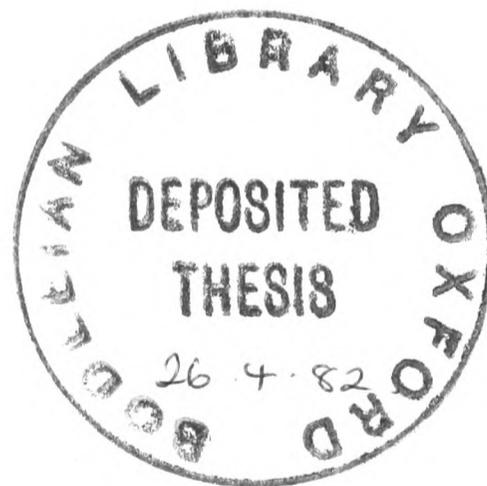THE DEVELOPMENT OF NOVEL METHODS FOR THE SYNTHESIS OF

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 DEVELOPMENT OF NOVEL METHODS FOR THE SYNTHESIS OF HISTIDINE-CONTAINING

## PEPTIDES.

### J.D. RICHARDS, St. JOHNS COLLEGE.

### SUBMITTED FOR THE DEGREE OF D.Phil., MICHAELMAS TERM, 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[1] establishing the importance of the location of the im-protecting group -N(π) being desirable are reviewed in the introduction.

In Chapter 1 the electrolytic deprotection of π-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 π-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- N(1,1,1-trichlorobut-2-enyl)imidazole-was prepared and subjected to screening tests. A number of undesirable side-reactions including 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 N(∝)-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-L-histidyl-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 N(π) was indispensable for its complete prohibition.

A new optimised preparation for N(∝)-t-butoxycarbonyl,N(π)-benzyloxymethyl-L-histidine[2] is described in Chapter 5, this being the first simple three-step synthesis of a N(π)-protected histidine derivative.

In Chapter 6 the use of N(∝)-t-butoxycarbonyl,N(π)-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

1. A.R. Fletcher, J.H. Jones, W.I. Ramage & A.V. Stachulski, J. Chem. Soc. Perkin I, 1979, 2216.

2. T. Brown & J.H. Jones, J. Chem. Soc. Chem. Comm., 1981, 648.

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 |
| $Bu^t$ | t-butyl |
| Ac | Acetyl |
| Glp | L-pyroglutamic acid (L-2-pyrrolidone-5-carboxylic a |
| 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

CONTENTS. (Cont'd)

CHAPTER 4   -   BROMINATION OF THE IMIDAZOLE RING AS A

METHOD OF SIDE-CHAIN PROTECTION FOR

HISTIDINE IN PEPTIDE SYNTHESIS

CHAPTER 5   -   OPTIMISATION OF THE PREPARATION OF N(ᴋ)-t-

BUTOXYCARBONYL,N(ᴨ)-BENZYLOXYMETHYL-L-

HISTIDINE

CONTENTS (Cont'd)

CONTENTS (Cont'd)

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.
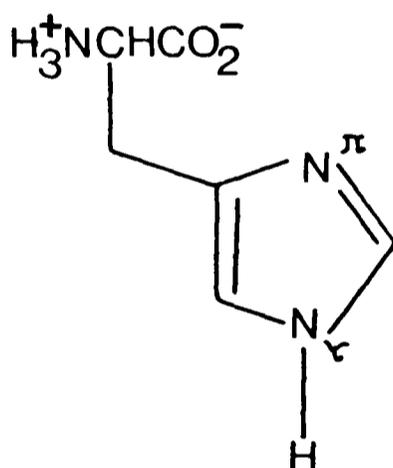


Figure 1

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[14]. Many peptide hormones, e.g.

corticotropin (ACTH)[15], angiotensin[16] and releasing factors, e.g.

thyroliberin (TRF)[17], luliberin (LH-RF)[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[19,20]. In many other proteins histidine

acts as a ligand to bind metal ions necessary for activity e.g.

$Zn^{2+}$ in carbonic anhydrase[21], and $Fe^{2+}$, in haemoglobin[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 incoporation of histidine into peptides.

## Unprotected Histidine.

N($\alpha$)-Acyl-histidines and N($\alpha$)-acyl-histidine peptides are

zwitterionic and, consequently their solubility in most organic solvents

is poor[23]. This has limited the use of standard derivatives such as

N($\alpha$)-benzyloxycarbonyl-L-histidine, with the result that incorporation of

histidine into peptides has tended to rely on the azide method[24] in which

the acid azide is readily obtainable from the more soluble N($\alpha$)-acyl-

L-histidine methyl ester via the hydrazide[25]. The presence of an unblocked

imidazole ring which is a weak base[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 $\underline{N}(\alpha)$-acylation and may even be favoured in some cases for example $\underline{N}(im)$-benzyloxycarbonyl-histidine, and not $\underline{N}(\alpha)$-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 $\underline{N}(\alpha)$-benzyloxycarbonylamino-acid derivatives), the Bamberger reaction[28] can occur, leading to an opening of the imidazole ring. $\underline{N}(im)$-acyl-histidines are often quite reactive in themselves, in fact $\underline{N}$-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 $\underline{N}(\tau)$- and $\underline{N}(\pi)$-monoalkylated species and the $\underline{N}(\tau,\pi)$-dialkylated material[32]. An unprotected imidazole ring can also react with $\underline{N},\underline{N}'$-dicyclohexylcarbodiimide[33] to form both isomeric $\underline{N}(im)$-dicyclohexyamidino-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).
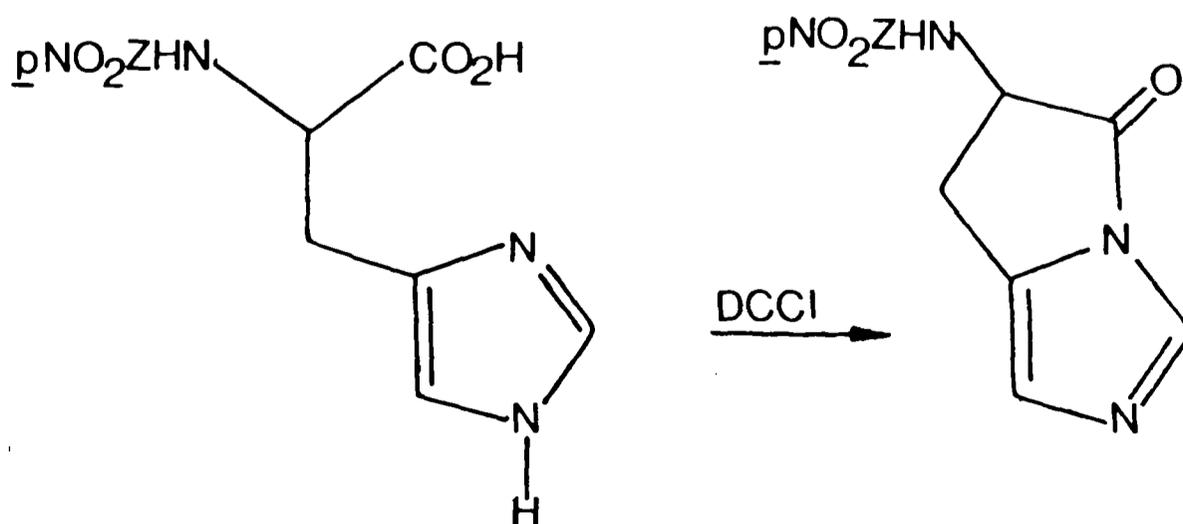


Scheme 1

Bodan<s>zky et al[37] 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[38-40]. This effect is enhanced by the presence of

tertiary bases and can be suppressed by the application of additives

such as 2,4-dinitrophenol[41]. Catalysis of racemisation by free imidazole

groups has been reported[42].

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[43]. When a two-fold excess of the activating

species (e.g. isobutylchloroformate) was used per equivalent of histidine

the excess mixed anhydride method gave a good yield of a product of high

optical purtiy. 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[44,45]. 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[46].

Verber[45] has studied the equilibrium that exists between histidine azides

and their corresponding cyclic imidaszolides. He has found that on

neutralisation of a solution of N(α)-t-butoxycarbonyl-L-histidine azide

hydrochloride, the imidazolide (detected by infra-red spectroscopy $\nu_{max}$

1780 cm$^{-1}$), was formed repidly and reversibly followed by a slower

racemisation of the chiral centre (scheme 2).

This observation of racemisation during the azide coupling of an
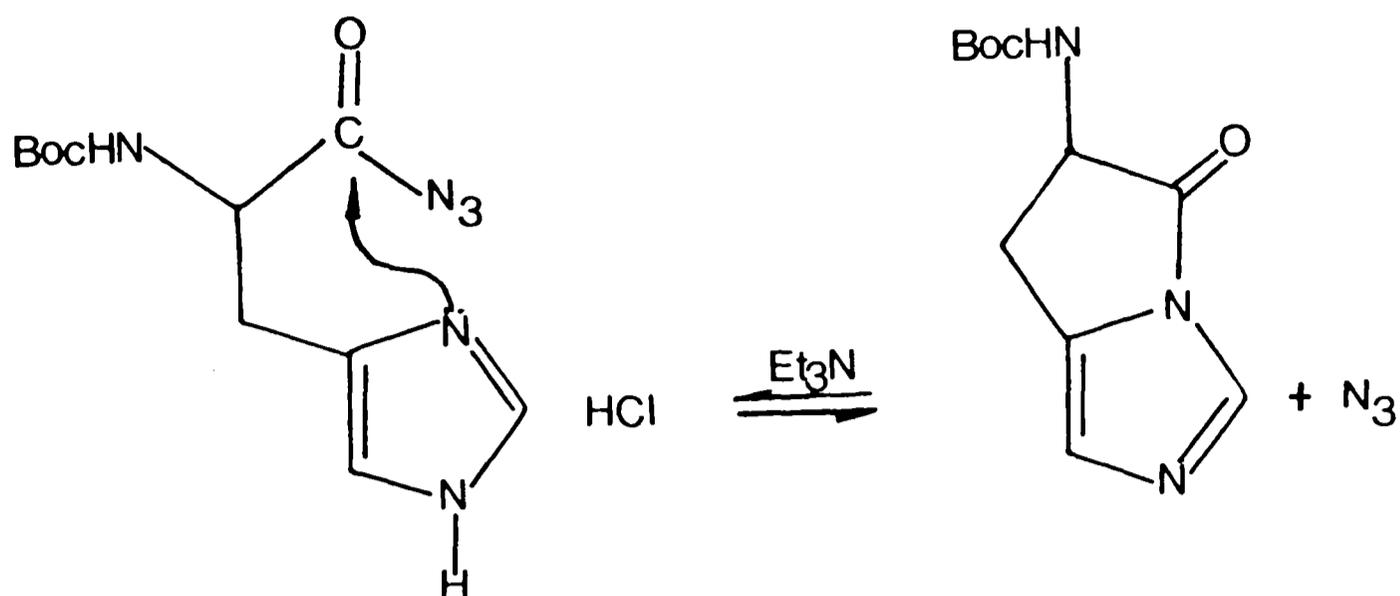
Scheme 2

$\underline{N}(\alpha)$-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[47], but the presence of an $\underline{N}(\alpha)$-alkoxycarbonyl group prevents cyclisation under normal conditions[48], and so amino acids protected in this way are not prone to racemisation. Kemp[49] 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[50]

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.
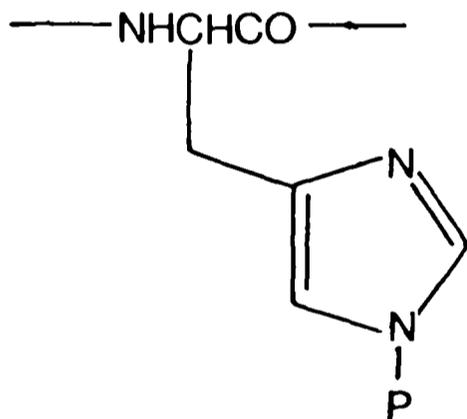


P=H

P=Alkyl: $-CH_2Ph$, $-CPh_3$

P=Aryl: -Dnp, Tnp

P=Acyl: -Z, -Boc, -Adoc,

piperidinecarbonyl etc.

P= -Tos

P= A derivative of a carbonyl

adduct: $-CH(CF_3)NHZ$,

$-CH(CF_3)NHBoc$, $-C(CF_3)_2OCHPh(p-Cl)$

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 $\tau$-position[51].

The distinction between $\underline{N}(\tau)$- and $\underline{N}(\pi)$-substitution may be important because Verber[45] has suggested that the position of protection might be crucial in determining the ease of racemisation of the protected hisidine 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) <u>Benzyl</u>: This was the first protecting group for the imidazole ring of histidine and was introduced by du Vigneaud and Behrens in 1937[52]. The benzyl group, however, does not reduce the basicity of the imidazole ring significantly and so derivatives of $\underline{N}$(im)-benzyl-histidine are still rather insoluble in organic solvents and purification by acid extraction is not possible. Active esters of $\underline{N}$(im)-benzyl-$\underline{L}$-histidine derivatives have been reported[53-55] 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[56] and in using $\underline{N}(\alpha)$-t-butoxycarbonyl,$\underline{N}$(im)-benzyl-$\underline{L}$-histidine both in solution[57] and solid phase[57-62] peptide synthesis, although in many cases the diastereoisometric material was removed in later purification steps[60-61]. The problem of racemisation can be greatly reduced in the presence of $\underline{N}$-hydroxysuccinimide or 1-hydroxybenzotriazole as additives[57,63,64]. 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[65,66]) but can be removed with sodium metal in liquid ammonia[67] or catalytic hydrogenation[68]. However, it has been observed

that sodium in liquid ammonia causes degradation of peptide bonds involving proline[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[59,63] for reduction of tyrosine and phenylalanine to take place.

b)  Triphenylmethyl (Trityl): N(im)-Triphenylmethyl histidine derivatives[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[69] and sodium in liquid ammonia[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)[70], but in mixed acids the group appears to be much more stable. N(im)-Triphenylmethyl-L-histidine can be recovered from N($\alpha$)-t-butoxycarbonyl,N(im)-triphenylmethyl-L-histidine after treatment with 1 M hydrogen chloride in acetic acid for 20h at room temperature[70]. The same authors also reported that the triphenylmethyl group was completely removed by 6M hydrogen bromide in acetic acid in 2h[23], however a histidine-containing sequential polypeptide[73] was successfully prepared using 6M hydrogen bromide in acetic acid for N($\alpha$)-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[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(∝)-t-butoxycarbonyl group is possible.

c) p-Toluenesulphony (tosyl): The use of the N(im)-tosyl group as protection for histidine was first proposed by Sakakibara and Fujii[74] 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[72], anhydrous hydrogen fluoride, aqueous sodium hydroxide, anhydrous hydrogen bromide or hydrogen chloride in acetic acid, 1-hydroxybenzotriazole[75] (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[43]. Thus although little racemisation has been observed in N(im)-tosyl-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(∝)-alkoxycarbonyl,N(im)-tosyl-L-histidine derivatives are themselves unstable and require storage as their dicyclohexylammonium salts[72].

d) 2,4-Dinitrophenyl: The observation by Shaltiel[76] that the N(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[77,78] and solid phase[79,80] peptide synthesis. N(im)-dinitrophenyl-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[77]. The dinitrophenyl group is stable to acidolysis (even

peptide hydrolysis conditions[81]) but is cleaved by bases and nucleophiles. The exact conditions are somewhat unclear-Shaltiel and Fridkin[77] observed that complete deprotection occurred in aqueous solution after 20 h at pH 12.2 and 22° whereas Losse and Krychowski[23] reported that no deprotection had occurred after 2 h in 2$\underline{M}$ sodium hydroxide and dioxane (1:1) at 20°. Hydrazine and mercaptoethanol also lead to complete cleavage. It has also been observed[77] that $\underline{N}$(im)-dinitrophenyl-$\underline{L}$-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 $\underline{N}$(im)-dinitrophenyl group to nucleophilic attack it is not surprising that yields in coupling reactions can be extremely low[43,75], 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 $\underline{N}$(α)-t-butoxycarbonyl,$\underline{N}$(im)-2,4-dinitrophenyl-$\underline{L}$-histidine as the carboxyl-component[43].

The related 2,4,6-trinitrophenyl substituent has been found to be even more unstable towards nucleophiles than the 2,4-dinitrophenyl analogue[23] and so is even less suitable as a protecting group for the imidazole side chain of histidine.

e)  Benzyloxycarbonyl: $\underline{N}$(α),$\underline{N}$(im)-Bisbenzyloxycarbonyl-$\underline{L}$-histidine has been prepard by treatment of histidine with benzylchloroformate in aqueous base[27,82,83]. This derivative has been used for mixed anhydride[43], active ester[84] and $\underline{N}$,$\underline{N}$'-dicyclohexylcarbodiimide mediated couplings without racemisation being observed[43] and the derivatives are readily soluble in the usual organic solvents. The fact that the

$\underline{N}(\alpha)$-benzyloxycarbonyl group may be removed by acidolysis (hydrogen bromide in acetic acid) in the presence of the $\underline{N}$(im)-benzyloxycarbonyl group[85,86] allows peptide elongation at the $\underline{N}$-terminus. However, in the presence of adjacent free carboxyl- or hydroxyl-groups, the $\underline{N}$(im)-benzyloxycarbonyl group is labile to hydrogen bromide deblocking. Catalytic hydrogenation[87] also effects deblocking but the most serious disadvantage of this protecting group is the facile removal of the $\underline{N}$(im)-benzyloxycarbonyl group by nucleophiles (amine, ammonia, sodium hydroxide, hydrazine[27,72,83]) 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 $\underline{N}$(im)-benzyloxycarbonyl group, the $\underline{N}$(im)-t-butoxycarbonyl group deactivates the imidazole ring sufficiently to allow couplings via active esters or mixed anhydrides. The derivatives[88,89] are soluble in organic solvents and no racemisation has been observed[43]. However, although an $\underline{N}(\alpha)$-t-butoxycarbonyl group can be removed by acidolysis in the presence of the $\underline{N}$(im)-t-butoxycarbonyl group[90] the latter group suffers from nucleophilic instability[91] and so is unsuitable for extended peptide synthesis, it is however widely used at present.

Similarly other acid labile $\underline{N}$(im)-alkoxycarbonyl substituents (e.g. 1-adamantyloxycarbonyl[92,93] isobutoxycarbonyl[43]) have been used in peptide synthesis for histidine side-chain protection. However, as for other alkoxycarbonyl derivatives all suffer from varying degrees of instability to nucleophilic attack[72].

g) Piperidinecarbonyl: The piperidinecarbonyl substituent as a protecting group for the imidazole side-chain of histidine (Figure 3) was introduced by Jager et al[94]. This substituent is stable to acid and hydrogenolysis and can therefore be used in combination with various
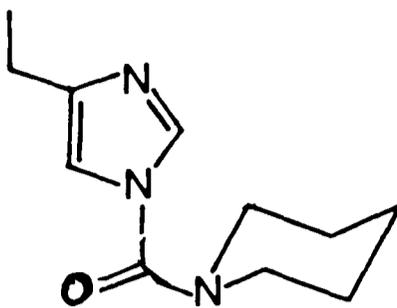
Figure 3

N(∝)-protecting groups. The derivatives, although not always crystalline, are soluble in organic solvents[23] and the imidazole ring is sufficiently deactived to allow the formation of active esters. Deblocking can be carried out with powerful nucleophilic reagents such as hydrazine or sodium hydroxide solution[94], 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)-piperidinecarbonyl-L-histidine have been reported but it is likely by analogy with the alkoxycarbonyl 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 piperidinecarbonyl group has been little used in peptide synthesis.

   h)   2,2,2-Trifluoro-1-acylaminoethyl: Weygand et al[50] 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-



R =Bzl
  = t-Bu
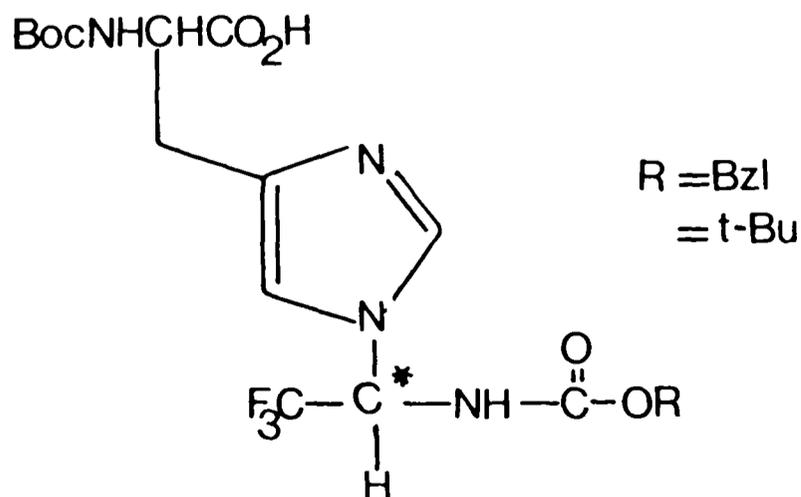
Figure 4

benzyloxycarbonylaminoethyl and $\underline{N}$(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[72], 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 $\underline{N}$($\alpha$)-t-butoxycarbonyl,

$\underline{N}$(im)-2,2,2-trifluoro-1-benzyloxycarbonyl-$\underline{L}$-histidine[79] - also some

racemisation has been reported in an excess mixed anhydride coupling where

the protected histidine derivative is the carboxyl component[43]. The lack

of crystallinity of these derivatives is also a problem leading to

difficulties in purification[95]. 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 $\underline{N}$($\tau$),$\underline{N}$($\pi$)-substitution

might have prevented crystallisation. In an attempt to improve crystallinity

by not introducing the asymmetric centre, Seltzman and Chapman[95,96]

synthesied derivatives of hexafluoroacetone. They have developed the

1,1,1,3,3,3-hexafluoro-2-($\underline{p}$-chlorophenoxymethoxy)-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.

ZNHCHCO₂H

$$\text{ZNHCHCO}_2\text{H}$$

(chemical structure)
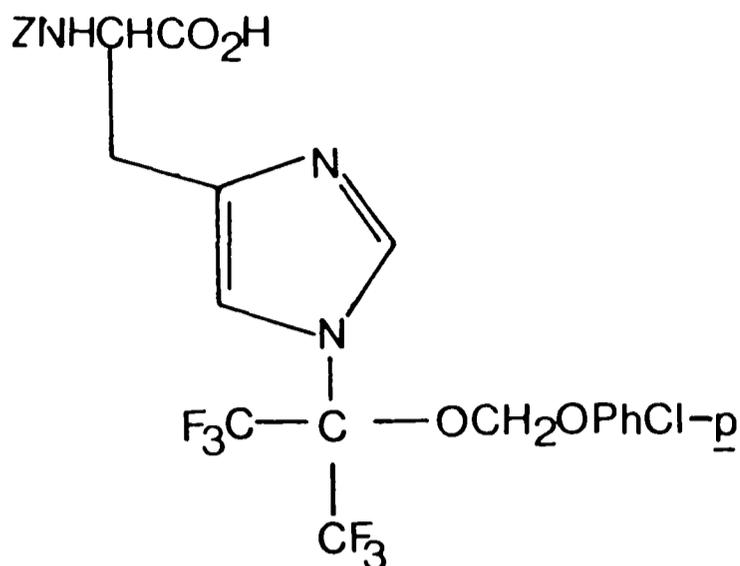
Figure 5

i)  o-Nitrobenzyl: Kalbag and Roeske[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 [98,99,100]. N($\alpha$)-Diphenyl-4-pyridylmethyl-L-

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 $\pi$-isomer has proved to be rather tedious[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[45] who suggested that since N-methylimidazole is an

effective acyl-transfer catalyst, any alkyl-substituent on the $\Upsilon$-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($\tau$)-benzyl-L-histidine derivatives and the lack of

racemisation in the derivatives of N($\Upsilon$)-t-butoxycarbonyl or N($\Upsilon$)-p-

toluenesulphonyl-L-histidine. Veber further suggested that specific

blockade of the $\Pi$-nitrogen might be advantageous in preventing racemisation

by this mechanism.

Jones et al[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

Histidine Imidazole Protecting Groups

| Protecting Groups | Reference | Removable by | Remarks |
|---|---|---|---|
| PhCH$_2$- | 52-68 | H$_2$/Pd, Na/NH$_3$(liq) | Gross racemisation on coupling, difficult to remove. |
| Ph$_3$C- | 23, 69-73 | Acids, H$_2$/Pd(slowly), Na/NH$_3$(liq) | Non crystalline derivatives, instability to acids a major disadvantage. |
| Ph$_2$CH- | 23, 70 | Acids, H$_2$/Pd, Na/NH$_3$(liq) | Slightly more acid stable than triphenylmethyl.but same problems apply. |
| Tos- | 23, 43, 72, 74, 75 | Na/NH$_3$(liq), HF, HBr/AcOH, NaOH | Suppresses racemisation well, very unstable to a wide range of conditions. |
| 2,4-NO$_2$Ph- | 23, 43, 72, 76-81 | Thiols, Bases | Very unstable to nucleophiles. |
| PhCH$_2$OCO- | 27, 43, 72, 82-87 | H$_2$/Pd, Acids (variable) | Very unstable to nucleophiles. |
| (CH$_3$)$_3$COCO- | 43, 72, 88-93 | Acids | Deactivates imidazole ring, suppresses racemisation, limited by its instability. |
| [piperidine]N-CO- | 23, 94 | NaOH, hydrazine | Only disadvantage appears to be prolonged treatment with base required to deprotect. |
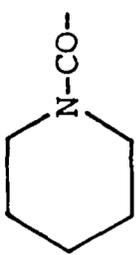
Table 1

Histidine Imidazole Protecting Groups (Continued)

| Protecting Groups | Reference | Removable by | Remarks |
|---|---|---|---|
| ROCONHCH(CF$_3$)- | 43, 50, 72, 79, 95 | R=Bzl: H$_2$/Pd, HBr/AcOH<br>R=(CH$_3$)$_3$C: TFA | Difficulty in purification thought to be due to second asymetric centre, could also be due to a τ- & π-mixture of isomers. |
| p-ClPhOCH$_2$OC- (CF$_3$, CF$_3$) | 95, 96 | HCl, TFA | Difficult to prepare and purify, possibly due to a mixture of τ & π- isomers. |
| o-NO$_2$Ph- | 97 | h , H$_2$/Pd | No examples of use, but will probably be poorly soluble and likely to racemise. |
| Ph$_2$C- (pyridine) | 98-100 | H$_2$/Pd, Zn/AcOH, electrolysis | Difficult to purify, not many examples of use, but likely to racemise. |
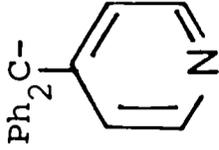
Table 1

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 Υ- and Π- protected isomers were

prepared from N(α)-alkyloxycarbonyl-histidine methyl ester. The Π- protected

isomer was obtained by a strategy of selective N(Υ)-blockade by

triphenylmethylation, highly selective for steric reasons, followed by

N(Π)-alkylation and finally removal of the N(Υ)-triphenylmethyl group

with weak acid and base saponification of the ester. The N(Υ)-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.
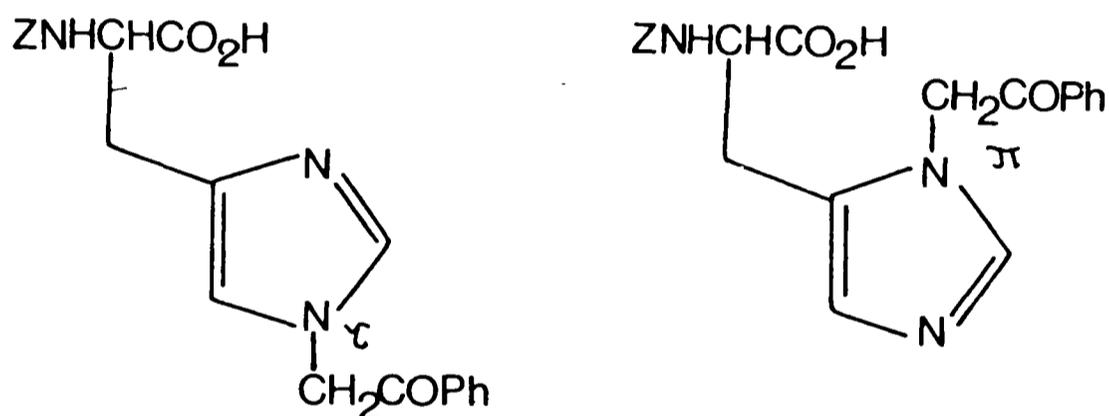


Figure 6

follows from the methods of synthesis but were also corroborated by

the cross ring coupling constant criterion of Mattews and Rapoport[104].

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[105], the N(Υ)-protected isomer had almost completely

racemised thus confirming Veber's suggestion that racemisation occurs

through the N(Υ)-nitrogen.

Further work by Jones et al showed that racemisation of N(α)-

benzyloxycarbonyl,<u>N</u>(Υ)-phenacyl-<u>L</u>-histidine, which occurs on activation

with dicyclohexylcarbodiimide in dimethylformamide takes place by the

action of the Π-nitrogen as an intramolecular base catalyst in the <u>O</u>-

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 <u>N</u>(π)-nitrogen lone pair is able to abstract the α-proton whilst it is

spacially impossible for the <u>N</u>(Υ)-nitrogen lone pair to abstract the

α-proton in the <u>N</u>(π)-protected isomer.



Racemeric peptide
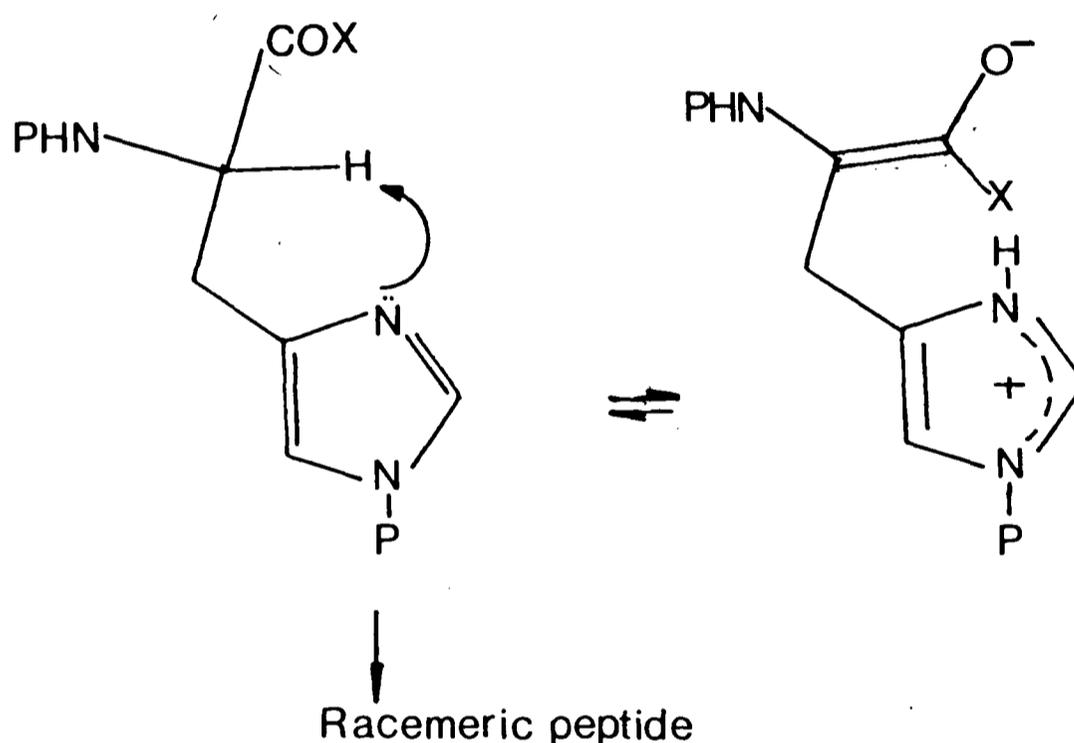
Scheme 3

## Conclusion.

Work performed by Jones <u>et</u> <u>al</u> 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 <u>N</u>(π)-

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
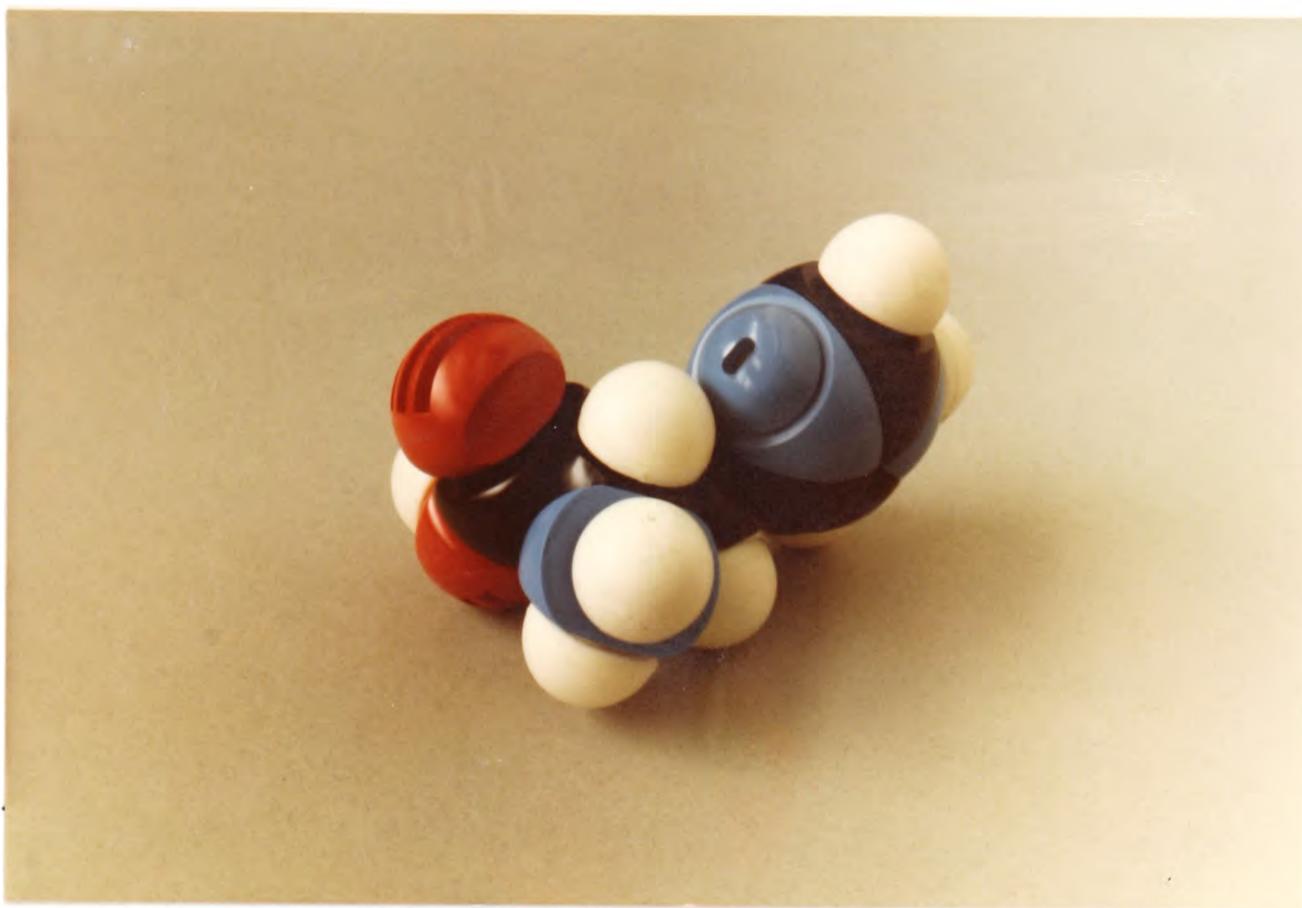
Lone pair on π-nitrogen



Lone pair on τ-nitrogen

Plate 1
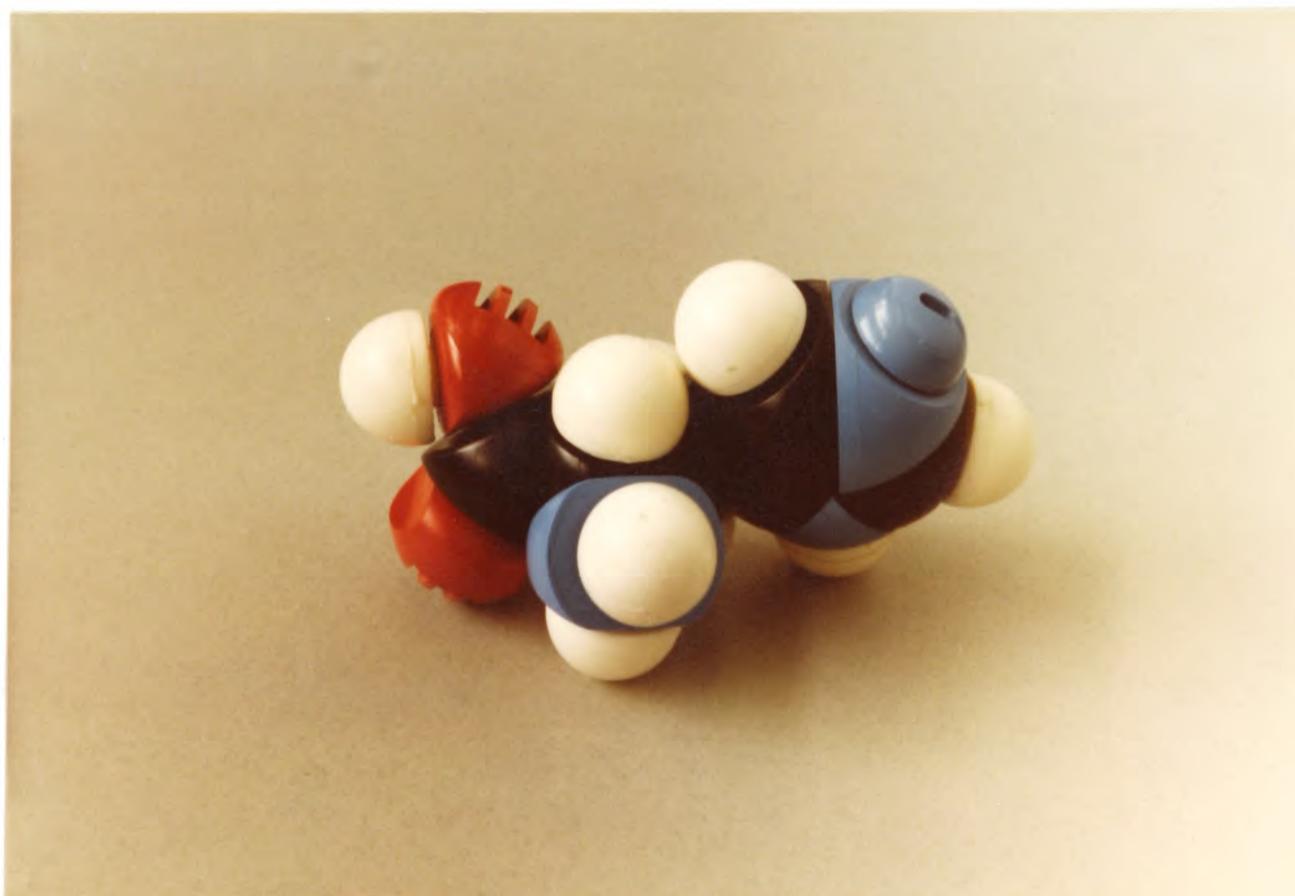
and photolysis[107]. Previous research in this field had tended to

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 $\underline{N}(\pi)$-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-L-histidine

### in Peptide Synthesis

## Introduction.

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

N($\pi$)-Nitrogen deprotection had been accomplished in most synthetic operations involving N($\pi$)-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[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[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 N($\pi$)-phenacyl-thyroliberin[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

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 N(π)-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 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-picolyl esters[117] and 3-picolyl esters[117], O-4-picolyltyrosine[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 N(π)-phenacyl-L-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 L-histidine and 25% of the reduced compound N(π)-2-hydroxy-2-phenylethyl-L-histidine. It has however also been shown that the products

from the electrolysis of alkylarylketones are very dependent on voltage, pH and solvent[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[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
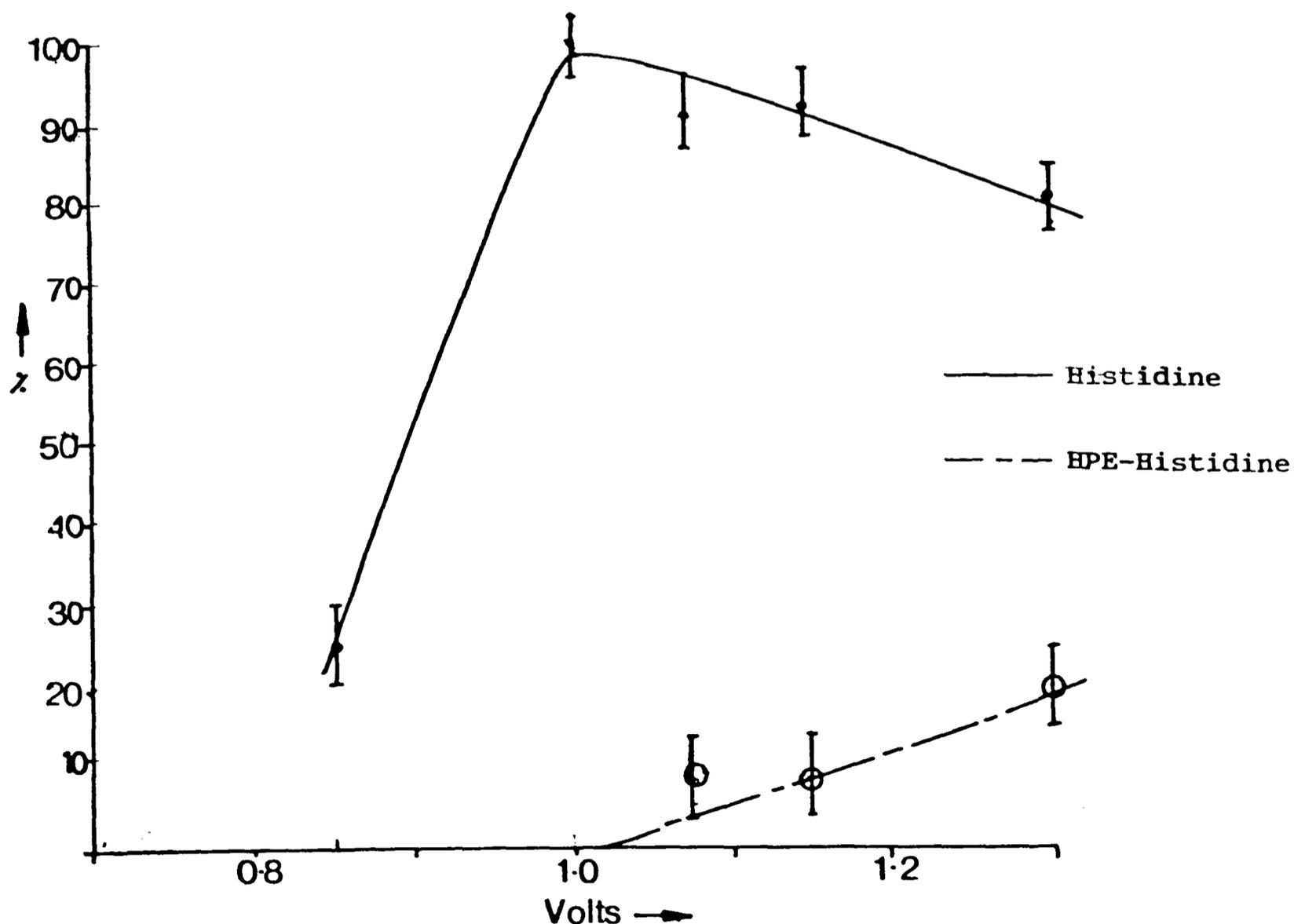


Figure 7

when the voltage was set at 1.00 V using a 0.5M sulphuric acid electrolyte.

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(π)-2-hydroxy-2-phenylethyl-L-

histidine.

This chapter deals with the extension of these studies to a

N(π)-phenacyl-histidine-containing peptide.

Attempted Electrolytic Deprotection of N(π)-Phenacyl-thyroliberin.

N(π)-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
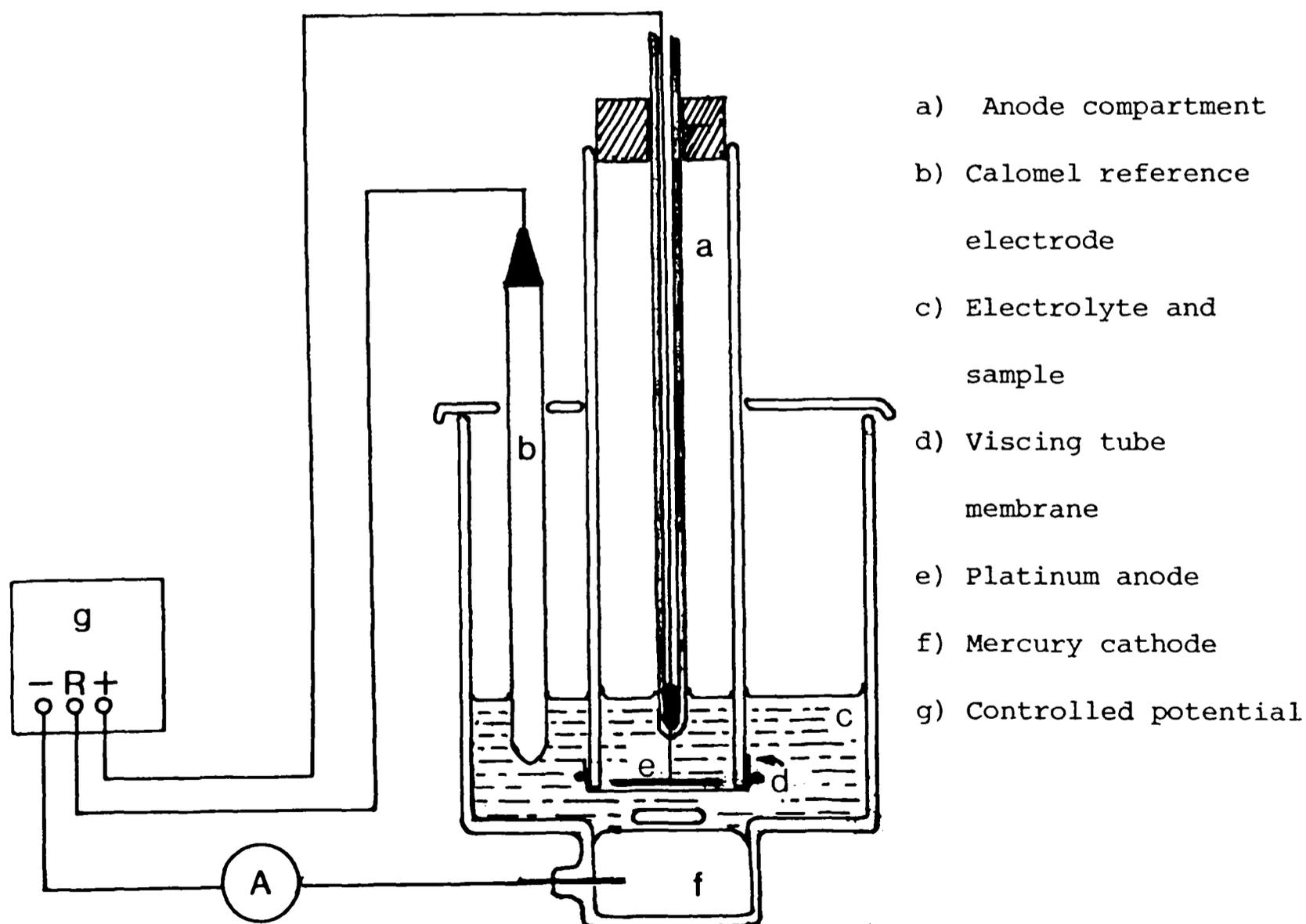
Figure 8

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[107] 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(π)-phenacyl-L-histidine and

not to the preparation of pure thyoliberin.

The most likely explanation for the presence of this by-product

is that although the elctrolysis of N(π)-phenacyl-L-histidine at 1.00 V

produced none of the reduced product N(π)-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(π)-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
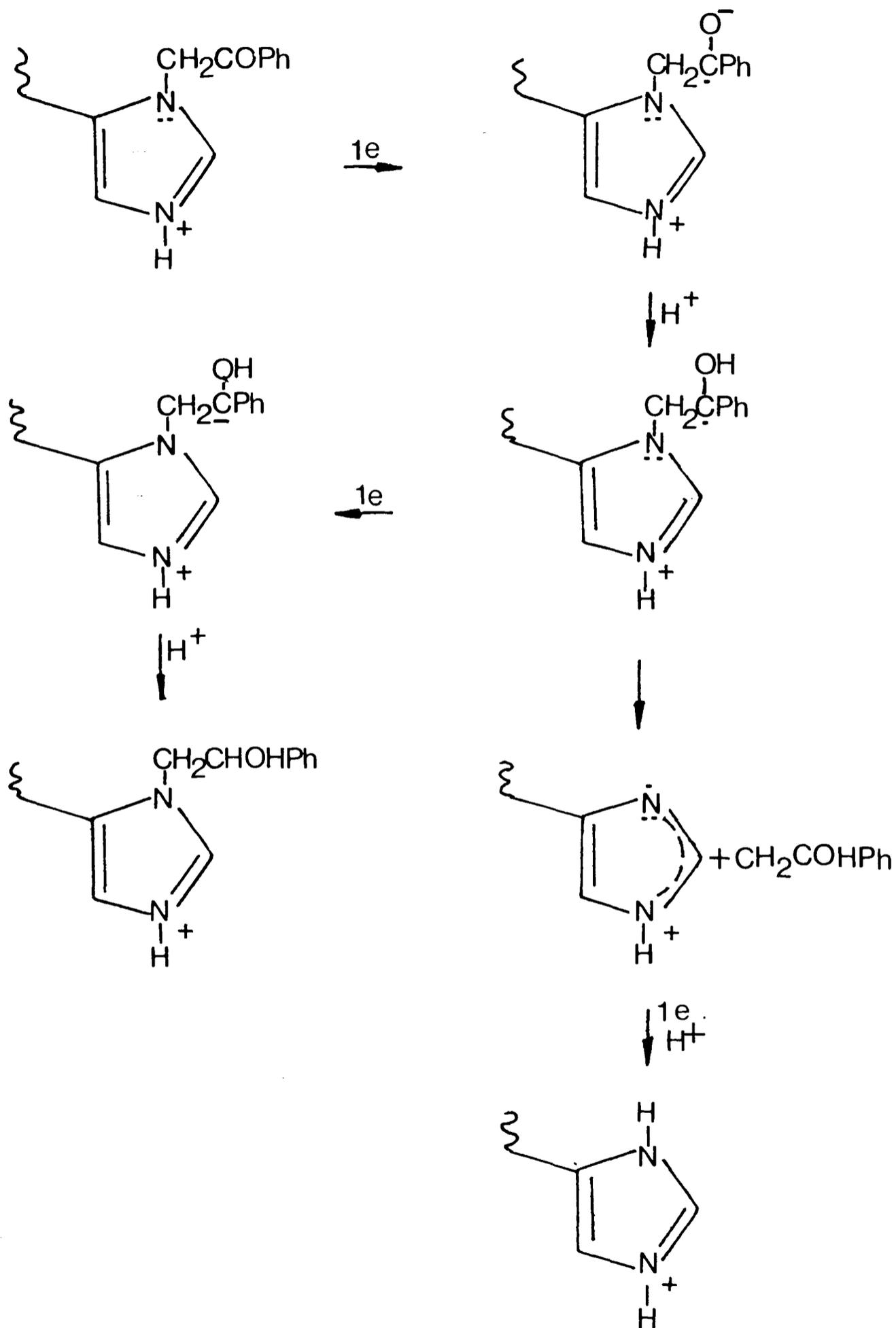


Scheme 4

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[114]. However, in the light of work by Young et al[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[107], but this explanation is unlikely due to the fact that the impurity gives a stong Pauly-positive test.

Results and Conclusion.

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

It might have been possible to establish conditions for clean cleavage after an exhausive investigation such as that required to establish the ideal conditions for the clean cleavage of N(π)-phenacyl-L-histidine. However such an investigation would probably have been necessary for each new N(π)-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(π)-phenacyl-L-histidine-containing peptides.

In parallel to this work further studies had been carried out

into the suitability of $\underline{N}(\pi)$-phenacyl-$\underline{L}$-histidine for use in peptide synthesis:

both Brown[108] and Mansfield[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[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[108] and characterise

the products fully when he reacted $\underline{N}$-(phenacyl)imidazole with di-(t-butyl)-

dicarbonate in dimethylformamide (Scheme 5).

CH$_2$COPh

$\overset{i}{\underset{ii}{\longrightarrow}}$

CH$_2$COOBu$^t$

+ PhCOOCH$_3$
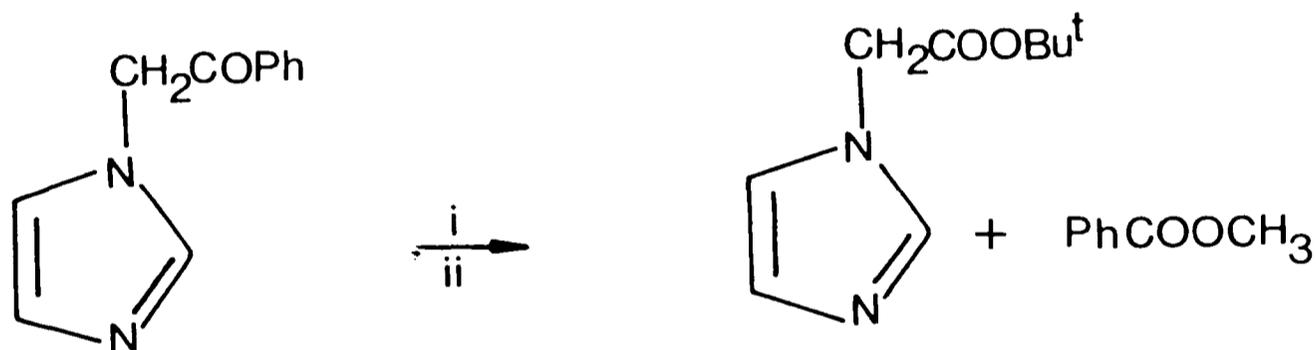
i) Boc$_2$O-DMF. ii) MeOH-CHCl$_3$, chromatography.

Scheme 5

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 $\underline{N}(\pi)$-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($\pi$)-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[125] proposed just such a novel strategy in which cleavage

of the protecting groups is accomplished by treatment with

supernucleophiles[126], often within a few seconds in essentially neutral

solutions at 0-20°. The protecting groups proposed were either $\beta$- or $\delta$-

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

$Co^I$-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.

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

A sample of 1,1,1,4-tetrachlorobut-2-ene was generously provided by

Professor Ugi of the Organish-Chemisches Institut, Technische Universitat,

München.

We initially attempted to prepare the imidazole derivative by the method used by Jones and Hysert[127] in the prepartion of N(but-2-enyl)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[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-tetrachlorobut-2-ene.

We next prepared N(trimethylsilyl)imidazole[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[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 decompostion of the product.

Preparation of N(1,1,1-Trichlorobut-2-enyl)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).
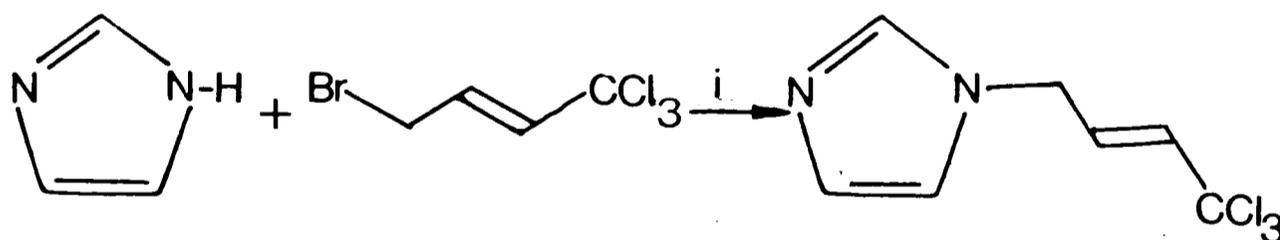


i) MeOH-Na$_2$CO$_3$-reflux 2.5 h.

Scheme 6

This material was crystallised and characterised as the oxalate salt.

Regeneration of N(1,1,1-trichlorobut-2-enyl)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-enyl)imidazole are summarised in Table 2. The N-chloroalkene

protecting group was rapidly and cleanly cleaved by zinc in aqueous acetic

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

Table 2

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 $\underline{N}(1,1,1$-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

starting material and the product had a similar molecular weight

and characteristic molecular ion for a compound containing three

chlorine atoms. The u.v. absorbtion 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[134], 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 intirgrated intensity of the resonances of spacially 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[129] 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-trichlorobut-2-enyl)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 spacially

more distant 4-proton (Figure 10). This result, although small, was

exactly reproduceable over a number of runs. So we can conclude that

the methylene group is in exactly the same spacial environment with

respect to the imidazole ring in both cases. The only possible conclusion
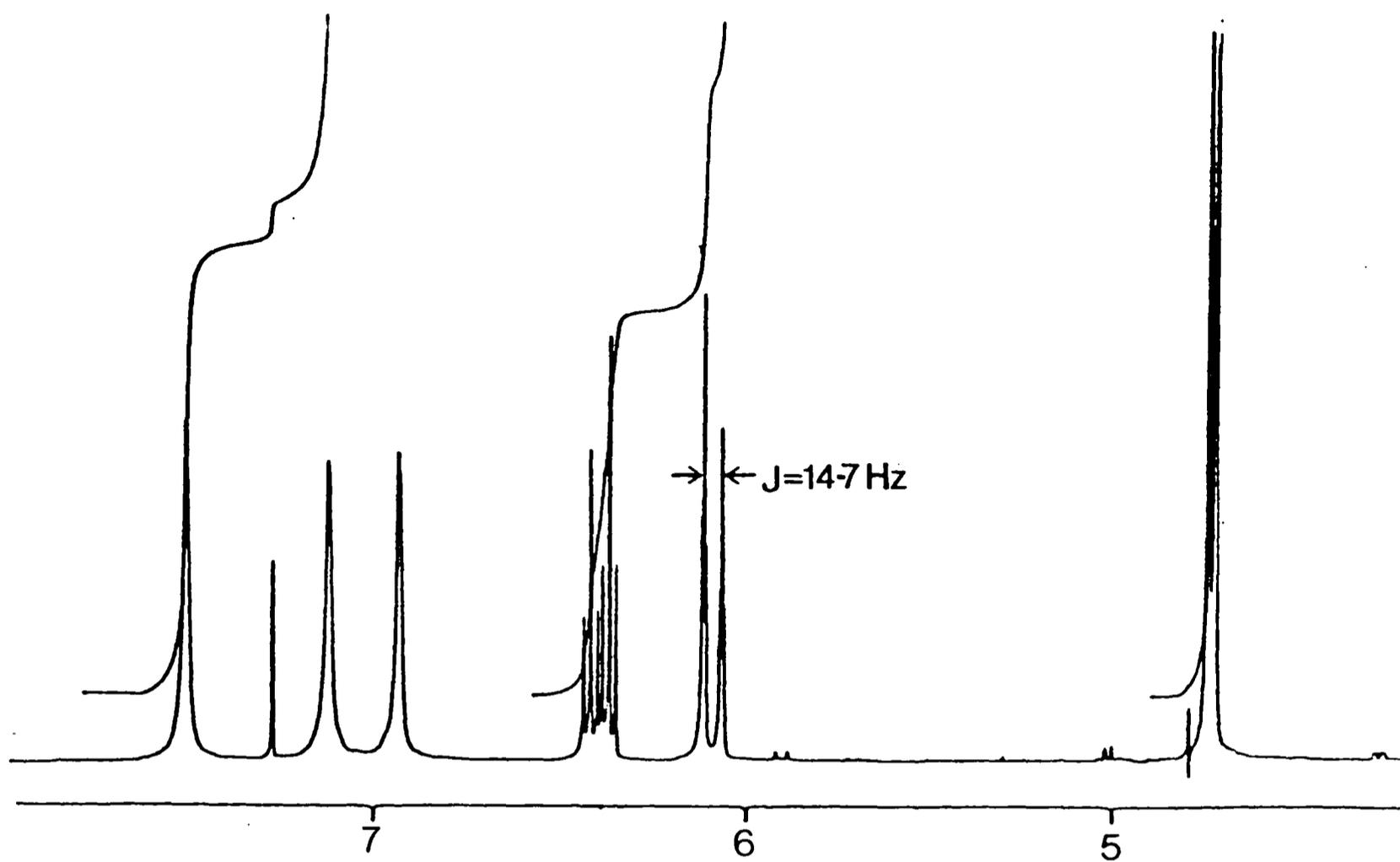
N(1,1,1-trichlorobut-2-enyl)imidazole
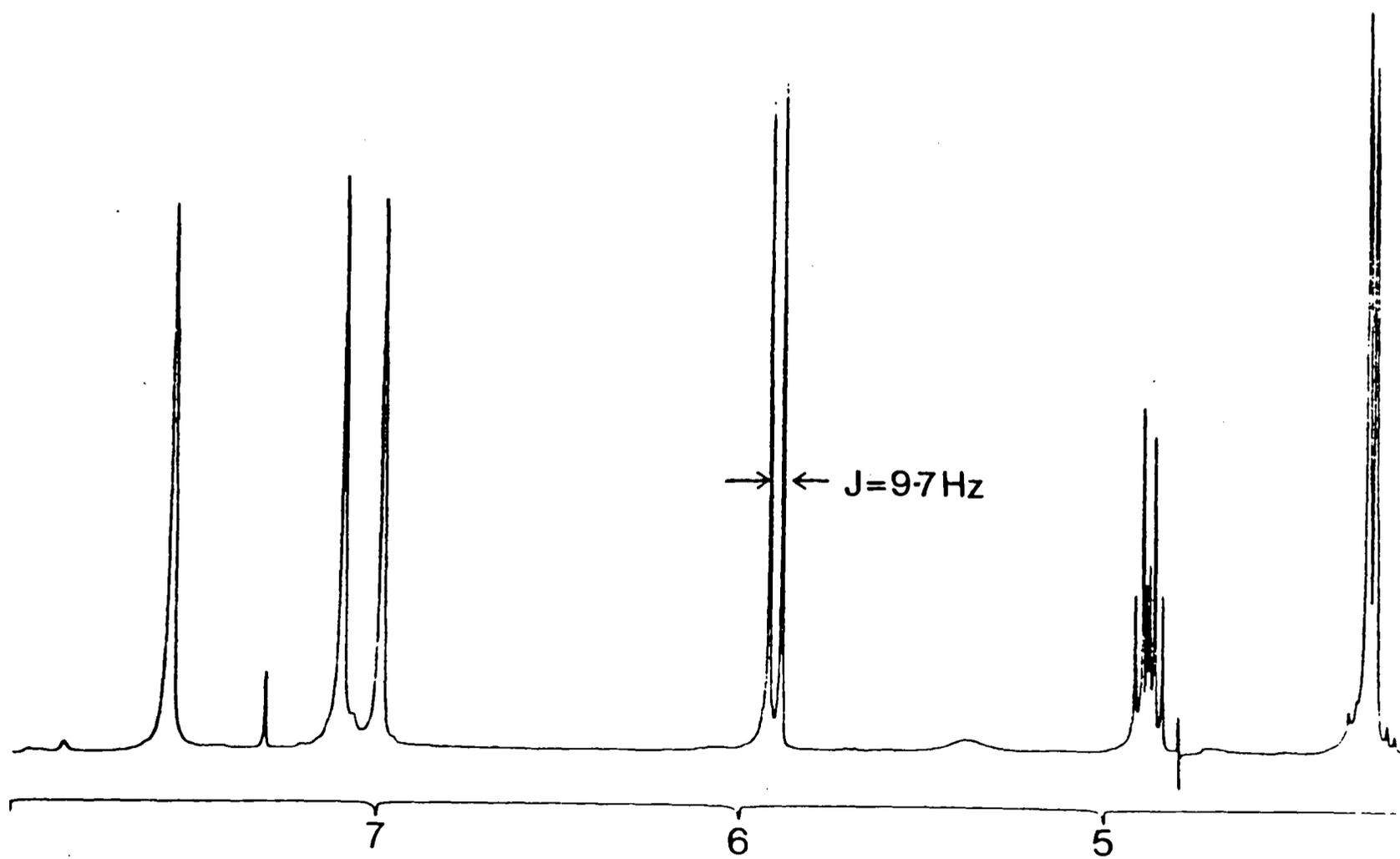
N(1,1,1-trichlorobut-2-enyl)imidazole after treatment with TFA

Figure 9
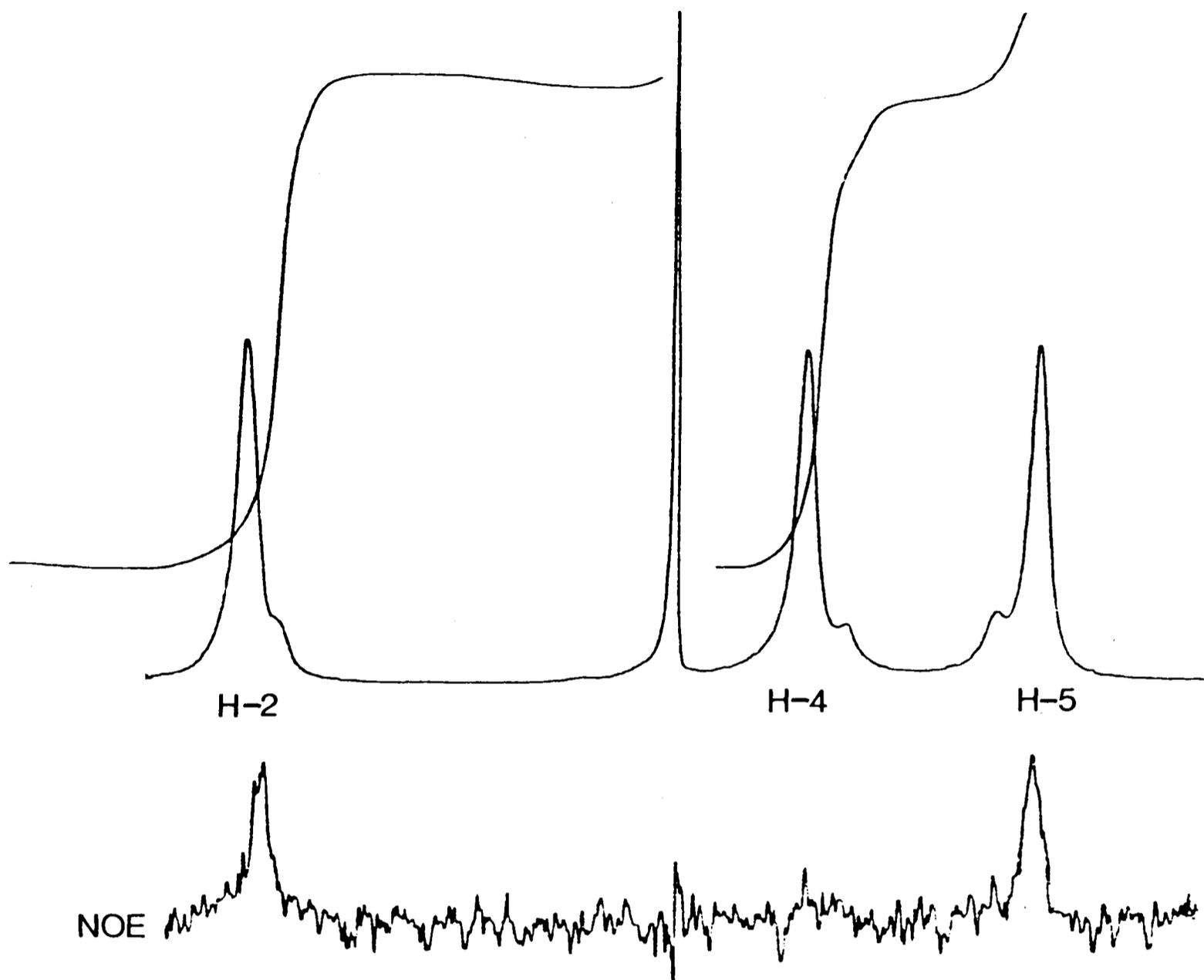
H-2  H-4  H-5

NOE

$\underline{N}(1,1,1\text{-trichlorobut-2-enyl})\text{imidazole}$

H-2  H-4  H-5

NOE
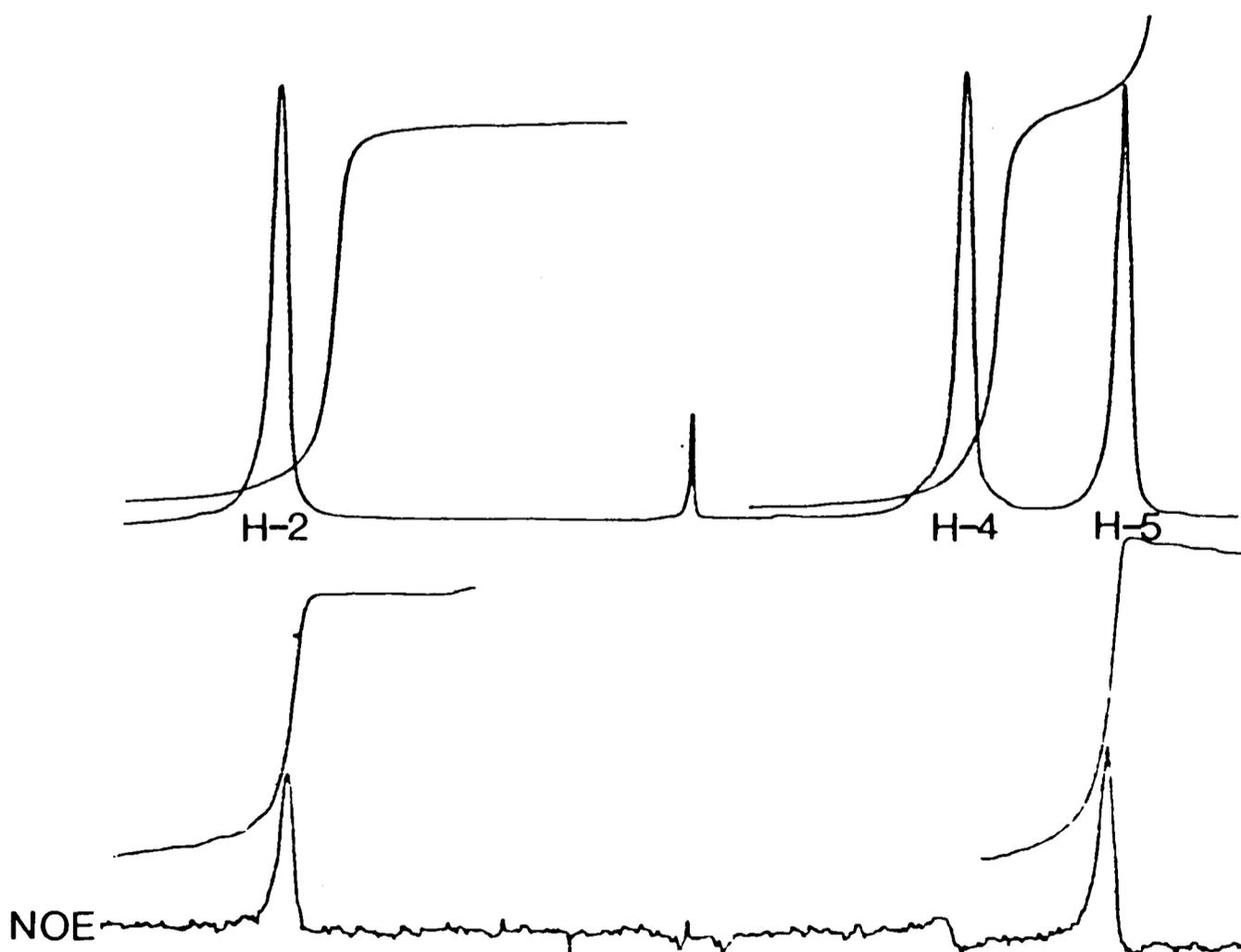
$\underline{N}(1,1,1\text{-trichlorobut-2-enyl})\text{imidazole after treatment with TFA}$

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).
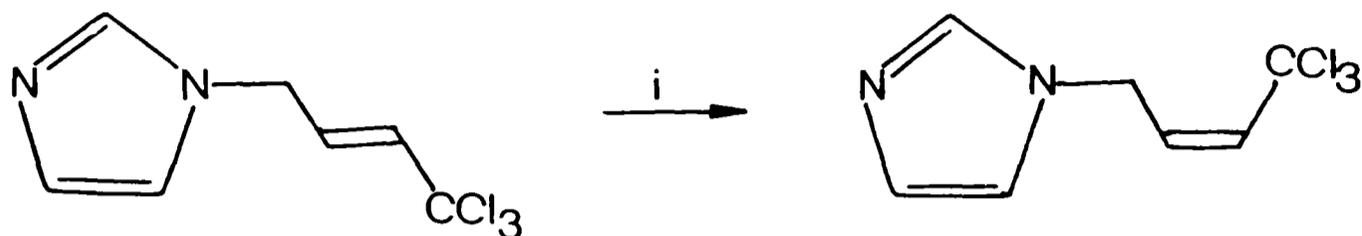
i) Trifluoroacetic acid-2 weeks

Scheme 7

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[130], decalins[131]. The most notable example in this case is the isomerisation of 1,2-dihalogenated ethylenes[132]. 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[132].

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 spectroscropy 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-dichloro-1,3-dienyl)imidazole

(Scheme 8).
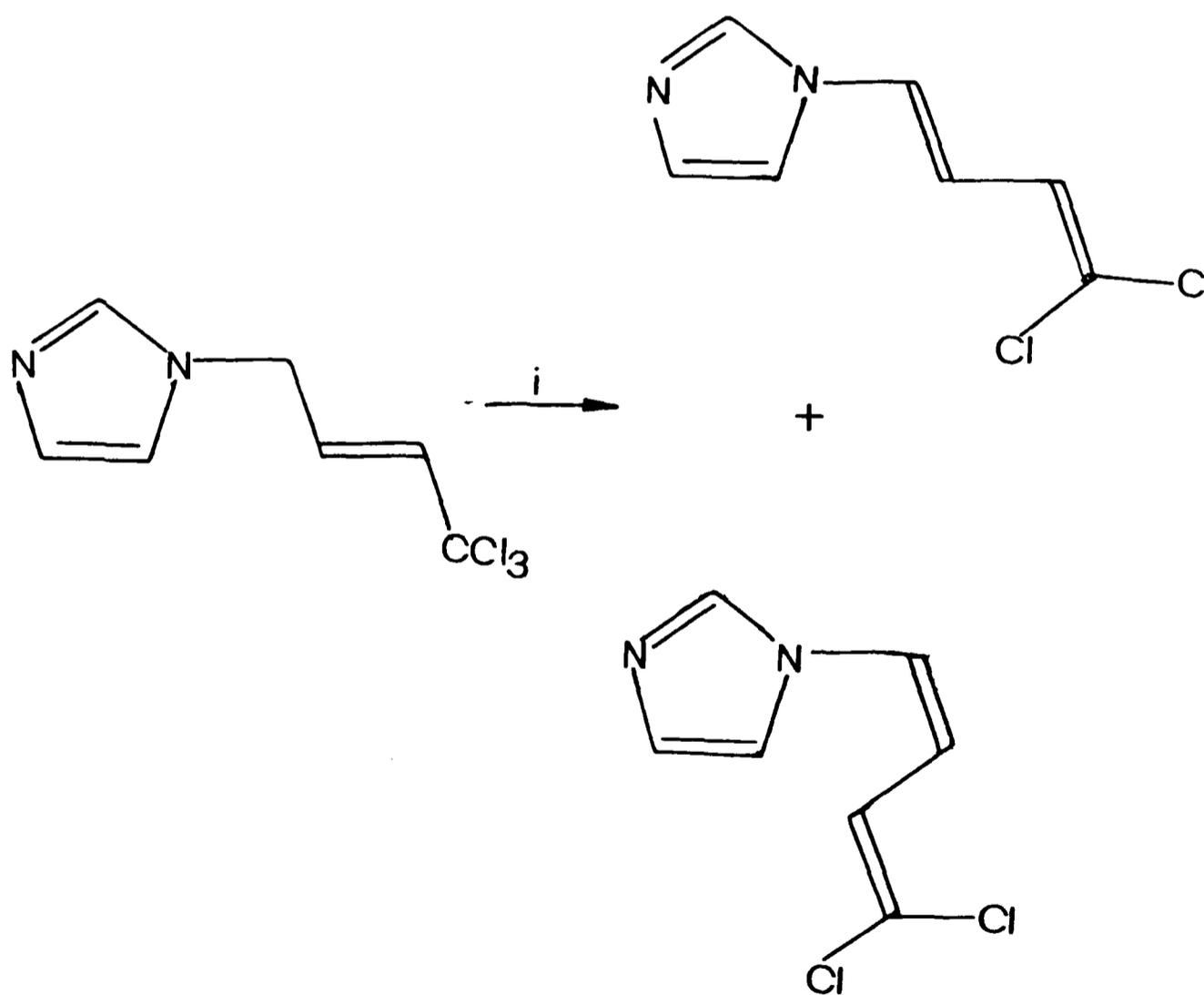
i) NaOH-$H_2$O-MeOH 1 h

Scheme 8

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(π)- 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[106] or via an optically labile imidazolium species, as proposed by Veber[45].

It has been shown by Fletcher et al[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 $\underline{N}(\tau)$-t-butoxycarbonyl or $\underline{N}(\gamma)$-$\underline{p}$-toluenesulphonyl-$\underline{L}$-histidine are also able to suppress racemisation effectively although for steric reasons they are thought to be blocking the $\tau$-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 subsitution of electron-donating groups on the $\tau$-nitrogen such as in $\underline{N}(\tau)$-benzyl or $\underline{N}(\tau)$-phenacyl-$\underline{L}$-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 $\tau$-nitrogen synthetic procedures for the preparation of $\underline{N}(\pi)$-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 $\underline{N}(\pi)$- substituents none of which has proved ideal. It was thus decided to look into the novel concept of deactivating the $\underline{N}(\pi)$- 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[135] which were later proved to contain both mono- and diiodohistidine[136]. Savoie et al[137] 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[138] 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[139]. Whilst iodination of histidine residues was first reported by Fraenkel-Conrat[140] it was not until much later that Cloelli and Wolff were able to demonstrate the first specific labelling of histidine with $^{131}$I in Ribonuclease A[141]. Since that time

many examples of iodination of histidine containing peptides have been reported[142-144].

For the study of metabolism the specific replacement of protons with radioactive tritium can be desirable. Thus after Birkofer and Hempel[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 et al[146] to label thyroliberin and then by Menez et al[147] in the labelling of the α-neurotoxin Nujunigricallis. This work was taken a stage further when Allen et al[148] synthesised a 1-24 ACTH 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[155]. Disulphide bridges did however poison the catalyst, preventing dehalogenation and necessitating special synthetic strategies[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 $pK_a$ values of mono- and diiodohistidine had been conducted by Bruning[149] (Table 3)

| | Histidine | Monoiodohistidine | Diiodohistidine |
|---|---|---|---|
| $pK_a$ ImH | 6.00 | 4.18 | 2.72 |

Table 3

which indicated that iodination would infact 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[149], however the initial product was impure and purification by reprecipition 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(∝)-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 purifing the product in a reproducibly good yield. It was decided that if N(∝)-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(∝)-protected amino-acid, the increased solubility of the N(∝)-protected methyl ester would make the reaction much easier to follow by t.l.c. Purfication 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(∝)-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).



i) $I_2$-CHCl$_3$-Na$_2$CO$_3$-H$_2$O   ii) NaOH-H$_2$O-CH$_3$OH
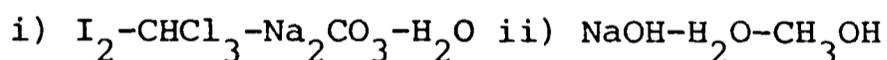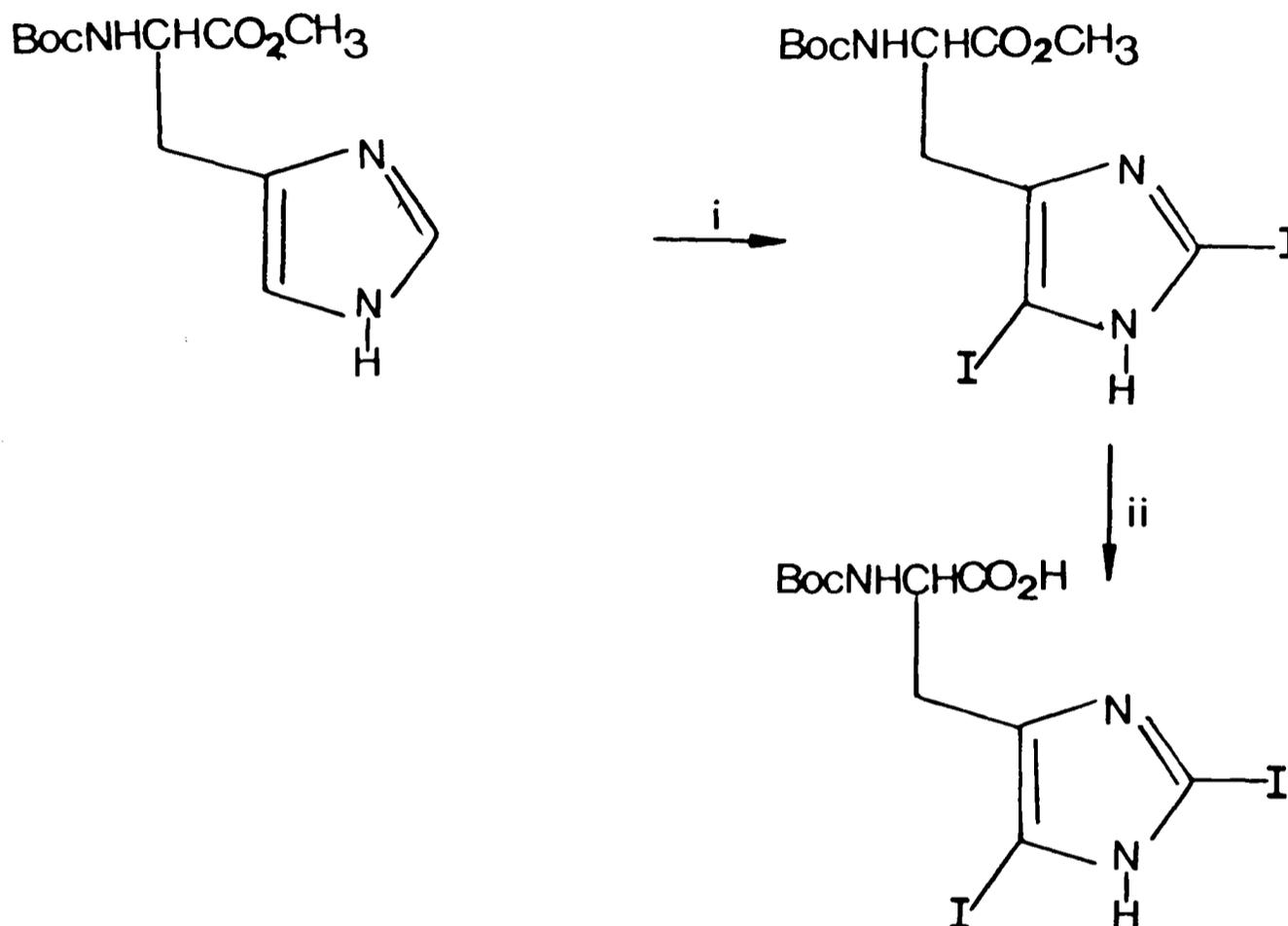
Scheme 9

The $\underline{N}(\alpha)$-protected amino-acid was shown, after deprotection and digestion with an $\underline{L}$-amino-acid oxidase using the method of Jones and Ramage[105], to contain less than 2% $\underline{D}$-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 cyrstalline and so was not used further as an intermediate.

Synthesis of $\underline{N}(\alpha)$-Benzyloxycarbonyl-$\underline{L}$-2,5-diiodohistidine.

The $\underline{N}(\alpha)$-benzyloxycarbonyl derivative was prepared and

characterized from N(∝)-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(∝)-t-Butoxycarbonyl-L-2,5-diiodohistidine Methyl Ester.

Deprotection of the imidazole ring of N(∝)-t-butoxycarbonyl-L-2,5-

diiodohistidine 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
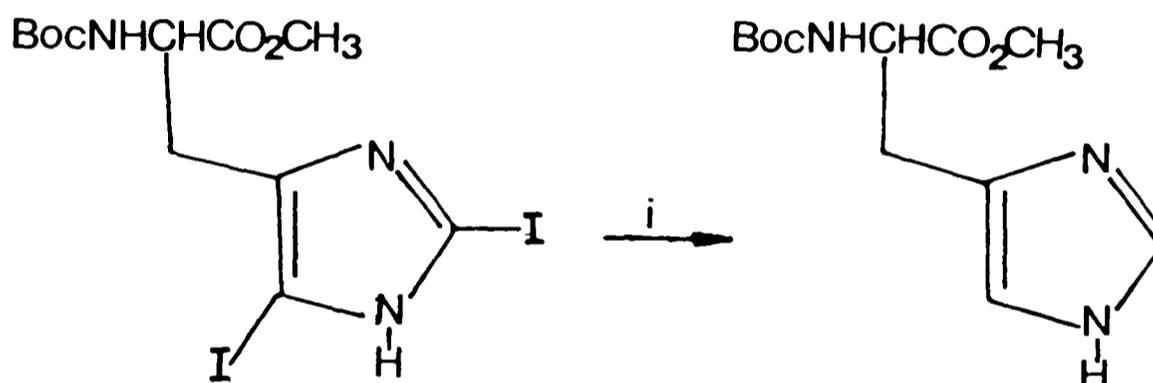
i) 5%Rh/CaCO$_3$-5%Pd/C-DMF-H$_2$-30 min

Scheme 10

within 30 min, and the product was shown by t.l.c. and amino-acid

analysis to be N(∝)-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(∝)-t-Butoxycarbonyl-L-5-iodohistidine Methyl Ester.

In early experiments where only two equivalents of iodine were

used in the iodination of N(∝)-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 N(∝)-t-butoxycarbonyl-L-monoiodohistidine methyl ester.

The literature is somewhat ambiguous as to the position of

substitution of iodine in directly iodinated monoiodohistidines. Bruning[149]

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[153] assumed 2-substitution, however Bensusan and

Naidu[151] and Holloway et al[152] claimed simultaniously to show that

substitution was actually occuring at the 5-position on the imidazole

ring. Both Bensusan et al[151] and Holloway et al[152] 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

constisting 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 occured 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.

N(α)-t-Butoxycarbonyl-L-monoiodohistidine methyl ester was deuterated under conditions similar to those established for the hydrogenolys 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 N(α)-t-butoxycarbonyl-L-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 infact occured at both the 2-and 5-positions on the imidazole ring. Confirmation was obtained by the deuteration of N(α)-t-butoxycarbonyl-L-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 monoiodinated 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 monoiodination 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.

Figure 11

BocNHCHCO$_2$Me

i →

BocNHCHCO$_2$Me

BocNHCHCO$_2$Me

i →

BocNHCHCO$_2$Me
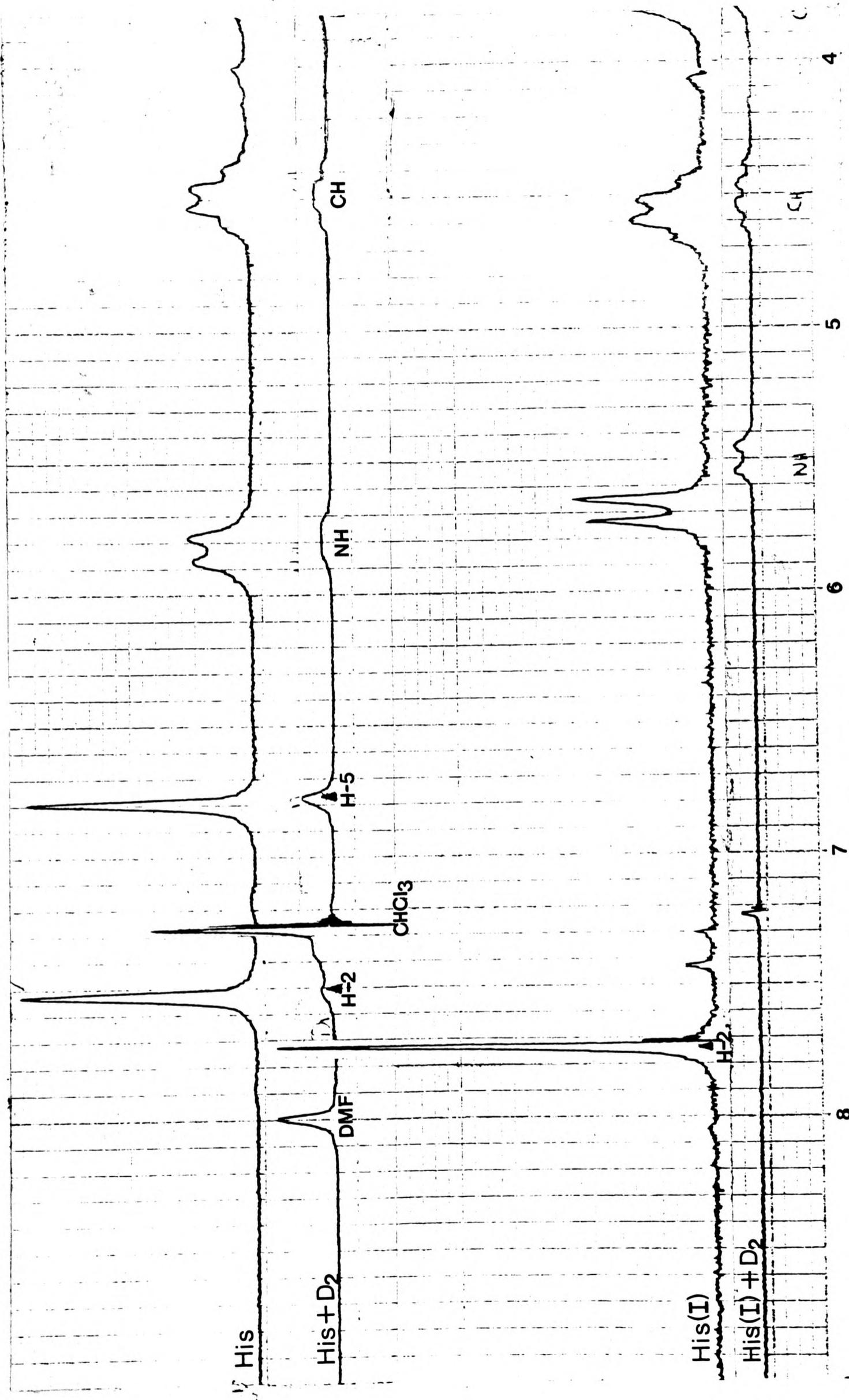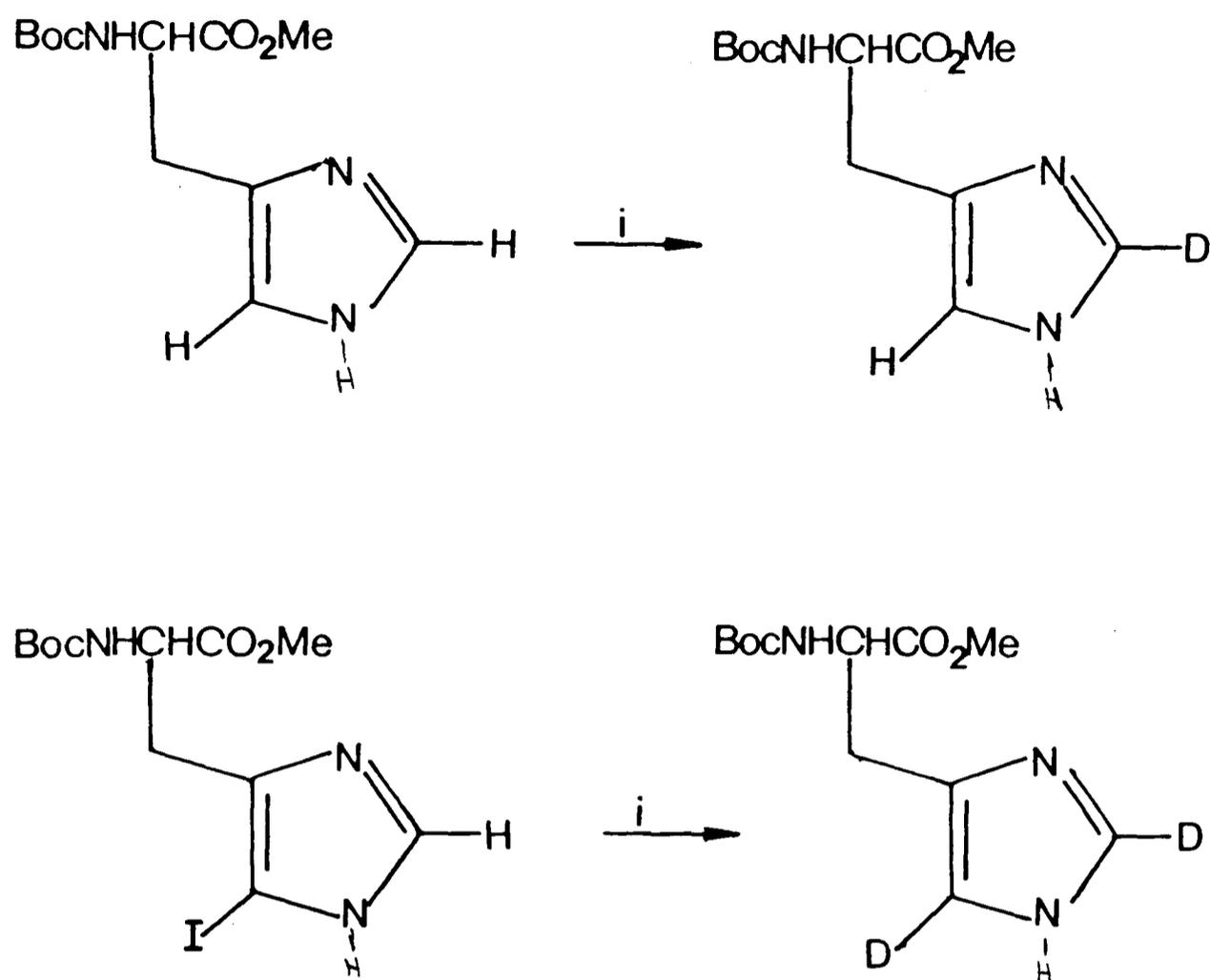
i) 5%Rh/CaCO$_3$-5%Pd/C-DMF-D$_2$-30 min

Scheme 11

## Synthesis of Thyroliberin.

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[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($\kappa$)-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[146] by direct iodination of

Glp                    His                      Pro

Boc————————$I_2$————OH    H————————————$NH_2$

Boc————————$I_2$————————————————————$NH_2$
                                              i

————OTcp    H————————$I_2$————————————$NH_2$
                                              ii

————————————————————$I_2$————————————$NH_2$
                                              iii

————————————————————————————————————$NH_2$
                                              iv
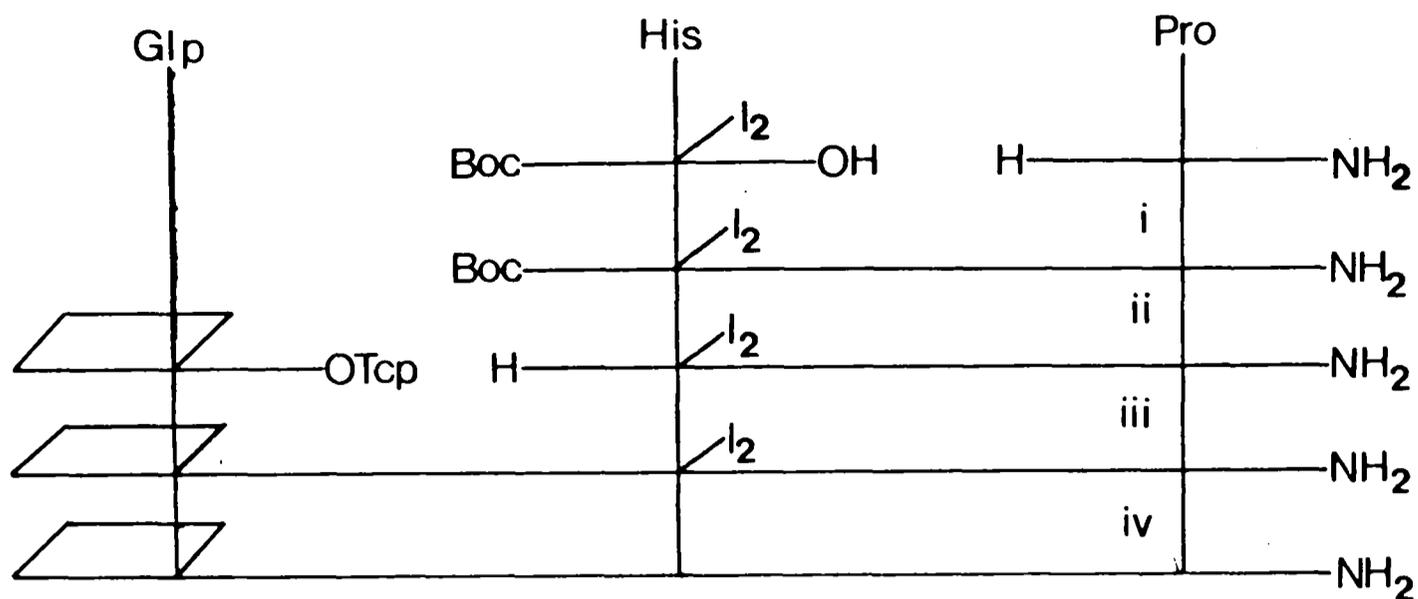
i) DCCI-HOBt-Et$_3$N-DMF  ii) TFA  iii) Et$_3$N-DMF  iv) 5%Rh/CaCO$_3$-5%Pd/C-DMF-H$_2$

Scheme 12

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-amino-acid oxidase[105] 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-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-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 inconsistant yields of pure product.

2)  A general method of producing N(α)-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(α)-t-Butoxycarbonyl-L-5-iodohistidine methyl ester obtained as

    a by product 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 the literature. However

5-bromoimidazole has a $pK_a$ of $3.60^{158}$ as opposed to the published value

of 4.18 for 5-monoiodohistidine[149], so it looked possible that bromination

of the imidazole ring might lower the basicity suffiently 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[167] 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(α)-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(∝)-t-Butoxycarbonyl-L-2,5-dibromohistidine.

Bromination of N(∝)-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 µm) 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(∝)-t-butoxycarbonyl-L-dibromohistidine

methyl ester. This compound was perfectly stable to air unlike Binkley's

derivative and saponification gave the N(∝)-protected acid in good yield

(Scheme 13).

BocNHCHCO₂Me



BocNHCHCO₂Me

i →

ii ↓

BocNHCHCO₂H
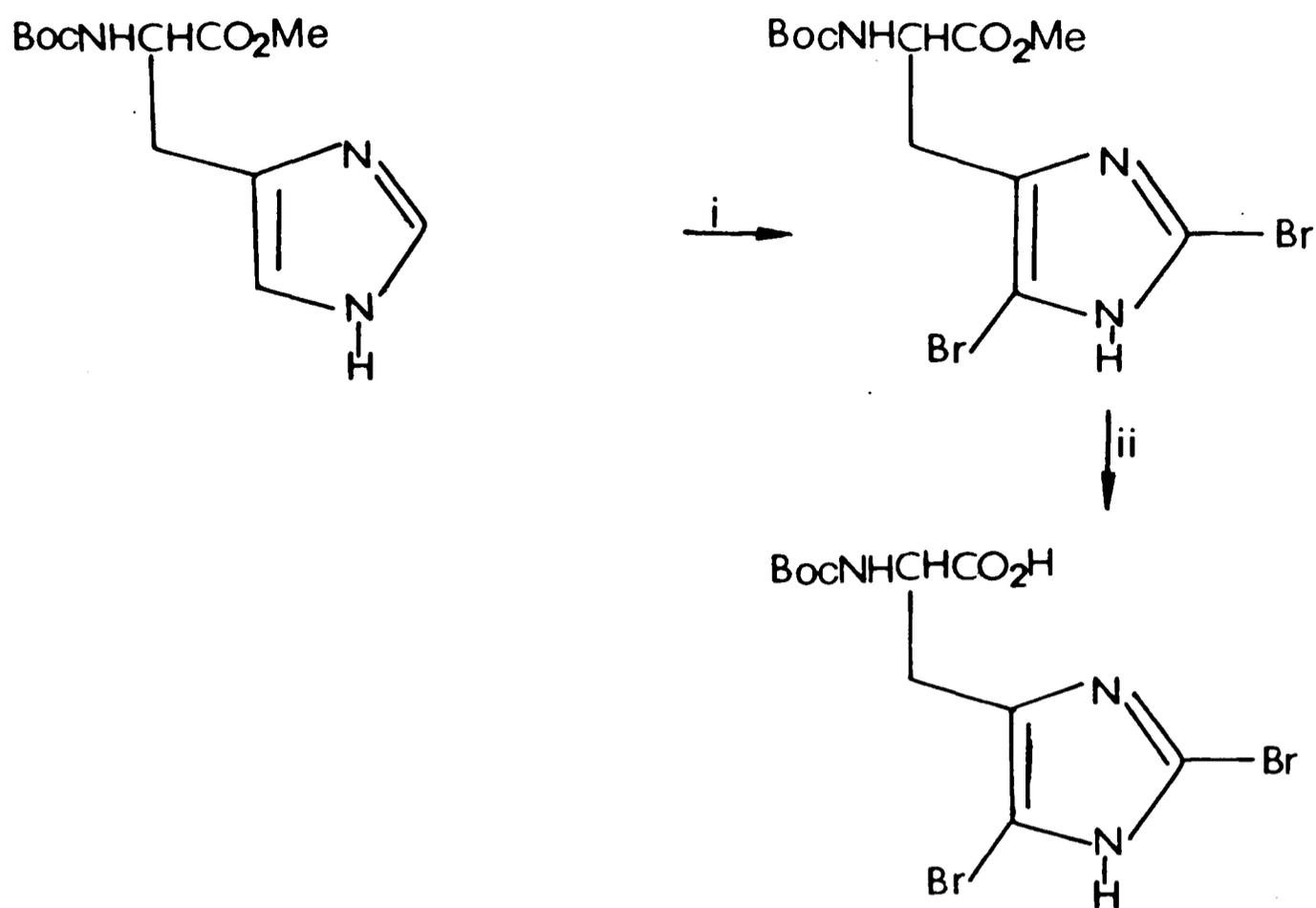
i) Br$_2$-CHCl$_3$-Et$_3$N-16h-flash chromatography (25% yield)    ii) NaOH-H$_2$O-MeOH (73% yield).

Scheme 13

Dehalogenation of N(α)-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(α)-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 $\underline{N}(\alpha)$-t-

butoxycarbonyl-$\underline{L}$-histidine methyl ester (Scheme 14). The catalyst and
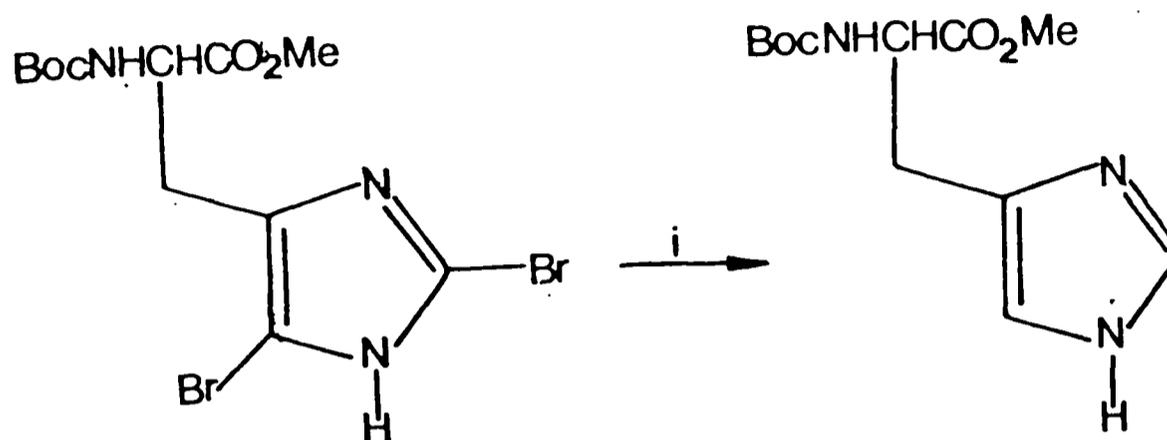


i) $H_2$-80%aq AcOH-5%Pd/C-5%Rh/C-IR 45(AcO$^-$)-35 min.

Scheme 14

solvent were removed and the product subjected to acid hydrolysis under-

standard conditions, after which amino-acid analysis showed histidine to

be the only amino-acid present in the hydrolysate.

### 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 $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-

2,5-dibromohistidine. The synthesis was carried out according to Scheme

15. $\underline{N}(\alpha)$ -t-butoxycarbonyl-$\underline{L}$-2,5-dibromohistidine was coupled to $\underline{L}$-

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 $\underline{L}$-amino-acid oxidase was shown to contain less than 4%

$\underline{D}$-histidine. After $\underline{N}(\alpha)$-deprotection with trifluoroacetic acid the crude
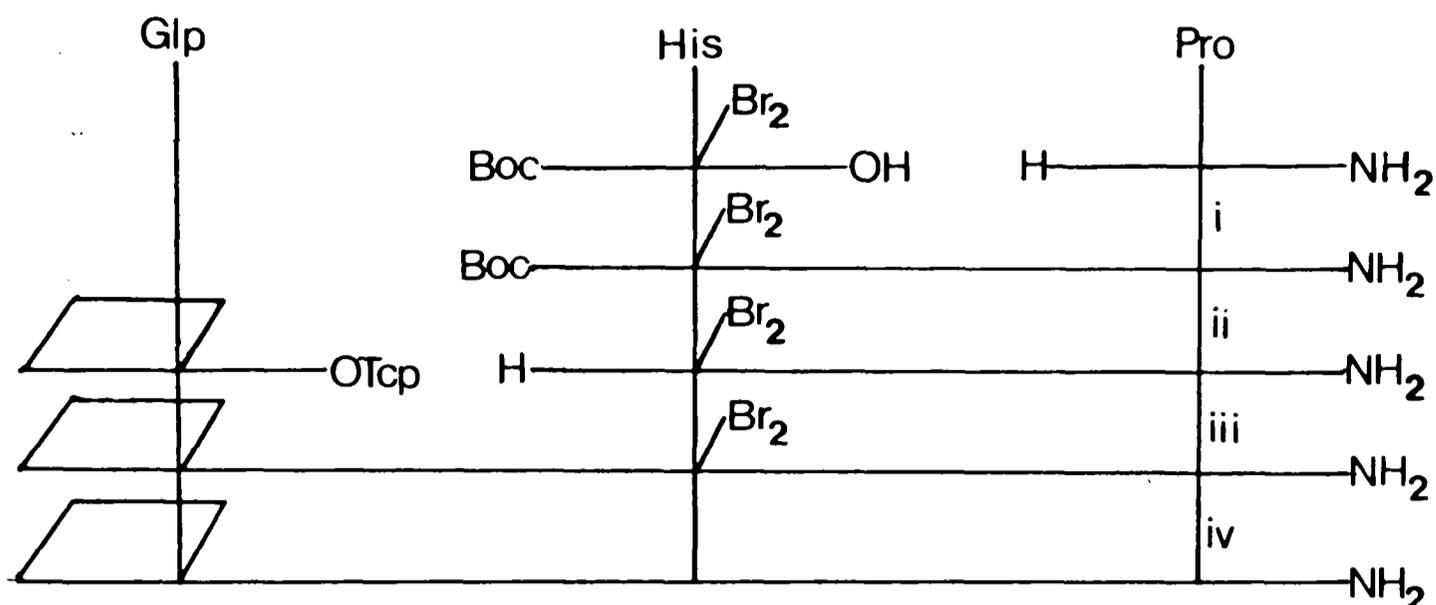
i) DCCI-HOBt-DMF (70% yield)   ii) TFA (Product not isolated)   iii) DMF-

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[103]. 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[154].

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

Having successfully demonstated 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 N(∝)-phenacyl-L-histidine-containing peptides by Turner[161] and Brown[108]. None of these syntheses had however proved to be entirely successful. This was thought to be principally due to difficulities caused by the previously-dicussed side-reactions of N(∝)-phenacyl-L-histidine rather than to equipment or methodology.

Hence it was decided to attempt the synthesis of the tripeptide glycyl-L-histidyl-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 cesium salt of N-t-butoxycarbonyl-L-phenylalanine was esterified with a chloromethylated 1% crosslinked Merrifield polystryene 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 N-t-butoxycarbonyl-L-phenylalanine resin was placed in the synthesis vessel where each additional amino-acid residue was added using

the following cycle of operations. $\underline{N}(\varkappa)$-Deblocking was initially

carried out using 40% trifluoroacetic acid in dichloromethane, followed

by neutralisation with triethylamine, and then coupling of the $\underline{N}(\alpha)$-

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
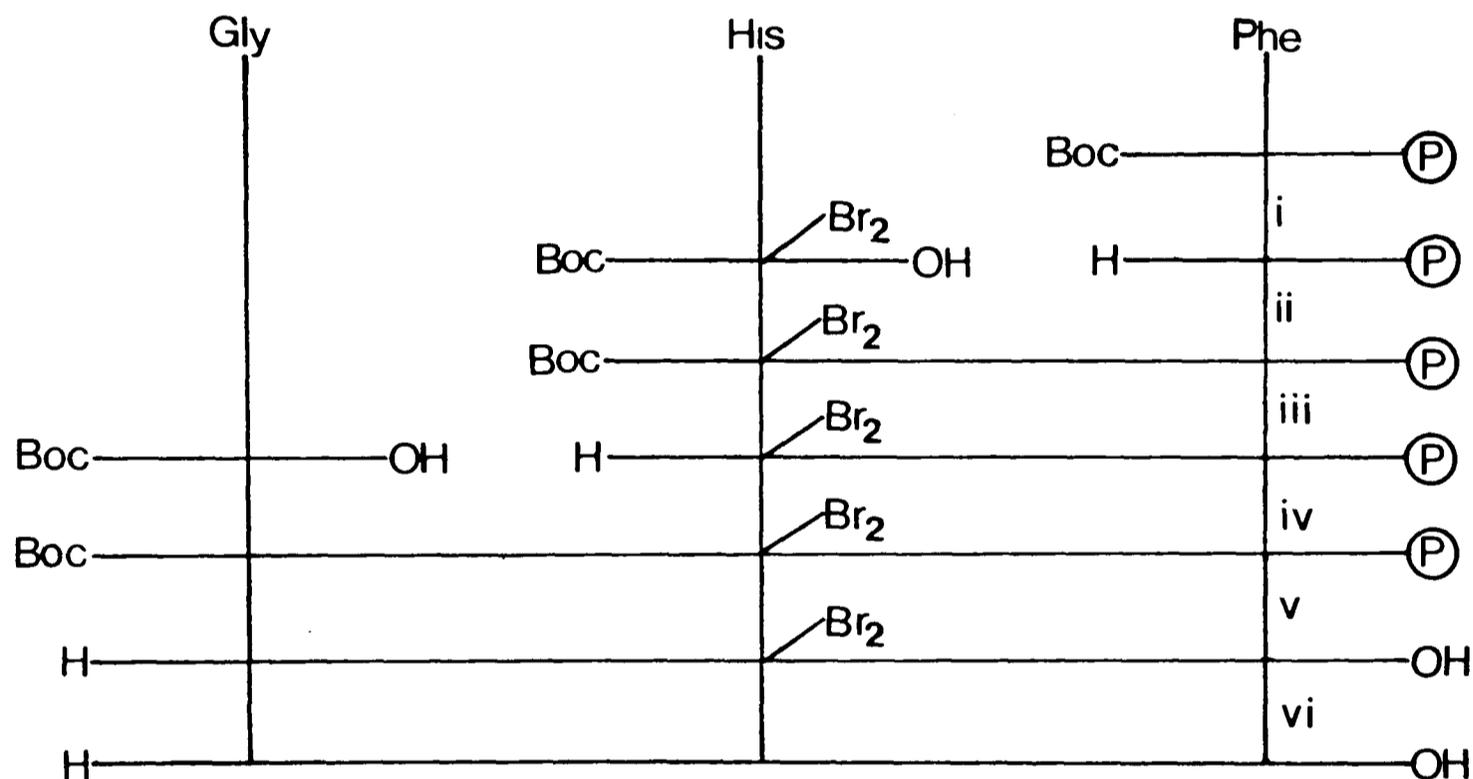


i) 40%v/v-TFA-CH$_2$Cl$_2$ ii) DCCI-CH$_2$Cl$_2$ iii) 40%v/v-TFA-CH$_2$Cl$_2$ iv) DCCI-

CH$_2$Cl$_2$ v) HBr-TFA vi) 5%Rh/C-5%Pd/C-80%aq AcOH-Amberlite IR 45(AcO$^-$)

(56% overall yield).

Scheme 16

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

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[166] 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
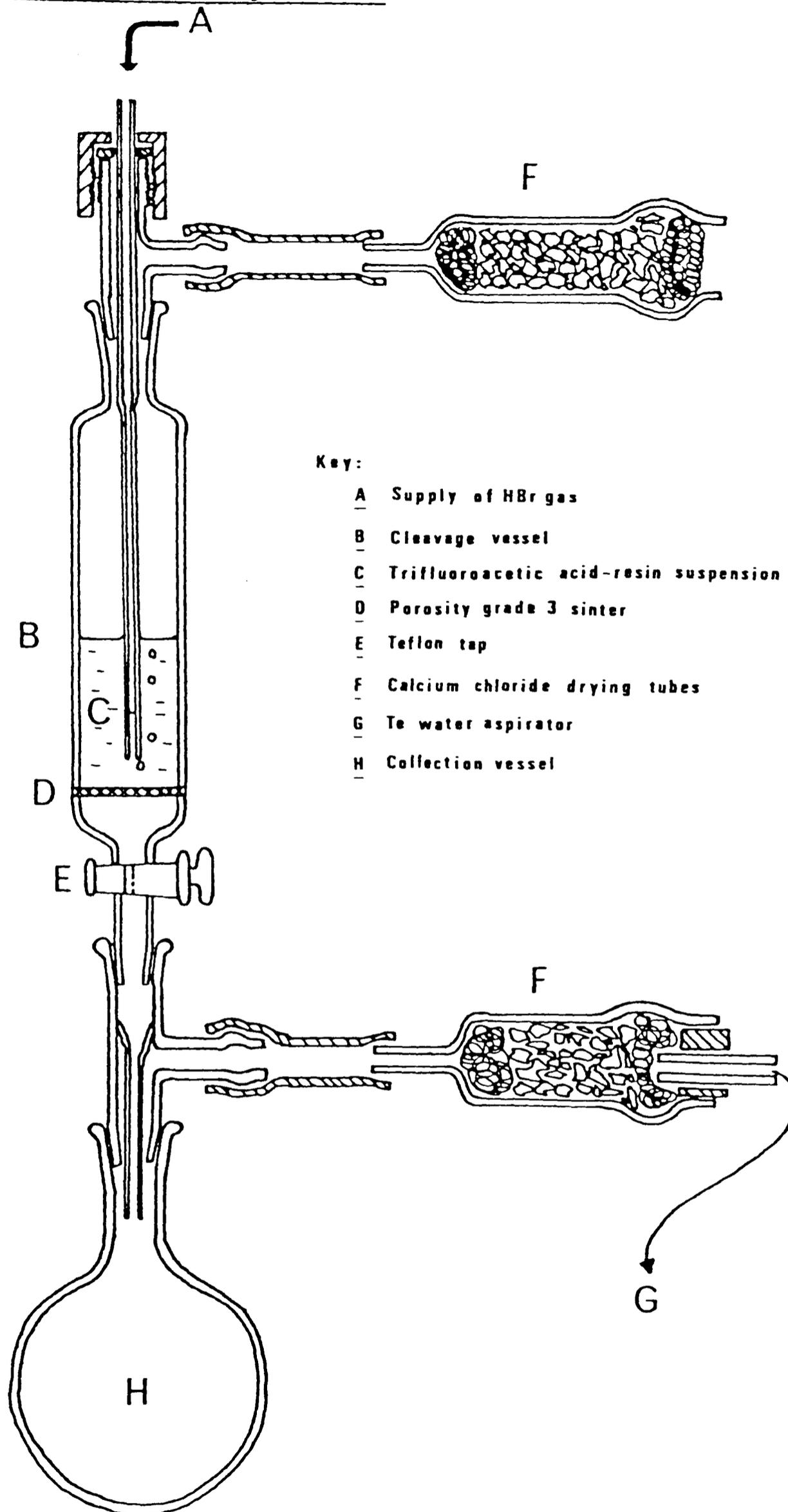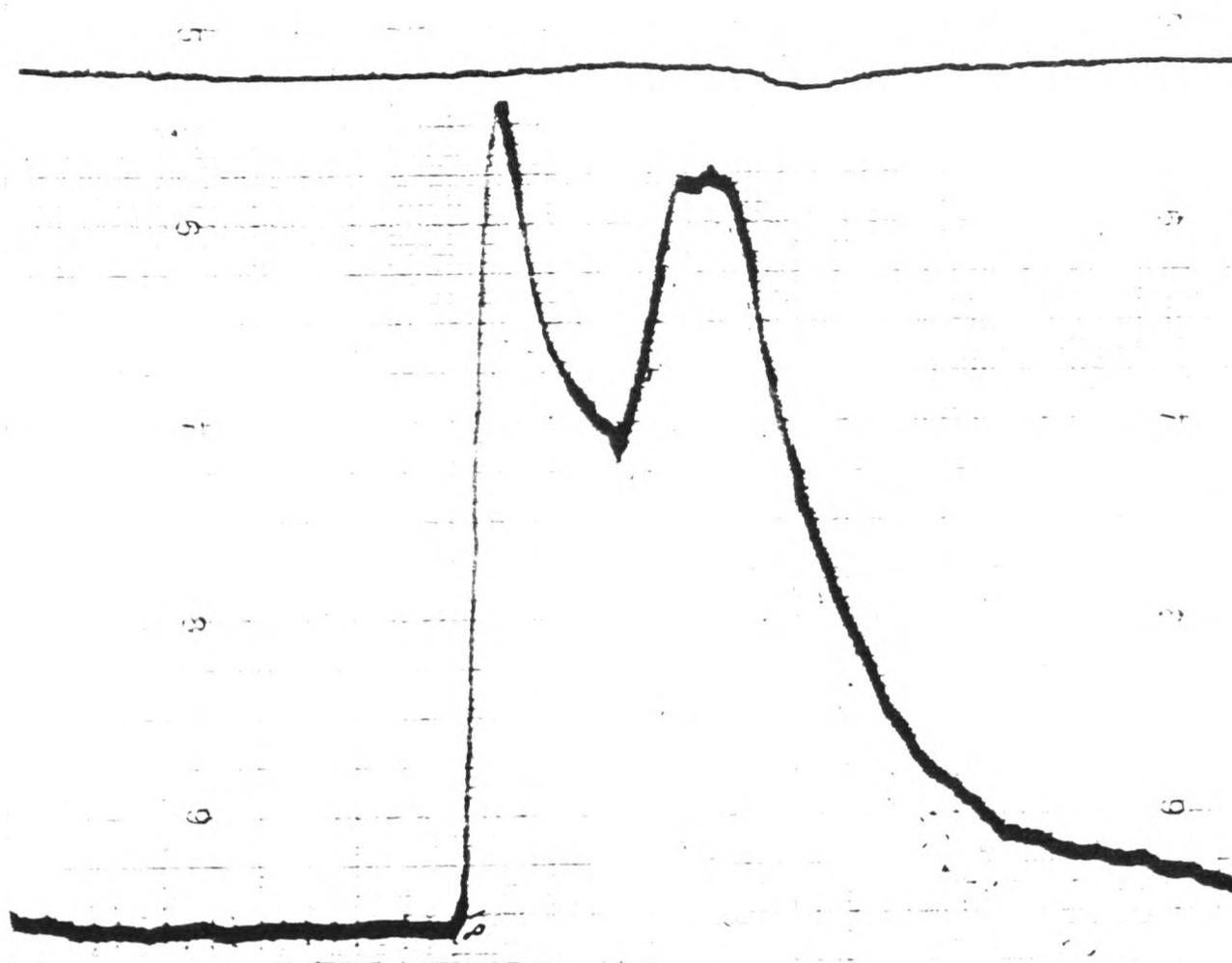
Figure 12

Figure 13

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.

$\underline{N}(\mathcal{A})$-t-Butoxycarbonyl-$\underline{\underline{L}}$-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

$\underline{\underline{L}}$-amino-acid oxidase indicated that (after correction for racemisation in

hydrolysis) 15% $\underline{\underline{D}}$-histidine was present.

Hence whilst halogenation significantly reduces the levels of

racemisation as compared with $\underline{N}(\mathcal{T})$-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

$\underline{N}(\alpha)$-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) $\underline{N}(\alpha)$-t-Butoxycarbonyl-$\underline{L}$-dibromohistidine was synthesied by a similar method to that used to prepare the diiodo-derivative. Unlike the reported preparation of 2,5-dibromohistidine the $\underline{N}(\alpha)$-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 $\underline{N}(\alpha)$-protected dibromohistidine derivative, the intermediate dipeptide was shown by enzyme digest to be optically pure.

4) The simple tripeptide glycyl-$\underline{L}$-histidyl-$\underline{L}$-phenylalanine was prepared by solid phase peptide synthesis in good yield from the $\underline{N}(\alpha)$-protected dibromohistidine derivative. The final product was exactly similar to authentic material. However after hydrolysis and digestion by $\underline{L}$-amino-acid oxidase the hydrolysate was shown to contain after correction 7% $\underline{D}$-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-histidine was found in the hydrolysate of the deprotected dipeptide after correction.

6) It was not possible to prepare N(α)-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 under go 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(τ)-protecting groups such as N(τ)-benzyl-L-histidine racemised significantly more. However the N(π)-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(π)-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(π)-protection, where no reasonable conditions have yet been established where racemisation occurs.

Hence N(π)-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(α)-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(∝)-t-Butoxycarbonyl,N(π)-

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 $\pi$-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 synthic pathways required for selective protection of the less available $\pi$-nitrogen, make $\underline{N}(\pi)$-protected derivatives complex and expensive to prepare.

Ramage[107] had tested a number of $\underline{N}$-imidazole derivatives none of which had appeared to be as useful for histidine protection as phenacyl, however Anderson and Groves[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 $\underline{N}(\alpha)$-t-butoxycarbonyl,$\underline{N}(\pi)$-$\underline{p}$-bromobenzyloxymethyl-$\underline{L}$-histidine via a synthic route analqgeous to that used in the preparation of the $\underline{N}(\pi)$-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 $\underline{N}$-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 $\underline{N}(\pi)$-protected material in trifluoroacetic acid as well as under amino-acid hydrolysis conditions. A number of simple tripeptides
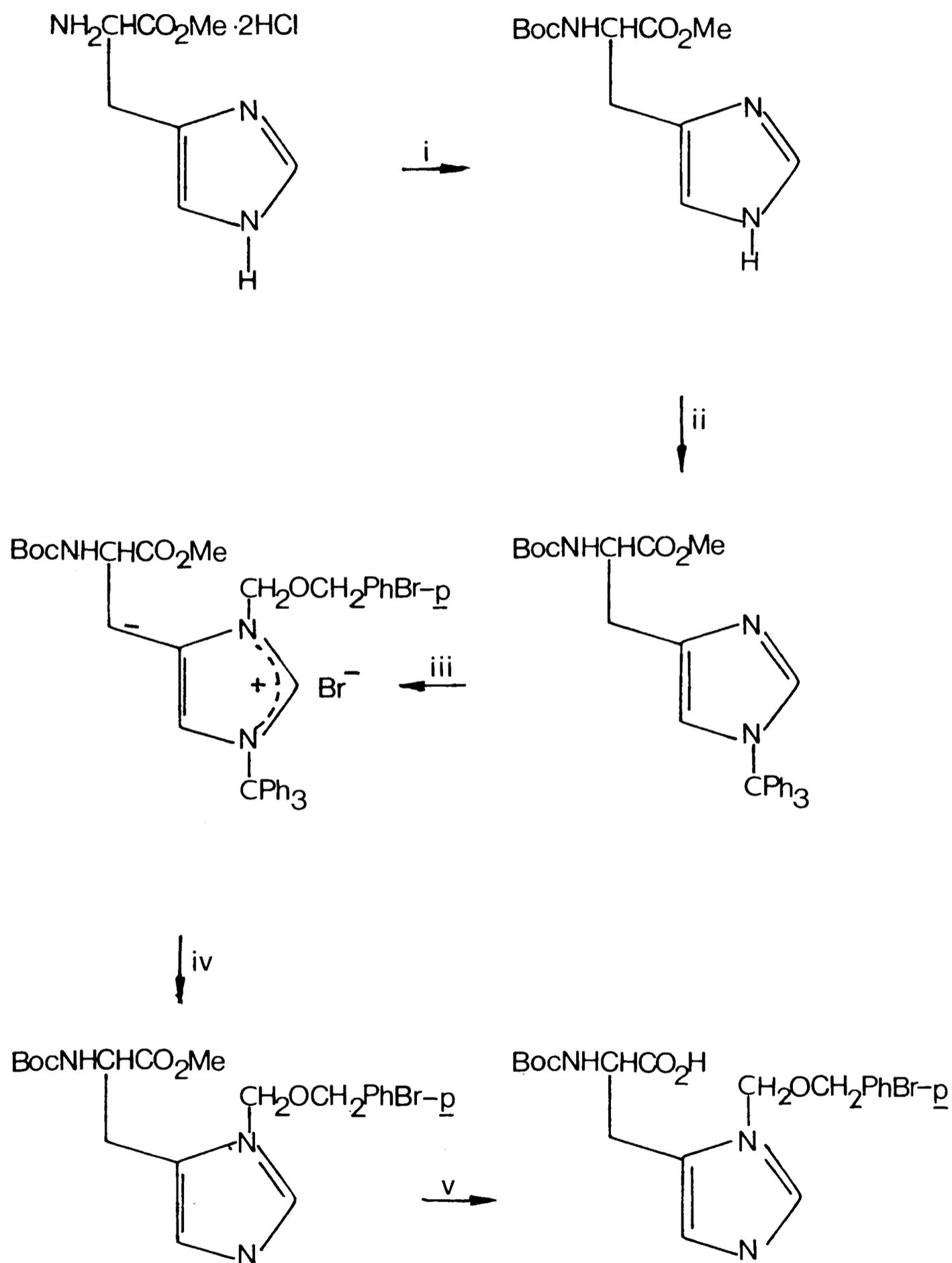
i) $Boc_2O$-MeOH-$Et_3N$   ii) $Et_2O$-$Ph_3CCl$   iii) $\underline{p}BrPhCH_2OCH_2Br$-$Et_2O$   iv) AcOH-

$H_2O$-IR 45(AcO$^-$)   v) NaOH-$H_2O$-MeOH

Scheme 17

were synthesised including thyroliberin and glycyl-$\underline{L}$-histidyl-$\underline{L}$-

phenylalanine, all of which were easily prepared with retention of

optical purity[166]. Both the $\underline{N}$($\pi$)-protected amino-acid, (which is

readily soluble in cold chloroform) and the peptide intermediates had

convenient physical properties.

However whilst $\underline{N}$($\pi$)-benzyloxymethyl 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

$\underline{N}$($\tau$)-histidine derivatives.

In the course of preparing $\underline{N}$($\alpha$)-t-butoxycarbonyl-$\underline{L}$-histidine

methyl ester Brown had isolated the $\underline{N}$($\alpha$),$\underline{N}$($\tau$)-bis-t-butoxycarbonyl-

$\underline{L}$-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 $\underline{N}$($\tau$)-

triphenylmethyl-histidine derivative. Benzyl-chloromethyl ether was

reacted with $\underline{N}$($\alpha$),$\underline{N}$($\tau$)-bis-t-butoxycarbonyl-$\underline{L}$-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 $\underline{N}$($\alpha$)-t-butoxycarbonyl,$\underline{N}$($\pi$)-benzyloxymethyl-

$\underline{L}$-histidine methyl ester as well as large amounts of material having a

lower $R_F$ value. Separation by 'flash' chromatography[159] gave the desired

product in 34% yield[166, 170] (Scheme 18).

The $\underline{N}$($\pi$)-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

$NH_2CHCO_2Me \cdot 2HCl$

$BocNHCHCO_2Me$

Boc

ii,iii

$BocNHCHCO_2Me$   $CH_2OCH_2Ph$
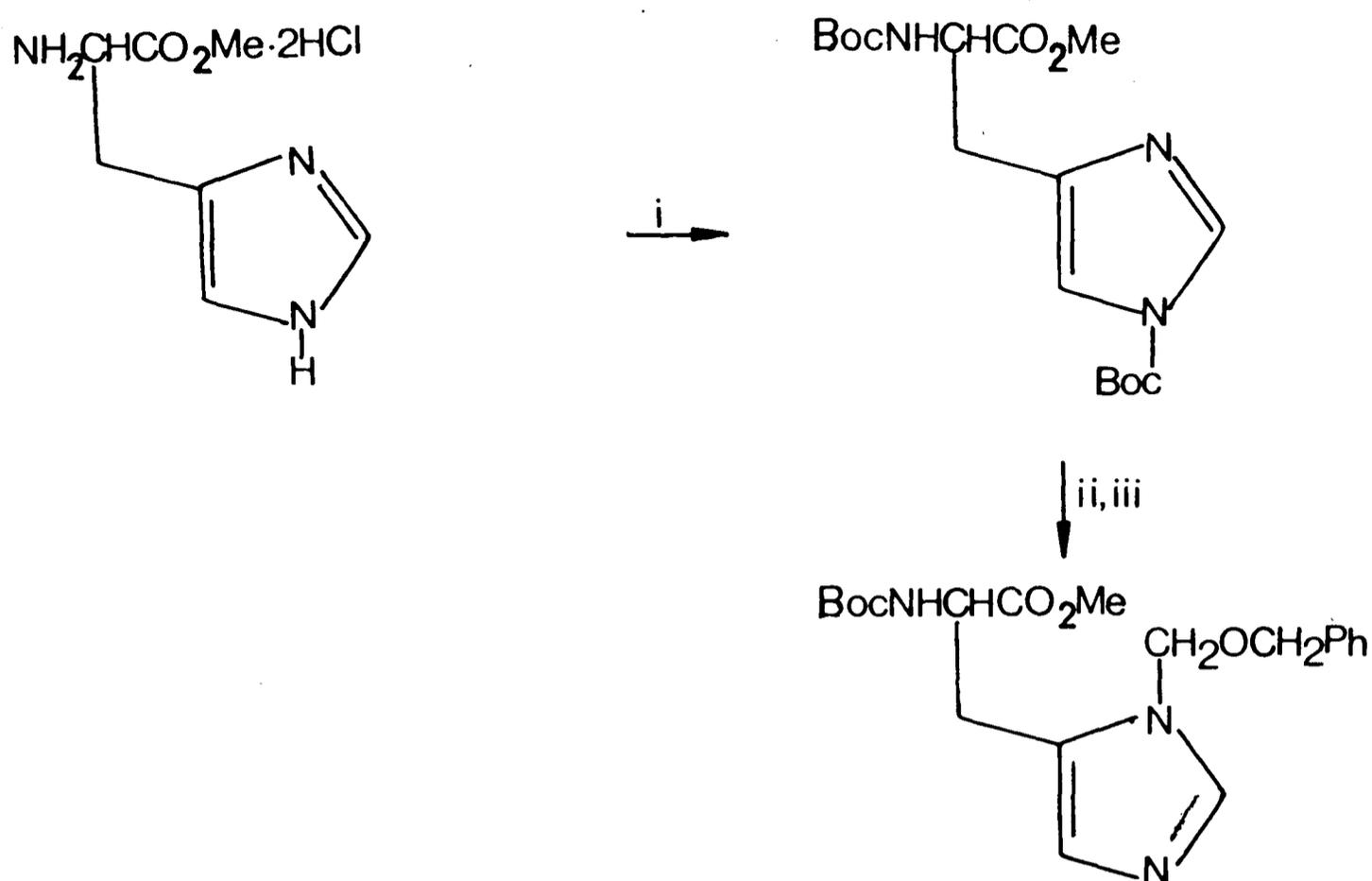
i) $Boc_2O-Et_3N-CH_3OH$   ii) $PhCH_2OCH_2Cl-CH_2Cl_2$   iii) $Et_3N-CH_3OH-Column$
chromatography (yield 34%)

Scheme 18

systematically to try and improve the yields at each step and also to

produce the N(π)-histidine protected methyl ester without the need for

column chromatography.

## Synthesis of N(α),N(τ)-Bis-t-butoxycarbonyl-L-histidine Methyl Ester.

N(α),N(τ)-Bis-t-butoxycarbonyl-L-histidine methyl ester has been

described and characterized in the literature as a by-product isolated

during the preparation of N(α)-t-butoxycarbonyl-L-histidine methyl ester[171].

Brown had obtained his sample of the N(α),N(τ)-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 N(α),N(τ)-protected histidine derivative as the sole

product.

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($\alpha$),N($\tau$)-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($\alpha$)-t-Butoxycarbonyl,N($\pi$)-benzyloxymethyl-L-histidine Methyl Ester.

It was decided to repeat Brown's synthesis of N($\alpha$)-t-butoxycarbonyl, N($\pi$)-benzyloxymethyl-L-histidine methyl ester with slight modifications to see if by carring 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[166] 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($\pi$)-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 $\underline{N}(\alpha),\underline{N}(\tau)$-bis-t-butoxycarbonyl-$\underline{\underline{L}}$-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 monitered, 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 $\underline{N}(\pi)$-protected derivative to form the stable $\underline{N}(\alpha)$-t-butoxycarbonyl,$\underline{N}(\tau),\underline{N}(\pi)$-bis-benzyloxymethyl-$\underline{\underline{L}}$-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 $\underline{N}$($\alpha$),$\underline{N}$($\tau$)-

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 $\underline{N}$($\alpha$)-t-Butoxycarbonyl,$\underline{N}$($\pi$)-benzyloxymethyl-$\underline{L}$-histidine.

On the basis of these results it was decided to attempt to prepare

the $\underline{N}$($\pi$)-histidine protected derivative by reacting 1.6 equivalents of the

alkylating agent with the $\underline{N}$($\alpha$),$\underline{N}$($\tau$)-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 precititated 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(α)-

t-butoxycarbonyl,N(π)-benzyloxymethyl-L-hisitidine 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).
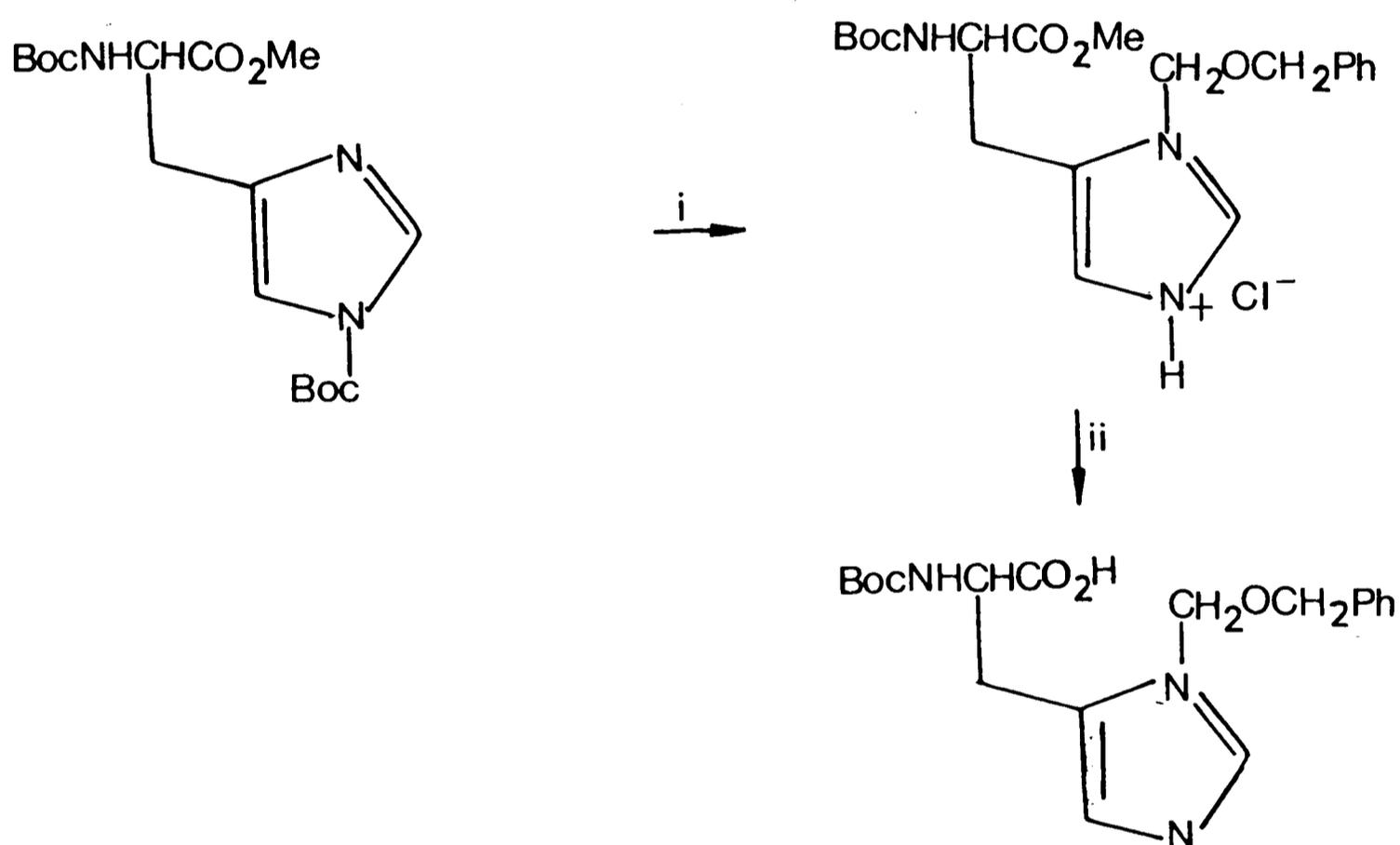


i) $PhCH_2OCH_2Cl-CH_2Cl_2$ (69% yield)   ii) $NaOH-H_2O-MeOH$ (87% yield)

Scheme 19

Results and Conclusion.

1)  N(α),N(τ)-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(α)-t-butoxycarbonyl,N(π)-

    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(α),N(τ)-bis-t-butoxycarbonyl-L-histidine methyl
ester was indefinitely stable if stored below 0°, however benzyl-
chloromethyl ether was liable to decompose unless freshly distilled
and then stored below 0°.

4) Using freshly-prepared starting materials N(α)-t-butoxycarbonyl,
N(π)-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(α),N(τ)-bis-t-butoxycarbonyl-L-histidine methyl
ester in dichloromethane.

We had therefore succeeded in preparing a N(π)-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.
Further more the N(π)-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(π)-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)-Benzyloxymethyl Histidine

Introduction.

Having demonstrated that N(∝)-t-butoxycarbonyl,N(π)-benzyloxymethyl-

L-histidine could be prepared in good yield by a simple three-stage

synthesis the incorporation of N(π)-benzyloxymethyl-protected histidine

derivatives into peptides had to be thoroughly investigated.

Brown had previously prepared thyroliberin using N(π)-

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(π)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(π)-phenacyl-

containing peptides.

Thus in this case it was felt that whilst N(π)-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[175] and on a solid support.[172-174] 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(π)-benzyloxycarbonyl protecting group.

In our previous attempts at solid phase peptide synthesis we had used the apparatus developed by Turner.[161] 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(α)-t-butoxycarbonyl-L-phenylalanine was esterified with a chloromethylated 1% crosslinked Merrifield polystyrene resin according to the method of Gisin[160] to give (after washing) a

Solid Phase Peptide Synthesis Apparatus
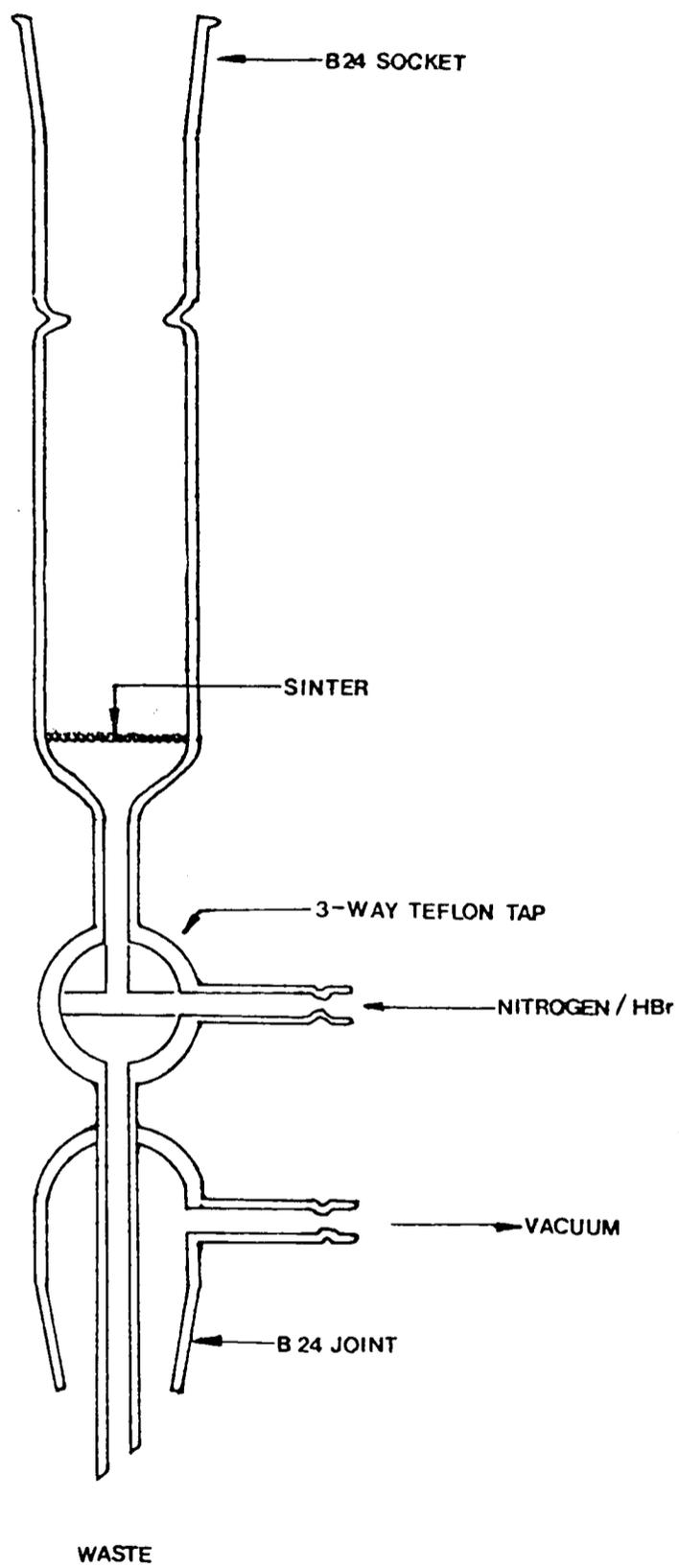


Figure 14
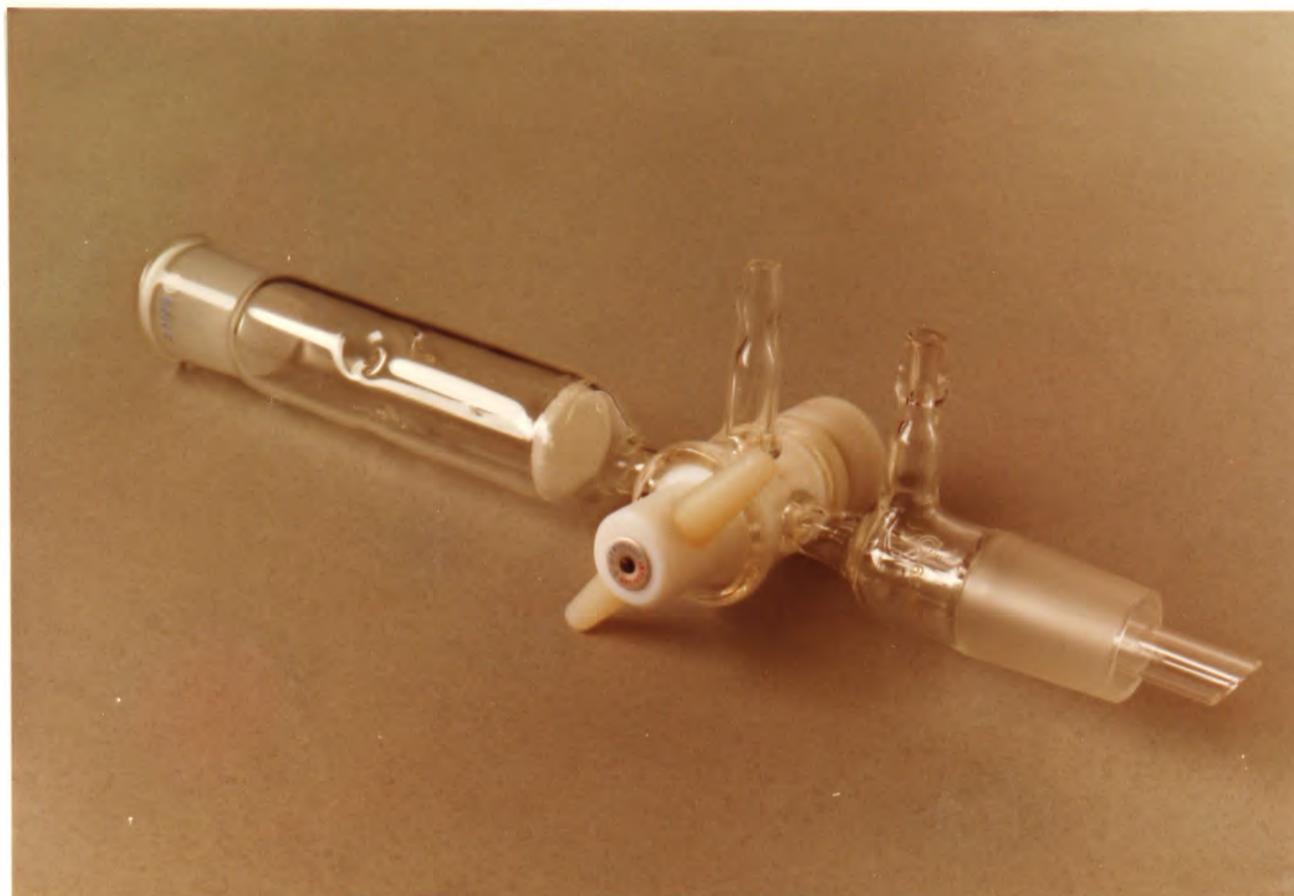
Plate 2

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. $N(\alpha)$-

Deblocking was effected using 40% trifluoroacetic acid in dichloromethane

which was followed by neutralisation with triethylamine. The $N(\alpha)$-

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[8]. 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[176] 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-benzyltyrosine[177].had been shown to occur under the standard conditions for removal of N($\alpha$)-t-butoxycarbonyl groups with trifluoroacetic acid in the case of O-benzyl-tyrosine[177]. Erickson and Merrifield demonstrated that the more stericlly hindered 2,6-dichlorobenzyl-protected derivative was much less susceptible to this rearrangement[177]. 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[178].

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

Phe  Pro  His  Ile  Tyr  Val  Arg  Asp

i       ii      iii     iv      v       vi      vii     viii    ix      x       xi      xii     xiii    xiv     xv      xvi

Boc  H  OH  Boc  H  πBom  OH  Bzl(Cl₂)  NO₂  Bzl  OH

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

Scheme 20

residues proceeded smoothly with amino-acid analysis indicating

complete incorporation of the terminal residues. After addition of

N(α)-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 reamainder 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(ω)-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 aquire 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

Phe

Gly

His

His

His

i
ii
iii
iv
v
vi
vii
viii
ix
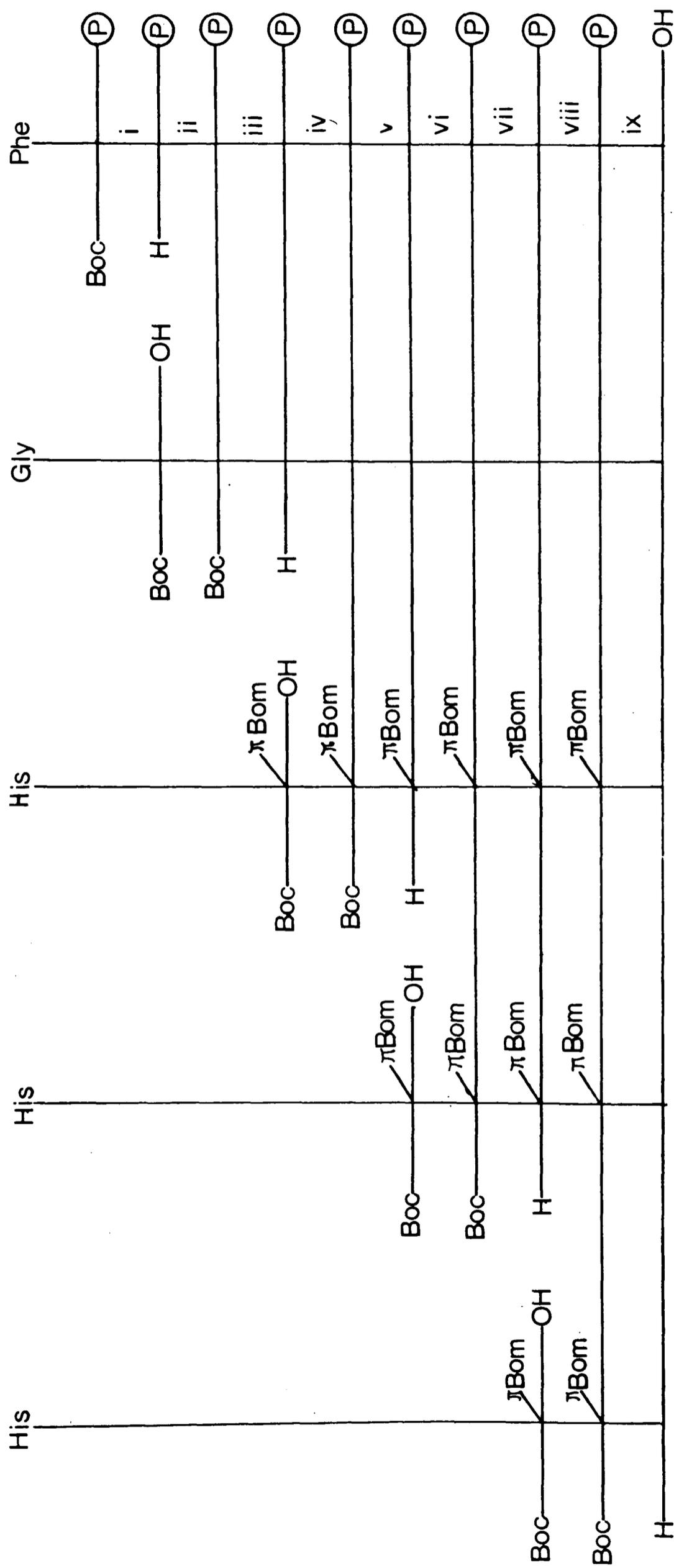
i) 40%v/v-TFA-CH$_2$Cl$_2$   ii) DCCI-CH$_2$Cl$_2$   iii) 40%v/v-TFA-CH$_2$Cl$_2$   iv) DCCI-CH$_2$Cl$_2$   v) 40%v/v-TFA-CH$_2$Cl$_2$

iv) DCCI-CH$_2$Cl$_2$   vii) 40%v/v-TFA-CH$_2$Cl$_2$   viii) DCCI-CH$_2$Cl$_2$   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 L-Histidyl-L-histidyl-L-histidyl-glycyl-L-phenylalanine
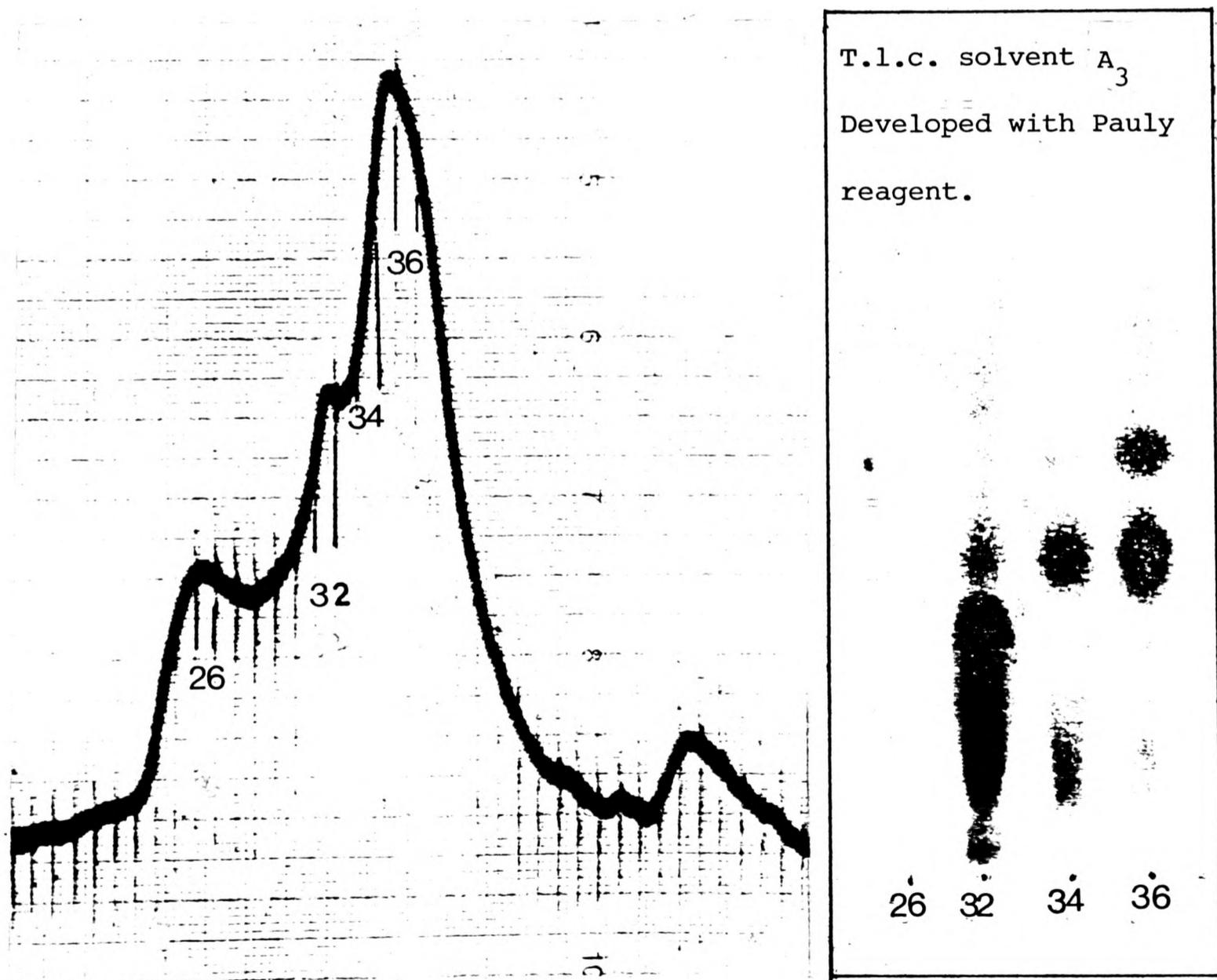


Figure 15

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 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 N(π)-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(∝)-t-Butoxycarbonyl,N(π)-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

synthesis. 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(π)-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(α)-t-butoxycarbonyl,N(π)-benzyloxymethyl-L-

histidyl-L-prolyl-L-phenylalanine benzyl ester had been prepared

according to Scheme 22.
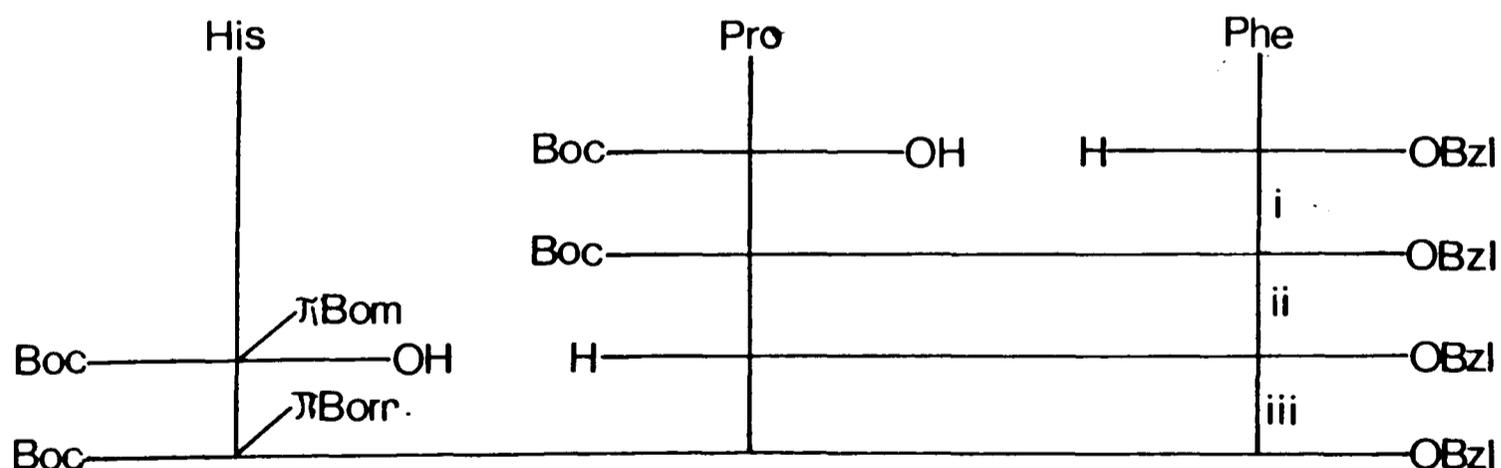


i) DCCI-CH$_2$Cl$_2$-HOBt-Et$_3$N   ii) TFA   iii) DCCI-HOBt-Et$_3$N-CH$_2$Cl$_2$

Scheme 22

L-Phenylalanine benzyl ester p-toluenesulphonate was prepared

in good yield by the method of Zervas et al[180] and coupled without

problem to N-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($\alpha$)-deblocking with

trifluoroacetic acid the dipeptide was coupled with the N($\pi$)-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($\pi$)-

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[181] 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($\pi$)-

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.[182] 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(π)-protected histidine and tryptophan in order

to ensure both that the reported reaction of free tryptophan with

formaldehyde[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(α)-t-Butoxycarbonyl,N(π)-benzyloxymethyl-L-histidyl-L-

tryptophan methyl ester was prepared and characterized in good yield

followed by the hydrogenolysis of the N(π)-protecting group to give

the partially deprotected dipeptide (Scheme 23).
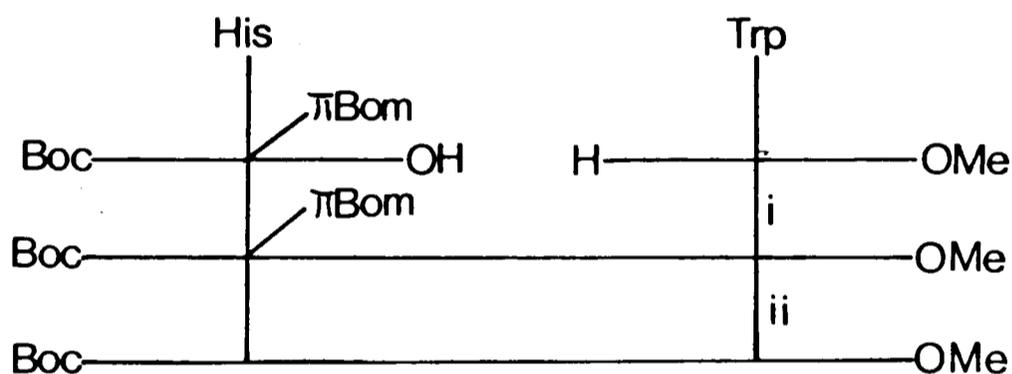


i) DCCI-HOBt-Et$_3$N-DMF   ii)H$_2$-Pd/C-80%aq AcOH

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(α)-Deblocking was accomplished

by treating the dipeptide with trifluoroacetic acid for 15 min. After

coupling with $\underline{N}(\alpha)$-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 $\underline{N}(\alpha)$-t-butoxycarbonyl-blocking group of histidine.

This reaction is well-documented in the literature[184] and would be

totally independent of the nature of the histidine side-chain protecting

group.

Parallel to this work Brown had prepared the $\underline{N}(\alpha)$-benzyloxycarbonyl

and the $\underline{N}(\alpha)$-fluorenylmethyloxycarbonyl,$\underline{N}(\pi)$-benzyloxymethyl

derivatives, as well as $\underline{L}$-histidyl-$\underline{L}$-histidine and $\underline{L}$-histidyl-$\underline{L}$-

histidyl-$\underline{L}$-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 $\underline{L}$-tryptophyl-$\underline{L}$-histidyl-$\underline{L}$-tryptophan

methyl ester.

Results and Conclusion.

1) $\underline{L}$-Tyrosyl-$\underline{L}$-isoleucyl-$\underline{L}$-histidyl-$\underline{L}$-prolyl-$\underline{L}$-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(α)-deblocked tetrapeptide in the solvents used in our coupling cycles.

4) N(α)-t-Butoxycarbonyl,N(π)-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(α)-t-Butoxycarbonyl-L-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(α)-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(α)-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 L- or D- amino-acid oxidase has proved the most straightforward and accurate technique.[107] Jones and Ramage[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 L-amino-acid oxidase, the peptide hydrolysate then undergoing amino-acid analysis with D-arginine added as an internal standard. This method (based on refinements of previous digestion methods using L-amino-acid oxidases[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 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[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.[105] After amino-

acid analysis it was possible to calculate the percentage of D-histidine present in each sample (Table 4).

| Derivative | % D-Histidine (±5%) | |
|---|---|---|
| | Measurement by Enzyme Digest | Measurement by Change in Optical Rotation |
| N(α)-t-Butoxycarbonyl,N(τ)-benzyl-L-histidine | 45 | 44 |
| N(α)-t-Butoxycarbonyl-L-2,5-diiodohistidine | 20 | 30 |
| N(α)-t-Butoxycarbonyl-L-2,5-dibromohistidine | 15 | – |

Table 4

It can be seen that N(α)-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 O-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 O-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 N-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
N-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 $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-histidine,

$\underline{N}(\alpha)$-t-butoxycarbonyl-2,5-diiodohistidine and $\underline{N}(\alpha)$-t-butoxycarbonyl,

$\underline{N}(\tau)$-benzyl-$\underline{L}$-histidine. In each case after 2 h a t.l.c. of the

activated mixture revealed a component that had a higher $R_F$ 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 $\tau$-benzyl-

derivative the value for the optical rotation dropped rapidly. After

2 h it had reached a level consistent with the 45% $\underline{D}$-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 $\underline{D}$-

histidine (30%) is present than was suggested by enzyme digest (20%).

In the case of the $\underline{N}(im)$-unprotected histidine very little decrease

in optical activity was observed; this is in accordance with Veber's

N($\alpha$)-t-Butoxycarbonyl-L-histidine

N($\alpha$)-t-Butoxycarbonyl-L-2,5-diiodohistidine

N($\alpha$)-t-Butoxycarbonyl,N($\tau$)-benzyl-L-histidine

● N($\alpha$)-t-Butoxycarbonyl-L-histidine

○ N($\alpha$)-t-Butoxycarbonyl-L-2,5-diiodohistidine

+ N($\alpha$)-t-Butoxycarbonyl,N($\tau$)-benzyl-L-histidine

Time min.

Figure 16

% of Initial Rotation
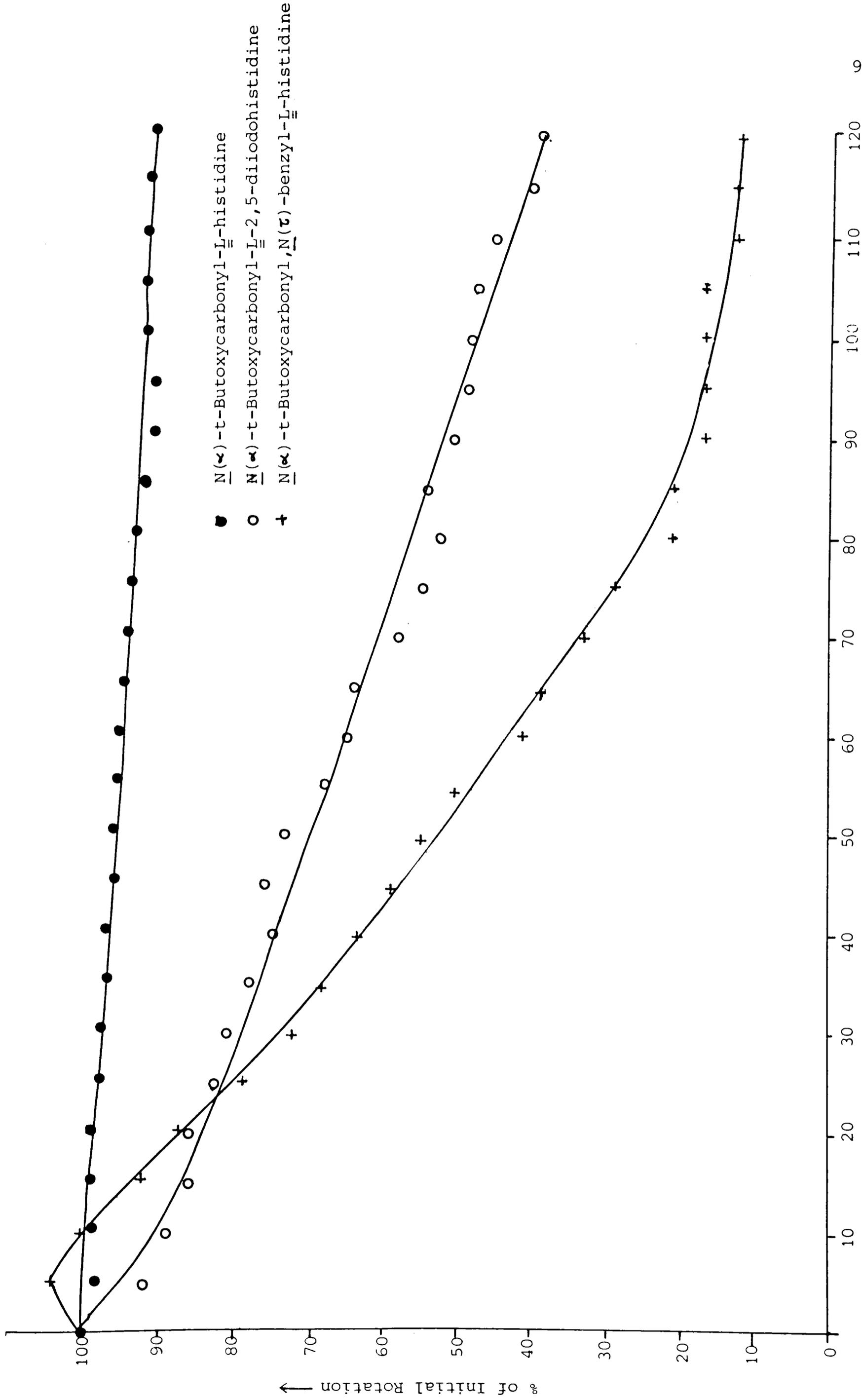
hypothesis that the imidazolium ring is formed which then undergoes comparatively slow racemisation.[45]

A further series of experiments was carried out to investigate the comparative rates of racemisation of $\underline{N}$(τ)- and $\underline{N}$(π)-protected histidine derivatives. Brown had prepared both the τ- and π-isomers of $\underline{N}$(α)-t-butoxycarbonyl,$\underline{N}$(im)-p-bromobenzyloxymethyl-$\underline{L}$-histidine and $\underline{N}$(α)- t-butoxycarbonyl,$\underline{N}$(im)-benzyloxymethyl-$\underline{L}$-histidine. The τ-isomers were both prepared by reaction of the alkylating agent with the unprotected imidazole ring on $\underline{N}$(α)-t-butoxycarbonyl-$\underline{L}$-histidine methyl ester. In each case the product was purified by column chromatography. The τ- and π-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 $\underline{N}$-acylurea. An attempted comparison of $\underline{N}$(τ)- and $\underline{N}$(π)-phenacyl-histidine using this method had to be abandoned when the $\underline{N}$(π)-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 $\underline{N}$-acylurea.

However in the other two comparisons the changes in optical rotation of the τ- and π-isomers produced very clear results (Figures 17 and 18). It can be seen that in both cases the rotation of the $\underline{N}$(π)-

N($\alpha$)-t-Butoxycarbonyl,N($\tau$)-p-bromobenzyloxymethyl-L-histidine

N($\alpha$)-t-Butoxycarbonyl,N($\tau$)-p-bromobenzyloxymethyl-L-histidine

Time min.
Figure 17

% of Initial Rotation

Time min.

N($\tau$)-t-Butoxycarbonyl,N($\pi$)-benzyloxymethyl-L-histidine

N($\pi$)-t-Butoxycarbonyl,N($\tau$)-benzyloxymethyl-L-histidine
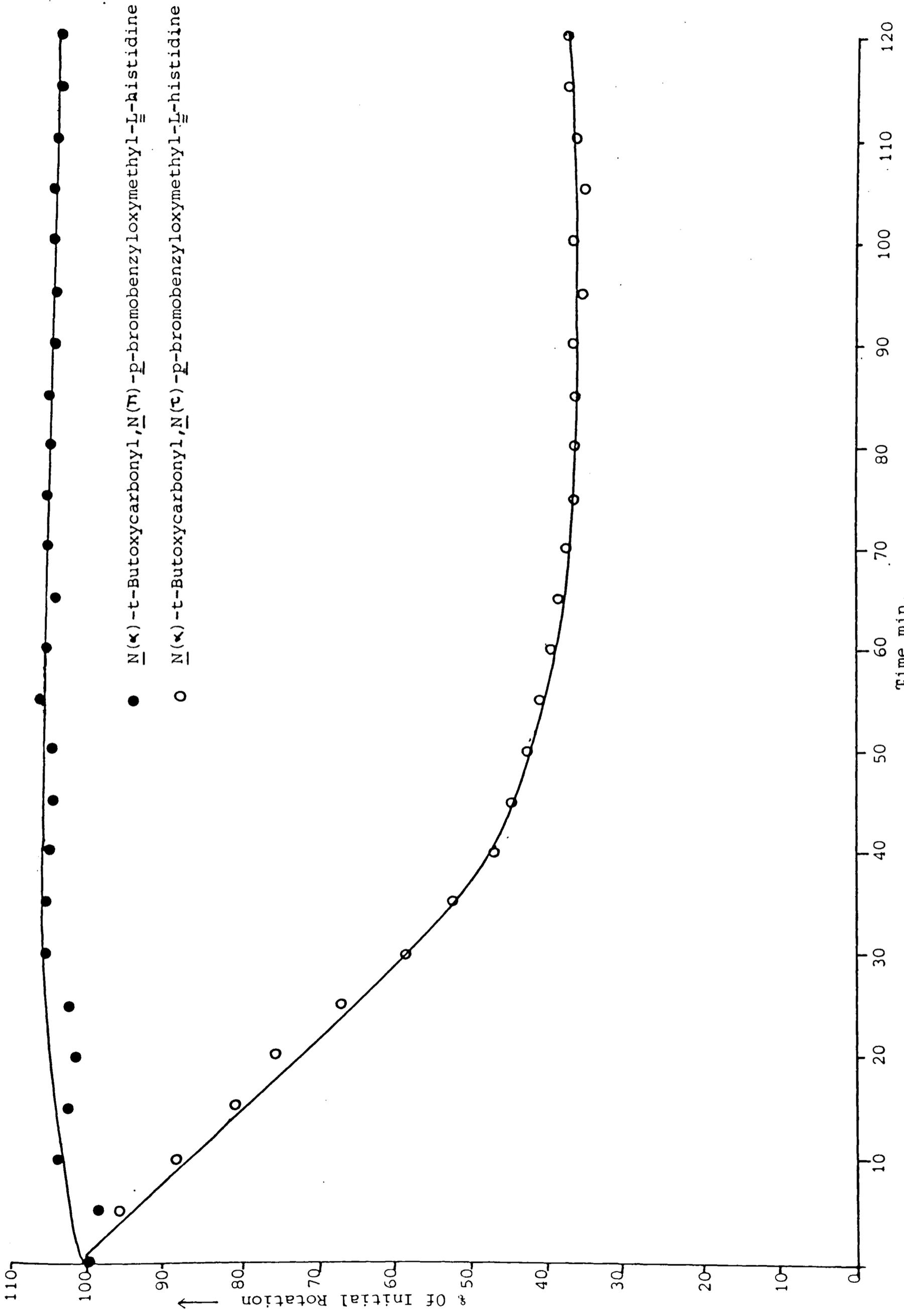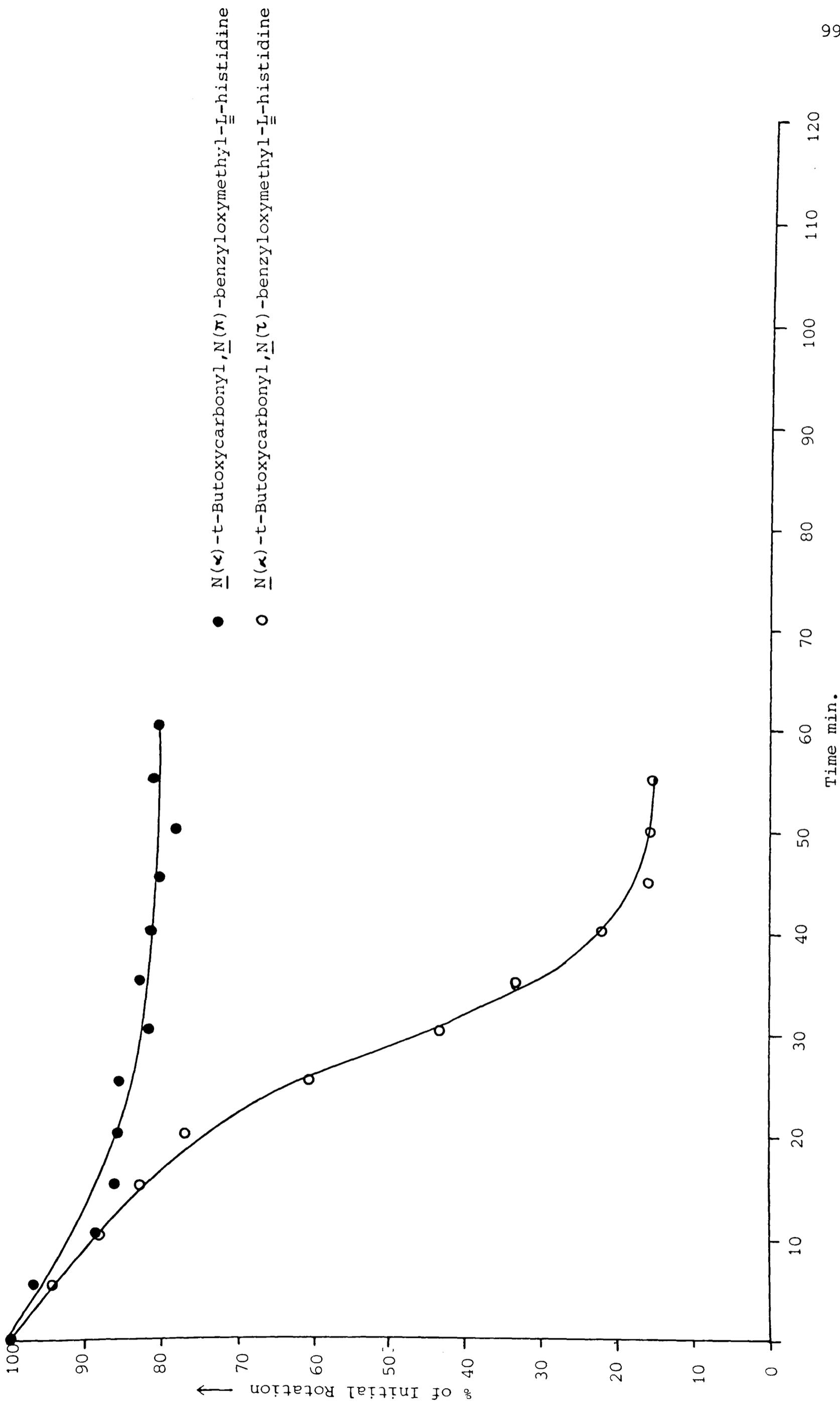
isomer changes a little initially but then remains remarkably stable,

whilst the optical acivity of the two $\underline{N}(\Upsilon)$-isomers drops rapidly.

These results confirm the trend discovered by Ramage[105] after

enzyme digest of the $\underline{N}(\tau)$- and $\underline{N}(\pi)$-phenacyl isomers indicated that

blockade of the $\underline{N}(\pi)$-nitrogen suppresses racemisation, whilst the

same group on the $\Upsilon$-position results in gross racemisation. Hence these

results are consistent with Veber's theory and preliminary work

conducted in this laboratory: that only the $\pi$-nitrogen of imidazole

catalyses intramolecular racemisation. Further evidence for this is

provided by molecular space-filling models of $\underline{N}(\tau)$-benzyloxymethyl-

$\underline{L}$-histidine and $\underline{N}(\pi)$-benzyloxymethyl-$\underline{L}$-histidine (Plate 3) which

demonstrate that whilst the $\underline{N}(\pi)$-nitrogen could easily abstract the

$\alpha$-proton or form the imidazolium ring, the $\underline{N}(\tau)$-nitrogen is situated

too far from the $\alpha$-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 $\underline{N}$-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 $\underline{N}$-

acylurea.

Results and Conclusion.

1) 2,5-Diiodohistidine was shown by enzyme digest to be significantly

less prone to racemisation on activation than $\underline{N}(\tau)$-benzyl-$\underline{L}$-

histidine.
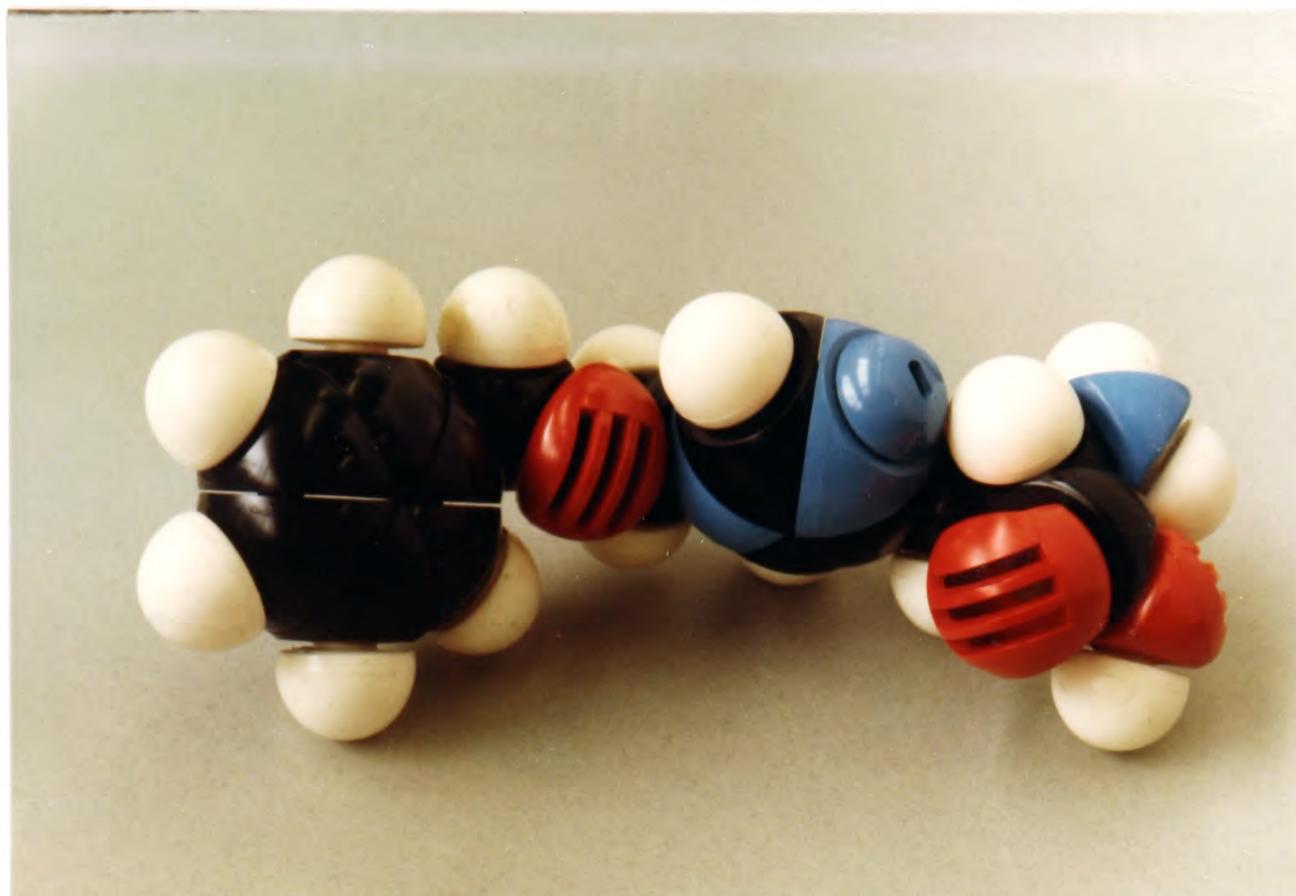
N(τ)-Benzyloxymethyl-L-histidine



N(π)-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 $\underline{N}$(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 $\pi$- and τ-isomers indicated that the $\pi$-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 Köfler hot-stage apperatus 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 $\delta$ 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 6$\underline{M}$ 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$^n$OH:AcOH:H$_2$O          4:1:5

(2) A$_3$      Bu$^n$OH:AcOH:H$_2$O          4:1:1

| | | | |
|---|---|---|---|
| (3) | $E_3$ | MeOH:CHCl$_3$ | 1:4 |
| (4) | $E_3$ | MeOH:CHCl$_3$ | 1:19 |
| (5) | $P_1$ | CHCl$_3$:MeOH:AcOH | 17:2:1 |
| (6) | $P_2$ | CHCl$_3$:MeOH:AcOH | 10:2:1 |
| (7) | CMAW | CH$_2$Cl$_2$:MeOH:AcOH:H$_2$O | 15:10:2:3 |
| (8) | X | EtOAc:MeOH:Et$_3$N | 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 imediately 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:
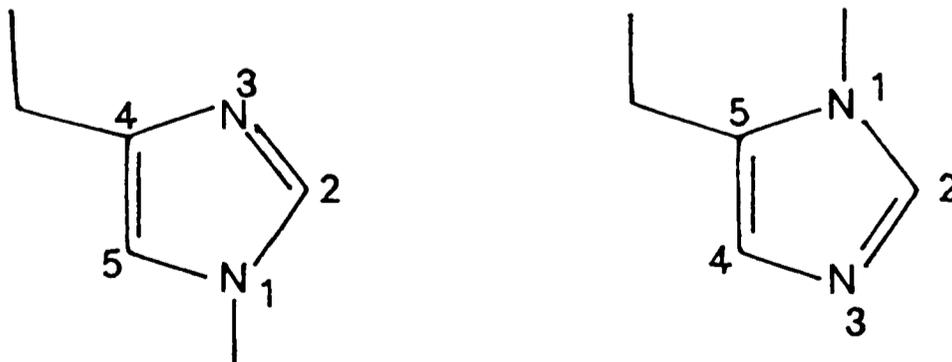
CHAPTER 1


Experimental Section


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


in Peptide Synthesis

Attempted Electrolytic Deprotection of L-Pyroglutamyl,N(π)-phenacyl-
L-histidyl-L-prolineamide.

The protected tripeptide as prepared by Ramage[107] 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.

| TIME | CURRENT |
|---------|---------|
| Start | 100 mA |
| 1 min | 10 mA |
| 10 min | 8 mA |
| 20 min | 1.5 mA |
| 60 min | 0.5 mA |
| 120 min | 0.5 mA |

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 $R_F$(CMAW) of 0.25 on t.l.c. to an authentic sample

of thyroliberin, significant amounts of another Pauly active contaminant

$R_F$(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(π)-Protecting Group for Histidine

Attemped 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 1$\underline{M}$ 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, $R_F$(X) 0.54;

$\delta$(CDCl$_3$) 4.1(2H, d, (J=5 Hz), -C$\underline{H}_2$Cl), 6.3(2H, complex, -C$\underline{H}$C$\underline{H}$-).

Attempt C   N(Trimethylsilyl)imidazole was prepared by the normal method

from imidazole[128] (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 reation 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[133] 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, 60mmol) 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°, $((CD_3)_2SO)$ 5.05(2H, d, J=5 Hz, $-C\underline{H}_2CH-)$, 6.6(2H, complex,

$-C\underline{H}C\underline{H}-)$, 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.

$C_9H_9N_2Cl_3O_4$ requires C, 34.25; H, 2.87; N. 8.87; Cl, 33.75%).

## $\underline{N}(1,1,1$-Trichlorobut-2-enyl)imidazole.

$\underline{N}(1,1,1$-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 $\underline{N}(1,1,1$-trichlorobut-2-enyl)imidazole

as a colourless oil (73 mg, 87%) of $R_F(E_3)$ 0.70; $M^+$ found 224, 226, 228,

230 $C_7H_7N_2Cl_3$ requires: 224, 226, 228, 230; $_{max}215$ nm; $\delta(CDCl_3)$ 4.7

(2H, dd, J=5.3 Hz and 1.7 Hz, $-C\underline{H}_2CH-)$, 6.0-6.1(1H, dt, J=14.7 Hz and

1.1 Hz, $-C\underline{H}CCl_3)$, 6.3-6.4(1H, dt, J=14.7 Hz and 5.3 Hz, $-CH_2C\underline{H}-)$, 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 $N(1,1,1$-Trichlorobut-2-enyl)imidazole using Zinc in Acetic Acid.

$\underline{N}(1,1,1$-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 $R_F(X)$ 0.57, and a component having an $R_F(X)$ 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

$\delta$ 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-enyl)imidazole

having $R_F(E_3)$ 0.76; $\nu_{max}$ 215 nm; $M^+$ found 224, 226, 228 $C_7H_7N_2Cl_3$

reqires: 224, 226, 228, 230; $(CDCl_3)$ 4.2(2H, dd, J=6.1 Hz and 0.92 Hz,

-CH$_2$CH-), 4.9(1H, dt, J=9.74 Hz and 6.0 Hz, -CHCH$_2$-), 5.90(1H, d,

J=9.74 Hz, -CHCCl$_3$), 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-enyl)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-enyl)imidazole

as a colourless oil. After dissolving in deuterochloroform (0.5 ml)

benzylamine (33 µl, 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-enyl)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.$(E_3)$ 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 $\underline{N}$(1,1,1-dichloro-1,3-

dienyl)imidazole, having $R_F(E_3)$ 0.64; $\nu_{max}$ 273 nm; $M^+$ found 188, 190, 192

$C_7H_6N_2Cl_2$ requires 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=7$H_2$, -CHC$H_2$-), 4.2(1H, m, -NDC$H$-).

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

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 μl) 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.

| Reagent | Stability |
|---------|-----------|
| 1$\underline{M}$ Sodium Hydroxide | Slight loss of starting amino-acid; no monoidohistidine or histidine formed |
| 90% aq TFA | Starting amino-acid unchanged |
| 45% w/v HBr/AcOH | Starting amino-acid unchanged |
| 6$\underline{M}$ Hydrochloric Acid | Partial loss of starting amino-acid; monoiodohistidine and histidine formed |

Table 6

$\underline{N}(\alpha)$-t-Butoxycarbonyl-$\underline{L}$-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 $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-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 1$\underline{M}$ sodium thiosulphate (5 x 50 ml), dried and evaporated to give a pale yellow oil. Trituration with ether gave $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-2, 5-diiodohistidine methyl ester as a yellow solid (4.99 g, 49%) of m.p. 89-91°; $[\alpha]_D^{20}$ +4.6° ($\underline{c}$ 1.0 in CHCl$_3$); R$_F$(E$_3$) 0.66; $\delta$(CDCl$_3$) 1.4(9H, s, (C$\underline{H}_3$)$_3$C-), 3.1(2H, d, -C$\underline{H}_2$CH-), 3.76(3H, s, C$\underline{H}_3$OCO-), 4.5(1H, complex, -NHC$\underline{H}$-), 5.5(1H, m, -N$\underline{H}$CH-); (Found: C, 28.01; H, 3.44; N, 7.98. C$_{12}$N$_3$O$_4$H$_{17}$I$_2$ requires C, 27.63; H, 3.26; N, 8.06%); M$^+$ found 521 C$_{12}$N$_3$O$_4$H$_{17}$I$_2$ requires: 521

$\underline{N}(\alpha)$-t-Butoxycarbonyl-$\underline{L}$-2,5-diiodohistidine.

1$\underline{M}$ Sodium hydroxide (6.2 ml) was added to a vigorously stirred

solution of $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-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 1$\underline{M}$ 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 $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-2,5-diiodohistidine monohydrate

(1.22 g, 78%) of m.p. 165-168° (Lit 181°)[148]; $[\alpha]_D^{20}$ +3.8° ($\underline{c}$ 1.0 in EtOH)

(Lit +4.2° $\underline{c}$ 0.6 in EtOH)[148]; $R_F(P_1)$ 0.48; $\delta(CD_3OD)$ 1.1(9H, s, $(C\underline{H}_3)_3C-$),

2.9(2H, d, $-C\underline{H}_2CH-$), 4.1(1$\underline{H}$, complex, $-NHC\underline{H}-$); (Found: C, 25.29; H, 3.40;

N, 7.72. $C_{11}H_{15}N_3O_4I_2.H_2O$ requires: C, 25.14; H, 3.04; N, 8.28%); $M^+$ found

507, 508 $C_{11}H_{15}N_3O_4I_2$ requires 507. Deprotection by catalytic hydrogenation

and digestion with $\underline{L}$-amino-acid oxidase showed the acid to contain 2% $\underline{D}$-

histidine

## $\underline{N}(\alpha)$-t-Butoxycarbonyl-$\underline{L}$-2,5-diiodohistidine Dicyclohexylammonium Salt.

Dicyclohexylamine (0.6 ml, 3.0 ammol) in methanol (1 ml) was added

to a solution of $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-2,5-diiodohistidine (250 mg, 0.49

mmol) in methanol (1 ml). The solution was cooled to 0°: no product

crystallised out, but on adding ether a white solid was precipitated.

Reprecipitation from methanol-ether gave $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-2,5-

diiodohistidine dicyclohexylammonium dihydrate (310 mg, 92%); m.p. 148-150°;

$[\alpha]_D^{20}$ +1.6° ($\underline{c}$ 1.0 in $CHCl_3$); $R_F(P)$ 0.72; $\delta(CD_3OD)$ 1.0-2.o(31H, complex,

$C_6\underline{H}_{11}-$ and $(C\underline{H}_3)_3C-$), 3.0(2H, d, $-C\underline{H}_2CH-$), 4.2(1H, m, $-NHC\underline{H}-$); (Found:

C, 38.22; H, 5.60; N, 8.17. $C_{23}H_{38}O_4I_2.2H_2O$ requires: C, 38.20; H, 5.50; N,

7.84%).

N(∝)-t-Butoxycarbonyl-L-monoiodohistidine 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(∝)-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°. 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 1$\underline{M}$ 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(∝)-t-butoxycarbonyl-L-monoiodohistidine methyl ester as a white solid (0.76 g, 10.3%) of m.p. 135-137°; $[\alpha]_D^{20}$ +4.8° (c 1.0 in $CHCl_3$); $R_F(E_3)$ 0.57; δ($CDCl_3$) 1.3(9H, s,($C\underline{H}_3$)C-), 2.75(2H, d, $-C\underline{H}_2$CH-), 3.45(3H, s, $C\underline{H}_3$OCO-), 4.2(1H, m, -NHC$\underline{H}$CO$_2$-), 5.25(1H, m, -N$\underline{H}$CH), 7.1(1H, s, imidazole); (Found: C, 36.75; H, 4.64; N, 10.45. $C_{12}H_{18}N_3O_4I$ requires: C, 36.4; H, 4.5; N, 10.6%) M$^+$ found 395, $C_{12}H_{18}N_3O_4I$ requires: 395

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

A solution of N(∝)-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 periòd 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 1$\underline{M}$ sodium thiosulphate (5 x 50 ml), dried

and the solvent evaporated to give a dark brown oil. Precipitation from

ethyl acetate-ether gave $\underline{N}$(∝)-benzyloxycarbonyl-$\underline{L}$-2,5-diiodohistidine

<u>methyl ester</u> as a pale yellow solid (1.19 g, 70.4%) of m.p. 68-71°;

$[\alpha]_D^{20}$ +9.9° ($\underline{c}$ 0.75 in CHCl$_3$); R$_F$(P) 0.52,   $\delta$(CDCl$_3$) 3.1(2H, d, -C$\underline{H}_2$CH-);

3.75(3H, s, C$\underline{H}_3$OCO-), 4.5(1H, m, -NHC$\underline{H}$CO$_2$-), 5.1(2H, s, -C$\underline{H}_2$Ph), 5.86(1H,

m, -N$\underline{H}$CH-), 7.35(5H, s, C$_6\underline{H}_5$-); (Found: C, 34.71; H, 3.39; N, 7.33.

C$_{15}$H$_{15}$N$_3$O$_4$I$_2$.$\frac{1}{2}$Et$_2$O requires: C, 34.57; H, 3.38; N, 7.11). M$^+$ found 555,

556, C$_{15}$H$_{15}$N$_3$O$_4$I$_2$ requires: 555.

## $\underline{N}$(∝)-Benzyloxycarbonyl-$\underline{L}$-2,5-diiodohistidine.

1$\underline{M}$ Sodium hydroxide (0.9 ml) was added to a vigorously stirred

solution of $\underline{N}$(∝)-benzyloxycarbonyl-$\underline{L}$-2,5-diiodohistidine methyl ester

(250 mg, 0.45 mmol) dissolved in methanol (1 ml). After 1 h t.l.c.

(E$_3$) 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 1$\underline{M}$ hydrochloric acid,

precipitating a brown solid. After filtration and reprecipitation

from methanol-ether $\underline{N}$(∝)-benzyloxycarbonyl-$\underline{L}$-2,5-diiodohistidine was

obtained as a white solid (132 mg, 54%) m.p. 118-120°; $[\alpha]_D^{20}$ +13.2 ($\underline{c}$

1 in MeOH); R$_F$(P) 0.48;   $\delta$(CD$_3$OD) 3.1(2H, d, -C$\underline{H}_2$CH-), 4.4(1H, m, -NHC$\underline{H}$-),

5.0(2H, s, C$\underline{H}_2$Ph), 7.3(5H, s, C$_6\underline{H}_5$-); M$^+$(F.D) found 542 C$_{14}$H$_{13}$N$_3$O$_4$I$_2$

requires 541. The material was contaminated with trapped ether which

could not be removed under vacumn. (Found C, 32.75; H, 2.34; N, 7.70

C$_{14}$H$_{13}$N$_3$O$_4$I$_2$.0.25 Et$_2$O 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-butoxycarbony-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 analyer: 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

methyl ester. $R_F(E_3)$ 0.42, $\delta$(CDCl$_3$) 1.4(9H, s, (C$\underline{H}_3$)$_3$C-), 3.05(2H, d, J=6Hz, -CHC$\underline{H}_2$-), 3.6(3H, s, -OC$\underline{H}_3$), 4.5(1H, m, -C$\underline{H}$NH-), 5.85(1H, d, J=10Hz, -N$\underline{H}$CH-).

### $\underline{N}(\alpha)$-t-Butoxycarbonyl-$\underline{L}$-2-$^2\underline{H}_1$-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 $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-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 $R_F(E_3)$ 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, (C$\underline{H}_3$)$_3$C-), 2.9(9H, d, J=11Hz, (C$\underline{H}_3$)$_2$NCOH), 3.05(2H, d, J=6Hz, -CHC$\underline{H}_2$-), 3.65(3H, s, -OC$\underline{H}_3$), 4.5(1H, s, -NHCH-), 5.85(1H, d, J=10Hz, -N$\underline{H}$CH-), 6.8(1H, s, H-4 of imidazole), 8.0(1.5H, s, (CH$_3$)$_2$NCO$\underline{H}$).

### $\underline{N}(\alpha)$-t-Butoxycarbonyl-$\underline{L}$-2,5-diiodohistdyl-$\underline{L}$-prolineamide.

1-Hydroxybenzotriazole (67.6 mg, 0.5 mmol) and dicyclohexylcarbodiimide (124 mg, 0.6 mmol) was added to a solution of $\underline{N}(\alpha)$-t-butoxycarbonyl-$\underline{L}$-2,5-diiodohistidine (252 mg, 0.5 mmol) and $\underline{L}$-prolineamide hydrochloride (67.2 mg, 0.5 mmol) dissolved in dimethylformamide (2 ml) stirred at 0° which 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 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° 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 N(ᴋ)-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° (c 1.0 in MeOH); $R_F(P_2)$ 0.58; $\delta$(CDCl$_3$)

1.3(9H, s, (CH$_3$)$_3$C-), 1.8-2.2(4H, complex, -CHCH$_2$CH$_2$- of Pro), 3.0-3.1

(2H, complex, -CHCH$_2$- of His), 3.3-3.7(2H, complex, -NHCH$_2$CH$_2$- 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, -NH$_2$), 6.4(1H, s, -NH$_2$) (Found: C, 31.21; H, 3.39;

N, 11.12. $C_{16}H_{21}N_5O_4I_2 \cdot 2H_2O$ requires C, 31.01; H, 3.7; N, 11.31%) $M^+$(F.D)

found; 603, $C_{16}H_{21}N_5O_4I_2$ requires 603.

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

The preceding dipeptide (96 mg, 0.16 mmol) was dissolved in

trifluroacetic 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 µl, 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 $R_F$(CMWA)

0.87; $[\alpha]_D^{20}$ -20.5° (c 1.0 in 1M AcOH), $[\alpha]_D^{20}$ -7.1° (c 0.25 in MeOH);

$\delta$ (CD$_3$OD) 1.2-2.5(8H, complex -NCH$_2$CH$_2$CH$_2$- of Pro and -COCH$_2$CH$_2$- of Glp),

3.0-3.1(2H, m, -CHCH$_2$- of His), 3.6-3.8(2H, complex, -NCH$_2$CH$_2$- of Pro),

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

NDCH- of His); M$^+$(F.D) found: 615, C$_{16}$H$_{20}$N$_6$O$_4$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$_6$O$_4$I$_2$.1.5 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$_F$(CMAW) 0.45; $\delta$(CD$_3$OD)

2.0-2.4(8H, complex -CHCH$_2$CH$_2$- of Pro and -COCH$_2$CH$_2$- of Glp), 3.05(2H,

complex, -CHCH$_2$- of His), 3.8(2H, complex, -NHCH$_2$CH - of Pro), 3.9(1H,

m, -NDCH- of Glp), 4.4(1H, m, -NCHCO- of Pro), 4.5(1H, m, -NDCH of His),

7.2(1H, s, H-4 of imidazole of His), 8.0(1H, s, H-2 of imidazole of His);

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

Lit[154] -68° (c 1.0.in 1M AcOH); 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

N(κ)-t-Butoxycarbonyl-L-2,5-dibromohistidine Methyl Ester.

A solution of N(α)-t-butoxycarbonyl-L-histidine 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 N(κ)-

t-butoxycarbonyl-L-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, $C_{12}H_{17}N_3O_4Br_2$·

0.25CH$_3$CO$_2$Et requires: C, 34.7; H, 4.23; N, 9.35%); M$^+$ found 425, 427,

429, $C_{12}H_{17}N_3O_4Br_2$ requires: 425, 427, 429.

N(α)-t-Butoxycarbonyl-L-2,5-dibromohistidine.

1M Sodium hydroxide (15 ml) was added to a vigorously stirred

solution of N(α)-t-butoxycarbonyl-L-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, N($\alpha$)-t-butoxycarbonyl-L-2,5-dibromohistidine as a pale yellow foam (2.18g, 73%) of m.p. 92-94°; R$_F$(P$_2$) 0.66; [$\alpha$]$_D^{20}$ -1.1° (c 1.0 in MeOH); $\delta$(CD$_3$OD) 1.4(9H, s, (CH$_3$)$_3$C-), 3.1(2H, d, -CH$_2$CH-), 4.4(1H, m, -CHNH-); (Found: C, 33.0; H, 3.94; N, 9.45; C$_{11}$H$_{15}$N$_3$O$_4$Br$_2$.0.25Et$_2$O requires: C, 33.37; H, 4.05; N, 9.74%); M$^+$ found 411, 413, 415. C$_{11}$H$_{15}$N$_3$O$_4$Br$_2$ requires 411, 413, 415.

## Hydrogenolysis of N($\alpha$)-t-butoxycarbonyl-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 N($\alpha$)-t-butoxycarbonyl-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 N($\alpha$)-t-butoxycarbonyl-L-2,5-dibromohistidine methyl ester had been converted to N($\alpha$)-t-butoxycarbonyl-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 consistant with N($\alpha$)-t-butoxycarbonyl-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(∝)-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(∝)-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(∝)-t-butoxycarbonyl-L-2,5-dibromohistidyl-L-prolineamide as a white hygroscopic foam (358 mg, 70%) of m.p. 98-105°; $R_F(P_2)$ 0.75; $[\alpha]_D^{20}$ +4.9° (c 1.0 in MeOH); $\delta(CDCl_3)$ 1.4(9H, s, $(CH_3)_3C-$), 1.9-2.2(4H, complex, $-CHCH_2CH_2-$ of Pro), 3.0-3.1(2H, complex, $-CHCH_2-$ of His), 3.3-3.7(2H, complex, $-NHCH_2CH_2-$ of Pro), 4.6(1H, m, $-NHCHCO-$ of His), 5.4(1H, d, $-NHCH$ of His), 6.0(1H, s, $-NH_2$), 6.85(1H, s, $-NH_2$); $M^+$(F.D) found 507, 509, 511. $C_{16}H_{23}N_5O_4Br_2$ requires 507, 509, 511;(N:C ratio found: 0.35, $C_{16}H_{23}N_5O_4Br_2$ 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% D-histidine, the result being

corrected for racemisation during hydrolysis of the peptide.

## 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 μl, 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; $[\alpha]_D^{20}$ -29.4° (c 0.5 in 1M AcOH); $\delta$(CD$_3$OD) 1.9-2.1(4H, complex, -COCH$_2$CH$_2$- of Glp), 2.2-2.5(4H, complex, -CHCH$_2$CH$_2$- of Pro), 3.1-3.2(2H, m, -CHCH$_2$- of His), 3.5-3.7(2H, complex, -NHCH$_2$CH$_2$- of Pro), 3.9(1H, m, -NHCH-of Glp), 4.3(1H, t, -CHCH$_2$- of Pro), 4.5(1H, t, -NHCH- of His); M$^+$(F.D) 518, 520, 522. $C_{16}H_{20}N_6O_4Br_2$ requires 518, 520, 522. (N:C ratio found 0.369. $C_{16}H_{20}N_6O_4Br_2$·1.5 AcOH requires 0.368)

## 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 $R_F$ to authenic 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; $C_{16}H_{22}N_6O_4$.4AcOH. $6H_2O$ requires: C, 40.56; H, 7.04; 11.82%; $R_F$(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- of Glp), 4.2(1H, m, -CHCH$_2$- of Pro), 4.5(1H, m, -NHCH- of His), 7.0(1H, s, H-4 in imidazole of His), 7.72(1H, s, H-2 in imidazole of His); $[\alpha]_D^{20}$ -69° (c 0.11 in 1M AcOH(corrected for water present in C,H,N analysis)) Lit[154]. $[\alpha]_D^{20}$ -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[160] 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 dicyclohexylcarbondiimide 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(α)-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 chromotographically

homogeneous Pauly-positive material were pooled and concentrated to give,

after reprecipitation from methanol-ether, glycyl-$\underline{L}$-histidyl-$\underline{L}$-

phenylalanine as a white solid identical to authentic samples prepared

by Brown[166] (52 mg, 56%) of m.p. 182-185°; $R_F$(CMAW) 0.25; $[\alpha]_D^{20}$ -2.3°

($\underline{c}$ 1.87 in 1$\underline{M}$ AcOH); $\delta$(CD$_3$OD) 1.9(3H, s, C$\underline{H}_3$CO$_2$H), 2.8-3.0(4H, complex,

-CHC$\underline{H}_2$- of His and -CHC$\underline{H}_2$- of Phe), 3.5(2H, s, -NDC$\underline{H}_2$- of Gly), 4.5(1H,

t, -C$\underline{H}$CH$_2$- of His) 7.0(1H, s, H-4 of imidazole), 7.05-7.2(5H, complex,

C$_6\underline{H}_5$CH$_2$- of Phe), 8.3(1H, s, H-2 of imidazole); M$^+$(F.D) found: 560

C$_{17}$H$_{21}$N$_5$O$_4$ requires 359. Amino-acid analysis ratios: Gly, 1.00; His,

1.05; Phe 0.97. Digestion of the hydrolysate with $\underline{L}$-amino-acid oxidase

showed the presence of 7% $\underline{D}$-histidine (after correction for racemisation

occurring during hydrolysis).

CHAPTER 5


Experimental Section


Optimisation of the Preparation of N($\alpha$)-t-Butoxycarbonyl,N($\pi$)-


Benzyloxymethyl-L-histidine

N(ᴋ),N(ᴛ)-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[171]. However we devised the following proceedure 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(ᴛ)-bis-t-

butoxycarbonyl-L-histidine methyl ester as a white solid (37.1 g, 96%)

of m.p. 92°; $[\alpha]_D^{20}$ +3.2° (c 1.1 in MeOH); $\delta$(CDCl$_3$) 1.4(9H, s,

(CH$_3$)$_3$COCONH-), 1.6(9H, s, (CH$_3$)$_3$COCO-im), 3.0(2H, d, -CHCH$_2$),

3.7(3H, s, CH$_3$O-), 4.5(1H, m, -CHCH$_2$-), 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$_6$N$_3$ requires: C, 55.28; H, 7.32; N, 11.38%).

N(α)-t-Butoxycarbonyl,N(ᴨ)-benzyloxymethyl-L-histidine Methyl Ester.

This was prepared according to the general method of Brown and

Jones[166] by adding benzyl-chloromethyl ether (3.9 ml, 28 mmol) to

a solution of N(ᴋ),N(ᴛ)-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° $\delta$(CDCl$_3$) 1.45(9H, s, (CH$_3$)$_3$C-), 3.0-3.2(2H, complex, -CHCH$_2$-), 3.7(3H, s, CH$_3$O-), 4.4 (1H, m, -CHCH$_2$-), 4.65(2H, s, -OCH$_2$N-), 5.3(2H, s, PhCH$_2$O-), 5.7(1H, d, -NHCH-), 6.85(1H, s, H-4 of imidazole), 6.1-6.5(5H, complex, C$_6$H$_5$CH$_2$-), 7.9(1H, s, H-2 of imidazole).

T.l.c. (E$_3$) 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[159]. The column was eluted under a slight pressure of nitrogen gas with 5% methanol-chloroform and those fractions containing only the product were indentified by t.l.c. (E$_3$) and concentrated. After trituration with ether a white solid was obtained (2.6 g, 25%) which t.l.c. (E$_3$) and n.m.r. spectroscopy showed to be a further crop of N($\alpha$)-t-butoxycarbobyl,N($\pi$)-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$_{(0.8)}$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($\alpha$)-t-Butoxycarbonyl, N($\pi$)-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 quantites 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 $\underline{N}(\varkappa).\underline{N}(\tau)$-bis-t-butoxycarbonyl-$\underline{L}$-histidine methyl ester (0.2 g, 0.54 mmol) was dissolved in the appropriate solvent (2 ml) to which was added benzyl-chloromethyl ether (82 µl,1.09 equiv.,120 µl,1.59 equiv.,157 µl,2.09 equiv.) and in a number of cases triethylamine (82 µl, 0.59 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 $\underline{N}(\varkappa),\underline{N}(\tau)$-bis-t-butoxycarbonyl-$\underline{L}$-histidine methyl ester and $\underline{N}(\varkappa)$-t-butoxycarbonyl,$\underline{N}(\pi)$-benzyloxymethyl-$\underline{L}$-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 benzyloxymethyl groups to the imidazole of histidine and running on the base line on a t.l.c. eluted with $E_3$.

$\underline{N}(\varkappa)$-t-Butoxycarbonyl,$\underline{N}(\pi)$-benzyloxymethyl-$\underline{L}$-histidine Methyl Ester Hydrochloride.

A solution of $\underline{N}(\varkappa),\underline{N}(\tau)$-bis-t-butoxycarbonyl-$\underline{L}$-histidine methyl ester (32 g, 0.082 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

| | 1.09 Equivalents of benzyl-chloromethyl ether | 1.59 Equivalents of benzyl-chloromethyl ether | 2.09 Equivalents of benzyl-chloromethyl ether |
|---|---|---|---|
| Solvent: Dichloromethane<br><br>Base: None | S.M.:- Strong<br><br>Product:- Strong<br><br>Quat. Salts:- Weak | S.M.:- None<br><br>Product:- Strong<br><br>Quat. Salts:- Weak | S.M.:- None<br><br>Product:- Medium<br><br>Quat. Salts:- Strong |
| Solvent: Dichloromethane<br><br>Base: Triethylamine | S.M.:- Strong<br><br>Product:- Weak<br><br>Quat. Salts:- Medium | S.M.:- Medium<br><br>Product:- Medium<br><br>Quat. Salts:- Medium | S.M.:- Medium<br><br>Product:- Medium<br><br>Quat. Salts:- Strong |
| Solvent: Methanol<br><br>Base: Triethylamine | S.M.:- Strong<br><br>Product:- None<br><br>Quat. Salts:- Medium | S.M.:- Strong<br><br>Product:- None<br><br>Quat. Salts:- Strong | S.M.:- Medium<br><br>Product:- None<br><br>Quat. Salts:- Strong |

S.M.=N($\alpha$),N($\tau$)-Bis-t-butoxycarbonyl-L-histidine methyl ester, Product=N($\alpha$)-t-Butoxycarbonyl,N($\pi$)-benzyloxymethyl-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 $Cl_2$/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 $\underline{N}(\alpha)$-t-butoxycarbonyl,$\underline{N}$(л)-

benzyloxymethy-$\underline{L}$-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°($\underline{c}$ 1.0 in MeOH): $\delta$(CDCl$_3$) 1.4(9H, s, (C$\underline{H}_3$)$_3$C-), 3.0-3.3(2H, complex,

-CHC$\underline{H}_2$-), 3.6(3H, s, C$\underline{H}_3$O-), 4.5(1H, m, -C$\underline{H}$CH$_2$-), 4.6(2H, s, -OC$\underline{H}_2$N-).

5.4(1H, d, -N$\underline{H}$CH-), 5.75(2H, s PhC$\underline{H}_2$O-), 7.1(1H, s, H-4 of imidazole),

7.2(5H, complex, C$_6$$\underline{H}_5$CH$_2$-), 9.7(1H, s, H-2 of imidazole); (Found:

C,56.50; H,6.72; N,9.57 C$_{20}$H$_{28}$N$_3$O$_5$Cl requires: C,56.40; H,6.58; N,9.87%).

$\underline{N}$($\alpha$)-t-Butoxycarbonyl,$\underline{N}$(л)-benzyloxymethyl-$\underline{L}$-histidine.

1$\underline{M}$ Sodium hydroxide (110 ml) was added to a solution of $\underline{N}$($\alpha$)-t-

butoxycarbonyl,$\underline{N}$(л)-benzyloxmethyl-$\underline{L}$-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 1$\underline{M}$ 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 $\underline{N}$($\alpha$)-t-butoxycarbonyl,$\underline{N}$(л)-

benzyloxymethyl-$\underline{L}$-histidine as a crystalline solid (19 g, 87%) of m.p.

155°, lit. 155°[166], $[\alpha]_D^{20}$ +6.9° ($\underline{c}$ 1.0 in MeOH) Lit.[166, 170] $[\alpha]_D^{20}$ +6.9°.

CHAPTER 6

Experimental Section

Practical Peptide Applications of N($\tau$)-Benzyloxymethyl Histidine

Section A: Solid Phase Synthesis

Synthesis of 5-Isoleucine Angiotensin II and L-Tyrosyl-L-isoleucyl-

L-histidyl-L-prolyl-L-phenylalanine using the Solid Phase Method.

N-t-Butoxycarbonyl-L-phenylalanine was coupled to a Merrifield

1% crosslinked polystyrene resin in dimethylformamide at 60° by the method

of Gisin[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 d chloromethane (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(∝)-t-butoxycarbonyl,N(π)-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),

dicylohexylcarbodiimide (2 x 0.3 g, 2.92 mmol).

Cycle 2:-   N(∝)-t-Butoxycarbonyl,N(π)-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,O-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(∝)-t-Butoxycarbonyl,N(ω)-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 sythesis 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,O-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

|          | Phe  | Pro  | His  | Ile  | Tyr     | Val  | Arg  | Asp  |
|----------|------|------|------|------|---------|------|------|------|
| Cycle 1  | –    | –    |      |      |         |      |      |      |
| Cycle 2  | 1.05 | 1.00 | 0.94 |      |         |      |      |      |
| Cycle 3  | 0.97 | 1.00 | 0.99 | 0.97 |         |      |      |      |
| Cycle 4  | 1.07 | 1.04 | 0.95 | 1.04 | $0.34^+$ |      |      |      |
| Cycle 5  | 1.03 | 1.01 | 1.02 | 1.01 | 0.98    | 1.04 |      |      |
| Cycle 6  | 0.96 | 0.99 | 1.14 | 0.98 | 1.05    | 1.09 | 1.03 |      |
| Cycle 7  | 0.95 | 0.91 | 1.09 | 0.95 | 1.03    | 1.00 | 0.97 | 0.98 |
| Cycle 4* | 1.02 | 0.98 | 1.06 | 0.98 | 0.95    |      |      |      |
| Cycle 7* | 1.00 | 0.99 | 1.00 | 0.99 | 1.00    | 1.01 | 1.01 | 1.06 |

*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 $\underline{in}$ $\underline{vacuo}$ $\underline{L}$-tyrosyl-$\underline{L}$-isoleucyl-$\underline{L}$-histidyl-$\underline{L}$-prolyl-$\underline{L}$-phenylalanine as a white solid (88 mg, 30%) of $[\alpha]_D^{20}$ -2.81° ($\underline{c}$ 0.25 in 1$\underline{M}$ AcOH (uncorrected)); $\delta$ ($D_2O$) 0.6(6H, complex, $C\underline{H}_3CH_2CHC\underline{H}_3$- of Ile), 0.8-1.3(2H, complex, $-C\underline{H}_2CH-$ of Ile), 1.5-2.0(10H, complex, $C\underline{H}_3CO_2H$ and $-CHC\underline{H}_2C\underline{H}_2-$ of Pro), 2.9-3.0(6H, complex $-CHC\underline{H}_2-$ of His, $-CHC\underline{H}_2-$ of Phe and $-CHC\underline{H}_2-$ of Tyr), 4.3-4.5(2H, complex, $-CH_2C\underline{H}_2N-$ of Pro), 4.0-4.4(4H, complex, $\alpha$-CH ptotons), 6.5-6.9 (4H, AB-q, $HOC_6\underline{H}_4CH_2-$ of tyr), 7.0-7.2(6H, complex, $C_6\underline{H}_5CH_2-$ of Phe and H-4 on imidazole of His), 8.2(1H, s, H-2 on imidazole of His); $M^+$ 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 $\underline{N}$($\omega$)-nitroarginine-5-isoleucine angiotensin II as a hygroscopic solid which was dissolved in 80% aqueous acetic acid (10 ml) in which was suspended 10% palladium on carbon catalyst (50 mg). The stirred suspension was hydrogenated for 24 h in order to deprotect the $\underline{N}$($\omega$)-nitroarginine, after this period t.l.c.($A_3$) showed one major component. The solvent was removed and the oily residue (180 mg) was dissolved in 25% 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 0.0185$\underline{M}$ 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 0.0185$\underline{M}$ pH 4.2 to 0.185$\underline{M}$ 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

$\underline{in}$ $\underline{vacuo}$ 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).

$[\alpha]_D^{20}$ -69° ($\underline{c}$ 0.5 in 1$\underline{M}$ HCl (corrected by quantitative amino-acid

analysis)), Lit[172] -67° (Found: C, 51.27; H, 6.52; N, 14.11 $C_{50}H_{71}N_{13}O_{12}$.

3.5AcOH,2.75$H_2O$ 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 $\underline{L}$-Histidyl-$\underline{L}$-histidyl-
$\underline{L}$-histidyl-glycyl-$\underline{L}$-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-$\underline{L}$-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:-  $\underline{N}(\alpha)$-t-Butoxycarbonyl,$\underline{N}(\pi)$-benzyloxymethyl-$\underline{L}$-histidine

(0.6 g then 0.3 g, 2.4 mmol), dicyclohexylcarbodiimide

(0.36 g then 0.18 g, 2.7 mmol).

Cycle 3:-  $\underline{N}(\alpha)$-t-Butoxycarbonyl,$\underline{N}(\pi)$-benzyloxymethyl-$\underline{L}$-histidine

(0.6.g then 0.3 g, 2.4 mmol), dicyclohexylcarbodiimide

(0.36 g then 0.18 g, 2.7 mmol).

Cycle 4:-  $\underline{N}(\alpha)$-t-Butoxycarbonyl,$\underline{N}(\pi)$-benzyloxmethyl-$\underline{L}$-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 6$\underline{M}$ hydrochloric acid in propionic acid

(with 0.1% phenol added) for 24 h. After evaporation of the solvent

the sample was dissolved in 0.01$\underline{M}$ 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':-  $\underline{N}(\alpha)$-t-Butoxycarnonyl,$\underline{N}(\pi)$-benzyloxymethyl-$\underline{L}$-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

| | Phe | Gly | His |
|---|---|---|---|
| Cycle 1 | – | – | |
| Cycle 2 | 1.00 | 1.04 | 0.96 |
| Cycle 3 | 0.94 | 0.98 | 2.08 |
| Cycle 4 | 1.00 | 1.02 | 2.40 |
| *Cycle 4' | 0.98 | 1.02 | 2.10 |

* 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. ($A_3$) 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 $R_F(A_3)$ 0.21

gave the ratios: His, 2.93; Gly, 1.02; Phe, 1.00, whilst material

$R_F(A_3)$ 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-histidyl-L-histidyl-L-histidyl-glycyl-L-phenylalanine and L-histidyl-L-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[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°); $[\alpha]_D^{20}$ -7.0° (c 1.0 in MeOH) Lit -7.2°; $\delta$(CDCl$_3$) 2.3

(3H, s, CH$_3$Ph-), 3.0-3.2(2H, complex, -CHCH$_2$-), 4.6(1H, complex,

-CHCH$_2$-), 4.9(2H, m, -OCH$_2$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, (CH$_3$)$_3$C-), 1.6-2.2(4H, complex,

-CH$_2$CH$_2$CH- of Pro), 3.0-3.4(4H, complex, -CH$_2$N- of Pro and -CHCH$_2$-

of Pro and -CHC$\underline{H}_2$- of Phe), 3.6(1H, m, -C$\underline{H}$NH- of Pro), 4.2(1H, m,

-C$\underline{H}$NH- of Phe), 4.8(1H, m, -CHN$\underline{H}$- of Pro), 5.1(2H, s, -OC$\underline{H}_2$Ph).

6.9-7.3(11H, complex, -N$\underline{H}$CH- of Phe, C$_6$$\underline{H}_5$- of Phe and C$_6$$\underline{H}_5$- of Bzl).

<u>N(∝)-t-Butoxycarbonyl,N(π)-benzyloxymethyl-L-histidyl-L-prolyl-L-</u>

<u>phenylalanine Benzyl Ester.</u>

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 trifluroacetate was dissolved in chloroform (5 ml)

with $\underline{N}$(∝)-t-butoxycarbonyl,$\underline{N}$(π)-benzyloxymethyl-$\underline{L}$-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

$\underline{N}$(∝)-t-butoxycarbonyl,$\underline{N}$(π)-benzyloxymethyl-$\underline{L}$-histidyl-$\underline{L}$-prolyl-

$\underline{L}$-phenylalanine benzyl ester. as homogeneous white crystals

(1.1 g, 66&) of m.p. 111-112°; R$_F$(P$_2$) 0.56; [∝]$_D^{20}$ -38° (c 1.0 in

MeOH); $\delta$(CDCl$_3$) 1.3(9H, s, (C$\underline{H}_3$)$_3$C-), 1.7-2.0(4H, complex, -CHC$\underline{H}_2$C$\underline{H}_2$-

of Pro), 2.8-3.0(2H, complex, -C$\underline{H}_2$OH- of citrate), 3.0-3.3(6H, complex,

-CHC$\underline{H}_2$- of His, -CHC$\underline{H}_2$- of Phe and -NC$\underline{H}_2$- of Pro), 4.4(1H, m,

-C$\underline{H}$C$\underline{H}_2$- of Pro), 4.6-4.9(4H, complex, -C$\underline{H}$C$\underline{H}_2$- of His, -C$\underline{H}$C$\underline{H}_2$- of Phe

and imC$\underline{H}_2$O-), 5.1(2H, m. PhC$\underline{H}_2$OCH$_2$-), 5.7(1H, d, -N$\underline{H}$CH- of His),

5.8-5.9(2H, m, -CO$_2$C$\underline{H}_2$Ph, 6.9(1H, d, -N$\underline{H}$CH- of Phe), 7.0-75(16H,

complex, H-4 on imidazole of His, C$_6\underline{H}_5$CH$_2$O-, -CO$_2$CH$_2$C$_6\underline{H}_5$ and

C$_6\underline{H}_5$CH$_2$- 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.


$\underline{N}$(∝)-t-Butoxycarbonyl,$\underline{N}$(ᴨ)-benzyloxymethyl-$\underline{L}$-histidyl-$\underline{L}$-tryptophan

Methyl Ester.

1-Hydroxybenzotriazole (243 mg, 1.60 mmol) and dicyclohexylcarbodiimide

(328 mg, 1.60 mmol) was added to a solution of $\underline{N}$(α)-t-butoxycarbonyl,

$\underline{N}$(ᴨ)-benzyloxymethyl-$\underline{L}$-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 $\underline{N}$(α)-t-butoxycarbonyl,$\underline{N}$(ᴨ)

benzyloxymethyl-$\underline{L}$-histidyl-$\underline{L}$-tryptophan methyl ester (476 mg, 61%)

of m.p. 90-92°; $[\alpha]_D^{20}$ +3.1° ($\underline{c}$ 1.0 in MeOH); $\delta$(CDCl$_3$) 1.4(9H, s,

(C$\underline{H}_3$)$_3$C-), 2.2(2H, br, H$_2$O), 3.0(2H, complex, -CHC$\underline{H}_2$- of His), 3.2(2H,

complex, -CHC$\underline{H}_2$- of Trp), 3.8(3H, s, -CO$_2$C$\underline{H}_3$), 4.4(2H, s, -NC$\underline{H}_2$O-),

4.45(1H, complex, -C$\underline{H}$CH$_2$- of His), 4.9(1H, m, -C$\underline{H}$CH$_2$- of Trp), 5.15(2H,

s, PhC$\underline{H}_2$O-), 5.2(1H, d, -N$\underline{H}$CH- of His), 6.8(1H, d, -N$\underline{H}$CH- of Trp),

6.9-7.5(12H, complex, indole of Trp, imidazole of His and C$_6$$\underline{H}_5$CH$_2$O-),

8.5(1H, s, indole N$\underline{H}$ of Trp); M$^+$ Found 576 C$_{31}$H$_{37}$O$_6$N$_5$ requires: 575;

(Found: C, 63.97; H, 6.33; N, 12.03 C$_{31}$H$_{37}$O$_6$N$_5$.0.5H$_2$O requires: C, 63.69;

H, 6.51; N, 11.98%).

## N($\alpha$)-t-Butoxycarbonyl-$\underline{\underline{L}}$-histidyl-$\underline{\underline{L}}$-tryptophan Methyl Ester.

$\underline{N}$($\alpha$)-t-Butoxycarbonyl,$\underline{N}$($\pi$)-benzyloxymethyl-$\underline{\underline{L}}$-histidyl-$\underline{\underline{L}}$-

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.

(P$_2$) 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 $\underline{N}$($\alpha$)-t-butoxycarbonyl-$\underline{\underline{L}}$-histidyl-$\underline{\underline{L}}$-

tryptophan methyl ester as a white solid (58 mg, 73%) of m.p. 164-165°;

$[\alpha]_D^{20}$ -3.7° ($\underline{c}$ 1.0 in MeOH); R$_F$(P$_2$) 0.36; $\delta$(CDCl$_3$) 1.3(9H, s,

(C$\underline{H}_3$)$_3$C-), 2.0(6H, s, C$\underline{H}_3$CO$_2$H), 2.95(2H, complex, -CHC$\underline{H}_2$- of His),

3.1(2H, complex, -CHC$\underline{H}_2$- of Trp), 3.8(3H, s, -CO$_2$CH$_3$). 4.4(1H, complex,

-C$\underline{H}$CH$_2$- of His), 4.85(1H, complex, - C$\underline{H}$CH$_2$- of Trp), 5.5(1H, complex,

-N$\underline{H}$CH- of His), 6.8(1H, complex, -N$\underline{H}$CH- of Trp), 6.9-7.5(7H, complex,

imidazole of His and indole of Trp), 9.5(1H, s, indole N$\underline{H}$ of Trp);

M$^+$ Found 455, 456 C$_{23}$H$_{29}$O$_5$N$_5$ requires: 455; (Found: C, 56.11; H, 6.95;

H, 6.95; N, 11.94 C$_{23}$H$_{29}$O$_5$N$_5$.2AcOH requires: C, 56.34; H, 6.59; N, 12.17%).

Attempted Preparation of N(α)-t-Butoxycarbonyl-L-tryptophoαyl-N(π)-

benzyloxymethyl-L-histidyl-L-tryptophan Methyl Ester.

N(α)-t-Butoxycarbonyl,N(π)-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(α)-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 μl, 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° 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.(P$_2$)

showed to contain a major product with a trace impurity. 300MHz N.m.r.

spectoscropy and F.D. mass spectroscopy M$^+$ 761, (M+H)$^+$ 762, (M+56)$^+$

817, (M+57)$^+$ 818 were consistent with the product containing a mixture

of N(∝)-t-butoxycarbonyl-L-tryptophyl-N(π)-benzyloxymethyl-L-histidyl-

L-tryptophan methyl ester and N(∝)-t-butoxycarbonyl-L-tryptophyl-N(π)-

benzyloxymethyl-L-histidyl-N(i)-t-butyl-L-tryptophan methyl ester.

CHAPTER 7

Experimental Section

Racemisation of Histidine Derivatives

Coupling of N(α)-t-Butoxycarbonyl,N(im)-benzyl-L-histidine with L-prolineamide Under Conditions Designed to Exaggerate Racemisation.

N(α)-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(κ)-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[105] (Table 10).

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

N(α)-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 μl, 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 μmol)

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(∝)-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[105]. (Table 10).

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

N(∝)-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.μl, 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.

($P_2$) (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(ᴋ)-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).

| | % D-histidine* |
|---|---|
| N(ᴋ)-Benzyloxycarbonyl,N(ᴝ)-phenacyl-L-histidine[107] | 49 |
| N(ᴋ)-t-Butoxycarbonyl,N(ᴝ)-benzyl-L-histidine | 45 |
| N(ᴋ)-t-Butoxycarbonyl-L-2,5-diiodohistidine | 20 |
| N(ᴋ)-t-Butoxycarbonyl-L-2,5-dibromohistidine | 15 |

*All results refer to the amount of D-histidine in the hydrolysate

of the hydrolᴜsed 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 dimethylormamide 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-Benzyloxycarbony,N(π)-phenacyl-L-histidine with N,N'-Diisopropylcarbodiimide.

On standing with diisopropylcarbodiimide (20 μl, 0.12 mmol) N(α)-benzyloxymethyl,N(π)-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

| Time | BocHisOH | BocHisOH | $I_2$ | BocHisOH | $\pi$-Pac | BocHisOH | $\tau$-Bzl |
|---|---|---|---|---|---|

| Time | BocHisOH | BocHisOH $I_2$ | BocHisOH $\pi$-Pac | BocHisOH $\tau$-Bzl |
|---|---|---|---|---|
| Start | -0.187 | -0.161 | +0.086 | +0.024 |
| 1-5 | -0.184 | -0.148 | +0.116 | +0.025 |
| 6-10 | -0.186 | -0.143 | +0.104 | +0.024 |
| 11-15 | -0.186 | -0.138 | +0.087 | +0.023 |
| 16-20 | -0.185 | -0.138 | +0.070 | +0.021 |
| 21-25 | -0.183 | -0.134 | +0.066 | +0.019 |
| 26-30 | -0.183 | -0.130 | +0.065 | +0.017 |
| 31-35 | -0.182 | -0.126 | +0.063 | +0.016 |
| 36-40 | -0.182 | -0.121 | +0.062 | +0.015 |
| 41-45 | -0.182 | -0.123 | +0.062 | +0.014 |
| 46-50 | -0.181 | -0.117 | +0.062 | +0.013 |
| 51-55 | -0.180 | -0.109 | +0.062 | +0.012 |
| 56-60 | -0.179 | -0.104 | +0.061 | +0.010 |
| 61-65 | -0.178 | -0.103 | +0.061 | +0.009 |
| 66-70 | -0.177 | -0.094 | +0.060 | +0.008 |
| 71-75 | -0.176 | -0.088 | +0.058 | +0.007 |
| 76-80 | -0.175 | -0.086 | +0.058 | +0.005 |
| 81-85 | -0.173 | -0.087 | +0.057 | +0.005 |
| 86-90 | -0.171 | -0.082 | +0.057 | +0.004 |
| 91-95 | -0.170 | -0.079 | +0.055 | +0.004 |
| 96-100 | -0.172 | -0.077 | +0.055 | +0.004 |
| 101-105 | -0.173 | -0.076 | +0.055 | +0.004 |
| 106-110 | -0.172 | -0.072 | +0.054 | +0.003 |
| 111-115 | -0.172 | -0.065 | +0.053 | +0.003 |
| 116-120 | -0.171 | -0.062 | +0.052 | +0.003 |
| % Change | -8.6% | -61.5% | -39.5% | -87.5% |

Table 11

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

Table 11 (Cont'd)

obtain meaningfull results. After 2 h t.l.c.($P_2$) indicated the

formation of only one Pauly active impurity haveing $R_F$($P_2$) 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

<u>N</u>(∝)-<u>benzyloxycarbonyl</u>,<u>N</u>(π)-<u>phenacyl</u>-<u>L</u>-<u>histidine</u>,<u>N-acyldiisopropylurea</u>

as a yellow solid (20 mg, 31%) of ${\delta}$(CDCl$_3$) 1.1-1.2(12H, m, (C$\underline{H}_3$)$_2$CH-),

2.9-3.0(2H, complex, -CHC$\underline{H}_2$-), 3.85(1H, br, (CH$_3$)$_2$C$\underline{H}$N$\underline{H}$-), 4.05(1H, br,

(CH$_3$)$_2$C$\underline{H}$NCO-), 4.5(1H, m, -C$\underline{H}$C$\underline{H}_2$-), 5.0(2H, q, PhC$\underline{H}_2$OCO-), 5.45

(2H, m, PhCOC$\underline{H}_2$-), 5.6(1H, d, -CHN$\underline{H}$-), 7.0(1H, d, H-4 of imidazole),

7.3-7.5(10H, complex, C$_6$$\underline{H}_5$-), 7.6(1H, complex, H-2 of imidazole),

8.0(1H, complex, -N$\underline{H}$CH(CH$_3$)$_2$); Mass spectra (F.D.): M$^+$ Found 534, 448

C$_{29}$H$_{35}$N$_5$O$_5$ requires: 533.

## References

1.  M. Bodanszky, Y.S. Klausner & M.A. Ondetti, 'Peptide Synthesis' 2nd edition, John Wiley and Sons, Inc., New York, 1976.

2.  J.P. Greenstein & M.W. Winitz, 'Chemistry of the Amino-Acids', Vol. 2, John Wiley & Sons, Inc., New York, 1961.

3.  E. Schröder & K. Lübke, 'The Peptides', Vol. 1, Academic Press, New York, 1965.

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

5.  H.D. Law, 'The Organic Chemistry of Peptides', John Wiley & Sons, Ltd., London, 1970.

6.  G.T. Young in 'Essays in Chemistry', Vol. 4, eds. J.N. Bradley, R.D. Gillard & R.F. Hudson, Academic Press, London, 1972, p. 115.

7.  J.H. Jones, Chem. and Ind., 1974, 723.

8.  M. Bodanszky & J. Martinez, Synthesis, 1981, 333.

9.  A.P. Mathias, A. Deavin & B.R. Rabin in 'The Structure and Function of Enzymes', Academic Press Inc., New York, 1964.

10. W.P. Jencks in 'Chemical Reactivity and Biological Role of Functional Groups in Enzymes', ed. R.M.S. Smellie, Academic Press Inc., London, 1970, p. 59.

11. G.M. Blackburn, H.L.H. Dodds & D.J. Shire in reference 10, p. 81.

12. M.L. Bender, 'Mechanism of Homogeneous Catalysis from Protons to Proteins', John Wiley & Sons, Inc., New York, 1971.

13. O.D. von Batenburg, J. Raap, K.E.T. Kerling & E. Haringa, Rec. Trav. Chim., 1976, 95, 278.

14. A. Sanda & M. Irie, Chem. Pharm. Bull., 1979, 27, 2310.

15. I.I. Geschwind, Am. Zool., 1967, 7, 89.

16. I.H. Page & F.M. Bumpus (eds.) 'Angiotensin Handbook of Experimental Pharmacology', Vol. XXXVII, Springer Verlag, Berlin, 1974.

17. C. Gual, A. Kastin & A.V. Schally, Rec. Prog. Horm. Res., 1972, 28, 173.

18. M. Amoss, R. Burgus, R. Blackwell, W. Vale, R. Fellows & R. Guillemin, Biochem. Biophys. Res. Comm., 1971, 44, 205.

19. L.L. Iversen, Nature, 1974, 252, 630.

20. C.J. Lote, J.P. Gent, J.H. Wolstencroft & M. Szelke, Nature, 1976, 264, 188.

21.  S. Lindskog, J. Biol. Chem., 1963, 238, 945.

22.  M.F. Perutz, Nature, 1970, 288, 726.

23.  G. Losse & U. Krychowski, Tet. Lett., 1971, 44, 4121,

24.  Y.S. Klausner & M. Bodanszky, Synthesis, 1974, 549.

25.  R.W. Holley & E. Sondheimer, J. Amer. Chem. Soc., 1954, 76, 1326.

26.  As Reference 2, pp. 461 & 1065.

27.  A.Patchornik, A. Berger & E. Katchalski, J. Amer. Chem. Soc.,
     1957, 79, 6416.

28.  E. Bamberger, Annalen, 1893, 273, 267.

29.  H.A. Staab & W. Rohr in 'New Methods of Preparative Organic
     Chemistry', Vol. V, ed. W. Forest, Verlag Chemie GmbH., Weinheim,
     1968, p. 61.

30.  M.A. Tilak & C.S. Hollinden, Tet. Lett., 1969, 6, 391.

31.  J.M. Stewart & J.D. Young, 'Solid Phase Peptide Synthesis',
     W.H. Freeman & Co. San Francisco, 1969, p. 8.

32.  J.B. Jones & D.W. Hysert, Canad. J. Chem., 1971, 49, 3012.

33.  J.C. Sheehan & G.P. Hess, J. Amer. Chem. Soc., 1955, 77, 1067.

34.  H. Rink & B. Riniker, Helv. Chim. Acta., 1974, 57, 831.

35.  J.C. Sheehan & K. Hasspacker & Y.L. Yeh, J. Amer. Chem. Soc.,
     1959, 81, 6086.

36.  B. Helterich & H. Böshagen, Chem. Ber., 1959, 92, 2813.

37.  M. Bodanszky, M.L. Fink, Y.S. Klausner, S. Natarajan, K. Tatemoto,
     A.E. Yiotakis & A. Bodanszky, J. Org. Chem., 1977, 42, 149.

38.  Y.S. Klausner, M. Chorev, J. Chem. Soc. Chem. Comm., 1977, 973.

39.  S. Sakakibara in 'Peptides', Proceedings of the 5th American
     Peptide Symposium, M. Goodman, J. Meinhofer, eds., John Wiley
     & Sons, New York, 1977, p. 436.

40.  S.K. Girin, Y.P. Shvachkin, J. Gen. Chem. USSR, 1977, 47, 1085,
     1950; 1978, 48, 823.

41.  J. Martinez, J.C. Tolle & M. Bodanszky, Int. J. Peptide. Protein
     Res., 1979, 13, 22.

42.  F. Weygand, A. Prox, L. Schmidhammer & W. König, Angew. Chem.
     Internat. Edn., 1963, 2, 184.

43. H.C. Beyerman, J. Hirt, P. Kranenburg, J.L.M. Syrier &
    A. van Zon, Rec. Trav. Chim., 1974, 93, 256.

44. E. Wünsch & A. Zwick, Chem. Ber., 1964, 97, 2497.

45. D.F. Veber in 'Peptides: Chemistry, Structure and Biology'
    (Proc. 4th American Peptide Sym.) eds. R. Walter & J. Meienhofer
    Ann Arbour Science Publications Inc., Ann Arbour, 1975, p. 307.

46. P. Sieber, R. Riniker, M. Brugger, B. Kamber & W. Rittel,
    Helv..Chim Acta., 1970, 53, 2135.

47. I. Antonovics & G.T. Young, J. Chem. Soc. (C),1967, 595.

48. M.J. Witty, D.Phil Thesis, University of Oxford, 1979.

49. D.S. Kemp, Peptides (N. Y.), 1979, 1, 315

50. F. Weygand, W. Steglish & P. Pietta, Chem. Ber., 1967, 100, 3841.

51. J.R. Bell & R. Geiger, J. Chem Soc. Perkin I, 1974, 2336.

52. V. du Vigneaud & O.K. Behrens, J. Biol. Chem.1937, 117, 27.

53. J. Pless & R.A. Boissonnas, Helv. Chim. Acta., 1963, 46, 1609.

54. .R. Boissonnas, S. Guttmann & J. Pless, Belg. P. 636, 667, C.A.,
    1965, 62, P633c.

55. B.O. Handford, T.A. Hylton, K.T. Wang & B. Wienstien, J. Org.
    Chem., 1968, 33, 4251.

56. M.C. Kosla, R.R. Smeby & F.M. Bumpus, in 'Chemistry and
    Biology of Peptides', ed. J. Meienhofer, Ann Arbour Science
    Publications Inc., Ann Arbour, 1972, p. 227.

57. G.C. Windridge & E.C. Jorgensen, Interscience Chem. Rep., 1971,
    5, 375.

58. R.B. Merrifield, J. Amer. Chem. Soc., 1963, 85, 2149.

59. E.C. Jorgensen, G.C. Windridge & T.C. Lee, J. Medicin. Chem.,
    1970, 13, 352.

60. E.C. Jorgensen, S.R. Rapacka, G.C. Windridge & T.C. Lee, J.
    Medicin. Chem., 1971, 14, 899.

61. M.C, Khosla, M.M. Hall, R.R. Smeby & F.M. Bumpus, J. Medicin.
    Chem., 1973, 16, 829.

62. M.C. Khosla, R.A. Leese, W.L. Maloy, A.T. Ferreria, R.R. Smeby &
    F.M. Bumpus, J. Medicin. Chem., 1972, 15, 792.

63. G.C. Windridge & E.C. Jorgensen, J. Amer. Chem. Soc., 1971, 93,
    6318.

64. J.L.M. Syrier & H.C. Beyerman, Rec. Trav. Chim., 1974, 93, 117.

65. A. Marglin & R.B. Merrifield, Ann. Rev. Biochem., 1970, 39, 841.

66. As Reference 31, p. 11.

67. As Referance 31, p. 48.

68. As Reference 31, p. 20.

69. G.L. Stelakotos, D.M. Theodoropoulos & L. Zervas, J. Amer. Chem. Soc., 1959, 81, 2884.

70. G. Losse & U. Krychowski, J. Prakt. Chem., 1970, 312, 1097.

71. G. Losse, H. Stange, B. Shwenzer, W. Naumann, M. Mauck, K.J. Schumacher & B. Meisegeier, Proc 15th European Peptide Sym., in the press.

72. T. Fujii & S. Sakakibara, Bull. Chem. Soc. Japan, 1974, 47, 3146.

73. J.H. Jones & R.B. Walker,unpublished results.

74. S. Sakakibara & T. Fujii, Bull. Chem. Soc. Japan, 1969, 42, 1466.

75. V.T. Ivanov- I.I. Mikhaleva, O.M. Volpina, M.A. Myagkova & V.I. Deigin, in 'Peptides 1976' (Proc. 14th European Peptide Sym.) ed. A. Loffet, Editions de l'Universite de Bruxelles, Bruxelles, 1976, p. 219.

76. S. Shaltiel, Biochem. Biophy. Res Comm., 1967, 29, 178.

77. S. Shaltiel & M. Friedkin, Biochemistry, 1970, 9, 5122.

78. S. Shaltiel & M. Friedkin, Arch. Biochem. Biophys., 1971, 147, 70.

79. F. Cillemi & R.B. Merrifield, Biochemistry, 1969, 8, 4344.

80. J.T.W.A.R.M. Buis, doctoral thesis, University of Nijmegen, 1973.

81. P. Henkart, J. Biol. Chem., 1971, 246, 2711.

82. S. Akabori, K. Okawa & F.Sakiyama, Nature, 1958, 181, 722.

83. F. Sakiyama, K. Okawa, T. Yamakawa & S. Akabori, Bull. Chem. Soc. Japan, 1958, 31, 926.

84. E. Schaich, A.M. Fretzdorff & F. Schneider, Z. physiol. Chem., 1973, 354, 897.

85. K. Inouye & H. Otsuka, J. Org. Chem., 1962, 27, 4236.

86. D.F. Elliott & D. Morris, Chima (Switz), 1960, 14, 373.

87. H. Kappeler, Helv. Chim. Acta., 1961, 44, 476.

88. E.H. Schnabel, H. Herzog, P. Hoffmann, E. Klaube & I. Ugi, *Annalen*, 1968, 716, 175.

89. M. Fridkin & H.J. Goren, *Canad. J. Chem.*, 1971, 49, 1578.

90. D. Yamashiro, J. Blake & C.H. Li, *J. Amer. Chem. Soc.*, 1972, 94, 2855.

91. W. Klee & M. Brenner, *Helv. Chim. Acta.*, 1961, 44, 2151.

92. W.L. Haas, E.V. Krumklans & K. Gerzon, *J. Amer. Chem. Soc.*, 1966, 88, 1988.

93. H. Eckstein, *Annalen*, 1976, 1289.

94. G. Jäger, R. Geiger & W. Siedel, *Chem. Ber.*, 1968, 101, 3537.

95. H.H. Seltzman & T.M. Chapman, *Tet. Lett.*, 1974, 30, 2637.

96. H.H Seltzman, Ph.D. Thesis, University of Pittsburg, 1973.

97. S.M. Kalbag & R.W. Roeske, *J. Amer. Chem. Soc.*, 1975, 97, 440.

98. S. Coyle & G.T. Young, *J. Chem. Soc. Chem. Comm.*, 1976, 980.

99. S. Coyle & G.T. Young, in reference 75, p. 205.

100. S. Coyle, A. Hallett, M.S. Munns & G.T. Young, *J. Chem. Soc. Perkin I*, 1981, 524.

101. A. Hallett, personal communication.

102. J.H. Jones & W.I. Ramage, *J. Chem. Soc. Chem. Comm.*, 1978, 472.

103. A.R. Fletcher, J.H. Jones, W.I. Ramage & A.V. Stachulski, *J. Chem. Soc. Perkin I*, 1979, 2261.

104. H.R. Matthews & H. Rapoport, *J. Amer. Chem. Soc.*, 1973, 95, 2297.

105. J.H. Jones & W.I. Ramage, *Int. J. Peptide.Protein Res.*, 1979, 14, 65.

106. J.H. Jones, W.I. Ramage & M.J. Witty, *Int. J. Peptide Protein Res.*, 1980, 15, 301.

107. W.I. Ramage, D. Phil Thesis, University of Oxford, 1978.

108. T. Brown, personal communication.

109. G. Hancock, Ph.D. Thesis, University of Liverpool, 1981.

110. A. Patchornik, B..Amit & R.B. Woodward, *J. Amer. Chem. Soc.*, 1970, 92, 6333.

111. A. Patchornik, B. Amit & R.B. Woodward, in 'Peptides, Proceedings of the Thirteenth European Peptide Sym., Kiryat Anavim, Israel, 1974. Y. Wolman, ed., Wiley, New York-Israel University Press, Jerusalem, 1975, p.379.

112. P.M. Scopes, K.B. Walshaw, M. Welford & G.T. Young, J. Chem. Soc., 1965, 126, 782.

113. M.E. Clubb, P.M. Scopes & G.T. Young, Chimia., 1960, 14, 373.

114. T.I. Orlova & N.I. Gavrilov, J. Gen. Chem. (USSR), 1959, 29, 56.

115. D. Stevenson & G.T. Young, J. Chem. Soc.(C)., 1969, 2389.

116. D. Stevenson, D. Phil Thesis, University of Oxford, 1968.

117. M.C. Holly, Part II Thesis, University of Oxford, 1972.

118. A. Gosden, D. Stevenson & G.T. Young, J. Chem. Soc. Chem. Comm., 1972, 1123.

119. R. Macrae & G.T. Young, J. Chem. Soc. Perkin I, 1975, 1185.

120. V.G. Mairanovski, Angew. Chem. Internat. Edn., 1976, 15, 281.

121. A.J. Fry, in 'Organic Electrochemistry', Harper and Row, Inc., New York, 1972.

122. J.H.P. Utley, in 'Essays in Chemistry', Vol. 6, eds.,J.N. Bradley, R.D. Gillard & R.F. Hudson, Academic Press, London, 1977, p. 83.

123. J.D. Richards, Part II Thesis, University of Oxford, 1979.

124. A. Mansfield, Part II Thesis, University of Oxford, 1979.

125. H. Eckert, W. Breuer, J. Geller, I. Lagerlund, M. Listl, D. Marguarding, S. Stüber, I. Ugi, S. Zahr & H.V. Zychlinski, Pure & Appl. Chem., 1979, 51, 1219.

126. G.N. Schrazezer & E. Deutsch, J. Amer. Chem. Soc., 1969, 91, 3341.

127. J.B. Jones & D.W. Hysert, Canad. J. Chem., 1971, 49, 325.

128. J.C. Reepmeyer, K.L. Kirk & L.A. Cohen, Tet. Lett., 1975, 47, 4107.

129. F.A.L. Anet & A.J.R. Bourn, J. Amer. Chem., 1965, 87, 5250.

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

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

132. N.C. Craig & E.A. Entemann, J. Amer. Chem. Soc., 1961, 83, 3047.

133. J. Baddiley, J. Chem. Soc., 1959, 2893.

134. J.H. Noggel & R.E. Schirmer, in ' The Nuclear Overhauser Effect, Chemical Applications', Academic Press, New York & London, 1971.

135. L.J. Groot & J.B. Stanbury, Am. J. Med., 1959, 27, 586.

136. J.C. Savoie, J.P. Massin & F. Savoie, J. Clin. Invest., 1973, 52, 116.

137. J.C. Savoie, P. Thomopoulos & F. Savoie, J. Clin. Invest., 1973, 52, 106.

138. J. Wolff & I. Covelli, Eur. J. Biochem., 1969, 9, 371.

139. C. Cha & H.H. Scheragu, J. Biol. Chem., 1963, 238, 2958.

140. H. Fraenkel-Conrat, Arch. Biochem., 1950, 27, 109.

141. I. Covelli & J. Wolff, J. Biol. Chem., 1966, 241, 4444.

142. I. Covelli & J. Wolff, J. Biol. Chem., 1967, 242, 881.

143. H.B. Bensusan, Arch. Biochem., 1966, 115, 77.

144. J.R. Chambert, R. Dedonder & G. Rapoport, Biochim. Biophys. Acta., 1967, 132, 330.

145. L. Birkofer & K. Hempel, Ber., 1963, 96, 1373.

146. J. Prudelles, J.L. Margot, P. Fromageot, C. Oliver, P. Jacquet, D. Gorrdji & A. Tixier-Vidal, F.E.B.S. Letters, 1972, 22, 19.

147. A. Menez, J.L. Margot, P. Fromageat, A-M. Ronseray, P. Boquet & J.P. Changeaux, F.E.B.S. Letters, 1972, 22, 19.

148. M.C. Allen, D.E. Brundish & R. Wade, J. Chem. Soc. Perkin I, 1979, 2057.

149. K.J. Bruning, J. Amer. Chem. Soc., 1947, 69, 205.

150. L. Schutte & E. Havinga, Rec. Trav. Chim. Pay-Bas, 1967, 86, 385.

151. H.B. Bensusan & M.S.R. Naidu, Biochem., 1967, 6, 12.

152. C.T. Holloway, R.P.M. Bond, I.G. Knight & R.B. Beechey, Biochem., 1967, 6, 19.

153. L.E. Perlgut & W.W. Wainio, Biochem., 1966, 5, 608.

154. J.L.M. Syrier & H.C. Beyerman, Rec. Trav. Chim., 1974, 93, 117.

155. D.E. Brundish & R. Wade, J. Chem. Soc. Perkin I, 1973, 2875.

156. D.E. Brundish & R. Wade, J. Chem. Soc. Perkin I, 1981, 31.

157. M.C. Allen, D.E.Brundish, J.R. Martin & R. Wade, J. Chem. Soc. Perkin I, 1981, 2040.

158. R.W. Cowgill & J. M. Clark, J. Biol. Chem., 1952, 198, 33.

159. W.C. Still, M. Kahn & A. Mitra, J. Org. Chem., 1978, 43, 2923.

160. B.F. Gisin, Helv. Chim. Acta., 1973, 56, 1476.

161. C.J. Turner, Part II Thesis, University of Oxford, 1979.

162. J. Meienhofer, in 'Hormonal Proteins and Peptides II', Ed.,C.H. Li, Academic Press, New York & London, 1973, p. 46.

163. R.B. Merrifield, J. Amer. Chem. Soc., 1963, 85, 2149.

164. A. Loffet & J. Close, in 'Peptides 1968', North Holland Publications, Amsterdam, p. 189.

165. V. Gut & J. Rudinger, in 'Peptides 1968', North Holland Publications, Amsterdam, p. 185.

166. T. Brown & J.H. Jones, J. Chem. Soc. Chem. Comm., 1981, 648.

167. W.W. Binkly, J. Amer. Chem. Soc., 1948, 70, 2010.

168. K.L. Kirk & L.A. Cohen, J. Amer. Chem. Soc., 1973, 95, 4619.

169. H.J. Anderson & J.K. Groves, Tet. Lett., 1971, 34, 3165.

170. T. Brown & J.H. Jones, British Patent Application No. 8110116.

171. B.O. Handford, T.A. Hylton, K.T. Wang & B. Weinstein, J. Org. Chem., 1968, 33, 4251.

172. M.C. Khosla, R.R. Smeby & F.M. Bumpus, Biochem., 1967, 6, 754.

173. G.R. Marshall & R.B. Merrifield, Biochem., 1965, 4, 2394.

174. W.K. Park, R.R. Smeby & F.M. Bumpus, Biochem., 6, 3458.

175. K. Arakawa, & F.M. Bumpus, J. Amer. Chem. Soc., 1961, 83, 728.

176. L. Benoition, Can, J. Chem., 1962, 40, 570.

177. B.W. Erickson & R.B. Merrifield, J. Amer. Chem. Soc., 1973, 95, 3750.

178. B. Iselin, in 'Peptides', Proceedings of the Sixth European Peptide Symposium, Athens 1973, L. Zervas, ed., Pergamon Press, Oxford, 1966, p. 27.

179. R.C. Sheppard, personal communication.

180. L. Zervas, M. Winitz & J.P. Greenstein, J. Org. Chem., 1957, 22, 1515.

181. D. French & J.T. Edsall, Adv. Protein Chem., 1945, 2, 227.

182. A.K. Smith, P. Hundler & J.N. Mrgudish, J. Phys. Chem., 1940, 44, 874.

183. W.A. Jacobs & C.C. Craig, J. Biol. Chem., 1936, 113, 759.

184. E. Wünsch, E. Jaeger, C. Kisfaludy & M. Löw, Angew. Chem. Int. Ed. Engl., 1977, 359, 1617.

185. A. Hallett, personal communication.

186. E.C. Jorgensen, G.C. Windridge & T.C. Lee, Rec. Trav. Chim., 1974, 93, 117.