

The initial resin was Fmoc-Ala (0.48 mmol g⁻¹; 1.04 g, 0.5 mmol). The side-chain amide group of asparagine was left unprotected. The weight of peptide-resin after chain assembly was 1.55 g. Resin-bound peptide (0.5 g) was cleaved for 1.5 h with a solution of 5% thioanisole and 5% water in TFA (20 cm³), giving crude peptide (0.19 g). Crude peptide (0.15 g) was dissolved in 50% aqueous acetonitrile (10 cm³) and purified in 10 portions by preparative HPLC (11 cm column; flow 5 cm³/minute; [(time, %acetonitrile), (0, 10), (25, 45), (26, 90), (27, 10)]). Appropriate fractions were combined and lyophilised. The main fraction contained purified peptide (0.047 g). The presence of a small amount of contaminating material was detected by analytical HPLC of this product. The peptide was redissolved in 50% aqueous acetonitrile (3 cm³) and further purified in 10 portions by preparative HPLC (11 cm column; flow 5 cm³/minute; isocratic 24% acetonitrile). Lyophilisation of the appropriate fractions gave the title compound as a fluffy white solid (6 mg).

FAB-MS m/z 1483.4; C₆₉H₉₀N₁₄O₂₃ requires 1483.6; Analytical HPLC (gradient B) Rt 10.0 minutes; Amino acid analysis (acid hydrolysis; 18 h) Asx₃3.07, Thr₁1.03, Glu₂2.17, Pro₁0.96, Ala₁0.99, Ile₁0.83, Phe₂1.77, Trp₁1.16.

This peptide was also prepared using side-chain protected asparagine derivatives in the same manner. The products of these syntheses were not isolated.

Amino-acid analysis of IL-1-β-pre 101-112 peptide-resin prepared using Fmoc-Asn-(trityl)-OH (acid hydrolysis; 18 h) Asx₃2.89, Thr₁1.03, Glu₂1.98, Pro₁n.d., Ala₁1.16, Ile₁0.70, Phe₂1.65, Trp₁1.24.
Acetyl-Interleukin-1-β-Precursor 106-117 (123)

Ac-Asp-Thr-Trp-Asp-Asn-Glu-Ala-Tyr-Val-His-Asp-Ala-OH

The initial resin was Fmoc-Ala-\(\text{R}\) (0.48 mmol g\(^{-1}\); 1.04 g, 0.5 mmol). The side-chain amide group of asparagine was left unprotected. Histidine was protected with a trityl group on the \(\tau\)-nitrogen of the imidazole ring (His-(Trt)). The weight of peptide-resin after chain assembly was 1.69 g. Peptide-resin (0.4 g) was cleaved for 1.5 h with a solution of 2.5% ethane-1, 2-dithiol (EDT), 5% thioanisole, and 5% water, in TFA (10 cm\(^3\)) to give crude peptide (0.13 g). Crude peptide (0.10 g) was dissolved in acetic acid (1.2 cm\(^3\)) and water (0.8 cm\(^3\)) added. This solution was passed in 20 portions down an 11 cm semi-preparative HPLC column eluted with 15% aqueous acetonitrile. Appropriate fractions were combined and lyophilised to give three products.

Product 1: FAB-MS: \(m/z\) 1477.6; \(C_{64}H_{84}N_{16}O_{25}\) requires 1477.4; Analytical HPLC (gradient E) Rt 9.2 minutes.

Product 2: FAB-MS: \(m/z\) 1477.6; Analytical HPLC (gradient E) Rt 9.2 minutes (50%), 12.0 minutes (50%).

Product 3: FAB-MS: \(m/z\) 1477.6; Analytical HPLC (gradient E) Rt 12.0 minutes.

Amino acid analysis (acid hydrolysis; 18 h):

<table>
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Amino acid analysis (proteolytic digestion):

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600 MHz $^1$H NMR on product 3 (90%H$_2$O, 10%D$_2$O)

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<td>117-Ala</td>
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**Interleukin-1-β-Precursor 111-122 (124)**

H-Glu-Ala-Tyr-Val-His-Asp-Ala-Pro-Val-Arg-Ser-Leu-OH

The initial resin was Fmoc-Leu (0.59 mmol g⁻¹; 1.03 g, 0.60 mmol). Histidine was protected with a trityl group. The weight of peptide-resin after chain assembly was 1.70g. Resin-bound peptide (0.4 g) was cleaved for 1.5 h with a solution of 5% thioanisole and 5% water in TFA (20 cm³), giving crude peptide (0.16 g). Crude peptide (0.10 g) was dissolved in 0.1% aqueous TFA (5 cm³), filtered to remove a small amount of insoluble material and purified in 6 portions by preparative HPLC (11 cm. column; flow 10 cm³/minute; gradient: (time, % acetonitrile) (0, 10), (1, 13), (9, 13), (12, 90), (14, 90), (16, 10)). Lyophilisation of the appropriate fractions gave the *title compound* as a fluffy white solid (42 mg).

FAB-MS (HRMS) m/z 1356.69111; C₆₀H₉₄N₁₇O₁₉ requires 1356.69113;
Analytical HPLC (gradient B) Rt 13.4 minutes; (gradient D) Rt 14.2 minutes;
Amino acid analysis (acid hydrolysis; 18 h) Asx₁1.01, Ser₁0.87, Glu₁1.13,
Pro₁1.01, Ala₂2.07, Val₂2.00, Leu₁1.03, Tyr₁0.97, His₁1.01, Arg₁0.95;
Sequencing gave unambiguous identification of the expected amino acid at each cycle.

**600 MHz ¹H NMR (90%H₂O, 10%D₂O)**

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<th>α-NH</th>
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Acetyl-Interleukin-1-β-Precursor 111-122-NH₂ (125)

Ac-Glu-Ala-Tyr-Val-His-Asp-Ala-Pro-Val-Arg-Ser-Leu-NH₂

The initial resin was 5-(Fmoc-amino)-dibenzosuberyl-resin (0.74 mmol g⁻¹; 0.54 g, 0.40 mmol). Histidine was protected with a trityl group. The weight of peptide-resin after chain assembly was 1.05 g. Resin-bound peptide (0.25 g) was cleaved for 2 h with a solution of 5% anisole and 5% water in TFA (20 cm³), giving crude peptide (0.12 g). Crude peptide (0.042 g) was dissolved in 10% aqueous acetonitrile (4 cm³) and purified in 8 portions by preparative HPLC (11 cm column; flow 3 cm³/minute; gradient: (time, % acetonitrile) (0, 15), (14, 20), (15, 15). Lyophilisation of the appropriate fractions gave three products as fluffy white solids.

Product 1: FAB-MS: m/z 1396.8; C₆₂H₉₆N₁₈O₁₉ requires 1396.7; Analytical HPLC (gradient D) Rt 8.2 minutes (95%), 9.3 minutes (5%).

Product 2: FAB-MS m/z 1396.8; Analytical HPLC (gradient D) Rt 8.2 minutes (50%), 9.3 minutes (50%).

Product 3: FAB-MS m/z 1396.8; Analytical HPLC (gradient D) Rt 8.2 minutes (3%), 9.3 minutes (97%).

Amino acid analysis (acid hydrolysis; 18 h):

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Amino acid analysis (proteolytic digestion):

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600 MHz $^1$H NMR of product 1 (90%H$_2$O, 10%D$_2$O)

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<th>$\beta$-CH</th>
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600 MHz $^1$H NMR on product 3 (90%H$_2$O, 10%D$_2$O)

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<td>122-Leu</td>
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<td>γ1.67; δ 0.88,0.95</td>
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N-(N',N'-dimethyl-5-aminonaphthalenesulphonyl)-11-aminoundecanoyl-Interleukin-1-$\beta$-Precursor 111-122 (126)

Dansyl-aminoundecanoyl-Glu-Ala-Tyr-Val-His-Asp-Ala-Pro-Val-Arg-Ser-Leu-OH

Peptide-resin (ca. 0.5 mmol) was prepared as for Interleukin-1-$\beta$-Precursor 111-122. Following removal of the N-terminal Fmoc group the resin was treated with a solution of Fmoc-aminoundecanoic acid (0.806 g, 2.0 mmol), HOBt (0.27 g, 2.0 mmol), and DIC (0.26 g, 2.0 mmol) in DMF (16 cm$^3$) which had been allowed to react at room temperature for 15 minutes. The mixture was sonicated for 1 hour then filtered and washed thoroughly with DMF. After removal of the Fmoc group one quarter of the peptide resin was treated with a solution of dansyl chloride (1.1 g, 4.0 mmol) in DMF (15 cm$^3$). The mixture was sonicated for 1 hour then filtered. The resin was washed well with DMF then diethyl ether and dried to give dansyl-aminoundecanoyl-peptide-resin (0.46 g). The resin gave a strong yellow-green fluorescence when placed under a UV lamp emitting at 366 nm. Peptide-resin (0.2g) was cleaved for 1.5 h with a solution of 5% anisole and 5% water in TFA (10 cm$^3$), giving crude peptide (0.087 g). Crude peptide (0.080 g) was
dissolved in 20% aqueous acetonitrile (6 cm³) and purified in 6 portions by preparative HPLC (22 cm column length, flow 7 cm³/minute, gradient (time, %acetonitrile): (0, 15), (20, 50), (21, 15)) to give two products.

Product 1 (*title compound* and epimer containing D-His): 11.2 mg; FAB-MS m/z 1772.9; C₈₃H₁₂₅N₁₉O₂₂S requires 1772.4; λₘₐₓ 202 nm (100%), 222 (76), 283 (6.6), 320 (2.0); Analytical HPLC (gradient A) Rt 12.3 minutes (50%), Rt 12.6 minutes (50%).

Product 2 (aminoundecanoyl-IL-1-β-pre-111-122 and epimer containing D-His); 12.4 mg; FAB-MS m/z 1539.9; C₇₁H₁₁₅N₁₈O₂₀ requires 1539.3; λₘₐₓ (20% aqueous acetonitrile) 202 nm (87% relative to A₂₀₂ for product 1), 277 (1.7); Analytical HPLC (gradient A) Rt 9.6 minutes (50%), Rt 9.9 minutes (50%).

Interleukin-1-β-Precursor 117-128 (127)

H-Ala-Pro-Val-Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-OH

The initial resin was Fmoc-Asp(OtBu)-R (0.58 mmol g⁻¹, 1.02 g, 0.50 mmol). The side-chain amide group amide group of asparagine was left unprotected. The thiol group of cysteine was protected with the trityl group. The weight of peptide-resin after chain assembly was 1.59 g. Resin-bound peptide (0.3 g) was cleaved for 1.5 h with a solution of 5% EMS, 5% thioanisole and 5% water in TFA (10 cm³), giving crude peptide (0.08 g). Crude peptide (0.08 g) was dissolved in 50% aqueous acetonitrile (5 cm³), and purified in 5 portions by preparative HPLC (11 cm. column; flow 5 cm³/minute; gradient: (time, % acetonitrile) (0, 15), (20, 50), (21, 15)). Lyophilisation of the appropriate fractions gave the *title compound* as a fluffy white solid (0.013 g).

FAB-MS m/z 1344.4; C₅₅H₉₇N₁₉O₁₈S requires 1344.6; Analytical HPLC (gradient A) Rt 11.2 minutes; Amino acid analysis (acid hydrolysis; 18 h) Asx22.03, Thr11.10, Ser11.12, Pro11.40, Cys10.42, Ala10.93, Val10.91, Leu22.30, Arg22.08.
Acetyl-Interleukin-1-β-Precursor 117-128-NH₂ (129)

Ac-Ala-Pro-Val-Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-NH₂

The initial resin was 5-(Fmoc-amino)dibenzosuberyl-2-resin (0.65 mmol g⁻¹; 0.82 g, 0.53 mmol). Cysteine was protected with a trityl group on the side-chain thiol group. The weight of peptide-resin after chain assembly was 2.42 g. Resin-bound peptide (0.5 g) was cleaved for 2 h with a solution of 5% thioanisole, 5% EDT, and 5% water in TFA (10 cm³), giving crude peptide (0.16 g). Crude peptide (0.16 g) was dissolved in 50% aqueous acetic acid (10 cm³), filtered to remove a small amount of insoluble material and purified in 6 portions by preparative HPLC (11 cm, column; flow 5 cm³/minute; gradient: (time, % acetonitrile) (0, 10), (13, 25), (15, 50), (17, 50), (18, 10)). Lyophilisation of the appropriate fractions gave the title compound as a fluffy white solid (0.06 g).

FAB-MS m/z 1385.5; C₅₇H₁₀₀N₂₀O₁₈S requires 1385.6; Analytical HPLC (gradient A) Rt 9.7 minutes; Amino acid analysis (acid hydrolysis; 18 h) Asx₂ 2.01, Thr₁ 0.91, Ser₁ 0.82, Pro₁ 1.74, Ala₁ 1.10, Cys₁ 0.38, Val₁ 1.00, Leu₂ 2.05, Arg₂ 2.01.

600 MHz ¹H NMR (90%H₂O, 10%D₂O)

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Interleukin-1-β-Precursor 121-132 (130)

H-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys-OH

The initial resin was Fmoc-Lys-(Boc)-\(\text{R} \) (0.44 mmol g\(^{-1}\); 0.90 g, 0.40 mmol). The side-chain amide groups of asparagine and glutamine were left unprotected. The thiol group of cysteine was protected with the trityl group. The weight of peptide-resin after chain assembly was 1.80 g. Resin-bound peptide (0.5 g) was cleaved for 1.5 h with a solution of 5% EDT, 5% thioanisole and 5% water in TFA (10 cm\(^3\)), giving crude peptide (0.26 g). Crude peptide (0.24 g) was dissolved in acetic acid (0.1 cm\(^3\)) and water (0.5 cm\(^3\)) added. This solution was purified in 10 portions by preparative HPLC (11 cm. column; flow 5 cm\(^3\)/minute; gradient: (time, % acetonitrile) (0, 10), (20, 30), (21, 10)). Lyophilisation of the appropriate fractions gave the title compound as a fluffy white solid (0.052 g).

FAB-MS m/z 1393.2; \(\text{C}_{55}\text{H}_{98}\text{N}_{19}\text{O}_{21}\text{S}\) requires 1392.6; Analytical HPLC (gradient (0, 10), (25, 44), (26, 10)) Rt 7.7 minutes; Amino acid analysis (acid hydrolysis; 18 h) Asx 2.06, Thr 1.07, Ser 2.1, Gln 2.02, Leu 2.95, Lys 1.1, Arg 0.88.

600 MHz \(^1\text{H}\) NMR (90\%\text{H}_2\text{O}, 10\%\text{D}_2\text{O})

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<td>(\gamma)1.45; (\delta)1.68; (\varepsilon)3.18; (\text{NH}:7.18)</td>
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Two other partial syntheses of this peptide were carried out using the side-chain amide protected glutamine derivatives Fmoc-Gln-(dibenzosuberenyl)-OH and Fmoc-Gln-(Trt)-OH. On each occasion the synthesis was suspended after the coupling of Asp-128. Neither cleavage of the resulting peptide-resin nor isolation of the product were carried out on either occasion.

**Acetyl-Interleukin-1-β-Precursor 127-138 (131)**

Ac-Arg-Asp-Ser-Gln-Gln-Lys-Ser-Leu-Val-Met-Ser-Gly-OH

The initial resin was Fmoc-Gly (0.51 mmol g⁻¹; 1.00 g, 0.51 mmol). The side-chain amide group of glutamine was left unprotected. Coupling cycles were modified to allow the use of 1-hydroxy-4-ethoxycarbonyl-5-methyltriazole as an activating reagent. This was used in exactly the same manner as HOBt (section 3.2.4ii). The weight of peptide-resin after chain assembly was 1.40 g. Resin-bound peptide (0.5 g) was cleaved for 1.5 h with a solution of 5% EDT, 5% EMS, 5% thioanisole and 5% water in TFA (10 cm³), giving crude peptide (0.29 g). Crude peptide (0.07 g) was dissolved in 50% aqueous acetonitrile (1.5 cm³). This solution was purified in 10 portions by preparative HPLC (11 cm. column; flow 3.5 cm³/minute; gradient: (isocratic conditions: 11% acetonitrile)). Lyophilisation of the appropriate fractions gave the **title compound** as a fluffy white solid (0.011 g).

FAB-MS m/z 1377.4; C₅₅H₉₆N₁₈O₂₁S requires 1377.6; Analytical HPLC (gradient A) Rt 7.3 minutes; Amino acid analysis (acid hydrolysis; 18 h) Asx₁0.72, Ser₃2.65, Glx₂2.23, Gly₁1.10, Val₁1.09, Met₁1.11, Leu₁1.09, Lys₁1.02, Arg₁0.65.
600 MHz $^1$H NMR (90%H$_2$O, 10%D$_2$O)

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$N^\alpha$-9-Fluorenylmethoxycarbonyl-$N^\gamma$-(5H-dibenzo[a,d]cyclohepten-5-yl)-L-glutamine (110)

Fmoc-Gln-(Dbse)-OH

$N^\alpha$-9-Fluorenylmethoxycarbonyl-L-glutamine (5.0 g, 13.6 mmol) and 5H-dibenzo[a,d]cyclohepten-5-ol (3.0 g, 14.4 mmol) were suspended in acetic acid (250 cm$^3$) and conc. sulphuric acid (0.3 cm$^3$) added. The bright pink mixture was stirred for 30 h, during which time a pale pink precipitate formed and the colour of the mixture faded considerably. After filtration the wet solid was resuspended in EtOAc (300 cm$^3$) and stirred for 2 h, then filtered again and washed with EtOAc (400 cm$^3$), water (until the filtrate was neutral to litmus paper) and diethyl ether (250 cm$^3$). The resulting white solid was dried in vacuo to give the title compound (6.6 g; 87%).

M.p. 215-219°C; TLC-A: Rf 0.78; $[\alpha]_D^{25}$ (c=1, DMF) -12.1°; FAB-MS: m/z=559 (5%), 191 (100%); HRMS m/z 559.22328; $C_{35}H_{31}N_2O_5$ requires 559.22333; $\lambda_{max}$ (DMF) 267 (e=26, 000), 277 (24, 000), 288 (17, 000), 300 nm (15, 000 mol$^{-1}$dm$^3$cm$^{-1}$); $\nu_{max}$ (KBr disc) 3300 (s, COOH, NH), 3030 (w,
arom. CH str.), 2940 (w, aliph. CH str.), 1690 (s, urethane C=O), 1640 (s, amide), 1530, 730 cm$^{-1}$ (s, arom. CH bend); $\delta_{H}$ (80 MHz, DMSO-d$_6$) 9.0 (d, J=9Hz, 1H, amide NH), 7.2-7.9 (m, 16H, aromatic CH), 7.1 (s, 2H, CH=CH), 5.6 (d, J=8Hz, 1H, urethane NH), 4.2 (bm, 4H, Fmoc CHCH$_2$, Dbse CH), 4.0 (b, 1H, $\alpha$CH), 2.4 (m, 2H, $\gamma$CH$_2$), 1.9 (m, 2H, $\beta$CH$_2$); $\delta_{C}$ (DMSO-d$_6$) 173.8 (q, COOH), 171.0 (q, amide), 156.3 (q, urethane), 143.9-133.6 (4 peaks, q, arom.), 131.3-120.3 (9 peaks, CH aromatic), 65.8 (CH$_2$, Fmoc), 53.7 ($\alpha$CH), 46.8 (CH, Fmoc), 32.0 ($\gamma$CH$_2$), 30.9 (CH, Dbse), 29.9 ($\beta$CH$_2$).

N$^\alpha$-9-Fluorenyl methoxycarbonyl-N$^\beta$-(5H-dibenzo[a,d]cyclohepten-5-yl)-L-asparagine (109)

Fmoc-Asn-(Dbse)-OH

N$^\alpha$-9-Fluorenyl methoxycarbonyl-L-asparagine (4.81 g, 13.6 mmol) and 5H-dibenzo[a,d]cyclohepten-5-ol (3.0 g, 14.4 mmol) were suspended in acetic acid (250 cm$^3$) and conc. sulphuric acid (0.3 cm$^3$) added. The bright pink mixture was stirred for 30 h, during which time a pale pink precipitate formed and the colour of the mixture faded considerably. After filtration the wet solid was resuspended in EtOAc (300 cm$^3$) and stirred for 2 hours, then filtered again and washed with EtOAc (400 cm$^3$), water (until the filtrate was neutral to litmus paper) and diethyl ether (250 cm$^3$). The resulting white solid was dried in vacuo to give the title compound (6.3 g; 85%).

M.p. 212-214°C; TLC-A: Rf 0.65; [$\alpha$]$_D^{25}$ (c=1, DMF) -10.0°; FAB-MS: m/z=545 (5%), 191 (100%); HRMS m/z 545.20766; C$_{34}$H$_{29}$N$_2$O$_5$ requires 545.20763; $\lambda_{\text{max}}$ (DMF) 266 (ε=27, 000), 277 (24, 000), 288 (17, 000), 300 nm (15, 000 mol$^{-1}$dm$^3$cm$^{-1}$); $\nu_{\text{max}}$ (KBr disc) 3300 (s, COOH, NH), 3030 (w, arom. CH str.), 2940 (w, aliph. CH str.), 1690 (s, urethane C=O), 1640 (s, amide), 1530, 730 cm$^{-1}$ (s, arom. CH bend); $\delta_{H}$ (80 MHz, DMSO-d$_6$) 9.0 (d, J=9Hz, 1H, amide NH), 7.2-7.8 (m, 16H, aromatic CH), 7.1 (s, 2H, CH=CH), 5.6 (d, J=9Hz, 1H, urethane NH), 4.3 (b, 1H, $\alpha$CH), 4.1 (b, 4H, Fmoc CHCH$_2$, Dbse CH), 2.7 (m, 2H, $\beta$CH$_2$); $\delta_{C}$ (DMSO-d$_6$) 173.3 (q, COOH), 168.9 (q, amide), 156.0 (q, urethane), 143.9-133.6 (4 peaks, q, arom.), 131.4-120.3 (9 peaks, CH aromatic), 65.9 (CH$_2$, Fmoc), 50.8 ($\alpha$CH), 46.8 (CH, Fmoc), 37.3 ($\beta$CH$_2$), 22.5 (CH, Dbse).
Nα-9-Fluorenylmethoxycarbonyl-Nγ-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-yl)-L-glutamine (112)

Fmoc-Gln-(Dbsa)-OH

Nα-9-Fluorenylmethoxycarbonyl-L-glutamine (1.60 g, 4.3 mmol) and 10, 11 dihydro-5H-dibenzo[a,d]cyclohepten-5-ol (1.76 g, 8.4 mmol) were suspended in acetic acid (40 cm³) and conc. sulphuric acid (0.075 cm³) added. The mixture was stirred for 30 h, during which time a precipitate formed. The mixture was poured into a stirred mixture of EtOAc/water (1:1; 150 cm³) then filtered. The resulting solid was washed thoroughly with EtOAc and cyclohexane then dried in vacuo to give the title compound (1.9 g; 78%).

M.p. 204-205°C; TLC-A: Rf 0.78; [α]D 25 (c=1, DMF) -4.0°; FAB-MS: m/z=561 (7%), 193 (100); HRMS m/z 561.23891; C35H33N2O5 requires 561.23893; λmax (DMF) 266 (ε=13, 000), 289 (4, 500), 300 nm (5, 200 mol⁻¹ dm³ cm⁻¹); νmax (KBr disc) 3300 (s, COOH, NH), 3030 (w, arom. CH str.), 2940 (w, aliph. CH str.), 1690 (s, urethane C=O), 1640 (s, amide), 1540 (s, amide), 1270(s), 730 cm⁻¹ (s, arom. CH bend); δH (80 MHz, DMSO-d6) 9.0 (d, J=9Hz, 1H, amide NH), 7.2-7.8 (m, 16H, aromatic CH), 6.4 (d, J=9Hz, 1H, urethane NH), 4.2 (b, 4H, Fmoc CHCH2, Dbsa CH), 4.0 (b, 1H, αCH), 3.1 (s, 4H, Dbsa CH2CH2), 2.4 (m, 2H, χCH2), 1.9 (m, 2H, βCH2).

Nα-9-Fluorenylmethoxycarbonyl-Nβ-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-yl)-L-asparagine (111)

Fmoc-Asn-(Dbsa)-OH

Nα-9-Fluorenylmethoxycarbonyl-L-asparagine (0.40 g, 1.1 mmol) and 10, 11 dihydro-5H-dibenzo[a,d]cyclohepten-5-ol (0.24 g, 1.1 mmol) were suspended in acetic acid (20 cm³) and conc. sulphuric acid (0.02 cm³) added. The mixture was stirred for 30 h, during which time a precipitate formed. The mixture was poured into a stirred mixture of EtOAc/water (1:1;
100 cm$^3$) then filtered. The resulting solid was washed thoroughly with EtOAc and cyclohexane then dried \textit{in vacuo} to give the \textit{title compound} (0.46 g; 74%).

M.p. 210-214°C; TLC-A: Rf 0.75; [\alpha]_D^{25} (c=1, DMF) -3.2°; FAB-MS: m/z=547 (5%), 193 (100); HRMS 547.22332; C$_{34}$H$_{31}$N$_2$O$_5$ requires 547.22328; $\lambda_{\text{max}}$ (DMF) 268 ($\epsilon=14,000$), 289 (4, 500), 300 nm (5, 300 mol$^{-1}$dm$^3$cm$^{-1}$); $\nu_{\text{max}}$ (KBr disc) 3300 (s, COOH, NH), 3020 (w, arom. CH str.), 2940 (w, aliph. CH str.), 1690 (s, urethane C=O), 1640 (s, amide), 1540 (s, amide), 1240 (s), 730 cm$^{-1}$ (s, arom. CH bend); $\delta_H$ (80 MHz, DMSO-d$_6$) 9.0 (d, J=9Hz, 1H, amide NH), 7.2-7.8 (m, 16H, aromatic CH), 6.4 (d, J=9Hz, 1H, urethane NH), 4.4 (b, 1H, $\alpha$CH), 4.2 (b, 4H, Fmoc CHCH$_2$, Dbsa CH), 3.1 (s, 4H, Dbsa CH$_2$CH$_2$), 2.7 (m, 2H, $\beta$CH$_2$).

\[ \text{N}^\alpha-(2,2-\text{Bis(4-nitrophenyl)ethoxycarbonyl-N}^\gamma-(5H-\text{dibenzo[a,d]cyclohepten-5-yl})-L\text{-glutamine (114)}} \]

N$^\alpha$-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)-L-glutamine (0.53 g, 1.2 mmol) and 5H-dibenzo[a,d]cyclohepten-5-ol (0.28 g, 1.2 mmol) were dissolved in acetic acid (3.0 cm$^3$) and conc. sulphuric acid (0.02 cm$^3$) added. The bright orange solution was stirred for 20 h, during which time a pale pink precipitate formed and the colour of the mixture faded considerably. After filtration the solid was washed with EtOAc (20 cm$^3$), water (until the filtrate was neutral to litmus paper) and diethyl ether (20 cm$^3$). The resulting white solid was dried \textit{in vacuo} to give the \textit{title compound} (0.46 g; 60%).

M.p. 165-172°C; TLC-A: Rf 0.73; [\alpha]_D^{25} (c=0.5, DMF) -2.5°; FAB-MS: m/z=651 (6%), 191 (100%); HRMS m/z 651.20905; C$_{35}$H$_{31}$N$_4$O$_9$ requires 651.20908; $\lambda_{\text{max}}$ (MeOH) 279 nm ($\epsilon=28,000$ mol$^{-1}$dm$^3$cm$^{-1}$); $\nu_{\text{max}}$ (KBr disc) 3300 (s, COOH, NH), 3040 (w, arom. OH str.), 2960 (w, aliph. OH str.), 1700 (s, urethane C=O), 1640 (m, amide), 1520 (s, C-NO$_2$), 1340 cm$^{-1}$ (s, C-NO$_2$); $\delta_H$ (80 MHz, CDCl$_3$) 8.1 (d, J=8Hz, 4H, Bnpeoc 3, 5H), 7.2-7.5 (m, 12H, Bnpeoc 2, 4H Dbsa CH), 7.1 (s, 2H, CH=CH), 6.1 (d, J=8Hz, 1H, urethane NH), 4.5 (b, 4H, Bnpeoc CHCH$_2$, Dbs CH), 4.1 (m, 1H, $\alpha$CH), 3.1 (s, 4H, Dbsa CH$_2$CH$_2$), 2.7 (m, 2H, $\beta$CH$_2$).
2.1 (m, 2H, γCH₂), 1.9 (m, 2H, βCH₂); δc (DMSO-d₆) 175.7 (q, COOH), 171.5 (q, amide), 155.6 (q, urethane), 146.7-133.1 (4 peaks, q, arom.), 131.3-120.3 (7 peaks, CH aromatic), 65.8 (CH₂, Bnpeoc), 53.2 (αCH), 49.3 (CH, Fmoc), 31.9 (γCH₂), 27.2 (βCH₂), 20.1 (CH, Dbse).

Nα–(2,2-Bis(4-nitrophenyl)ethoxycarbonyl-Nβ-(5H-dibenzo[a,d]cyclohepten-5-yl)-L-asparagine (113)

Bnpeoc-Asn-(Dbse)-OH

Nα–(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)-L-asparagine (0.60 g; 1.3 mmol) and 5H-dibenzo[a,d]cyclohepten-5-ol (0.28; 1.3 mmol) were dissolved in acetic acid (3.0 cm³) and conc. sulphuric acid (0.02 cm³) added. The bright orange solution was stirred for 20 h, during which time a pale pink precipitate formed and the colour of the mixture faded considerably. After filtration the solid was washed with EtOAc (20 cm³), water (until the filtrate was neutral to litmus paper) and diethyl ether (20 cm³). The resulting white solid was dried in vacuo to give the title compound (0.52 g; 61%).

M.p. 161-171°C; TLC-A: Rf 0.80; [α]D²⁵ (c=0.5, DMF) -2.0°; FAB-MS: m/z=637 (6%), 191 (100); HRMS m/z 637.19346; C₃₄H₂₉N₄O₉ requires 637.19344; λmax(DMF) 278 nm (ε=29,000 mol⁻¹dm³cm⁻¹); νmax (KBr disc) 3300 (s, COOH, NH), 3040 (w, arom. CH str.), 2940 (w, aliph. CH str.), 1700 (s, urethane C=O), 1640 (s, amide), 1515 (s, C-NO₂), 1340 cm⁻¹ (s, C-NO₂); δH (80 MHz, CDCl₃) 8.1 (d, J=8Hz, 4H, Bnpeoc 3, 5H), 7.2-7.5 (m, 12H, Bnpeoc 2, 4H Dbse CH), 7.1 (s, 2H, CH=CH), 6.2 (d, J=9Hz, 1H, urethane NH), 4.5 (bm, 3H, Bnpeoc CH₂CH₂), 4.3 (b, 2H, αCH, Dbs CH), 2.5 (m, 2H, βCH₂); δc (DMSO-d₆) 171.1 (q, COOH), 168.2 (q, amide), 154.7 (q, urethane), 147.1-137.4 (4 peaks, q, arom.), 132.9-122.8 (7 peaks, CH aromatic), 64.8 (CH₂, Bnpeoc), 53.6 (βCH₂), 49.7 (αCH), 48.6 (CH, Fmoc), 36.0 (CH, Dbse).
**Nα-Benzylloxycarbonyl-L-glutamine (132)**

Z-Gln-OH

This compound was prepared by the method of Beacham et al.\textsuperscript{166}. To a cooled suspension of L-glutamine (36.5 g; 0.25 mol) in 1 M sodium hydrogen carbonate was added benzylchloroformate (37.5 cm\textsuperscript{3}; 0.26 mol) dropwise such that the reaction temperature did not exceed 5°C. The mixture was allowed to warm to room temperature then stirred for 20 h and extracted with diethyl ether (2x200 cm\textsuperscript{3}). The aqueous solution was carefully brought to pH=1 with 10 M HCl, keeping the temperature below 5°C. The resulting white solid was recrystallised from water and dried \textit{in vacuo} to give the title compound as a white solid (55.3 g; 70%).

M.p. 138-139°C (lit.\textsuperscript{166} 139-141°C); TLC-B Rf 0.62; $\lambda_{max}$ (THF) 257 nm ($\varepsilon$=220 mol\textsuperscript{-1}dm\textsuperscript{3}cm\textsuperscript{-1}); $\nu_{max}$ (nujol mull) 3470 (s, COOH, NH), 3330 (s, NH), 1690 (s, urethane C=O), 1620 (s, amide), 1530 cm\textsuperscript{-1} (s); $\delta_{H}$ (80 MHz, MeOH-d4) 7.5 (d, J=8Hz, 1H, amide NH), 7.3 (bs, 5H, Z aromatic CH), 6.8 (b, urethane NH), 5.0 (s, 2H, benzylic CH\textsubscript{2}), 4.1 (b, 1H, $\alpha$CH), 2.2 (m, 2H, $\gamma$CH\textsubscript{2}), 1.8 (m, 2H, $\beta$CH\textsubscript{2}).

**Nα-Benzylloxycarbonyl-N$_\gamma$-bis(4-methoxyphenyl)methyl-L-glutamine (133)**

Z-Gln-(Mbh)-OH

This compound was prepared by the method of König and Geiger\textsuperscript{123}. To a solution of Nα-benzylloxycarbonyl-L-glutamine (11.2 g, 0.04 mol) and 4,4'-dimethoxybenzhydrol (9.6 g, 0.04 mol) in acetic acid was added conc. sulphuric acid (0.5 cm\textsuperscript{3}) giving an orange solution. After stirring for 20 h the solution was poured into water (250 cm\textsuperscript{3}). The mixture was filtered and the solid washed with water, then taken up in EtOAc (100 cm\textsuperscript{3}). This solution was dried over anhydrous magnesium sulphate then concentrated \textit{in vacuo} to a yellow oil. Trituration with diethyl ether and recrystallisation of the resulting solid from THF/ light petroleum gave the title compound as a white solid (13.0 g; 64%).
Ny-Bis(4-methoxyphenyl)methyl-L-glutamine (134)

H-Gln-(Mbh)-OH

This compound was prepared by the method of König and Geiger\textsuperscript{123}. A solution of N\textsuperscript{\alpha}-benzyloxycarbonyl-Ny-bis(4-methoxyphenyl)methyl-L-glutamine (13.0 g, 25.6 mmol) in THF (50 cm\textsuperscript{3}) was cooled to -78\textdegree C and flushed with dry nitrogen during the addition of 10% palladium on carbon (0.65 g; 5% by weight). The flask was evacuated then placed under 1 atm. hydrogen gas for 48 h. Glacial acetic acid (100 cm\textsuperscript{3}) was added to dissolve a white precipitate and the resulting suspension filtered through celite and concentrated to an oil. Trituration with diethyl ether gave a pale brown solid which was recrystallised from water and dried to give the title compound (7.77 g; 82%).

M.p. 190-192\textdegree C (lit.\textsuperscript{123} 205-206\textdegree C); TLC-A Rf 0.35; FAB-MS m/z= 373 (10\%), 227 (100); HRMS m/z 373.17631; C\textsubscript{20}H\textsubscript{25}N\textsubscript{2}O\textsubscript{5} requires 373.17633; \(\delta\text{H} (80 \text{ MHz, DMSO-d6/CD}_{3}\text{COOD 1:1})\) 7.2, 6.8 (AB, J=8Hz, 8H, Mbh aromatic), 6.0 (s, 1H, Mbh CH), 4.0 (b, 1H, \(\alpha\text{CH}\)), 3.7 (s, 6H, OMe), 2.3 (m, 2H, \(\gamma\text{CH}\)), 1.9 (m, 2H, \(\beta\text{CH}\)).
Nα-9-Fluorenylmethoxycarbonyl-Nγ-bis(4methoxyphenyl)methyl-L-glutamine (135)

Fmoc-Gln-(Mbh)-OH

To a suspension of Nγ-bis(4-methoxyphenyl)-methyl-L-glutamine (4.0 g, 10.8 mmol) in 10% aqueous sodium carbonate (150 cm³) and dimethoxyethane (80 cm³) was added a solution of N-(9-fluorenylmethoxycarbonyl)-O-succinimide (5.6 g, 16.2 mmol) in dimethoxyethane (40 cm³). The resulting solution was stirred for 20 h then diluted with water (200 cm³) and extracted with diethyl ether (2x250 cm³) and EtOAc (2x250 cm³). The aqueous solution was carefully acidified with 2 M potassium hydrogensulphate and extracted with EtOAc (2x300 cm³). The organic extracts were dried over anhydrous magnesium sulphate then concentrated to ca. 10 cm³ and passed down a column of silica (60H; 15 cm diam. x 15 cm deep) eluted with a gradient of methanol in chloroform (0-20%, total volume 1.5 dm³). The appropriate fractions were combined and concentrated to ca. 15 cm³ then pipetted slowly into cold, stirred light petroleum (1 dm³). The resulting white precipitate was filtered and dried to give the title compound (3.8 g; 58%).

M.p. 155-160°C (lit.123 158-164°C); TLC-A Rf 0.75; [α]D²⁵ (c=1.0, DMF) - 8.9°; FAB-MS m/z= 586 (13%), 227 (100); λmax (THF) 262 (ε=17, 000), 300 nm (5000 mol⁻¹dm³cm⁻¹); δH (80 MHz, DMSO-d6) 8.7 (d, J=8Hz, 1H, amide NH), 7.0-7.8 (m, 16H, Fmoc and Mbh aromatic CH), 6.0 (m, 2H, urethane NH, Mbh CH), 4.3 (b, 3H, Fmoc CHCH₂), 4.1 (b, 1H, αCH), 3.7 (s, 6H, OMe), 2.2 (m, 2H, γCH₂), 1.9 (m, 2H, βCH₂).

Nα-Benzylxocarbonyl-Nγ-triphenylmethyl-L-asparagine (136)

Z-Asn-(Trt)-OH

This compound was prepared by the method of Sieber and Riniker156. To a solution of Nα-benzylxocarbonyl-L-asparagine (10.6 g, 0.04mol) and triphenylmethanol (20.8 g, 0.08mol) in acetic acid (120 cm³) was added acetic anhydride (7.5 cm³, 0.08mol) and conc. sulphuric acid (0.5 cm³). The resulting solution was heated to 50°C for 90 minutes then treated with water (100 cm³) and EtOAc (200 cm³). After separation the aqueous
solution was further extracted with EtOAc (200 cm\(^3\)). The combined organic extracts were washed with water (200 cm\(^3\)) and brine (200 cm\(^3\)) then dried over anhydrous magnesium sulphate and concentrated to an oil. Trituration with diethyl ether gave a pale brown solid which was recrystallised from THF/diethyl ether to give the title compound as a white solid (6.8 g, 55%).

**M.p. 192-193°C (lit.\(^{156}\) 195-196°C); TLC-A Rf 0.76; FAB-MS m/z= 509 (40%), 243 (100); \(\lambda_{\text{max}}\) (THF) 260 nm (\(\epsilon=930\) mol\(^{-1}\)dm\(^3\)cm\(^{-1}\)); \(\delta_H\) (80 MHz, CDCl\(_3\)) 8.5 (d, J=9Hz, 1H, amide NH), 7.3 (bs, 5H, Z aromatic CH), 7.1 (s, 15H, trityl CH), 4.9 (s, 2H, benzylic CH\(_2\)), 4.3 (b, 1H, \(\alpha\)CH), 3.0 (m, 2H, \(\beta\)CH\(_2\)).**

**N\(^\gamma\)-Triphenylmethyl-L-asparagine (137)**

**H-Asn-(Trt)-OH**

This compound was prepared by the method of Sieber and Riniker\(^{156}\). A solution of \(N^\alpha\)-benzylxocarbonyl-N\(^\gamma\)-triphenylmethyl-L-asparagine (9.3 g, 18.2 mmol) in THF (150 cm\(^3\)) and methanol (150 cm\(^3\)) was cooled to -78°C and flushed with dry nitrogen during the addition of 10% palladium on carbon (0.47 g; 5% by weight). The flask was evacuated then placed under 1 atm. hydrogen gas for 72 h. The resulting suspension was filtered through celite and concentrated to an oil. Trituration with diethyl ether gave a white solid which was recrystallised from water and dried to give the title compound (5.24 g; 77%).

**M.p. >220°C (lit.\(^{156}\) >220°C); TLC-A Rf 0.20; FAB-MS m/z= 375 (7%), 243 (100); \(\delta_H\) (80 MHz, DMSO-d\(_6\)) 8.6 (d, J=5Hz, 1H, amide NH), 7.1(s, 15H, trityl CH), 4.3 (b, 1H, \(\alpha\)CH), 2.3 (m, 2H, \(\beta\)CH\(_2\)).**

**N\(^\alpha\)-9-Fluorenylmethoxycarbonyl-N\(^\gamma\)-triphenylmethyl-L-asparagine (138)**

**Fmoc-Asn-(Trt)-OH**

To a suspension of \(N^\gamma\)-triphenylmethyl-L-asparagine (4.9 g, 13.1 mmol) in water (60 cm\(^3\)) was added a solution of triethylamine (4.6 cm\(^3\); 33 mmol) in dioxan (60 cm\(^3\)). To the resulting solution was added \(N\)-(9-fluorenylmethoxycarbonyl)-O-succinimide (4.5 g, 13.1 mmol). The resulting
solution was stirred for 12 h then diluted with water (150 cm$^3$), acidified with 2 M potassium hydrogensulphate and extracted with EtOAc (2x300 cm$^3$). The organic extracts were washed with water (200 cm$^3$) and brine (200 cm$^3$), dried over anhydrous magnesium sulphate then concentrated to ca. 10 cm$^3$ and passed in three portions down a column of silica (60H; 7.5 cm diam. x 15 cm deep) eluted with a gradient of methanol in chloroform (0-10%, total volume 400 cm$^3$). The appropriate fractions were combined and concentrated to ca. 15 cm$^3$ then pipetted slowly into cold, stirred light petroleum (1dm$^3$). The resulting white precipitate was filtered and dried to give the title compound (4.6 g; 60%).

M.p. 180-187°C (lit.\textsuperscript{156} 211-213°C); TLC-A Rf 0.66; [$\alpha$]$_D^{25}$ (c=1.0, DMF) -9.8 (lit.\textsuperscript{156} -14.8°); FAB-MS m/z = 611 (7%), 243 (100); \nu$_{max}$(KBr disc) 3300 (s, COOH, NH), 3040 (w, arom. CH str.), 2940 (w, aliph. CH str.), 1690 (s, urethane C=O), 1640 (s, amide), 1560 (s), 1480 (s), 1320 (m), 730 (s), 690 cm$^{-1}$(s); $\lambda_{max}$ (THF) 268 nm ($\varepsilon$=17, 000 mol$^{-1}$dm$^3$cm$^{-1}$); $\delta$H (80 MHz, CDCl$_3$) 7.0-7.8 (m, 23H, Fmoc and Trt aromatic CH), 6.3 (b, 1H, urethane NH), 4.3 (bm, 4H, Fmoc CHCH$_2$, CH), 2.7 (m, 2H, $\beta$CH$_2$).

**Coupling of Fmoc-GIn/Asn-(Dbse/Dbsa)-OH**

To a stirred solution of DIC (0.5 M in DMF; 2.0 cm$^3$) and HOBt (0.5 M in DMF; 2.0 cm$^3$) in DMF (4 cm$^3$) was added in small portions the protected amino acid (1.0 mmol). After 20 minutes the mixture was filtered and the filtrate transferred to an Applied Biosystems reagent cartridge. The activated amino acid was taken up to the activator vessel and the cycle continued as normal.

**Comparative Deprotection of Side-Chain-Amide Protecting Groups**

To the protected amino acid (20 mg) was added 95% TFA (1.2 cm$^3$). The mixture was quickly shaken to achieve dissolution, then analysed by TLC. Further analyses were performed at intervals of 5 minutes up to 15 minutes. The upper and lower limits for the half-reaction point (t$^{1/2}$) were determined by estimating the intensities of the appropriate spots following elution of the plate (eluant A) and detection with ninhydrin. Rf: 0.4 (Fmoc-Gln-OH); 0.7-
0.8 (Fmoc-Gln-(PG)-OH). After 10 minutes reaction a portion of the reaction mixture (0.6 cm\(^3\)) was withdrawn, evaporated to dryness and triturated with diethyl ether to give a white solid. This solid was taken up in acetonitrile/water (1:1; 1 cm\(^3\)) and analysed by TLC. The amount of Fmoc-Gln-OH was calculated from the estimated intensities of the appropriate spots. The results are summarised below.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>(t_0) (mins)</th>
<th>%Fmoc-Gln (t=10mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Gln-OH</td>
<td>---</td>
<td>100</td>
</tr>
<tr>
<td>Fmoc-Gln-(Mbh)-OH</td>
<td>5-10</td>
<td>90</td>
</tr>
<tr>
<td>Fmoc-Gln-(Dbse)-OH</td>
<td>0-5</td>
<td>100</td>
</tr>
<tr>
<td>Fmoc-Gln-(Dbsa)-OH</td>
<td>10-15</td>
<td>60</td>
</tr>
</tbody>
</table>

**Comparative Solubility of Asn/Gln Derivatives**

To stirred solvent (0.5 cm\(^3\)) was added small portions of a weighed amount of the protected amino acid until the added material would not dissolve. Weighing the remaining solid placed an upper limit on the solubility of that derivative.

<table>
<thead>
<tr>
<th>Upper limit of Solubility in 1 cm(^3) of:</th>
<th>DMF</th>
<th>Dioxan/DMF (1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Asn-(Trt)-OH</td>
<td>&gt;0.2g</td>
<td>&gt;0.2g</td>
</tr>
<tr>
<td>Fmoc-Gln-(Trt)-OH</td>
<td>&gt;0.2g</td>
<td>&gt;0.2g</td>
</tr>
<tr>
<td>Fmoc-Asn-(Dbse)-OH</td>
<td>0.08g</td>
<td>insol.</td>
</tr>
<tr>
<td>Fmoc-Gln-(Dbse)-OH</td>
<td>&gt;0.2g</td>
<td>&lt;0.05g</td>
</tr>
<tr>
<td>Bnpeoc-Asn-(Dbse)-OH</td>
<td>&gt;0.2g</td>
<td>&gt;0.2g</td>
</tr>
<tr>
<td>Bnpeoc-Gln-(Dbse)-OH</td>
<td>&gt;0.2g</td>
<td>&gt;0.2g</td>
</tr>
</tbody>
</table>

**IL-1-β-pre 233-238 (139)**

Phe-Pro-Asn-Trp-Tyr-Leu-OH

The initial resin was Fmoc-Leu\(\text{H}(0.55 \text{ mmol g}^{-1} ; 1.00 \text{ g}, 0.55 \text{ mmol}). The side-chain amide group of asparagine was protected by the dibenzosuberenyl group (Fmoc-Asn-(Dbse)-OH). This asparagine
derivative was activated outwith the peptide synthesiser as described above. The weight of peptide-resin after chain assembly was 1.32g. Resin-bound peptide (0.2 g) was treated for 1.5 h with a solution of 5% thioanisole, 5% N-acetyltryptamine and 5% water in TFA (10 cm³). The resulting solution was concentrated in vacuo to ca. 2 cm³, then treated with water/diethyl ether (1:1; 30 cm³). After separation the aqueous extract was washed with two further portions of diethyl ether (2x15 cm³) and purified in 10 portions by preparative HPLC (11 cm. column; flow 10 cm³/minute; gradient: (time, % acetonitrile) (0, 10), (20, 40), (21, 10)). Lyophilisation of the appropriate fractions gave the title compound as a white solid (0.035 g; 34% by proportion from starting resin..

FAB-MS calculated for C_{44}H_{54}N_{8}O_{9} m/z 839.0; observed 839.0; Analytical HPLC (gradient A) Rt 13.0 minutes; Amino acid analysis (acid hydrolysis; 18 h) Asx_{1}0.91, Pro_{1}1.01, Ala_{2}2.07, Leu_{1}1.05, Tyr_{1}0.98, Phe_{1}1.04, Trp_{1}0.84.

Substance P (140)

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂

Chain assembly of this peptide was repeated five times. On each occasion the glutamine residues were incorporated with different side-chain amide protecting group. Peptide-resin (0.4 g) was cleaved in each case for 90 minutes with a solution of 5% thioanisole, 5% anisole, 5% ethylmethylsulphide and 5% water in TFA (10 cm³) to give crude peptide (0.16-0.19 g). The crude peptide was analysed by FAB-MS.

Relative Peak Intensity When Amide Protected By:

<table>
<thead>
<tr>
<th>m/z</th>
<th>Dbse</th>
<th>Dbsa</th>
<th>Trt</th>
<th>Unprotected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1348</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>1234</td>
<td>55</td>
<td>55</td>
<td>15</td>
<td>100</td>
</tr>
</tbody>
</table>

Note. Crude peptide prepared using Fmoc-Gln-(Mbh)-OH not analysed.
Notes and References


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Courses Attended


Advances in Organic Chemistry, various speakers, Department of Chemistry, University of Edinburgh, 1989-91.

Industrial Chemistry, Members of ICI Grangemouth and Department of Chemical Engineering, University of Edinburgh, 1990.

R.S.C. Peptide and Protein Group, spring meeting 1990, Exeter University
