THE DEVELOPMENT OF NOVEL METHODS FOR THE SYNTHESIS OF

HISTIDINE-CONTAINING PEPTIDES

A THESIS SUBMITTED TO THE BOARD OF
THE FACULTY OF PHYSICAL SCIENCES FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

J.D. RICHARDS, B.A.

St. Johns College and the
Dyson Perrins Laboratory,
University of Oxford.

October 1981.
The role of the amino-acid histidine in biologically important molecules and the problems encountered in its incorporation whether in protected or unprotected form into peptides, and recent work establishing the importance of the location of the \textit{im}-protecting group \( \text{N(M)} \) being desirable are reviewed in the introduction.

In Chapter 1 the electrolytic deprotection of \( \text{N}-\text{phenacyl-thyroliberin} \) is described: it was found to produce a complex mixture. This and other difficulties encountered by co-workers led to the conclusion that \( \text{N}-\text{phenacyl} \) was unsuitable for general use as a histidine protecting group.

In Chapter 2, the investigation of the 1,1,1-trichlorobut-2-enyl group for histidine protection is described. A model compound \( \text{N}(1,1,1\text{-trichlorobut-2-enyl})\text{imidazole} \) was prepared and subjected to screening tests. A number of undesirable side-reactions including \textit{cis-trans} isomerisation of the double bond were observed indicating that this is not a practical blocking group.

A novel strategy of histidine protection involving reducing the basicity of the imidazole ring by temporary substitution with electron-withdrawing groups is outlined in Chapter 3. A new synthesis of \( \text{N(M)} \)-protected diiodohistidine derivatives and tests demonstrating their stability to routine conditions encountered in peptide synthesis and deprotection by hydrogenolysis are described. A synthesis of thyroliberin indicated the potential of this strategy.

In Chapter 4 preparations of the corresponding 2,5-dibromo-derivatives are described, and a solid phase synthesis of glycyl-\( \text{L} \)-histidyl-\( \text{L} \)-phenylalanine and a classical synthesis of thyroliberin are outlined. It was found however that although diiodination inhibited racemisation and dibromination was even more effective that direct blockade of the \( \text{N(M)} \) was indispensable for its complete prohibition.

A new optimised preparation for \( \text{N(M)}-\text{t-butoxycarbonyl,N(H)-benzyloxymethyl-L-histidine} \) is described in Chapter 5, this being the first simple three-step synthesis of a \( \text{N(M)} \)-protected histidine derivative.

In Chapter 6 the use of \( \text{N(M)}-\text{t-butoxycarbonyl,N(M)-benzyloxymethyl-L-histidine} \) as a protected intermediate in a number of demanding exercises, including the solid phase synthesis of angiotensin II and a solution synthesis of a histidyl-tryptophan dipeptide are demonstrated. No problems due to the histidine derivatives were encountered.

Methods of evaluating the degree of racemisation occurring in activated histidine derivatives are discussed in Chapter 7.

References
To Ruth
Acknowledgements

I wish to record my sincere thanks to Dr. J.H. Jones for his inspiration and guidance during the course of this work.

My thanks are also due to many colleagues in the Dyson Perrins Laboratory, particularly Dr. T. Brown for helpful and enjoyable discussions, and to the service staff for their assistance.

Finally, I should like to thank my mother and my fiancée for the typing of this thesis.
ABBREVIATIONS

The abbreviations used in this thesis follow current IUPAC-IUB commission recommendations as listed in *J. Biol. Chem.*, 1972, **247**, 977.

Other abbreviations are listed below.

- **DMF**: dimethylformamide
- **TFA**: trifluoroacetic acid
- **DCCI**: dicyclohexylcarbodiimide
- **HOBt**: 1-hydroxybenzotriazole
- **-OTcp**: 2,4,5-trichlorophenyl ester
- **Z**: benzyloxycarbonyl
- **Boc**: t-butoxycarbonyl
- **Bom**: benzyloxymethyl
- **Pac**: phenacyl
- **Me**: methyl
- **Et**: ethyl
- **Ph**: phenyl
- **Bzl**: benzyl
- **Bu**: t-butyl
- **Ac**: Acetyl
- **Glp**: L-pyroglutamic acid (L-2-pyrrolidone-5-carboxylic acid)
- **t.l.c.**: thin layer chromatography
- **n.m.r.**: nuclear magnetic resonance
- **u.v.**: ultraviolet
- **s**: singlet
- **d**: doublet
- **t**: triplet
- **q**: quartet
- **m**: multiplet
- **br**: broad
CONTENTS

INTRODUCTION

Background 1
Unprotected Histidine 2
Protected Histidine 5
Conclusion 19

RESULTS AND DISCUSSION

CHAPTER 1 - FURTHER INVESTIGATIONS INTO THE USE OF N(M)-PHENACYL-L-HISTIDINE IN PEPTIDE SYNTHESIS

Introduction 22
Attempted Electrolytic Deprotection of N(M)-Phenacyl-thyroliberin 25
Results and Conclusion 28

CHAPTER 2 - INVESTIGATIONS INTO THE POSSIBILITY OF USING 1,1,1-TRICHLOROBUT-2-ENYL AS A N(M)-PROTECTING GROUP FOR HISTIDINE

Introduction 30
Attempted Preparation of N(1,1,1,-Trichlorobut-2-etyl)imidazole using 1,1,1,4-Tetrachlorobut-2-ene 30
Preparation of N(1,1,1-Trichlorobut-2-etyl)imidazole and Investigation of its Stability Under Conditions Likely to be Encountered in Peptide Synthesis 32
Results and Conclusion 39

CHAPTER 3 - IODINATION OF THE IMIDAZOLE RING AS A METHOD OF SIDE-CHAIN PROTECTION FOR HISTIDINE IN PEPTIDE SYNTHESIS

Introduction 40
Synthesis of L-2,5-Diiodohistidine and Investigation of its Stability Under Various Conditions 43
CONTENTS (Cont'd)

Synthesis of $N(\omega)$-t-Butoxycarbonyl-$L$-2,5-diiodohistidine 44
Synthesis of $N(\omega)$-Benzyloxy carbonyl-$L$-2,5-diiodohistidine 45
Dehalogenation of $N(\omega)$-t-Butoxycarbonyl-$L$-2,5-diiodohistidine Methyl Ester 46
Isolation and Characterization of $N(\omega)$-t-Butoxycarbonyl-$L$-5-iodohistidine Methyl Ester 46
Synthesis of Thyroliberin 50
Results and Conclusion 52

CHAPTER 4 - BROMINATION OF THE IMIDAZOLE RING AS A METHOD OF SIDE-CHAIN PROTECTION FOR HISTIDINE IN PEPTIDE SYNTHESIS

Introduction 53
Synthesis of $N(\omega)$-t-Butoxycarbonyl-$L$-2,5-dibromohistidine 54
Dehalogenation of $N(\omega)$-t-Butoxycarbonyl-$L$-2,5-dibromohistidine Methyl Ester 55
Synthesis of Thyroliberin 56
Solid Phase Synthesis of Glycyl-$L$-histidyl-$L$-phenylalanine 57
Results and Conclusion 63

CHAPTER 5 - OPTIMISATION OF THE PREPARATION OF $N(\omega)$-t-BUTOXYCARBONYL.$N(\eta)$-BENZYLOXYMETHYL-$L$-HISTIDINE

Introduction 65
Synthesis of $N(\omega)$.,$N(\tau)$-Bis-t-butoxycarbonyl-$L$-histidine Methyl Ester 68
Optimisation of the Preparation of $N(\omega)$-t-Butoxycarbonyl,$N(\eta)$-benzyloxymethyl-$L$-histidine Methyl Ester 69
Synthesis of $N(\omega)$-t-Butoxycarbonyl,$N(\eta)$-benzyloxymethyl-$L$-histidine 71
Results and Conclusion 72
## CONTENTS (Cont'd)

### CHAPTER 6 - PRACTICAL PEPTIDE APPLICATIONS OF N(\(\text{N}\))-BENZYLOXYMETHYL HISTIDINE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>74</td>
</tr>
<tr>
<td>Solid Phase Synthesis of 5-Isoleucine Angiotensin II</td>
<td>75</td>
</tr>
<tr>
<td>Attempted Solid Phase Synthesis of L-Histidyl-L-histidyl-glycyl-L-phenylalanine</td>
<td>81</td>
</tr>
<tr>
<td>Synthesis of N((\text{N}))-t-Butoxycarbonyl,N((\text{N}))-benzyloxyethyl-L-histidyl-L-prolyl-L-phenylalanine Benzyl Ester</td>
<td>84</td>
</tr>
<tr>
<td>Synthesis of Peptides Containing Histidine and Tryptophan</td>
<td>86</td>
</tr>
<tr>
<td>Results and Conclusion</td>
<td>88</td>
</tr>
</tbody>
</table>

### CHAPTER 7 - RACEMISATION OF HISTIDINE DERIVATIVES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>91</td>
</tr>
<tr>
<td>Estimation of Racemisation in Histidine Derivatives by Digestion with an L-Amino-acid Oxidase</td>
<td>92</td>
</tr>
<tr>
<td>Investigation into the use of Optical Rotation as a Method of Measuring Racemisation in Activated Amino-Acids</td>
<td>94</td>
</tr>
<tr>
<td>Results and Conclusion</td>
<td>100</td>
</tr>
</tbody>
</table>

### EXPERIMENTAL SECTION

#### METHODS AND MATERIALS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 1 - FURTHER INVESTIGATIONS INTO THE USE OF N((\text{N}))-PHENACYL-L-HISTIDINE IN PEPTIDE SYNTHESIS</td>
<td>106</td>
</tr>
<tr>
<td>CHAPTER 2 - INVESTIGATIONS INTO THE POSSIBILITY OF USING 1,1,1-TRICHLOROBUT-2-ENYL AS A N((\text{N}))-PROTECTING GROUP FOR HISTIDINE</td>
<td>108</td>
</tr>
<tr>
<td>CHAPTER 3 - IODINATION OF THE IMIDAZOLE RING AS A METHOD OF SIDE-CHAIN PROTECTION FOR HISTIDINE IN PEPTIDE SYNTHESIS</td>
<td>114</td>
</tr>
</tbody>
</table>
## CONTENTS (Cont'd)

<table>
<thead>
<tr>
<th>CHAPTER 4</th>
<th>BROMINATION OF THE IMIDAZOLE RING AS A METHOD OF SIDE-CHAIN PROTECTION FOR HISTIDINE IN PEPTIDE SYNTHESIS</th>
<th>124</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 5</td>
<td>OPTIMISATION OF THE PREPARATION OF (N(\alpha)-t)-BUTOXYCARBONYL,(N(\text{t})-)BENZYLOXYMETHYL-L-HISTIDINE</td>
<td>131</td>
</tr>
<tr>
<td>CHAPTER 6</td>
<td>PRACTICAL PEPTIDE APPLICATIONS OF (N(\text{t}))-BENZYLOXYMETHYL HISTIDINE</td>
<td>136</td>
</tr>
<tr>
<td>CHAPTER 7</td>
<td>RACEMISATION OF HISTIDINE DERIVATIVES</td>
<td>150</td>
</tr>
<tr>
<td>REFERENCES</td>
<td></td>
<td>157</td>
</tr>
</tbody>
</table>
Introduction
Background.

The vast field of peptide synthesis has often been extensively reviewed\(^1\-^7\) and further additions to the literature are reviewed annually in the Specialist Periodical Reports of the Chemical Society: "Amino-acids, Peptides and Proteins". However it is clear from the literature that despite the considerable amount of work carried out in this field the synthesis of even relatively small peptides can prove difficult, especially if amino-acid residues with heterocyclic side-chains are to be incorporated\(^8\) and in many cases the synthesis of biologically active peptides containing these awkward residues has been avoided. Histidine (Figure 1) is clearly one of these difficult amino-acids and only the aspects of the subject directly relevant to histidine will be considered here.

![Histidine structure](image)

Figure 1

The histidine residue plays a vital role in the function of many biologically active peptides and proteins. These include proteolytic enzymes e.g. trypsin, chymotrypsin, papain and subtilisin\(^9\-^{13}\) in which histidine is present at the active site where the imidazole ring can function as an acid/base or nucleophilic catalyst. It has recently
been shown that RNase Rh, isolated from glucozyme, also contains histidine at the active site\textsuperscript{14}. Many peptide hormones, e.g. corticotropin (ACTH)\textsuperscript{15}, angiotensin\textsuperscript{16} and releasing factors, e.g. thyroliberin (TRF)\textsuperscript{17}, luliberin (LH-RF)\textsuperscript{18} are histidine containing and replacement of the histidine by another amino-acid usually reduces biological activity drastically, or in some cases abolishes it completely. Peptides containing histidine have also been shown to be involved as neurotransmitters and inhibitors in the central nervous system of mammals\textsuperscript{19,20}. In many other proteins histidine acts as a ligand to bind metal ions necessary for activity e.g. Zn\textsuperscript{2+} in carbonic anhydrase\textsuperscript{21}, and Fe\textsuperscript{2+}, in haemoglobin\textsuperscript{22}. The synthesis of these biologically-important molecules and their analogues is often difficult due to the complications introduced by the presence of an imidazole side-chain. The latter, if left unprotected, participates in and catalyses undesirable side-reactions including racemisation, causes insolubility of intermediates and products and limits the permissible strategies for the incorporation of histidine into peptides.

**Unprotected Histidine.**

\textit{N(\&)}-Acyl-histidines and \textit{N(\&)}-acyl-histidine peptides are zwitterionic and, consequently their solubility in most organic solvents is poor\textsuperscript{23}. This has limited the use of standard derivatives such as \textit{N(\&)}-benzyloxycarbonyl-\textit{L}-histidine, with the result that incorporation of histidine into peptides has tended to rely on the azide method\textsuperscript{24} in which the acid azide is readily obtainable from the more soluble \textit{N(\&)}-acyl-\textit{L}-histidine methyl ester via the hydrazide\textsuperscript{25}. The presence of an unblocked imidazole ring which is a weak base\textsuperscript{26}, restricts the use of an acid wash as a purification step so that the isolation of pure histidine
derivatives can be difficult. Side reactions of the unblocked imidazole ring are also potentially troublesome. Acylation by some reactive acyl components at the im-nitrogen can compete with \( N(\omega) \)-acylation and may even be favoured in some cases for example \( N(\omega) \)-benzyloxycarbonyl-histidine, and not \( N(\omega) \)-benzyloxycarbonyl-histidine is the only monobenzyloxycarbonyl derivative detected in the reaction of histidine with two equivalents of benzylchloroformate\(^{27} \). In the presence of excess acylating agent and aqueous base (typical conditions for the preparation of \( N(\omega) \)-benzyloxycarbonylamino-acid derivatives), the Bamberger reaction can occur, leading to an opening of the imidazole ring. \( N(\omega) \)-acylimidazoles are often quite reactive in themselves, in fact \( N \)-acylimidazole derivatives are commercially available as acylating agents\(^{29} \). Alkylation of the imidazole ring can also occur\(^{30} \), for example by reaction with the chloromethyl group of the Merrifield resin\(^{31} \) and alkylation under quite mild conditions leads to the formation of a mixture of \( N(\gamma) \)- and \( N(\pi) \)-monoalkylated species and the \( N(\pi,\pi) \)-dialkylated material\(^{32} \). An unprotected imidazole ring can also react with \( N,N' \)-dicyclohexylcarbodiimide\(^{33} \) to form both isomeric \( N(\im) \)-dicyclohexylamidino-histidines\(^{34} \). In fact Sheehan et al\(^{35} \) have shown that activation of the histidine carboxyl group in the presence of an unprotected imidazole side-chain leads to intramolecular cyclisation to form a cyclic imidazole (Scheme 1).

\[
\begin{align*}
\text{CO}_2\text{H} & \quad \text{pNO}_2\text{ZHN} \\
\text{DCCI} & \quad \text{pNO}_2\text{ZHN}
\end{align*}
\]

Scheme 1
Bodanisky et al. have shown that the presence of a free imidazole group can catalyse the alcoholysis of active esters, and the presence of an unprotected histidine has been shown to lead to extensive O-acylation of unprotected serine. This effect is enhanced by the presence of tertiary bases and can be suppressed by the application of additives such as 2,4-dinitrophenol. Catalysis of racemisation by free imidazole groups has been reported.

The types of coupling procedure which are applicable in the presence of unprotected histidine are clearly restricted. Active esters of free histidine have not been obtained, presumably because of the intramolecular cyclisation process and yields in mixed anhydride syntheses have been very poor for similar reasons. When a two-fold excess of the activating species (e.g. isobutyl chloroformate) was used per equivalent of histidine the excess mixed anhydride method gave a good yield of a product of high optical purity. However the isolated product consisted of the N(im)-isobutyloxycarbonyl derivative of histidine instead of the required anhydride.

As mentioned previously, the coupling method of choice for unprotected histidine has been the azide procedure. However, it now seems that this coupling method can be accompanied by substantial racemisation even when the amount and type of tertiary base is carefully controlled. Verber has studied the equilibrium that exists between histidine azides and their corresponding cyclic imidazolides. He has found that on neutralisation of a solution of N(ω)-t-butoxycarbonyl-L-histidine azide hydrochloride, the imidazolide (detected by infra-red spectroscopy ν = 1780 cm⁻¹), was formed rapidly and reversibly followed by a slower racemisation of the chiral centre (scheme 2).

This observation of racemisation during the azide coupling of an
N(ω)-alkoxycarbonylamino-acid derivative was most surprising as for many years such couplings had been considered "racemisation-free" within the limits of the tests available. This had been a fundamental factor in the strategy of peptide synthesis. Racemisation has been shown to occur in activated intermediates, usually via oxazolone formation, but the presence of an N(ω)-alkoxycarbonyl group prevents cyclisation under normal conditions, and so amino acids protected in this way are not prone to racemisation. Kemp produced a comprehensive review of racemisation in relation to peptide chemistry, but this did not explain the uniqueness of histidine in its ease of racemisation.

In view of all the problems associated with the use of unprotected histidine, it is not surprising that much effort has been applied to the development of various protecting groups for the imidazole ring.

**Protected Histidine.**

The ideal protecting group for the imino-nitrogen of histidine should:

1) lower the basicity and nucleophilicity of the imidazole ring
2) be stable to conditions used in peptide synthesis
3) be readily introduced and upon completion of the synthesis be removable under mild conditions which do not adversely affect the peptide
4) give derivatives which are readily soluble in solvents normally encountered in peptide synthesis
5) be free from the problems of racemisation.

In a histidine derivative with all these properties, the risks of side-reactions would be minimised, the solubility of the derivative would be increased and a greater variety of coupling methods would be available. Some of the more important protecting groups which have been devised to date are summarised in Figure 2. The actual position of substitution has

**Protection of Histidine Side Chains in Peptide Synthesis.**

\[
\begin{align*}
\text{P} &= \text{H} \\
\text{P} &= \text{Alkyl: } \text{-CH}_2\text{Ph, -CPh}_3 \\
\text{P} &= \text{Aryl: } \text{-Dnp, Tnp} \\
\text{P} &= \text{Acyl: } \text{-Z, -Boc, -Adoc, piperidinecarbonyl etc.} \\
\text{P} &= \text{-Tos} \\
\text{P} &= \text{A derivative of a carbonyl adduct: } \text{-CH(CF}_3\text{)NHZ, -CH(CF}_3\text{)NHBoc, -C(CF}_3\text{)OCHPh(p-Cl)}
\end{align*}
\]

Figure 2

has only been proved in the case of N(im)-2,4-dinitrophenyl-histidine where the protecting group was shown to be located at the T-position.
The distinction between $N(T)$- and $N(W)$-substitution may be important because Vezber has suggested that the position of protection might be crucial in determining the ease of racemisation of the protected histidine derivative. By analogy with the 4-alkylimidazoles and steric conditions, it is probable, however, that all these protecting groups shown are also located at the $\gamma$-nitrogen. A detailed survey of the protecting groups available shows that although significant progress has been made, no protecting group is available that fulfills all the criteria for an ideal protecting group.

a) Benzyl: This was the first protecting group for the imidazole ring of histidine and was introduced by du Vigneaud and Behrens in 1937. The benzyl group, however, does not reduce the basicity of the imidazole ring significantly and so derivatives of $N$(im)-benzyl-histidine are still rather insoluble in organic solvents and purification by acid extraction is not possible. Active esters of $N$(im)-benzyl-$L$-histidine derivatives have been reported but their physical constants vary greatly and there is some doubt about the optical and structural purity of these derivatives. A large number of examples of racemisation have been observed in coupling reactions involving an active ester and in using $N(\omega)$-t-butoxycarbonyl,$N$(im)-benzyl-$L$-histidine both in solution and solid phase peptide synthesis, although in many cases the diastereoisometric material was removed in later purification steps. The problem of racemisation can be greatly reduced in the presence of $N$-hydroxysuccinimide or 1-hydroxybenzotriazole as additives.

The other main disadvantage of benzyl protection is the difficulty of its removal. The group is stable to bases and acids (even anhydrous hydrogen fluoride) but can be removed with sodium metal in liquid ammonia or catalytic hydrogenation. However, it has been observed
that sodium in liquid ammonia causes degradation of peptide bonds involving proline\textsuperscript{67} such that selective removal of the benzyl group is not possible. The rate of hydrogenolysis of the N(im)-benzyl group is often sufficiently low\textsuperscript{59,63} for reduction of tyrosine and phenylalanine to take place.

b) **Triphenylmethyl (Trityl):** N(im)-Triphenylmethyl histidine derivatives\textsuperscript{69-71} are usually soluble in organic solvents but are not in general crystalline, so removal of impurities can be a serious problem. The triphenylmethyl group is stable to nucleophiles and bases but is slowly removed by catalytic hydrogenation\textsuperscript{69} and sodium in liquid ammonia\textsuperscript{72}. The question of acid stability is less clear; the N(im)-triphenylmethyl group can be removed by treatment with trifluoroacetic acid (30min, room temperature), concentrated formic acid (2 min, room temperature) or 50% aqueous acetic acid (30 min, reflux)\textsuperscript{70}, but in mixed acids the group appears to be much more stable. N(im)-Triphenylmethyl-L-histidine can be recovered from N(ω)-t-butoxycarbonyl,N(im)-triphenylmethyl-L-histidine after treatment with 1 M hydrogen chloride in acetic acid for 20h at room temperature\textsuperscript{70}. The same authors also reported that the triphenylmethyl group was completely removed by 6M hydrogen bromide in acetic acid in 2h\textsuperscript{23}, however a histidine-containing sequential polypeptide\textsuperscript{73} was successfully prepared using 6M hydrogen bromide in acetic acid for N(ω)-debenzyloxycarbonylation with apparently little cleavage of the triphenylmethyl group. Whatever the exact conditions necessary for removal of the group, the facile cleavage under some acidic conditions and the lack of crystallinity has limited the use of this otherwise very efficient protecting group, particularly in extended synthesis.

The closely related diphenylmethyl (benzhydryl) substituent has also been examined as a possible protecting group\textsuperscript{23,70}. This group has
very similar properties to the triphenylmethyl group but is slightly
more stable to acids and selective acidolysis of the $N(\kappa)$-t-butoxycarbonyl
group is possible.

c)  $p$-Toluenesulphony (tosyl): The use of the $N(\text{im})$-tosyl group as
protection for histidine was first proposed by Sakakibara and Fujii in
1969. They found that the tosyl group was stable to trifluoroacetic acid
but could be removed by treatment with sodium hydroxide, sodium in liquid
ammonia, anhydrous hydrogen fluoride, aqueous sodium hydroxide, anhydrous
hydrogen bromide or hydrogen chloride in acetic acid, 1-hydroxybenzotriazole
(a common additive in dicyclohexylcarbodiimide mediated couplings), and
even primary amino groups. More recently it has been shown that pyridine
hydrochloride can also effect deprotection. Thus although little
racemisation has been observed in $N(\text{im})$-tosyl-$\text{L}$-histidine derivatives
during activation, and although the derivatives are usually crystalline
and readily soluble in organic solvents, the tosyl group is not a suitable
protecting group for histidine in extended synthesis in view of the wide
range of commonly used conditions which give rise to cleavage. In fact
$N(\kappa)$-alkoxycarbonyl,$N(\text{im})$-tosyl-$\text{L}$-histidine derivatives are themselves
unstable and require storage as their dicyclohexylammonium salts.

d)  2,4-Dinitrophenyl: The observation by Shaltiel that the $N(\text{im})$-
dinitrophenyl group could be quantitatively removed by thiols under mild
conditions (aqueous medium, pH 8, 22°C) opened up the potential use of
this group as protection for the side-chain of histidine. This group has
since found application in classical and solid phase peptide
synthesis. $N(\text{im})$-dinitrophenyl-$\text{L}$-histidine derivatives are crystalline
and soluble in organic solvents and the basicity of the imidazole ring is
sufficiently reduced to allow the preparation of the $N$-hydroxysuccinimide
active ester. The dinitrophenyl group is stable to acidolysis (even
peptide hydrolysis conditions but is cleaved by bases and nucleophiles. The exact conditions are somewhat unclear—Shaltiel and Fridkin observed that complete deprotection occurred in aqueous solution after 20 h at pH 12.2 and 22° whereas Losse and Krychowski reported that no deprotection had occurred after 2 h in 2M sodium hydroxide and dioxane (1:1) at 20°. Hydrazine and mercaptoethanol also lead to complete cleavage. It has also been observed that N\(^{(im)}\)-dinitrophenyl-L-histidine derivatives decompose in solution in organic solvents in the dark, and that the rate of decomposition is increased in the presence of light. In view of the instability of the \(N^{(im)}\)-dinitrophenyl group to nucleophilic attack it is not surprising that yields in coupling reactions can be extremely low, probably as a result of the transfer of the dinitrophenyl group to the amino-component. This problem is particularly serious in solid phase peptide synthesis where chain termination would result but nevertheless the dinitrophenyl substituent is still commonly used. Finally, significant racemisation of histidine has been observed in one case in a mixed anhydride coupling involving \(N^{(*)}\)-t-butoxycarbonyl,\(N^{(im)}\)-2,4-dinitrophenyl-L-histidine as the carboxyl-component.

The related 2,4,6-trinitrophenyl substituent has been found to be even more unstable towards nucleophiles than the 2,4-dinitrophenyl analogue and so is even less suitable as a protecting group for the imidazole side chain of histidine.

e) **Benzylxoycarbonyl**: \(N^{(*)},N^{(im)}\)-Bisbenzylxoxycarbonyl-L-histidine has been prepared by treatment of histidine with benzylchloroformate in aqueous base. This derivative has been used for mixed anhydride, active ester and \(N,N'\)-dicyclohexylcarbodiimide mediated couplings without racemisation being observed and the derivatives are readily soluble in the usual organic solvents. The fact that the
N(ω)-benzyloxycarbonyl group may be removed by acidolysis (hydrogen bromide in acetic acid) in the presence of the N(ω)-benzyloxycarbonyl group allows peptide elongation at the N-terminus. However, in the presence of adjacent free carboxyl- or hydroxyl-groups, the N(ω)-benzyloxycarbonyl group is labile to hydrogen bromide deblocking. Catalytic hydrogenation also effects deblocking but the most serious disadvantage of this protecting group is the facile removal of the N(ω)-benzyloxycarbonyl group by nucleophiles (amine, ammonia, sodium hydroxide, hydrazine) and transfer of the group from the imidazole ring to the amino-component in a coupling reaction has been observed.

f) t-Butoxycarbonyl: As in the case of the N(ω)-benzyloxycarbonyl group, the N(ω)-t-butoxycarbonyl group deactivates the imidazole ring sufficiently to allow couplings via active esters or mixed anhydrides. The derivatives are soluble in organic solvents and no racemisation has been observed. However, although an N(ω)-t-butoxycarbonyl group can be removed by acidolysis in the presence of the N(ω)-t-butoxycarbonyl group the latter group suffers from nucleophilic instability and so is unsuitable for extended peptide synthesis, it is however widely used at present.

Similarly other acid labile N(im)-alkoxycarbonyl substituents (e.g. 1-adamantyloxycarbonyl, isobutoxycarbonyl) have been used in peptide synthesis for histidine side-chain protection. However, as for other alkoxy carbonyl derivatives all suffer from varying degrees of instability to nucleophilic attack.

g) Piperidinecarbonyl: The piperidinecarbonyl substituent as a protecting group for the imidazole side-chain of histidine (Figure 3) was introduced by Jager et al. This substituent is stable to acid and hydrogenolysis and can therefore be used in combination with various
N(ω)-protecting groups. The derivatives, although not always crystalline, are soluble in organic solvents and the imidazole ring is sufficiently deactivated to allow the formation of active esters. Deblocking can be carried out with powerful nucleophilic reagents such as hydrazine or sodium hydroxide solution, but no cleavage occurs with primary amino-groups and so the group is not transferred to the amino-component in coupling reactions. No studies on the racemisation of N(im)-piperidinocarbonyl-L-histidine have been reported but it is likely by analogy with the alkoxy carbonyl derivatives that the racemisation should be minimal. The only obvious disadvantage with this group appears to be the strongly basic reagents required to effect deprotection but surprisingly the piperidinocarbonyl group has been little used in peptide synthesis.

h) 2,2,2-Trifluoro-1-acylaminoethyl: Weygand et al. developed derivatives of the trifluoracetaldehyde for the protection of the imidazole ring (Figure 4) of histidine. In both N(im)-2,2,2-trifluoro-1-
benzyloxycarbonylaminoethyl and N(im)-2,2,2-trifluoro-1-t-butoxycarbonylaminoethyl histidine derivatives the basicity of the imidazole ring is greatly reduced and the derivatives are soluble in organic solvents. The protecting groups are stable to the conditions used in peptide synthesis, except that they are cleaved under the conditions required for the removal of the appropriate acyl group, e.g. the benzyloxycarbonyl derivative can be removed by hydrogenolysis or hydrogen bromide in acetic acid, whilst the t-butoxycarbonyl derivative can be cleaved by trifluoroacetic acid followed by treatment with water in each case. The disadvantage of these derivatives lies in the marginal differential stability of the t-butoxycarbonyl and benzyloxycarbonyl groups in N(\(\alpha\))-t-butoxycarbonyl, N(im)-2,2,2-trifluoro-1-benzyloxycarbonyl-L-histidine - also some racemisation has been reported in an excess mixed anhydride coupling where the protected histidine derivative is the carboxyl component. The lack of crystallinity of these derivatives is also a problem leading to difficulties in purification. This was thought initially to be due to the introduction of a second asymmetric centre into the molecule although it is also possible that a mixture of N(\(\tau\)),N(\(\pi\))-substitution might have prevented crystallisation. In an attempt to improve crystallinity by not introducing the asymmetric centre, Seltzman and Chapman synthesised derivatives of hexafluoroacetone. They have developed the 1,1,1,3,3,3-hexafluoro-2-(p-chlorophenoxy)methoxy)-propyl group for imidazole protection (Figure 5). In a complicated synthesis these authors prepared an active ester which, however was an oil, so absence of a second asymmetric centre seems to have little effect on the crystallinity.
i) o-Nitrobenzyl: Kalbag and Roeske\textsuperscript{97} have introduced an imidazole protecting group which is stable under all conditions normally encountered in peptide synthesis, but which can be removed very specifically by irradiation with a mercury vapour lamp with a pyrex filter in dioxane after 1h. The $N$(im)-o-nitrobenzyl substituent is also slowly removed by catalytic hydrogenation but no example of peptide synthesis has been reported using this group, and by analogy to $N$(im)-benzyl derivatives it is likely that lack of solubility in organic solvents and ease of racemisation will limit its usefulness.

j) Diphenyl-4-pyridylmethyl: This recent addition to the collection of protecting groups for the imidazole ring of histidine was introduced in 1976 by Coyle and Young\textsuperscript{98,99,100}. $N$(α)-Diphenyl-4-pyridylmethyl-$\textepsilon$-histidine was reported to be soluble in organic solvents but the lack of crystallinity makes purification difficult and removal of a small amount of the $\textepsilon$-isomer has proved to be rather tedious\textsuperscript{101}. The protecting group is stable to acid and basic conditions but can be cleaved by reductive methods, e.g. catalytic hydrogenolysis, zinc in acetic acid or electrolysis.
As this group has not yet been applied in a sufficient number of cases it is difficult to assess its usefulness adequately; its stability to racemisation also remains to be fully investigated.

The use of the various protecting groups mentioned above has not simplified the problem of the incorporation of histidine into peptides (Table 1). The derivatives are often either poorly soluble in organic solvents or are not sufficiently crystalline for easy purification. They are either insufficiently stable to be useful in extended synthesis or are so stable that forcing conditions are required for their removal. Most of these protecting groups do reduce the basicity of the imidazole ring enough to permit the synthesis of active esters, but the optical purity of these in at least one case is questionable. In fact, racemisation has been a serious problem in the presence of several protecting groups but until recently the only rationale had been proposed by Veber who suggested that since N-methylimidazole is an effective acyl-transfer catalyst, any alkyl-substituent on the Y-nitrogen does not necessarily prevent cyclisation to an imidazolium species which may be optically labile. Strongly electron-withdrawing substituents would, on the other hand prevent this process. This explained the observation of racemisation in N(Y)-benzyl-L-histidine derivatives and the lack of racemisation in the derivatives of N(Y)-t-butoxycarbonyl or N(Y)-p-toluenesulphonyl-L-histidine. Veber further suggested that specific blockade of the Y-nitrogen might be advantageous in preventing racemisation by this mechanism.

Jones et al.\textsuperscript{102,103} working in this laboratory decided to investigate systematically the problems of histidine protection with a view to discovering the mechanism of racemisation of activated protected histidine derivatives and hence a better protecting group. After screening
<table>
<thead>
<tr>
<th>Protecting Groups</th>
<th>Reference</th>
<th>Removable by</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhCH₂⁻</td>
<td>52-68</td>
<td>H₂/Pd, Na/NH₃(liq)</td>
<td>Gross racemisation on coupling, difficult to remove.</td>
</tr>
<tr>
<td>Ph₃C⁻</td>
<td>23, 69-73</td>
<td>Acids, H₂/Pd(slowly), Na/NH₃(liq)</td>
<td>Non crystalline derivatives, instability to acids a major disadvantage.</td>
</tr>
<tr>
<td>Ph₂CH⁻</td>
<td>23, 70</td>
<td>Acids, H₂/Pd, Na/NH₃(liq)</td>
<td>Slightly more acid stable than triphenylmethyl. but same problems apply.</td>
</tr>
<tr>
<td>Tos⁻</td>
<td>23, 43, 72, 74, 75</td>
<td>Na/NH₃(liq), HF, HBr/AcOH, NaOH</td>
<td>Suppresses racemisation well, very unstable to a wide range of conditions.</td>
</tr>
<tr>
<td>2,4-NO₂Ph⁻</td>
<td>23, 43, 72, 76-81</td>
<td>Thiols, Bases</td>
<td>Very unstable to nucleophiles.</td>
</tr>
<tr>
<td>PhCH₂CO⁻</td>
<td>27, 43, 72, 82-87</td>
<td>H₂/Pd, Acids (variable)</td>
<td>Very unstable to nucleophiles.</td>
</tr>
<tr>
<td>(CH₃)₂COCO⁻</td>
<td>43, 72, 88-93</td>
<td>Acids</td>
<td>Deactivates imidazole ring, suppresses racemisation, limited by its instability.</td>
</tr>
<tr>
<td>N-CO⁻</td>
<td>23, 94</td>
<td>NaOH, hydrazine</td>
<td>Only disadvantage appears to be prolonged treatment with base required to deprotect.</td>
</tr>
</tbody>
</table>

Table 1
<table>
<thead>
<tr>
<th>Protecting Groups</th>
<th>Reference</th>
<th>Removable by</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROCONHCH(CF₃)</td>
<td>43, 50, 72, 79, 95</td>
<td>R=Bzl: H₂/Pd, HBr/AcOH</td>
<td>Difficulty in purification thought to be due to second asymmetric centre, could also be due to a ( \tau )- &amp; ( \pi )-mixture of isomers.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R=(CH₃)₃C: TFA</td>
<td></td>
</tr>
<tr>
<td>O-NO₂Ph-</td>
<td>95, 96</td>
<td>HCl, TFA</td>
<td>Difficult to prepare and purify, possibly due to a mixture of ( \tau ) &amp; ( \pi )-isomers.</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>h/H₂/Pd</td>
<td>No examples of use, but will probably be poorly soluble and likely to racemise.</td>
</tr>
<tr>
<td>Ph₂C-</td>
<td>98-100</td>
<td>H₂/Pd, Zn/AcOH, electrolysis</td>
<td>Difficult to purify, not many examples of use, but likely to racemise.</td>
</tr>
</tbody>
</table>

Table 1
a wide range of possible N(im) protecting groups they decided to
investigate further the properties of phenacyl as a possible protecting
group for histidine. Both the γ- and π- protected isomers were
prepared from N(ω)-alkyloxycarbonyl-histidine methyl ester. The π- protected
isomer was obtained by a strategy of selective N(γ)-blockade by
triphenylmethylation, highly selective for steric reasons, followed by
N(ω)-alkylation and finally removal of the N(π)-triphenylmethyl group
with weak acid and base saponification of the ester. The N(π)-isomer
was prepared directly from the silver salt of the protected histidine.
The positions of the isomers (Figure 6) which were different on t.l.c.

![Figure 6](image)

follows from the methods of synthesis but were also corroborated by
the cross ring coupling constant criterion of Mattews and Rapoport. Comparison of the racemisation-susceptibility of the isomers
under coupling conditions designed to exaggerate the danger produced the
result that while the N(π)-protected isomer was optically pure within the
limit of the assay, the N(π)-protected isomer had almost completely
racemised thus confirming Veber's suggestion that racemisation occurs
through the N(π)-nitrogen.

Further work by Jones et al showed that racemisation of N(ω)-
benzyloxy carbonyl, \(N(\Upsilon)-\)phenacyl-L-histidine, which occurs on activation with dicyclohexylcarbodiimide in dimethylformamide takes place by the action of the \(\pi\)-nitrogen as an intramolecular base catalyst in the 0-acylisourea adduct which is in reversible equilibrium with the reactants (Scheme 3), rather than by formation of an optically labile imidazolium species.\(^{106}\) It can be seen by using space-filling models (Plate 1) that the \(N(\Upsilon)\)-nitrogen lone pair is able to abstract the \(\alpha\)-proton whilst it is spacially impossible for the \(N(\Pi)\)-nitrogen lone pair to abstract the \(\alpha\)-proton in the \(N(\Pi)\)-protected isomer.

![Racemeric peptide](image)

Scheme 3

Conclusion.

Work performed by Jones et al had brought about great progress in the understanding of the mechanisms of racemisation in histidine derivatives and led to the preparation of the first specifically \(N(\Pi)\)-blocked histidine derivative. This derivative had been used in a number of exercises including the classical synthesis of thyroliberin.\(^{103}\) It was fairly soluble in organic solvents and easily cleaved by zinc-acetic acid.
Lone pair on $\text{\textepsilon}$-nitrogen

Lone pair on $\text{\textgamma}$-nitrogen

Plate 1
and photolysis. Previous research in this field had tended to be somewhat haphazard; protecting groups were proposed which appeared to satisfy a number of the criteria set out for a good protecting group, but little follow-up work was done to determine the ramifications with regard to the other criteria. The result being that the authors were often the only workers to make use of such protecting groups which had been launched with such high hopes. In some cases they were never used at all for the purpose for which they had been developed. The purpose of this research was thus to investigate N(\text{N})-phenacyl protection further in order to ensure that a protecting group was developed that satisfied all criteria, and in particular was easy to prepare, minimised the danger of racemisation when activated for coupling, was stable to most conditions encountered in peptide synthesis but could be cleaved cleanly - preferably under conditions which would harmonise with general side-chain deprotection strategies and which was readily soluble in a range of solvents.
CHAPTER 1

Results and Discussion

Further Investigations into the use of $N$(π)-Phenacyl-$\alpha$-histidine

in Peptide Synthesis
Introduction.

Preliminary work conducted by Ramage\textsuperscript{107} in this laboratory had indicated that \( \text{N}(\text{N}) \)-phenacyl-L-histidine might be a valuable intermediate in peptide synthesis\textsuperscript{103}. He had demonstrated this with the classical solution synthesis of thyroliberin.

\( \text{N}(\text{N}) \)-Nitrogen deprotection had been accomplished in most synthetic operations involving \( \text{N}(\text{N}) \)-phenacyl-L-histidine-containing peptides by reductive cleavage using zinc in acetic acid. Whilst Ramage achieved high yields of pure product on this step, later workers found that they could only achieve moderate yields which were often contaminated with impurities\textsuperscript{108}. The problems had arisen both due to side-reactions on the reductive cleavage and in achieving reasonable levels of product recovery from the precipitated zinc sulphide whilst also ensuring the removal of all zinc ions.

It has been suggested that replacement of zinc by cadmium powder would, (due to the lower redox potential of cadmium), result in milder reaction conditions greater selectivity and hence a purer product. This concept has been demonstrated by Hancock\textsuperscript{109} in cleavage of trifluoroethyl protecting groups, but this method was not tried in this laboratory and due to the toxic nature of cadmium dust it is unlikely that it would ever become a general method of deprotection.

When proposing phenacyl as a protecting group Ramage had considered the possibility that cleavage could also be accomplished by photolysis or electrolytic reductive cleavage. Stachulski demonstrated the photolytic deprotection of a small sample of \( \text{N}(\text{N}) \)-phenacyl-thyroliberin\textsuperscript{103} using a medium pressure mercury lamp with a pyrex filter for 6 h. However, whilst thyroliberin was recovered in good yield (75%) a rather complex mixture of by-products had to be removed first. A number of
\(\text{\textit{o}-nitrobenzyl derivatives including 6-nitroveratryloxycarbonyl and 2-nitrobenzyloxycarbonyl have been suggested by Patchornik et al.}^{110,111}\) over a number of years as possible amino and carboxy blocking groups that are easily cleavable by photolysis. However none of these groups has ever been generally used, probably due to difficulties encountered in deprotecting peptides containing tyrosine and tryptophan, both of which absorb ultraviolet light near the frequencies employed for cleavage. For similar reasons it seems unlikely that the photolytic deprotection of \(\text{N(\text{\textit{m}})-phenacyl-histidine}\) would ever gain widespread usage.

Electrolytic reduction as a method for deprotecting amino-acids was used by Scopes et al.\(^{112,113}\) to remove protecting groups from both nitroarginine and nitroarginine-containing peptides, cleavage occurring at the mercury cathode. It was demonstrated that under carefully controlled conditions cleavage proceeded readily to give \(\text{L-arginine}\), and that the reported electrolytic fission of the peptide bond in proline-containing peptides\(^{114}\) was not observed over the time for deprotection to take place. This work was followed by the observations that piperidino-oxycarbonyl groups\(^{115,116}\), 4-picoly esters\(^{117}\) and 3-picoly esters\(^{117}\), \(\text{0-4-picolytyrosine}\)\(^{118}\), 4-picolyloxycarbonyl hydrazides\(^{119}\) and most recently diphenyl-4-pyridylmethylhistidine\(^{100}\) were all readily cleaved by electrolytic reduction at a mercury cathode.

Ramage and Stachulski carried out an electrolytic reduction on a sample of \(\text{N(\text{\textit{m}})-phenacyl-L-\text{\textit{m}}-histidine}\) using similar conditions to those favoured by previous workers i.e. 0.5M sulphuric acid electrolyte at a potential of 1.3 V. After 3 h aliquots were withdrawn and subjected to amino-acid analysis which showed that the products consisted of 73% deprotected \(\text{L-\text{\textit{m}}-histidine}\) and 25% of the reduced compound \(\text{N(\text{\textit{m}})-2-hydroxy-2-phenylethyl-L-\text{\textit{m}}-histidine}\). It has however also been shown that the products
from the electrolysis of alkylarylketones are very dependent on voltage, pH and solvent \textsuperscript{120-122}.

Thus further work was carried out by the present author in order to establish whether satisfactory conditions could be found for clean deprotection \textsuperscript{123}. A number of different electrolytes were examined including aqueous acetic acid, potassium chloride solution and dry dimethylformamide. However none of these conditions significantly improved the ratios of cleavage to reduction. Further studies were conducted into the effect of varying the voltage whilst retaining a 0.5M sulphuric acid electrolyte. These studies were based on initial polarometric studies using the model \(N\)-(phenylacyl)imidazole and showed that the by-product could be eliminated by lowering the voltage (Figure 7). However as the voltage is lowered so the rate of cleavage drops rapidly. Thus in order to balance these effects it was decided that the optimum conditions for this reaction occurred

![Figure 7](image-url)
The delicacy of control required in this reaction was illustrated when the effect of temperature on the rate of cleavage was investigated under the conditions we had determined to be optimum. It was observed that an increase in the temperature of the electrolyte from 18° to 40° resulted in the formation of 8% of the by-product N(\(\pi\))-2-hydroxy-2-phenylethyl-L-histidine.

This chapter deals with the extension of these studies to a N(\(\pi\))-phenacyl-histidine-containing peptide.

**Attempted Electrolytic Deprotection of N(\(\pi\))-Phenacyl-thyroliberin.**

N(\(\pi\))-Phenacyl-thyroliberin was dissolved in 0.5M sulphuric acid and placed in the cathode compartment of a simple electrolysis apparatus (Figure 8).

- **a** Anode compartment
- **b** Calomel reference electrode
- **c** Electrolyte and sample
- **d** Viscing tube membrane
- **e** Platinum anode
- **f** Mercury cathode
- **g** Controlled potential

Figure 8
The solution was then electrolysed at the mercury cathode at a potential of 1.00 V. The current was monitored and the reaction followed by thin layer chromatography. After 2.5 h the current had fallen to a steady 0.5 mA and all the starting material had been consumed.

Although t.l.c. showed that the product was not pure no relative change had occurred in the proportions of the products present over the final 0.5 h. It was thought unwise to continue further with the electrolysis due to the long-term decrease in material noted by Ramage and attributed to the destruction of the imidazole ring. After treatment with an ion exchange resin to remove sulphate ions an oily residue was obtained which was filtered through a Sephadex gel column to remove low molecular weight impurities. Concentration of the appropriate fractions followed by trituration with ether gave a brown solid. Although t.l.c. indicated that the major product was similar to authentic thyroliberin, a significant quantity of a by-product was also present which did not correspond to the starting material. No attempt was made to purify the thyroliberin further as the exercise had been attempted to investigate the generality of the application of electrolytic deprotection to \( N^\alpha \)-phenacyl-L-histidine and not to the preparation of pure thyroliberin.

The most likely explanation for the presence of this by-product is that although the electrolysis of \( N^\alpha \)-phenacyl-L-histidine at 1.00 V produced none of the reduced product \( N^\alpha \)-2-hydroxy-2-phenylethyl-L-histidine, when the peptide was electrolysed, reduction did in fact occur and the impurity was due to the presence of \( N^\alpha \)-2-hydroxy-2-phenylethyl-thyroliberin. The reason for this delicate balance between cleavage and reduction can be appreciated in terms of a possible mechanism for this reaction (Scheme 4). Assuming that both the initial one-electron reduction
Possible Mechanism for Electrolytic Reduction

Scheme 4
to give the radical anion and the later addition of a second electron to
give the carbanion of the alcohol are both possible at this voltage, then
the product distribution will be very dependent upon the lifetime of the
radical anion prior to its cleavage or reduction. Hence it is possible
that by altering the peptide chain the rate of diffusion to and from the
electrode might be changed and thus the average lifetime of the radical
anion before it is reduced further might be affected, so altering the
balance between reduction and cleavage.

The product distribution might also be explained by the cleavage
of the proline peptide bond\(^\text{114}\). However, in the light of work by Young et al\(^\text{112,113}\) demonstrating the stability of the bond to electrolysis over the
timescale of this reaction then this explanation seems unlikely. It is
also possible that the reaction proceeded too far and that destruction of
the imidazole ring occurred as observed by Ramage\(^\text{107}\), but this explanation
is unlikely due to the fact that the impurity gives a strong Pauly-positive
test.

Results and Conclusion.

Electrolytic reductive cleavage of the phenacyl group from \(N(\text{aryl})\)-phenacyl-L-histidine-containing peptides does not proceed cleanly, unlike
the deprotection of \(N(\text{aryl})\)-phenacyl-L-histidine.

It might have been possible to establish conditions for clean
cleavage after an exhaustive investigation such as that required to
establish the ideal conditions for the clean cleavage of \(N(\text{aryl})\)-phenacyl-
L-histidine. However such an investigation would probably have been
necessary for each new \(N(\text{aryl})\)-phenacyl-L-histidine-containing peptide,
necessitating the waste of valuable protected peptide. We therefore
conclude that electrolytic cleavage cannot be put forward as a general
method of deprotection for \(N(\text{aryl})\)-phenacyl-L-histidine-containing peptides.
In parallel to this work further studies had been carried out into the suitability of \textit{N(\textit{H})-phenacyl-L-histidine} for use in peptide synthesis: both Brown\textsuperscript{108} and Mansfield\textsuperscript{124} had attempted solution synthesis of simple peptides and obtained low yields and complex mixtures. An attempted solid phase synthesis of angiotensin II had also resulted in less than 50% incorporation of residues after histidine\textsuperscript{108}, hence the behaviour of the protected derivative had been investigated further and it was found that the acidic methylene group in phenacyl reacted rapidly with acylating agents. Brown was able to demonstrate this reaction\textsuperscript{108} and characterise the products fully when he reacted \textit{N-(phenacyl)imidazole} with di-(t-butyl)-dicarbonate in dimethylformamide (Scheme 5).

\begin{center}
\begin{tikzpicture}
  \node (a) at (0,0) {\CH_2\text{COPh}};
  \node (b) at (2,0) {\CH_2\text{COOBU}^+};
  \node (c) at (1,-1.5) {\text{N}};
  \node (d) at (1,-2) {\text{N}};
  \node (e) at (2,-1.5) {\text{N}};
  \node (f) at (2,-2) {\text{N}};
  \draw (a) -- (c);
  \draw (c) -- (d);
  \draw (d) -- (e);
  \draw (e) -- (f);
  \draw (f) -- (b);
  \draw (a) -- (i) node [midway, above] \text{i) Boc}_2\text{O-DMF. ii) MeOH-CHCl}_3, chromatography.};
\end{tikzpicture}
\end{center}

Thus it is possible to conclude that phenacyl provided a valuable insight into the mechanisms of the racemisation of histidine and established the necessity of \textit{N(\textit{H})}-protection. However due to the limitations in its use resulting from difficulties in deprotection and the relative chemical complexity of the phenacyl group leading to numerous side-reactions, phenacyl is unsuitable as a general protecting group for histidine in peptide synthesis.
CHAPTER 2

Results and Discussion

Investigations into the Possibility of using 1,1,1-Trichlorobut-2-enyl

as a N(π)-Protecting Group for Histidine
Introduction.

In the design of protecting groups the objective is often to find a group which can be removed under standard conditions, such that all protecting groups can be removed by a single operation. However it is also advantageous to have in one's arsenal protecting groups which can be selectively removed under mild conditions whilst leaving the rest of the blocking groups intact. The development of completely new protection strategies in which deprotection can be accomplished under milder conditions than those normally encountered in peptide synthesis is a constant endeavour of the peptide chemist.

Eckert et al. proposed just such a novel strategy in which cleavage of the protecting groups is accomplished by treatment with supernucleophiles, often within a few seconds in essentially neutral solutions at 0-20°. The protecting groups proposed were either β- or δ-halogenated alkyl species which were generally stable to quite strongly acidic or basic conditions as well as to many reagents that are used in peptide synthesis. The supernucleophile of choice for deprotection was the Co-phthalocyanine anion, the lithium salt of which is indefinitely stable in solution.

The 1,1,1-trichlorobut-2-ene derivative seemed to be the group favoured by Eckert for use as a side-chain protecting group for amino-acids. It was decided to attempt the synthesis of the model compound N(1,1,1-trichlorobut-2-enyl)imidazole and then to investigate its suitability as a N(im)-protecting group for histidine.

Attempted Preparation of N(1,1,1-Trichlorobut-2-enyl)imidazole using 1,1,1,4-Tetrachlorobut-2-ene.

A sample of 1,1,4-tetrachlorobut-2-ene was generously provided by Professor Hugi of the Organisch-Chemisches Institut, Technische Universität,
München.

We initially attempted to prepare the imidazole derivative by the method used by Jones and Hysert\textsuperscript{127} in the preparation of \(\text{N}(\text{but-2-enyl})\text{imidazole}\). \(1,1,1,4\)-Tetrachlorobut-2-ene was refluxed with excess imidazole and base in methanol. The reaction was monitored by t.l.c. until all the chloroalkene had been consumed. However the rate of reaction was very much slower than that reported by Jones and Hysert\textsuperscript{127} and after work-up to remove excess imidazole, a brown oil was obtained which was shown to contain a complex mixture of products.

Thus it appeared that the chloroalkene was unstable over the long period of reflux required in this case. We decided to attempt to induce a reaction under milder conditions by using more reactive imidazole derivatives.

Initially a solution of the chloroalkene and mercuric acetate imidazolium salt in toluene was stirred overnight, but the only material recovered from the organic layer after work-up was \(1,1,1,4\)\text{-tetrachlorobut-2-ene.}

We next prepared \(\text{N}(\text{trimethylsilyl})\text{imidazole}\)\textsuperscript{128} and stirred it with the chloroalkene in toluene for 24 h after which t.l.c. indicated that no reaction had occurred. Catalytic quantities of mercuric acetate were added to the solution but after a further 6 h of stirring, still no reaction had been observed. Finally the mixture was refluxed for 1 h but after work-up only a complex mixture of products was recovered.

Silver imidazolide\textsuperscript{133} was prepared and dissolved in dimethylsulphide and the chloroalkene was added. After 3 h the reaction mixture was worked-up but the only product recovered from the organic layer was \(1,1,1,4\)-tetrachlorobut-2-ene.

From these experiments we concluded that \(1,1,1,4\)-tetrachlorobut-2-ene
is not a sufficiently good alkylating agent to react with imidazole or its activated derivatives under conditions sufficiently mild to prevent the decomposition of the product.

Preparation of N(1,1,1-Trichlorobut-2-eny1)imidazole and Investigation of its Stability Under Conditions Likely to be Encountered in Peptide Synthesis.

After learning of the results of our experiments with 1,1,1,4-tetrachlorobut-2-ene Professor Ugi was able to supply us with a sample of the potentially better alkylating agent 1,1,1-trichloro-4-bromobut-2-ene. A sample of this was reacted with excess imidazole under the conditions of Jones and Hysert\(^{127}\). After less than 2.5 h all the chloroalkene had been consumed. The reaction mixture was worked up to give crude N(1,1,1-trichlorobut-2-ene)imidazole as a colourless oil (Scheme 6).

![Scheme 6](image_url)

i) MeOH-Na\(_2\)CO\(_3\)-reflux 2.5 h.

This material was crystallised and characterised as the oxalate salt. Regeneration of N(1,1,1-trichlorobut-2-eny1)imidazole from its oxalate salt by partitioning between ether and sodium hydrogen carbonate solution proved to be very straightforward. The full spectral details of the free base were recorded.

The results of an investigation into the stability of N(1,1,1-trichlorobut-2-eny1)imidazole are summarised in Table 2. The N-chloroalkene
protecting group was rapidly and cleanly cleaved by zinc in aqueous acetic acid to give imidazole and a by-product assumed to be the cleavage product of the protecting group. The protected imidazole was found to be completely stable to nucleophilic benzylamine over an extended period and also stable to hydrogen bromide in acetic acid for 1 hour.

However, upon allowing N(1,1,1-trichlorobut-2-enyl)imidazole to stand in trifluoroacetic acid for 2 h it was obvious from t.l.c. and n.m.r. that a substantial change had occurred. The reaction mixture was put aside for a further 2 weeks after which conversion of the starting material to a new product was complete. After work-up this product was obtained as an oil which was pure on t.l.c. but which had a different R_f from the starting material. The mass spectra revealed that both the

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Length of exposure</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc in aqueous acetic acid</td>
<td>2 min</td>
<td>Complete cleavage of protecting group</td>
</tr>
<tr>
<td>HBr-Acetic acid</td>
<td>1 h</td>
<td>No reaction</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>2 h</td>
<td>Substantial impurities on n.m.r.</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>2 weeks</td>
<td>Complete conversion to new product</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>1 week</td>
<td>No reaction</td>
</tr>
<tr>
<td>1M Sodium hydroxide</td>
<td>1 h</td>
<td>Complete conversion to new product</td>
</tr>
</tbody>
</table>

Table 2
starting material and the product had a similar molecular weight and characteristic molecular ion for a compound containing three chlorine atoms. The u.v. absorption spectra of both compounds were identical. The 300 mHz n.m.r. spectra were, however, completely different (Figure 9) and an examination of the coupling constants indicated that the starting material contained a trans double bond (\(J:CH=CH; 14.7\) Hz) whilst the product after treatment with trifluoroacetic acid, contained a cis double bond (\(J:CH=CH; 9.7\) Hz). In order to establish the position of this double bond the methylene protons on both compounds were irradiated overnight and the Nuclear Overhauser effect on the imidazole ring protons investigated. The Nuclear Overhauser effect and its application to organic chemistry has been reviewed elsewhere, but it can be briefly summarised in its application to proton n.m.r. by saying that if the resonance frequency of a proton or protons is saturated then a degree of change in the integrated intensity of the resonances of spatially adjacent protons will occur. This effect is generally small but the increase in availability of advanced Fourier transform n.m.r. spectrometers has made it a practical investigative procedure. The potential of this effect for providing information on the configuration and confirmation of molecules was first demonstrated by Anet and Bourne and has since been used by numerous authors in structure determination studies.

In this case, after irradiation of the methylene protons both the \(N(1,1,1\text{-trichlorobut-2-enyl})\text{imidazole}\) and the material produced following treatment with trifluoroacetic acid, exhibited a 1% enhancement on the 2- and 5-imidazole protons whilst there was no enhancement on the spatially more distant 4-proton (Figure 10). This result, although small, was exactly reproducible over a number of runs. So we can conclude that the methylene group is in exactly the same spatial environment with respect to the imidazole ring in both cases. The only possible conclusion
\[ N(1,1,1\text{-trichlorobut-2-enyl})\text{imidazole} \]

\[ N(1,1,1\text{-trichlorobut-2-enyl})\text{imidazole after treatment with TFA} \]

Figure 9
NOE

\[ N(1,1,1\text{-trichlorobut-2-yl})\text{imidazole} \]

\[ N(1,1,1\text{-trichlorobut-2-yl})\text{imidazole after treatment with TFA} \]

Figure 10
to be drawn from these results is that on treatment with trifluoroacetic acid, trans N(1,1,1-trichlorobut-2-enyl)imidazole undergoes complete conversion to give the cis geometrical isomer (Scheme 7).

![Scheme 7]

i) Trifluoroacetic acid-2 weeks

Further support for this conclusion was obtained from the discovery that the cis N(1,1,1-trichlorobut-2-enyl)imidazole was cleaved rapidly and cleanly by zinc in acetic acid in exactly the same manner as the starting material.

Isomerisation of a double bond from the trans to cis geometrical isomer are fairly rare in the literature, although well-documented in some cases such as stilbenes\(^1\), decalins\(^2\). The most notable example in this case is the isomerisation of 1,2-dihalogenated ethylenes\(^3\). It is possible that the trans to cis geometrical isomerisation observed in the N(1,1,1-trichlorobut-2-enyl)imidazole derivatives could be explained by similar dipole interactions as those invoked to explain the trans-cis isomerisation in 1,2-dichloroethylene\(^3\).

When a mixture of N(1,1,1-trichlorobut-2-enyl)imidazole and 1M sodium hydroxide was stirred vigorously with methanol for 1 h a dense white precipitate was observed and t.l.c. showed all the starting material to have been consumed. After filtering, the white precipitate (which was soluble in water but not in organic solvents) gave a positive test for chloride and burned with a yellow flame, and thus was thought to be
sodium chloride. The organic material, after work-up, was an oil and had two closely spaced spots on t.l.c. Mass spectroscopy of the product indicated that HCl had been lost from the starting material, the peaks for the molecular ion now being characteristic of a compound containing only two chlorine atoms. The product also absorbed u.v. light at lower frequencies than the starting material, but the 300 MHz n.m.r. spectrum was very complex and the products ran much too closely together on t.l.c. to contemplate separating them.

We concluded that the base had abstracted one of the methylene protons with loss of a chloride ion to give a mixture of the two possible geometric isomers of $N(1,1\text{-dichloro-1,3\text{-dienyl}})\text{imidazole}$ (Scheme 8).

\[ \text{Scheme 8} \]

\[ \text{i) } \text{NaOH-}H_2O\text{-MeOH }1\text{ h} \]
Further investigations such as the effect of catalytic hydrogenation on the double bond and the ease of cleavage using a supernucleophile were not pursued as lack of suitability in other circumstances illustrated clearly the unsuitability of this group for histidine side-chain protection. A sample of 1,1,1-trichloro-4-bromobut-2-ene was however successfully, reacted with N(triphenylmethyl)imidazole to form a rather unstable quaternary imidazolium salt which was not characterised, demonstrating that it would, in principle, be possible to prepare selectively the N(M)-protected histidine derivative.

Results and Conclusion.

1) 1,1,1-Tetrachlorobut-2-ene is not a sufficiently good alkylating agent to be used to prepare N(1,1,1-trichlorobut-2-enyl)imidazole under conditions sufficiently mild to avoid side-reactions.

2) 1,1,1-Trichloro-4-bromobut-2-ene can be used to prepare N(1,1,1-trichlorobut-2-enyl)imidazole in good yield. The product was characterised as its oxalate salt.

3) When subjected to conditions commonly encountered in peptide synthesis N(1,1,1-trichlorobut-2-enyl)imidazole proved to be stable to nucleophiles and to a strong mineral acid for a short period of time and to be readily cleaved by zinc in aqueous acetic acid. However it rapidly lost hydrogen chloride in the presence of a strong base (1M sodium hydroxide) to give a diene and underwent geometrical isomerisation over a period of time in the presence of acid.

4) From these results we concluded that 1,1,1-trichlorobut-2-ene was unstable over too wide a range of conditions to be of general use as a histidine protecting group.
CHAPTER 3

Results and Discussion

Iodination of the Imidazole Ring as a Method of Side-Chain Protection

for Histidine in Peptide Synthesis
Introduction.

Work published to date on the unusual tendency of histidine to racemise has all indicated that the basicity of the imidazole ring is the primary cause of the problem. This is equally true independently of whether racemisation occurs via $\alpha$-proton abstraction by the imidazole $\pi$-nitrogen, as argued by Jones and Ramage\textsuperscript{106} or via an optically labile imidazolium species, as proposed by Veber\textsuperscript{45}.

It has been shown by Fletcher et al\textsuperscript{103} that the active site in the $\alpha$-proton abstraction is the $\pi$-nitrogen of the imidazole ring and that if that position is blocked then racemisation is suppressed even under forcing conditions.

However a number of protecting groups, particularly $N(\varepsilon)$-t-butoxycarbonyl or $N(\gamma)$-$p$-toluenesulphonyl-$L$-histidine are also able to suppress racemisation effectively although for steric reasons they are thought to be blocking the $\varepsilon$-nitrogen on the imidazole ring. For reasons previously discussed neither of these groups is an ideal side-chain protecting group for histidine but both are good electron-withdrawing agents and it is thought that they act to decrease racemisation by reducing the basicity of the $\pi$-nitrogen such that it can no longer catalyse racemisation. Conversely the substitution of electron-donating groups on the $\varepsilon$-nitrogen such as in $N(\varepsilon)$-benzyl or $N(\varepsilon)$-phenacyl-$L$-histidine increases the rate of racemisation by increasing the basicity of the imidazole ring.

Thus whilst our work on $\pi$-nitrogen protection produced a derivative in which all imidazole catalysed racemisation was suppressed, then due to the necessity of first blocking the more accessible $\varepsilon$-nitrogen synthetic procedures for the preparation of $N(\pi)$-protected derivatives were protracted. We therefore decided to investigate the possibility of reducing
racemisation and improving the physical properties of a histidine intermediate by reducing the basicity of the imidazole ring.

Thus an electron-withdrawing species which could easily be substituted on the imidazole ring, would be stable under normal conditions of peptide synthesis but which could be readily and quantitatively cleaved was required. As previously noted in the introduction, a large amount of work has been done on N(\(\cap\))-substituents none of which has proved ideal. It was thus decided to look into the novel concept of deactivating the N(\(\cap\))-position by substituting electron-withdrawing groups on to the 2- and 5- imidazole carbon atoms.

Substitution with iodine was a logical first choice as iodine is a good electron-withdrawing substituent and a lot of literature information on the iodination of histidine and histidine-containing peptides is available. It has long been known that congenital goitres contain large amounts of iodo-compounds which were later proved to contain both mono- and diiodohistidine. Savoie et al. have shown that normal thyroid glands in both humans and rats contain very small quantities of mono- and diiodohistidines in thyroglobulin and thyralbumin, and Wolff and Covelli that monoiodohistidine is present in the normal rat thyroid.

The concept of using the reactivity of aromatic peptide side-chains towards iodinating agents has been known for some time. The differential rate of iodine uptake by tyrosine residues between exposed and buried sites provided a great deal of information about active sites in tyrosine containing peptides. Whilst iodination of histidine residues was first reported by Fraenkel-Conrat it was not until much later that Cloelli and Wolff were able to demonstrate the first specific labelling of histidine with \(^{131}\text{I}\) in Ribonuclease A. Since that time
many examples of iodination of histidine containing peptides have been reported\textsuperscript{142-144}.

For the study of metabolism, the specific replacement of protons with radioactive tritium can be desirable. Thus after Birkofer and Hempel\textsuperscript{145} had demonstrated that it was possible to catalytically tritiate 2,5-diiodohistidine, completely replacing the iodines with tritium, iodination of a histidine-containing peptide, followed by tritiation became an ideal method of specifically replacing the imidazole protons by tritium. This method was first reported by Prudelles \textit{et al.}\textsuperscript{146} to label thyroliberin and then by Menez \textit{et al.}\textsuperscript{147} in the labelling of the \(\alpha\)-neurotoxin Nujunigrillacis. This work was taken a stage further when Allen \textit{et al.}\textsuperscript{148} synthesised a 1-24 Ac\beta fragment containing 2,5-diiodohistidine and then catalytically tritiated the final product to ensure complete specificity of incorporation of the tritium label.

Catalytic dehalogenation of iodine-substituted peptides was shown to proceed well even in the presence of methionine, where the sulphur might have been expected to poison the catalyst\textsuperscript{155}. Disulphide bridges did however poison the catalyst, preventing dehalogenation and necessitating special synthetic strategies\textsuperscript{156,157}.

The initial introduction of iodine into histidine and histidine-containing peptides as well as its eventual removal were thus well established techniques. An investigation of the relative pK\textsubscript{a} values of mono- and diiodohistidine had been conducted by Bruning\textsuperscript{149} (Table 3)

<table>
<thead>
<tr>
<th></th>
<th>Histidine</th>
<th>Monoiodohistidine</th>
<th>Diiodohistidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK\textsubscript{a} ImH</td>
<td>6.00</td>
<td>4.18</td>
<td>2.72</td>
</tr>
</tbody>
</table>

Table 3
which indicated that iodination would in fact reduce the basicity of the imidazole ring quite considerably.

On the basis of information obtained from the literature it was thought that 2,5-diiodohistidine fulfilled many of the criteria demanded of a protecting group for histidine. Thus we decided to prepare a sample of iodinated histidine and systematically test its suitability as a protected histidine intermediate in peptide synthesis.

**Synthesis of L-2,5-Diiodohistidine and Investigation of its Stability Under Various Conditions.**

Initially 2,5-diiodohistidine was prepared by the method of Bruning, however the initial product was impure and purification by reprecipitation resulted in a very low yield (28%).

The material that we obtained was pure and we were able to investigate its stability to conditions commonly encountered in peptide synthesis. L-2,5-Diiodohistidine and a L-lysine hydrochloride standard were dissolved in the reagent under test. An aliquot of the solution was immediately withdrawn for comparison with aliquots withdrawn after the desired exposure time, by amino-acid analysis.

It was found that 2,5-diiodohistidine was stable both to trifluoroacetic acid and hydrogen bromide in acetic acid for 3 h. However, after treatment under standard amino-acid hydrolysis conditions for 24 h a purple colour thought to be due to iodine was observed in the hydrolysis tube and amino-acid analysis of the hydrolysate showed that decomposition had occurred to give both monoiodohistidine and histidine. Thus it is not possible to measure directly the ratio of 2,5-diiodohistidine in a peptide by amino-acid analysis without first deprotecting the imidazole ring. In the presence of sodium hydroxide a slight decrease in the amount of 2,5-diiodohistidine relative to lysine was observed after 3 h. The
decrease was however small and was probably due to a deviation in the relative peak heights, or small amounts of reaction with trace impurities. No trouble ever occurred in later saponifications of the methyl ester with sodium hydroxide, so it appeared that 2,5-diiodohistidine was stable to the strongly acidic and basic conditions encountered in peptide synthesis.

Synthesis of \( N(\alpha)\)-t-Butoxycarbonyl-L-2,5-diiodohistidine.

Although Bruning's method of preparation of diiodohistidine worked we, as had previous authors\(^{137,141}\) experienced difficulties in purifying the product in a reproducibly good yield. It was decided that if \( N(\alpha)\)-t-butoxycarbonyl-L-histidine methyl ester was used as the starting material, then although an extra saponification step would be required for the preparation of the \( N(\alpha)\)-protected amino-acid, the increased solubility of the \( N(\alpha)\)-protected methyl ester would make the reaction much easier to follow by t.l.c. Purification of the methyl ester by column chromatography in organic solvents would also be possible.

Initially the iodination was carried out using a two-phase reaction mixture, with iodine and \( N(\alpha)\)-t-butoxycarbonyl-L-histidine methyl ester being dissolved in chloroform, and vigorously stirred with aqueous sodium carbonate (Scheme 9). This two-phase system was used in the hope that polar impurities might go into the aqueous phase, however this was found to be unnecessary and later experiments were carried out in a homogeneous organic solution with triethylamine as the base.

At first we found that if two equivalents of iodine were added then after standing overnight two components appeared as a double spot on t.l.c., whilst if 2.5 equivalents of iodine was added then degradation to give a complex mixture of products was observed\(^{150}\). However it was found that the addition of 2.3 equivalents of iodine produced complete reaction to give, after standing overnight, a chromatographically
homogeneous product. After workup the product was characterized as a pale yellow amorphous solid. Saponification with aqueous sodium hydroxide gave the acid in good yield (78%) which was characterized as the hydrate after neutralisation and workup. (Scheme 9).

![Chemical Structure](image)

**Scheme 9**

i) \( I_2-\text{CHCl}_3-\text{Na}_2\text{CO}_3-\text{H}_2\text{O} \)  
ii) \( \text{NaOH-}\text{H}_2\text{O-CH}_3\text{OH} \)

The \( \text{N}(\alpha) \)-protected amino-acid was shown, after deprotection and digestion with an \( \text{L} \)-amino-acid oxidase using the method of Jones and Ramage\(^{105}\), to contain less than 2% \( \text{D} \)-histidine.

In an effort to obtain a crystalline derivative the dicyclohexylammonium salt of the acid was prepared and characterized in very high yield (92%); but the product was not crystalline and so was not used further as an intermediate.

**Synthesis of \( \text{N}(\alpha) \)-Benzylxycarbonyl-L-2,5-diiodohistidine.**

The \( \text{N}(\alpha) \)-benzylxycarbonyl derivative was prepared and
characterized from $N(\alpha)-$benzyloxycarbonyl-$L$-histidine methyl ester in an analogous manner to the $t$-butoxycarbonyl compound. No problems were encountered and the relatively low yield on the saponification step was attributed to the small scale of the reaction leading to poor recovery of the product.

Dehalogenation of $N(\alpha)-t$-Butoxycarbonyl-$L$-$2,5$-diodohistidine Methyl Ester.

Deprotection of the imidazole ring of $N(\alpha)-t$-butoxycarbonyl-$L$-$2,5$-diodohistidine methyl ester was demonstrated by hydrogenolysis in the presence of a mixed catalyst combination of rhodium on calcium carbonate and palladium on carbon (Scheme 10). The reaction went cleanly to completion within 30 min, and the product was shown by t.l.c. and amino-acid analysis to be $N(\alpha)-t$-butoxycarbonyl-$L$-histidine methyl ester. Attempts to deprotect using only the more conventional palladium catalyst failed to produce any significant reaction within a reasonable time scale.

Isolation and Characterization of $N(\alpha)-t$-Butoxycarbonyl-$L$-$5$-iodohistidine Methyl Ester.

In early experiments where only two equivalents of iodine were used in the iodination of $N(\alpha)-t$-butoxycarbonyl-$L$-histidine methyl ester
a mixture of two products were observed. The one having the greater $R_F$ was later shown to be the diiodo-derivative. It was decided to attempt the isolation and characterization of the material with the smaller $R_F$. The impure material was separated twice on a silica gel chromatography column eluting with 5% methanol-chloroform. After the chromatographically homogeneous material corresponding to the lower spot on t.l.c. had been concentrated, a crystalline solid was obtained which was characterized and shown to be $\text{N}^{(\omega)}-\text{t-butoxycarbonyl-}\text{I}^-\text{-monoiodohistidine methyl ester}$.

The literature is somewhat ambiguous as to the position of substitution of iodine in directly iodinated monoiodohistidines. Bruning in his paper outlining the preparation of iodohistidines suggested that, by analogy with the iodination of 4-methylimidazoles, the iodine should be in the 2-position on the imidazole ring. Whilst Bruning himself commented on the scarcity of evidence for this assignment later authors, especially Perlgut and Wainio assumed 2-substitution, however Bensusan and Naidu and Holloway et al claimed simultaneously to show that substitution was actually occurring at the 5-position on the imidazole ring. Both Bensusan et al and Holloway et al based their assignment on the observation that upon iodination the resonance corresponding to the 5-proton in the n.m.r. spectra of monoiodohistidine disappeared.

We felt that this evidence, whilst possibly correct, could be disputed on the grounds that the chemical shift of the remaining proton on a monosubstituted imidazole ring might be significantly altered by the electron-withdrawing iodine relative to the analogous chemical shift in a non-substituted imidazole ring. Thus it was felt that evidence consisting of analogies in chemical shifts of differently substituted aromatic rings was unsound.

A simple experiment to assign the position of the iodine in monoiodohistidines unequivocally occurred to us. This involved the
replacement of the iodine with deuterium by catalytic deuteration to give a product which would be chemically identical to the analogous histidine derivative so that direct comparison of the imidazole chemical shifts to fix the position of substitution would be valid.

Deuterium gas was kindly provided by Dr. M.L.H. Green of the Inorganic Chemistry Laboratory, Oxford.

$\text{N}(\alpha)$-t-Butoxycarbonyl-$\text{I}_2$-monoiiodohistidine methyl ester was deuterated under conditions similar to those established for the hydrogenolysis of diiodohistidine derivatives. After 30 min t.l.c. indicated that all the starting material had been consumed and the product had an identical $R_F$ to $\text{N}(\alpha)$-t-butoxycarbonyl-$\text{I}_2$-histidine methyl ester. The catalyst and solvent were removed and the n.m.r. spectrum of the product examined. It was discovered that whilst the rest of the spectrum was unchanged no imidazole resonances were present (Figure 11). This implied that exchange had in fact occurred at both the 2- and 5-positions on the imidazole ring.

Confirmation was obtained by the deuteration of $\text{N}(\alpha)$-t-butoxycarbonyl-$\text{I}_2$-histidine methyl ester for 30 min after which an n.m.r. spectrum showed that the resonance due the 2-imidazole proton had considerably decreased relative to that for the 5-proton (Figure 11). Hence we were able to conclude that the 2-position is labile to deuteration whilst the 5-position is stable, and as in the monoiiodinated compound both positions had been deuterated then the iodine must be in the 5-position (Scheme 11).

We were thus able to confirm the assignments of Bensusan and Holloway that monoiiodination produces the 5-iodohistidine derivative. The fact that the 2-proton exchanges under catalytic hydrogenation conditions in non-substituted histidine derivatives is rather surprising and should be borne in mind when selective tritiation of other residues in histidine containing peptides is attempted, as additional tritium might be taken up by the histidine imidazole ring.
Satisfied by this preliminary investigation that 2,5-diiodohistidine was a practical method of histidine protection, it was decided to attempt the synthesis of thyroliberin (TRF, L-pyroglutamyl-L-histidyl-L-prolineamide) a biologically important tripeptide to demonstrate the general utility of iodinated histidine derivatives in peptide synthesis. Many syntheses of thyroliberin have been reported but it has been recorded by Syrier and Beyerman\textsuperscript{154} that yields are often low and products poorly characterized.

Thyroliberin was prepared by us as outlined in scheme 12. The dipeptide which was isolated and characterized in moderate yield had convenient physical properties. The N(\text{K})-protection was removed with trifluoroacetic acid and the crude deprotected dipeptide was coupled with L-pyroglutamic acid 2,4,5-trichlorophenyl ester to give after purification diiodothyroliberin in moderate overall yield. Diiodothyroliberin had previously been prepared by Prudelles et al\textsuperscript{146} by direct iodination of
thyroliberin, as an intermediate prior to tritiation. The compound had not however been characterized, the only identification reported being t.l.c. evidence that the product was different from the non-iodinated starting material.

Deprotection to give thyroliberin was accomplished by catalytic hydrogenation. However the final product was found to be contaminated by small quantities of calcium iodide, produced when the basic calcium carbonate rhodium support neutralised the hydrogen iodide evolved in the deprotection. The last traces of this impurity stuck tenaciously to the tripeptide and although the material was otherwise pure full characterization proved impossible.

The tripeptide was hydrolysed and the hydrolysate digested by an $L_e$-amino-acid oxidase and shown to contain less than 5% $D$-histidine. Since up to 5% $D$-amino-acid has been shown to be produced on hydrolysis of a histidine containing peptide it is possible to say that the thyroliberin was optically pure.

However when coupled to $L_e$-prolineamide under conditions designed to exaggerate racemisation, e.g. 2 h preactivation of the histidine carboxy function, then after deprotection by hydrogenolysis of the iodo-groups and hydrolysis, the hydrolysate was shown by $L_e$-amino-acid oxidase
to contain 20% D-histidine.

Results and Conclusion.

1) 2,5-Diiodohistidine was prepared by the method of Bruning. In our hands this reaction produced inconsistent yields of pure product.

2) A general method of producing $N(\xi)$-protected 2,5-diiodohistidine derivatives from the readily available methyl ester was devised.

3) An initial investigation into the stability of 2,5-diiodohistidine to some of the more extreme conditions encountered in peptide synthesis indicated that it was stable under most conditions, but rapidly cleaved by hydrogenolysis.

4) $N(\xi)$-t-Butoxycarbonyl-L-5-iodohistidine methyl ester obtained as a byproduct in the preparation of the diiodohistidine derivative was isolated by silica column chromatography and characterized. The position of iodination was unambiguously assigned by catalytic deuteration.

5) Diiodothyroliberin was prepared in good yield with intermediates having convenient physical properties, however final catalytic hydrogenolysis produced thyroliberin contaminated with traces of calcium iodide, which conventional purification techniques failed to remove.

6) No racemisation was observed under standard coupling conditions, although under forcing conditions (2 h preactivation with dicyclohexylcarbodiimide) up to 20% D-histidine was detected.

7) The reversible substitution of the imidazole side chain of histidine by iodine seems to be a promising way of protecting histidine in peptide synthesis. A number of problems were identified including purification and removal of inorganic by-products after deprotection and the racemisation detected under forcing conditions.
CHAPTER 4

Results and Discussion

Bromination of the Imidazole Ring as a Method of Side-Chain Protection for Histidine in Peptide Synthesis
Introduction.

In the previous chapter investigations into the use of iodohistidines as intermediates in peptide synthesis were outlined. Overall, these investigations produced quite promising results as well as exposing a number of problems, including difficulty in purifying the final product from inorganic by-products after the deprotection of the imidazole ring and the fact that after 2 h preactivation 20% D-histidine was present in the hydrolysate of a deprotected dipeptide prepared from a diiodohistidine intermediate.

Thus it was decided that it might be useful to investigate whether the use of a more strongly electron-withdrawing imidazole substituent had any additional effect on racemisation, in conjunction with further experiments to attempt to demonstrate the general utility of halogenated histidine derivatives in peptide synthesis. Bromine is a suitable electron-withdrawing substituent, but the effect of bromination of the imidazole ring on the $pK_a$ of the unprotected nitrogen is difficult to gauge from the somewhat fragmentary data to be found in the literature. However, 5-bromoimidazole has a $pK_a$ of 3.60 as opposed to the published value of 4.18 for 5-monoiodohistidine, so it looked possible that bromination of the imidazole ring might lower the basicity sufficiently to reduce the rate of racemisation further compared with the iodo-derivative.

Unlike iodination, references to the bromination of histidines and histidine containing peptides in the literature are rare, and the only reference to the preparation of dibromohistidine is in a paper by Binkly in which he reports the preparation of 2,5-dibromohistidine as a solid which decomposed rapidly on exposure to air (no characterization data was included).

We therefore decided to attempt to prepare and characterize the $N(\alpha)$-protected dibromo-derivative of histidine using a similar method to
that used to prepare the 2,5-diiodohistidine derivative from the methyl ester and to investigate further the use of halogenated intermediates in peptide synthesis.

Synthesis of \( N(\omega)-t\)-Butoxycarbonyl-L-2,5-dibromohistidine.

Bromination of \( N(\omega)-t\)-butoxycarbonyl-L-histidine methyl ester was carried out with bromine in chloroform using triethylamine as the base. However unlike the iodination of the imidazole ring, addition of 2.3 equivalents of bromine resulted in a mixture of products. T.l.c. showed a major spot due to what was thought (by analogy with the iodo-derivative) to be the 2,5-dibromo-derivative as well as a smaller spot immediately below thought to be the monobrominated material; there were however a number of spots caused by low \( R_F \) polar material. Addition of more bromine resulted in complete decomposition of the product to give low \( R_F \) polar material.

Thus it was decided to attempt the purification of the mixture produced by the addition of 2.5 equivalents of bromine. The crude material was washed with sodium thiosulphate solution to remove excess bromine and any water-soluble polar material. The mixture was separated by the rapid 'flash' chromatography technique proposed by Still et al\(^{159}\). This involved the use of a short column of high quality Merck Kieselgel 60 (40-63 \( \mu \)m) silica dry packed and eluted under a slight pressure of nitrogen gas. Excellent separation occurred and after passing fractions containing the major product through the column a second time it was possible to isolate and fully characterize the major product as \( N(\omega)-t\)-butoxycarbonyl-L-dibromohistidine methyl ester. This compound was perfectly stable to air unlike Binkley's derivative and saponification gave the \( N(\omega) \)-protected acid in good yield (Scheme 13).
Dehalogenation of $N^\omega$-t-Butoxycarbonyl-L-2,5-dibromohistidine Methyl Ester.

Previously problems had occurred after the final hydrogenolysis to deprotect the imidazole ring of the iodinated peptides owing to difficulty in removing the last traces of calcium iodide. We had also experienced problems in obtaining rhodium on calcium carbonate catalysts in the small quantities normally required for this type of work.

Thus it was decided to examine the possibility that rhodium on calcium carbonate might be replaced with the much more commonly available rhodium on carbon catalyst, with the addition of an anion exchange resin to remove bromide ions from the solution. A sample of $N^\omega$-t-butoxycarbonyl-L-2,5-dibromohistidine methyl ester was hydrogenated in aqueous acetic acid with a mixture of rhodium and palladium on carbon, catalysts in the presence
of a small amount of Amberlite IR 45 (acetate-form) anion exchange resin. After 35 min t.l.c. indicated that all the starting material had been consumed and the only product had a similar \( R_F \) to \( \text{N}(\epsilon)-\text{t}-\)butoxycarbonyl-\( \text{L} \)-histidine methyl ester (Scheme 14). The catalyst and

![Scheme 14](image)

i) \( \text{H}_2-\text{80\%aq AcOH-5\%Pd/C-5\%Rh/C-IR 45 (AcO^-)} \)-35 min.

Synthesis of Thyroliberin.

Having proved that it is possible to prepare dibromohistidine derivatives and then to deprotect the imidazole ring under conditions such that no inorganic impurities were produced it was decided to repeat the attempted synthesis of thyroliberin using \( \text{N}(\alpha)-\text{t}-\)butoxycarbonyl-\( \text{L} \)-2,5-dibromohistidine. The synthesis was carried out according to Scheme 15. \( \text{N}(\alpha)-\text{t}-\)butoxycarbonyl-\( \text{L} \)-2,5-dibromohistidine was coupled to \( \text{L} \)-prolineamide in good yield and purified by conventional techniques to give a dipeptide with convenient physical properties. Deprotection and hydrolysis of a portion of the dipeptide gave a hydrolysate which after digestion with \( \text{L} \)-amino-acid oxidase was shown to contain less than 4% \( \text{D} \)-histidine. After \( \text{N}(\epsilon) \)-deprotection with trifluoroacetic acid the crude
i) DCCI-HOBt-DMF (70% yield) ii) TFA (Product not isolated) iii) DMF-Et$_3$N (79% yield) iv) 5%Rh/C-5%Pd/C-80%aq AcOH-Amberlite IR 45 (AcO$^-$) (76% yield).

Scheme 15

dipeptide was allowed to react with L-pyroglutamic acid 2,4,5-trichlorophenyl ester to give dibromothyroliberin as a very hygroscopic white solid. Hydrogenolysis to remove the bromine imidazole protection proceeded in good yield and the product was easily purified to give thyroliberin which was indistinguishable from an authentic sample as prepared by Stachalski. The use of a rhodium on carbon catalyst in place of rhodium on calcium carbonate proved successful with deprotection proceeding cleanly and rapidly, the product was free from inorganic impurities. After correction for the acetate-water content of the peptide as calculated using both elemental analysis data and information from a quantitative amino-acid analysis carried out in the presence of a known amount of a lysine standard, the optical rotation was shown to be somewhat above the average value for thyroliberin as calculated by similar methods for other syntheses by Syrier and Beyerman.

Solid Phase Synthesis of Glycyl-L-histidyl-L-phenylalanine.

Having successfully demonstrated the applicability of 2,5-
dibromohistidine derivatives in classical solution synthesis it was decided to attempt to prepare a simple histidine-containing tripeptide using the solid phase method of peptide synthesis.

Work had previously been carried out in this laboratory on the solid phase synthesis of \( \text{N}(-\text{phenacyl-}\text{L-histidine-containing peptides by Turner}^{161} \text{ and Brown}^{108} \). None of these syntheses had however proved to be entirely successful. This was thought to be principally due to difficulties caused by the previously-discussed side-reactions of \( \text{N}(-\text{phenacyl-}\text{L-histidine} \) rather than to equipment or methodology.

Hence it was decided to attempt the synthesis of the tripeptide glycyl-\text{L-histidyl-}\text{L-phenylalamine} using the apparatus and wash cycle developed by Turner\(^{161}\). Solid phase synthesis had first been put forward in a practical form by Merrifield in the early sixties. Since that time numerous different methods have been applied both to the problem of thoroughly washing the peptide-polymeric support to remove all impurities and to the efficient agitation of the resin during synthesis\(^{162}\). The most common method of agitation has been that of simple external shaking as proposed by Merrifield\(^{163}\). However Turner chose to agitate the reaction mixture with an upward flow of nitrogen gas, through a sinter in the bottom of a cylindrical reaction vessel, a method first employed by Loffet et al\(^{164}\) and Gut et al\(^{165}\).

The cerium salt of \( \text{N-t-butoxycarbonyl-}\text{L-phenylalanine} \) was esterified with a chloromethylated 1\% crosslinked Merrifield polystyrene resin according to the method of Gisin\(^{160}\), to give after washing a resin, which nitrogen analysis showed to contain 0.82 mmol of protected amino-acid per gram of resin.

The \( \text{N-t-butoxycarbonyl-}\text{L-phenylalanine} \) resin was placed in the synthesis vessel where each additional amino-acid residue was added using
the following cycle of operations. N(\(\alpha\))-Deblocking was initially carried out using 40% trifluoroacetic acid in dichloromethane, followed by neutralisation with triethylamine, and then coupling of the N(\(\alpha\))-t-butoxycarbonyl-protected amino-acid using a four fold excess of the acid with dicyclohexylcarbodiimide. Between each operation the resin was repeatedly washed with dichloromethane and isopropanol which swell and shrink the resin respectively in order to facilitate the washing out of residual reagents.

Synthesis was carried out as in Scheme 16 with each amino-acid being left to couple overnight. All additions of reagents and wash solutions were carried out manually, with removal being effected by suction through the sinter after the required time. A number of design faults in the apparatus which had hindered earlier syntheses also caused problems in

Scheme 16
this preparation. The long runs of narrow bore Teflon tubing tended to become blocked with dicyclohexylurea as it crystallised out. However, the most notable difficulty was that the sinter in the bottom of the synthesis vessel was much too fine and consequently was very easily clogged by dicyclohexylurea and resin fines making it almost impossible to remove wash and reaction solutions. This blocking was in fact so severe that the resin had to be removed from the synthesis vessel several times in order to clean the sinter with chromic acid which must have lowered the yield to some extent.

After completion of the second coupling cycle to give the tripeptide the resin was removed from the synthesis vessel and put in a cleavage vessel as used by Turner (Figure 12) with trifluoroacetic acid. Hydrogen bromide gas was bubbled slowly through the mixture for forty-five minutes after which the cleaved peptide was filtered off. The crude tripeptide was then subjected to catalytic hydrogenolysis to remove the bromine protecting groups. When the hydrogenolysis solution showed only one Pauly-positive spot on t.l.c. the crude mixture was filtered through a Sephadex G10 gel which showed an initial peak due to high molecular weight non-peptide impurities and the second peak containing Pauly-active material (Figure 13).

Those fractions containing Pauly-active material were refiltered through Sephadex G10 and after pooling chromatographically homogeneous fractions the tripeptide was obtained as a hygroscopic white solid in good overall yield. This material was shown to be identical with a similar tripeptide which had been synthesised by three different routes by Brown in this laboratory using a different protected histidine intermediate.

An enzyme digest on the hydrolysate of the tripeptide showed that after correction for racemisation upon hydrolysis there was still 7% D-histidine present.Whilst it is difficult to quote peak ratios
Solid Phase Cleavage Vessel.

**Key:**

- **A** Supply of HBr gas
- **B** Cleavage vessel
- **C** Trifluoroacetic acid-resin suspension
- **D** Porosity grade 3 sinter
- **E** Teflon tap
- **F** Calcium chloride drying tubes
- **G** To water aspirator
- **H** Collection vessel

**Figure 12**
accurately where, as in this case, one is so small the figure of 7\% is above that which could be explained by random base line and measurement error on the amino-acid analysis. Thus it seems likely that a small amount of racemisation occurred on coupling the histidyl residue to the peptide under standard solid phase coupling conditions.

\( N(\omega)-t\)-Butoxycarbonyl-\( L \)-2,5-dibromohistidine was coupled to prolineamide under conditions designed to exaggerate racemisation, i.e. 2 h preactivation of the carboxy-component with dicyclohexylcarbodiimide. After hydrogenolysis and hydrolysis, digestion of the hydrolysate with \( L \)-amino-acid oxidase indicated that (after correction for racemisation in hydrolysis) 15\% D-histidine was present.

Hence whilst halogenation significantly reduces the levels of racemisation as compared with \( N(\tau) \)-electron-donating protecting groups such as benzyl, it does not completely eliminate it, and the difference between bromo- and iodo-protection is small.

An attempt was made to prepare the dichloro-derivative from the
\(\text{N}(\alpha)\)-protected methyl ester in a analogous manner to the iodo- and bromo-compounds. However after the addition of only one equivalent of chlorine substantial decomposition occurred and after the addition of two equivalents of chlorine no material corresponding to the dichlorohistidine derivative was observed on t.l.c. Thus although it might be possible to introduce two chlorine or fluorine as has been done with the synthesis of monofluorohistamine\(^{168}\) then the complex synthetic pathway required would render the method unsuitable for the easy introduction of protecting groups for peptide synthesis.

Results and Conclusion.

1) \(\text{N}(\alpha)\)-t-Butoxycarbonyl-L-dibromohistidine was synthesied by a similar method to that used to prepare the diiodo-derivative. Unlike the reported preparation of 2,5-dibromohistidine the \(\text{N}(\alpha)\)-protected compound was indefinitely stable in air.

2) Deprotection of the dibromo-derivative was accomplished cleanly by hydrogenolysis in the presence of a mixed pallidium and rhodium on carbon catalyst with the addition of an anion exchange resin.

3) Thyroliberin was synthesised and fully characterized in good yield from the \(\text{N}(\alpha)\)-protected dibromohistidine derivative, the intermediate dipeptide was shown by enzyme digest to be optically pure.

4) The simple tripeptide glycyl-L-histidyl-L-phenylalanine was prepared by solid phase peptide synthesis in good yield from the \(\text{N}(\alpha)\)-protected dibromohistidine derivative. The final product was exactly similar to authentic material. However after hydrolysis and digestion by L-amino-acid oxidase the hydrolysate was shown to contain after correction 7\% D-histidine, indicating that under standard solid phase synthesis conditions a small degree of racemisation of 2,5-dibromohistidine occurs.
5) After coupling with prolineamide under conditions designed to exaggerate racemisation 15% $D_2$-histidine was found in the hydrolysate of the deprotected dipeptide after correction.

6) It was not possible to prepare $N_{(\omega)}$-t-butoxycarbonyl-$L_{-}$-$2,5$- dichlorohistidine methyl ester by the same general method as had been used for the preparation of the other dihalo-derivatives.

It is possible to conclude that dihalogenation of the imidazole ring of histidine produces intermediates of convenient physical properties for peptide synthesis which do not undergo side-reactions. However both the diiodo- and dibromo-derivatives racemised significantly under conditions designed to exaggerate racemisation, whilst under similar conditions the equivalent derivatives with electron-donating $N_{(\gamma)}$-protecting groups such as $N_{(\gamma)}$-benzyl-$L_{-}$-histidine racemised significantly more. However the $N_{(M)}$-protected derivatives remained optically pure under these conditions. More significantly the tripeptide glycyl-$L_{-}$-histidyl-$L_{-}$-phenylalanine synthesised under standard solid phase peptide synthesis conditions was found to be slightly racemised. Thus apart from ease of preparation dihalogen protection offered no significant advantages over $N_{(\pi)}$-protection where as it had the disadvantage that whilst racemisation would normally be absent under standard conditions it could possibly occur under certain circumstances unless special precautions were taken, unlike $N_{(\pi)}$-protection, where no reasonable conditions have yet been established where racemisation occurs.

Hence $N_{(\pi)}$-protection is still the method of choice for protection of the imidazole ring of histidine although dihalogen protection offered may advantages over present protecting groups and might be useful in certain preparations- for instance in the preparation of the $N_{(\omega)}$-fluorenylmethyloxycarbonyl-derivative where the ability to prepare the free 2,5-diiodohistidine would be of special interest.
CHAPTER 5

Results and Discussion

Optimisation of the Preparation of $N(\omega)$-t-Butoxycarbonyl,$N(\eta)$-benzyloxymethyl-L-histidine
Introduction.

All our studies had indicated that the only certain way of ensuring the complete optical integrity of histidine in peptide synthesis was to protect the \( \mathcal{N} \)-nitrogen of the imidazole ring. However two major problems had to be solved before this strategy could be viable. Firstly a suitable protecting group had to be found, phenacyl having proved to be far too reactive under conditions encountered in peptide synthesis. Secondly, the relative length and complexity of synthetic pathways required for selective protection of the less available \( \mathcal{N} \)-nitrogen, make \( \mathcal{N}(\mathcal{N}) \)-protected derivatives complex and expensive to prepare.

Ramage\textsuperscript{107} had tested a number of \( \mathcal{N} \)-imidazole derivatives none of which had appeared to be as useful for histidine protection as phenacyl, however Anderson and Groves\textsuperscript{169} had found benzyloxymethyl to be a good protecting group for the nitrogen in pyrrole. Unlike phenacyl, this group was a chemically simple ether which proved to be non-reactive and stable to strong mineral acids and bases. It could be cleaved from pyrrole by hydrogenation to give 1-hydroxymethylpyrrole which decomposed to pyrrole and formaldehyde upon refluxing in an organic base.

Brown prepared \( \mathcal{N}(\omega) \)-t-butoxycarbonyl,\( \mathcal{N}(\mathcal{N}) \)-\( \beta \)-bromobenzyloxymethyl-\( \mathcal{L} \)-histidine via a synthetic route analogous to that used in the preparation of the \( \mathcal{N}(\mathcal{N}) \)-phenacyl-histidine derivatives (Scheme 17). The product was characterized and found to be stable to most conditions encountered in peptide synthesis. However it was readily cleaved by hydrogenolysis where, unlike the \( \mathcal{N} \)-benzyloxymethylpyrrole the formaldehyde spontaneously cleaved from the heterocyclic ring giving deprotection in one step. Rapid deprotection was also observed when hydrogen bromide was bubbled through a solution of the \( \mathcal{N}(\mathcal{N}) \)-protected material in trifluoroacetic acid as well as under amino-acid hydrolysis conditions. A number of simple tripeptides
i) Boc₂O-MeOH-Et₃N  ii) Et₂O-Ph₃CCl  iii) pBrPhCH₂OCH₂Br-Et₂O  iv) AcOH-H₂O-IR 45(AcO⁻)  v) NaOH-H₂O-MeOH

Scheme 17
were synthesised including thyroliberin and glycyl-L-histidyl-L-phenylalanine, all of which were easily prepared with retention of optical purity. Both the N(\(\Pi\))-protected amino-acid, (which is readily soluble in cold chloroform) and the peptide intermediates had convenient physical properties.

However whilst N(\(\Pi\))-benzyloxyethyl protection looked promising chemically it would be unlikely to ever be widely used unless the long synthetic pathway required for its preparation could be simplified so that it compared more favourably with that required for the popular N(\(\Pi\))-histidine derivatives.

In the course of preparing N(\(\omega\))-t-butoxycarbonyl-L-histidine methyl ester Brown had isolated the N(\(\omega\)),N(\(\Pi\))-bis-t-butoxycarbonyl-L-histidine methyl ester which is always formed in small amounts as a by-product. He then investigated the use of this compound to provide the temporary blockade of the \(\gamma\)-nitrogen in the place of the N(\(\Pi\))-triphenylmethyl-histidine derivative. Benzyl-chloromethyl ether was reacted with N(\(\omega\)),N(\(\Pi\))-bis-t-butoxycarbonyl-L-histidine methyl ester in dichloromethane and after neutralisation with triethylamine t.l.c. indicated that the crude reaction mixture contained a considerable quantity of the desired N(\(\omega\))-t-butoxycarbonyl,N(\(\Pi\))-benzyloxyethyl-L-histidine methyl ester as well as large amounts of material having a lower \(R_p\) value. Separation by 'flash' chromatography gave the desired product in 34% yield (Scheme 18).

The N(\(\Pi\))-protected methyl ester was easily saponified to give the acid in good yield, hence the preparation was now in principle much simpler, although the yields were still low and any scaling up of the process limited by the necessity for purification of the methyl ester by column chromatography. It was then decided to look at this preparation
Synthesis of $\text{N}^{(\alpha)},\text{N}^{(\gamma)}$-Bis-t-butoxycarbonyl-$L$-histidine Methyl Ester.

$\text{N}^{(\omega)},\text{N}^{(\gamma)}$-Bis-t-butoxycarbonyl-$L$-histidine methyl ester has been described and characterized in the literature as a by-product isolated during the preparation of $\text{N}^{(\alpha)}$-t-butoxycarbonyl-$L$-histidine methyl ester. Brown had obtained his sample of the $\text{N}^{(\alpha)},\text{N}^{(\gamma)}$-protected histidine by a similar method, but it was thought that the synthesis of large quantities of this material would be facilitated by a preparative route that produced the $\text{N}^{(\alpha)},\text{N}^{(\gamma)}$-protected histidine derivative as the sole product.

Scheme 18

systematically to try and improve the yields at each step and also to produce the $\text{N}^{(\gamma)}$-histidine protected methyl ester without the need for column chromatography.

i) $\text{Boc}_2\text{O-Et}_3\text{N-CH}_3\text{OH}$ ii) $\text{PhCH}_2\text{OCH}_2\text{Cl-CH}_2\text{Cl}$ iii) $\text{Et}_3\text{N-CH}_3\text{OH}$-Column chromatography (yield 34%)
Two equivalents of di-(t-butyl)-dicarbonate were added to a previously-neutralised solution of L-histidine methyl ester dihydrochloride in methanol and left to stand overnight. After evaporation of the solvent the residue was partitioned between citric acid solution and ether, and the required product was recovered from the ether layer in excess of 90% yield. Since the product was pure and readily characterized, this proved a route to the starting material which is both simple and high-yielding. 

\[ N(\omega), N(\tau)-\text{Bis-t-butoxycarbonyl-L-histidine methyl ester} \]

is however unstable at room temperature over a period of days. We found that it was advisable either to use the bis-protected methyl ester immediately or to store it below 0°, at which temperature it appeared to be stable indefinitely.

Optimisation of the Preparation of \( N(\omega)-t\)-Butoxycarbonyl, \( N(\tau) \)-Benzyloxy methyl-\( L \)-Histidine Methyl Ester.

It was decided to repeat Brown's synthesis of \( N(\omega)-t\)-butoxycarbonyl, \( N(\tau) \)-Benzyloxy methyl-\( L \)-Histidine methyl ester with slight modifications to see if by carrying out the preparation on a larger scale it would be possible to crystallise the product from the crude reaction mixture. The reaction was carried out by the method of Brown and Jones with the exception that the product was washed with water after the neutralisation step in order to remove any water-soluble by-products. After dissolving the crude mixture in ethyl acetate and adding petroleum ether (40-60) to turbidity the \( N(\tau) \)-protected methyl ester identical to that purified by Brown crystallised out in 33% yield. However t.l.c. indicated that large quantities of product remained in solution, further crystallisation was not possible and the remainder of the product (25%) was recovered by a 'flash' chromatographic separation.

Whilst the overall yield had been substantially improved it was still difficult to attain high yields without column chromatography.
and large amounts of low \( R_F \) by-products were still present, so it was decided to optimise conditions for this reaction systematically.

Initially it was decided to investigate the purity of the benzyl-chloromethyl ether that we had been using. This had been purchased from Fluka and examined by n.m.r. spectroscopy on arrival and found to be acceptably pure. However over a period of time we noted that the n.m.r. spectra of crude reaction mixtures had a much wider range of impurities than had been observed in earlier experiments. Upon re-examining the benzyl-chloromethyl ether it was discovered that gross decomposition had occurred. This could have been due to storage at room temperature during transit producing small amounts of impurities including hydrogen chloride which might then catalyse the continued decomposition of the material. The benzyl-chloromethyl ether was then distilled and stored at 0°. In all future experiments the n.m.r. spectrum of the benzyl-chloromethyl ether was examined before use, however it did not undergo further decomposition over a period of one month.

Thus having ensured the purity of starting materials a number of small-scale experiments were conducted in which varying amounts of benzyl-chloromethyl ether were added to \( \text{N}^{(w)}, \text{N}^{(y)} \)-bis-t-butoxycarbonyl-L-histidine methyl ester in polar and non-polar solvents with and without the presence of triethylamine.

For each solvent based combination 1.1, 1.6 and 2.1 equivalents of benzyl-chloromethyl ether was added and the reaction followed on t.l.c. The intensity of spots associated both with the starting material and the desired product were monitored, as well as the quantity of material on the base line thought to be produced by reaction of a further molecule of benzyl-chloromethyl ether with the \( \text{N}^{(w)} \)-protected derivative to form the stable \( \text{N}^{(m)} \)-t-butoxycarbonyl,\( \text{N}^{(y)}, \text{N}^{(m)} \)-bis-benzyloxyethyl-L-histidine
methyl ester chloride salt. From these experiments we established that the addition of a base tended to increase the complexity of the t.l.c. of the reaction mixture with proportionally smaller quantities of the desired product. It was also shown that on adding 1.1 equivalents of benzyl-chloromethyl ether to a neutral solution of the \( \text{N}(\omega),\text{N}(\tau) \)-bis-protected methyl ester in dichloromethane significant quantities of both the starting material and quaternary salt were present, although the desired product was the major component in the mixture. However upon adding a further 0.5 equivalents of benzyl-chloromethyl ether all the starting material was consumed with only a slight increase in the amount of quaternary salt present.

**Synthesis of \( \text{N}(\omega)-\text{t-Butoxycarbonyl,N(\tau)}\)-benzyloxymethyl-L-histidine.**

On the basis of these results it was decided to attempt to prepare the \( \text{N}(\tau)\)-histidine protected derivative by reacting 1.6 equivalents of the alkylating agent with the \( \text{N}(\omega),\text{N}(\omega) \)-protected methyl ester in dichloromethane then to attempt to isolate the product as its hydrochloride salt without further neutralisation.

This reaction was carried out and a t.l.c. of the crude reaction mixture showed a much greater proportion of desired product than had previously been observed. The reaction mixture was left to stand over night before removing the solvent, the last traces of which were evaporated under high vacuum leaving the crude mixture as a stiff foam. This was then dissolved in a small quantity of methanol and ether was added until slight turbidity was observed. It was found to be very important that only the minimum of ether was added as the hydrochloride salt of the product crystallised very easily and if it precipitated too rapidly it was usually found to be contaminated with quaternary salts. However if the ether was added until a slight turbidity was observed and the solution was left to
stand at room temperature for 2 h and then at 0° overnight the \(N(\alpha)-t\)-butoxycarbonyl,\(N(\gamma)\)-benzyloxymethyl-\(L\)-histidine methyl ester hydrochloride salt crystallised out in 69% yield as a pure and readily characterized product.

Saponification proceeded in good yield to give the acid as a crystalline solid in an easy three-stage synthesis with a high yield of pure crystalline material at each stage (Scheme 19).

\[
\begin{align*}
\text{BocNHCHCO}_2\text{Me} & \xrightarrow{i} \text{BocNHCHCO}_2\text{Me} \\
& \xrightarrow{\text{ii}} \text{BocNHCHCO}_2\text{H} \\
\end{align*}
\]

i) \(\text{PhCH}_2\text{OCH}_2\text{Cl-CH}_2\text{Cl}_2\) (69% yield) ii) \(\text{NaOH-H}_2\text{O-MeOH}\) (87% yield)

\text{Scheme 19}

Results and Conclusion.

1) \(N(\alpha),N(\gamma)\)-Bis-\(t\)-butoxycarbonyl-\(L\)-histidine methyl ester was prepared by a novel method in very high yield.

2) It was possible to crystallise some \(N(\alpha)-t\)-butoxycarbonyl,\(N(\gamma)\)-benzyloxymethyl-\(L\)-histidine methyl ester from the crude reaction mixture after neutralisation but the free base did not crystallise
readily and large amounts of unrecovered product remained in the liquors.

3) Thorough investigation of the purity of the starting materials revealed that $N(\alpha),N(\tau)$-bis-t-butoxycarbonyl-$L$-histidine methyl ester was indefinitely stable if stored below $0^\circ$, however benzyl-chloromethyl ether was liable to decompose unless freshly distilled and then stored below $0^\circ$.

4) Using freshly-prepared starting materials $N(\alpha)$-t-butoxycarbonyl, $N(\tau)$-benzyloxymethyl-$L$-histidine methyl ester hydrochloride salt was prepared in good yield as a pure crystalline solid by the addition of 1.6 equivalents of benzyl-chloromethyl ether to a solution of $N(\alpha),N(\tau)$-bis-t-butoxycarbonyl-$L$-histidine methyl ester in dichloromethane.

We had therefore succeeded in preparing a $N(\tau)$-protected histidine derivative by a simple synthetic pathway. Only three-steps were necessary all of which proceeded in good yield to give pure crystalline material without the need for chromatographic purification. Furthermore the $N(\tau)$-benzyloxymethyl-protected derivative was readily soluble in non-polar organic solvents, was stable to conditions normally encountered in peptide synthesis yet readily removed by catalytic hydrogenation or hydrogen bromide in trifluoroacetic acid, and appeared to be free from the problems of racemisation. Hence it appeared from these preliminary studies that $N(\tau)$-benzyloxymethyl-$L$-histidine protected derivatives fulfilled all our requirements for useful protected histidine intermediates.
CHAPTER 6

Results and Discussion

Practical Peptide Applications of N(π)-Benzylloxymethyl Histidine
Introduction.

Having demonstrated that \(N(\omega)\)-t-butoxycarbonyl,\(N(\pi)\)-benzyloxymethyl-\(L\)-histidine could be prepared in good yield by a simple three-stage synthesis the incorporation of \(N(\pi)\)-benzyloxymethyl-protected histidine derivatives into peptides had to be thoroughly investigated.

Brown had previously prepared thyroliberin using \(N(\pi)\)-benzyloxymethyl-protected histidine as an intermediate. The synthesis of thyroliberin has been used in this laboratory to demonstrate the potential use in peptide synthesis of protected histidine derivatives that have looked promising after initial screening of the protected amino-acid and imidazole models. Thyroliberin was chosen because it is a small peptide with well-characterised biological and physical properties but its preparation using previous histidine side-chain protecting groups has proved difficult. However the successful synthesis of thyroliberin only indicated that the protected derivative is potentially useful. This was amply demonstrated in the case of the \(N(\pi)\)-phenacyl-protected histidine derivative where the synthesis of thyroliberin was accomplished and the protected derivative was put forward as a useful intermediate although later syntheses then highlighted a number of problems, both with side-reactions and the deprotection of \(N(\pi)\)-phenacyl-containing peptides.

Thus in this case it was felt that whilst \(N(\pi)\)-benzyloxymethyl protection looked promising, an extensive series of syntheses using it as a protected histidine intermediate should be carried out both to demonstrate its general use in peptide synthesis and to investigate possible side-reactions in the presence of other difficult amino-acids.

Brown has successfully prepared the simple tripeptide glycyl-\(L\)-histidyl-\(L\)-phenylalanine by both classical and solid phase methods,
so it was decided to investigate applications in more demanding syntheses.

**Solid Phase Synthesis of 5-Isoleucine Angiotensin II.**

Initially it was decided to attempt the synthesis of the biologically important octapeptide 5-isoleucine angiotensin II by both solid phase and classical methods. The synthesis of this peptide had been demonstrated in solution and on a solid support. It was felt that a synthetic strategy involving early incorporation of our protected histidine derivative would provide a severe test of the utility of the \(N(\alpha)-\)benzyloxy carbonyl protecting group.

In our previous attempts at solid phase peptide synthesis we had used the apparatus developed by Turner. This apparatus was clearly inadequate for an extended synthesis, being over-complex in design and having a small fine sinter in the reaction vessel that made it virtually impossible to remove wash solutions and reagents.

A completely new apparatus was developed, with a large, coarse sinter to facilitate the removal of solvents etc. by vacuum. The apparatus whilst still working on the principle of agitating the resin with an upward flow of nitrogen gas (Figure 14, Plate 2) was designed to be much more compact and simple to operate than previous examples. This system has the further advantage that it could also be used for the cleavage of the peptide from the resin simply by replacing the nitrogen flow with hydrogen bromide and the waste collection flask with a clean flask to collect the crude cleaved peptide in trifluoroacetic acid solution.

The cesium salt of \(N(\alpha)-t\)-butoxycarbonyl-\(\beta\)-phenylalanine was esterified with a chloromethylated 1% crosslinked Merrifield polystyrene resin according to the method of Gisin to give (after washing) a
Solid Phase Peptide Synthesis Apparatus

Figure 14
resin which nitrogen analysis showed to contain 0.82 mmol of protected amino-acid per gramme of resin. The N-t-butoxycarbonyl-L-phenylalanine was placed in the synthesis vessel and each additional amino-acid residue was introduced using the following cycle of operations. N(α)-Deblocking was effected using 40% trifluoroacetic acid in dichloromethane which was followed by neutralisation with triethylamine. The N(α)-protected amino-acid was added in fourfold excess with a similar excess of dicyclohexylcarbodiimide and the mixture was allowed to react for four hours to ensure complete coupling. The resin was then washed and the coupling procedure was repeated, leaving the reaction mixture overnight. Between each operation the resin was repeatedly washed with dichloromethane and isopropanol which swell and shrink the resin respectively, to ensure complete removal of residual reagents.
After each complete coupling cycle a small sample of the resin was removed from the synthesis vessel, hydrolysed and the hydrolysate then subjected to amino-acid analysis to determine the degree of incorporation of the terminal amino-acid onto the growing chain.

The synthetic strategy chosen is set out in Scheme 20. It was decided to use maximum protection, with the side-chains of histidine, tyrosine, arginine and aspartic acid all being blocked as all these residues are liable to participate in side reactions if left unprotected. In choosing a strategy for the blockade of side-chains other than that of histidine our main criteria were that ideally all our protecting groups should be cleaved by hydrogen bromide in trifluoroacetic acid at the same time as the peptide was removed from the resin, and that there should be the minimum of complications and side reactions due to the chosen protected derivative. Thus we chose benzyl protection for the side-chain of aspartic acid and 2,6-dichlorobenzyl protection for the phenol function of tyrosine. The dichlorinated derivative was used for the protection of tyrosine because cleavage and rearrangement to the irreversibly substituted 3-benzylytyrosine had been shown to occur under the standard conditions for removal of N(\(\alpha\))-t-butoxycarbonyl groups with trifluoroacetic acid in the case of \(\beta\)-benzyl-tyrosine. Erickson and Merrifield demonstrated that the more sterically hindered 2,6-dichlorobenzyl-protected derivative was much less susceptible to this rearrangement. Whilst there are several possible protecting groups for arginine we decided to use well tried \(N(\omega)\)-nitroarginine as it was readily available and its side-reactions are well-documented.

\(N\)-t-Butoxycarbonyl-L-phenylalanine resin was added to the reaction vessel and the synthesis commenced. Addition of the first three
Scheme 20

i) 40%v/v-TFA-CH₂Cl₂ ii) DCCI-CH₂Cl₂ iii) 40%v/v-TFA-CH₂Cl₂ iv) DCCI-CH₂Cl₂ v) 40%v/v-TFA-CH₂Cl₂ vi) DCCI-CH₂Cl₂
vii) 40%v/v-TFA-CH₂Cl₂ viii) DCCI-CH₂Cl₂ ix) 40%v/v-TFA-CH₂Cl₂ x) DCCI-CH₂Cl₂ xi) 40%v/v-TFA-CH₂Cl₂ xii) DCCI-DMF
xiii) 40%v/v-TFA-CH₂Cl₂ xiv) DCCI-CH₂Cl₂ xv) HBr-TFA xvi) 5%Pd/C-80%aq AcOH-H₂
residues proceeded smoothly with amino-acid analysis indicating complete incorporation of the terminal residues. After addition of \(N_\text{oc}\)-t-butoxycarbonyl, O-2,6-dichlorobenzyl-L-tyrosine amino-acid analysis indicated a very low degree of incorporation. This later proved to have been due to omitting to add phenol to the hydrolysis solution, causing the tyrosine to decompose; a repeat analysis indicated that complete incorporation had in fact occurred. At this stage cleavage and characterization of the peptide from a portion of the resin was deemed advantageous as the pentapeptide contained no difficult groups apart from histidine and it could also be established unambiguously that total incorporation of tyrosine had taken place. Thus a sample of the peptide was cleaved and deprotected using hydrogen bromide in trifluoroacetic acid with anisole added as a scavenger. The pentapeptide L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine was characterized as an amorphous white solid in good yield after separation on a Sephadex G25 gel column. The remainder of the resin was replaced in the reaction vessel and no further problems were encountered in the coupling of the remaining amino-acids. The octapeptide was cleaved from the resin by hydrogen bromide in trifluoroacetic acid, conditions which simultaneously deprotected the aspartyl, tyrosyl and histidyl residues. The crude \(N_\text{oc}\)-nitroarginine-5-isoleucine angiotensin II was hydrogenated in 80% aqueous acetic acid over a palladium on charcoal catalyst for 24 h until t.l.c. showed only a single major component which gave positive tests with both Pauly and Sataguchi reagents. The product was separated on a Sephadex gel column and purified by gradient elution ion-exchange chromatography. 5-Isoleucine angiotensin II was fully characterized in good overall yield and shown to be indistinguishable by t.l.c. and
n.m.r. spectroscopy from an authentic sample as supplied by Sigma Chemicals.

The solid phase synthesis-cleavage vessel performed well and can be recommended to other workers who wish to experiment in solid phase peptide synthesis but have neither the time nor the resources to acquire and set up conventional automated solid phase equipment.

**Attempted Solid Phase Synthesis of L-Histidyl-L-histidyl-L-histidyl-glycyl-L-phenylalanine.**

Having been successful in the previous relatively ambitious synthesis we decided to investigate the possibility of preparing a pentapeptide on a solid phase resin containing three adjacent histidyl residues - such a preparation had never been attempted before due to the difficulties of incorporating even a single histidyl residue on solid phase.

The C-terminal residue was provided by the same L-phenylalanyl-resin as used in the angiotensin synthesis. Synthesis was attempted according to Scheme 21 with glycyl being added as the second residue to provide a good reference for amino-acid analysis.

After each complete coupling cycle a sample was removed and hydrolysed and the hydrolysate subjected to amino-acid analysis to determine the degree of incorporation of the N-terminal residue. Glycine and the first two histidyl residues were shown to have been fully incorporated. However amino-acid analysis after the fourth coupling cycle showed that incorporation of the final histidyl residue had been negligible. It was decided to repeat the coupling cycle (omitting the trifluoroacetic acid washes which would have deprotected any pentapeptide) to see if further coupling increased the degree of incorporation. The
i) 40%v/v-TFA-CH₂Cl₂, ii) DCCI-CH₂Cl₂, iii) 40%v/v-TFA-CH₂Cl₂, iv) DCCI-CH₂Cl₂, v) 40%v/v-TFA-CH₂Cl₂, vi) DCCI-CH₂Cl₂, vii) 40%v/v-TFA-CH₂Cl₂, viii) DCCI-CH₂Cl₂, ix) HBr-TFA

Scheme 21
extent of incorporation after amino-acid analysis remained as low and
the resin still gave a strongly positive fluorescamine test. It was
also noted that the resin did not swell significantly in dichloromethane.

At this stage it was decided to cleave the peptide from the
resin and examine the products. Cleavage and deprotection was effected
by the normal procedure using trifluoroacetic acid and hydrogen bromide.
The peptide residue was shown by t.l.c. to contain a number of products.
Separation on a Sephadex G25 column partially resolved these (Figure 15).

**Attempted Purification of \( \text{L-Histidyl-L-histidyl-L-histidyl-glycyl-L-phenylalanine} \)**

![T.l.c. solvent A \(_3\)
Developed with Pauly
reagent.](image-url)
All major components were Pauly-positive and so it was decided that the mixture probably consisted of the penta- and tetrapeptides with a small amount of the monohistidine-containing tripeptide.

Sheppard\(^{179}\) was able to inform us that lack of incorporation and the change in the swelling properties of the resin that we observed are quite common occurrences in solid phase peptide synthesis and were due to the growing peptide chain becoming insoluble in the reaction solvents and thus coagulating and in effect becoming a part of the insoluble polymer support.

Whilst conducting the coupling reactions in a more polar solvent such as dimethylformamide might solve the problem in terms of the actual incorporation of the histidyl residues, then the growing peptide would still be insoluble in the solvents used in our washing procedure. Thus removal of reagents would become a considerable problem.

Brown had succeeded in preparing \(\text{L-histidyl-L-histidyl-L-histidine}\) in good yield by classical solution synthesis demonstrating that the problem did in fact originate in the solid phase technique rather than in any particular side-reaction of the \(\text{N(M)-benzyloxymethyl-L-histidine}\) derivatives or in multiple histidine chains. It was decided that the problems encountered in solid phase methodology were outside the scope of this work and the synthesis was not repeated.

**Synthesis of N(\(\kappa\))-\(\text{t-Butoxycarbonyl,N(M)-benzyloxymethyl-L-histidyl-L-prolyl-L-phenylalanine Benzyl Ester}\).**

We had originally intended to prepare 5-isoleucine angiotensin II by a conventional solution synthesis in parallel with the solid phase synthesis. However the latter proceeded so smoothly that there seemed little to be gained by carrying out the less demanding conventional
It is generally believed that if a synthesis gives a pure product in good yield using solid phase under circumstances when the purification and characterization of all the intermediates has not been possible, then in solution phase (where any impurity can be removed before proceeding to the next step) the synthesis will present no problems. That the inverse is not true was demonstrated in the preparation of trihistidine compounds; these could be prepared readily from the N(ω)-protected intermediates in solution but not at all on a solid phase resin.

Whilst we did not prepare 5-isoleucine angiotensin by a conventional solution phase synthesis, a synthetic strategy involving benzyl protection of the C-terminal carboxy group had been designed and the tripeptide \(N(\xi)-t\)-butoxycarbonyl,\(N(\eta)-\)benzylxymethyl-\(L\)-histidyl-\(L\)-prolyl-\(L\)-phenylalanine benzyl ester had been prepared according to Scheme 22.

![Diagram of peptide structure](image)

\(i)\) DCCI-CH\(_2\)Cl\(_2\)-HOBt-Et\(_3\)N  \(ii)\) TFA  \(iii)\) DCCI-HOBt-Et\(_3\)N-CH\(_2\)Cl\(_2\)

Scheme 22

\(L\)-Phenylalanine benzyl ester \(p\)-toluenesulphonate was prepared
in good yield by the method of Zervas et al. and coupled without problem to t-butoxycarbonyl-proline. The dipeptide was purified as an oil that was homogeneous on t.l.c. and had an n.m.r. spectrum consistent with the requisite structure. After N-deblocking with trifluoroacetic acid the dipeptide was coupled with the N-protected histidine derivative and the resulting tripeptide was purified and characterized in good yield as a white crystalline solid. No further work was carried out on this compound.

Synthesis of Peptides Containing Histidine and Tryptophan.

Whilst we had not observed any problems in the use of N-benzyloxymethyl-protected derivatives at this stage, it was still possible that adverse side-reactions could occur between the side-chains of deprotected amino-acids and the formaldehyde produced on cleavage of the benzyloxymethyl group. French and Edsall reviewed the reactions of peptides and peptide side-chains with formaldehyde and observed that formaldehyde reacted with many side-chains on free amino-acids over a range of conditions to form methylene bridges. However the reactions often required elevated temperatures and excess formaldehyde. Throughout our experiments with the deprotection of the N-benzyloxymethyl-protected histidine derivatives we saw no evidence of the methylene-bridged compound 1,2,3,4-tetrahydropyridio-3,4-imidazole-6-carboxylic acid as observed by Smith et al. No sign of reaction of formaldehyde with other residues such as phenylalanine, tyrosine and arginine where reaction had been reported with the free amino-acids was observed. Thus we felt able to conclude that when incorporated into peptides the side-chains of poly-functional amino-acids did not appear to react with the very tiny amounts of formaldehyde present.
Nevertheless it was decided to undertake the preparation of a dipeptide containing N(\text{\textit{n}})-protected histidine and tryptophan in order to ensure both that the reported reaction of free tryptophan with formaldehyde\textsuperscript{183} did not in fact take place in this case and also to demonstrate the synthesis of a dipeptide containing these two very difficult amino-acids.

N(\text{\textit{\alpha}})-t-Butoxycarbonyl, N(\text{\textit{n}})-benzyloxymethyl-L-histidyl-L-tryptophan methyl ester was prepared and characterized in good yield followed by the hydrogenolysis of the N(\text{\textit{n}})-protecting group to give the partially deprotected dipeptide (Scheme 23).

![Diagram of dipeptide structure]

i) DCCI-HOBt-Et\textsubscript{3}N-DMF  ii) H\textsubscript{2}-Pd/C-80\%aq AcOH

Scheme 23

The N-terminal and C-terminal were deliberately left protected to give a product which would have more convenient physical properties, be more stable and would therefore be easier to characterize than the completely deprotected dipeptide. The dipeptide was fully characterized in good overall yield as a white solid and no trace of the methylene-bridged compound was found.

It was decided to attempt to couple a further tryptophan residue to the protected dipeptide. N(\text{\textit{\alpha}})-Deblocking was accomplished
by treating the dipeptide with trifluoroacetic acid for 15 min. After coupling with N(α)-t-butoxycarbonyl-tryptophan using dicyclohexylcarbodiimide in dimethylformamide, the product was purified by conventional extraction techniques. However a trace of impurity was observed. Separation on a Sephadex gel column failed to purify the product further. F.D. Mass spectroscopy and n.m.r. spectroscopy indicated that the product consisted of a mixture of the desired tripeptide and material corresponding to the tripeptide with an additional t-butyl group. This is almost certainly due to the t-butylation of the indole ring of tryptophan during the acidolytic cleavage of the N(α)-t-butoxycarbonyl-blocking group of histidine. This reaction is well-documented in the literature and would be totally independent of the nature of the histidine side-chain protecting group.

Parallel to this work Brown had prepared the N(α)-benzyloxyacarbonyl and the N(α)-fluorenylmethyloxycarbonyl,N(α)-benzyloxymethyl derivatives, as well as L-histidyl-L-histidine and L-histidyl-L-histidyl-L-histidine without difficulty. Thus the only problems encountered in this work had been due either to methodology, as in the attempted solid phase synthesis of a pentapeptide containing three histidine residues or in side-reactions of other difficult amino-acids as in the attempted synthesis of L-tryptophyl-L-histidyl-L-tryptophan methyl ester.

Results and Conclusion.

1) L-Tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine had been prepared and characterized in good overall yield by solid phase synthesis.
2) 5-Isoleucine angiotensin II had been prepared and characterized in good yield by solid phase peptide synthesis.

3) The attempted synthesis of L-histidyl-L-histidyl-L-histidyl-glycyl-L-phenylalanine by solid phase methods failed due to the insolubility of the N(ω)-deblocked tetrapeptide in the solvents used in our coupling cycles.

4) N(ω)-t-Butoxycarbonyl,N(ω)-benzyloxymethyl-L-histidyl-L-prolyl-L-phenylalanine benzyl ester was prepared and characterized in good yield as an intermediate in the projected classical synthesis of 5-isoleucine angiotensin II. This synthesis was not continued due to the success of the preparation of the same compound by a solid phase synthesis.

5) N(ω)-t-Butoxycarbonyl-N(ω)-histidyl-L-tryptophan methyl ester was prepared to demonstrate that deprotection of the imidazole ring of histidine did not produce side-reactions with the formaldehyde liberated. No such reactions were observed and the product was characterized in good yield.

6) The attempted preparation of N(ω)-t-butoxycarbonyl-L-tryptophyl-N(π)-benzyloxymethyl-L-histidyl-L-tryptophan methyl ester failed to produce an analytically pure tripeptide due to a small degree of t-butylation of the indole ring of tryptophan during the N(ω)-deblocking of the dipeptide.

Thus we have been unable to observe any detrimental side-reactions of the N(π)-benzyloxymethyl group during the synthesis of a number of peptides of varying sizes. It was felt that further work to synthesize additional peptides would serve little purpose and that we could now recommend the use of this histidine derivative in general peptide synthesis where any particular problems in specific systems of
interest would be rapidly exposed. Other workers have already incorporated the protected derivative into peptides of interest to them without difficulty.¹⁸⁵
CHAPTER 7

Results and Discussion

Racemisation of Histidine Derivatives
Introduction.

In the course of preparing and assessing protected histidine derivatives the accurate measurement of the degree of racemisation occurring during coupling reactions has often been necessary.

In the determination of the optical purity of synthetic peptides, hydrolysis followed by digestion with either an \( \text{L} \)- or \( \text{D} \)-amino-acid oxidase has proved the most straightforward and accurate technique. Jones and Ramage\textsuperscript{105} devised a general method for the determination of the optical purity of histidine-containing peptides based on digestion of a carefully buffered solution of the peptide with an \( \text{L} \)-amino-acid oxidase, the peptide hydrolysate then undergoing amino-acid analysis with \( \text{D} \)-arginine added as an internal standard. This method (based on refinements of previous digestion methods using \( \text{L} \)-amino-acid oxidases\textsuperscript{107,154}) worked very well providing great care was taken both with the purity of reagents and the precise execution of the enzyme digest.

However an enzyme digest on a protected amino-acid is time-consuming to conduct; also one is often examining small quantities of \( \text{D} \)-histidine and their accurate estimation can be difficult after allowing for the non-negligible racemisation that occurs during the hydrolysis of a peptide bond. We attempted to overcome this problem by deliberately exaggerating racemisation in order to make trends between different protected derivatives more obvious. The exaggerated racemisation was achieved by preactivation of the carboxy function of the histidine derivative under test for 1-2 h with a carbodiimide without the addition of any substances such as 1-hydroxybenzotriazole which are believed to suppress racemisation. After the requisite preactivation
period the activated carboxy function was coupled to a second amino-acid before the optical purity of the dipeptide thus produced was determined by hydrolysis and enzyme digest. This method had been used by Ramage\textsuperscript{107} to estimate the degree of racemisation present in a number of protected histidine derivatives after varying periods of preactivation.

It was decided to use this method to investigate the optical stability of a number of the histidine derivatives produced during the course of this work and also to see if it would be possible to evolve a simpler method for the rapid determination of the optical stabilities of histidine-containing peptides under activating conditions.

**Estimation of Racemisation in Histidine Derivatives by Digestion with an L-Amino-acid Oxidase.**

Initially a series of experiments was conducted using the conventional enzyme digest technique to measure the percentage of D-histidine produced after preactivation of the carboxy component of a number of histidine derivatives with dicyclohexylcarbodiimide for 2 h. The histidine derivative under test was dissolved in dimethylformamide and incubated at 0° for 2 h with dicyclohexylcarbodiimide after which the activated compound was coupled by the addition of a precooled neutralised solution of prolineamide in dimethylformamide. The mixture was left overnight before the dipeptide in each case was isolated as an uncharacterized solid using conventional methods. The imidazole side-chain was deprotected using the appropriate technique after which the dipeptide was hydrolysed under standard conditions. Enzyme digest of the hydrolysate was carried out with an added D-arginine internal standard using the method of Jones and Ramage.\textsuperscript{105} After amino-
acid analysis it was possible to calculate the percentage of D-histidine present in each sample (Table 4).

<table>
<thead>
<tr>
<th>Derivative</th>
<th>% D-Histidine (±5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measurement by Enzyme Digest</td>
</tr>
<tr>
<td>N(ω)-t-Butoxycarbonyl,N(τ)-Benzyl-L-histidine</td>
<td>45</td>
</tr>
<tr>
<td>N(ω)-t-Butoxycarbonyl-L-2,5-diiodohistidine</td>
<td>20</td>
</tr>
<tr>
<td>N(ω)-t-Butoxycarbonyl-L-2,5-dibromohistidine</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 4

It can be seen that N(ω)-t-butoxycarbonyl,N(τ)-benzyl-L-histidine underwent gross racemisation, corresponding to the results predicted for a N(τ)-substituted electron-donating histidine 'protecting' group. The 2,5-diiodo-derivative appeared to racemise considerably less and the 2,5-dibromo-derivative racemised slightly less still. It must be noted that such small differences as those observed between the two halogen derivatives must be treated with caution due to the difficulty in the exact measurement of the areas under such small peaks and in assessing the size of the correction factor for racemisation on hydrolysis.
Investigation into the use of Optical Rotation as a Method of Measuring Racemisation in Activated Amino-Acids.

In the previous experiments we were essentially investigating the effect of incubation of the protected histidine derivative with a carbodiimide on the optical purity of the histidine derivative. If the activation of the carboxy component of an amino-acid in dimethylformamide results in the formation of only small equilibrium concentrations of the activated $\alpha$-acylurea then the effect upon the optical rotation of the amino-acid should be small and equilibrium should be reached rapidly if the amino-acid is optically stable. Thus if the optical rotation of the activated amino-acid is monitored then after the initial equilibrium concentration of $\alpha$-acylurea has been established any change will either be caused by loss of optical activity due to racemisation or the formation of a non-equilibrium product such as the $\eta$-acylurea.

If the rate of formation of non-equilibrium products was small then it should be possible to determine the effect of activation upon optical purity simply by monitoring the optical rotation of the activated species.

It was decided to investigate the change in rotation over a period of 2 h for a number of histidine derivatives activated by diisopropylcarbodiimide whilst also investigating the quantities of $\eta$-acylurea present after each experiment by t.l.c. The histidine derivatives were dissolved in dry dimethylformamide to give solutions of similar concentrations to those found in conventional peptide synthesis. The initial rotation was established over a period of several mins in a polarimeter cell cooled to 4° by a circulating water
jacket. This temperature was chosen to simulate that used in coupling reactions but considerable problems were caused as atmospheric moisture rapidly condensed on the cell windows producing a sharp drop in transmittance and random readings unless they were continually wiped clean.

When a constant initial rotation had been established the solution was removed from the cell, mixed with diisopropylcarbodiimide and immediately returned to the cell. Rotation was then monitored at 64 s intervals for 2 h.

Initially we looked at N(α)-t-butoxycarbonyl-L-histidine, N(α)-t-butoxycarbonyl-2,5-diiodohistidine and N(α)-t-butoxycarbonyl, N(τ)-benzyl-L-histidine. In each case after 2 h a t.l.c. of the activated mixture revealed a component that had a higher Rf value than the amino-acid derivative, although it was apparently only present in trace quantities.

The results, expressed as a percentage of the initial rotation at any particular time (Figure 16), show that in the case of the τ-benzyl-derivative the value for the optical rotation dropped rapidly. After 2 h it had reached a level consistent with the 45% D-histidine revealed by the enzyme digest analysis. In the case of the 2,5-diiodo-derivative the rotation drops much more slowly as would also be expected from the enzyme digest, however in this case the level of rotation after 2 h suggests that if the drop is solely due to racemisation, more D-histidine (30%) is present than was suggested by enzyme digest (20%). In the case of the N(im)-unprotected histidine very little decrease in optical activity was observed; this is in accordance with Veber's
% of Initial Rotation

\[
\begin{align*}
\text{\textbullet} & \quad N(\alpha)-\text{t-Butoxycarbonyl-}L\text{-histidine} \\
\text{\textcircled{O}} & \quad N(\alpha)-\text{t-Butoxycarbonyl-}L\text{-2,5-diiodohistidine} \\
\text{\textasciitilde} & \quad N(\alpha)-\text{t-Butoxycarbonyl,}N(\tau)\text{-benzyl-}L\text{-histidine}
\end{align*}
\]

Figure 16
hypothesis that the imidazolium ring is formed which then undergoes comparatively slow racemisation.

A further series of experiments was carried out to investigate the comparative rates of racemisation of $N(\text{t})$- and $N(\pi)$-protected histidine derivatives. Brown had prepared both the $\gamma$- and $\pi$-isomers of $N(\omega)$-t-butoxycarbonyl-$N(\text{im})$-p-bromobenzoyloxymethyl-$L$-histidine and $N(\omega)$-t-butoxycarbonyl-$N(\text{im})$-benzyloxymethyl-$L$-histidine. The $\gamma$-isomers were both prepared by reaction of the alkylating agent with the unprotected imidazole ring on $N(\omega)$-t-butoxycarbonyl-$L$-histidine methyl ester. In each case the product was purified by column chromatography. The $\gamma$- and $\pi$-isomers were both fully characterized and shown to have different $R_F$ values on t.l.c.

These experiments were conducted in exactly the same way as the previous runs. In each case t.l.c. of the final activated mixtures showed traces of material having a higher $R_F$ value than the original amino-acid and thought to be the $N$-acylurea. An attempted comparison of $N(\gamma)$- and $N(\pi)$-phenacyl-histidine using this method had to be abandoned when the $N(\pi)$-derivative rapidly became deeply coloured and transmission dropped to a level such that accurate results could no longer be obtained. Large quantities of a material with a high $R_F$ value were shown to be present. After standing for 24 h this substance was the major product and was isolated as a crude oil which was shown by n.m.r. spectroscopy and mass spectroscopy to be the $N$-acylurea.

However in the other two comparisons the changes in optical rotation of the $\gamma$- and $\pi$-isomers produced very clear results (Figures 17 and 18). It can be seen that in both cases the rotation of the $N(\pi)$-
% Of Initial Rotation

- N(α)-t-Butoxycarbonyl,N(β)-p-bromobenzoyloxymethyl-L-histidine
- N(α)-t-Butoxycarbonyl,N(γ)-p-bromobenzoyloxymethyl-L-histidine

Time min.  
Figure 17
isomer changes a little initially but then remains remarkably stable, whilst the optical acivity of the two N(τ)-isomers drops rapidly.

These results confirm the trend discovered by Ramage after enzyme digest of the N(T)- and N(π)-phenacyl isomers indicated that blockade of the N(π)-nitrogen suppresses racemisation, whilst the same group on the τ-position results in gross racemisation. Hence these results are consistent with Veber's theory and preliminary work conducted in this laboratory: that only the π-nitrogen of imidazole catalyses intramolecular racemisation. Further evidence for this is provided by molecular space-filling models of N(τ)-benzyloxymethyl-L-histidine and N(π)-benzyloxymethyl-L-histidine (Plate 3) which demonstrate that whilst the N(π)-nitrogen could easily abstract the ω-proton or form the imidazolium ring, the N(τ)-nitrogen is situated too far from the ω-proton to participate in such an intramolecular reaction.

We felt that while these results were consistent with results from previous experiments, due to the unpredictable rate of N-acylurea formation this could not be recommended as a general screening method for racemisation of amino-acids under activating conditions unless very careful checks were made on the product distribution after activation with special reference to significant quantities of N-acylurea.

Results and Conclusion.

1) 2,5-Diiodohistidine was shown by enzyme digest to be significantly less prone to racemisation on activation than N(τ)-benzyl-L-histidine.
$N(\tau)$-Benzyloxymethyl-$l$-histidine

$N(\pi)$-Benzyloxymethyl-$L$-histidine

Plate 3
2) 2,5-Dibromohistidine appeared to be slightly less prone to racemisation than 2,5-diiodohistidine.

3) Similar results were obtained after a study of the change in rotation with time of activated 2,5-diiodohistidine and N(τ)-benzyl-histidine.

4) Histidine was shown to be relatively stable to rapid racemisation under activating conditions.

5) A comparison of the rates of racemisation of a series of α- and τ-isomers indicated that the α-isomer was stable to racemisation whilst the equivalent τ-isomer racemised rapidly.

6) Measurement of racemisation by following changes in optical rotation can only be qualitative due to formation of non-equilibrium impurities and great care should be taken in the interpretation of results.
Experimental Section

Methods and Materials
I Instruments and Techniques.

Melting points were determined on a Kőfier hot-stage apparatus and are quoted uncorrected.

Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter in a 1 dm cell.

$^1$H nuclear magnetic resonance spectra were recorded on one of the following spectrometers:

1. Hitachi Perkin-Elmer R 24A or R 24B (60 MHz),
2. Perkin-Elmer R 32 (90 MHz),
3. Bruker WH 300 (300 MHz).

Tetramethysilane (TMS) was used as an internal standard and chemical shifts from TMS are quoted in δ ppm. All spectra of new peptides were recorded on the Bruker WH 300.

Infrared spectra were recorded on a Perkin-Elmer 257.

Ultraviolet spectra were recorded on a Unicam SP 800A.

Mass spectra were recorded on one of the following spectrometers:

1. Varian CH 7,
2. V.G. Micromass 16F,
3. V.G. Micromass ZAB 1F.

For amino-acid analyses samples were hydrolysed in 6M hydrochloric acid under vacuum at 110° for 24 h unless stated otherwise, and run on either a Jeol JLC 5AH or Locart amino-acid analyser.

Thin layer chromatography was performed on Merck DC-Plastikfolien Kieselgel 60F$_{254}$ (100 x 20 x 0.2 mm) or DC-Fertigplatten Kieselgel 60F$_{254}$ (2000 x 500 x 0.25 mm) precoated plates using the following solvent systems:

1. $A_1$ Bu'OH:AcOH:H$_2$O 4:1:5
2. $A_3$ Bu'OH:AcOH:H$_2$O 4:1:1
(3) E₃ MeOH:CHCl₃ 1:4
(4) E₃ MeOH:CHCl₃ 1:19
(5) P₁ CHCl₃:MeOH:AcOH 17:2:1
(6) P₂ CHCl₃:MeOH:AcOH 10:2:1
(7) CMAW CH₂Cl₂:MeOH:AcOH:H₂O 15:10:2:3
(8) X EtOAc:MeOH:Et₃N 5:1:1:

Detection was by quenching of ultraviolet fluorescence, chlorine followed by starch/iodide solution, Pauly reagent or Sakaguchi reagent.

Column chromatography was performed using relevant media and eluants, with the eluate being monitored by ultraviolet absorption at 280 or 254 nm using a LKB 8300 Uvicord II detector with in some cases rotation being monitored by a Perkin-Elmer 141 polarimeter.

Solvent removal was by rotary film evaporator under reduced pressure.

II Solvents.
Ethyl acetate, methanol, acetic acid, toluene, benzene and light petroleum were all of AnalaR grade.
Ether, chloroform and dichloromethane were dried over calcium chloride or magnesium sulphate and filtered before use.
Dimethylformamide was distilled under reduced pressure.
Water was distilled.
All other solvents were as commercially available and were used without further purification.

III Reagents.
Triethylamine was dried over potassium hydroxide and distilled.
Anisole was distilled and stored over sodium wire.
Trifluoroacetic acid was distilled before use.
Benzyl chloromethyl ether was distilled before use and stored at 0°.
Other reagents were used as commercially available.

IV Amino-acids.

Unless otherwise stated these were prepared by standard methods in this laboratory or purchased from either Fluka A.G of Buchs, Switzerland or Sigma Chemical Company of St. Louis, U.S.A.

Notes.

1. The enzyme used in histidine racemisation experiments was the $L$-amino-acid oxidase from Crotalus Adamanteus venom (5.1 mg protein/ml, Sigma Chemical Company of St.Louis, U.S.A.).

2. The Pauly reagent - sodium nitrite (5% in water), sulphanilic acid (0.5% in 1M hydrochloric acid) and sodium carbonate (10% in water) in the ratios 1:1:2 - was mixed immediately before use.

3. The Sakaguchi reagent - 8-hydroxyquinoline (0.1% in acetone) was sprayed on to the plate followed by bromine (0.4% in 1.0M sodium hydroxide).

4. Solutions were dried over magnesium sulphate.

5. Voltages were recorded relative to a standard calomel electrode.

6. The following convention was adopted for designating the n.m.r. spectra of histidine derivatives with side chains:

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{4} & \quad \text{2} \\
\text{3} & \quad \\
\text{1} & \quad \\
\text{5} & \quad \\
\end{align*}
\]
CHAPTER 1

Experimental Section

Further Investigations into the use of N(P)-Phenacyl-L-histidine

in Peptide Synthesis
Attempted Electrolytic Deprotection of $\text{L-Pyroglutamyl,\text{N(\text{I})-phenacyl-}}$
$\text{L-histidyl-L-prolineamide.}$

The protected tripeptide as prepared by Ramage was dissolved in 0.5M sulphuric acid (10 ml) and placed in the cathode compartment of a simple electrolysis apparatus (Figure 8). The electrolysis was carried out at a potential of 1.0 V at a mercury cathode. The current was monitored (Table 5) and at intervals a sample of the electrolyte was removed and examined by t.l.c. (CMAW). After 2.5 h all the starting material had been consumed and the current had fallen to a steady 0.5 mA.

<table>
<thead>
<tr>
<th>TIME</th>
<th>CURRENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td>100 mA</td>
</tr>
<tr>
<td>1 min</td>
<td>10 mA</td>
</tr>
<tr>
<td>10 min</td>
<td>8 mA</td>
</tr>
<tr>
<td>20 min</td>
<td>1.5 mA</td>
</tr>
<tr>
<td>60 min</td>
<td>0.5 mA</td>
</tr>
<tr>
<td>120 min</td>
<td>0.5 mA</td>
</tr>
</tbody>
</table>

Table 5

The solution was removed from the cathode compartment and the mercury washed with water (2x5 ml). The combined aqueous solution was then passed several times down an Amberlite IR 45 (acetate-form) anion exchange resin until a negative test for sulphate ions was obtained (Barium Chloride). After evaporation of the solvent the brown oily residue was dissolved in 25% acetic acid and filtered through a Sephadex G10 gel column (2.5x100 cm). Those fractions containing mainly one Pauly active component were combined and the solvent removed, to give after trituration with ether a very hygroscopic brown solid. However whilst the major component
had an identical $R_p$ (CMAW) of 0.25 on t.l.c. to an authentic sample of thyroliberin, significant amounts of another Pauly active contaminant $R_p$ (CMAW) were also present. This side-product did not correspond to the starting material.
CHAPTER 2

Experimental Section

Investigations into the Possibility of using 1,1,1-Trichlorobut-2-enyl

as a N(T)-Protecting Group for Histidine
Attempted preparation of N(1,1,1-trichlorobut-2-enyl)imidazole with 1,1,1,4-tetrachlorobut-2-ene.

Attempt A 1,1,1,4-Tetrachlorobut-2-ene (490 mg, 2.53 mmol) was added to a solution of imidazole (850 mg, 12.5 mmol) in methanol (5 ml) in which sodium hydrogen carbonate (210 mg, 2.5 mmol) was suspended. After refluxing for 16 h t.l.c. (X) indicated complete consumption of the alkene. Evaporation of the solvent gave an oily residue which was dissolved in water (5 ml) and extracted with chloroform (4 x 10 ml). The combined organic phase was washed with brine (2 x 10 ml), dried and the solvent evaporated to give a brown oil which t.l.c. and n.m.r. showed to contain a complex mixture of products.

Attempt B 1,1,1,4-Tetrachlorobut-2-ene (500 mg, 0.55 mmol) was added to a suspension of mercuric acetate imidazolium salt (842 mg, 2.5 mmol) in toluene (5 ml). After stirring for 13 h the solid was removed by filtration and dissolved in 1M hydrochloric acid (10 ml). Residual solid was removed by further filtration and the solution was neutralised by the addition of sodium hydrogen carbonate. The aqueous phase was extracted with chloroform (3 x 10 ml), and the combined organic phase was washed with water (2 x 10 ml) dried and evaporated. The oily residue was shown by t.l.c. and n.m.r. to contain only 1,1,1,4-tetrachlorobut-2-ene, R<sub>f</sub> (X) 0.54; 
§(CDCl<sub>3</sub>) 4.1(2H, d, (J=5 Hz), -CH<sub>2</sub>Cl), 6.3(2H, complex, -CHCH-).

Attempt C N(Trimethylsilyl)imidazole was prepared by the normal method from imidazole (400 mg, 5.8 mmol) and chloromethylsilane (629 mg, 5.8 mmol) in disilazone (15 ml). After refluxing for 1 h the solvent was evaporated and without further purification the oily N(Trimethylsilyl)imidazole was dissolved in toluene (10 ml) to which was added 1,1,1,4-tetrachlorobut-2-ene (1.125g, 5.8 mmol). After 24 h at room temperature t.l.c. (X) indicated that no reaction had occurred. A catalytic quantity of mercuric
acetate (25 mg) was added to the solution but after a further 6 h of vigorous stirring reaction had still not begun, hence the mixture was refluxed for 1 h after which t.l.c. indicated that all the starting material had been consumed. The solvent was evaporated and the residual oil dissolved in water (20 ml) which was extracted with chloroform (3 x 20 ml). The combined organic extracts were washed with brine (10 ml), dried and the solvent evaporated to give a brown oily residue which t.l.c. and n.m.r. showed to contain a complex mixture of products.

Attempt D Silver imidazolide salt was prepared on a 30 mmol scale from imidazole and ammoniacal silver nitrate as a white microcrystalline solid which rapidly decolourised on standing (2.2 g, 42%). 1,1,1,4-Tetrachlorobut-2-ene (100 mg, 0.51 mmol) in chloroform (10 ml) was added dropwise to silver imidazolide salt (90 mg, 0.51 mmol) in dimethylsulphoxide (2 ml). After 3 h the solution was washed with water (3 x 5 ml) to remove dimethylsulphoxide, dried and the solvent evaporated, the oily residue was shown by n.m.r. and t.l.c. to be 1,1,1,4-tetrachlorobut-2-ene.

Preparation of N(1,1,1-trichlorobut-2-enyl)imidazolide oxalate salt using 1,1,1-trichloro-4-bromobut-2-ene.

1,1,1-Trichloro-4-bromobut-2-ene (2.85 g, 12 mmol) was added to a solution of imidazole (4.1 g, 60 mmol) in methanol (100 ml) containing sodium hydrogen carbonate (1.02 g, 12 mmol). After refluxing for 2.5 h all the alkene had reacted. The methanol was removed and the reaction mixture was partitioned between water (50 ml) and ether (50 ml). After drying and removal of the solvent the colourless oily residue was dissolved in methanol (10 ml), to which oxalic acid (1.3 g, 12 mmol) was added. Upon standing N(1,1,1-trichlorobut-2-enyl)imidazole oxalate salt crystallised as a homogeneous white solid (1.53 g, 47.5%) of m.p.
148-149°, ((CD$_3$)$_2$SO) 5.05(2H, d, J=5 Hz, -CH$_2$CH-), 6.6(2H, complex, -CH-CH-), 7.5(1H, s, H5 of imidazole), 7.6(1H, s, H4 of imidazole), 8.9(1H, s, H2 of imidazole). (Found: C, 34.33; H, 3.01; N, 8.95; Cl, 34.06. C$_9$H$_9$N$_2$Cl$_3$O$_4$ requires C, 34.25; H, 2.87; N, 8.87; Cl, 33.75%).

_\ce{N(1,1,1-Trichlorobut-2-enyl)imidazole}.

_\ce{N(1,1,1-Trichlorobut-2-enyl)imidazole} oxalate salt (100 mg, 0.37 mmol) was partitioned between saturated sodium hydrogen carbonate solution (10 ml) and ether (5 ml) and the aqueous layer was then extracted with ether (5 x 5 ml). The organic extracts were combined and dried after which the solvent was removed to give \_\ce{N(1,1,1-Trichlorobut-2-enyl)imidazole} as a colourless oil (73 mg, 87%) of R$_F$(E) 0.70; M$^+$ found 224, 226, 228, 230 C$_9$H$_9$N$_2$Cl$_3$ requires: 224, 226, 228, 230; $\delta$(CDCl$_3$) 4.7 (2H, dd, J=5.3 Hz and 1.7 Hz, -CH$_2$CH-), 6.0-6.1(1H, dt, J=14.7 Hz and 1.1 Hz, -CHCCl$_3$), 6.3-6.4(1H, dt, J=14.7 Hz and 5.3 Hz, -CH$_2$CH-), 6.92 (1H, s, H-5 of imidazole), 7.12(1H, s H-4 of imidazole), 7.51(1H, s, H-2 of imidazole). If the methylene protons at $\delta$4.7 were irradiated over a period of several hours a 1% Nuclear Overhauser effect was observed on the imidazole ring protons at the 2- and 5-positions whilst no enhancement was observed on the proton in the 4-position proving that the methylene protons are adjacent to the imidazole ring.

Investigation of the Deprotection of \_\ce{N(1,1,1-Trichlorobut-2-enyl)imidazole}

using Zinc in Acetic Acid.

\_\ce{N(1,1,1-Trichlorobut-2-enyl)imidazole} (30 mg, 0.13 mmol) was dissolved in 50% aqueous acetic acid (2 ml) to which was added zinc dust (100 mg). After shaking for 2 min excess zinc was removed by filtration. The solution was saturated with hydrogen sulphide gas and the precipitated zinc was removed by filtration through a Millipore HA, 0.45 µm membrane.
The solution was shown by t.l.c. to contain a mixture of imidazole $R_p(X) \approx 0.57$, and a component having an $R_p(X) \approx 0.73$, which gave a positive chlorine/starch/potassium iodide test, but which was not Pauly-positive and, unlike the starting material, did not absorb ultraviolet light at 254 nm. No starting material remained.

Investigation of the stability of $N(1,1,1$-trichlorobut-2-enyl)imidazole to conditions commonly encountered in peptide synthesis.

1) Hydrogen bromide - acetic acid:- $N(1,1,1$-trichlorobut-2-enyl)imidazole oxalate salt (50 mg, 0.16 mmol) was dissolved in 45% w/v hydrogen bromide-acetic acid (2 ml). After standing for 1 h at room temperature the solvent was removed and the oily residue was partitioned between ether (5 ml) and saturated sodium hydrogen carbonate solution (5 ml). The aqueous phase was further extracted with ether (4 x 5 ml) and the combined organic phase was dried and the solvent removed to give a colourless oil. This was shown by t.l.c. (X) and n.m.r. to contain only $N(1,1,1$-trichlorobut-2-enyl)imidazole.

2) Trifluoroacetic acid:- $N(1,1,1$-trichlorobut-2-enyl)imidazole oxalate salt (0.94 g, 42 mmol) was dissolved in trifluoroacetic acid (30 ml). A sample (0.3 ml) was removed and its stability investigated by n.m.r. spectroscopy. After 2 h the sample showed a substantial change, with the resonances due to the side-chain protons decreasing in intensity and additional resonances appearing. The solution was set aside and its n.m.r. spectrum recorded at regular intervals until after 2 weeks all the starting material had been converted to the product which was homogeneous on t.l.c. (E$^3$). The trifluoroacetic acid was removed and the oily residue partitioned between ether (20 ml) and saturated sodium hydrogen carbonate solution (20 ml). The aqueous phase was further
extracted with ether (5 x 20 ml) and the combined organic extract was
dried and evaporated to give a chromatographically homogeneous
colourless oil. This was shown by t.l.c. and n.m.r. to be different
from the starting material, although mass spectroscopy showed it to
have a similar molecular weight. The 300 MHz n.m.r. spectrum of the
compound was examined in detail and when the methylene protons at
§4.2 were irradiated over a period of several hours, a 1% Nuclear
Overhauser effect was observed on the H-5 and H-2 imidazole ring protons.
This investigation indicated that the double bond was still in the same
position relative to the imidazole ring but that isomerisation had
occurred to give the cis form of N(1,1,1-trichlorobut-2-ynyl)imidazole
having $R_F(E_3)$ 0.76; $\nu_{max} 215$ nm; $M^+$ found 224, 226, 228 $C_7H_4N_2Cl_3$
requires: 224, 226, 228, 230; (CDCl$_3$) 4.2(2H, dd, $J$=6.1 Hz and 0.92 Hz,
$-\text{CH}_2\text{CH}$), 4.9(1H, dt, $J$=9.74 Hz and 6.0 Hz, $-\text{CHCH}_2$), 5.90(1H, d,
$J$=9.74 Hz, $-\text{CHCl}_3$), 6.96(1H, s, H-5 of imidazole), 7.06(1H, s, H-4 of
imidazole), 7.52(1H, s, H-2 of imidazole). Treatment with zinc powder
and acetic acid gave imidazole and an unidentified product as shown
by t.l.c. (X).

3) Benzylamine: N(1,1,1-Trichlorobut-2-ynyl)imidazole oxalate salt
(54 mg, 0.17 mmol) was partitioned between ether (5 ml) and saturated
sodium hydrogen carbonate solution (5 ml). The organic phase was dried
and the solvent removed to give N(1,1,1-trichlorobut-2-ynyl)imidazole
as a colourless oil. After dissolving in deuterochloroform (0.5 ml)
benzylamine (33 $\mu l$, 0.3 mmol) was added. The mixture was examined by
n.m.r. spectroscopy and t.l.c. for 1 week, after which the starting
material remained unchanged.

4) 1M Sodium hydroxide: N(1,1,1-Trichlorobut-2-ynyl)imidazolide oxalate
salt (51 mg, 0.16 mmol) was dissolved in 1M sodium hydroxide (1 ml) and
methanol (1 ml). After stirring vigorously for 1 h t.l.c. (E) indicated that most of the starting material had been consumed and a product exhibiting a strongly ultraviolet-positive double spot on t.l.c. had been formed. A white precipitate was removed by filtration and shown to contain chloride ions (silver nitrate). The filtrate was evaporated and partitioned between chloroform (5 ml) and water (5 ml). The aqueous phase was further extracted with chloroform (2 x 5 ml) and the combined organic extract was dried and the solvent removed to give a colourless oil whose physical properties were consistent with \( \text{N}(1,1,1\text{-dichloro-1,3-dienyl})\text{imidazole} \), having \( R_f (E) \) 0.64; \( \lambda_{\text{max}} \) 273 nm; \( M^+ \) found 188, 190, 192. The material had a complex n.m.r. (CDCl\(_3\)) consistent with an isomeric mixture of the dienes.
CHAPTER 3

Experimental Section

Iodination of the Imidazole Ring as a Method of Side-Chain Protection

for Histidine in Peptide Synthesis
L-2,5-Diiodohistidine.

This was prepared by the method of Bruning \(^{149}\) on a 6 mmol scale, by the reaction of L-histidine monohydrochloride dissolved in aqueous sodium carbonate with iodine in hexane. The reaction mixture was worked up by acidification of the aqueous phase, addition of potassium iodate and exhaustive extraction with hexane. Careful neutralisation of the aqueous phase with aqueous ammonia produced and off white precipitate. Crystallisation from solution in aqueous hydrochloric acid by addition of aqueous ammonia gave the amino-acid as a white solid (0.55 g, 28%) of m.p. 214-216° (lit. 220°); \( R_f \) (CMAW) 0.74; \( (D_2O) \) 3.25(2H, d, \( J=7H \), \(-\text{CHCl}(-) \), 4.2(1H, m, \(-N\text{DCH}(-) \).}

Investigation of the stability of L-2,5-diiodohistidine under various conditions.

L-2,5-Diiodohistidine (21 mg, 0.05 mmol) and L-lysine hydrochloride (9 mg, 0.05 mmol) were dissolved in the reagent under test (10 ml). Except in the case of 6M hydrochloric acid in which the solution was heated in a sealed tube under vacuum at 110° for 24 h, all the tests were carried out at room temperature for a period of 3 h. Upon completion of the test an aliquot (100 \( \mu l \) was removed and dissoved in 0.01M hydrochloric acid (2 ml). This solution was subjected to amino-acid analysis in order to determine the amount of starting material remaining relative to the known amount of lysine added and also whether any degradation to monoiodohistidine or histidine had occurred by comparison with known amino-acid analyser retention times for these amino-acids. The results are set out in Table 6.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Sodium Hydroxide</td>
<td>Slight loss of starting amino-acid; no monoidohistidine or histidine formed</td>
</tr>
<tr>
<td>90% aq TFA</td>
<td>Starting amino-acid unchanged</td>
</tr>
<tr>
<td>45% w/v HBr/AcOH</td>
<td>Starting amino-acid unchanged</td>
</tr>
<tr>
<td>6M Hydrochloric Acid</td>
<td>Partial loss of starting amino-acid; monoiodohistidine and histidine formed</td>
</tr>
</tbody>
</table>

Table 6

N(ω)-t-Butoxycarbonyl-L-2,5-diiodohistidine Methyl Ester.

Iodine (12.7 g, 50 mmol) in chloroform (300 ml) was added dropwise over a period of 2 h to a vigorously stirred mixture of N(ω)-t-butoxycarbonyl-L-histidine methyl ester (5.2 g, 22.3 mmol) dissolved in chloroform (100 ml) and sodium carbonate (10.6 g, 100 mmol) dissolved in water (100 ml) at 0°. The reaction mixture was allowed to attain room temperature and stirred for a further 15 h. T.l.c. indicated the presence of a single organic product. The organic layer was separated, washed with 1M sodium thiosulphate (5 x 50 ml), dried and evaporated to give a pale yellow oil. Trituration with ether gave N(ω)-t-butoxycarbonyl-L-2,5-diiodohistidine methyl ester as a yellow solid (4.99 g, 49%) of m.p. 89-91°; [α]_D^20 +4.6° (c 1.0 in CHCl₃); R_f(E) 0.66; δ(CDCl₃) 1.4(9H, s, (CH₃)₃C-), 3.1(2H, d, -CH₂CH-), 3.76(3H, s, CH₃OCO-), 4.5(1H, complex, -NHCH-), 5.5(1H, m, -NHCH-); (Found: C, 28.01; H, 3.44; N, 7.98. C₁₂N₃O₄H₁₇I₂ requires C, 27.63; H, 3.26; N, 8.06%); M⁺ found 521

C₁₂N₃O₄H₁₇I₂ requires: 521

N(ω)-t-Butoxycarbonyl-L-2,5-diiodohistidine.

1M Sodium hydroxide (6.2 ml) was added to a vigorously stirred
solution of \( \text{N}(\omega)-t\text{-butoxycarbonyl-L-2,5-diiodohistidine methyl ester} \) (1.61 g, 3.1 mmol) in methanol (2 ml). After 1 h t.l.c. (E\(_3\)) indicated that all the starting material had been consumed. Water (20 ml) was added and the methanol evaporated before the pH was adjusted to 5 by the addition of 1M hydrochloric acid. The aqueous phase was exhaustively extracted with chloroform (5 x 50 ml) and the combined organic phase was dried and evaporated to give a yellow oil. Precipitation from methanol-ether gave \( \text{N}(\omega)-t\text{-butoxycarbonyl-L-2,5-diiodohistidine monohydrate} \) (1.22 g, 78%) of m.p. 165-168\(^\circ\) (Lit 181\(^\circ\)). \( \left[ \text{\(\omega\)} \right]_{\text{D}}^{20} +3.8^\circ \) (c 1.0 in EtOH) (Lit +4.2\(^\circ\) c 0.6 in EtOH) \( \left[ \text{\(\omega\)} \right]_{\text{D}}^{20} +3.8^\circ \) (c 1.0 in EtOH) \( \left[ \text{\(\omega\)} \right]_{\text{D}}^{20} +3.8^\circ \) (c 1.0 in EtOH) \( \left[ \text{\(\omega\)} \right]_{\text{D}}^{20} +3.8^\circ \) (c 1.0 in EtOH) \( \left[ \text{\(\omega\)} \right]_{\text{D}}^{20} +3.8^\circ \) (c 1.0 in EtOH) \( R_f (P_1) 0.48 ; \left( \text{CD}_3\text{OD} \right) 1.1(9\text{H}, \text{s}, (\text{CH}_3)_3\text{C}^-), 2.9(2\text{H}, d, -\text{CH}_2\text{CH}^-), 4.1(1\text{H}, \text{complex}, -\text{NHCH}^-) ; \) (Found: C, 25.29; H, 3.40; N, 7.72. \( \text{C}_{11}\text{H}_{15}\text{N}_3\text{O}_4\text{I}_2\cdot\text{H}_2\text{O} \) requires: C, 25.14; H, 3.04; N, 8.28%); M\(^+\) found 507, 508 \( \text{C}_{11}\text{H}_{15}\text{N}_3\text{O}_4\text{I}_2 \) requires 507. Deprotection by catalytic hydrogenation and digestion with \( \text{L-amino-acid oxidase} \) showed the acid to contain 2% \( \text{D-} \)histidine

\( \text{N}(\omega)-t\text{-butoxycarbonyl-L-2,5-diiodohistidine Dicyclohexylammonium Salt.} \)

Dicyclohexylamine (0.6 ml, 3.0 ammol) in methanol (1 ml) was added to a solution of \( \text{N}(\omega)-t\text{-butoxycarbonyl-L-2,5-diiodohistidine} \) (250 mg, 0.49 mmol) in methanol (1 ml). The solution was cooled to 0\(^\circ\): no product crystallised out, but on adding ether a white solid was precipitated. Reprecipitation from methanol-ether gave \( \text{N}(\omega)-t\text{-butoxycarbonyl-L-2,5-diiodohistidine dicyclohexylammonium dihydrate} \) (310 mg, 92%); m.p. 148-150\(^\circ\); \( \left[ \text{\(\omega\)} \right]_{\text{D}}^{20} +1.6^\circ \) (c 1.0 in CHCl\(_3\)); \( R_f (P_1) 0.72 ; \left( \text{CD}_3\text{OD} \right) 1.0-2.0(31\text{H}, \text{complex}, \) \( C_{6\text{-I}} \) and \( (\text{CH}_3)_3\text{C}^-), 3.0(2\text{H}, d, -\text{CH}_2\text{CH}^-), 4.2(1\text{H}, m, -\text{NHCH}^-) ; \) (Found: C, 38.22; H, 5.60; N, 8.17. \( \text{C}_{23}\text{H}_{38}\text{O}_4\text{I}_2\cdot\text{H}_2\text{O} \) requires: C, 38.20; H, 5.50; N, 7.84%).
N(\(\omega\))-t-Butoxycarbonyl-L-moniiodohistidine Methyl Ester.

Iodine (9.92 g, 39 mmol) dissolved in chloroform (100 ml) was added dropwise over a period of 2 h to a vigorously stirred mixture of N(\(\omega\))-t-butoxycarbonyl-L-histidine methyl ester (5 g, 18.6 mmol) dissolved in chloroform (100 ml) and sodium carbonate (8.4 g, 80 mmol) dissolved in water (50 ml) at 0\(^\circ\). The reaction mixture was allowed to attain room temperature and stirred for a further 15 h. T.l.c. indicated complete consumption of the starting material.

The organic phase was washed with 1M sodium thiosulphate (2 x 50 ml), dried and the solvent evaporated giving a yellow solid (7.0 g) which was shown by t.l.c. to comprise of two components of \(R_F(E_3)\) 0.66 and 0.57. The product was dissolved in 5\% methanol-chloroform (2 ml) and applied to a 60-120 mesh silica gel chromatography column. The column was eluted with 5\% methanol-chloroform and the fractions comprising predominantly of the material of \(R_F(E_3)\) 0.57 were pooled, concentrated and subjected to repeated chromatography under the same conditions. The chromatographically homogeneous fractions were pooled and the solvent was evaporated giving after crystallisation from methanol-chloroform N(\(\omega\))-t-butoxycarbonyl-L-moniiodohistidine methyl ester as a white solid (0.76 g, 10.3\%) of m.p. 135-137\(^\circ\); \([\alpha]_D^{20}\) +4.8\(^\circ\) (c 1.0 in CHCl\(_3\)); \(R_F(E_3)\) 0.57; \(\delta(CDCl_3)\) 1.3(9H, s, (CH\(_3\))C-), 2.75(2H, d, -CH\(_2\).CH-), 3.45(3H, s, CH\(_3\).OCO-), 4.2(1H, m, -NHCHCO\(^-\)), 5.25(1H, m, -NHCH), 7.1(1H, s, imidazole);

(Found: C, 36.75; H, 4.64; N, 10.45. \(C_{12}H_{18}N_{3}O_{4}\) I requires: C, 36.4; H, 4.5; N, 10.6\%) \(M^+\) found 395, \(C_{12}H_{18}N_{3}O_{4}\) I requires: 395

N(\(\omega\))-Benzyloxycarbonyl-L-2,5-diiodohistidine Methyl Ester.

A solution of \(N(\omega)-\)benzyloxycarbonyl-L-histidine methyl ester (1.0 g, 3.3 ammol) dissolved in chloroform (20 ml) was stirred vigorously with a solution of sodium carbonate (2.12 g, 20 mmol) dissolved in water
(20 ml) at 0°. Iodine (2.45 g, 10 mmol) dissolved in chloroform (62 ml) was added dropwise over a period of 2 h. The reaction mixture was allowed to warm to room temperature and stirred for a further 16 h. T.l.c. indicated the presence of a single organic product. The organic phase was separated, washed with 1M sodium thiosulphate (5 x 50 ml), dried and the solvent evaporated to give a dark brown oil. Precipitation from ethyl acetate-ether gave \( \text{N(\(\alpha\))-benzyloxycarbonyl-L-2,5-diiodohistidine methyl ester} \) as a pale yellow solid (1.19 g, 70.4%) of m.p. 68-71°; \( [\alpha]_{D}^{20} +9.9° \) (c 0.75 in CHCl₃); \( R_{F}(P) 0.52, \delta(CDC₁₃) 3.1(2H, d, -CH₂CH-)\), 3.75(3H, s, CH₃OCO⁻), 4.5(1H, m, -NHCH₂CO⁻), 5.1(2H, s, -CH₂Ph), 5.86(1H, m, -NHCH⁻), 7.35(5H, s, C₆H₅⁻); (Found: C, 34.71; H, 3.39; N, 7.33. \( C_{15}H_{15}N_{0.1}O_{1.4}I_{2.0} \) requires: C, 34.57; H, 3.38; N, 7.11). M⁺ found 555, 556, C₁₅H₁₅N₀.1O₁.4I₂ requires: 555.

\( \text{N(\(\alpha\))-Benzyloxycarbonyl-L-2,5-diiodohistidine} \):

1M Sodium hydroxide (0.9 ml) was added to a vigorously stirred solution of \( \text{N(\(\alpha\))-benzyloxycarbonyl-L-2,5-diiodohistidine methyl ester} \) (250 mg, 0.45 mmol) dissolved in methanol (1 ml). After 1 h t.l.c. \( (R₃) \) indicated that all the starting material had been consumed. Water (5 ml) was added and the methanol evaporated. The pH of the aqueous solution was adjusted to 5 by the addition of 1M hydrochloric acid, precipitating a brown solid. After filtration and reprecipitation from methanol-ether \( \text{N(\(\alpha\))-benzyloxycarbonyl-L-2,5-diiodohistidine} \) was obtained as a white solid (132 mg, 54%) m.p. 118-120°; \( [\alpha]_{D}^{20} +13.2° \) (c 1 in MeOH); \( R_{F}(P) 0.48, \delta(CD₃OD) 3.1(2H, d, -CH₂CH-)\), 4.4(1H, m, -NHCH⁻), 5.0(2H, s, CH₂Ph), 7.3(5H, s, C₆H₅⁻); M⁺(F.D) found 542. \( C_{14}H_{13}N_{0.1}O_{1.0}I_{2.0} \) requires 541. The material was contaminated with trapped ether which could not be removed under vacuum. (Found C, 32.75; H, 2.34; N, 7.70 \( C_{14}H_{13}N_{0.1}O_{1.0}I_{2.0} \) requires: C, 32.17; H, 2.77; N, 7.51%).
Hydrogenolysis of $N(\alpha)$-t-Butoxycarbonyl-L-2,5-diiodohistidine Methyl Ester.

5% Rhodium on calcium carbonate (9.5 mg) and 5% palladium on carbon (10.7 mg) was added to a gently stirred solution of $N(\alpha)$-t-butoxycarbonyl-L-2,5-diiodohistidine (12.2 mg, 0.025 mmol) dissolved in dimethylformamide (1 ml). Hydrogen was bubbled through the reaction mixture for 1 h after which t.l.c. ($E_3$) showed that all the $N(\alpha)$-t-butoxycarbonyl-L-diiodohistidine methyl ester had been consumed. The catalyst was removed by filtration and the filtrate was evaporated to give a yellow oil, which was shown by t.l.c. ($E_3$) to have a similar $R_F$ value to authentic $N(\alpha)$-t-butoxycarbonyl-L-histidine methyl ester, $\delta$(CDCl$_3$) 1.49(9H, s, (CH$_3$)$_3$C-), 3.05(2H, d, (J=6Hz),C-CHCH$_2$-), 4.5(1H, m, -NHCH-), 5.85(1H, d, (J=10Hz), -NHCH-), 6.8(1H, s, H-4 of imidazole), 7.52(1H, s, H-2 of imidazole).

A sample of oil was hydrolysed and applied to an amino-acid analyser: only histidine was detected.

$N(\alpha)$-t-Butoxycarbonyl-L-2,5-$^2$H$_2$-histidine Methyl Ester.

5% Rhodium on calcium carbonate (40 mg) and 5% palladium on carbon (40 mg) was added to a gently agitated solution of $N(\alpha)$-t-butoxycarbonyl-L-monoiodohistidine methyl ester (40 mg, 0.1 mmol) dissolved in dimethylformamide (2 ml). The reaction vessel was evacuated and cooled in liquid nitrogen to remove all traces of air before being filled to atmospheric pressure with deuterium gas (100 cm$^3$, 4.5 mmol). After 30 min the vessel was evacuated and t.l.c. ($E_3$) showed that all the starting material had been converted to a compound with a similar $R_F$ value to $N(\alpha)$-t-butoxycarbonyl-L-histidine methyl ester. The catalyst was removed by filtration and the filtrate was evaporated to give an oil which was then triturated with ether and dried under vacuum to remove dimethylformamide. An n.m.r. of the product indicated that both imidazole ring protons had exchanged with deuterium giving $N(\alpha)$-t-butoxycarbonyl-L-2,5-$^2$H$_2$-histidine
N\(_{3}\)-t-Butoxycarbonyl-L-2,5-diiiodohistidine Methyl Ester.

5% Rhodium on calcium carbonate (40 mg) and 5% palladium on carbon (40 mg) was added to a gently agitated solution of N\(_{3}\)-t-butoxycarbonyl-L-histidine methyl ester (38.4 mg, 0.15 mmol) dissolved in dimethylformamide (2 ml) and the mixture was allowed to react with deuterium gas in a manner analogous to that described in the previous experiment. The oil produced after work-up had had a similar \( R_f \) to that of the starting material but n.m.r. showed that the proton at the 2 position on the imidazole ring had largely been exchanged for deuterium \( \delta \) (CDCl\(_3\)) 1.4(9H, s, (CH\(_3\))\(_3\)C\(-\)), 2.9(9H, d, J=11Hz, (CH\(_3\))\(_2\)NCOH), 3.05(2H, d, J=6Hz, -CHCH\(-\)), 3.65(3H, s, -OCH\(_3\)), 4.5(1H, m, -CHNH\(-\)), 5.85(1H, d, J=10Hz, -NHCH\(-\)), 6.8(1H, s, H-4 of imidazole), 8.0(1.5H, s, (CH\(_3\))\(_2\)NCOH).

N\(_{3}\)-t-Butoxycarbonyl-L-2,5-diiodohistidyl-L-prolineamide.

1-Hydroxybenzotriazole (67.6 mg, 0.5 mmol) and dicyclohexylcarbodiimide (124 mg, 0.6 mmol) was added to a solution of N\(_{3}\)-t-butoxycarbonyl-L-2,5-diiodohistidine (252 mg, 0.5 mmol) and L-prolineamide hydrochloride (67.2 mg, 0.5 mmol) dissolved in dimethylformamide (2 ml) stirred at 0\(^\circ\)C which had its pH adjusted to 9 by the addition of triethylamine. The reaction mixture was stirred at 0\(^\circ\)C for 2 h before being allowed to warm at room temperature, and then stirred for a further 16 h. After removal of dicyclohexylurea by filtration and evaporation of the solvent a yellow oil was obtained which was dissolved in chloroform (25 ml) and cooled to 0\(^\circ\)C for 1 h after which a second crop of dicyclohexylurea was removed by filtration. The solution was washed with saturated sodium bicarbonate solution (2 x 25 ml). The aqueous washings were extracted with chloroform (4 x 25 ml).
and the organic extracts combined and evaporated to give a pale yellow oil. Precipitation from ethyl acetate-ether gave \( \text{N}(\omega)-\text{t-butoxycarbonyl-L-2,5-diiodohistidyl-L-prolineamide} \) as a pale yellow solid (184 mg, 62%) of m.p. 160-165°; \([\alpha]_{D}^{20} -13.3^\circ \) (c 1.0 in MeOH); \( R_{F}(P) \) 0.58; \( \gamma (\text{CDCl}_3) 1.3(9H, s, (\text{CH}_3)_3 C-) \), 1.8-2.2(4H, complex, -CHCH=CH- of Pro), 3.0-3.1 (2H, complex, -CHCH- of His), 3.3-3.7(2H, complex, -NHCH=CH- of Pro), 4.4(1H, m, -NHCH- of Pro), 4.5(1H, complex, -NHCH- of His), 5.3(1H, d, -NHCH- of His), 5.9(1H, s, -NH₂), 6.4(1H, s, -NH₂) (Found: C, 31.21; H, 3.39; N, 11.12. \( \text{C}_{16}\text{H}_{21}\text{N}_0\text{I}_2\text{H}_2\text{O} \) requires C, 31.01; H, 3.7; N, 11.31%) \( M^+ \text{(F.D}) \) found; 603, \( \text{C}_{16}\text{H}_{21}\text{N}_0\text{I}_2 \) requires 603.

L-Pyroglutamyl-L-2,5-diiodohistidyl-L-prolineamide.

The preceding dipeptide (96 mg, 0.16 mmol) was dissolved in trifluoroacetic acid (5 ml) at room temperature. After 0.5 h the trifluoroacetic acid was evaporated and the residue was triturated with ether. The crude dipeptide trifluoroacetate was dissolved in dimethylformamide (2 ml) at 0°. L-Pyroglutamic acid 2,4,5-trichlorophenyl ester (52.4 mg, 0.17 mmol) was added and the pH was adjusted to 8 by the addition of triethylamine (70 \( \mu \)l, 0.5 mmol). The mixture was left at room temperature for 16 h after which the solvent was evaporated and the residue was triturated with ether. The resulting solid was dissolved in 25% aqueous acetic acid (2 ml) and applied to a column of Sephadex G15 (2.5 x 100 cm), which was swollen and eluted with 25% aqueous acetic acid. The appropriate Pauly-positive fractions were pooled and reapplied to a G15 column and eluted as above. Those fractions which contained a single Pauly-positive component were pooled and concentrated to give, after evaporation and reprecipitation from methanol-ether, L-pyroglutamyl-L-2,5-diiodohistidyl-L-prolineamide as a hygroscopic white solid, (63.4 mg, 65%) of \( R_{F}(\text{CMWA}) \) 0.87; \([\alpha]_{D}^{20} -20.5^\circ \) (c 1.0 in \( 1M \) AcOH), \([\alpha]_{D}^{20} -7.1^\circ \) (c 0.25 in MeOH);
\( \text{(CD}_3\text{OD}) \ 1.2-2.5(8\text{H}, \text{complex } -\text{NCH}_2\text{CH}_2- \text{ of Pro and } -\text{COCH}_2\text{CH}_2- \text{ of Glp}), \)

3.0-3.1(2\text{H}, \text{m}, -\text{CHCH}_2- \text{ of His}), 3.6-3.8(2\text{H}, \text{complex}, -\text{NCH}_2\text{CH}_2- \text{ of Pro}),

4.16(1\text{H}, \text{complex}, -\text{NDCH-} \text{ of Glp}), 4.25(1\text{H}, \text{m}, -\text{NCH-} \text{ of Pro}), 4.45(1\text{H}, \text{m},

-\text{NDCH-} \text{ of His}); M^+ (F.D) found: 615, C_{16}H_{20}N_{2}O_{1}I_{2} requires 614. The sample

proved to be too hygroscopic for a good elemental analysis. (N:C ratio

found 0.367, C_{16}H_{20}N_{2}O_{1}I_{2}.1.5 \text{ AcOH requires 0.368}).

L-Pyroglutamyl-L-histidyl-L-prolineamide (Thyroliberin).

5% Rhodium on calcium carbonate (35 mg) and 5% palladium on charcoal

(35 mg) was added to a gently stirred solution of L-pyroglutamyl-L-2,5-diiodohistidyl-L-prolineamide (34 mg, 0.05 mmol) dissolved in

dimethylformamide (1 ml). Hydrogen was bubbled through the mixture for

6 h after which time t.l.c. (CMWA) indicated that all the starting material

had been consumed to give a product with a similar \( R_F \) to authentic

thyroliberin as prepared by Stachulski. The catalyst was removed by

filtration and the filtrate was evaporated to give a gum. After

dissolving in 25% aqueous acetic acid (1 ml) the sample was applied to a

column of Sephadex G10 (2.5 x 100 cm) which was swollen and eluted with

25% aqueous acetic acid. Pooling of the appropriate fractions and

evaporation gave, after lyophilization, L-pyroglutamyl-L-histidyl-L-

prolineamide contaminated with calcium iodide \( R_F \) (CMWA) 0.45; \( \delta \) (CD_3OD)

2.0-2.4(8\text{H}, \text{complex } -\text{CHCH}_2\text{CH}_2- \text{ of Pro and } -\text{COCH}_2\text{CH}_2- \text{ of Glp}), 3.05(2\text{H},

complex, -\text{CHCH}_2- \text{ of His}), 3.8(2\text{H}, \text{complex}, -\text{NHCH}_2\text{CH}_2- \text{ of Pro}), 3.9(1\text{H},

m, -\text{NDCH-} \text{ of Glp}), 4.4(1\text{H}, \text{m}, -\text{NCHCO-} \text{ of Pro}), 4.5(1\text{H}, \text{m}, -\text{NDCH of His}),

7.2(1\text{H}, \text{s}, H-4 of imidazole of His), 8.0(1\text{H}, \text{s}, H-2 of imidazole of His);

[\alpha]_D^{20} -61.3^\circ \ (c 0.48 \text{ (from quantitative amino acid analysis) in } 1\text{M AcOH}),

Lit^{154} 68^\circ \ (c 1.0 \text{ in } 1\text{M AcOH}); \text{ Amino-acid ratios: Glu, 1.00; His, 1.05;}

Pro, 0.99.
After hydrolysis the hydrolysate of the tripeptide was shown
to contain 95% L-histidine, by digestion with a L-amino-acid oxidase
using the method of Jones et al.¹⁰⁵
CHAPTER 4

Experimental Section

Bromination of the Imidazole Ring as a Method of Side-Chain Protection for Histidine in Peptide Synthesis
A solution of $\text{N}(-\text{t})$-$\text{t}$-\text{butoxycarbonyl}$-$\text{L}$-\text{2,5-dibromohistidine methyl ester}$ (8.0g, 28.6 mmol) in chloroform (100 ml) was stirred vigorously with triethylamine (12 ml, 86 mmol) at 0°. Bromine (4.0 ml, 76 mmol) dissolved in chloroform (50 ml) was added dropwise over a period of 2 h. The reaction mixture was then allowed to warm to room temperature and stirred for a further 16 h before t.l.c. ($E_3$) indicated that all the starting material had been consumed. The organic solution was washed with 1M sodium thiosulphate (3 x 50 ml), water (50 ml), dried and evaporated. A yellow foam was obtained which t.l.c. showed to contain a major product with several contaminants. The foam was dissolved in 5% methanol-chloroform (10 ml) and applied to a Merck Kieselgel 60 (3 x 14 cm) flash column. The column was eluted with 5% methanol-chloroform, the eluate was examined by t.l.c. ($E_6$) and those fractions containing the major product were pooled, concentrated and reapplied to the column. After being eluted with 5% methanol-chloroform once more those fractions which t.l.c. ($E_6$) showed to contain only chromatographically homogeneous material were pooled and concentrated. After repeated evaporation from ether $\text{N}(-\text{t})$-$\text{t}$-\text{butoxycarbonyl}$-$\text{L}$-\text{2,5-dibromohistidine methyl ester}$ was reprecipitated from ethyl acetate-ether as a pale yellow solid (3.09g, 25%) of m.p. 65-69°; $R_F(E_3)$ 0.75; $[\alpha]_D^{20}$ -6.7° ($c$ 1.0 in MeOH); $\delta$(CDCl$_3$) (9H, s, (CH$_3$)$_3$C-), 3.0(2H, d, -CHCH$_2$-), 3.7(3H, s, CH$_3$OCO-), 4.5(1H, m, -NHCH-), 5.5(2H, d, NHCH-); (Found: C, 34.57; H, 4.29; N, 9.36, C$_{12}$H$_{17}$N$_3$O$_4$Br$_2$. 0.25CH$_3$CO$_2$Et requires: C, 34.7; H, 4.23; N, 9.35%); $M^+$ found 425, 427, 429, C$_{12}$H$_{17}$N$_3$O$_4$Br$_2$ requires: 425, 427, 429.

$\text{N}(-\text{t})$-$\text{t}$-\text{butoxycarbonyl}$-$\text{L}$-\text{2,5-dibromohistidine}$.

1M Sodium hydroxide (15 ml) was added to a vigorously stirred solution of $\text{N}(-\text{t})$-$\text{t}$-\text{butoxycarbonyl}$-$\text{L}$-\text{2,5-dibromohistidine methyl ester}$.
(3.08g, 7.2 mmol) in methanol (4 ml). After 1 h t.l.c. \( (E_3) \) indicated that all the starting material had been consumed. Water (50 ml) was added and the methanol was evaporated and the aqueous phase was washed with chloroform (2 x 20 ml). The pH of the aqueous solution was adjusted to 5 by the addition of 1M aq. hydrochloric acid (15 ml) which was then extracted with chloroform (5 x 20 ml). The combined extracts were dried and evaporated to give, after repeated evaporation from ether, \( \text{N}(\omega)-t\)-butoxycarbonyl-\( \text{L}-2,5\)-dibromohistidine as a pale yellow foam (2.18g, 73%)

of m.p. 92-94°; \( R_f\) (P₂) 0.66; \([\omega]_{D}^{20} = -1.1^\circ \) (c 1.0 in MeOH); \( \delta\)\( (\text{CD}_{3}\text{OD})\) 1.4\( (9H, s, (\text{CH}_3)\text{C}-)\), 3.1\( (2H, d, -\text{CH}_2\text{CH}^-)\), 4.4\( (1H, m, -\text{CHNH}^-)\); (Found: C, 33.0; H, 3.94; N, 9.45; \( \text{C}_{11}\text{H}_{15}\text{N}_0\text{Br}_2\cdot 0.25\text{Et}_2\text{O} \) requires: C, 33.37; H, 4.05; N, 9.74%); \( M^+\) found 411, 413, 415. \( \text{C}_{11}\text{H}_{15}\text{N}_0\text{Br}_2 \) requires 411, 413, 415.

**Hydrogenolysis of \( \text{N}(\omega)-t\)-butoxycarbonyl-\( \text{L}-2,5\)-dibromohistidine Methyl Ester.**

5% Rhodium on carbon (20 mg), 5% palladium on carbon (20 mg) and a few beads of Amberlite IR 45 (acetate form) anion exchange resin were added to a gently stirred solution of \( \text{N}(\omega)-t\)-butoxycarbonyl-\( \text{L}-2,5\)-dibromohistidine methyl ester (20 mg, 0.05 mmol) in 80% aqueous acetic acid (3 ml). Hydrogen was bubbled through the reaction mixture for 35 min after which t.l.c. \( (E_3) \) comparison with authentic samples showed that all the \( \text{N}(\omega)-t\)-butoxycarbonyl-\( \text{L}-2,5\)-dibromohistidine methyl ester had been converted to \( \text{N}(\omega)-t\)-butoxycarbonyl-\( \text{L}-\)histidine methyl ester. The catalysts were removed by filtration to give the product, after evaporation, as a yellow oil whose n.m.r. spectrum was consistent with \( \text{N}(\omega)-t\)-butoxycarbonyl-\( \text{L}-\)histidine methyl ester. A sample of the product was hydrolysed under standard conditions for 18 h after which amino-acid analysis showed the hydrolysate to contain only a product having an exactly similar retention time and elution profile to histidine.
N(α)-t-Butoxycarbonyl-L-2,5-dibromohistidyl-L-prolineamide.

1-Hydroxybenzotriazole (183 mg, 1.2 mmol) and dicyclohexylcarbodiimide (247 mg, 1.2 mmol) were added to a solution of N(α)-t-butoxycarbonyl-L-2,5-dibromohistidine (413 mg, 1.0 mmol) and prolineamide hydrochloride (151 mg, 1.0 mmol) in dimethylformamide (2 ml) at 0° after which the pH was adjusted to 9 by the addition of triethylamine (150 μl, 1.1 mmol). The solution was stirred at 0° for 2 h before being allowed to warm to room temperature, and then stirred for a further 16 h. After removal of dicyclohexylurea by filtration and evaporation of the solvent, a yellow oil was obtained which was dissolved in ethyl acetate (5 ml) and cooled to 0° for 1 h after which a second crop of dicyclohexylurea was removed by filtration. The solution was washed with saturated sodium bicarbonate solution (2 x 10 ml) and then extracted with 5% citric acid (4 x 5 ml). The combined aqueous extracts were covered with an equal volume of ethyl acetate and the pH was adjusted to 8.5 by the portionwise addition of sodium bicarbonate. After a further extraction of the aqueous phase with ethyl acetate (3 x 20 ml) the combined organic extracts were dried and evaporated to give a pale yellow oil. Repeated evaporation from ether gave N(α)-t-butoxycarbonyl-L-2,5-dibromohistidyl-L-prolineamide as a white hygroscopic foam (358 mg, 70%) of m.p. 98-105°; Rp(P2) 0.75; [α]20

D +4.9° (c 1.0 in MeOH);

δ(CDCl₃) 1.4(9H, s, (CH₃)₃C-), 1.9-2.2(4H, complex, -CHCH₂CH₂- of Pro), 3.0-3.1(2H, complex, -CH₂CH₃ of His), 3.3-3.7(2H, complex, -NHCH=CH of Pro), 4.6(1H, m, -NHCHCO- of His), 5.4(1H, d, -NHCH of His), 6.0(1H, s, -NH₂), 6.85(1H, s, -NH₂); M⁺ (F.D) found 507, 509, 511. C₁₆H₂₃N₅O₄Br₂

requires 507, 509, 511; (N:C ratio found: 0.35, C₁₆H₂₃N₅O₄Br₂ requires: 0.36). The optical purity of the dipeptide was determined with L-amino-acid oxidase. After deprotection and hydrolysis the hydrolysate of the dipeptide was found to contain less than 4% L-histidine, the result being
corrected for racemisation during hydrolysis of the peptide.

\[ \text{L-Pyroglutamyl-L-2,5-dibromohistidyl-L-prolineamide.} \]

The preceding dipeptide (96 mg, 0.16 mmol) was dissolved in trifluoroacetic acid (5 ml) at room temperature. After 0.5 h the trifluoroacetic acid was evaporated and the residue was triturated with ether. The crude dipeptide trifluoroacetate was dissolved in dimethylformamide (2 ml) at 0° and L-pyroglutamic acid 2,4,5-trichlorophenyl ester (52.4 mg, 0.17 mmol) was added and the pH adjusted to 8 by addition of triethylamine (90 |l, 0.65 mmol). The mixture was left at room temperature for 16 h after which the solvent was evaporated and the residue was triturated with ether. The resulting solid was dissolved in 25% aqueous acetic acid (2 ml) and applied to a Sephadex G15 column (2.5 x 100 cm). The column was swollen and eluted with 25% aqueous acetic acid; the eluate was monitored and the appropriate fractions were pooled and evaporated to give a solid. The solid was reapplied to the column and eluted once more as above to give, after pooling of the appropriate chromatographically homogeneous fractions, evaporation of the solvent and reprecipitation from methanol-ether, L-pyroglutamyl-L-2,5-dibromohistidyl-L-prolineamide as a hygroscopic white solid (161 mg, 79%) of m.p. 179-181°; \( R_f \) (CMWA) 0.86; \( [\omega]^2_{D} \) -29.4° (c 0.5 in 1M AcOH); \( \delta \) (CD\(_3\)OD) 1.9-2.1(4H, complex, \(-\text{COCH}_2\text{CH}_2\) of Glp), 2.2-2.5(4H, complex, \(-\text{CHCH}_2\text{CH}_2\) of Pro), 3.1-3.2(2H, m, \(-\text{CHCH}_2\) of His), 3.5-3.7(2H, complex, \(-\text{NHCH}_2\text{CH}_2\) of Pro), 3.9(1H, m, \(-\text{NHCH}_2\) of Glp), 4.3(1H, t, \(-\text{CHCH}_2\) of Pro), 4.5(1H, t, \(-\text{NHCH}_2\) of His); \( M^+ \) (F.D) 518, 520, 522.

C\(_{16}\)H\(_{20}\)N\(_{2}\)O\(_{4}\)Br\(_2\) requires 518, 520, 522. (N:C ratio found 0.369.

C\(_{16}\)H\(_{20}\)N\(_{2}\)O\(_{4}\)Br\(_2\)\cdot 1.5\ AcOH requires 0.368)

\[ \text{L-Pyroglutamyl-L-histidyl-L-prolineamide (Thyroliberin).} \]

5% Rhodium on carbon (20 mg), palladium on carbon (20 mg) and
a few beads of Amberlite IR 45 (acetate form) anion exchange resin were added to a solution of L-pyroglutamyl-L-2,5-dibromohistidyl-L-prolineamide (27 mg, 0.05 mmol) dissolved in 80% aqueous acetic acid (1 ml). Hydrogen was bubbled through the stirred solution for 6 h after which t.l.c. (CMAW) indicated that all the protected tripeptide had been converted into a material with a similar R$_p$ to authentic thyroliberin.

The catalyst was removed by filtration and the filtrate was evaporated to give an oily residue. After dissolving in 25% aqueous acetic acid (1 ml) the sample was applied to a Sephadex G10 column (2.5 x 100 cm) which was eluted with 25% aqueous acetic acid. The eluate was monitored by ultra violet absorption at 280 nm and optical rotation at 436 nm. Pooling and evaporation of the appropriate chromatographically homogeneous fractions gave L-pyroglutamyl-L-histidyl-L-prolineamide as a white solid (14 mg, 76%) which was indistinguishable from authentic material; amino-acid analysis ratios: Glu, 1.04; His, 1.00; Pro, 1.07 (Found: C, 40.48; H, 6.80; N, 12.28; C$_{16}$H$_{22}$N$_6$O$_4$·4AcOH. 6H$_2$O requires: C, 40.56; H, 7.04; 11.82%; R$_p$(CMAW) 0.30; $\delta$(CD$_3$OD) 1.9-2.0 (4H, complex, -COCH$_2$CH$_2$ of Glp), 2.2 (12H, s, CH$_3$CO$_2$H), 2.3-2.6 (4H, complex, -CHCH$_2$CH$_2$ of Pro), 3.1 (2H, m, -CHCH$_2$ of His), 3.9 (1H, m, -NHCH$_2$ of Glp), 4.2 (1H, m, -CHCH$_2$ - of Pro), 4.5 (1H, m, -NHCH$_2$ - of His), 7.0 (1H, s, H-4 in imidazole of His), 7.72 (1H, s, H-2 in imidazole of His); $\left[\alpha\right]^{20}_D$ -69° (c 0.11 in 1M AcOH (corrected for water present in C, H, N analysis)) Lit$^{154}$ $\left[\alpha\right]^{20}_D$ -68° (c ca. 1 in 1M AcOH). Glycyl-L-histidyl-L-phenylalanine.

N-t-Butoxycarbonyl-L-phenylalanine was coupled to Merrifield 1% crosslinked polystyrene resin in dimethylformamide at 60° to give a resin that, after washing was shown to contain 0.82 mmol/g of protected amino-acid by nitrogen analysis. The resin was placed in a glass vessel
and agitated by passing a stream of nitrogen gas upwards through a glass sinter. Peptide synthesis was carried out manually using the following sequence of operations for each coupling (all solvent volumes 5 ml);

a) dichloromethane, 3 x 2 min; b) isopropanol, 3 x 2 min;
c) dichloromethane, 3 x 2 min; d) trifluoroacetic acid in dichloromethane (40% v/v), 1 x 1 min; e) trifluoroacetic acid in dichloromethane (40% v/v), 1 x 30 min; f) dichloromethane, 3 x 2 min; g) isopropanol, 3 x 2 min;
h) dichloromethane, 3 x 2 min; i) triethylamine in dichloromethane (10% v/v), 2 x 2 min; j) dichloromethane, 5 x 2 min; k) coupling with 4 equivalents of the t-butoxycarbonyl protected amino-acid and 4 equivalents of dicyclohexylcarbodiimide in dichloromethane (5 ml) or dichloromethane-dimethylformamide (50% v/v) in the case of the protected diiodohistidine.

N-t-Butoxycarbonyl-L-phenylalanyl resin (0.32 g, 0.26 mmol) was added to the synthesis vessel and the cycle commenced at operation a). In the first cycle N\((\omega)\)-t-butoxycarbonyl-L-2,5-diiodohistidine (0.43 g, 1.04 mmol) was used with dicyclohexylcarbodiimide (0.25 g, 1.04 mmol). In the second cycle N-t-butoxycarbonyl-glycine (0.18 g, 1.0 mmol) was used with dicyclohexylcarbodiimide (0.25 g, 1.04 mmol). At the end of the second cycle the resin was washed with trifluoroacetic acid (2 x 5 ml) and suspended in trifluoroacetic acid (10 ml). A stream of hydrogen bromide gas was passed slowly though the suspension for 45 min and then the filtrate was collected and evaporated to dryness. The residue was triturated with ether, to leave a brown solid (130 mg). 5% Rhodium on carbon (70 mg), 5% palladium on carbon (70 mg) and a few beads of Amberlite IR 45 (acetate form) anion exchange resin were added to a solution of the crude tripeptide in 80% aqueous acetic acid (10 ml) and hydrogen was bubbled through the mixture for 16 h. The catalyst mixture was filtered off, washed with 80% aqueous acetic acid (40 ml)
and the filtrate was evaporated to give a yellow oil. A solution of the oil in 25% acetic acid (2 ml) was applied to a Sephadex G10 gel filtration column and eluted with 25% acetic acid. The eluate was monitored by ultra-violet absorption 280 nm, and the fractions that were Pauly-positive were pooled and the solvent evaporated. The brown oil produced was dissolved in 25% acetic acid and reapplied to the G10 column, being eluted as above. Those fractions containing chromatographically homogeneous Pauly-positive material were pooled and concentrated to give, after reprecipitation from methanol-ether, glycyl-L-histidyl-L-phenylalanine as a white solid identical to authentic samples prepared by Brown\(^\text{166}\) (52 mg, 56%) of m.p. 182-185°; \(R_f\) (CMAN) 0.25; \([\alpha]_D^{20}\) -2.3° (\(c\) 1.87 in 1M AcOH); \(\delta (\text{CD}_{3} \text{OD})\) 1.9(3H, s, \(\text{CH}_{3} \text{CO}_{2} \text{H}\)), 2.8-3.0(4H, complex, \(-\text{CHCH}_{2}\) of His and \(-\text{CHCH}_{2}\) of Phe), 3.5(2H, s, \(-\text{NDCH}_{2}\) of Gly), 4.5(1H, t, \(-\text{CHCH}_{2}\) of His) 7.0(1H, s, H-4 of imidazole), 7.05-7.2(5H, complex, \(\text{C}_{6} \text{H}_{5} \text{CH}_{2}\) of Phe), 8.3(1H, s, H-2 of imidazole); \(M^+\) (F.D) found: 560 \(C_{17} \text{H}_{21} \text{N}_{2} \text{O}\) requires 359. Amino-acid analysis ratios: Gly, 1.00; His, 1.05; Phe 0.97. Digestion of the hydrolysate with \(L\)-amino-acid oxidase showed the presence of 7% \(D\)-histidine (after correction for racemisation occurring during hydrolysis).
CHAPTER 5

Experimental Section

Optimisation of the Preparation of N(ω)-t-Butoxycarbonyl,N(N)-Benzyloxymethyl-L-histidine
N(α),N(γ)-Bis-t-butoxycarbonyl-L-histidine Methyl Ester.

This compound can be obtained as a side product in the preparation of N(α)-t-butoxycarbonyl-L-histidine methyl ester and has been described by Handford et al. However, we devised the following procedure which gave the title compound as the sole product. Triethylamine (30 ml, 0.21 mol) was added to a stirred suspension of L-histidine methyl ester dihydrochloride (25 g, 0.105 mol) in methanol (75 ml). After 10 min di-(t-butyl)-dicarbonate (45.6 g, 0.21 mol) was added to the suspension which was stirred at room temperature for 16 h. After evaporation of the solvent the residue was suspended in 10% citric acid (250 ml) and extracted with ether (4 x 250 ml). The combined organic extract was dried and evaporated to give after trituration with light petroleum (40-60) N(α),N(γ)-bis-t-butoxycarbonyl-L-histidine methyl ester as a white solid (37.1 g, 96%) of m.p. 92°; [α]_{D}^{20} +3.2° (c 1.1 in MeOH); δ(DCl_{3}) 1.4(9H, s, (CH_{3})_{3}COCONH-), 1.6(9H, s, (CH_{3})_{3}COCO-im), 3.0(2H, d, -CHCH-), 3.7(3H, s, CH_{3}O-), 4.5(1H, m, -CHCH-), 5.6(1H, d, -NHCH-), 7.1(1H, s, H-4 of imidazole), 7.9(1H, s, H-2 of imidazole); (Found: C, 55.14; H, 7.24; N, 11.26 C_{17}H_{27}O_{3}N_{2} requires: C, 55.28; H, 7.32; N, 11.38%).

N(α)-t-Butoxycarbonyl,N(γ)-benzylxoxymethyl-L-histidine Methyl Ester.

This was prepared according to the general method of Brown and Jones by adding benzyl-chloromethyl ether (3.9 ml, 28 mmol) to a solution of N(α),N(γ)-bis-t-butoxycarbonyl-L-histidine methyl ester (9.7 g, 26.2 mmol) in dichloromethane (100 ml). After stirring for 16 h at room temperature the solution was evaporated and dissolved in methanol (25 ml) to which was added triethylamine (4.0 ml), 29 mmol). The solution was set aside for 30 min after which the solvent was evaporated and the oily residue dissolved in chloroform (100 ml). The organic solution was washed with water (2 x 50 ml) and brine (50 ml) before being dried and
evaporated to give a clear oily residue. Crystallisation from ethyl acetate-light petroleum (40-60) gave the title compound as a white microcrystalline solid (3.4 g, 33%) of m.p. 105° \( ^\circ \) (CDCl\textsubscript{3}) 1.45(9H, s, (CH\textsubscript{3})\textsubscript{3}C\textsuperscript{2}C\textsuperscript{2}-), 3.0-3.2(2H, complex, \(-CHCH\textsubscript{2}C\textsuperscript{2}-\)), 3.7(3H, s, CH\textsubscript{3}O\textsuperscript{2}-), 4.4 (1H, m, \(-CHCH\textsubscript{2}C\textsuperscript{2}-\)), 4.65(2H, s, \(-OCH\textsubscript{2}N\textsuperscript{2}-\)), 5.3(2H, s, PhCH\textsubscript{2}O\textsuperscript{2}-), 5.7(1H, d, \(-NH\textsubscript{2}C\textsuperscript{2}-\)), 6.85(1H, s, H-4 of imidazole), 6.1-6.5(5H, complex, C\textsubscript{6}H\textsubscript{5}CH\textsubscript{2}-), 7.9(1H, s, H-2 of imidazole).

T.l.c. (E\textsubscript{3}) indicated that the liquors contained a large amount of unrecovered product, hence the solvent was evaporated and the residue dissolved in 5% methanol-chloroform and applied to a Merck Kieselgel 60 (3 x 14 cm) 'flash' chromatography column. The column was eluted under a slight pressure of nitrogen gas with 5% methanol-chloroform and those fractions containing only the product were identified by t.l.c. (E\textsubscript{3}) and concentrated. After trituration with ether a white solid was obtained (2.6 g, 25%) which t.l.c. (E\textsubscript{3}) and n.m.r. spectroscopy showed to be a further crop of N(\textsuperscript{o})-t-butoxycarbonyl,N(\textsuperscript{N})-benzyloxymethyl-L-histidine methyl ester.

Investigation of the Long Term Stability of Benzyl-chloromethyl Ether.

Commercial benzyl-chloromethyl ether (Fluka) was examined by n.m.r. spectroscopy on purchase and found to be acceptably pure, however over a period of time doubts as to its continued purity grew. N.m.r. spectroscopy showed up to 40% impurity to be present in the liquid. Distillation under reduced pressure b.p.\( ^\circ \)\textsubscript{60} gave the title liquid as a pure liquid which was stored at 0° and examined by n.m.r. before use in all subsequent experiments.

Systematic Investigation into the Preparation of N(\textsuperscript{o})-t-Butoxycarbonyl, N(\textsuperscript{N})-benzyloxymethyl-L-histidine Methyl Ester.
In order to determine the optimum conditions for the preparation of the protected histidine derivative a number of parallel experiments were conducted using different quantities of benzyl-chloromethyl ether, different solvents, as well as with and without the addition of a base. Nine experiments were conducted in all and in each case \(N(\alpha)N(\gamma)-\text{bis-t-butoxycarbonyl-L-histidine methyl ester} (0.2 \text{ g}, 0.54 \text{ mmol})\) was dissolved in the appropriate solvent (2 ml) to which was added benzyl-chloromethyl ether (82 \(\mu\text{l}, 1.09 \text{ equiv.}, 120 \mu\text{l}, 1.59 \text{ equiv.}, 157 \mu\text{l}, 2.09 \text{ equiv.}) and in a number of cases triethylamine (82 \(\mu\text{l}, 0.59 \text{ mmol}) was added. The reaction mixtures were examined by t.l.c. (\(E_3\)) for up to three days and compared with authentic samples of \(N(\alpha)N(\gamma)-\text{bis-t-butoxycarbonyl-L-histidine methyl ester} and N(\alpha)-\text{t-butoxycarbonyl,N(\gamma)-benzyloxymethyl-L-histidine methyl ester}, the relative intensities of the spots produced on t.l.c., after developing with starch/KI/Cl\(_2\) are set out in Table 7. These results clearly indicated that optimum conditions appeared to be 1.5 equivalents of benzyl-chloromethy ether in a non-polar solvent with no base added. In all other cases either large amounts of starting material remained unreacted or if additional benzyl-chloromethyl ether was added then the major product consisted of the quaternary salt formed by the addition of two benzyloxyethyl groups to the imidazole of histidine and running on the base line on a t.l.c. eluted with \(E_3\).

\(N(\alpha)-\text{t-Butoxycarbonyl,N(\gamma)-benzyloxymethyl-L-histidine Methyl Ester Hydrochloride.}\)

A solution of \(N(\alpha),N(\gamma)-\text{bis-t-butoxycarbonyl-L-histidine methyl ester} (32 \text{ g}, 0.082 \text{ mol})\) and benzyl-chloromethyl ether (18 ml, 0.13 mol) in dichloromethane (200 ml) was allowed to stand at room temperature overnight. The solvent was evaporated under high vacuum to remove all traces of unreacted benzyl-chloromethyl ether giving an oil which was
<table>
<thead>
<tr>
<th>Solvent: Dichloromethane</th>
<th>1.09 Equivalents of benzyl-chloromethyl ether</th>
<th>1.59 Equivalents of benzyl-chloromethyl ether</th>
<th>2.09 Equivalents of benzyl-chloromethyl ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base: None</td>
<td>S.M.: Strong</td>
<td>S.M.: None</td>
<td>S.M.: None</td>
</tr>
<tr>
<td></td>
<td>Product: Strong</td>
<td>Product: Strong</td>
<td>Product: Medium</td>
</tr>
<tr>
<td>Base: Triethylamine</td>
<td>Product: Weak</td>
<td>Product: Medium</td>
<td>Product: Medium</td>
</tr>
<tr>
<td>Base: Triethylamine</td>
<td>Product: None</td>
<td>Product: None</td>
<td>Product: None</td>
</tr>
</tbody>
</table>

S.M. = \(N(\alpha),N(\tau)\)-Bis-t-butoxycarbonyl-L-histidine methyl ester, Product = \(N(\alpha)\)-t-Butoxycarbonyl,\(\text{N}(\text{H})\)-benzylxoxymethyl-L-histidine methyl ester, Quat. Salts = Material having a low \(R_F\) value in \(E_3\).

All designations are made on t.l.c. \((E_3)\) plates developed with \(\text{Cl}_2\)/Starch/KI.

Table 7
dissolved in methanol (30 ml). Ether (400 ml) was added and the solution which was slightly turbid was set aside at room temperature for 2 h, and then at 0° overnight, after which \( N(\alpha)\)-t-butoxycarbonyl,N(\alpha) \-benzyloxy methyl-L-histidine methyl ether hydrochloride was isolated by filtration as a white crystalline solid (24 g, 69%) of m.p. 152°; \([\alpha]_D^{20}\) -19.1°(c 1.0 in MeOH): \( \delta(\text{CDCl}_3) \) 1.4(9H, s, \((\text{CH}_3)_3\text{C}-\)), 3.0-3.3(2H, complex, \(-\text{CHCH}_2\)), 3.6(3H, s, \text{CH}_3\text{-O}-), 4.5(1H, m, \(-\text{CHCH}_2\)), 4.6(2H, s, \(-\text{OCH}_2\text{N}-\)). 5.4(1H, d, \(-\text{NHCH}_2\)), 5.75(2H, s \text{PhCH}_2\text{O}-), 7.1(1H, s, H-4 of imidazole), 7.2(5H, complex, \( \text{C}_6\text{H}_5\text{CH}_2\)), 9.7(1H, s, H-2 of imidazole); (Found: C,56.50; H,6.72; N,9.57 \( \text{C}_{20}\text{H}_{28}\text{N}_3\text{O}_5\text{Cl} \) requires: C,56.40; H,6.58; N,9.87%).

\( N(\alpha)\)-t-Butoxycarbonyl,N(\alpha) \-benzyloxy methyl-L-histidine.

1M Sodium hydroxide (110 ml) was added to a solution of \( N(\alpha)\)-t-butoxycarbonyl,N(\alpha) \-benzyloxy methyl-L-histidine methyl ester hydrochloride (22 g, 0.052 mol) in methanol (50 ml). After 15 min at room temperature the solution was diluted with water (1 l) and the pH was adjusted to 4.5 by the addition of 1M hydrochloric acid. The solution was extracted with chloroform (3 x 100 ml) and the combined extracts dried. The solvent was removed to give an oil which was redissolved in ethyl acetate (50 ml). Evaporation of the solvent gave \( N(\alpha)\)-t-butoxycarbonyl,N(\alpha) \-benzyloxy methyl-L-histidine as a crystalline solid (19 g, 87%) of m.p. 155°, lit. 155°166, \([\alpha]_D^{20}\) +6.9° (c 1.0 in MeOH) Lit. 166, 170 \([\alpha]_D^{20}\) +6.9°.
Experimental Section

Practical Peptide Applications of \( \text{N(\text{\&})-Benzylloxymethyl Histidine} \)
Section A: Solid Phase Synthesis


N-t-Butoxycarbonyl-L-phenylalanine was coupled to a Merrifield 1% crosslinked polystyrene resin in dimethylformamide at 60° by the method of Gisin\textsuperscript{160} to give a resin which after washing with dimethylformamide was shown to contain 0.8 mmol/g of protected amino-acid by nitrogen analysis. The resin was placed in a glass vessel and agitated by passing nitrogen gas upward through a glass sinter (Figure 14). Peptide synthesis was carried out using a manual procedure. One cycle of the synthesis consisting of the following operations (all solvent volumes 5 ml): (a) dichloromethane 3 x 2 min. (b) isopropanol 3 x 2 min. (c) dichloromethane 3 x 2 min. (d) trifluoroacetic acid in dichloromethane (40% v/v) 1 x 1 min. (e) trifluoroacetic acid in dichloromethane (40% v/v) 1 x 3 min. (f) dichloromethane 3 x 2 min. (g) isopropanol 3 x 2 min. (h) dichloromethane 3 x 2 min. Repeat (d),(e),(f),(g). and (h) then (i) triethylamine in dichloromethane 3 x 2 min. (j) dichloromethane 5 x 2 min. (k) coupling with 4 equivalents of t-butoxycarbonyl amino-acid and 4.4 equivalents of dicyclohexylcarbodiimide in dichloromethane (except in the case of nitroarginine where dimethylformamide was used) [5 ml] 1 x 4 h and 1 x 16 h. t-Butoxycarbonyl-L-phenylalanine resin (0.42 g) was added to the synthesis vessel and the cycle commenced at operation (a). The cycle was repeated seven times with each amino-acid being coupled for 4 h followed by a second coupling for 16 h with the exception of N(\(\alpha\))-t-butoxycarbonyl,N\(\beta\)-benzyloxymethyl-L-histidine which was coupled for 16 h. The quantities of reagents used in each cycle are listed below.
Cycle 1: - t-Butoxycarbonyl-L-proline (2 x 0.287 g, 2.66 mmol),
dicyclohexylcarbodiimide (2 x 0.3 g, 2.92 mmol).

Cycle 2: - N(ω)-t-Butoxycarbonyl,N(η)-benzyloxymethyl-L-histidine
(1 x 0.5 g, 1.33 mmol), dicyclohexylcarbodiimide (1 x 0.3 g,
1.46 mmol).

Cycle 3: - t-Butoxycarbonyl-L-isoleucine (2 x 0.32 g, 2.66 mmol),
dicyclohexylcarbodiimide (2 x 0.3 g, 2.92 mmol).

Cycle 4: - N-t-Butoxycarbonyl,0-2,6-dichlorobenzyl-L-tyrosine
(2 x 0.54 g, 2.67 mmol), dicyclohexylcarbodiimide
(2 x 0.3 g, 2.92 mmol).

Cycle 5: - t-Butoxycarbonyl-L-valine (2 x 0.14 g, 1.32 mmol),
dicyclohexylcarbodiimide (2 x 0.15 g, 1.46 mmol).

Cycle 6: - N(ω)-t-Butoxycarbonyl,0-nitro-L-arginine (2 x 0.21 g,
1.33 mmol), dicyclohexylcarbodiimide (2 x 0.15 g, 1.46 mmol).

Cycle 7: - t-Butoxycarbonyl-L-aspartic acid-4-benzyl ester (2 x 0.21 g,
1.32 mmol), dicyclohexylcarbodiimide (2 x 0.15 g, 1.46 mmol).

After each cycle (except the first) a sample of resin (2 mg) was
removed from the synthesis vessel and hydrolysed at 110° in a sealed
tube with a 6M hydrochloric acid in propionic acid solution for 24 h.
After evaporation of the solvent the sample was dissolved in 0.01M
hydrochloric acid and applied to the column of an amino-acid analyser
in the conventional manner. Results after each cycle are shown in
Table 8. It can be seen that at the pentapeptide stage the incorporation
of N-t-butoxycarbonyl,0-2,6-dichlorobenzyl-L-tyrosine appeared to be
very low. This was subsequently shown to be due to loss during the
hydrolysis and after the addition of 0.1% phenol to the hydrolysis
solution a repeat analysis showed 100% incorporation of the protected
tyrosine. However it was decided at that stage to split the resin into
<table>
<thead>
<tr>
<th>Cycle</th>
<th>Phe</th>
<th>Pro</th>
<th>His</th>
<th>Ile</th>
<th>Tyr</th>
<th>Val</th>
<th>Arg</th>
<th>Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 2</td>
<td>1.05</td>
<td>1.00</td>
<td>0.94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 3</td>
<td>0.97</td>
<td>1.00</td>
<td>0.99</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 4</td>
<td>1.07</td>
<td>1.04</td>
<td>0.95</td>
<td>1.04</td>
<td>0.34*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 5</td>
<td>1.03</td>
<td>1.01</td>
<td>1.02</td>
<td>1.01</td>
<td>0.98</td>
<td>1.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 6</td>
<td>0.96</td>
<td>0.99</td>
<td>1.14</td>
<td>0.98</td>
<td>1.05</td>
<td>1.09</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>Cycle 7</td>
<td>0.95</td>
<td>0.91</td>
<td>1.09</td>
<td>0.95</td>
<td>1.03</td>
<td>1.00</td>
<td>0.97</td>
<td>0.98</td>
</tr>
<tr>
<td>Cycle 4*</td>
<td>1.02</td>
<td>0.98</td>
<td>1.06</td>
<td>0.98</td>
<td>0.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 7*</td>
<td>1.00</td>
<td>0.99</td>
<td>1.00</td>
<td>0.99</td>
<td>1.00</td>
<td>1.01</td>
<td>1.01</td>
<td>1.06</td>
</tr>
</tbody>
</table>

*Amino-acid analysis carried out after cleavage from the resin and purification.
+ Result low due to omission of phenol in hydrolysis solution.

Table 8

two halves. One half was temporarily set aside and later subjected to coupling cycles 5 to 7 to give the octapeptide, whilst the remaining half was washed with trifluoroacetic acid (10 ml) before being suspended in trifluoroacetic acid (5 ml) in the presence of excess anisole. A stream of hydrogen bromide gas was passed slowly through the suspension for 1 h. before the resin was filtered off and the filtrate evaporated to give the crude pentapeptide hydrogen bromide salt as an oil. The crude product was dissolved in 25% aqueous acetic acid and passed several times through an Amberlite IR 45 (acetate-form) ion exchange resin to remove hydrogen bromide. After evaporation of the solvent a white solid was obtained on trituration with ether, which appeared to be homogeneous on t.l.c. (CMAW). However in order to remove traces of resin artifacts and anisole the peptide was
dissolved in 25% aqueous acetic acid and filtered through a Sephadex G25 gel column. Pauly active fractions were combined and evaporated to give a white solid. Reprecipitation from methanol-ether gave after drying in vacuo L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine as a white solid (88 mg, 30%) of \([\alpha]_{D}^{20} -2.81^\circ (c 0.25 \text{ in } 1\text{M }\text{AcOH (uncorrected)}); \delta(D_2O) 0.6(6H, \text{ complex, } CH_3CH_2CHCH_3 - \text{ of Ile}), 0.8-1.3(2H, \text{ complex, } -CH_2CH- \text{ of Ile}), 1.5-2.0(10H, \text{ complex, } CH_3CO_2H and -CHCH_2CH_2 - \text{ of Pro}), 2.9-3.0(6H, \text{ complex } -CHCH_2 - \text{ of His, } -CHCH_2 - \text{ of Phe and } -CHCH_2 - \text{ of Tyr}), 4.3-4.5(2H, \text{ complex, } -CH_2CH_2N- \text{ of Pro}), 4.0-4.4(4H, \text{ complex, } \alpha-\text{CH protons}), 6.5-6.9 (4H, AB-\text{q, } HOC_6H_4CH_2 - \text{ of tyr}), 7.0-7.2(6H, \text{ complex, } C_6H_5CH_2 - \text{ of Phe and } H-4 \text{ on imidazole of His}), 8.2(1H, s, H-2 on imidazole of His); M^+ \text{ Found } 676 C_{35}H_{45}N_7O_7 requires: 675; (Found: C, 49.77; H, 6.19; N, 10.33 C_{35}H_{45}N_7O_7 (2AcOH. 8H_2O requires: C, 49.84; H, 6.49; N, 10.43%); amino-acid analysis ratios: Tyr, 0.95; Ile, 0.98; His, 1.06; Pro, 0.98; Phe, 1.02.

The half of the resin which had been subjected to all seven coupling cycles was cleaved in trifluoroacetic acid with hydrogen bromide gas in an exactly analogous manner to the preceding pentapeptide. The crude octapeptide was then washed with ether, dissolved in 25% aqueous acetic acid and passed several times through an Amberlite IR 45 (acetate-form) anion exchange exchange resin to remove hydrogen bromide. Evaporation gave crude \(N(\omega)-\text{nitroarginine-5-isoleucine angiotensin II as a hygroscopic solid which was dissolved in } 80\% \text{ aqueous acetic acid (10 ml) in which was suspended } 10\% \text{ palladium on carbon catalyst (50 mg). The stirred suspension was hydrogenated for 24 h in order to deprotect the } N(\omega)-\text{nitroarginine, after this period t.l.c.}(A_3) \text{ showed one major component. The solvent was removed and the oily residue (180 mg) was dissolved in } 25\% \text{ aqueous acetic acid


and filtered through a Sephadex G25 gel column. The Pauly active fractions were combined and the filtration repeated a further two times. T.l.c. still showed trace impurities so the peptide was dissolved in 0.0185M trimethylammonium acetate buffered to pH 4.2 and applied to a Whatman CM 52 carboxymethyl cellulose cation exchange column (0.9 x 30 cm) and eluted with a linear pH and concentration gradient of 0.0185M pH 4.2 to 0.185M pH 5.2 trimethylammonium acetate. The major Pauly active component was collected and the solvent evaporated. The residue was repeatedly evaporated from water and dried in vacuo to give 5-Isoleucine Angiotensin II as a hygroscopic white solid (80 mg, 34%) indistinguishable by t.l.c.($A_1$) and n.m.r. spectroscopy from and authentic sample (Sigma Chemical Company).

$[\alpha]^2_0$ -69° (c 0.5 in 1M HCl (corrected by quantitative amino-acid analysis)), Lit -67° (Found: C, 51.27; H, 6.52; N, 14.11. C$_{50}$H$_{71}$N$_{13}$O$_{12}$ 3.5AcOH,2.75H$_2$O requires: C, 51.68; H, 6.99; N, 1375%); Amino-acid analysis ratios; Asp, 1.06; Arg, 1.01; Val, 1.01; Tyr, 1.00; Ile, 0.99; His, 1.00; Pro, 0.99; Phe, 1.06.

**Attempted Synthesis by the Solid Phase Method of L-Histidyl-L-histidyl-L-histidyl-glycyl-L-phenylalanine.**

The synthesis was attempted in the same apparatus that was used in the angiotensin II synthesis (Figure 14), with the same cycle of washing operations. t-Butoxycarbonyl-L-phenylalanine resin (0.5 g, 0.8 mmol/g of resin) was placed in the synthesis vessel and the cycle was commenced at operation (a). The cycle was repeated four times with each amino-acid being coupled for 4 h and then 16 h. The first coupling of each cycle was carried out with 4 equivalents of t-butoxycarbonyl protected amino-acid and 4.4 equivalents of dicyclohexylcarbodiimide, whilst the second coupling was done in the presence of only 2 equivalents.
of t-butoxycarbonyl protected amino-acid and 2.2 equivalents of dicyclohexylcarbodiimide. The quantities of reagents used in each cycle are listed below.

Cycle 1:- t-Butoxycarbonyl-glycine (0.28 g then 0.14 g, 2.4 mmol), dicyclohexylcarbodiimide (0.36 g then 0.18 g, 2.7 mmol).

Cycle 2:- N(\text{\textsuperscript{\textalpha}})-t-Butoxycarbonyl,N(\text{\textsuperscript{\textbeta}})-benzyloxymethyl-L-histidine (0.6 g then 0.3 g, 2.4 mmol), dicyclohexylcarbodiimide (0.36 g then 0.18 g, 2.7 mmol).

Cycle 3:- N(\text{\textsuperscript{\textalpha}})-t-Butoxycarbonyl,N(\text{\textsuperscript{\textbeta}})-benzyloxymethyl-L-histidine (0.6 g then 0.3 g, 2.4 mmol), dicyclohexylcarbodiimide (0.36 g then 0.18 g, 2.7 mmol).

Cycle 4:- N(\text{\textsuperscript{\textalpha}})-t-Butoxycarbonyl,N(\text{\textsuperscript{\textbeta}})-benzyloxymethyl-L-histidine (0.6 g then 0.3 g, 2.4 mmol), dicyclohexylcarbodiimide (0.36 g then 0.18 g, 2.7 mmol).

After each cycle (except the first) a sample of resin (2 mg) was removed from the synthesis vessel and hydrolysed at 110° in a sealed tube under vacuum with 6M hydrochloric acid in propionic acid (with 0.1% phenol added) for 24 h. After evaporation of the solvent the sample was dissolved in 0.01M hydrochloric acid and applied to the column of an amino-acid analyser in the conventional manner. Results after each cycle are shown in Table 9. It can be seen that after cycle 4 incorporation of the third histidyl residue was minimal, thus the coupling procedure was repeated (cycle 4') using the following reagents:

Cycle 4':- N(\text{\textsuperscript{\textalpha}})-t-Butoxycarnonyl,N(\text{\textsuperscript{\textbeta}})-benzyloxymethyl-L-histidine (0.6 g then 0.3 g, 2.4 mmol), dicyclohexylcarbodiimide (0.36 g then 0.18 g, 2.7 mmol).

However further coupling resulted in a lowering of the level
<table>
<thead>
<tr>
<th>Cycle</th>
<th>Phe</th>
<th>Gly</th>
<th>His</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>1.00</td>
<td>1.04</td>
<td>0.96</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>0.94</td>
<td>0.98</td>
<td>2.08</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>1.00</td>
<td>1.02</td>
<td>2.40</td>
</tr>
<tr>
<td>*Cycle 4'</td>
<td>0.98</td>
<td>1.02</td>
<td>2.10</td>
</tr>
</tbody>
</table>

* Cycle involved only operations (f) to (k)

Table 9

of incorporation of the third histidine residue. The peptide was cleaved from the resin with hydrogen bromide in trifluoroacetic acid and the resin removed by filtration as before. The filtrate was evaporated to give the product as an oily residue. The residue was dissolved in 25% aqueous acetic acid and passed several times through an Amberlite IR 45 (acetate-form) anion exchange resin to remove hydrogen bromide. After evaporation of the solvent a solid was obtained which t.l.c. (A<sub>3</sub>) showed to contain at least two major Pauly active components. The residue was dissolved in 25% aqueous acetic acid and filtered through a Sephadex G25 gel column. The fractions were monitored by t.l.c. and those containing mainly one Pauly active component were evaporated and hydrolysed in the normal manner before being applied to the column of an amino acid analyser. The amino-acid analysis for the fraction containing mainly material of R<sub>F</sub>(A<sub>3</sub>) 0.21 gave the ratios: His, 2.93; Gly, 1.02; Phe, 1.00, whilst material R<sub>F</sub>(A<sub>3</sub>) 0.29 had the ratios: His, 1.74; Gly, 1.00; Phe, 1.00. This indicates that whilst the glycine residue had been fully incorporated then the histidine had (at least in the final coupling) not been fully
incorporated and we had a mixture containing mainly $L_\text{H}-\text{histidyl-}L_\text{H}-\text{histidyl-}L_\text{H}-\text{histidyl-glycyl-L-phenylalanine}$ and $L_\text{H}-\text{histidyl-L-}\text{histidyl-glycyl-L-phenylalanine}$. 
Section B: Solution Phase Synthesis

L-Phenylalanine Benzyl Ester p-Toluenesulphonate.

This was prepared by the method of Zervas et al.\textsuperscript{180} on a 0.25 molar scale, by refluxing L-phenylalanine with benzyl alcohol and p-toluenesulphonic acid in benzene using a Stark and Dean trap to aziotropically remove the liberated water. Crystallisation from benzene-ether gave the title compound (70.1 g, 65\%) of m.p. 167-169° (Lit. 170-171°); [\(\alpha\)]\textsubscript{D}\textsuperscript{20} -7.0° (c 1.0 in MeOH) Lit -7.2°; \(\delta\)(CDCl\(_3\)) 2.3 (3H, s, CH Ph-), 3.0-3.2(2H, complex, -CHCH -), 4.6(1H, complex, -CHCH -), 4.9(2H, m, -OCH Ph), 7.0-8.4(17H, complex, aromatic protons).

t-Butoxycarbonyl-L-prolyl-L-phenylalanine Benzyl Ester.

1-Hydroxybenzotriazole (0.781 g, 5.1 mmol) and dicyclohexylcarbodiimide (1.05 g, 5.1 mmol) were added to a solution of t-butoxycarbonyl-L-proline (1.00 g, 4.65 mmol) and L-phenylalanine benzyl ester p-toluenesulphonate (1.99 g, 4.65 mmol) in dichloromethane (10 ml) at 0°, in which the pH had been adjusted to 9 by the addition of triethylamine (1.4 ml, 9 mmol). The solution was stirred at 0° for 2 h, before being allowed to warm to room temperature, and then stirred for a further 16 h. After removal of precipitated dichyclohexylurea by filtration, the solution was set aside at 0° for 16 h, after which a further crop of dicyclohexylurea was filtered off. The filtrate was washed with saturated sodium hydrogen carbonate solution (2 x 20 ml), 10\% citric acid (2 x 20 ml) and brine (20 ml); after drying the solvent was removed to give t-butoxycarbonyl-L-prolyl-L-phenylalanine benzyl ester as a chromatographically homogeneous colourless oil (1.41 g, 67\%) of \(R_F\) (P\(_2\)) 0.84; \(\delta\)(CDCl\(_3\)) 1.4(9H, s, \((\text{CH}_3)_3\)C-), 1.6-2.2(4H, complex, -CH\(_2\)CH- of Pro), 3.0-3.4(4H, complex, -CH\(_2\)N- of Pro and -CHCH -
of Pro and -CHCH₂ - of Phe), 3.6(1H, m, -CHNH- of Pro), 4.2(1H, m, -CHNH- of Phe), 4.8(1H, m, -CHNH- of Pro), 5.1(2H, s, -OCH₂ Ph).

6.9-7.3(11H, complex, -NHCH- of Phe, C₆H₅- of Phe and C₆H₅- of Bzl).

N(α)-t-Butoxycarbonyl,N(γ)-benzyloxymethyl-L-histidyl-L-prolyl-L-phenylalanine Benzyl Ester.

The preceding dipeptide (1.06 g, 2.35 mmol) was dissolved in trifluoroacetic acid (5 ml) at room temperature. After 15 min the acid was evaporated and the residue was repeatedly triturated with ether. The crude dipeptide trifluoroacetate was dissolved in chloroform (5 ml) with N(α)-t-butoxycarbonyl,N(γ)-benzyloxymethyl-L-histidine (0.86 g, 2.3 mmol) and the pH was adjusted to 9 by the addition of triethylamine (1.5 ml, 11 mmol). 1-Hydroxybenzotriazole (0.43 g, 2.8 mmol) and dicyclohexylcarbodiimide (0.58 g, 2.8 mmol) was added to the mixture which was stirred at 0° for 2 h. After being allowed to warm to room temperature the solution was stirred for a further 16 h before dicyclohexylurea was removed by filtration. The filtrate was diluted with chloroform (40 ml) and washed with saturated sodium hydrogen carbonate solution (2 x 50 ml), 10% citric acid (2 x 50 ml) and brine (50 ml). The organic layer was dried and the solvent evaporated to give a white solid. Crystallisation from chloroform-ether gave N(α)-t-butoxycarbonyl,N(γ)-benzyloxymethyl-L-histidyl-L-prolyl-L-phenylalanine benzyl ester as homogeneous white crystals (1.1 g, 66%) of m.p. 111-112°; Rₑ(Pₑ₂) 0.56; [α]_D²⁰ -38° (c 1.0 in MeOH); δ(CDCl₃) 1.3(9H, s, (CH₃)₃C⁻), 1.7-2.0(4H, complex, -CHCH₂ of Pro), 2.8-3.0(2H, complex, -CH₂OH- of citrate), 3.0-3.3(6H, complex, -CHCH₂- of His, -CHCH₂ - of Phe and -NCH₂- of Pro), 4.4(1H, m, -CHCH₂- of Pro), 4.6-4.9(4H, complex, -CHCH₂- of His, -CHCH₂- of Phe and imCH₂O-), 5.1(2H, m, PhCH₂OCH₂-), 5.7(1H, d, -NHCH- of His),
5.8-5.9 (2H, m, -CO$_2$H$_2$Ph, 6.9 (1H, d, -NHCH- of Phe), 7.0-7.5 (16H, complex), H-4 on imidazole of His, C$_6$H$_5$CH$_2$O$\cdot$, -CO$_2$CH$_2$C$_6$H and C$_6$H$_2$- of Phe), 9.4 (1H, s, H-2 on imidazole of His); M$^+$ Found 710 C$_{40}$H$_{47}$N$_5$O$_7$ requires: 709; (Found: C, 61.60; H, 6.26; N, 8.81 C$_{40}$H$_{47}$N$_5$O$_7$.0.3 citrate.2.3H$_2$O requires: C, 61.84; H, 6.66; N, 8.59%).

Amino-acid analysis ratios: His, 1.03; Pro, 0.97; Phe, 1.03.

**N(\text{\textgamma})-t-Butoxycarbonyl,N(\text{\textmu})-benzyloxymethyl-L-histidyl-L-tryptophan Methyl Ester.**

1-Hydroxybenzotriazole (243 mg, 1.60 mmol) and dicyclohexylcarbodiimide (328 mg, 1.60 mmol) was added to a solution of N(\text{\textgamma})-t-butoxycarbonyl, N(\text{\textmu})-benzyloxymethyl-L-histidine (500 mg, 1.33 mmol) and tryptophan methyl ester (339 mg, 1.40 mmol) in dimethylformamide (2 ml) and chloroform (8 ml) stirred at 0°, which had previously had its pH adjusted to 9 by the addition of triethylamine. The reaction mixture was stirred at 0° for 2 h before being allowed to warm to room temperature and then stirred for a further 16 h. After removal of the dicyclohexylurea by filtration, evaporation of the solvent gave a yellow oil which was dissolved in chloroform (10 ml) and cooled to 0° for 1 h after which a second crop of dicyclohexylurea was removed by filtration. The filtrate was diluted by the addition of chloroform (10 ml) and washed with saturated sodium hydrogen carbonate solution (2 x 10 ml) before being extracted with 10% citric acid (4 x 20 ml). The aqueous extracts were combined and the pH adjusted to 8.5 by the portionwise addition of solid sodium hydrogen carbonate. The aqueous phase was extracted with chloroform (4 x 50 ml) and the combined organic extract was dried and evaporated to give after trituration with ether a white solid. Crystallisation from methanol-ether gave N(\text{\textgamma})-t-butoxycarbonyl,N(\text{\textmu})-benzyloxymethyl-L-histidyl-L-tryptophan methyl ester (476 mg, 61%).
of m.p. 90-92°; [α]_D^{20} +3.1° (c 1.0 in MeOH); δ(CDCl₃) 1.4(9H, s, (CH₃)₂-C-), 2.2(2H, br, H₂O), 3.0(2H, complex, -CHCH₂- of His), 3.2(2H, complex, -CHCH₂- of Trp), 3.8(3H, s, -CO₂CH₃), 4.4(2H, s, -NCH₂O-), 4.45(1H, complex, -CHCH₂- of His), 4.9(1H, m, -CHCH₂- of Trp), 5.15(2H, s, PhCH₂O-), 5.2(1H, d, -NHCH- of His), 6.8(1H, d, -NHCH- of Trp), 6.9-7.5(12H, complex, indole of Trp, imidazole of His and C₆H₃CH₂O-), 8.5(1H, s, indole NH of Trp); M⁺ Found 576 C₃₁H₃₇O₅N₅ requires: 575; (Found: C, 63.97; H, 6.33; N, 12.03 C₃₁H₃₇O₅N₅-0.5H₂O requires: C, 63.69; H, 6.51; N, 11.98%).

N(α)-t-Butoxycarbonyl-L-histidyl-L-tryptophan Methyl Ester.

N(α)-t-Butoxycarbonyl,N(β)-benzyloxymethyl-L-histidyl-L-tryptophan methyl ester (100 mg, 0.17 mmol) was dissolved in 80% aqueous acetic acid (5 ml) to which was added 5% palladium on carbon (30 mg). The suspension was hydrogenated for 2 h after which t.l.c. (P₂) indicated that all the starting material had been consumed. The catalyst was removed by filtration and the filtrate evaporated to give after trituration with ether N(α)-t-butoxycarbonyl-L-histidyl-L-tryptophan methyl ester as a white solid (58 mg, 73%) of m.p. 164-165°; [α]_D^{20} -3.7° (c 1.0 in MeOH); Rₚ(P₂) 0.36; δ(CDCl₃) 1.3(9H, s, (CH₃)₂-C-), 2.0(6H, s, CH₃CO₂H), 2.95(2H, complex, -CHCH₂- of His), 3.1(2H, complex, -CHCH₂- of Trp), 3.8(3H, s, -CO₂CH₃), 4.4(1H, complex, -CHCH₂- of His), 4.85(1H, complex, -CHCH₂- of Trp), 5.5(1H, complex, -NHCH- of His), 6.8(1H, complex, -NHCH- of Trp), 6.9-7.5(7H, complex, imidazole of His and indole of Trp), 9.5(1H, s, indole NH of Trp); M⁺ Found 455, 456 C₂₃H₂₉O₅N₅ requires: 455; (Found: C, 56.11; H, 6.95; H, 6.95; N, 11.94 C₂₃H₂₉O₅N₅-2AcOH requires: C, 56.34; H, 6.59; N, 12.17%).
Attempted Preparation of \(N(\omega)\)-t-Butoxycarbonyl-\(L\)-tryptophoyl-\(N(\eta)\)-benzyloxymethyl-\(L\)-histidyl-\(L\)-tryptophan Methyl Ester.

\(N(\omega)\)-t-Butoxycarbonyl,\(N(\eta)\)-benzyloxymethyl-\(L\)-histidyl-\(L\)-tryptophan methyl ester (74 mg, 0.13 mmol) was dissolved in trifluoroacetic acid (5 ml) at room temperature. After 15 min the acid was evaporated and the residue repeatedly triturated with ether. The crude dipeptide trifluoroacetate salt was dissolved in dimethylformamide (2 ml) without further purification. \(N(\omega)\)-t-Butoxycarbonyl-\(L\)-tryptophan (42 mg, 0.14 mmol) was added to the solution and the pH was adjusted to 9 by the addition of triethylamine (110 \(\mu\)l, 0.8 mmol). 1-Hydroxybenzotriazole (22 mg, 0.14 mmol) and dicyclohexylcarbodiimide (29 mg, 0.14 mmol) was added to the solution which was stirred at 0\(^\circ\) for 2 h. The solution was then allowed to warm to room temperature and stirred for a further 16 h before dicyclohexylurea was removed by filtration. The filtrate was evaporated and the residue dissolved in chloroform (20 ml), washed with saturated sodium hydrogen carbonate solution (2 x 20 ml) and extracted with 10% citric acid (4 x 20 ml). The pH of the combined aqueous extract was adjusted to 8.5 by the addition of solid sodium hydrogen carbonate. The aqueous phase was extracted with chloroform (4 x 50 ml) and the combined organic extract was dried and evaporated to give after trituration with ether the crude tripeptide as a white solid (57 mg, 58%). The product was dissolved in 25% aqueous acetic acid and filtered through a Sephadex G15 gel column. The eluate was monitored by ultraviolet absorption and the appropriate fractions were pooled and evaporated to give after trituration with ether a white solid which t.l.c.\(\left(P_2\right)\) showed to contain a major product with a trace impurity. 300MHz N.m.r. spectroscopy and F.D. mass spectrosopy \(M^+\) 761, \((M+H)^+\) 762, \((M+56)^+\) 817, \((M+57)^+\) 818 were consistent with the product containing a mixture
of \( N(\omega) - \text{t-butoxycarbonyl-L-tryptophyl-N(\eta)} - \text{benzyloxymethyl-L-histidy-L-tryptophan methyl ester} \) and \( N(\omega) - \text{t-butoxycarbonyl-L-tryptophyl-N(\eta)} - \text{benzyloxymethyl-L-histidy-N(i)-t-butyl-L-tryptophan methyl ester} \).
CHAPTER 7

Experimental Section

Racemisation of Histidine Derivatives
Coupling of $N(\alpha)$-t-Butoxycarbonyl,$N$(im)-benzyl-$L$-histidine with $L$-
prolineamide Under Conditions Designed to Exaggerate Racemisation.

$N(\alpha)$-t-Butoxycarbonyl,$N$(im)-benzyl-$L$-histidine (172 mg, 0.50 mmol) was added to a solution of dicyclohexylcarbodiimide (113 mg, 0.55 mmol) in dimethylformamide (4 ml) and stirred at 0°. After 120 min a pre-cooled solution of $L$-prolineamide hydrochloride (75 mg, 0.50 mmol) and triethylamine (70 µl, 0.50 mmol) in dimethylformamide (2 ml) was added. The solution was stirred for a further 2 h before being allowed to warm to room temperature and stirred for 16 h. After filtration to remove the precipitated dicyclohexylurea the filtrate was evaporated and the oily residue dissolved in chloroform (25 ml), washed with saturated sodium hydrogen carbonate solution (2 x 30 ml) and brine (20 ml). The organic phase was dried and the solvent removed to give the dipeptide as a white solid chromatographically homogeneous ($P_2$), (154 mg, 86%). The product was dissolved without further purification in 80% aqueous acetic acid (5 ml), in which was suspended a 5% palladium on carbon catalyst (150 mg). The sample was hydrogenated and after 72 h t.l.c. ($P_2$) indicated that all the starting material had been consumed. The catalyst was removed by filtration and the filtrate evaporated to give crude $N(\alpha)$-t-butoxycarbonyl-$L$-histidyl-$L$-prolineamide as a white solid (132 mg). The optical purity of the dipeptide thus isolated was determined with $L$-amino-acid oxidase by the method of Jones et al. (Table 10).

Coupling of $N(\alpha)$-t-Butoxycarbonyl-$L$-2,5-diiodohistidine with $L$-
prolineamide Under Conditions Designed to Exaggerate Racemisation.

$N(\alpha)$-t-Butoxycarbonyl-$L$-2,5-diiodohistidine (50 mg, 0.10 mmol) was added to a solution of dicyclohexylcarbodiimide (24 mg, 0.11 mmol) in dimethylformamide (1 ml) and stirred at 0°. After 120 min a pre-
cooled solution of \( L \)-prolineamide hydrochloride (16 mg, 0.10 mmol) and triethylamine (14 \( \mu l \), 0.10 mmol) was added. The solution was stirred at 0° for a further 2 h before being allowed to warm to room temperature and stirred for 16 h. After removal of dicyclohexylurea by filtration and evaporation of the solvent, the yellow oily residue was dissolved in chloroform (5 ml) and cooled to 0° for 1 h after which a second crop of dicyclohexylurea was removed by filtration. After washing with 10% sodium hydrogen carbonate solution (2 x 5 ml) and 10% citric acid solution (2 x 5 ml) the organic phase was dried and the solvent removed to give the dipeptide as a white solid homogeneous on t.l.c. (\( P_2 \)). A portion of the dipeptide (3 mg, 5 \( \mu \)mol) was added to a suspension of 5% palladium on carbon (4 mg) and 5% rhodium on calcium carbonate (3 mg) in dimethylformamide (1 ml) and hydrogenated for 2 h. After removal of the catalyst by filtration and evaporation of the solvent crude \( N(\omega) \)-t-butoxycarbonyl-\( L \)-histidyl-\( L \)-prolineamide was obtained as a yellow solid. The optical purity of the dipeptide thus isolated was determined with \( L \)-amino-acid oxidase by the method of Jones et al. (Table 10).

**Coupling of \( N(\omega) \)-t-Butoxycarbonyl-\( L \)-2,5-dibromohistidine with \( L \)-prolineamide Under Conditions Designed to Exaggerate Racemisation.**

\( N(\omega) \)-t-Butoxycarbonyl-\( L \)-2,5-dibromohistidine (41.3 mg, 0.10 mmol) was added to a solution of dicyclohexylcarbodiimide (24 mg, 0.11 mmol) in dimethylformamide (1 ml) and stirred at 0°. After 120 min a pre-cooled solution of \( L \)-prolineamide hydrochloride (15 mg, 0.1 mmol) and triethylamine (14 \( \mu l \), 0.10 mmol) was added. The solution was stirred at 0° for 2 h before being allowed to warm to room temperature and stirred for a further 16 h. Dicyclohexylurea was removed by filtration and the filtrate was evaporated to give an oily residue which was
dissolved in chloroform (5ml) and set aside at 0° for 1 h. After removing a further crop of dicyclohexylurea by filtration the filtrate was washed with saturated sodium hydrogen carbonate solution (2 x 5 ml) and 10% citric acid (2 x 5 ml). The organic phase was dried and the solvent evaporated to give the dipeptide as an oil homogeneous on t.l.c. (P₂) (29 mg, 57%). A portion of the product (3 mg, 5 μmol) was dissolved in 80% aqueous acetic acid in which was suspended 5% rhodium on carbon (5 mg) and 5% palladium on carbon (5 mg) along with a few beads of Amberlite IR 45 (acetate-form) anion exchange resin. The stirred suspension was hydrogenated for 2h after which the catalyst was removed by filtration and the filtrate was evaporated to give the crude N(α)-t-butoxycarbonyl-L-histidyl-L-prolineamide as an oil. The optical purity of the dipeptide thus isolated was determined with L-amino-acid oxidase by the method of Jones et al.¹⁰⁵ (Table 10).

<table>
<thead>
<tr>
<th>Histidine Derivative</th>
<th>% D-histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>N(α)-Benzyloxy carbonyl,N(γ)-phenacyl-L-histidine</td>
<td>49</td>
</tr>
<tr>
<td>N(α)-t-Butoxycarbonyl,N(γ)-benzyl-L-histidine</td>
<td>45</td>
</tr>
<tr>
<td>N(α)-t-Butoxycarbonyl-L-2,5-diodohistidine</td>
<td>20</td>
</tr>
<tr>
<td>N(α)-t-Butoxycarbonyl-L-2,5-dibromohistidine</td>
<td>15</td>
</tr>
</tbody>
</table>

*All results refer to the amount of D-histidine in the hydrolysate of the hydrolysed dipeptide after correction for racemisation during acid hydrolysis.

Table 10

Investigation of the Change in Optical Rotation of Histidine Derivatives upon Activation with Diisopropylcarbodiimide.

In each case a solution of the histidine derivative (0.1 mmol)
in dimethylformamide was made up and its optical rotation was measured over a period of 5 min in a polarimeter cell surrounded by a thermostated water jacket at 4°. The solution was removed from the cell and diisopropylcarbodiimide (20 μl, 0.11 mmol) was added. After mixing the solution was immediately returned to the polarimeter cell and its rotation was recorded automatically at 64 s intervals for the next 2 h whilst the temperature of the jacket was maintained at 4°. The percentage transmission through the cell was monitored and the cell windows were frequently wiped as condensation on the glass caused a drop in transmission and random variations in the rotation. The results as presented in Table 11 are averaged over five readings in order to smooth out these random variations. However a number of other problems were encountered. It was found in the case of the phenacyl derivatives that upon incubation with diisopropylcarbodiimide the solutions became coloured and the percentage transmission progressively dropped throughout the experiment. Also after each run the solutions were examined by t.l.c. (P$_2$) and in each case traces of a second spot thought to be due to the N-acylurea were observed above the main product. As the intensity of this spot varied from derivative to derivative and the optical properties of the N-acylureas were not known then it is only possible to draw very empirical conclusions from Table 11.

Investigation of the Products Produced by the Activation of N(α)-t-
Benzylloxycarbonyl,N(η)-phenacyl-L-histidine with N,N'-
Diisopropylcarbodiimide.

On standing with diisopropylcarbodiimide (20 μl, 0.12 mmol)
N(α)-benzyloxymethyl,N(η)-phenacyl-L-histidine (50 mg, 0.12 mmol) rapidly discoloured to such an extent that light transmission through the polarimeter cell at any of the available wavelengths was too low to
<table>
<thead>
<tr>
<th>Time</th>
<th>BocHisOH</th>
<th>BocHisOH</th>
<th>BocHisOH</th>
<th>BocHisOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_2$</td>
<td>$\eta$-Pac</td>
<td>$\tau$-Bzl</td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>-0.187</td>
<td>+0.086</td>
<td>+0.024</td>
<td></td>
</tr>
<tr>
<td>1-5</td>
<td>-0.184</td>
<td>+0.116</td>
<td>+0.025</td>
<td></td>
</tr>
<tr>
<td>6-10</td>
<td>-0.186</td>
<td>+0.104</td>
<td>+0.024</td>
<td></td>
</tr>
<tr>
<td>11-15</td>
<td>-0.186</td>
<td>+0.087</td>
<td>+0.023</td>
<td></td>
</tr>
<tr>
<td>16-20</td>
<td>-0.185</td>
<td>+0.070</td>
<td>+0.021</td>
<td></td>
</tr>
<tr>
<td>21-25</td>
<td>-0.183</td>
<td>+0.066</td>
<td>+0.019</td>
<td></td>
</tr>
<tr>
<td>26-30</td>
<td>-0.183</td>
<td>+0.065</td>
<td>+0.017</td>
<td></td>
</tr>
<tr>
<td>31-35</td>
<td>-0.182</td>
<td>+0.063</td>
<td>+0.016</td>
<td></td>
</tr>
<tr>
<td>36-40</td>
<td>-0.182</td>
<td>+0.062</td>
<td>+0.015</td>
<td></td>
</tr>
<tr>
<td>41-45</td>
<td>-0.182</td>
<td>+0.062</td>
<td>+0.014</td>
<td></td>
</tr>
<tr>
<td>46-50</td>
<td>-0.181</td>
<td>+0.062</td>
<td>+0.013</td>
<td></td>
</tr>
<tr>
<td>51-55</td>
<td>-0.180</td>
<td>+0.062</td>
<td>+0.012</td>
<td></td>
</tr>
<tr>
<td>56-60</td>
<td>-0.179</td>
<td>+0.061</td>
<td>+0.010</td>
<td></td>
</tr>
<tr>
<td>61-65</td>
<td>-0.178</td>
<td>+0.061</td>
<td>+0.009</td>
<td></td>
</tr>
<tr>
<td>66-70</td>
<td>-0.177</td>
<td>+0.060</td>
<td>+0.008</td>
<td></td>
</tr>
<tr>
<td>71-75</td>
<td>-0.176</td>
<td>+0.058</td>
<td>+0.007</td>
<td></td>
</tr>
<tr>
<td>76-80</td>
<td>-0.175</td>
<td>+0.058</td>
<td>+0.005</td>
<td></td>
</tr>
<tr>
<td>81-85</td>
<td>-0.173</td>
<td>+0.057</td>
<td>+0.005</td>
<td></td>
</tr>
<tr>
<td>86-90</td>
<td>-0.171</td>
<td>+0.057</td>
<td>+0.004</td>
<td></td>
</tr>
<tr>
<td>91-95</td>
<td>-0.170</td>
<td>+0.055</td>
<td>+0.004</td>
<td></td>
</tr>
<tr>
<td>96-100</td>
<td>-0.172</td>
<td>+0.055</td>
<td>+0.004</td>
<td></td>
</tr>
<tr>
<td>101-105</td>
<td>-0.173</td>
<td>+0.055</td>
<td>+0.004</td>
<td></td>
</tr>
<tr>
<td>106-110</td>
<td>-0.172</td>
<td>+0.054</td>
<td>+0.003</td>
<td></td>
</tr>
<tr>
<td>111-115</td>
<td>-0.172</td>
<td>+0.053</td>
<td>+0.003</td>
<td></td>
</tr>
<tr>
<td>116-120</td>
<td>-0.171</td>
<td>+0.052</td>
<td>+0.003</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>-8.6%</td>
<td>-61.5%</td>
<td>-39.5%</td>
<td>-87.5%</td>
</tr>
</tbody>
</table>

Table 11
<table>
<thead>
<tr>
<th>Time</th>
<th>BocHisOH</th>
<th>BocHisOH</th>
<th>BocHisOH</th>
<th>BocHisOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>π-p-BrBom</td>
<td>γ-p-BrBom</td>
<td>π-Bom</td>
<td>γ-Bom</td>
</tr>
<tr>
<td>Start</td>
<td>-0.849</td>
<td>+0.185</td>
<td>-0.417</td>
<td>+0.018</td>
</tr>
<tr>
<td>1-5</td>
<td>-0.844</td>
<td>+0.178</td>
<td>-0.404</td>
<td>+0.017</td>
</tr>
<tr>
<td>6-10</td>
<td>-0.885</td>
<td>+0.166</td>
<td>-0.372</td>
<td>+0.016</td>
</tr>
<tr>
<td>11-15</td>
<td>-0.875</td>
<td>+0.151</td>
<td>-0.361</td>
<td>+0.015</td>
</tr>
<tr>
<td>16-20</td>
<td>-0.872</td>
<td>+0.141</td>
<td>-0.360</td>
<td>+0.014</td>
</tr>
<tr>
<td>21-25</td>
<td>-0.873</td>
<td>+0.125</td>
<td>-0.360</td>
<td>+0.011</td>
</tr>
<tr>
<td>26-30</td>
<td>-0.897</td>
<td>+0.110</td>
<td>-0.345</td>
<td>+0.008</td>
</tr>
<tr>
<td>31-35</td>
<td>-0.900</td>
<td>+0.097</td>
<td>-0.350</td>
<td>+0.006</td>
</tr>
<tr>
<td>36-40</td>
<td>-0.898</td>
<td>+0.087</td>
<td>-0.345</td>
<td>+0.004</td>
</tr>
<tr>
<td>41-45</td>
<td>-0.890</td>
<td>+0.083</td>
<td>-0.341</td>
<td>+0.003</td>
</tr>
<tr>
<td>46-50</td>
<td>-0.890</td>
<td>+0.081</td>
<td>-0.330</td>
<td>+0.003</td>
</tr>
<tr>
<td>51-55</td>
<td>-0.905</td>
<td>+0.077</td>
<td>-0.343</td>
<td>+0.003</td>
</tr>
<tr>
<td>56-60</td>
<td>-0.903</td>
<td>+0.074</td>
<td>-0.341</td>
<td></td>
</tr>
<tr>
<td>61-65</td>
<td>-0.898</td>
<td>+0.072</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66-70</td>
<td>-0.903</td>
<td>+0.070</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71-75</td>
<td>-0.902</td>
<td>+0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76-80</td>
<td>-0.900</td>
<td>+0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>81-85</td>
<td>-0.900</td>
<td>+0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>86-90</td>
<td>-0.895</td>
<td>+0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>91-95</td>
<td>-0.891</td>
<td>+0.068</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96-100</td>
<td>-0.895</td>
<td>+0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>101-105</td>
<td>-0.894</td>
<td>+0.068</td>
<td></td>
<td></td>
</tr>
<tr>
<td>106-110</td>
<td>-0.888</td>
<td>+0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>111-115</td>
<td>-0.882</td>
<td>+0.071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>116-120</td>
<td>-0.876</td>
<td>+0.071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>+3.2%</td>
<td>-61.6%</td>
<td>-18.2%(1h)</td>
<td>-83.4%(1h)</td>
</tr>
</tbody>
</table>

Table 11 (Cont'd)
obtain meaningful results. After 2 h t.l.c. ($R_f(P_2)$) indicated the formation of only one Pauly active impurity having $R_f(P_2)$ 0.67 compared to $R_f(P_2)$ 0.45 for the initial histidine derivative. The reaction mixture was monitored by t.l.c. for 2 days over which time most of the starting material was converted to the impurity, no further Pauly active impurities were observed. The solvent was evaporated and the residue partitioned between 10% citric acid (5 ml) and ethyl acetate (5 ml). T.l.c. indicated that the Pauly active material was in the aqueous phase. The pH of the aqueous phase was adjusted to 8.5 by the portionwise addition of solid sodium hydrogen carbonate in the presence of an equal volume of ethyl acetate. The organic phase was washed with brine (5 ml) dried and the solvent evaporated to give N(ω)-benzyloxycarbonyl, N(η)-phenacyl-L-histidine, N-acyldiisopropylurea as a yellow solid (20 mg, 31%) of $\delta$(CDCl$_3$) 1.1-1.2(12H, m, (CH$_3$)$_2$CH—), 2.9-3.0(2H, complex, -CHCH$_2$—), 3.85(1H, br, (CH$_3$)$_2$CHN—), 4.05(1H, br, (CH$_3$)$_2$CHNCO—), 4.5(1H, m, -CHCH$_2$—), 5.0(2H, q, PhCH$_2$OCO—), 5.45 (2H, m, PhCOCH$_2$—), 5.6(1H, d, -CHNH—), 7.0(1H, d, H-4 of imidazole), 7.3-7.5(10H, complex, C$_6$H$_5$—), 7.6(1H, complex, H-2 of imidazole), 8.0(1H, complex, -NHCH(CH$_3$)$_2$); Mass spectra (F.D.): $M^+$ Found 534, 448 C$_{29}$H$_{35}$N$_5$O$_5$ requires: 533.
References


4. 'Methoden der Organischen Chemie (Houben Weil)', Band XV/1, ed. E. Wünsch, Georg Thieme Verlag, Stuttgart, 1974.


44. E. Wünsch & A. Zwick, Chem. Ber., 1964, 97, 2497.
49. D.S. Kemp, Peptides (N. Y.), 1979, 1, 315
52. V. du Vigneaud & O.K. Behrens, J. Biol. Chem. 1937, 117, 27.
66. As Reference 31, p. 11.
68. As Reference 31, p. 20.
86. D.F. Elliott & D. Morris, Chima (Switz), 1960, 14, 373.


101. A. Hallett, personal communication.


108. T. Brown, personal communication.


130. R.B. Williams, ibid., 1942, 64, 1395.

131. N.L. Alliger & J.L. Coke, ibid., 1942, 64, 1395.


179. R.C. Sheppard, personal communication.


185. A. Hallett, personal communication.
