SYNTHESIS OF HISTIDINE DERIVATIVES PRESENT IN CARDIAC MUSCLE

A thesis presented in part fulfilment of the requirement for the Degree of Doctor of Philosophy

by

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November, 1991

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ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisor Professor D.J. Robins for the guidance and encouragement demonstrated during both the course of this work and in the preparation of this thesis.

I should also like to thank Dr. D.J. Miller (Department of Physiology) for the testing of the compounds synthesised during the course of this work and for his advice during the preparation of this thesis. My gratitude is extended to Dr. J.J. O'Dowd and Dr. A. O'Dowd for their work on the HPLC systems developed during this project.

I am also grateful to the following people: Dr. D.S. Rycroft, Mr. J. Gall, and Mr. J. McIvor for NMR spectra; Mr. A. Ritchie for mass spectra; Mr. G. McCulloch for IR spectra; Dr. A.A. Freer for X-ray diffraction work; Dr. D.J. Cole for GC work; Dr. J. Nally for proof reading this thesis; my friends and colleagues in the Henderson lab for helpful discussion.

I wish to acknowledge the financial support from the British Heart Foundation and the Loudon Bequest for this work.

Finally, I want to express my gratitude to my family and friends, in particular my nana and papa for their endless support and encouragement throughout my university career. It is to them I dedicate this thesis.

TABLE OF CONTENTS

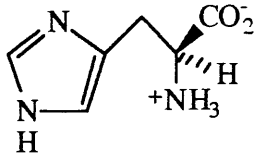
ABBREVIATIONS

A	-	actin
Ac	-	acetyl
Ans	-	anserine
ATP	-	adenosine triphosphate
Bal	-	balenine
t-BOC	-	*t*-butoxycarbonyl
Car	-	carnosine
CBZ	-	carbobenzoxy
βCD	-	β-cyclodextrin
CMOS	-	(*S*)-[(carboxymethyl)oxy]succinic acid
DABCO	-	diazobicyclo[2.2.2]octane
DCC	-	dicyclohexylcarbodiimide
DEPT	-	distortionless enhancement by polarisation transfer
DMF	-	*N,N*-dimethylformamide
EDDS	-	(*S,S*)-ethylenediamine-*N,N'*-disuccinic acid
Fmoc	-	9-fluorenylmethoxycarbonyl
GLC	-	gas chromatography
HC	-	homocarnosine
HPLC	-	high performance liquid chromatography
iBu	-	isobutyl
IR	-	infra red
Me	-	methyl
NAA	-	*N*-acetylanserine
NAC	-	*N*-acetylcarnosine
NAH	-	*N*-acetylhistidine
NAHC	-	*N*-acetylhomocarnosine

NAMH	-	*N*-acetyl-1-methylhistidine
N(im)	-	used to denote a histidine compound derivatised on the imidazole ring when the position of the substitution has not been specified
NMR	-	nuclear magnetic resonance
NOE	-	Nuclear Overhauser Enhancement
ODS	-	octadecyl-silica
PG	-	protecting group (general)
Ph	-	phenyl
THF	-	tetrahydrofuran
TLC	-	thin layer chromatography
Tm	-	tropomysin
Tn	-	troponin
Tosyl/pTSA	-	*p*-toluenesulphonate
Trt/trityl	-	triphenylmethyl
UV	-	ultraviolet
benzoxymethyl	-	$PhCH_2OCH_2$- benzyloxymethyl

SUMMARY

N-Acyl-L-histidine derivatives, a number of *N*-acyldipeptides containing L-histidine (A), and dipeptides containing 1-methyl-L-histidine (B) or 3-methyl-L-histidine (C) are thought to be present in cardiac muscle. These compounds are believed to play an important role in the healthy functioning of the heart. The work presented in this thesis investigates the synthesis of optically active forms of some of these L-histidine derivatives. The results obtained when these compounds were tested by the physiologists for their individual effects on cardiac muscle are presented.



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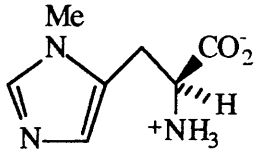

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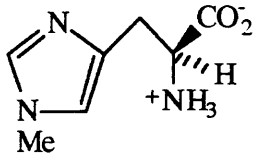


(C)

(A). *N*-Acyl-L-histidine Derivatives and *N*-Acyldipeptides Containing L-Histidine

N-Acetyl-L-histidine and *N*-propionyl-L-histidine were successfully synthesised in good yield and high optical purity by the direct acylation of L-histidine with acetic or propionic anhydride. Two routes aimed at providing a range of *N*-

acyldipeptides containing L-histidine in chemically and optically pure form were studied. A series of *N*-acylamino acids were coupled to L-histidine protected on the carboxyl function. The first route used the benzyl ester of L-histidine but a number of problems were encountered which made this route unattractive. The second route involved L-histidine protected as a metal salt. The mixed anhydride method of peptide coupling was employed to produce the compounds in chemically pure form.

A number of methods were used in an attempt to determine the optical purities of the histidine derivatives synthesised. The most promising method involved the coupling of (*R*)-α-methylbenzylamine to the carboxyl function of the *N*-acyldipeptides. The diastereoisomeric mixtures formed were then analysed using reverse-phase high performance liquid chromatography (HPLC). The solvent mixture used to elute the column was adjusted to find a composition which would allow the separation of the diastereoisomers. The areas under the peaks were then used to estimate the optical purities of the compounds under investigation.

The L-histidine derivatives were examined by physiologists at Glasgow University. They discovered, using HPLC techniques, that *N*-acetyl-L-histidine, *N*-acetyl-L-carnosine and *N*-acetyl-L-homocarnosine were present in cardiac muscle. The *N*-propionyl derivatives synthesised had an adverse effect on the Ca-sensitising apparatus of cardiac muscle. *N*-Acetyl-L-histidine, *N*-acetyl-L-alanyl-L-histidine and *N*-acetyl-D-alanyl-L-histidine were weak Ca-sensitisers. *N*-Acetylglycyl-L-histidine, *N*-acetyl-L-carnosine and *N*-acetyl-L-homocarnosine produced more positive action on the Ca-sensitising apparatus of the heart muscle.

(B). 1-Methyl-L-histidine, 3-Methyl-L-histidine and Dipeptides Containing L-Histidine Methylated on the Imidazole Ring

Because the imidazole function of L-histidine has two possible sites for *N*-alkylation to take place, routes were developed to obtain selectively the desired *N*-methylated histidine derivatives as pure isomers.

A synthesis of 1-methyl-L-histidine, reported in the literature, was attempted. Problems were encountered in introducing the protecting group onto the 3-position of the imidazole ring. This could not be achieved in the manner described in the literature, thus an alternative route was studied. *N*(α),3-bis(*t*-Butoxycarbonyl)-L-histidine methyl ester was alkylated using methyl iodide. The residue obtained from the reaction was found to be a mixture of the desired 1-methyl compound and the 1,3-dimethyl compound. This mixture was successfully separated. The final stage was to remove the protecting groups. A number of different methods were used. Each procedure removed the protecting groups without any difficulties but each attempt yielded 1-methylhistidine which was not optically active.

The synthesis of 3-methyl-L-histidine, reported in the literature, was attempted. The synthetic sequence was successful until the final deprotection stage which yielded 3-methylhistidine in chemically pure form, but contrary to the data published, the product was not optically active. An alternative route was investigated. *N*(α)-*t*-Butoxycarbonyl-1-benzoxymethyl-3-methyl-L-histidinium methyl ester iodide was synthesised. 3-Methyl-L-histidine should then be obtained upon removal of the protecting

groups. The removal of the methyl ester resulted in a racemic product. Attempts to remove the benzoxymethyl group were consistently unsuccessful. 3-Methylhistidine could not be obtained in chemically or optically pure form from this route.

A route to dipeptides containing 1-methylhistidine was established. 1-Methylglycylhistidine, 1-methylcarnosine and 1-methylhomocarnosine were synthesised in chemically pure form. The intermediates in this route all possessed some degree of optical purity but the hydrolysis of the methyl esters at the end of the route resulted in racemic products.

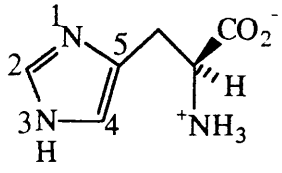
The route established for the synthesis of dipeptides containing 3-methylglycylhistidine and 3-methylcarnosine produced these compounds as pure isomers. Problems in removing the methyl esters in the final stage resulted in the products being racemic. This route failed to yield 3-methylhomocarnosine, thus an alternative route to these 3-methyl compounds was investigated. The benzoxymethyl group was introduced onto the 1-position of the imidazole ring of *N*(α)-carbobenzoxy-L-carnosine methyl ester. Methylation of the 3-position was attempted but failed to produce reasonable amounts of the *N*-methylated product. Time did not allow further investigation into the routes leading to the dipeptides containing 1- or 3-methyl-L-histidine.

1-Methylhistidine, 1-methylglycylhistidine, 1-methylcarnosine, 1-methylhomocarnosine, 3-methylhistidine, 3-methylglycylhistidine and 3-methylcarnosine were all obtained as pure isomers but none of the compounds possessed any optical activity.

CHAPTER 1

Introduction

It has long been established that three main dipeptides containing L-histidine (1) occur in muscle.[1] These are carnosine (β-alanyl-L-histidine) (2), anserine (β-alanyl-1-methyl-L-histidine) (3) and balenine (β-alanyl-3-methyl-L-histidine) (4). The numbering of the imidazole ring in L-histidine (1) and its derivatives in this thesis is carried out according to the IUPAC-IUB convention.[2] The system according to *Chemical Abstracts*,[3] numbers the nitrogens of the ring in the opposite order. It was decided to follow the biological convention since it would avoid confusion in communications with the physiologists. Furthermore, much of the literature available on histidine chemistry also follows the biological convention.



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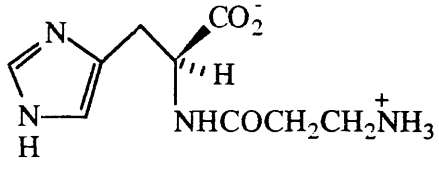

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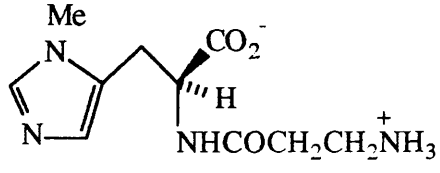

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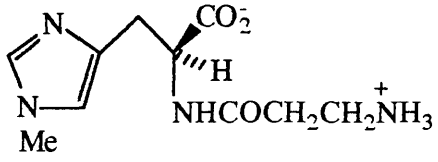


(4)

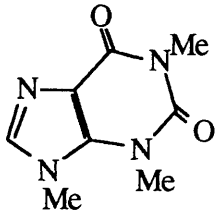
Other histidine derivatives exist that are acylated on the terminal amino group or monomethylated on the imidazole ring of histidine. Thus, *N*-acetylcarnosine and *N*-acetylhomocarnosine have been detected in muscle and brain respectively.[4] Free *N*-

acetylhistidine has been isolated from amphibian cardiac muscle.[5] It was found previously in the central nervous system and the eyes of frogs,[6] and subsequently in those of fish.[7,8] It can therefore be said that histidine dipeptides, the acyl forms of these derivatives, and the *N*-methylated histidine derivatives are widely distributed in the animal kingdom.

The full biological roles of these histidine derivatives, especially in muscle, is still subject to speculation. Imidazole derivatives occur at millimolar concentrations in muscle cytoplasm and undoubtedly contribute to pH buffering capacity.[1,9] Carnosine and its methylated analogues may play a role in the regulation of glycolysis. These compounds may also be involved in other reactions of energy transduction.[1,10] Carnosine has also been shown to possess at least some of the properties of a neurotransmitter in the olfactory system where its release from synaptosomes appears to be calcium dependent.[11]

Caffeine (5) has long been known to induce calcium release from the sarcoplasmic reticulum.[12] Caffeine is a methylxanthine and includes an imidazole ring in its structure. Sulmazole (6), a cardiotonic drug, is an imidazole derivative and behaves as a more potent calcium sensitiser.[13,14] Previous work[15] has shown that the additional lone pair on the nitrogen in the imidazole ring not required for aromaticity is the key feature necessary to cause contraction in cardiac and skeletal muscle. Dr. D. J. Miller and co-workers, physiologists at Glasgow University, sought an explanation of the enhancing effect of the compounds (5) and (6) on the contractile apparatus of the heart. They considered whether analogous compounds containing the imidazole ring exist in mammalian muscle tissue in sufficient quantities. It interested

them to find out if (5) and (6) were mimicking the action of such natural compounds.



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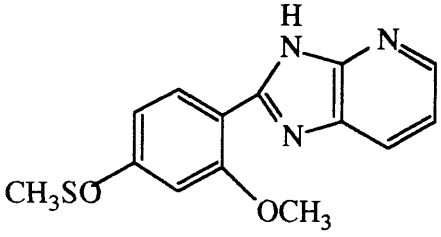
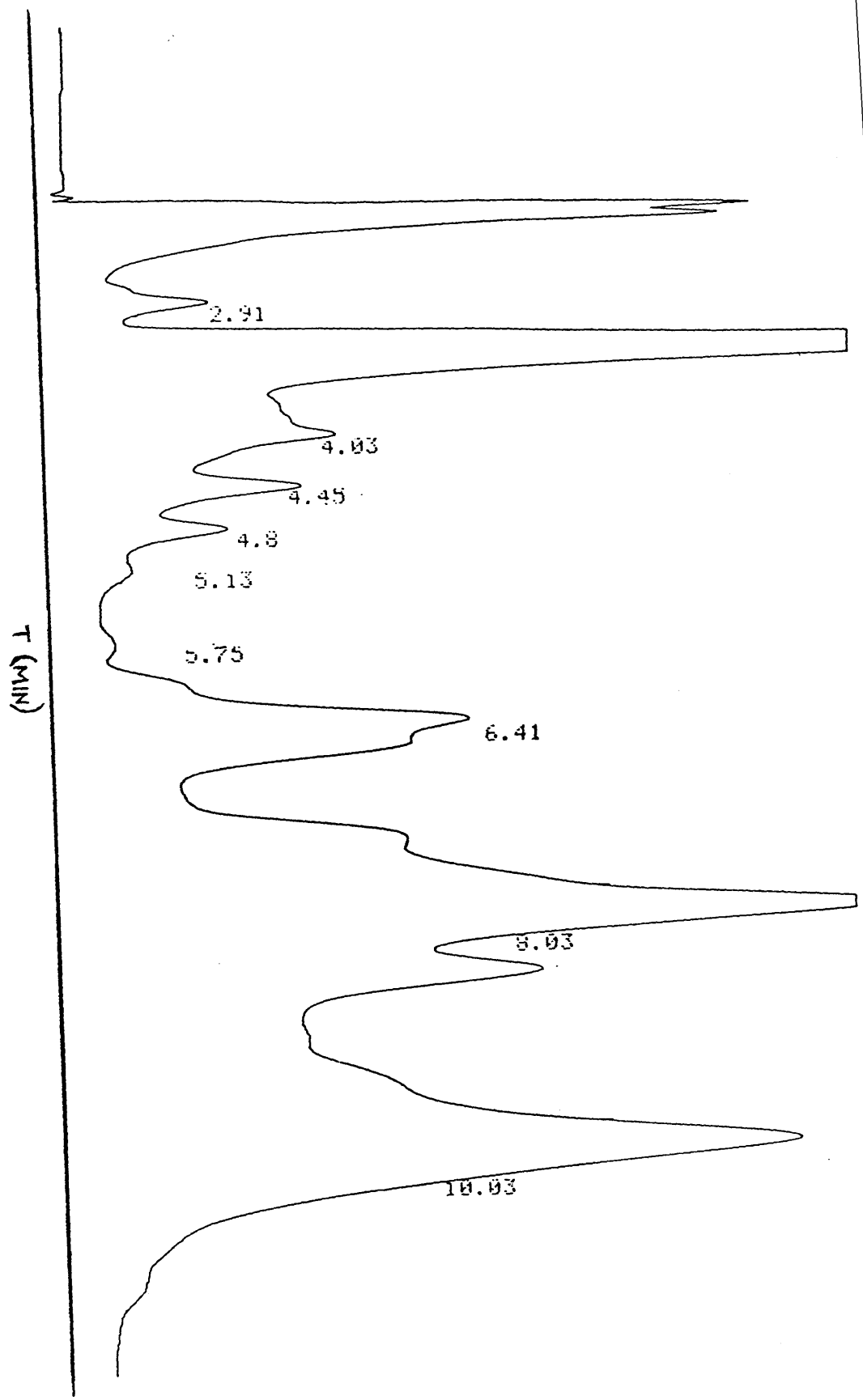


(6)

Preliminary work showed that carnosine and *N*-acetylhistidine enhance the calcium sensitivity of rat muscle contractile proteins.[16] Further data indicated that *N*-acetylhistidine and its 1-methyl derivative are present in skeletal muscles and that these, along with *N*-acetyl forms of carnosine and anserine, could be significant contributors to the imidazole pools of cardiac muscle.[17]

In order to obtain more substantial results, novel high performance liquid chromatography (HPLC) methods were developed by O'Dowd *et al.*[18] to analyse extracts taken from the cardiac muscle of frogs. This was intended to allow the identification and quantification of the histidine derivatives present. Elution of muscle extracts with a phosphate buffer mobile phase from columns packed with hypersil octadecyl-silica (ODS) (5 μm) resulted in good separation of the histidine derivatives as shown in Figure 1. The conditions used for HPLC are discussed in more detail in Section 4.4.1. Fractions corresponding to the chromatographic peaks were collected and spotted onto filter paper.

Figure 1. HPLC trace of extracts from cardiac muscle of frogs. See text for conditions.



Imidazole compounds both with substituted and unsubstituted rings were visualised using a reaction between imidazole and bromine.[18] Another visualisation system distinguished which fractions contained compounds with unsubstituted rings and ninhydrin was used to test for the presence of compounds with a free terminal amino group. Cardiac failure was induced in some hearts (collaborative work with scientists at the Dr Karl Thomae company in Germany) to look for any deviation in the composition of histidine derivatives from a healthy heart. On HPLC analysis there were very significant alterations in the relative abundance and the occurrence of the histidine derivatives. This gave an indication that histidine derivatives are important for the healthy function of the heart and that this area warranted further investigation.

Since the beginning of this work histidine derivatives have been identified as constituents in brain, vascular smooth muscle, and plasma. The derivatives have a variety of physiological roles, some of which have been discussed in the preceding paragraphs. In the course of this work it has also been discovered that some of the histidine derivatives have the ability to increase the calcium ion sensitivity of the contractile proteins of cardiac and skeletal muscle. It is also now known that some of the derivatives have actions on tone in vascular smooth muscle.

Because of these multiple and potentially important actions, alterations in either the concentration or variety of the histidine derivatives could be of very significant physiological importance in the cardiovascular system.

In order to identify the peaks present in the previously mentioned HPLC trace of cardiac muscle, it was necessary in this

work to synthesise a range of L-histidine compounds for comparison. Pure compounds in optically active form were required so that they could be tested to establish whether they behave as calcium sensitisers and to determine what other effects they have on heart muscle.

The physiology involved in these procedures will be discussed in detail in Chapter 2. Since most of the compounds required are commercially unavailable, general synthetic schemes to a range of these compounds were developed. The synthetic methods employed in the peptide synthesis will be discussed in Chapter 3. The routes to the *N*-acylhistidine dipeptides will be covered in Chapter 4 and the test data for these compounds is discussed in Section 4.4. The routes to the 1- and 3-*N*-methylated derivatives will be discussed in Chapter 5.

CHAPTER 2

Physiology of the Heart

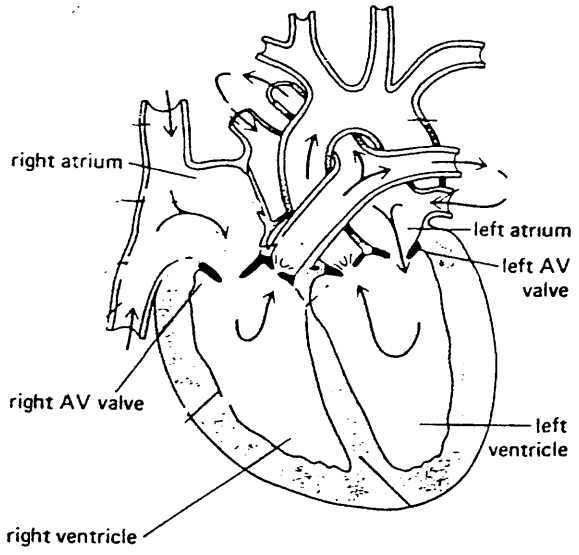
The function of the heart is to generate the pressures which produce blood flow.[19] The cardiac muscle apparatus contracts and relaxes rhythmically and without fatigue. Its power output constantly adapts to cater for the variable demands made on it. For example, during physical exercise various tissues and organs require a greater flow of blood if they are to maintain normal function and hence higher cardiac output is required. The mechanisms involved in the contraction of the heart will be discussed.

2.1 Anatomy of the Heart

The human heart is divided into two halves (Figure 2).[20] Each of these halves has two chambers, namely the atrium and the ventricle. The atrium receives blood from the veins and passes it on to the ventricle in the same half. The two cavities are separated by the atrioventricular valves. These valves permit blood flow from the atrium to the ventricle but not the reverse process. The opening and closing of these valves is a passive mechanism caused by the difference in pressure across them.

Myocardium is composed of layers of cardiac muscle which are tightly bound together and completely enclose the blood filled chambers.

Figure 2.20 Diagramatic section of the heart. The arrows indicate the direction of blood flow.



The individual cells of cardiac muscle are joined end to end at intercalated discs within which are gap junctions. These gap junctions allow the spread of currents from one cardiac muscle cell to another via low resistance pathways which trigger action potentials. Contraction of cardiac muscle, like that of many other muscle types, is triggered by depolarisation of the plasma membrane. If the threshold of stimulation is not reached then the cardiac muscle will not react at all. Normally, it responds with all its fibres being activated or none at all.

2.2 Origin of the Heartbeat

Certain areas of the heart contain specialised muscle fibres which are essential for normal excitation of the heart. These areas are known as the conducting system which are in contact with the

normal muscle fibres via the gap junctions. The specialised conducting system allows the rapid and coordinated spread of excitation. Several areas of the conducting system of the heart are capable of self excitation. This has the effect of causing the membrane potential to reach threshold through depolarisation, at which point an action potential is initiated. The area with the fastest intrinsic rhythm becomes the pacemaker. Normally the area known as the sinoatrial node is the pacemaker for the whole heart. From the sinoatrial node, the wave of excitation spreads through the atrium via the low resistance pathways provided by the gap junctions. It propagates through the atrioventricular node along a conducting link to the working myocardium of the ventricles and finally makes contact with the normal cardiac muscle cells through which the impulse spreads from cell to cell in the remaining myocardium. The rapid conduction ensures a single coordinated wave of contraction.

2.3 Role of Calcium in Muscle Excitation-contraction Coupling

The depolarisation of cardiac muscle triggers contraction. The importance of calcium for heart muscle contraction has been recognised since Ringer's discovery[21] in 1883 that an isolated heart from a frog ceases to beat in the absence of calcium in the bathing solution. Indeed, the mechanism coupling excitation and contraction in all types of muscle is an increase in the intracellular concentration of ionised calcium.

A muscle fibre contains actin and myosin; these are the two proteins crucial to contraction. Cross-bridges extend from the

myosin filament. Contraction occurs when these cross-bridges make contact with binding sites on the actin filament and as a result generates a force on the actin filaments. Adenosine triphosphate (ATP) is the source of chemical energy for the contraction process. Muscles are obviously not in a continuous state of contraction; in the resting muscle fibre cross-bridge binding is inhibited. This inhibition is due to two regulator proteins, troponin and tropomyosin, which are bound to the actin filament.

Tropomysin molecules are arranged end to end along the chains of actin, so that they partially cover the myosin binding sites. The result of this relationship is that the cross-bridges cannot make contact with their binding sites on actin. Troponin holds the tropomysin molecules in place (Figure 3).[22] In order for the cross-bridges to bind to actin, the binding sites must be uncovered.

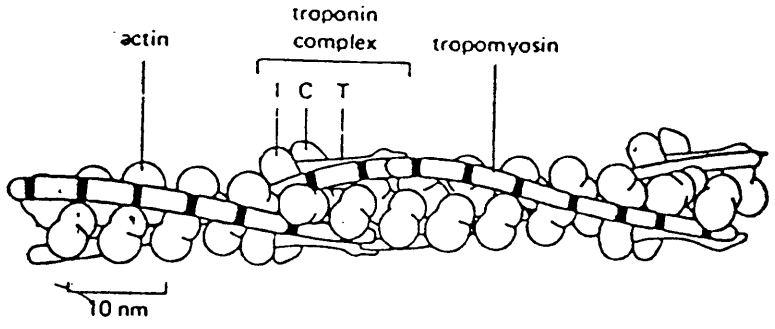


Figure 3.[22] Muscle thin filament showing the position of tropomysin and the troponin complex on actin.

The initial membrane depolarisation causes voltage-gated calcium channels in the cardiac cell membrane to open, which results in a flow of calcium ions into the cell. This results in a

rapid rise in the concentration of calcium ions which provokes a much larger amount of calcium to be released from the sarcoplasmic reticulum.

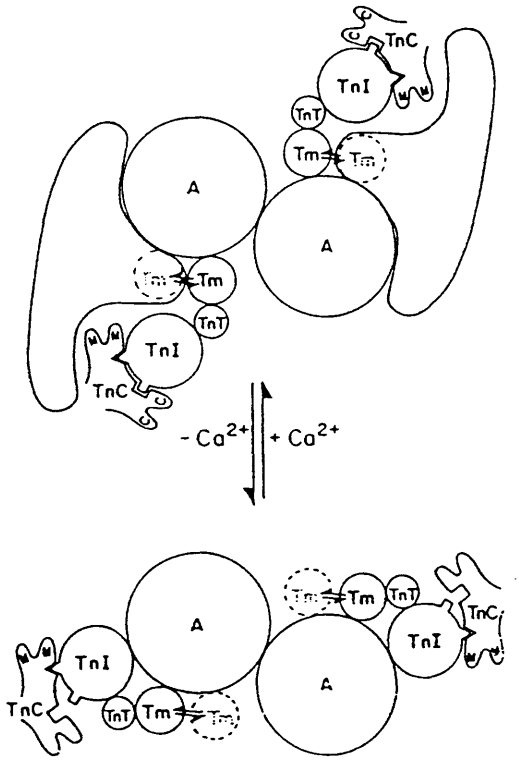


Figure 4.[23] This is a cross section through the filaments seen in Figure 3 to indicate the spatial relationships when calcium is bound and when it is not.

Calcium ions bind to specific sites on troponin. As in skeletal muscle, cardiac troponin is composed of three polypeptide chains (Figure 4).[23] It consists of a calcium binding subunit known as troponin C (TnC), an inhibitory subunit (TnI) and a tropomysin-binding peptide (TnT).

Within the troponin C crystal (Figure 5),[22] the molecules are extended structures, each with two domains connected by a single

helical strand. Each domain has two calcium ion binding sites. At the carboxy terminal domain there are two high affinity sites which bind either calcium or magnesium ions. These high affinity sites are required to maintain the integrity of the troponin complex binding sites. The amino terminal domain contains two low affinity sites, specific to calcium ions. These lower affinity sites are the sites which are involved in the regulation of muscle contraction.

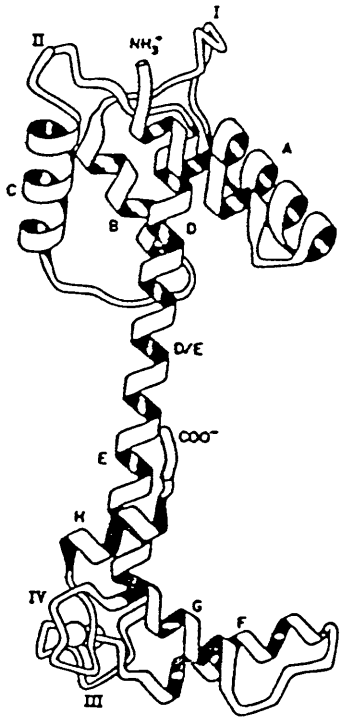


Figure 5.[22] The fold of the Troponin C crystal.

The E-F hand model (Figure 6)[24] is the model used to describe the common structure of intracellular calcium binding proteins. Six oxygen ligands are formed from the peptide chain to coordinate to a calcium ion. These ligands are arranged by a

helix:loop:helix configuration of the polypeptide chain. The α-helical segments in troponin C are labelled A-F. Each calcium binding site is flanked by two of these segments.

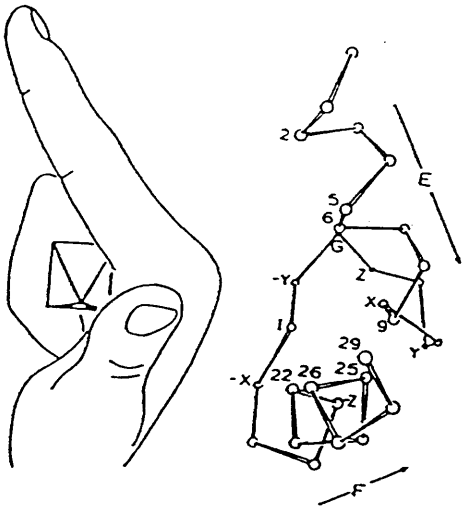


Figure 6.[24] The EF hand model for the common structure of intracellular calcium binding proteins.

The two helices work as lever arms, and their relative orientation depends on the presence of calcium ions. In the resting muscle ($[Ca^{2+}]$ about 10^{-8} to 10^{-7} M), the high affinity sites are thought to be always occupied. It is believed that the occupation of the low affinity sites with calcium ions causes the conformation of the troponin molecule to change. This change in conformation leads to an interaction with troponin I which in turn leads to the binding sites on actin being uncovered. The net result is that cross-bridging can take place and hence contraction. The removal of the calcium ions reverses the process. The entire cycle of contraction is represented in Figure 7.[20]

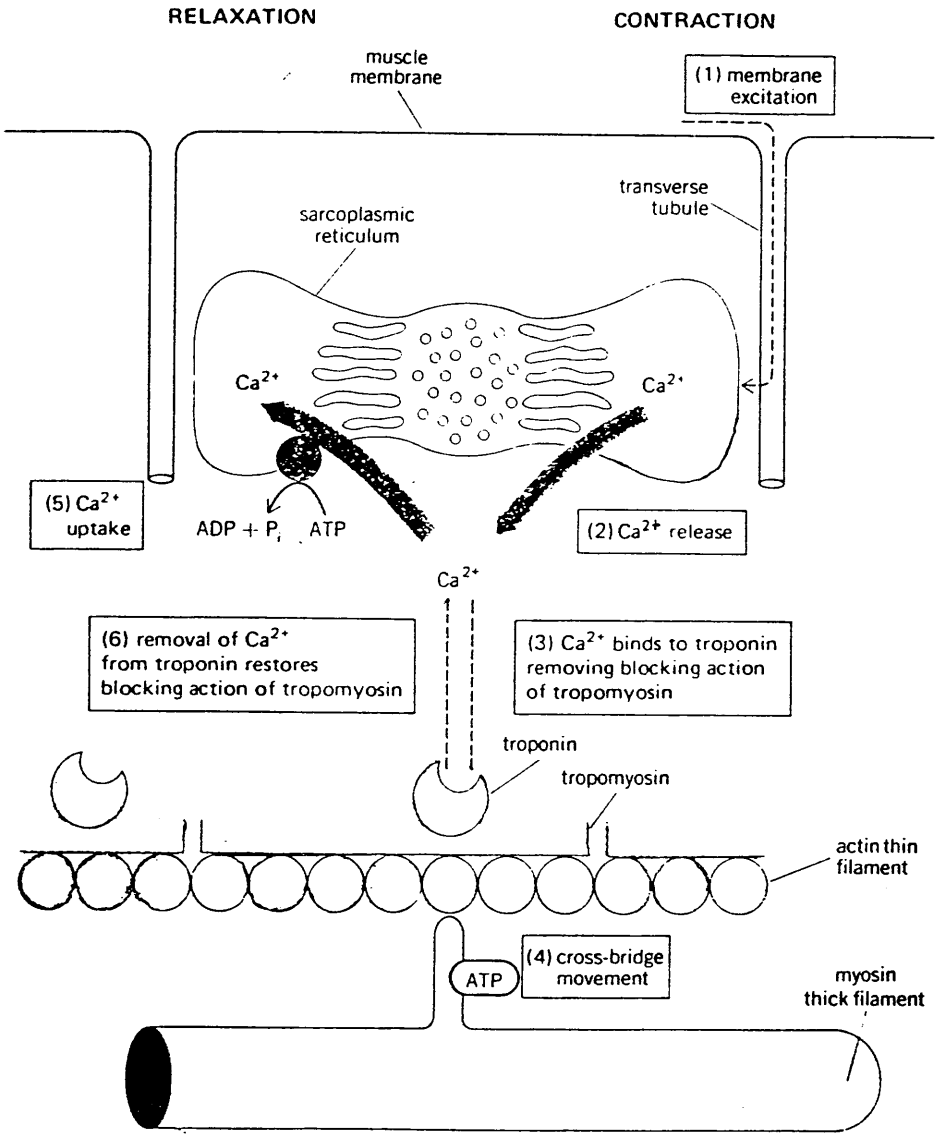


Figure 7.[20] Role of calcium in muscle excitation-contraction coupling.

2.4 Alterations to the Force of Contraction

The force of contraction is in direct proportion to the fraction of troponin binding sites occupied.[19] Heart muscle has a large contractile reserve, which means that its performance can be

increased by a number of means. These include increasing the extracellular calcium concentration, increasing the frequency of beating, and the application of compounds with a positive inotropic action. In the majority of these cases the increase in force or positive inotropy is due ultimately to an increase in the intracellular calcium concentration reached during contraction. If the frequency of stimulation is increased, the filling of the sarcoplasmic reticulum is improved, so that each stimulus induces a greater release of calcium. Due to the higher calcium concentration reached during the period when the ventricles of the heart are contracting, the contractile force also increases, but can take longer to develop fully.

The relationship between peak force and intracellular calcium ion concentration is virtually the same regardless of whether the force has been changed by altering the frequency of stimulation or by altering the extracellular calcium concentration. Even during positive inotropic intervention, it seems that force development is submaximal. Results obtained by Fabiato[25] suggest that, even under optimal conditions, living cardiac muscle is never more than about half to two-thirds maximally activated. Force can therefore be easily varied by increasing or decreasing the free maximum calcium ion concentration achieved during activation.

The binding of calcium ions to troponin receptor sites activates contraction and the degree of occupancy of troponin determines the contractile force. Thus contractility may also be altered by changing the calcium sensitivity of the regulator protein, troponin, so that at a given concentration of calcium ions more troponin molecules are occupied. Amongst the factors

known to alter the apparent affinity of the regulatory proteins to calcium are: the length of the sarcomere (i.e. the repeating structural unit of a muscle),[26] intracellular pH,[19] the concentration of cyclic nucleotides,[19] and calcium sensitising, cardiotonic drugs.[27,28,29]

The apparent affinity, K_{app}, of troponin C for calcium ions represents the ratio between 'on' and 'off' rates, (1-2 x 10^8 sec^{-1}), for Ca^{2+} binding. It is generally believed that the rate at which the troponin sites become occupied cannot be increased since it is diffusion limited.[30] An increase in K_{app}, thus implies a slowing of the 'off' rate. The removal of the calcium ions reverses the activation process. It is this removal which is the rate determining step for calcium binding. It is alterations to the speed of dissociation of the calcium ions which could cause changes to calcium sensitivity.

The relationship between the sarcomere length and the force produced on contraction is shown in Figure 8.[31] When the sarcomere length is less than optimum (area 1), the thin filaments will overlap. This could mean that, in some way, the cross-bridges do not make normal contact with the binding sites on the actin filament. As the length of the sarcomere is increased, cross-bridging is more efficient in producing force until an optimum length, (l_o), is reached where force production is maximal (area 2). At l_o, therefore, all of the cross-bridges have access to binding sites on actin and there is no steric hindrance to tension development at the filament ends. If the sarcomere length is increased past this optimum, the thin filaments are aligned in such a way that some of the cross-bridges are unable to reach the binding sites on actin (area 3). In this situation less cross-bridges

can form and the force produced is reduced in direct proportion to the amounts of overlap between the actin and myosin filaments.

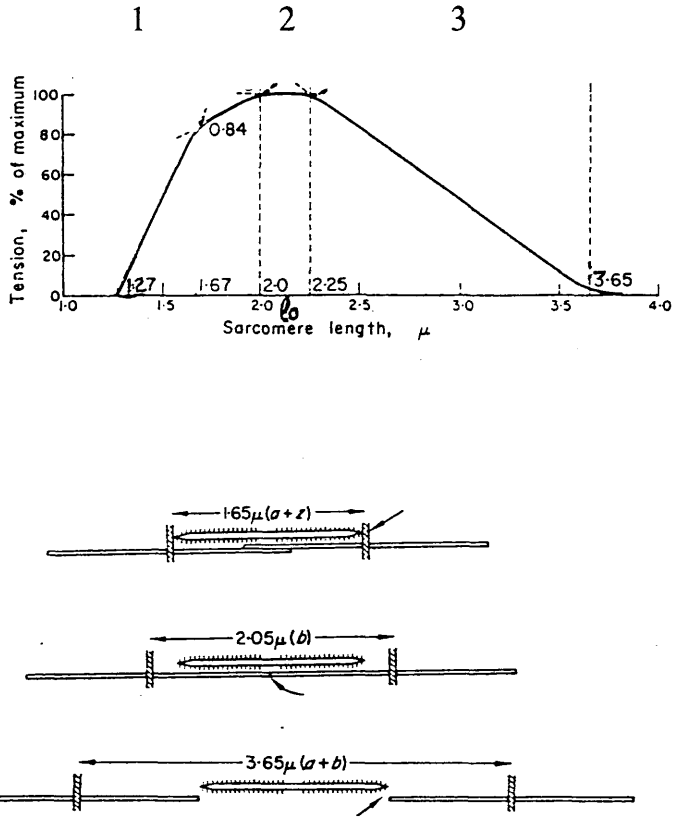


Figure 8.[31] Tension-length curve from part of a single muscle fibre.

The sarcomere length in the working heart is normally less than the optimum. This means that even if all the cross-bridges are active, the cardiac muscle is only able to produce less than 100% tension on activation. At a set sarcomere length, factors such as pH, temperature, and certain cardiotonic drugs can alter the tension produced at a given concentration of calcium ions. The qualitative effect of these factors on the tension is shown in Figure 9.

This action is to be understood as an alteration to the force produced by each cross-bridge or, if cross-bridge kinetics are altered, by force being transmitted more successfully through the filaments. This means that 'stiffness' is increased as a result of cross-bridges remaining attached for a longer part of each cycle.

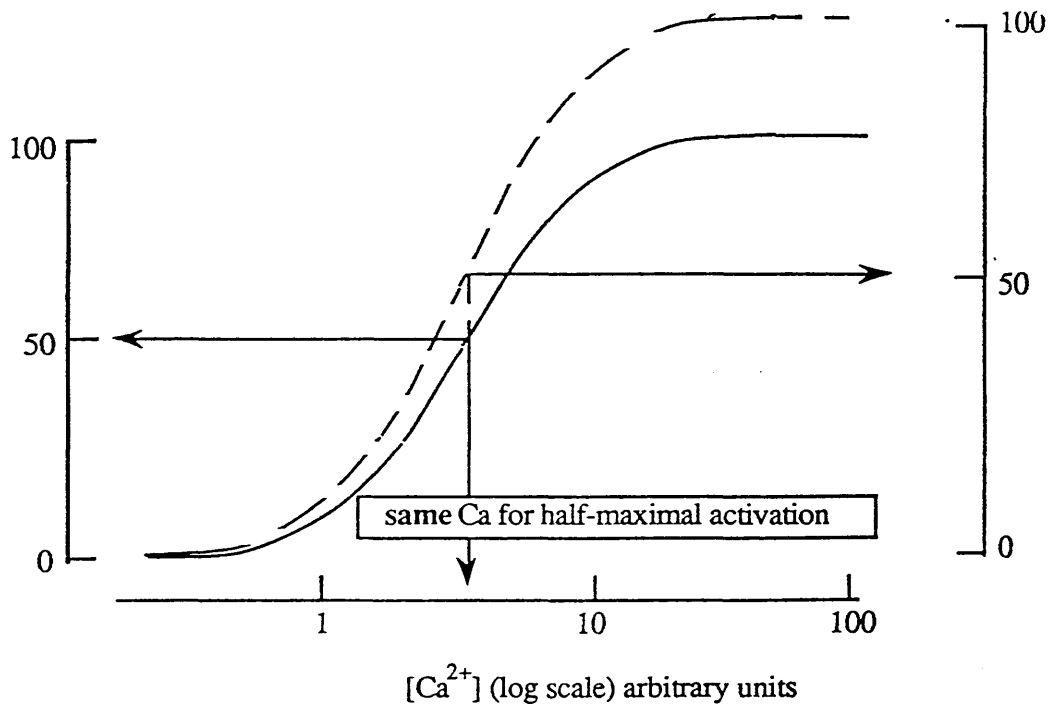


Figure 9 Simple peak force enhancement.

2.5 Testing of the Sensitising Effects of L-Histidine Derivatives on Cardiac Muscle Fibres

L-Histidine derivatives synthesised in the course of this work were tested by Dr D. J. Miller and his co-workers.[32] The tests were carried out on muscle fibres isolated from hearts taken from rats. These fibres were chemically skinned with either

Triton X-100 or saponin. Triton completely disrupts the membrane while saponin selectively skins the cells leaving the sarcolemma perforated with holes large enough to permit the free passage of small ions and molecules such as ATP, etc. The holes are small enough though to retain the diffusible sarcoplasmic enzymes. This means the mitochondrial and the sarcoplasmic reticulum membranes remain functional, permitting the study of the Ca-sensitising effects of these histidine derivatives under conditions of dynamic tension development. Skinned preparations have the advantage of having many of the complicating factors acting in the intact cell brought under experimental control.

Experiments were carried out on small, free running trabeculae isolated from the right ventricle of the rat and mounted for isometric force measurement. The animals were killed by a blow to the head, the heart was rapidly excised and flushed with a saline solution at room temperature.

Accurate knowledge of the sarcomere length of the isolated muscle fibre is critical in these experiments for the following reasons:

(a) the well established effect of reduced Ca-sensitivity of the contractile proteins at shorter sarcomere length;

(b) the efficacy of Ca-sensitising by caffeine (5) and sulmazole (6) may be affected by sarcomere length; and

(c) the effect of sarcomere length on the extent of calcium uptake and / or release by the sarcoplasmic reticulum.

In this study, the sarcomere length was set at 2.1-2.2 μm (equivalent to l_o in Figure 8). The fibre was fixed at one end and attached to a force transducer at the other. The ionic environment around the fibre approximates to that in the intact cell. When the

concentration of calcium ions is altered, the muscle generates a force which reaches an equilibrium level which is a simple function of the concentration of calcium ions. The fibre was exposed to buffer solutions containing a range of concentrations of the histidine derivative under investigation at a range of calcium concentrations and the tension produced was measured for each one. The results were collected as steady-state force and normalised. A relationship between the relative tension and pCa was plotted to show a curve similar to that in Figure 10.

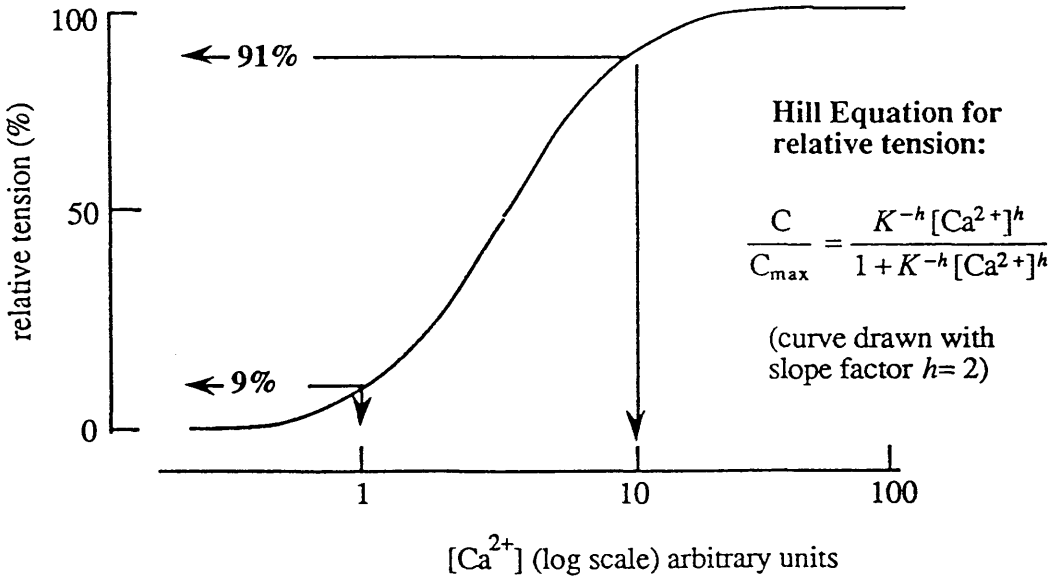


Figure 10. Basic calcium sensitivity

The curve plotted for each compound tested was compared to the control curve obtained with no test compounds present. The effect expected for an L-histidine derivative which sensitises the heart muscle is shown in Figure 11. The curve was shifted to

the left so that at the same concentration of calcium ions a larger force is produced when a sensitising derivative is present.

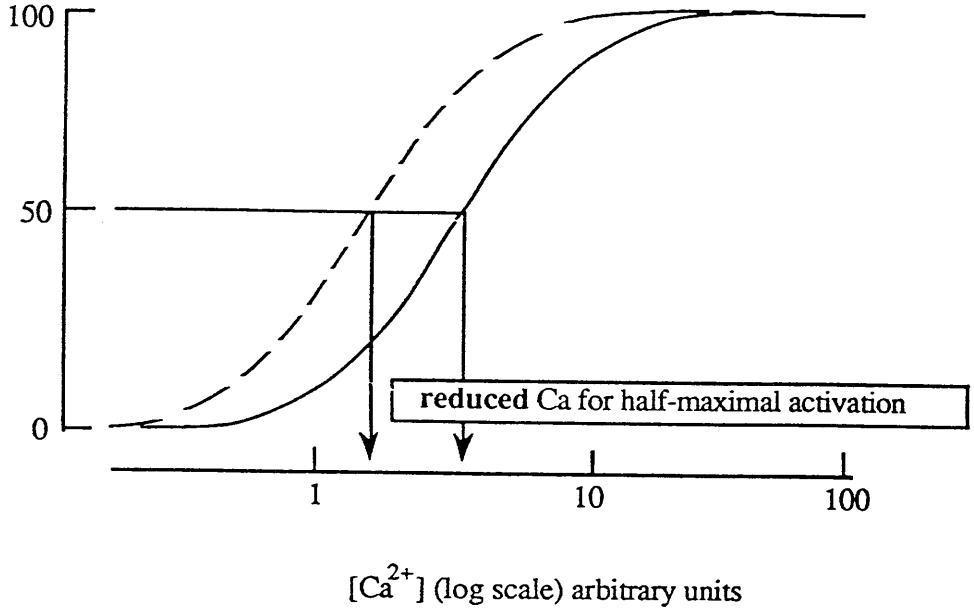


Figure 11. Simple calcium sensitising.

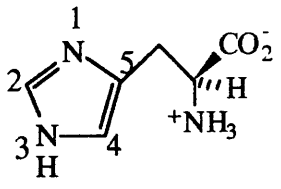
Two measurements were recorded. Firstly, the Ca-sensitising effects of the L-histidine derivatives on cardiac muscle expressed as a reduction in the concentration of calcium ions required to produce half maximum tension was noted. The second measurement was the effect of these derivatives on the peak force expressed as the change in force produced by the compound under investigation in a maximally Ca-activated preparation. The results obtained for each L-histidine derivative will be discussed in Section 4.4.2.

CHAPTER 3

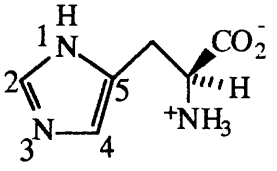
L-Histidine is a trifunctional α-amino acid. As well as the α-amino function and the α-carboxyl function, L-histidine has an imidazole side chain. This imidazole moiety has a pKa value of 6.0 which is close to neutrality.[33] This moiety therefore has the ability to function both as a proton donor and a proton acceptor at pH values found in many biological fluids. The imidazole portion can also behave as a nucleophile and as a ligand in the coordination of metal ions.[34] L-Histidine is therefore endowed with a wide range of potential functions. As the free amino acid, L-histidine has been implicated in the mechanism of a number of enzyme catalysed reactions. It is known that L-histidine is part of the active site of many of these enzymes[35,36] and it is thought that the imidazole ring plays a role in their catalytic function.

3.1 Tautomerism of L-Histidine

In aqueous solutions of pH greater than the pKa of the imidazole ring, two tautomeric structures are possible for the zwitterionic form of L-histidine (Figure 12).



3-H



1-H

Figure 12

In biologically active molecules, the predominance of one or other of the two forms may well have a direct effect on the mode of action or the activity of a given substance.

The nature of the tautomerism involved in the imidazole ring of L-histidine is known as annular tautomerism.[37] This type of tautomerism occurs when the hydrogen atom can move between sites within the ring system. Since the mechanism involves the breaking of one N-H bond and the forming of another, the rate of exchange is extremely fast. Due to the difficulties in determining the degree of population of each tautomer, for a long time there was no evidence to support the fact that two individual species existed.

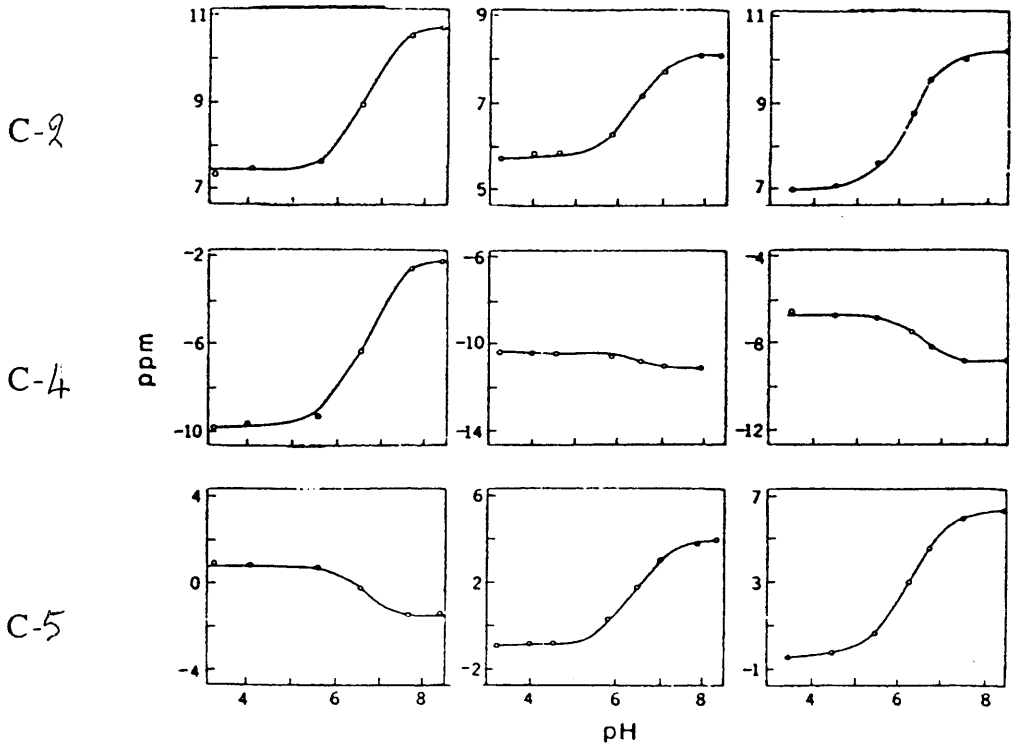
Over the past ten to fifteen years a number of papers have appeared concerning the determination of the preferred tautomer of L-histidine and its derivatives. The research published has encompassed a range of both physical and theoretical methods used to study the tautomerism.

Reynolds and co-workers[33] used ^{13}C NMR spectroscopy to determine the dominant tautomeric form of the imidazole ring of L-histidine in basic solution. They studied the pH dependence of ^{13}C chemical shifts of L-histidine in comparison with imidazole itself. They discovered that while the C-4 and C-5 resonances in imidazole both move to higher field upon protonation, the equivalent C-5 resonance in L-histidine shifted to higher field but the C-4 resonance moved to lower field. They went on to carry out theoretical calculations of ^{13}C NMR chemical shifts for the two compounds in an attempt to interpret the observed results. The theoretical calculations accurately predicted the observed shifts for imidazole when the calculated chemical shifts for C-4 and C-5

in the neutral form were averaged. On comparison, the theoretical values calculated for the 3-H tautomer of imidazole, without averaging, were close to the observed shifts for L-histidine. Similarly the theoretical results of the 3-H tautomer of L-histidine were closer to the observed values than those of the 1-H tautomer. These results indicated a predominance of the 3-H tautomer in basic solution.

Due to the approximations involved in the calculations, Reynolds and co-workers[33] went on to confirm the results by using 1-methyl and 3-methyl-L-histidine as model compounds. These can be regarded as frozen forms of the tautomers. The ^{13}C NMR chemical shift titration curves for the two model compounds were determined. The profiles they obtained are shown in Figure 13.[33]

Figure 13[33] 1-Me-L-histidine L-Histidine 3-Me-L-histidine



The titration ^{13}C NMR chemical shifts for all of the imidazole carbons of 3-methyl-L-histidine were identical in sign and similar in magnitude to those for L-histidine. The shifts for C-4 and C-5 of 1-methyl-L-histidine were quite different from those for L-histidine. This was viewed as conclusive proof that the 3-H tautomer was indeed the predominant tautomer in basic solution, in an estimated 4:1 ratio. A number of L-histidine derivatives were also diagnosed to have the 3-H tautomer predominant.

Blomberg and co-workers[38] used the pH dependence of ^{15}N and ^{13}C resonances of L-histidine and the various couplings between the different nuclei to describe the tautomeric equilibrium. The results they obtained were consistent with those previously described. Wasylishen and Tomlinson[34] estimated the predominant tautomer in L-histidine and related compounds using three bond ^{13}C, ^{1}H nuclear spin-spin coupling constants. Again the results were in accordance with those previously discussed. It would be safe to assume, therefore, that the prevalent tautomer of L-histidine and related compounds in basic solution is indeed the 3-H tautomer.

Madden and co-workers[39] have studied the crystal structure of L-histidine. Since the crystal lattice prevents interconversion, the crystal is composed of only one of the tautomeric species. The results these workers obtained show that the 3-H tautomer is also the prevalent tautomer in the solid state form of L-histidine.

All of the evidence presented indicates that the 3-H tautomer is the prevalent species. For the remainder of this thesis, therefore, only the 3-H tautomer will be represented.

3.2 The Differentiation Between *N*(1)- and *N*(3)-Derivatised L-Histidine Compounds

The differentiation of L-histidine compounds derivatised on the 1- or the 3-position of the imidazole ring has long been a problem.[40] By far the most common method of making assignments has been to assume that the main product in the reaction of L-histidine with an equimolar amount of electrophilic reagent will be that isomer arising from attack at the least hindered site. This leads to the assumption that the L-histidine will be derivatised preferentially at the 3-position. Various experimental approaches to the problem can be found in the literature.

The structures of compounds such as anserine (3) and the *N*-methylhistidines were originally assigned by degradation to the corresponding 1,4- or 1,5-dimethylimidazoles.[41] The structural assignments of these degradation products were in turn determined by their conversion into acyclic compounds or on comparison with synthesised compounds. Differentiation using chemical methods imposes severe limitations for many biologically important compounds of limited availability. A method was required which needed only small amounts of material with little chemical manipulation involved. The desired method would need to be widely applicable.

Matthews and Rapoport[40] evaluated several spectral methods which could possibly be used to solve the problem. The use of mass spectrometry had previously been reported.[42] Matthews and Rapoport obtained the spectra of four isomeric pairs of 1,4- and 1,5-disubstituted imidazoles. On analysis,

differences were observed between the members of each pair. Unfortunately these differences were not significant enough to be of any general diagnostic value.

These workers went on to look at the ultraviolet spectra of several 1,4- and 1,5-disubstituted imidazoles including *N*(1)- and *N*(3)-methylhistidine. They looked for three kinds of distinguishing differences between the isomers:

(a) the relative difference in the wavelength of the absorption maxima of the free acids and the conjugate acids;

(b) differences in curve shapes; and

(c) relative differences in shifts in the maxima upon protonation.

For non-conjugating substituents such as in the case of histidine derivatives, no consistent relative differences were observed.

It has been reported[43] that 1,4- and 1,5-disubstituted imidazoles can sometimes be differentiated by their infrared (IR) spectra. Matthews and Rapoport confirmed a previous report[44] that *N*(im)-methyl- and *N*(im)-carboxymethylhistidines exhibited an absorption maximum at 12μ in the IR spectra of the 1,5-disubstituted isomers. The 1,4-disubstituted isomers exhibited a minimum at that point. They could not find consistent differences between isomers of other disubstituted imidazoles. This led them to speculate that there could be differences in the solid state spectra and the solution spectra. A study of the solution spectra of the same compounds was therefore made. There were differences in the solution IR spectra between members of each pair of isomers. These differences were not, however, sufficient to be of any diagnostic value.

The aromatic protons of 1,4- and 1,5-disubstituted imidazoles are coupled to each other across the ring giving rise to the coupling constants $J_{2,5}$ and $J_{2,4}$ respectively. Matthews and Rapoport measured the coupling constants of several imidazole derivatives. On studying the results obtained, they concluded that under specified conditions the cross-ring coupling constants can be used to distinguish 1,4- and 1,5-disubstituted imidazoles. The differences between the coupling constants can be rationalised by the geometry of the imidazole system. At least two geometric factors may produce differences in the J values:

(a) the angles the carbon-hydrogen bonds make to each other; and

(b) the relative separations of the carbons.

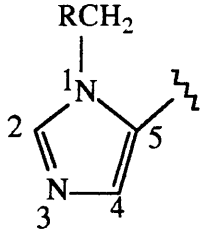
Both of these factors will influence the interactions of the two protons involved. The cross-ring coupling constants can only be observed in aprotic solvents. In all protic solvents the aromatic protons appeared as broad singlets.

Of the several spectral methods they investigated, Matthews and Rapoport concluded that only the method involving the cross-ring coupling constants is of general applicability. This method proved to be highly reliable and other workers[45] have since applied it to determine which derivatised L-histidine isomer they have synthesised.

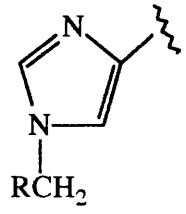
A crystal structure has been reported[45] for an L-histidine compound derivatised on the imidazole function. This is another physical method for determining the substitution pattern. This method has obvious restrictions and can only therefore be of use when a suitable crystal can be obtained. Jones and his coworkers[45] used the evidence they obtained from the crystal structure of $N(\alpha)$-*t*-butoxycarbonyl-1-benzoxymethyl-L-histidine

to substantiate the rule on cross ring coupling constants developed by Matthews and Rapoport.[40]

Colombo *et al.*[46] synthesised a range of L-histidine compounds derivatised on the imidazole ring. They used two methods to determine which nitrogen of the imidazole ring was derivatised. The first method involved conversion of these derivatives into *N*-methyl-L-histidines. These *N*-methyl derivatives were then assigned by comparison with commercially available 1- and 3-methyl-L-histidine. This is a fairly reliable method so long as the derivatives are easily converted into *N*-methyl-L-histidines and if the L-histidine is only derivatised on the imidazole ring. It is, therefore, not generally applicable. The second method involved the measurement of Nuclear Overhauser Enhancements (NOEs). This was applied to the derivatives which had an RCH_2- type substituent. The two aromatic imidazole protons were separately irradiated. If the substituent was in the 1-position (7), then the signal due to the protons of the CH_2 group would be enhanced only when the proton in the 2-position was irradiated.



(7)



(8)

If the substituent was in the 3-position (8), the signal due to the protons of the CH_2 group would be enhanced no matter what proton was irradiated. This is a good method for determining the

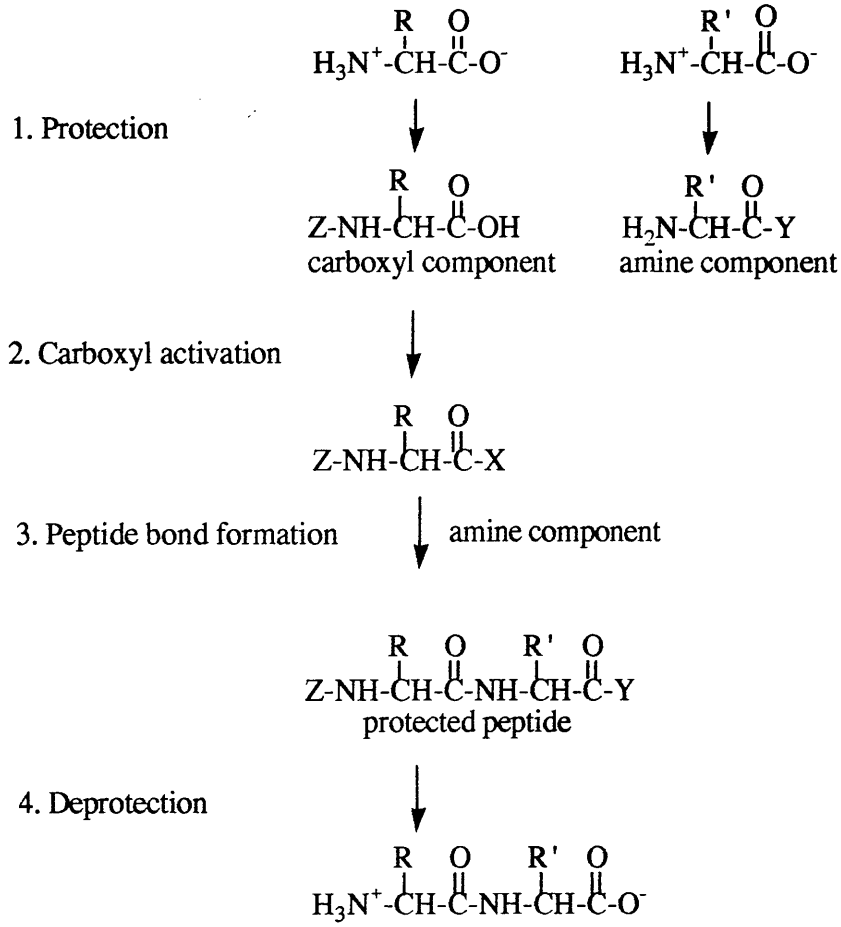
substitution pattern on the imidazole ring. It is widely applicable and does not depend on the substitution on the primary amino function.

3.3 Peptide Synthesis

The principal reaction in the synthesis of a peptide is the acylation of the amino group of an amino acid by the carboxyl group of a second amino acid resulting in the formation of an amide bond.

The presence of several functional groups in amino acid molecules and the necessity to maintain the integrity of the α-carbon chirality centres during coupling makes peptide synthesis more complicated than simple amide formation. Condensation of two amino acids formally provides peptides with elimination of water. To obtain a defined product, A-B, the amine function of A and the carboxyl function of B must be temporarily blocked. This is the first step of four consecutive operations generally employed in peptide synthesis (Scheme 1).[47] In the second stage the carboxyl group of the *N*(α)-protected carboxyl component is activated. Peptide bond formation results in condensation of this activated carboxyl component with the carboxyl-protected amino component. The final stage involves removal of the protecting groups. Each of these stages will be discussed in detail in the following text, with particular application to L-histidine chemistry.

Scheme 1[47]



3.3.1 Reversible Blocking of Amino and Carboxyl Groups

In order that the blocking substituent may be satisfactorily employed for the masking of an amino acid function in peptide synthesis, it should conform to a number of criteria:[48]

(a) the protected derivative should be conveniently available through the use of simple organic chemical techniques;

(b) the protection step should proceed in high yield;

(c) the conditions employed for the derivatisation step should not lead to racemisation of optically active amino acids;

(d) the protected compound should be easily purified;

(e) the blocking group should be stable under conditions utilised in the coupling step; and
(f) the blocking group should be easily and selectively removable without leading to side reactions.

(a) Masking of the Amino Function

Several protecting groups for the masking of the amino functions of L-histidine have been reported in the literature. The majority of these reports have been concerned with selectively masking one of the nitrogens of the imidazole ring. There are two reasons why this is desired:
(a) masking of the nitrogen in the 1-position has been shown to suppress racemisation of L-histidine during peptide synthesis;[47] and
(b) protecting one of the nitrogen positions allows selective alkylation at the other position.

Several protecting groups are no longer considered to provide suitable protection of L-histidine since they do not fulfil the criteria previously mentioned. The *N*(im)-benzyl[50] protecting group, although stable to nucleophiles, requires drastic conditions in the deprotection stage for its removal. The *N*(im)-*p*-toluenesulphonyl and *N*(im)-dinitrophenol derivatives are too susceptible to nucleophilic displacement to be of any synthetic use.[51] Those protecting groups which have found practical application will now be discussed in greater detail.

The Trityl Group

Trityl derivatives of L-histidine have been known for a long time.[52] When reacted with the imidazole function of L-histidine, the trityl group locates in the less sterically hindered 3-position. Barlos *et al.*[53] reported an efficient one pot synthesis of *N*-trityl amino acids including L-histidine. Sieber and Riniker[54] published a reassessment of the trityl group as a protecting group for L-histidine in peptide synthesis. The trityl group lowers the basicity of the imidazole ring and its bulk helps to prevent the base catalysed rearrangement which leads to racemisation. Trityl groups in the *N*(α)- and *N*(im)-positions are both completely stable in neutral and alkaline conditions and towards nucleophiles. The *N*(im)-trityl group can be cleaved by trifluoroacetic acid while the *N*(α)-trityl group is much more susceptible to acidolysis and can be removed selectively. Due to the steric hindrance caused by the bulk of the trityl group, selective alkylation in the 1-position can prove to be difficult.

The Phenacyl Group

Fletcher *et al.*[51] introduced the phenacyl group as suitable protection of the imidazole side chain of L-histidine. When the phenacyl group was located in the 1-position no detectable racemisation was observed under conditions which normally promote racemisation. The phenacyl group was introduced by reacting the silver salt of the *N*(α)-protected L-histidine derivative with phenacyl bromide. The major product was that with the 3-position protected. They went on to improve the method by protecting the 1-position selectively with the phenacyl group by first introducing a trityl group into the 3-position. The phenacyl

group is stable in strongly acidic and basic conditions. It is cleaved by treating the protected compound with zinc in acetic acid, although the last traces of zinc are difficult to remove. Cleavage can also be achieved by photolysis or electrolysis but these methods are more complex. Side reactions of the phenacyl group in the presence of reactive acylating agents have been observed.

The t-*Butoxymethyl Group*

Colombo and co-workers[55] introduced the *t*-butoxymethyl group for the protection of carbobenzoxy (CBZ), or 9-fluoromethoxycarbonyl (Fmoc), derivatives of L-histidine. The *t*-butoxymethyl group is stable to hydrogenolysis and base but is cleaved under mildly acidic conditions. The reagent used to synthesise *t*-butoxymethyl derivatives is *t*-butyl chloromethyl ether, which is unstable. This is a fairly new protecting group and it has not yet been established whether it will achieve popularity.

The Carbobenzoxy (CBZ) Group

Bergmann and Zervas[56] studied the carbobenzoxy group as a selectively removable blocking substituent. It has proved more versatile and more popular than any other in the preparation of synthetic peptides. The carbobenzoxy derivatives of amino acids are readily obtainable. They are usually obtained under Schotten-Baumann conditions, the general procedure for which has been described in *Organic Syntheses*.[57] In contrast to the racemisation sometimes observed with other acylating agents such as acetic anhydride or benzoic anhydride, the use of benzyl chloroformate induces no detectable racemisation when optically active amino

acids such as L-histidine are employed.[58] The protected derivatives are stable compounds usually obtained in excellent yield. The protecting group can be removed by a variety of methods, including the following: catalytic hydrogenation,[56] aqueous hydrochloric acid,[59] boron tribromide,[60,61] liquid hydrogen bromide,[62] and triethylsilane.[63] The carbobenzoxy group protects the amino acid against unwanted acylation and against racemisation. The importance of its effect on suppressing racemisation will be discussed in Section 3.4.

The t-*Butoxycarbonyl Group*

Carpino[64] introduced the *t*-butoxycarbonyl (*t*-BOC) group as a general amino protecting group. McKay and Albertson[65] and Anderson and McGregor[66] applied it to peptide synthesis. The introduction of the *t*-BOC group is not as straightforward as that of the carbobenzoxy group, since *t*-butyl chloroformate is unstable. A number of reagents have been introduced over the years to facilitate the synthesis of these derivatives. Di-*t*-butyl dicarbonate is the reagent most commonly used in the synthesis of L-histidine derivatives.[49,67] The cleavage of the *t*-butoxycarbonyl group by acids proceeds through the formation of *t*-butyl cations, which are converted into isobutene by the elimination of a proton.[68] The other products are carbon dioxide and a salt of the free amino acid or peptide. Acidic conditions which have been used for the cleavage include trifluoroacetic acid,[69] boron tribromide[61] and hydrochloric acid.[70] Since the *t*-butoxycarbonyl group is a urethane type of blocking substituent, it also possesses the ability to suppress racemisation.

(b) Masking of the Carboxyl Function

Salt Formation

The simplest form of protection for the carboxyl function of an amino acid is by salt formation.[68] The salt can be formed with a strong base such as potassium or lithium hydroxide, or with carbonate or bicarbonate. Organic bases such as triethylamine or other tertiary amines can also be used. The choice of base depends of course on the reaction solvent which in turn depends on the solubility properties of the amino acid.

Methyl Ester

It is fairly easy to prepare methyl esters of amino acids. In addition to the classical method of esterification whereby HCl gas is introduced into a suspension of the amino acid in methanol,[71] many variations on this have appeared in the literature. For example, the addition of the amino acid to a cold mixture of thionyl chloride and methanol[72] or the treatment of amino acids with acetone dimethyl ketal and aqueous hydrochloric acid have been employed.[73] Removal of methyl esters is not straight forward. The simplest and most common method is saponification with aqueous alkali in organic solvents such as methanol. Even at low temperatures and in the absence of excess alkali, racemisation is common. Several syntheses involving the methyl ester of L-histidine have been reported in the literature. The methods used for the deprotection of L-histidine methyl ester derivatives include cleavage of the ester using 6M hydrochloric acid,[74] as well as saponification of the ester with sodium hydroxide.[49] In all instances the products are reported to have optical activity.

There has also been a report in the literature of the use of boron tribromide to cleave methyl esters with the preservation of optical activity.[61]

Benzyl Ester

The use of the benzyl ester as a carboxyl protecting group is attractive since it can be removed by reduction thus avoiding the problems associated with hydrolysis. The procedure for the esterification of L-histidine with benzyl alchohol in the presence of *p*-toluenesulphonic acid was first reported by Akaboro *et al.*[75] in 1958. They reacted L-histidine hydrochloride monohydrate with benzyl alcohol in the presence of *p*-toluenesulphonic acid monohydrate. They obtained both the product and a hydrated form in a crystalline state in good yield. They reported that the hydrated form was easier to handle since it was not so hygroscopic.

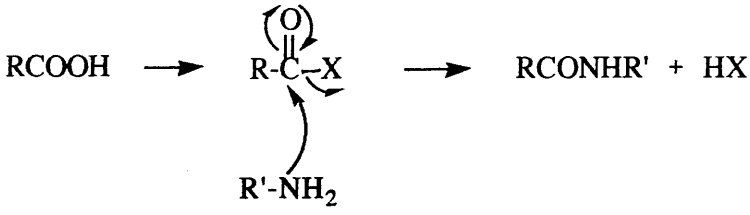
In 1970 Felix and Winter[76] reported an improved synthesis of L-histidine benzyl ester. These workers found the method of Akaboro *et al.* consistently unsuccessful. Felix and Winter reacted *N*-*t*-butoxycarbonyl-L-histidine with ethereal phenyl-diazomethane to yield *N*-*t*-butoxycarbonyl-L-histidine benzyl ester in excellent yield. L-Histidine benzyl ester dihydrochloride was isolated after treatment of the *t*-butoxycarbonyl derivative with acid. The dihydrochloride salt was also hygroscopic.

More recently, Jones and Wood[77] disputed the findings of Felix and Winter with regard to their claims that the method of Akaboro *et al.* was consistently unsuccessful. Jones and Wood varied the procedure reported by Akaboro *et al.* only slightly by changing the solvent from benzene to chloroform. They used a

Dean and Stark separator designed for use with solvents denser than water in place of the Wieland apparatus employed by Akaboro *et al.* They found that this procedure was simpler compared to the indirect method of synthesising the benzyl ester proposed by Felix and Winter. They managed to obtain the product satisfactorily and in good yield (93%). Removal of the benzyl ester can be achieved through reduction with hydrogen in the presence of a palladium catalyst.[48]

3.3.2 Coupling Between Amino Acids: an Overview

Practically all of the techniques which have ever been used for constructing peptide bonds are of the type outlined in Scheme 2. Many leaving groups, and the reactions for attaching them to the electrophilic carbon atom of the carboxyl component, have been investigated. Only a small number of these are regularly practised since good yields of optically and chemically pure products are required as well as convenience and economy. The principal coupling methods will be discussed.



Scheme 2

(a) The Use of Acyl Halides and Pseudohalides

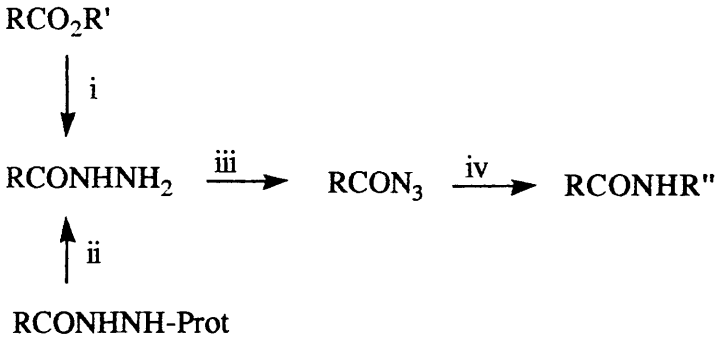
Acyl Chlorides

Perhaps the simplest approach to peptide synthesis is the activation of acylamino acids by conversion into the corresponding acyl chlorides followed by reaction with amino acids or amino acid esters under Schotten-Baumann conditions.[47] Methods based on this principle did indeed play an important role in the early days of peptide synthesis. This type of method is however unsuitable for a number of reasons. Reagents which are used for the acyl chloride formation such as thionyl chloride and phosphorus pentachloride are too vigorous to be used with sensitive substrates. Also, simple acylamino acid chlorides cyclise spontaneously to give oxazolones and hence racemisation occurs as discussed in Section 3.4. Several amino blocking groups have been employed in conjunction with this method of peptide coupling. CBZ-protected amino acid chlorides are unstable,[78] although the chlorides of trifluoroacetyl-, phthaloyl- and tosyl amino acids are reasonably stable.[79] Since more convenient and efficient methods are available, the acid chloride method can be regarded as no longer useful for conventional peptide synthesis.

Acyl Azides

The use of acyl azides for peptide bond formation was first introduced by Curtius in 1902.[80] With the introduction of more efficient methods of peptide bond formation in the 1950s, the use of *N*(α)-protected amino acid azides declined sharply. Nevertheless, it remains an important procedure. The hydrazides from which the azides are generally made may be obtained by

hydrazinolysis of a protected amino acid ester. In the early days, conversion into the azide was usually achieved by adding sodium nitrite to a solution of the hydrazide in a mixture of acetic and aqueous hydrochloric acids at around 0 ^{o}C.[81] Honzl and Rudinger[82] studied the influence of temperature, solvents, acidity, type of acid and the nitrosating agent on azide formation. On the basis of their results, they defined a more refined method involving dry acid and an organic nitrite ester in a dry organic solvent at a lower temperature. This method is now generally preferred. The azides are subsequently reacted with the amine component without delay. This approach is summarised in Scheme 3.



conditions: i, NH_2NH_2; ii, deprotection; iii, $NaNO_2$ in aqueous AcOH-HCl or an alkyl nitrite with dry H^+; iv, $R"NH_2$

Scheme 3

Numerous side reactions have been recognised in the formation and the use of amino acid azides.[83] As a result of this, the azide method has largely been replaced by cleaner procedures since 1950. The azide method remained popular for a number of reasons: it is reliable in assembling chains of up to sixty amino acids, only minimal side chain protection is required, and it is easy

to convert ester protected carboxyl functions into activated azides. The most important reason for its continuing popularity was that for years it appeared that racemisation during the coupling of acyl amino acid azides never took place. It is now known that racemisation does take place with excess base in polar solvents. This tendency is greater when L-histidine with an unprotected imidazole function is the carboxyl component; with triethylamine in dimethyl formamide even *t*-butoxycarbonyl-L-histidine azide was racemised.[84]

The azide method is more time consuming since the reaction is in three stages.[47] Product yields are inferior to those obtained by other methods of peptide coupling.[83] The rate of acylation is also considerably slower than either the mixed anhydride method or dicylohexylcarbodiimide (DCC) mediated coupling.

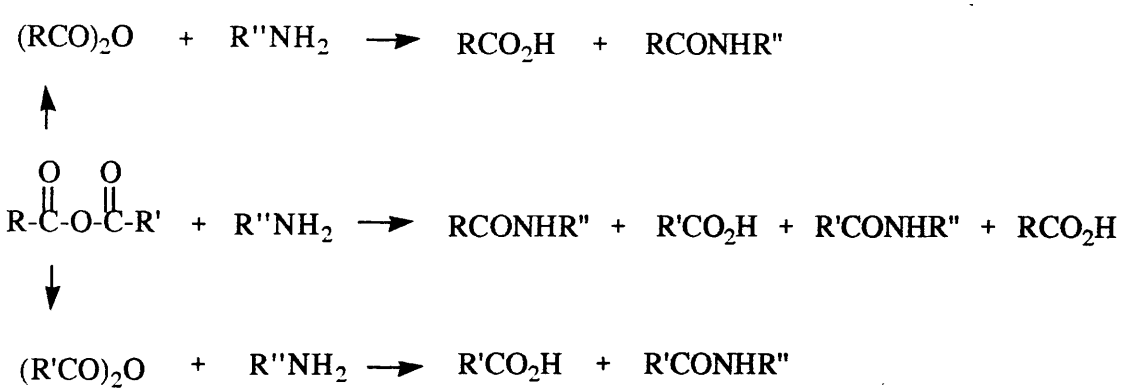
(b) The Use of Anhydrides

Symmetrical Anhydrides

Acylamino acid anhydrides can be prepared from the corresponding acylamino acids by use of a number of reagents such as ethoxyacetylene and DCC.[47] Aminolysis of a symmetrical anhydride proceeds without the formation of side products. This is an advantage over some mixed anhydride procedures but only half of the acylamino acid taken is incorporated into the product. The symmetrical anhydrides of *t*-butoxycarbonyl-, carbobenzoxy- and phthaloyl amino acids are generally reasonably stable, clean acylating agents.

Mixed Anhydrides with Carboxylic Acids

There are two main problems with the use of a mixed anhydride of an *N*-acylamino acid with a carboxylic acid in peptide synthesis.[47] First, the anhydride has two electrophilic sites and therefore two possible sites for reaction with the amine component; and second, mixed anhydrides with carboxylic acids tend to disproportionate (Scheme 4). The selectivity can be improved by introducing steric hindrance and inductive depression of electrophilicity so as to direct the attack to the carboxyl component carbonyl. Mixed pivalic anhydrides are the only intermediates in this class to have attained any significant popularity.[85]

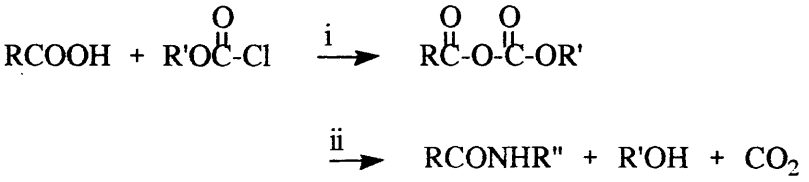


Scheme 4

Mixed Anhydrides with Carbonic Acids

The most generally successful mixed anhydride method involves the reaction of the carboxyl component with an alkyl chloroformate to produce the mixed anhydride.[47] The mixed anhydride is reacted with the amine component immediately as outlined in Scheme 5.

One of the carbonyl groups in the activated intermediate is flanked by two oxygens, hence its reactivity is diminished. Nucleophilic attack is therefore directed toward the carbonyl of the original carboxyl component. Side reactions can be minimised by the careful control of the reaction temperature and the activation time. The value of this technique lies in its speed and economy.



conditions: i, 1 equiv tertiary base/unreactive dry solvent/-10 °C/ 2 min; ii, $R''NH_2$

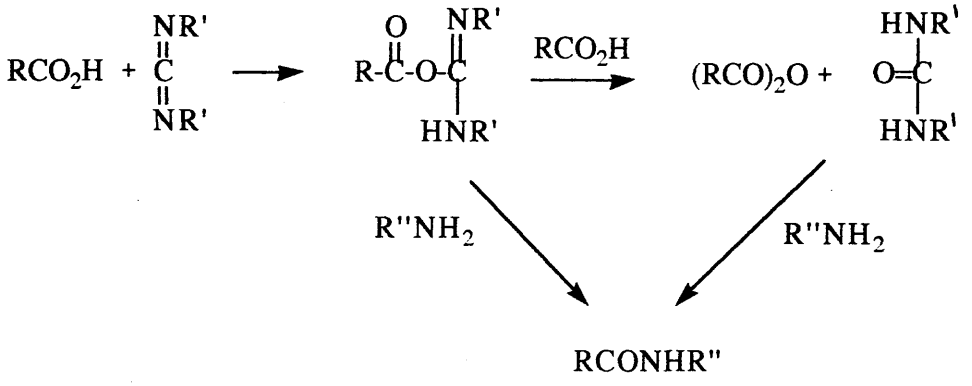
Scheme 5

(c) The Activation of Carboxyl Groups by Addition Reactions

The nucleophilic addition of carboxylic acids to cumulated double bond or triple bond systems gives adducts which are, in general, reactive acylating agents because acyl-oxygen fission leads to a carbonyl compound.[47] The main method which falls into this class is the use of carbodiimides.

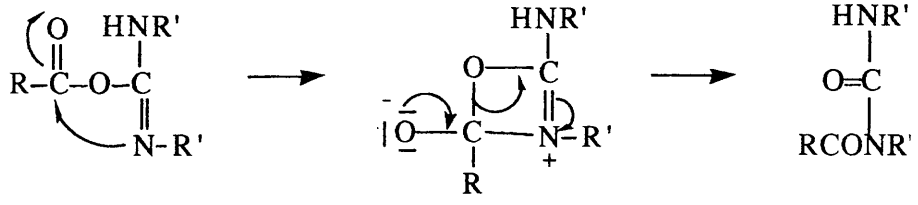
Dicyclohexylcarbodiimide (DCC) has been used in peptide synthesis since 1955[86] and is still the most important single reagent. This is especially true since the advent of solid phase synthesis, where it is used to the near exclusion of other coupling methods. The procedure involves the mixing of the amine and carboxyl components with DCC in equimolar amounts. When *O*-acylisourea formation takes place, the peptide is obtained either

by direct aminolysis or via a symmetrical anhydride with dicyclohexylurea as the by-product as shown in Scheme 6.[47]



Scheme 6

The intermediates are highly reactive and side reactions are very common. Extensive racemisation is the outcome with susceptible carboxyl components. Collapse of the *O*-acylisourea by intramolecular acyl transfer (Scheme 7) is known to compete significantly with the desired reaction. This results in a much less reactive *N*-acylurea which reduces the yield and is difficult to remove.[47]



Scheme 7

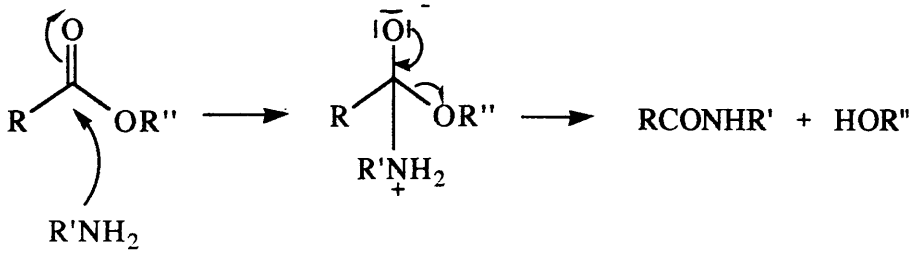
Both of these problems have been reduced significantly with the introduction of additives such as *N*-hydroxysuccinimide.[87] This nucleophile suppresses *N*-acylurea formation and gives a procedure which is sufficiently free from racemisation.

Certain side chains can react with DCC in an undesirable manner. Unprotected imidazole groups give *N*(im)-amidino derivatives,[88] and so unprotected L-histidine is therefore susceptible. This side reaction can be avoided either by protection of the imidazole function or negated by subsequent nucleophilic displacement of the amidino group.

In some cases the peptides formed have solubilities comparable to that of dicyclohexylurea. This will of course lead to problems with purification. The water soluble diimide reagent *N*-ethyl-*N'*-dimethylaminopropylcarbodiimide can be used in the same way as DCC but has different solubility properties.[89] It has not as yet been widely used; this may be due to the comparatively high cost of the reagent.

(d) The Use of Active Esters

Ester aminolysis has been used in peptide synthesis since the turn of the last century.[90] Gordon *et al.*[91] revealed that aminolysis rates of esters are related to the electron withdrawing properties of the acid or the alcohol component of the ester. The withdrawal of electrons enhances the electrophilic character of the carbonyl carbon. This facilitates the formation of the tetrahedral intermediate with the amine. More recently, Menger and Smith[92] revealed that in contrast to ester saponification, aminolysis of phenyl esters in organic solvents proceeds with rate limitation by the collapse of the tetrahedral adduct, not its formation (Scheme 8). The leaving ability of the ester is therefore probably the most important consideration. A large number of active esters have been investigated.



Scheme 8

A number of methods have been developed to form the activated esters. The most common method for their preparation involves the use of condensing agents. An example of this is DCC mediated coupling between a protected acid and the active ester.[93] The popular active esters are generally crystalline, stable compounds. This is advantageous since the reactive intermediates can be isolated and are stable on storage, ready for use. They are at a low level of activation and therefore the active esters react selectively with the amine component in coupling. Side reactions including racemisation are generally not a problem. There is however a serious danger of racemisation when most active esters are prepared if the carboxyl component is susceptible, especially in the presence of base. Bodanszky and co-workers[94] reported that the presence of L-histidine in the amine component can greatly enhance *O*-acylation. This side reaction is catalysed by the imidazole function in the side chain of L-histidine.

The individuality of amino acids means that no generalisation can be made as to the best active ester in peptide coupling. The esters are all reactive systems simply by being *O*-acyl derivatives of moderately strong acids. *N*-hydroxysuccinimide esters are highly reactive, crystalline compounds.[47] They can be prepared easily and the water soluble

by-product makes purification easier. They react cleanly and rapidly to give an easily isolable product. *p*-Nitrophenyl esters are popular since *p*-nitrophenol is very cheap and the esters can generally be synthesised in high yield.[47] The products are stable, crystalline compounds. *p*-Nitrophenyl esters were the first to become commercially available. Pentachlorophenyl esters[47] were developed since the chlorine groups are resistant to catalytic hydrogenation while nitro groups are not. These esters are crystalline and easy to prepare but there are some steric problems due to the two substituents in the ortho positions. These difficulties can be overcome by using pentafluorophenyl esters or trichlorophenyl esters.

The major drawback of the active ester method is the comparatively slow rates of acylation. This becomes increasingly significant when steric factors impede acylation. The reaction rate can be significantly increased with the addition of catalysts into the system. Several workers studied the effect of the addition of weak acids and bases as catalysts.[47] Konig and Geiger[95] introduced 1-hydroxybenzotriazole to catalyse the reactions of *p*-nitrophenol esters and 2,4,5-trichlorophenyl esters.

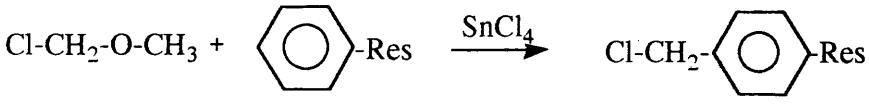
(e) Reagents for Solid Phase Peptide Synthesis

Merrifield[96] introduced the attachment of protected peptide intermediates to an insoluble polymeric support. The purpose of the Merrifield solid phase approach is to enable quantitative separation of the peptide under construction from all excess reagents and coproducts after each step by filtration. The peptide is totally insolubilised by attachment to a polymer but the

reagents and coproducts are in solution. Some workers[97,98] have also investigated the possibility of binding the reagents to the polymer and hence on filtration at the end of the reaction the peptide is left alone in solution.

In most instances of solid phase peptide synthesis, the solid supports are copolymers prepared from a mixture of styrene and divinylbenzene.[68] These cross-linked polymers are insoluble but swell to gels in solvents such as dichloromethane. The swelling is important since it allows the diffusion of the soluble reactants into the inside of the gel particles. The reactions, such as acylation and deprotection, can hence be carried out on amino acids and peptides attached to the polymer.

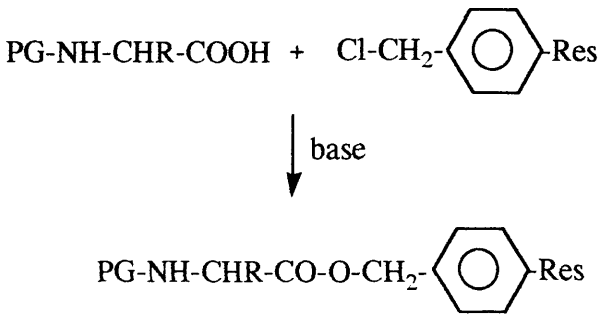
The linking of the first residue to the resin requires a reactive group on the polymer. Chloromethylation of the aromatic nuclei was the first, and still most used, solution to this problem (Scheme 9).



Scheme 9

An *N*-protected amino acid is allowed to react with the chloromethylated resin in the presence of a base to form an ester bond thus attaching the carboxyl group to the polymer. The amino protecting group is removed and the amino function is available for coupling. Dicyclohexylcarbodiimide (DCC) is the reagent most commonly used in solid phase peptide coupling. The attachment is represented in Scheme 10. After each operation the excess reagents and by-products are removed by repeated

washings, simply and efficiently. The final step to remove the completed chain from the polymer requires exposure to strong acids such as hydrobromic acid in trifluoroacetic acid.



Scheme 10

The elimination of isolation, purification and characterisation of intermediates is probably necessary if large peptides are to be constructed within a smaller time frame. There is however the risk that complex mixtures will be produced when there is no purification between steps. Careful study of the purity of the final products is essential.

Conclusions

All of the coupling methods of enduring popularity give adequate results most of the time and they are chemically simple. Although the active ester intermediates can be isolated and stored, the slow rates of acylation make this method unattractive for general use. This method is particularly unsuitable due to the *O*-acylation induced by the imidazole function of L-histidine. The azide method of peptide coupling is not recommended due to the many side reactions which can occur. The rate of acylation of the azides is slower than that in mixed anhydride or DCC mediated

couplings. The yields obtained by this method are also inferior to those obtained by other methods. The solid state method for peptide synthesis is really only advantageous for the construction of large peptide chains. Upon examination of these methods, it would appear that the DCC and mixed anhydride methods are the most effective and convenient for the synthesis of L-histidine-containing dipeptides. On application to this work however, the DCC method was not useful, since it was difficult to remove the dicyclohexylurea from the products at the end of the reactions. The water soluble carbodiimide mentioned in Section 3.3.2 (c) was applied successfully in some cases, as discussed in Section 4.3. However, due to its expense, it was impossible to use this reagent in all cases. The mixed anhydride method has proved to be a very effective peptide coupling method in the synthesis of the L-histidine dipeptides required in this work. This method will be discussed in more detail in the following section.

3.3.3 The Mixed Carbonic Anhydride Method of Peptide Coupling

The formation of peptide bonds by the mixed carbonic anhydride method was developed simultaneously in 1951 by Wieland and Bernhard,[99] Boissonnas[100] and Vaughan.[101] The historical aspects of this method have been discussed by Greenstein and Winitz[47] and by Bodanszky *et al.*[68] Reviews on the chemistry of the mixed carbonic anhydrides method and their application to peptide synthesis have been compiled.[102,103]

There are several reasons why the mixed carbonic anhydride method is attractive in peptide synthesis.[104] In

particular it is possible to achieve high reaction rates at low temperatures with this method. The reaction is simple to perform and the purity of the products is high compared to other methods. The mixed anhydride method is also by far the most economical process of peptide synthesis.

The major drawback of this method is the high tendency of mixed anhydrides to undergo racemisation, due to the strong activation of the carboxyl carbonyl. This has not been a great problem in this work since the carboxyl component has very rarely been an α-amino acid. In the few cases where the carboxyl component has been an optically active amino acid precautions were taken to preserve the optical purity. For example *N*(α)-urethane protective groups are known to suppress racemisation.

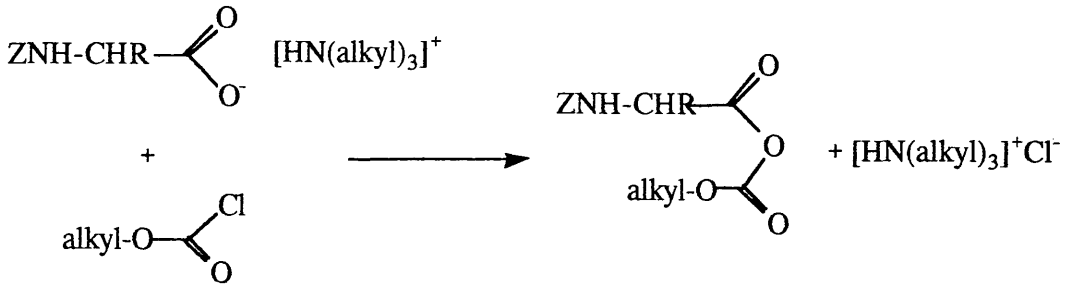
Since the mixed anhydrides belong to the group of most highly activated acyl derivatives in peptide synthesis, they are prone to undergo a variety of rearrangements and decomposition reactions. Carefully controlled and defined conditions have been described in the literature to prevent or minimise these side reactions.[105]

The mixed anhydride method of peptide bond formation involves two separate stages. The first stage is the activation stage and the second is the coupling stage. These stages will be discussed in turn.

(a) Activation

The alkyl chloroformate is added to a cold solution of the carboxyl component anion in anhydrous organic solvent in the presence of a tertiary base (Scheme 11).[47] This process is

sensitive to the nature of the solvent, the reagent, and is prone to base catalysed racemisation.[105] The activation time is the period between the addition of the alkyl chloroformate and the addition of the amine component once the mixed anhydride formation is complete.



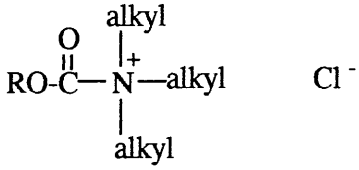
Scheme 11

Anhydride formation must be carried out in a manner such that the alkyl chloroformate is completely reacted since any excess would attack the amine component. This reaction would lead to irreversible urethane formation and hence lower yields. Fast reaction ensures side reactions such as decomposition, racemisation and disproportionation are minimised.

Activation is influenced by the nature of the reagent, the tertiary amine, the side chain of the carboxyl component, the temperature and the reaction time.

Isobutyl chloroformate is the best reagent for the formation of mixed anhydrides.[104,105] The branched alkyl group increases the electron density at the adjacent carbonyl in the mixed anhydride. This therefore reduces attack by the nucleophile and the carbonyl of the carboxyl function becomes the predominant point of attack. Better yields are hence obtained compared to the use of ethyl chloroformate as the reagent.

Evaluation of the effects of many bases led Anderson and his co-workers[105] to the conclusion that the tertiary amine is not only a hydrogen chloride acceptor, but that it reacts with the chloroformate, for example isobutyl chloroformate, to form an alkyloxycarbonylammonium ion complex (9). This complex subsequently reacts with the carboxylate. Evidence supporting this mechanism was the low yields obtained when a sterically hindered base such as ethyl diisopropylamine was used. This was in contrast to the high yields obtained when triethylamine was the base.



(9)

Anderson and co-workers[105] recommended *N*-methylmorpholine as the tertiary base. It is a weak base and hence has little effect on promoting racemisation; it is not sterically hindered; and it is sufficiently strong for rapid reaction. In situations where racemisation is not an issue, triethylamine is as good a choice.

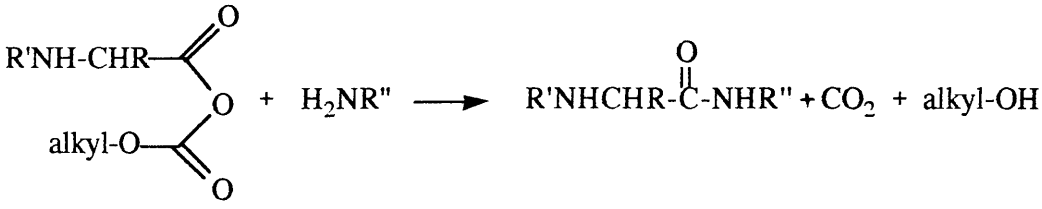
Strictly anhydrous solvents of high purity are essential for successful mixed anhydride formation. Solvents have been reported to affect reactions of some simple unsymmetrical anhydrides. Their influence in peptide synthesis has not been thoroughly studied. Ethyl acetate, tetrahydrofuran,[105] toluene,[106] *t*-butanol[107] and acetonitrile[108] are recommended as the solvents which will optimise overall yields. The use of chloroform and

dichloromethane results in impure products and lower yields.[105,106] When more polar solvents are required for the solvation of the carboxyl component, dimethylacetamide gives better yields than *N,N*-dimethylformamide.[105]

An activation time of 1-2 min at -15 °C is recommended for mixed anhydride formation. At lower temperatures yields are considerably lower and at temperatures higher than 0 °C disproportionation of the mixed anhydride can occur.[99] The synthesis of the mixed anhydride is very exothermic and adequate heat dissipation is essential. Temperature control within the reaction vessel must be strictly controlled by stirring and by controlling the rate of addition of the alkyl chloroformate.

(b) Coupling

The mixed carbonic anhydrides are not usually isolated but are reacted immediately *in situ* with the amine component. The reaction proceeds quickly to form the peptide bond with carbon dioxide and a volatile alchohol as the by-products (Scheme 12).[47]



Scheme 12

As the second stage is not very sensitive to the nature of the solvent, the coupling reaction may be carried out in either aqueous media or in organic solvents. In an aqueous environment

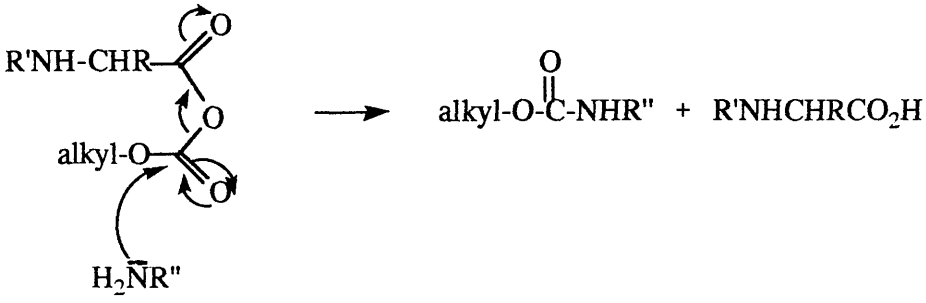
the yields of the coupled product are lower than those obtained in organic solvent, probably due to competing hydrolysis of the anhydride.[104]

If base is required to neutralise hydrogen halides of amine components, it should be mixed with the amine component shortly before coupling is to take place.[105] Care must be taken to ensure there is no excess of base present as this will promote racemisation of the product.

The temperature of the reaction is generally maintained at -10 °C for up to an hour. The mixture is then allowed to warm to room temperature until the reaction is complete.

(c) Side Reactions

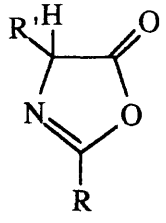
Under the commonly used low-temperature reaction conditions in the mixed anhydride method, only two side reactions have given concern in peptide synthesis. Attack of the amine component at the carbonyl of the alkyl formate part of the mixed anhydride (Scheme 13) leads to the formation of a urethane side product.[47] This urethane can also arise from reaction of any excess chloroformate from incomplete anhydride formation with the amine component.



Scheme 13

It was not proven until 1969[109] that the side product was derived from the mixed anhydride. More recently, Bodanszky and Tolle[110] studied the urethane side product formation in detail. The branched alkyl group of isobutyl chloroformate increases the electron density at the adjacent carbonyl of the mixed anhydride. Since this diminishes attack by the nucleophile at this centre it will also diminish the tendency toward urethane formation more effectively than ethyl chloroformate. It was found that the only other real influence on the formation of the urethane side product was the steric bulk in the carboxyl component. The mixed anhydride method is therefore not a suitable peptide coupling method for the coupling of sterically hindered carboxyl components.[110]

The other principal side reaction is the formation of 5(4H)-oxazolones.[47] The mixed carbonic anhydrides belong to the most highly activated acylating species in peptide synthesis. Strong electron withdrawal from the carbonyl carbon creates a high risk of racemisation at the chiral centre of the carboxyl component. One of the mechanisms of racemisation in peptide synthesis proceeds via the 5(4H)-oxazolone formation (10) (*cf.* Section 3.4).



(10)

This side reaction is therefore of great importance. Vaughan's studies[106] showed that the extent of racemisation in

mixed anhydride coupling can be influenced by the nature of the solvent, the reagent, the activation time and the temperature. The extent of this side reaction can therefore be controlled by taking these factors into control.

(d) Conclusions

The mixed carbonic anhydride method is attractive for several reasons; it combines rapid reaction at low temperature with frequently very high coupling yields. The by-products are volatile which greatly improves purification of the product. Side reactions are few and their extent is often negligible. It is undoubtedly the most economic method of peptide synthesis.

3.4 Racemisation in Peptide Synthesis

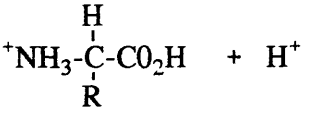
The single most important side reaction in peptide synthesis and α-amino acid derivatisation is racemisation. It has been shown to occur during all methods of peptide coupling when the necessary precautions are not taken. The subject has therefore been extensively reviewed by several authors.[47,68,111]

L-Histidine is a vulnerable amino acid since it bears an aromatic ring in the β-position. This serves to stabilise enolate formation to some extent. It is important therefore to understand the racemisation processes which can take place before embarking on any syntheses involving L-histidine.

(a) Racemisation of Free Amino Acids

Racemisation of α-amino acids is dependent upon enolate formation. Enolate formation results from the attack of base or acid on the α-amino acid species.

In aqueous solution racemisation has been known to occur over the full pH range. There are four processes by which racemisation can take place in an aqueous environment: At very low pH racemisation will proceed via protonation of the conjugate acid (11).



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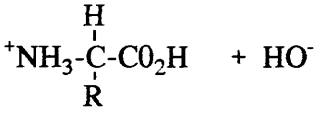

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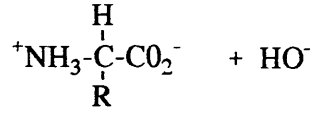

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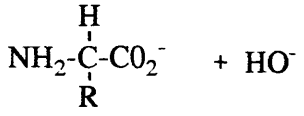


(14)

Near neutrality, hydroxide ions from the water can react with the amino acid conjugate acid (12). At higher pH the hydroxide ions will react with the zwitterion of the amino acid, leading to racemisation (13). Finally at a very high pH the mechanism probably involves hydroxide-catalysed enolisation of the amino acid conjugate base (14). These processes are not equally viable. For example at very high pH, it will be difficult to extract the α-proton from the amino acid species since it already possesses a negative charge.

Several studies have been made on the extent of racemisation of a number of amino acids at various pH values and conclusions have been drawn from the results. Under aqueous conditions, inductive and resonance stabilisation of the developing enolate anion at the α-carbon of an α-amino acid has a major effect on racemisation. Thus at neutral pH, acceleration of racemisation was observed with α-amino acids bearing an electron withdrawing substituent in the side chain. A large increase in the rate of racemisation was observed in all the cases studied when the temperature of the solution was increased.[112]

Manning[113] studied both the tritium α-exchange and the racemisation of amino acids in 6M hydrochloric acid at 110 °C. He established that for normal amino acids the rates are equal. Bada[112] observed that 6M hydrochloric acid caused relatively little change in rate as compared to that observed at pH 7.

Racemisation has long been noted as being a rapid process in aqueous alkaline solution.[114] This is particularly true for vulnerable amino acids bearing an electron withdrawing substituent in the β-position.

A special case has been observed when vulnerable amino acids are dissolved in glacial acetic acid. Matsuo *et al.*[115] studied the effects on some amino acids heated in acetic acid. They observed that the rate of racemisation was significantly slowed by dilution of the acetic acid with water. Addition of sodium acetate to acetic acid was found to increase greatly the rate of racemisation of *N*-acetylphenylglycine. These workers therefore proposed that the racemisation of amino acids in acetic acid solution results when an acetate ion encounters a carboxylic acid

bearing an α-ammonium ion. The acetate ion behaves as a basic catalyst.

In organic solutions, α-amino acids are vulnerable to racemisation in the presence of bases; in particular tertiary amines. This creates a problem when the amino acids are present as hydrohalide salts which must be neutralised before a reaction involving the amino function can take place. The amount of base added must be strictly controlled; in the presence of excess base the α-amino acid is prone to racemisation. Several studies[116,117] have been made on the effects of a number of tertiary amines on the racemisation of amino acids. Triethylamine is the worst offender in causing racemisation. *N*-Methylmorpholine is the base which most workers have recommended in peptide synthesis.

(b) Racemisation of Amino Acid Derivatives

Two different mechanisms have been proposed to explain the loss of optical purity in reactions involving *N*-acylamino acids and peptides:

(a) the formation of oxazolinone intermediates. These are known to racemise easily, and

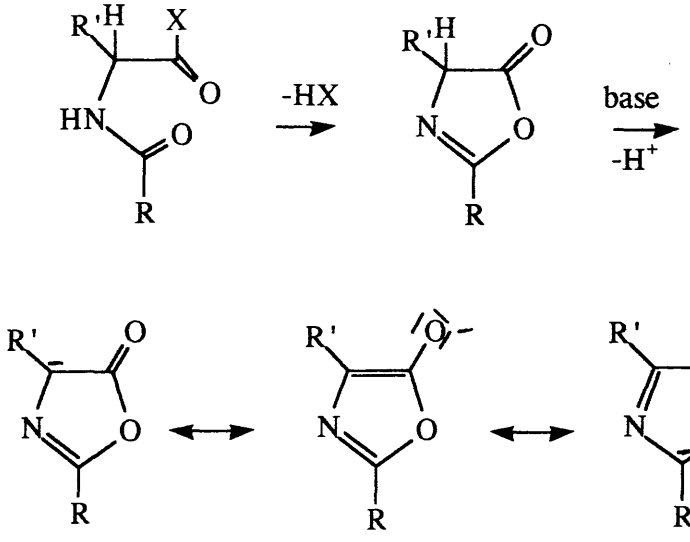
(b) the direct proton abstraction from the asymmetric α-carbon atom.

The formation of oxazolinones was suggested by Bergmann and Zervas as early as 1928[118] to explain the racemisation observed during the acetylation of amino acids. The 5(4H)-oxazolone mediated racemisation mechanism (Scheme 14)[68] involves a rate determining cyclisation that is base catalysed. The

5(4H)-oxazolone is then proposed to racemise via a resonance stabilised tautomer.

When X is an electronegative group or atom, the enhancement in the electrophilic character of the carbonyl carbon of the carboxyl function will facilitate the attack by the carbonyl oxygen of the acyl substituent. This is a problem in peptide synthesis since the activated intermediates in the coupling reactions have this situation. The resulting cyclic intermediate is stabilised by the elimination of HX.

Scheme 14



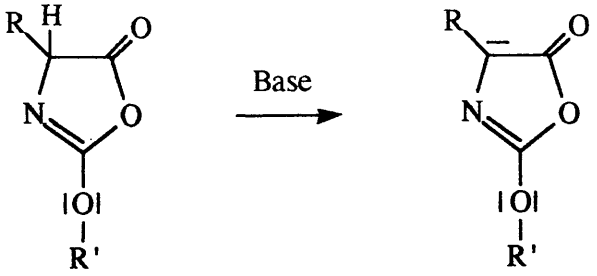
The oxazolinones thus formed have a strong tendency, in the presence of base, to lose a proton and acquire the resonance structures as shown. If R is an electronegative group, such as a phenyl group, the probability of ring closure is enhanced, since this group will increase the delocalisation of the anion (Scheme 14).

Since the report by Bergmann and Zervas,[118] much evidence has been reported to support the existence of oxazolinone

intermediates. In fact, some workers[119] have managed to isolate them and they can be detected in reaction mixtures by their characteristic IR absorption at 1832 cm^{-1}.

Factors which have been found to influence the racemisation of oxazolinones include the nucleophilicity to basicity ratio of the amino-component in a peptide coupling reaction mixture. A large ratio favours the opening of the oxazolinone ring as opposed to abstraction of the α-proton. Solvents, such as dioxan, which enhance the separation of charges favour racemisation. It has been found that *N*-acylamino acid oxazolinones racemise faster than peptide oxazolinones. This suggests that steric hindrance of the amino-component has an effect.[68]

Until recently, it was generally assumed that urethane-protected amino acids and peptides do not produce azlactones. Isolation[120] of optically pure 5(4H)-oxazolones contradicts this assumption. It is now believed that the beneficial effects of urethane type protecting groups rests on the electron release provided by them. Due to this electron release, the anion which would be formed by proton abstraction is destabilised (Scheme 15).

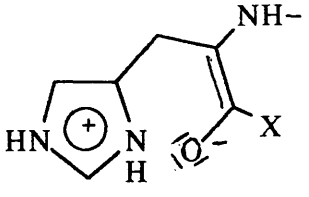


Scheme 15

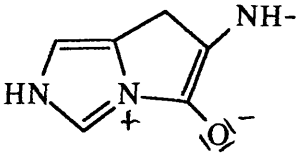
Considerable experimental evidence indicates an alternative mechanism of racemisation. Even though urethane-type protected amino acids show no tendency to racemise through oxazolinone intermediates, some CBZ-amino acid esters undergo base catalysed racemisation. *t*-Butoxycarbonyl-*N*(im)-benzyl-L-histidine was found to racemise appreciably when activated by the DCC method.[121] A mechanism of proton abstraction from the α-carbon atom was advanced by Neuberger,[122] who suggested that in mixed anhydrides, hydrogen bonding with one of the acyl oxygens would weaken the C-H bond. This favours ionisation.

(c) Racemisation of L-Histidine

It has been recognised that acyl imidazoles formed from peptide acids undergo unusually easy racemisation.[104] It has since been clear that the presence of a free imidazole function in a peptide coupling reaction increases the risk of racemisation.[117] In acylation with activated derivatives of L-histidine significant racemisation can take place.[121] This has been explained by base catalysed enolisation (15), or by cyclisation and enolisation (16).[111]



(15)



(16)

Support for these mechanisms is seen in substituents such as the *p*-toluenesulfonyl group, which lower the basicity of the

imidazole nucleus and also reduce the extent of racemisation.[123,124] Complete protection against loss of optical purity in L-histidine derivatives is only expected when the 1-nitrogen is protected.

(d) Conclusions

It has become evident that the extent of racemisation is determined by a series of factors. Until the discovery of a completely racemisation-free coupling method, conservation of chiral integrity requires optimisation in the choice of protecting groups, reagents and solvents to name only a few of the factors to be considered.

3.5 Determination of Optical Purity

(a) Assays for Optical Purity

There are five principal assays for measuring the extent of racemisation of amino acids and their derivatives. The Anderson test[125] is based on the measurement of the specific rotation of the crude racemic product when glycine ethyl ester is coupled to *N*-protected glycylphenylalanine. The racemate is isolated on fractional crystallisation and its weight is determined to measure the extent of racemisation. This test has a sensitivity limit of 1-2%. The Young tests[126,127,128] involve the coupling of glycine ethyl ester to an *N*-protected α-amino acid. They depend on the measurement of the specific rotation of the amide product and have the same sensitivity limits as the Anderson test. Kemp[129]

described how, in both cases, the sensitivity limit can be bettered on the application of a multiple isotope dilution procedure. He used ^{14}C-labelled carboxy components.

Weygand and his associates[117] developed a number of tests based on gas chromatographic (GLC) separations of diastereoisomers. The carboxyl component was converted into its *N*-trifluoroacetyl derivative and the carboxyl function of the amino component was converted into an ester. The two components were coupled together and the resulting diastereoisomeric mixture was assayed by GLC. There also exists a procedure whereby *N*-trifluoroacetyl amino acid esters are separated as enantiomers on a chiral column.[130]

The Izumiya test[131] employs the ion exchange resin of an amino acid analyser to effect separation of diastereomeric peptides after coupling and deprotection. The sensitivity limit of this test is 0.1-1%.

These are the well known general tests. There also exists a large number of assays which have been used in particular instances. A list of these can be found in the book of Gross and Meienhoffer.[47] One important recent development is the use of high performance liquid chromatography (HPLC) techniques to determine the optical purity of amino acid derivatives.[47]

(b) The Use of Chiral Lanthanide Shift Reagents

Nuclear magnetic resonance (NMR) spectroscopy is a valuable technique for the study of molecular structures. The use of NMR spectroscopy is sometimes restricted due to insufficient sensitivity of proton chemical shifts to changes in the

stereochemical environment. Lanthanide shift reagents are effective in reducing this equivalence of nuclei.[132] There are two mechanisms that can lead to enantiomeric resolution in the NMR spectra of racemic substrates in the presence of chiral shift reagents. The first is a difference in the equilibrium constant for association between the enantiomers and the shift reagent. Secondly, the geometry of the complexes between each enantiomer and the shift reagent may be different.

Chiral lanthanide shift reagents were separately introduced by Whitesides[133] and Goering[134] and have been widely used to distinguish enantiomers in proton NMR spectra. The majority of work involving chiral shift reagents has been performed in organic solvents and very few chiral shift reagents are known that can be used in water.

Peters[135] used lanthanide derivatives of (*S*)-[(carboxymethyl)oxy]succinic acid (CMOS) to separate the ^{1}H NMR spectroscopic signals of the enantiomeric nuclei of amino acids and hydroxycarboxylic acids. This ligand has however been shown not to be a good choice due to the self association tendency observed with lanthanide-CMOS complexes. Kabuto and Sasaki[136,137] observed a highly consistent correlation between the absolute configuration of α-amino acids and the shift of their α-proton signals induced by the chiral lanthanide shift reagent, europium(III) propane-1,2-diaminetetra-acetate. They examined twenty two substrates of wide variety, including histidine. In all cases the α-proton signals due to the L-enantiomers always showed larger upfield shifts than those due to the D-isomers in the presence of the (*R*)-form of the chiral shift reagent. In the case of histidine, however, the signal broadened and it was

difficult to assign the configuration accurately. More recently, Kido and Okamoto[132] reported (*S,S*)-ethylenediamine-*N,N'*-disuccinic acid (EDDS) as a water soluble chiral shift reagent. They showed it to be a useful chiral shift reagent for aqueous work, as long as the solution pH was maintained between 9 and 11. Their study was however limited to four amino acids and it has not therefore been established if it is widely applicable.

(c) The Use of Cyclodextrins

A cyclodextrin has a central cavity into which organic molecules can enter to form inclusion complexes.[138] The nature of this association has been studied by various techniques, including solution state NMR spectroscopy. Cyclodextrins are chiral molecules. If a molecule containing a chiral centre is placed into a cyclodextrin cavity then diastereomeric pairs may be produced. These would probably have different NMR spectra.

There are three cyclodextrins used in chiral NMR spectroscopic work.[138] The most common cyclodextrin reported is β-cyclodextrin (βCD) probably due to economic considerations. βCD has a torus shape formed by seven amylose units. It has a central width of about seven angstroms. α-Cyclodextrin is one sugar moiety smaller than β-cyclodextrin while γ-cyclodextrin is one sugar moiety larger than β-cyclodextrin. Different effects will be found with the different cyclodextrins due to the variation in the cavity size and hence different interactions with added compounds. For optical purity measurements the most effective reagent should be found by experimentation.

Casy and Mercer[139] reported the application of cyclodextrins to analysis of optical purity by proton NMR spectroscopy. They drew the conclusions that the use of cyclodextrins has several advantages over methods involving derivatisation with optically active reagents or the application of chiral shift reagents, namely:
(a) the material is water (and D_2O) soluble and so can be used with water soluble chiral molecules;
(b) cyclodextrins have no broadening effects on proton resonances while the effects of lanthanide chiral shift reagents are sometimes obscured due to the paramagnetic relaxation effects associated with them; and
(c) the proton NMR chemical shift range of cyclodextrins is narrow.

(d) Enzymatic Methods

Enzymic procedures of resolution, when successful, yield isomers whose absolute configurations are known from the procedure itself. This is one advantage over chemical procedures. Another advantage is that once the specificity of an enzyme toward amino acids of known absolute configuration has been established, its action towards amino acids of unknown absolute configuration can, with some accuracy, be predicted.

Many enzymes have been used to resolve racemic amino acids.[48] Few of these are suitable for amino acid or peptide derivatives. For example amino acid decarboxylase requires both the amino- and the carboxyl- function to be unsubstituted. Another drawback with this method is that with L-amino acid decarboxylase, the chirality of the L-isomer is lost in the

formation of the corresponding amine compound. Davis[140] has reported on the action of proteolytic enzymes on some peptides and derivatives containing histidine.

Renal acylases have been used to resolve *N*-acylated α-amino acids. It is therefore conceivable that if the reaction could be followed and the products quantified then this could be a method for determining the optical purity of *N*-acyl derivatives of amino acids. Chenault *et al.*[141] reported the enantioselective hydrolysis of *N*-acyl amino acids catalysed by Acylase I. These workers found that Acylase I was specific for the position and chemical nature of the amide bond in substrates. They found that Acylase I was specific to α-amino acids although it did accept a range of substituted acetyl and propionyl derivatives. Chenault *et al.* found that one disadvantage was the incomplete hydrolysis of the L-enantiomer.

(e) Conclusions

Many methods are available for the determination of the optical purities of amino acid and peptide derivatives. Of the methods discussed, no particular method appears to be completely general for the range of compounds to be synthesised in the course of this project.

CHAPTER 4

Synthesis of *N*-Acylated L-Histidine Derivatives

It has been discovered (Chapter 1) that derivatives of L-histidine (1) appear to have an important role in the healthy functioning of heart muscle. Many of the L-histidine derivatives required for testing by the physiologists are commercially unavailable. It was necessary to synthesise those L-histidine derivatives thought to be present in heart muscle, in order to confirm their presence and to enable the physiologists to determine the effects of individual compounds on cardiac muscle.

4.1 Direct *N*-Acylation Reactions

The first target compounds were optically active *N*-acetyl derivatives of L-histidine and dipeptides containing L-histidine shown in Figure 14. Although *N*-propionyl derivatives of L-histidine and the dipeptides are not thought to be present in cardiac muscle in nature, it was decided to synthesise these derivatives as simple analogues (Figure 14). L-Histidine (1) and L-carnosine (2) are both commercially available. The first acylation reactions carried out were then the direct acylations of these compounds using either acetic or propionic anhydride.

	n	R
(17)	1	Me
(18)	2	Me
(19)	3	Me
(20)	1	Et
(21)	2	Et
(22)	3	Et

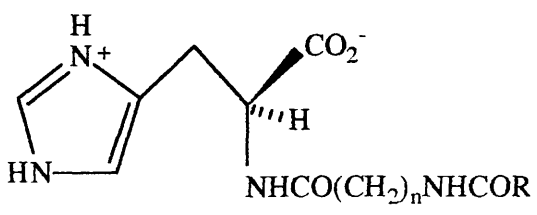
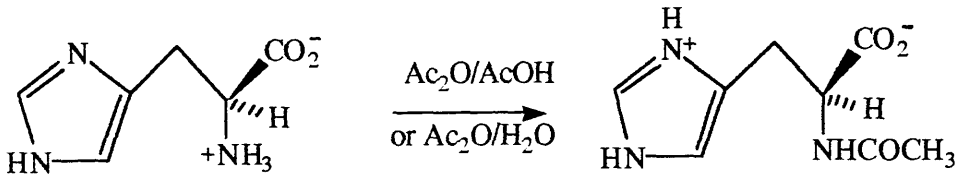


Figure 14

In 1928 Bergmann and Zervas published a method for the direct acylation of L-histidine (1) which led to an optically active product.[118] They used one equivalent of acetic anhydride as the acylating agent and glacial acetic acid as the solvent (Scheme 16). All attempts to copy the procedure of these workers failed to give *N*-acetyl-L-histidine (23) with as high an optical activity as they had obtained. Bergmann and Zervas[118] heated their reaction mixture over a boiling water bath. In an effort to obtain material with a higher optical purity, the reaction was carried out at various temperatures ranging from 0 °C to heating at the same temperature that these workers had reported. Each time, the optical activity was less than that reported, ranging from zero to half that of the reported value.



(1) **Scheme 16** (23)

Several groups have reported that vulnerable amino acids racemise unusually easily when heated in glacial acetic acid. Matsuo *et al.*[115] made a study of the racemisation of amino acids in glacial acetic acid (Section 3.4). They concluded that the acetate ion acts as a basic catalyst when it encounters a carboxylic acid bearing an α-ammonium ion.

On the basis of the observations of Matsuo *et al.*,[115] it was decided to attempt the acylation of L-histidine (1) and L-carnosine (2) using water as the solvent at room temperature. This method was successful and the *N*-acetyl- and *N*-propionyl- derivatives of

L-histidine (23,24) and L-carnosine (18,21) were obtained in yields typically in excess of 80%. More importantly, the optical purities of the compounds were greatly increased. The only optical rotation available for comparison was that for *N*-acetyl-L-histidine (23). The value for the synthesised compound was +39.5° (*c*, 0.9 in H_2O) compared to the reported value of +44.7° (*c*, 1.0 in H_2O).[118]

4.2 Peptide Coupling Reactions

The next stage in the work was to obtain the *N*-acylated dipeptide derivatives. The general idea was to couple *N*-acyl forms of glycine, β-alanine, L-alanine, D-alanine and 4-aminobutyric acid to L-histidine to form the acylated dipeptides. Coupling of the *N*-acyl-β-alanine compounds to L-histidine (1) would of course yield the L-carnosine derivatives (18) and (21) synthesised by the direct acylation reactions discussed in Section 4.1. Generating the same compounds in optically active form by two routes should test the integrity of each route.

β-Alanine and 4-aminobutyric acid were acylated with acetic anhydride or propionic anhydride in either glacial acetic or propionic acid. The yields of products obtained were always in excess of 70%. Only *N*-propionyl-β-alanine could not be obtained in crystalline form. Glycine was acetylated according to the method described by Herbst and Shemin.[142] It was obtained in 88% yield and had an identical melting point (207-208 °C) to the reported value.[142] *N*-Propionylglycine was obtained in 83% yield by the reaction of propionic anhydride and glycine in water. L- And D-alanine were both acylated with acetic anhydride in water to yield

58%. *N*-acetyl-L-alanine and 53% *N*-acetyl-D-alanine respectively. *N*-acetyl-L-alanine was isolated with m.p. 123-125 °C and $[\alpha]_D^{20}$ -58° (*c*, 1.3 in water), compared to the literature values[143] of m.p. 125 °C and $[\alpha]_D^{20}$ -66.2° (*c*, 1.0 in water). *N*-acetyl-D-alanine was obtained with m.p. 124-126 °C and $[\alpha]_D^{20}$ +56.6° (*c*, 1.4 in water), compared to the reported values[143] of m.p.125 °C and $[\alpha]_D^{20}$ +66.2° (*c*, 1.0 in water).

In order to obtain the *N*-acylated dipeptide derivatives in pure form, the first aim was to protect the carboxyl group of L-histidine to prevent it from interfering in the peptide coupling stage. When considering the options available, the alkyl esters and particularly the readily available methyl ester, were considered to be unsuitable. There were three reasons why this was so.

(a) The methyl ester is available as the dihydrochloride salt. This salt is insoluble in suitable organic solvents commonly employed in peptide coupling reactions.

(b) Strong bases are required to liberate the free base form. This could cause problems of racemisation of the L-histidine residue if the amounts of base added were not strictly controlled.

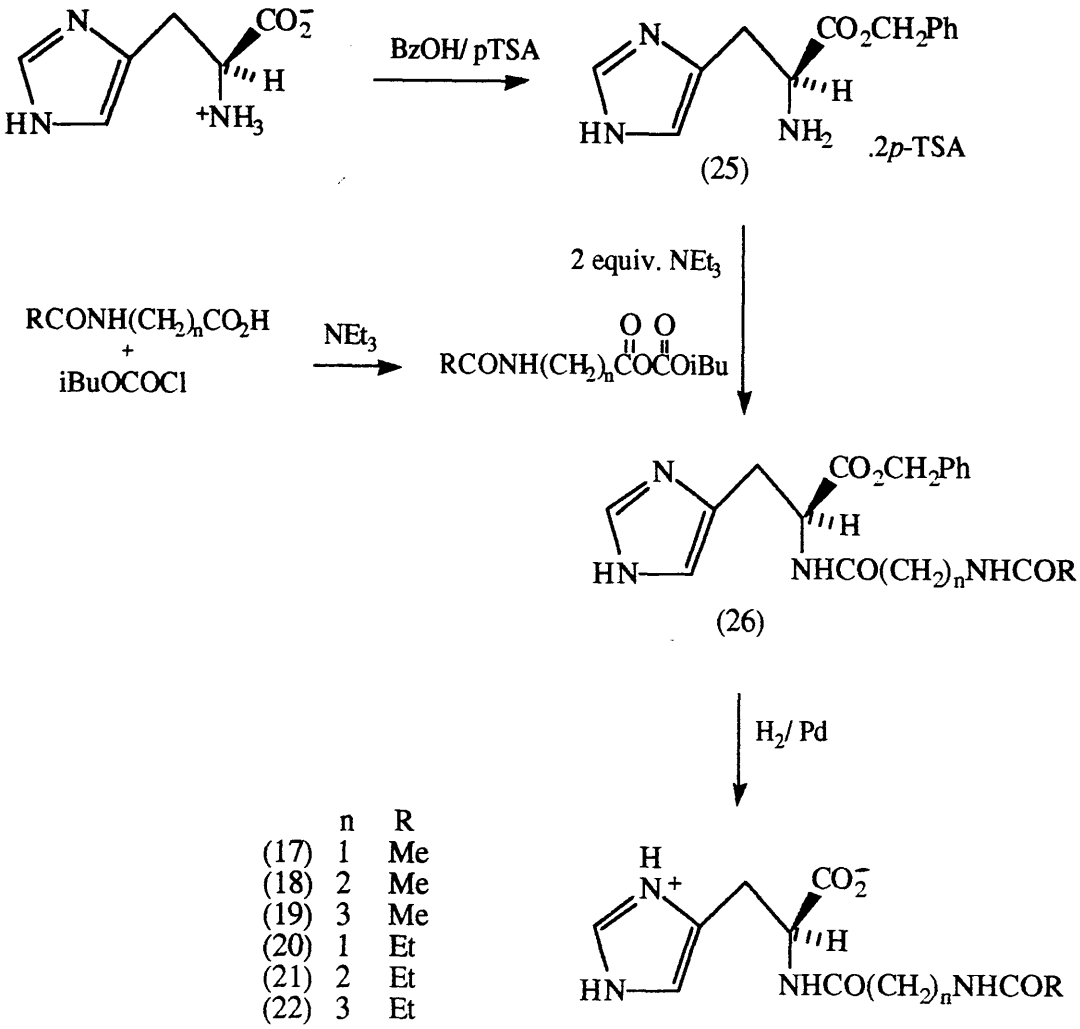
(c) The methods commonly employed for the removal of methyl esters, such as base hydrolysis, promote racemisation.

The choice of ester was then for one which could be synthesised easily. It should be stable to the conditions employed in peptide synthesis and it should be removed cleanly without promoting racemisation. The synthesis of the benzyl ester of L-histidine has been reported by several groups in the literature,[75,76,77] and was discussed in more detail in Section 3.3.1 (b).

In theory, the benzyl ester of L-histidine fulfilled all the criteria required of an effective protecting group for the carboxyl function of L-histidine. In practice however, a number of complications occurred. On following the procedure set out by Jones and Wood,[77] the yields and the chemical as well as the optical purities of L-histidine benzyl ester di-*p*-toluenesulphonate (25) obtained differed greatly on each application. Even when the conditions were strictly controlled for a number of preparations, the yield of the product (25) varied from 45% to 70%. Yields could be improved to a maximum of around 80% by filling the Dean and Stark apparatus with silica gel and anhydrous sodium carbonate. The water was thus removed from the reaction vessel more efficiently. The yields were still variable but at least they were higher. Since the optical rotation value for L-histidine benzyl ester di-*p*-toluenesulphonate (25) is low, $[\alpha]_D^{20}$ -4.13° (*c*, 0.94 in H_20), it was difficult to determine the optical purities of the products obtained in the course of this work.

Satisfied that the reaction could not be improved further, the next stage was to couple the *N*-acylated second amino acid to the benzyl ester of L-histidine (Scheme 17). Two peptide coupling methods were investigated, namely the dicyclohexylcarbodiimide (DCC) method and the mixed anhydride method. The DCC method was soon abandoned since the product could not be isolated from the dicyclohexylurea by-product. The mixed anhydride method was then explored.

The mixed anhydride was formed on reaction of the *N*-acylated amino acid with ethyl chloroformate. Triethylamine was the base and dry dichloromethane was the solvent.



Scheme 17

The free base was obtained from L-histidine benzyl ester di-*p*-toluenesulphonate (25) on addition of two equivalents of triethylamine to a solution of the salt in dichloromethane. In the first instance, both the solutions were filtered before mixing. The yields of the coupled products were frequently low, but better yields were obtained when the solutions were mixed without previously filtering them. Yields were also improved when isobutyl chloroformate was used in place of ethyl chloroformate to form the mixed anhydride. This is due to the better capability of

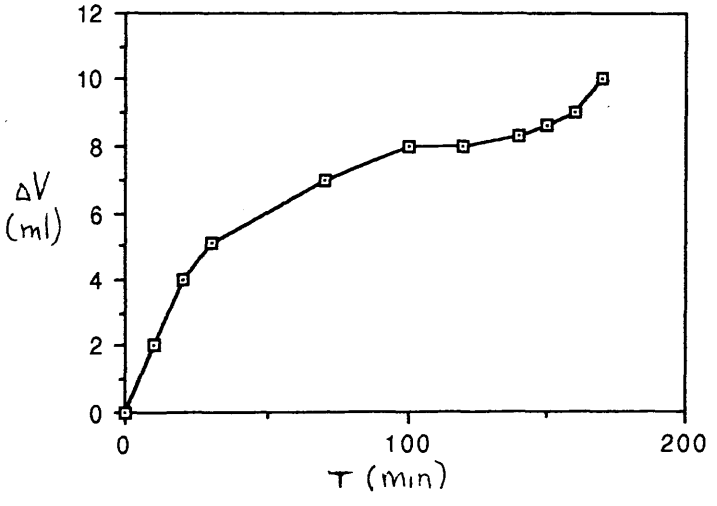
the isobutyl chloroformate to promote nucleophilic attack at the carbonyl group of the *N*-acylated amino acid.

The yields of the protected dipeptide products (26) were frequently in the range of 60 to 70%. All of the products (26) were highly coloured oils and accurate rotation values could not therefore be measured. A mixed anhydride was also formed between propionic anhydride and ethyl chloroformate. This was reacted with the benzyl ester of L-histidine to yield *N*-propionyl-L-histidine benzyl ester which could be isolated as a solid and was optically active with $[\alpha]_D^{20}$ -17° (*c*, 0.32 in ethanol).

The final stage involved the removal of the benzyl esters to yield the *N*-acyldipeptides (17)-(22) and *N*-propionyl-L-histidine (24). This was the chief reason that the benzyl ester was chosen to protect the carboxyl group of L-histidine: it should be easily removed on reduction with hydrogen in the presence of a catalyst, thus avoiding harsh conditions which could promote racemisation. Hydrogen was maintained over a solution containing each benzyl ester in ethanol in the presence of 10% Pd/C and the reaction was left overnight. Frequently very little product was obtained and the residual material had the benzyl ester intact. It appeared that the hydrogenolysis was inefficient. It was possible that the catalyst was being poisoned by the presence of basic nitrogen atoms in the compounds. To alleviate this, a couple of drops of dilute hydrochloric acid were added to each reaction. Again the reactions were allowed to run overnight. On examination of the proton NMR spectra of the mixtures at the end of this time, the imidazole protons in the aromatic region of each spectrum were absent and on integration, more aliphatic protons were present than there should have been. This indicated that the imidazole

ring in each L-histidine derivative was being reduced, which was quite surprising. The next step was to monitor the uptake of hydrogen, and the results were plotted graphically as a function of the change in volume *vs* time. A typical graph for the reduction of *N*-acetyl-L-homocarnosine benzyl ester (26; n=3, R=Me) is shown in Figure 15.

Figure 15 Typical graph for the removal of the benzyl ester of L-histidine derivative (26; n=3, R=Me) by catalytic reduction.



At the beginning when hydrogen is first passed into the reaction vessel, the active sites on the catalyst are saturated. The curve then rises steeply indicating the uptake of hydrogen as the cleavage of the benzyl ester takes place. The curve then levels out when the reaction at the benzyl ester is complete. If the compound (26; n=3, R=Me) is left in the presence of hydrogen for longer then the curve rises steeply once more, this is when the imidazole ring becomes reduced. In general the reduction of the benzyl ester in (26; n=3, R=Me) was complete within two hours.

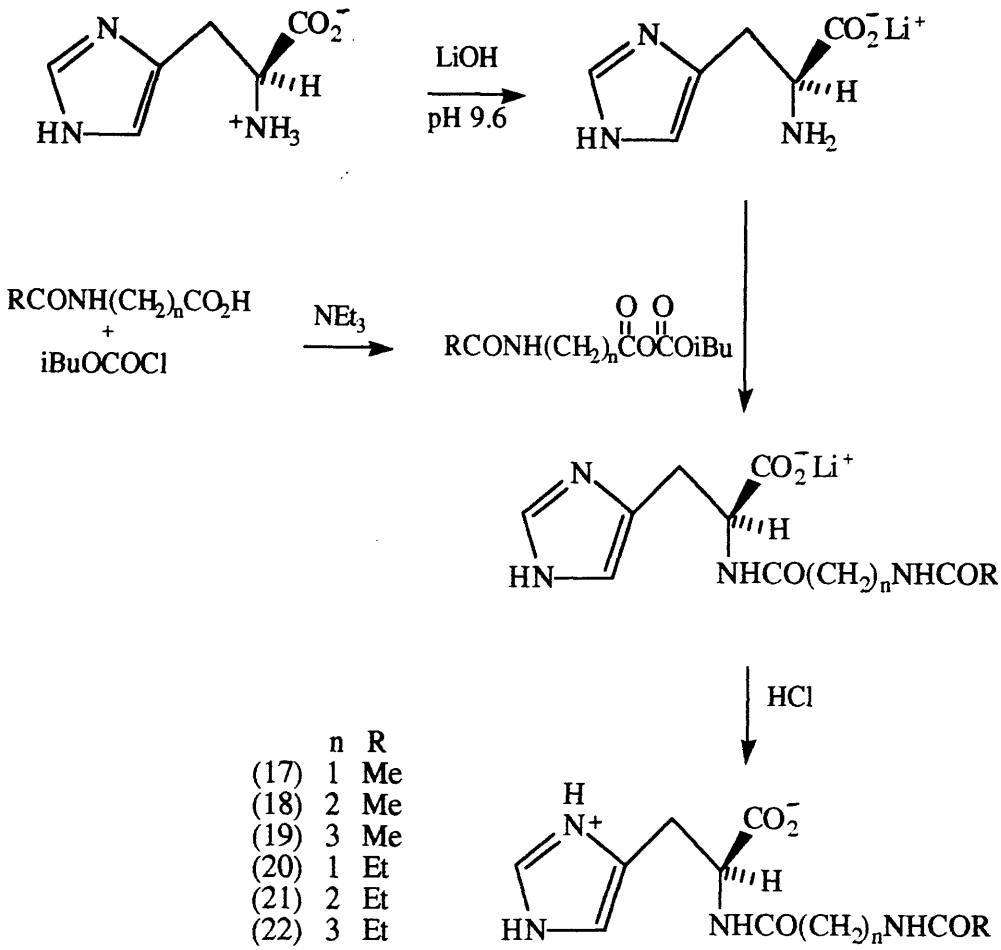
These conditions were then used to prepare compounds (17)-(22) and (24).

In all cases the *N*-acyl dipeptide derivatives (17)-(22) and *N*-propionylhistidine (24) could only be isolated as highly coloured oils and again it could not be determined whether they possessed any optical activity. Attempts were made to remove the yellow colour by treating these compounds in ethanol with activated charcoal. These attempts were unsuccessful and the intense yellow colour prevailed in each case.

When taking into account the difficulties encountered in the first and the final steps of this route in particular, it was decided that it was not suitable as a general route to chemically and optically active products.

In 1964 Rinderknecht *et al.* published a synthesis of L-carnosine (2) and its methylated derivatives as optically active compounds.[144] The interesting feature in this paper was that they protected the acid function of L-histidine as a metal salt. It was decided to follow an adaptation of their procedure to set up a new general route to the *N*-acylated dipeptides (17)-(22) (Scheme 18).

Aqueous lithium hydroxide was the first alkali solution to be used. The same mixed anhydride method of peptide coupling was used since it had been successful in the previous route and Rinderknecht *et al.*[144] had used a similar procedure in their work with no apparent problems. There was little concern about racemisation occurring when the carboxyl group was protected as the alkali metal salt since it should be difficult to abstract a further proton from the α-position.



Scheme 18

Various concentrations of the alkali solution were tried and the best turned out to be 0.33 M lithium hydroxide in terms of yield. The initial pH of the L-histidine solution was varied between 9 and 10. The optimum pH was found to be around 9.6. At lower pH not all of the acid was protected as the metal salt and this led to lower yields of desired product. At higher pH the extra base could interfere with the mixed anhydride before it reacted with the protected L-histidine and again the result was a lower yield.

At the end of the reaction the solution was acidified and the product was isolated. In general it was a much simpler reaction to

carry out than the route involving the benzyl ester (Scheme 17). It was difficult however to isolate the product at the end of the reaction. The by-product, lithium chloride, is hygroscopic which caused some handling difficulties. The lithium chloride also has similar solubility properties to some of the *N*-acyldipeptides. For these reasons the base used was changed to potassium hydroxide with some success. No general method could be found to isolate the *N*-acyldipeptides as crystalline solids. A different method was required for each product. Samples of each product were burned to check that no metal salts remained. In addition to this crude method, the compounds were each applied to a column and analysed by reverse phase HPLC. In all cases only one peak was present indicating the compounds (17)-(22) were free from contamination.

The yields of the isolated products (17)-(22) were higher than 50% in all cases. These yields were good considering the mixed anhydride coupling stage was carried out in an aqueous environment. The yields were also tolerable since the method was essentially a one step procedure.

All of the L-histidine derivatives (17)-(22) synthesised had certain similarities in the different types of spectra recorded. In the proton NMR spectra, all of the compounds exhibited two singlets in the aromatic region, each integrating as one proton. These signals were due to the two imidazole protons. The signal in the lower field position (H-2) was sometimes seen to be a doublet. The coupling constant was very small, and the splitting was due to cross-ring coupling between the two aromatic protons. The other imidazole proton (H-4) was never seen as a doublet but broadening was observed.[40] The other common feature in the

proton NMR spectra of these compounds was the appearance of an ABX system. This system was due to coupling between the protons shown in Figure 16.

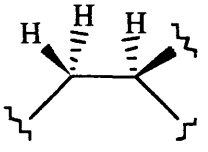
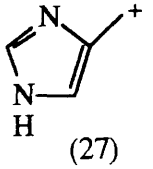


Figure 16

It was not possible to obtain accurate analytical data for these compounds (17)-(22). This was due to the different number of moles of water present in the crystalline solids. Even after extensive drying of the compounds, analytical data were unreliable.

In the mass spectra of these *N*-acylated derivatives (17)-(22), the parent ion peak was present in every case. The peak which all the derivatives showed in their spectra had *m/z* 81. This peak corresponded to the ion (27).



(27)

The derivatives (17)-(22) showed many similarities in the infra red spectra. All of the IR spectra showed a sharp peak in the region 3300-3500 cm^{-1} which corresponded to an N-H stretch. A group of signals typical of a carboxylic acid could be seen in the region 2900-3200 cm^{-1}. Amide stretches were present in the region 1600-1640 cm^{-1}, while signals corresponding to a carboxylate anion were observed in the region 1540-1600 cm^{-1}.

A synthesis of *N*-acetylglycyl-L-histidine (17) has been reported in the literature. Bryce *et al.*[145] coupled *N*-acetylglycine to *N*(im)-benzyl-L-histidine benzyl ester using the active ester method of peptide coupling. They isolated the product after removal of the benzyl group and the benzyl ester upon treatment with hydrogen in the presence of a catalyst. The product Bryce *et al.* isolated was a hygroscopic solid. Although they claim to have synthesised *N*-acetylglycyl-L-histidine (17), Bryce *et al.*[145] did not report an optical rotation value for their product. Indeed the only evidence they offered was an R_F value. The product obtained in the course of this work was a crystalline solid and it was in no way hygroscopic. It was obtained in 72% yield and had $[\alpha]_D^{20}$ +31.1° (*c*, 1.1 in H_2O). The proton NMR spectrum of (17) showed a singlet at δ 2.10 which on integration corresponded to the 3 protons of the acetyl group. The singlet at δ 3.93 integrated as 2 protons. This signal was due to the two protons of the CH_2 group of the glycyl residue. An accurate mass measurement of this compound (17) was made. A value of 254.1021 was obtained while (17) required a mass of 254.1015.

N-Propionylglycyl-L-histidine (20) was obtained in 64% yield with $[\alpha]_D^{20}$ +22.8° (*c*, 0.94 in H_2O). The propionyl group was shown on the proton NMR spectrum of (20) as a triplet at δ 1.17 and a quartet at δ 2.37. A singlet for the two protons of the glycyl residue showed at δ 3.93. The mass spectrum showed peaks corresponding to the parent ion as well as the loss of the amino side chain. Accurate mass measurements gave a mass of 268.1172 which is the exact mass *N*-propionylglycyl-L-histidine (20) required.

N-Acetyl-L-carnosine (18) was synthesised both by direct acylation of L-carnosine (2) and by coupling *N*-acetyl-β-alanine to L-histidine (1). By the direct acylation procedure the product was obtained in 80% yield with $[\alpha]_D^{20}$ +14.4° (*c*, 1.0 in H_2O). This compares to a yield of 59% and $[\alpha]_D^{20}$ +20.1° (*c*, 1.14 in H_2O) for the product obtained by the mixed anhydride method. Although the mixed anhydride route gave a lower yield of product, it was considered to be superior since the optical activity of the product was higher than that obtained by the direct acylation route. In all other respects the products obtained from the two routes were identical. A study of the proton and ^{13}C NMR spectra of underivatised carnosine (2) has been reported in the literature.[146] This study allowed the unambiguous assignment of proton and ^{13}C resonance signals using a variety of NMR spectroscopic techniques. These involved a ^{13}C NMR titration. Measurements of three bond ^1H-^1H and ^{13}C-^1H spin-spin coupling constants, and of the ^{13}C NMR chemical shifts for carnosine in the solid state were made. The information obtained can be used to aid the assignment of the resonances observed for derivatives of carnosine such as those obtained in this work. Figure 17 shows *N*-acetyl-L-carnosine (18) and Table 1 assigns the proton and ^{13}C NMR chemical shifts recorded for this compound. A synthesis of *N*-acetyl-L-carnosine has been reported but an optical rotation value has not been quoted.[147]

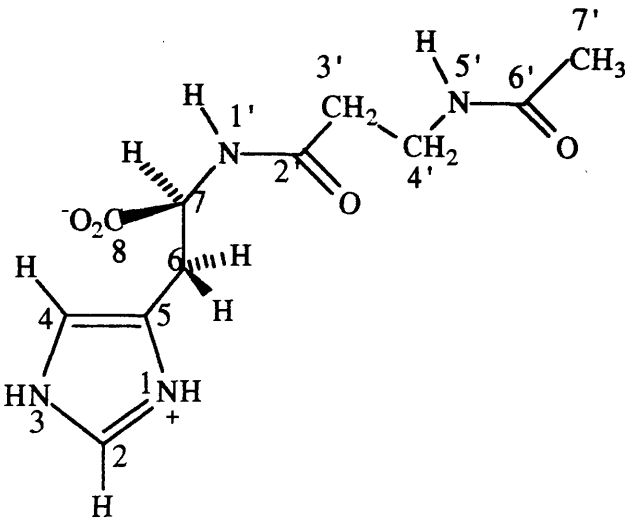


Figure 17

Table 1 NMR spectral data for *N*-acetyl-L-carnosine (18).

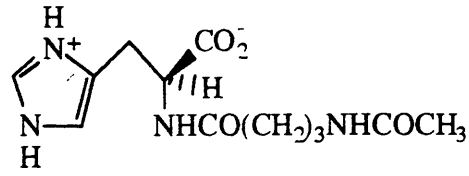
Position	^{13}C shifts (ppm)	^{1}H shifts (ppm)
2	133.2	8.58
4	116.7	7.24
5	129.8	-
6	27.3	2.99-3.28
7	54.0	4.46-4.52
8	176.4	-
2'	174.1	-
3'	35.1	2.44
4'	35.9	3.35
6'	173.4	-
7'	21.6	1.91

N-Propionyl-L-carnosine (21) was also synthesised in two ways. The direct acylation of L-carnosine (2) with propionic anhydride afforded a 71% yield of product (21) with $[\alpha]_D^{20}$ +14.6° (*c*, 1.2 in H_2O). The method involving the mixed anhydride coupling of *N*-propionyl-β-alanine to L-histidine (1) gave a 53% yield of product (21) with $[\alpha]_D^{20}$ +15.5° (*c*, 1.2 in H_2O). Again the mixed anhydride route produced the superior product with

respect to optical purity, although the difference was not great. No reference to this compound could be found in the literature. The proton NMR spectrum of (21) showed the propionyl group to be present with a triplet at δ 1.12 and a quartet at δ 2.25. Again the methylene groups of the β-alanine residue could be assigned. The mass spectrum showed the parent ion as well as the loss of the amino side chain. Accurate mass measurement gave a mass of 282.1326 while *N*-propionyl-L-carnosine required a value of 282.1328.

N-Acetyl-4-aminobutyric acid was coupled to L-histidine (1) using the mixed anhydride procedure to produce *N*-acetyl-L-homocarnosine (19) in 61% yield. The product was optically active with $[\alpha]_D^{20}$ +14.7° (*c*, 0.8 in H_2O). The proton NMR spectrum of (19) was similar to that for *N*-acetyl-L-carnosine (18) with an extra multiplet at the lowfield position of δ 1.77. This is due to the protons which are coupled to two other methylene groups *i.e.* $CH_2C\underline{H}_2CH_2$. This residue was seen in the ^{13}C NMR spectrum at δ 29.4, δ 34.9 and δ 40.6. An accurate mass measurement gave a mass of 282.1332 while *N*-acetyl-L-homocarnosine (19) requires a mass measurement of 282.1328. *N*-Acetyl-L-homocarnosine (19) was crystallised from ethanol. One of the crystals was subjected to X-ray diffraction by Dr. A. A. Freer.[148] The structure obtained is shown in Figure 18. From the structure it is possible to see that the compound (19) is present as the zwitterion with the ring protonated. Figure 19 shows the crystal packing of this compound. It is interesting to see the bending present in the side chain and how well this bent structure packs in the crystal. No reference to the synthesis or the crystal structure of *N*-acetyl-L-homocarnosine (19) has been found in the literature.

Figure 18 X-ray structure of *N*-acetyl-L-homocarnosine (19)



(19)

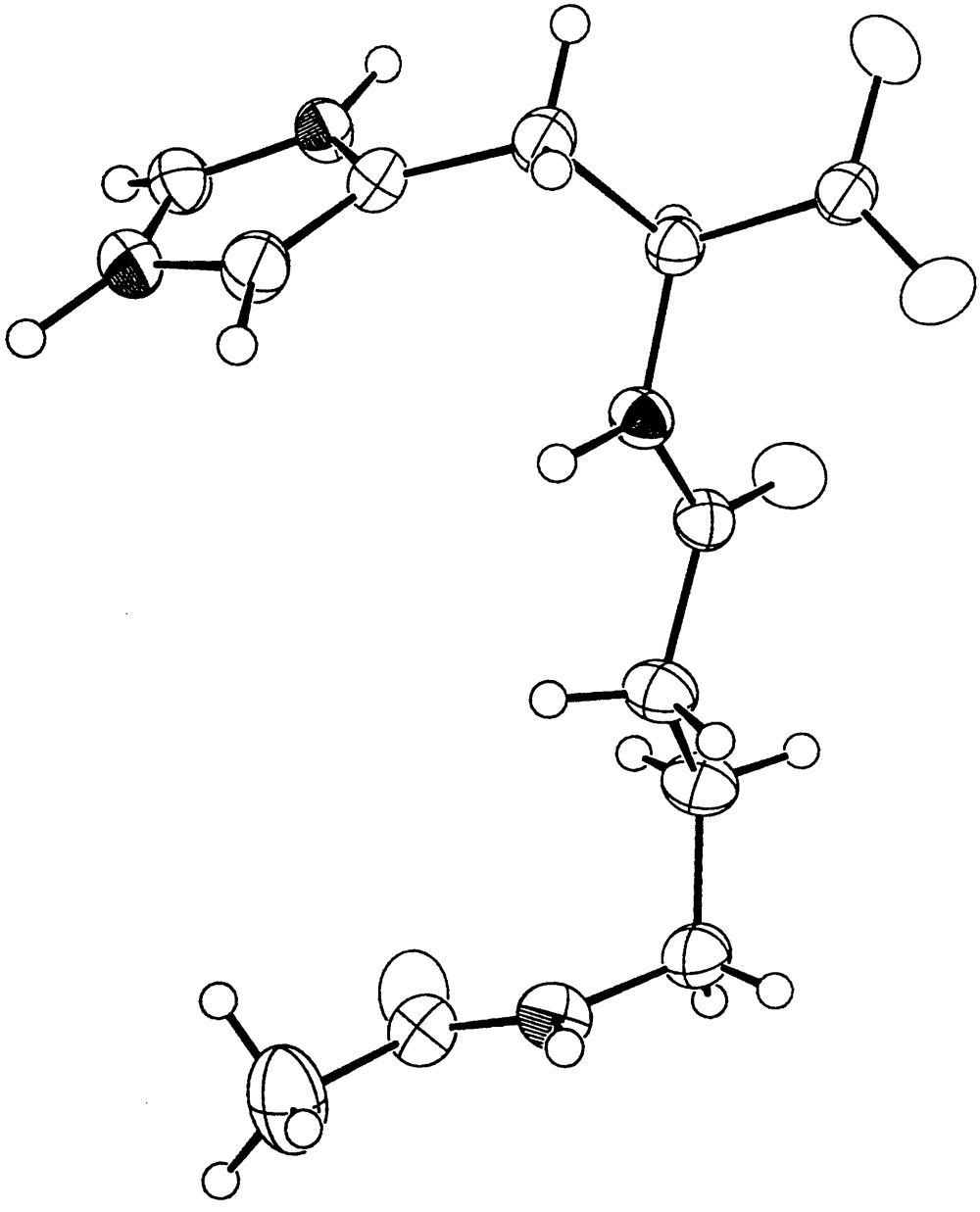
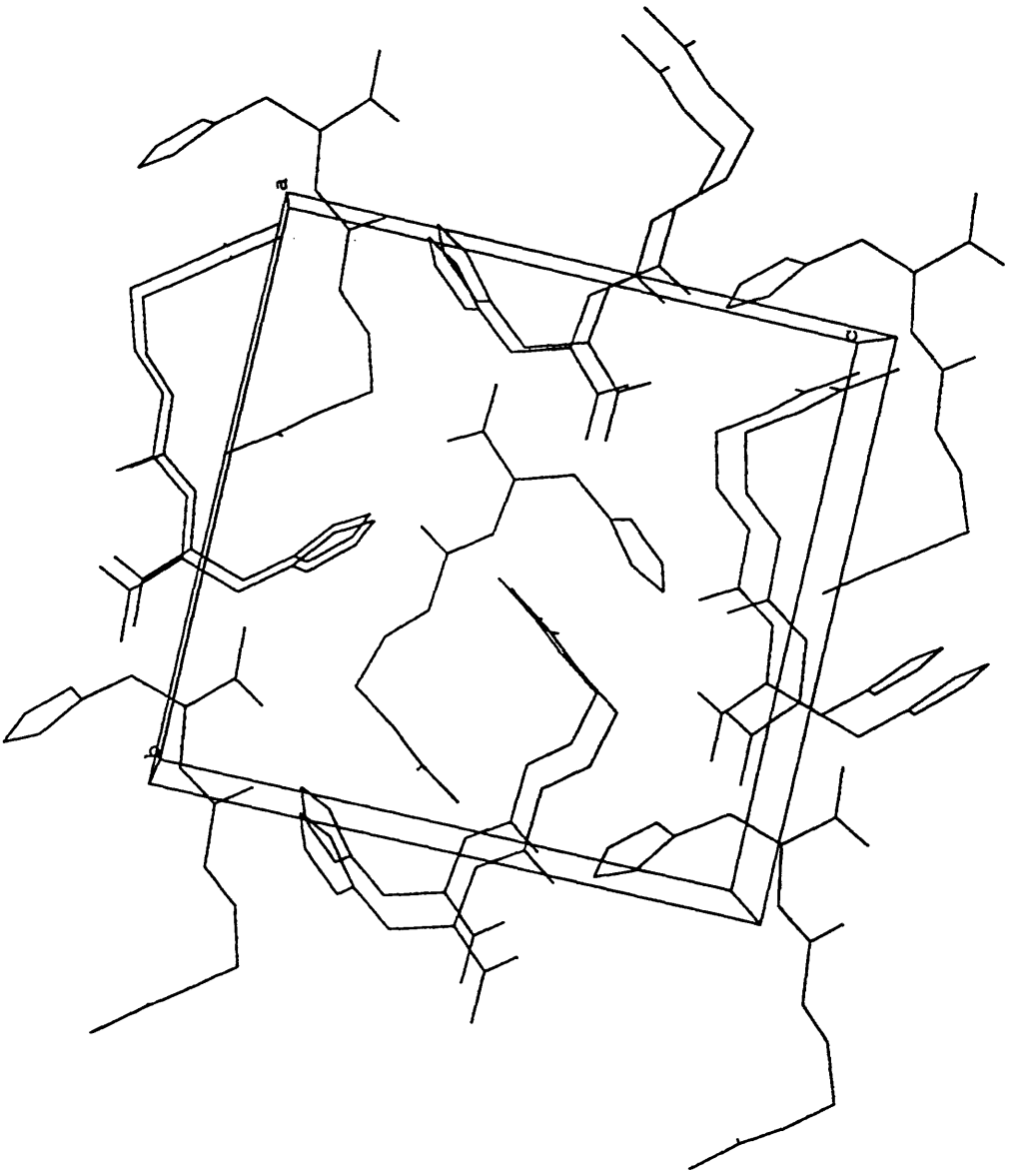


Figure 19 Crystal packing of *N*-acetyl-L-homocarnosine (19)



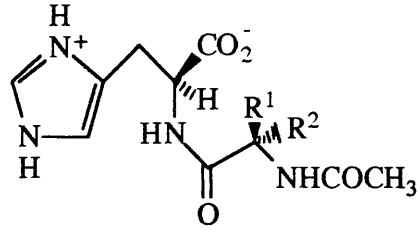
N-Propionyl-L-homocarnosine (22) was obtained in 61% yield when *N*-propionyl-4-aminobutyric acid was coupled to L-histidine. The product had $[\alpha]_D^{20}$ +10.3° (*c*, 1.3 in H_2O). There were some problems associated with the isolation of this product

as it is hygroscopic. Attempts were made to isolate it as a hydrate by trying to crystallise it from a mixture containing water since it was thought that a hydrated form of the product would not be so hygroscopic. Many solvent mixtures were tried but the product could only be isolated as an oil when any water was present. A hygroscopic solid was obtained from an isopropanol solution on addition of acetone. Once more no reference to this compound could be found in the literature. The proton and ^{13}C NMR spectra of (22) were similar to those obtained for the *N*-acetyl derivative (19). The propionyl function was shown as a triplet at δ 0.89 and a quartet at δ 2.02 in the proton NMR spectrum. An accurate mass measurement was made. The value obtained was 296.1479; *N*-propionyl-L-homocarnosine (22) required a value of 296.1484.

So far the dipeptide derivatives (17)-(22) have been of a series in which only the length of the alkyl chain of the second amino acid has been varied. None of the second amino acids have possessed any chirality. If an optically pure amino acid derivative is coupled to L-histidine then any racemisation which occurs in the peptide coupling process should be detectable by examination of the ^{13}C NMR spectrum of the dipeptide derivative since diastereoisomers would be formed.

Both *N*-acetyl-L-alanine and *N*-acetyl-D-alanine were coupled to L-histidine (1). There was a fear that the *N*-acylalanyl residue would be racemised in the activation stage of the mixed anhydride coupling procedure. This could provide misleading information regarding the optical integrity of the coupling step. *N*-Acetyl-L-alanyl-L-histidine (28) was obtained in 59% yield with $[\alpha]_D^{20}$ -32.8° (*c*, 0.9 in 6M HCl). *N*-Acetyl-D-alanyl-L-histidine (29) was obtained in 58% yield with $[\alpha]_D^{20}$ -42.1° (*c*, 0.8 in 6M HCl).

Both the proton and ^{13}C NMR spectra of (28) and (29) showed only one diastereoisomer was present in each case (Figure 20). These results were encouraging and suggested that the coupling method was not inducing racemisation at the chiral centre of L-histidine.



(28) R^1=H, R^2=Me

(29) R^1=Me, R^2=H

Conclusions

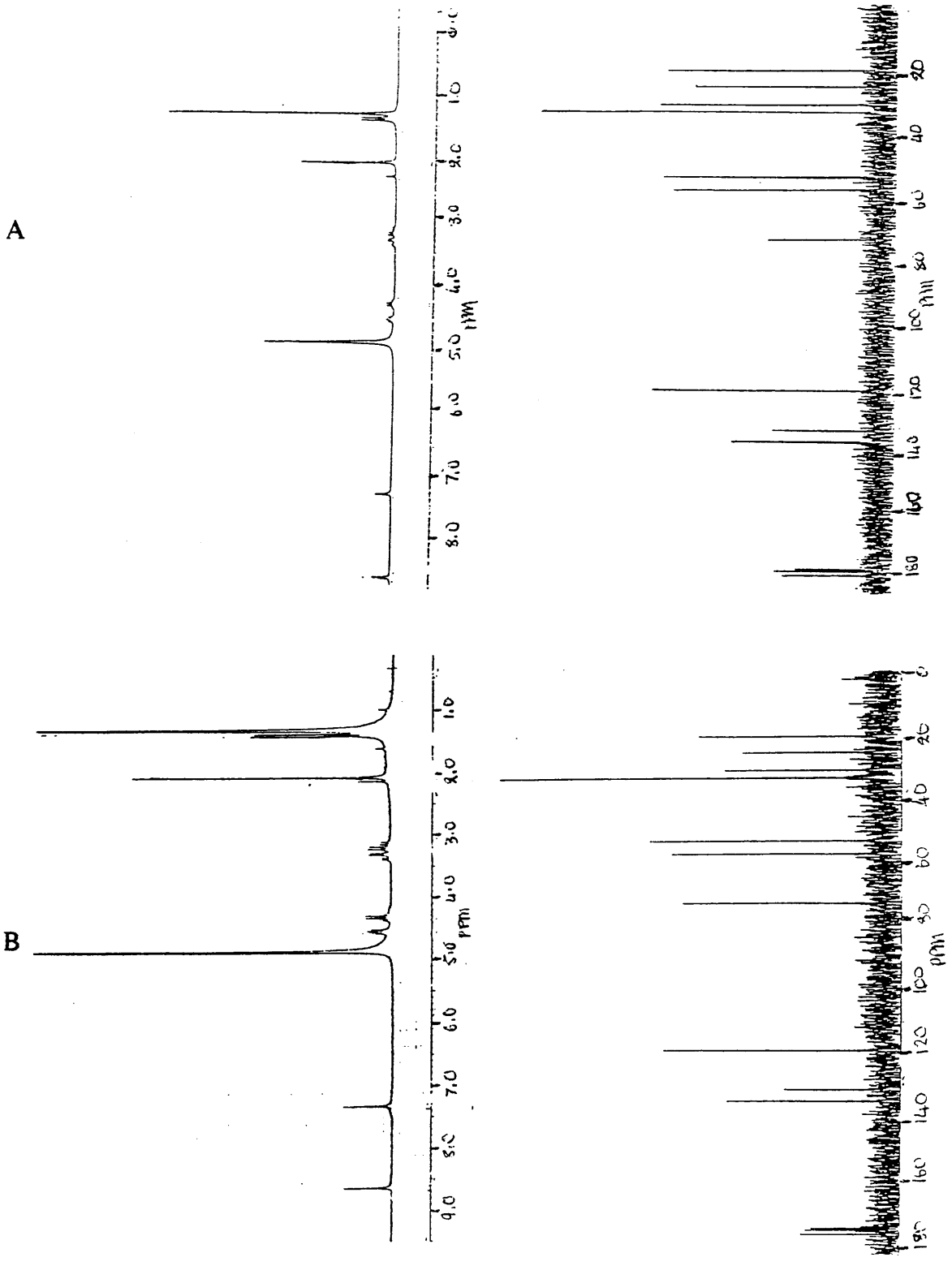
The route involving the carboxyl group protected as a metal salt shown in Scheme 18 had a number of advantages over the route involving the benzyl ester of L-histidine (Scheme 17):

(a) simplicity of the reaction scheme;

(b) consistent yields; and

(c) formation of crystalline, optically active products.

Figure 20 Proton and ^{13}C NMR spectra of (A) N-acetyl-L-alanyl-L-histidine (28) and (B) N-acetyl-D-alanyl-L-histidine (29).



4.3 Determination of the Optical Purity of the Compounds Synthesised

All of the target compounds (17)-(22), (24), (28) and (29) were synthesised as discussed in Section 4.2. They all possessed optical activity, but it was impossible in most cases to determine the optical purity of the products. The most straightforward method would be to compare the optical rotation values of the synthesised compounds to reported values; however, since *N*-acetyl-L-histidine (23) was the only product to have any optical rotation data reported in the literature,[118] it was necessary to try other means.

A method was required which would use only small amounts of the compound under investigation. It would need to be generally applicable and sensitive to small amounts of any D-isomer present. A number of methods for determining the optical purity of the L-histidine derivatives were investigated. Partially racemic mixtures made from *N*-acetyl-DL-histidine and commercially available *N*-acetyl-L-histidine (23) were used as the control compounds to test the effectiveness of each method.

The first method tried was the use of cyclodextrins as resolving agents. Dr. D. S. Rycroft tried both α- and β-cyclodextrin with a racemic mixture of *N*-acetylhistidine. On looking at the proton NMR spectra of the mixtures in D_2O, some broadening of the signals was observed, but no useful effect on the signals due to the histidine derivative was seen. These results indicated that this method would not be useful in determining the optical purity of the compounds synthesised in this project.

N-acetyl-DL-histidine was converted into the isopropyl ester by Dr. D. J. Cole and the resulting racemate was applied to a column and analysed by gas chromatography. On a normal column one peak showed as was expected. When the racemate was applied to a chiral valine column, it was found that it was not volatile enough. This meant that the racemate required temperatures far in excess of those which could be used with the chiral column. This technique was not therefore useful.

A number of chiral lanthanide shift reagents have been reported to be useful in resolving some of the signals in the proton NMR spectra of racemic amino acids. The chiral lanthanide shift reagent, europium(III) propane-1,2-diaminetetra-acetate, was synthesised.[136,137] It has been reported that for DL-histidine itself, broadening of the signals occurred, as opposed to separation. This was indeed found to be the case. When the chiral lanthanide shift reagent was tried in the presence of *N*-acetyl-DL-histidine, no effect on the ^1H NMR spectrum was observed. It appears therefore that the shift reagent requires a free α-amino function as well as the free carboxyl function for coordination. It was hoped that it would coordinate to the imidazole ring but this did not appear to happen.

Acylases are known to cleave the amide bonds of *N*-acyl-L-amino acids while leaving *N*-acyl-D-amino acids intact [Section 3.5(d)]. Racemic *N*-acetylhistidine was incubated in D_2O at 28° at pD 8 for 24 hours with Hog Kidney Acylase I (EC 3.5.1.14). The reaction was monitored by proton NMR spectroscopy. As the reaction progressed the signal corresponding to the acetyl group of *N*-acetyl-L-histidine disappeared while at the same time a signal with a different chemical shift appeared corresponding to

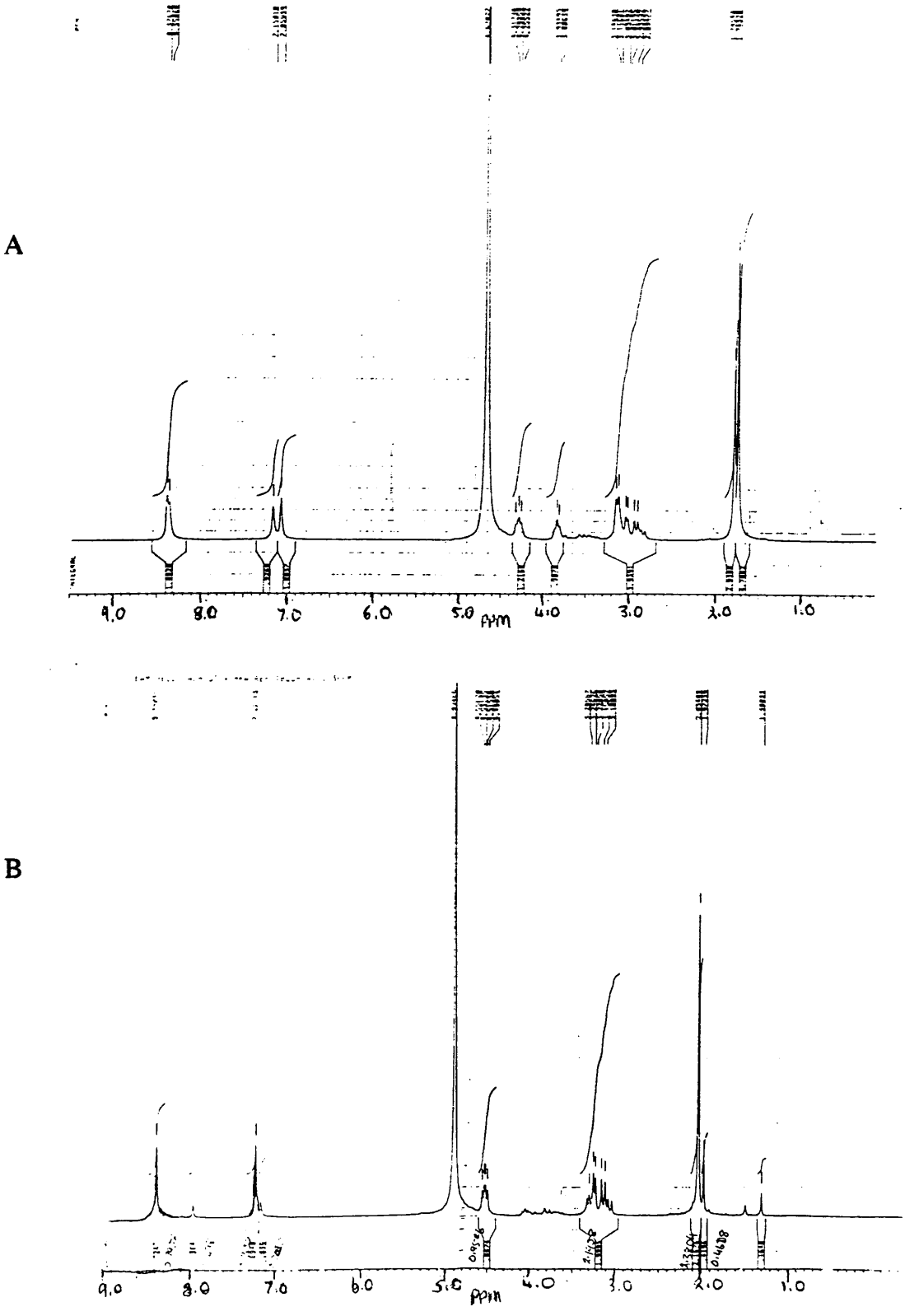
the formation of acetate as a by-product of the enzymatic cleavage. The signal corresponding to the acetyl group of acetyl-D-histidine remained unchanged. The amounts of L- and D-isomers present could be quantified by integration of the two different acyl signals at the end of the reaction.

A control experiment was run at pD 8 with *N*-acetyl-L-histidine (23) with no enzyme present. This experiment was to check that the amide bond was not being hydrolysed at this pD. No change in the proton NMR spectrum of the *N*-acetyl-L-histidine was observed after 24 hours, indicating that any cleavage occurring in the reactions with enzyme present was purely due to the action of the enzyme. Acylase I was added into this reaction mixture to determine whether the basic conditions had caused racemisation. The proton NMR spectra showed that the experimental conditions did not induce racemisation since the acyl peak had completely disappeared. This meant that only *N*-acetyl-L-histidine (23) had been present.

A number of experiments were run using a variety of mixtures of D- and L- *N*-acetylhistidine with varying optical purities. A selection of the results obtained is shown in Figure 21 to illustrate how useful this method is for determining the optical purities of these acetylhistidine mixtures.

This method should also be applicable for the *N*-propionyl-L-histidine (24), *N*-acetyl-L-alanyl-L-histidine (28) and *N*-acetyl-D-alanyl-L-histidine (29) synthesised in this project since all three compounds have a terminal α-acylamino acid. These compounds should then be substrates for Acylase I. The experiments with the *N*-acylalanyl residues present should then give an indication of the extent of racemisation taking place in the mixed anhydride

Figure 21 ^1H NMR spectra of *N*-acetylhistidine mixtures of different optical purity after treatment with Acylase I. A. Initial $[\alpha]_D^{20}$ 0°. B. Initial $[\alpha]_D^{20}$ +39.5°.



activation stage. They will not, however, provide any information on racemisation at the L-histidine residue on coupling.

The reactions were carried out under identical conditions to those used with the *N*-acetylhistidine mixtures. For *N*-propionyl-L-histidine (24) it was expected that the peaks due to the *N*-propionyl group of the L-isomer would disappear with the simultaneous appearance of peaks due to propionate. Any peaks due to the D-isomer would remain unchanged. No difference in the proton NMR spectra before and after treatment with Acylase I was observed. In the case of *N*-acetyl-L-alanyl-L-histidine (28), it was expected that the signal due to the *N*-acetyl group would disappear completely in the proton NMR spectrum if the compound was optically pure. Any of the peak due to the *N*-acetyl group remaining would then be due to some *N*-acetyl-D-alanyl residue present. Again no change in the proton NMR spectra was observed. It would be reasonable to assume therefore that neither *N*-propionyl-L-histidine nor *N*-acetyl-L-alanyl-L-histidine were substrates for the enzyme.

For *N*-acetyl-D-alanyl-L-histidine (29), it was initially hoped that any *N*-acetyl-L-alanyl residue present would be hydrolysed by the enzyme leaving the peak due to the *N*-acetyl-D-alanyl residue unchanged. If the derivative was optically pure at the alanyl residue, then no change in the proton NMR spectra would be expected after treatment with Acylase I. No difference in the proton NMR spectra was observed. This is not surprising since *N*-acetyl-L-alanyl-L-histidine (28) did not appear to be a substrate for the enzyme.

The length of time, the amount of enzyme used, the temperature of the reaction and the pH were varied in an attempt

to find conditions under which these compounds would be substrates for the enzyme. The results were always negative. A fresh batch of enzyme was also used but again no changes in the NMR spectra were observed.

This method was not universally applicable since it only works with *N*-acylated α-amino acid derivatives and even some of those have been shown not to be substrates for Acylase I. When the enzyme was applied to solutions containing *N*-acyl derivatives of glycyl-L-histidine, L-carnosine or L-homocarnosine (17)-(22), no reaction was observed. The enzymes lipase and peptidase were also challenged with these L-histidine dipeptide derivatives but neither enzyme cleaved the peptide or the amide bond.

Racemic *N*-acetylhistidine was mixed with brucine to form a mixture of the two diastereoisomers. This mixture was examined using proton NMR spectroscopy but no differences in the signals due to *N*-acetylhistidine were observed. The mixture was also applied to a reverse phase HPLC column in an attempt to separate the diastereoisomers. Although broadening of the peak was observed upon varying the conditions of elution, a separation into two peaks was not achieved.

(*R*)-α-Methylbenzylamine was coupled to the carboxyl function of the L-histidine derivative using the water soluble carbodiimide, *N*-ethyl-*N'*-dimethylaminopropylcarbodiimide. The diastereoisomers obtained were analysed on reverse phase HPLC. The results obtained for *N*-acetylhistidine are shown in Figure 22. It can be clearly seen when any of the D-isomer is present. Although the signals are not completely resolved, it is still possible to estimate the ratio of isomers using the area under each peak. When applied to other L-histidine derivatives, the same

type of separation was observed. The results obtained are listed in Table 2. There appears to be a direct correlation between the optical purities of the compounds and the ease with which they were obtained as crystalline solids. For example, *N*-acetylglycyl-L-histidine (17) appears to have full optical purity compared to *N*-propionyl-L-carnosine (21) which was much more difficult to crystallise. The values quoted are approximate but at least give an indication of the optical purities of the compounds synthesised.

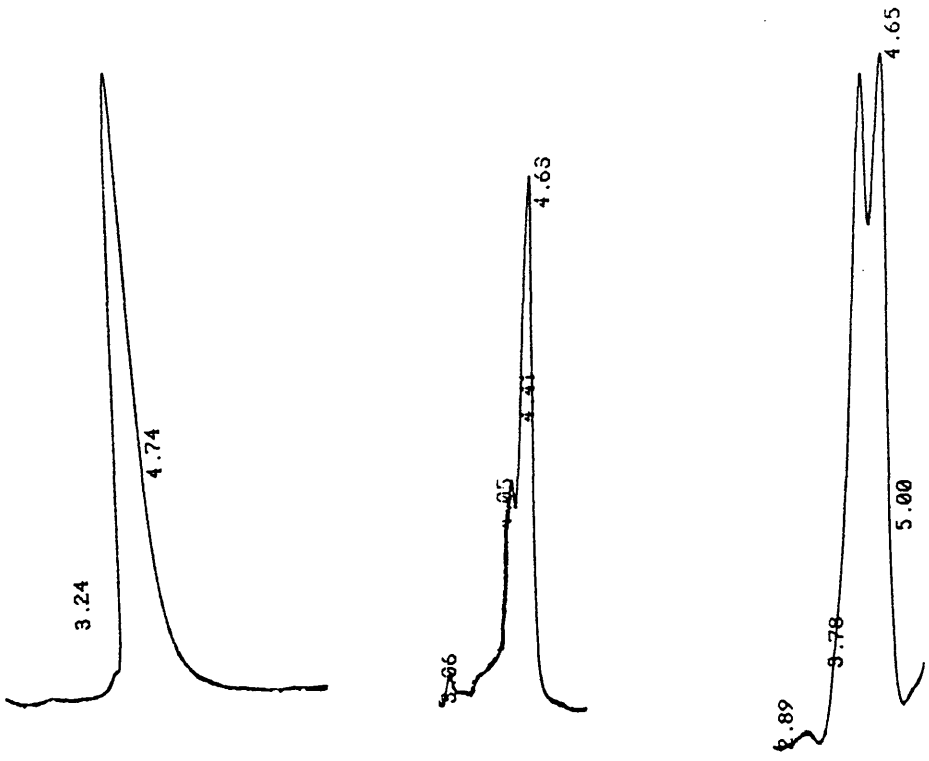
Table 2 Results from optical purity measurements by HPLC.

Derivative	$[\alpha]_D^{20}$	% L-isomer
N-Acetyl-DL-histidine (23)	0°	50
N-Acetyl-L-histidine (23)	+24.3°	80
N-Acetyl-L-histidine (23)	+46.0°	100
N-Propionyl-L-histidine (24)	+29.9°	75
N-Acetylglycyl-L-histidine (17)	+31.1°	100
N-Propionylglycyl-L-histidine (20)	+22.8°	82
N-Acetyl-L-carnosine (18)	+20.1°	71
N-Propionyl-L-carnosine (21)	+15.5°	58
N-Acetyl-L-homocarnosine (19)	+14.7°	77
N-Propionyl-L-homocarnosine (22)	+10.3°	69

N-Acetyl-L-alanyl-L-histidine (28) and *N*-acetyl-D-alanyl-L-histidine (29) were run on the same HPLC system. A chromatograph was obtained for each compound as well as a

mixture of the two compounds. Only one peak was present for each diastereoisomer. The peak due to (28) had retention time 2.18 while the peak due to (29) had retention time 2.05. These results suggest that (28) and (29) have good optical purity.

Figure 22 HPLC traces of the products of the coupling of (*R*)-α-methylbenzylamine with *N*-acetylhistidine mixtures of varying optical purity



Initial $[\alpha]_D^{20}$ +46.0^0 Initial $[\alpha]_D^{20}$ +24.3^o Initial $[\alpha]_D^{20}$ 0^o

Conclusions

Many methods have been used to try to determine the optical purity of the derivatives synthesised during the course of this work. The most useful method has been to convert the *N*-acyl

derivatives (17)-(24) into diastereoisomers and to separate these using HPLC. It should have been possible to develop this method to find conditions which would allow the complete separation of the diastereoisomers had time allowed. It is clearly the most promising method for determining the optical purities of the types of compounds synthesised during the course of this work.

4.4 Test Results For The Compounds Synthesised

The L-histidine derivatives (17)-(24), (28) and (29), synthesised during this work were tested by Dr. D.J. Miller and his co-workers in the Physiology Department at Glasgow University. The methods employed were discussed in Section 2.5. The L-histidine derivatives were required for three purposes:

1. to determine whether each individual compound was present in cardiac muscle and if so in what concentrations,
2. to test the derivatives as calcium sensitisers of the contractile apparatus of the heart, and
3. To determine the effect of these L-histidine compounds on maximum calcium activated force.

4.4.1 Determination of the Presence of Each Derivative in Cardiac Muscle by HPLC Analysis

Two distinct HPLC methods were developed by the physiologists at Glasgow University[18,149] to analyse cardiac muscle-derived L-histidine derivatives, some of which were *N*-acylated and novel. The first method (reverse-phase HPLC) relies on the elution from a non polar stationary phase (octadecyl-silica)

with a polar mobile phase (aqueous phosphate buffer at low pH) of a number of closely related L-histidine derivatives. These compounds differ slightly in a number of respects, for example; aliphatic chain length, methylation or not of the imidazole ring, and *N*-acylation or not of the terminal amino function. This method relies upon the separation of the compounds due to their individual hydrophobicities.

The second method (reverse-phase/ion-pair HPLC) relies upon the interaction of a hydrophobic surfactant anion (1-heptane sulphonic acid) with a solute cation (the pH dependent protonated terminal amino group) to form an ion-pair which then absorbs onto the non-polar stationary phase. This ion-pair may be eluted from the column in a temperature dependent manner. *N*-Acetyl derivatives are incapable of ion pair formation with a terminal amino group and it has been found that their retention times are independent of the concentration of the hydrophobic surfactant anion.

These two independent HPLC methods were used to examine extracts from cardiac muscle. The *N*-acyldipeptides (17)-(24), (28) and (29), synthesised during the course of this work were used as reference compounds to identify the peaks present in each trace. Identification was based on elution time relative to standards, co-elution with reference compounds, and fraction collection and re-analysis with the alternative method.

The biological extracts showed identical properties to a number of reference compounds on each chromatographic system. Figure 23 shows the results of this work using method 1, while Figure 24 shows the results obtained when method 2 was employed. From the two figures it is possible to see that *N*-

acetylcarnosine (18) and *N*-acetylhomocarnosine (19) can be identified as constituents of cardiac muscle. *N*-Acetylhistidine (23) can be seen as a constituent using method 1 but it cannot be separated from the peak due to *N*-acetyl-1-methylhistidine using method 2. Method 2 allowed the separation of carnosine (2), 3-methylcarnosine (4) and homocarnosine. This separation was not possible with method 1. The two methods, therefore, complement each other. Further work will reveal whether any of the other derivatives synthesised in this work are present in the heart. Work is continuing to isolate enough of each histidine derivative present in cardiac muscle in order to confirm its identity by NMR spectroscopy.

HPLC chromatographs of human serum extracts from different regions of the body were run. This allowed the identification of histidine derivatives (a) in the serum from the aorta, the start of the arterial blood supply, which carries blood from the left ventricle of the heart ultimately to all of the body tissues; (b) in the serum from the veins which carry blood from the heart muscle; and (c) in the serum in the large vein which carries blood from the upper half of the body and the head to the right atrium of the heart. Figure 25 shows the three chromatographs obtained and indicates the different distribution of these histidine derivatives in these regions. It also indicates the different concentrations at which these compounds occur in these regions. As yet a large number of the peaks in these traces remain unidentified. Work is continuing to identify these peaks, particularly those present in large concentrations.

Figure 23 HPLC chromatographs obtained by Method 1. (A). Extracted perfusate from Langendorff rabbit heart. (B). Authentic reference compounds. Abbreviations are listed on page V.

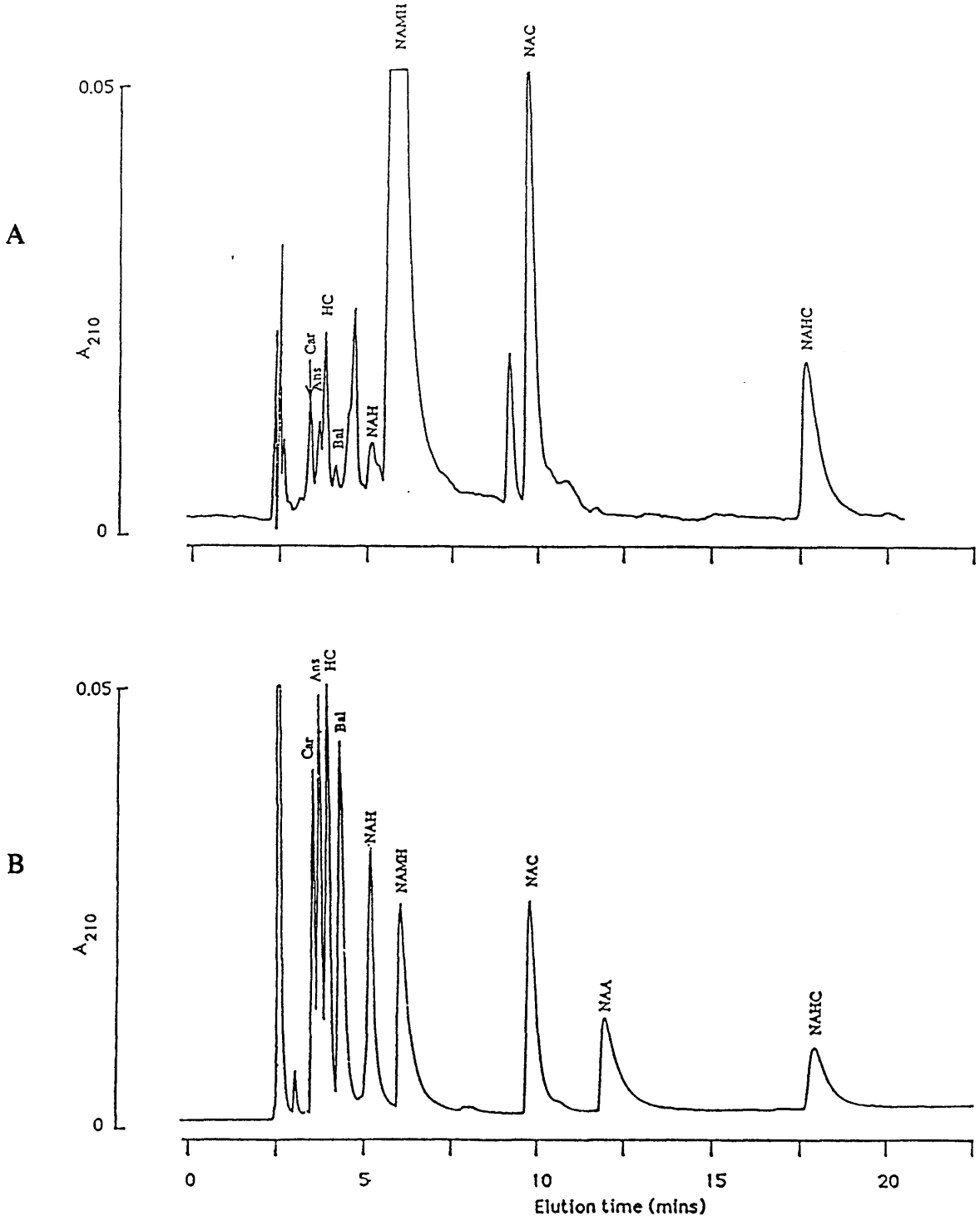


Figure 24 HPLC chromatographs obtained with Method 2. (A). Extracted perfusate as in Figure 23. (B). Authentic reference compounds. Abbreviations are listed on page V.

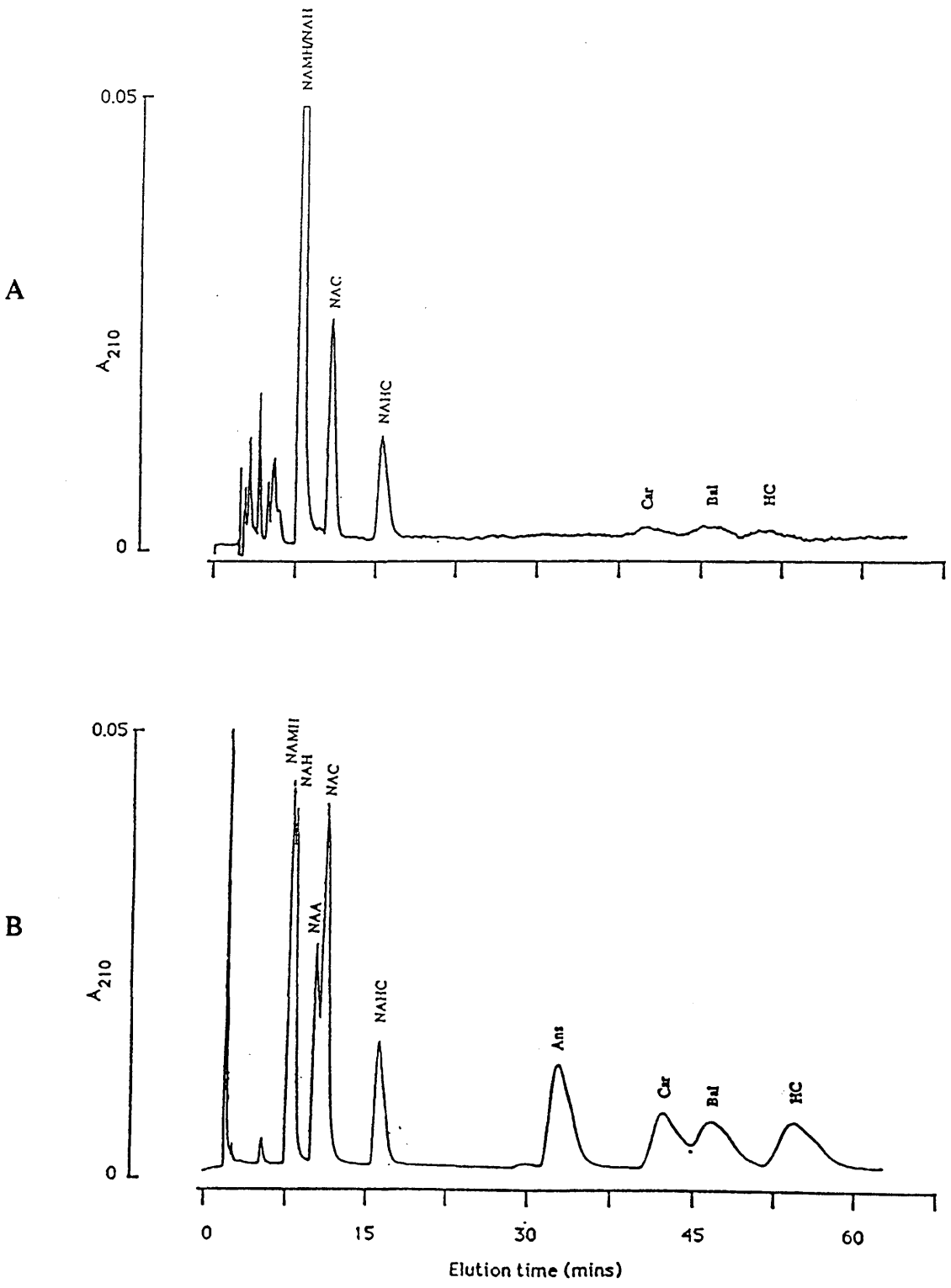
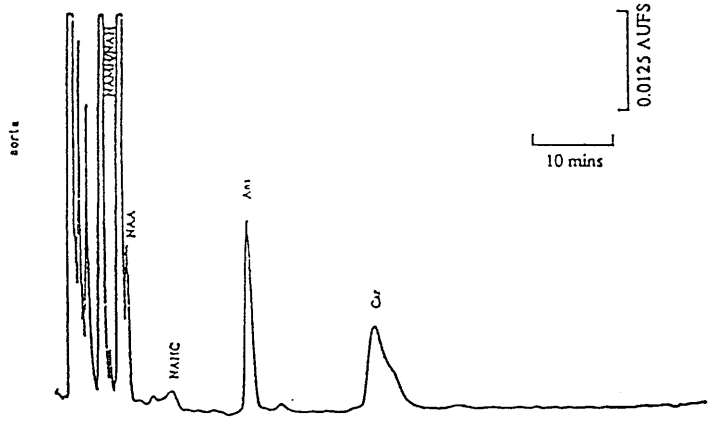
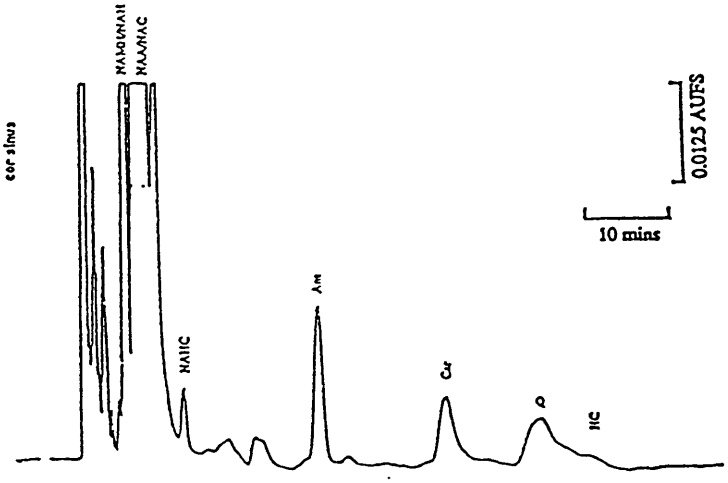


Figure 25 HPLC chromatographs of human serum extracts. (A.) Aortic serum. (B). Coronary sinus serum. (C). Superior vena cava serum. Abbreviations are listed on page V.

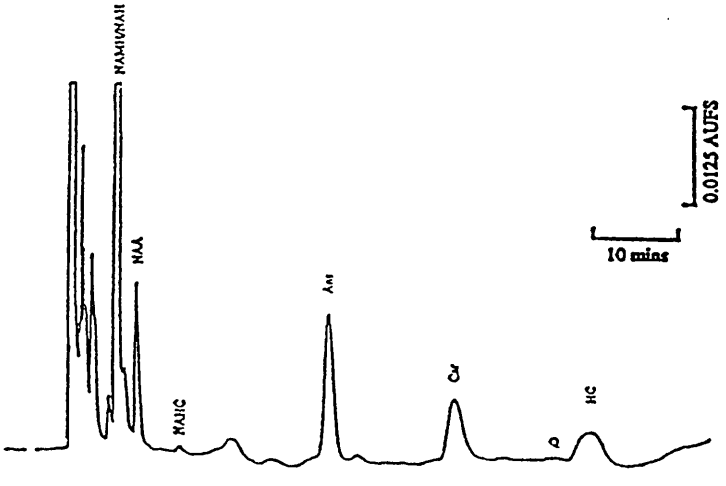
A



B



C



4.4.2 Results From Calcium-Sensitising and Peak Force Experiments

The L-histidine derivatives (17)-(24), (28) and (29), synthesised during the course of this work were tested for their effects on the calcium sensitivity of cardiac muscle. They were also tested for their effects on maximum calcium-activated force. The procedures used were discussed in Section 2.5.

The *N*-propionyl derivatives (20)-(22) and (24) all depressed the force immediately (within seconds) upon application to the Ca-activated preparation. Thereafter, no further tension responses could be obtained. No reason has been found to explain these observations.

N-acetyl-L-histidine (23), *N*-acetyl-L-alanyl-L-histidine (28) and *N*-acetyl-D-alanyl-L-histidine (29) had very little effect on either the calcium sensitivity or the peak force measurements. At low levels of activation (<30% of maximum Ca-activated force), these compounds promoted force consistently, but only by a small amount. At higher levels of activation no effect was observed. These compounds did not increase the maximum Ca-activated force.

The results for the remaining three compounds are listed in Table 3. The results obtained for carnosine and homocarnosine are included for comparison. The results collected at pCa 5.52 are those which indicate the effectiveness of the derivative as a Ca-sensitiser. The results obtained at pCa 4.0 indicate whether or not the derivatives increase the maximum Ca-activated force (these are the peak force measurements). From the table it can be seen that both *N*-acetylglycyl-L-histidine (17) and *N*-acetyl-L-carnosine

(18) have a fairly potent Ca-sensitising effect. *N*-Acetyl-L-homocarnosine (19) does have an effect on Ca-sensitivity but to a much lesser degree. *N*-Acetylglycyl-L-histidine (17) and *N*-acetyl-L-homocarnosine have a comparable effect on increasing the maximum Ca-activated force while *N*-acetyl-L-carnosine (18) causes only a small increase in the maximum Ca-activated force.

Table 3 Enhancement of tension production by L-histidine derivatives.

mean ±s.e.m. (n preparations)

	pCa 5.52	pCa 5.22	pCa 4.0	
Steady-state tension (normalised, % of C_{max})	24.8 ±7.8 (8)	64.7 ±6.4 (8)	100.0 (def)	Control
Mean tension increase (% of control force)				***compound(10mM)***
	79.6 ±8.6 (6)	28.0 ±4.2 (6)	14.6 ±0.8 (6)	Homocarnosine
	14.0 ±2.6(4)	2.9 ±0.4(4)	1.4 ±1.6 (5)	Carnosine
	36.5 ±11.7(4)	12.8 ±2.5(5)	1.4 ±0.68(5)	N-acetylcarnosine
	8.0 ±3.5(3)	0.5 ±3.0(3)	5.3 ±1.5(3)	N-acetylhomocarnosine
	27.2 ±4.5(4)	6.6 ±0.6(4)	5.7 ±1.7(5)	N-acetylglycylhistidine

Conclusions

The results obtained from the testing of these compounds have confirmed the presence of L-histidine derivatives in cardiac muscle as well as in blood being carried to all parts of the body. The actions of the L-histidine derivatives (17)-(24), (28) and (29), synthesised during this work on cardiac muscle were investigated. Preliminary results indicate two possible modes of action for these derivatives: they can behave as Ca-sensitisers to cardiac muscle or they can increase the maximum Ca-activated force. Some of the compounds such as *N*-acetylglycyl-L-histidine (17) are capable of both actions. Further work testing different mixtures of these compounds is planned in order to obtain more detailed information.

CHAPTER 5

N-Methylated Derivatives

A number of constituents of cardiac muscle are thought to be *N*-methylated L-histidine derivatives. It has already been established that anserine (1-methyl-β-alanyl-L-histidine) (3) and balenine (3-methyl-β-alanyl-L-histidine) (4) occur in muscle. Of the derivatives required for testing purposes, only 1- and 3-methyl-L-histidine are commercially available. These compounds are, however, expensive to buy. It was necessary, then, to devise general synthetic routes to the range of *N*-methyl-L-histidine derivatives required.

The imidazole ring of L-histidine possesses two nitrogens which can be alkylated. The first methods of synthesising histidine derivatives alkylated on the imidazole ring were designed to produce a mixture of isomers which were then separated, for example, by ion exchange chromatography. This was not a desirable situation since even if the separation was effective, the yields obtained of each isomer were frequently very low (14%).[150]

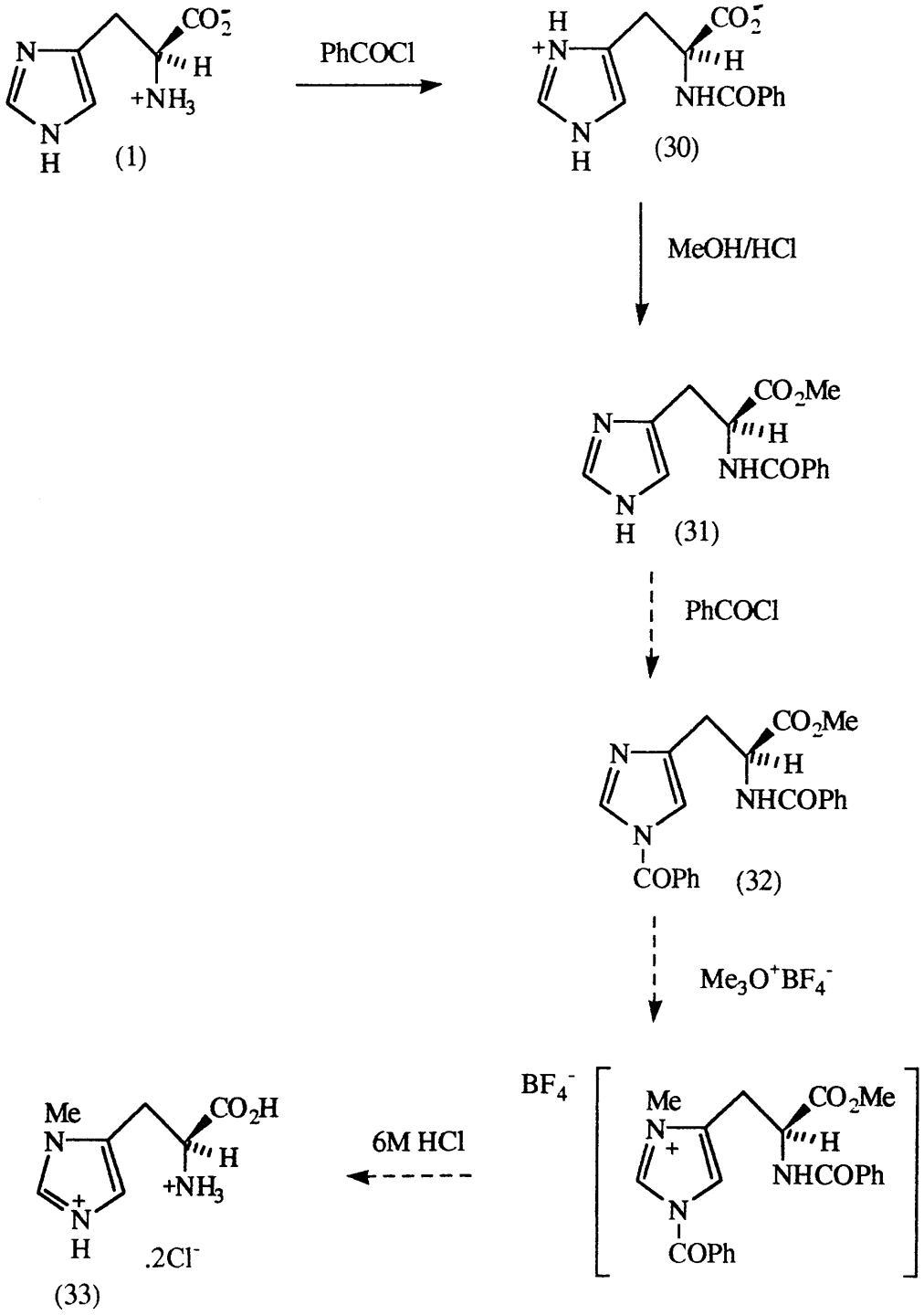
To obtain compounds in any quantity and to avoid the need for difficult purification steps, a synthetic sequence leading to one isomer was required. Since there are two possible sites of alkylation on the imidazole ring, in order to methylate one of the nitrogens selectively, the other one needs to be protected. Several protecting groups offer themselves as candidates and some literature is available on the subject (Section 3.3.1).

5.1 1-Methyl-L-Histidine (33) Dihydrochloride

Beyerman and his associates published a synthesis of 1-methyl-L-histidine (33) dihydrochloride in 1972.[74] L-Histidine (1) was reacted with benzoyl chloride to yield *N*(α)-benzoyl-L-histidine (30) (Scheme 20). This was subsequently converted into the methyl ester (31) in order to improve the solubility of the series of compounds in organic solvents. A second benzoyl group was introduced selectively on the 3-position to give (32). The benzoyl group was chosen since it is sufficiently acid resistant and can be easily introduced and removed. Beyermann *et al.*[74] methylated the 1-position with trimethyloxonium tetrafluoroborate. In the final step they removed the protecting groups by hydrolysing the ester and the amide bonds with 6M hydrochloric acid to afford 1-methyl-L-histidine (33) dihydrochloride. The yields for all of the steps were high and the product, on converting into the free base, at the end of the reaction had optical activity which was comparable to commercially available 1-methyl-L-histidine (33).

Due to the high yields and the high optical purity of the product obtained from this reaction sequence, there seemed to be good reason to use this method to obtain 1-methyl-L-histidine (33) dihydrochloride and subsequently other 1-methylated histidine derivatives. The first step proceeded without any difficulties to yield the crystalline *N*(α)-benzoyl-L-histidine (30). The product was identified on examination of the spectra obtained. The proton NMR spectrum of (30) showed the signals typical of histidine derivatives, that is, the two singlets in the aromatic region corresponding to the imidazole protons, and the

ABX system for -$CHCH_2$-. In addition to these signals a multiplet, which integrated as five protons, was present in the aromatic region of the spectrum. This multiplet was identified as the signals corresponding to the benzoyl group.



Scheme 20

The ^{13}C NMR spectrum of (30) showed a signal associated with the quaternary carbon of the amide carbonyl as well as the signals due to the quaternary and tertiary carbons of the phenyl ring. The IR spectrum of (30) showed a resonance for the amide at 1630 cm^{-1} in addition to a carboxylate anion stretch at 1580 cm^{-1}. An accurate mass measurement was made. *N*(α)-Benzoyl-L-histidine (30) required a mass of 259.0957, while the value obtained was 259.0956.

The next stage involved the synthesis of the methyl ester (31) of *N*(α)-benzoyl-L-histidine. The procedure used was published in 1919 by Gerngross.[151] HCl gas was introduced into a suspension of *N*(α)-benzoyl-L-histidine (30) in methanol. At the end of the reaction, the product (31) was isolated as a crystalline solid with m.p. 158-160 °C and $[\alpha]_D^{20}$ -27.5° (*c*, 0.8 in 6M HCl), this compared to the literature value,[151] m.p. 159-160 °C. Gerngross[151] did not quote an optical rotation for (31). The proton NMR spectrum was referenced on the peak due to the water present at δ 4.67. It was necessary to add DCl to the D_2O solvent in order to dissolve the product. The chemical shifts of the signals are, therefore, at a higher field than they would normally be. The signal due to the $-CH_3$ of the ester (31) was seen as a singlet at δ 1.87, and this singlet integrated as three protons. The ^{13}C NMR spectrum of (31) showed a signal at δ 52.2 corresponding to the $-CH_3$ of the ester. In the IR spectrum of (31), the ester carbonyl was seen at 1750 cm^{-1}. An accurate mass measurement gave a mass of 273.1106, while *N*(α)-benzoyl-L-histidine methyl ester (31) required 273.1113.

The procedure of Beyerman *et al.*[74] was followed to introduce a second benzoyl group onto the 3-position of the

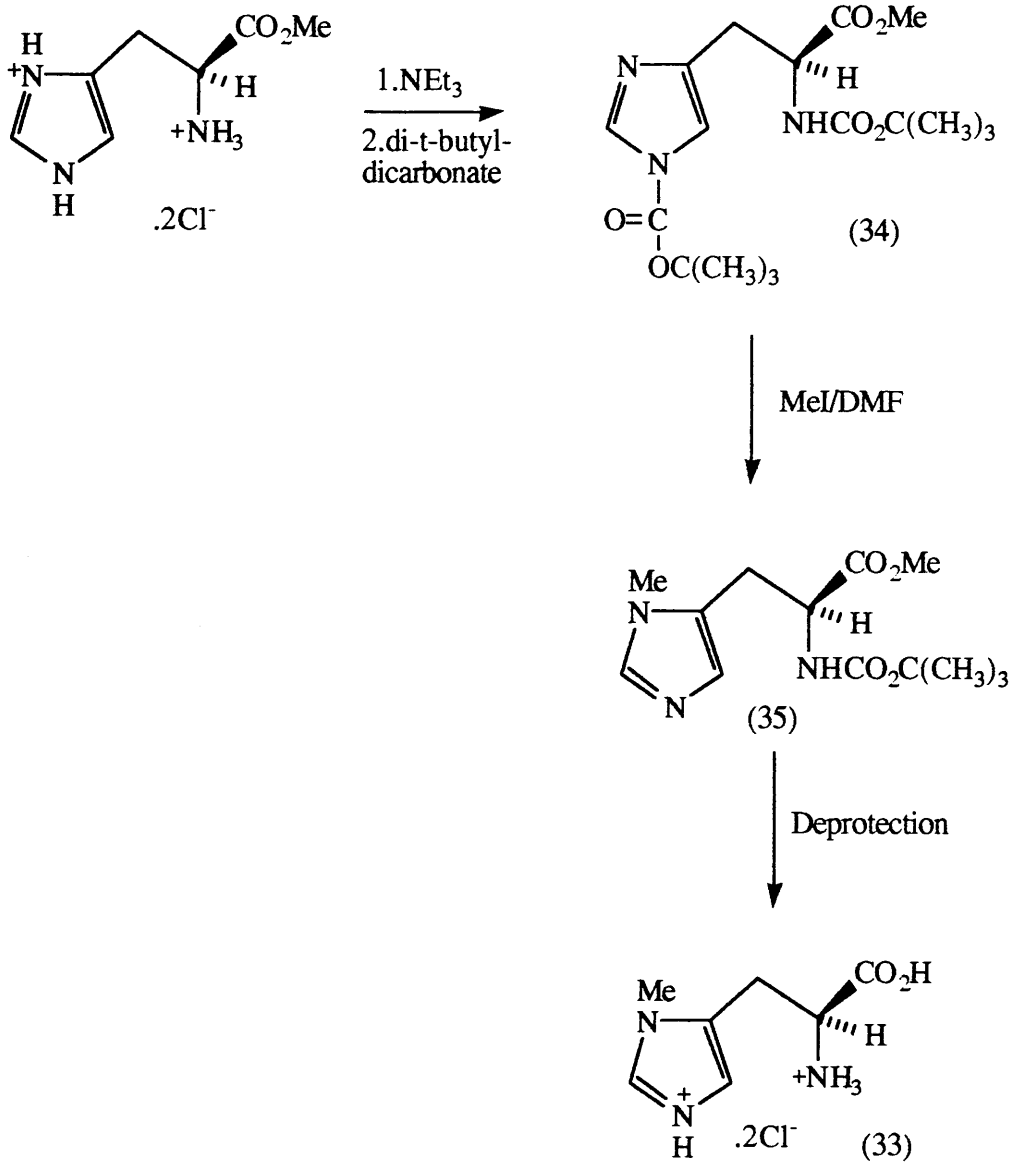
imidazole ring (Scheme 20). This was attempted several times. When the residue obtained at the end of the reaction was examined by proton NMR spectroscopy and TLC, it was found that there was a little product but mainly starting material and dicyclohexylamine hydrochloride present. Separation of this mixture could not be achieved by chromatography. The reason for the failure of this reaction to give good yields of the dibenzoyl product (32) could be due to the imidazole ring being a good acylating agent. Any *N*-acyl imidazole compound is likely then to be unstable. Due to the problems encountered in this step, the remaining steps of this route (Scheme 20) could not be attempted.

On looking for an alternative route, the first consideration was the choice of protecting groups and their ease of preparation. Two reports of a synthesis of *N*(α),3-bis(*t*-butoxycarbonyl)-L-histidine methyl ester (34) have appeared in the literature.[49,67] The procedure (Scheme 21) involves one step starting from commercially available L-histidine methyl ester dihydrochloride. This compound was reacted with di-*t*-butyl dicarbonate in the presence of two equivalents of triethylamine. The two reported procedures differ in the duration of the reaction and in the work up. Both of these were followed in the course of this work. It was found that they were comparable for conserving optical purity of the product but the procedure reported by Brown *et al.*[49] was found to be the superior method in terms of yield and chemical purity. At the end of the reaction Brown *et al.* washed the residue in chloroform with a solution of citric acid to remove the by-products. It was discovered in the course of this work that not all of the triethylammonium chloride was removed in this washing procedure. The residual material was then hampering the

purification and crystallisation of the *N*(α),3-bis(*t*-butoxycarbonyl)-L-histidine methyl ester (34). A better purification was obtained when the residue was first dissolved in acetone, filtered and concentrated. The oil obtained was then dissolved in chloroform and the procedure of Brown *et al.*[49] was followed.

N(α)-3-bis(*t*-Butoxycarbonyl)-L-histidine methyl ester (34) was isolated in 87% yield with m.p. 88-90 °C and $[\alpha]_D^{20}$ +25.9° (*c*, 0.9 in CCl_4). This compared with the literature values[67] for the m.p. 85-88 °C and $[\alpha]_D^{25}$ +19.9° (*c*, 1.16 in $CHCl_3$) and the literature values[49] for the m.p. 96 °C and $[\alpha]_D^{20}$ +25.6° (*c*, 1.0 in CCl_4). The two *t*-BOC-groups were recognised on examination of the proton NMR spectrum by the presence of two singlets, each integrating as 9 protons, at δ 1.21 and δ 1.40. The other signals expected of a histidine methyl ester derivative were also present. The signals due to the CH_3 carbons of the *t*-butyl groups were seen at δ 28.0 and δ 28.5 in the ^{13}C NMR spectrum of (34), while the signals due to the quaternary carbons of the *t*-butyl group were present at δ 79.7 and δ 85.7. The urethane carbonyls were present at δ 147.0 and δ 155.7. In both the proton and ^{13}C NMR spectra of (34), the peaks in the lower field positions should be due to the *t*-butoxycarbonyl group on the imidazole ring. The IR spectrum showed a signal at 1750 cm^{-1} due to the ester and a signal due to the urethanes at 1710 cm^{-1}. An accurate mass measurement was made. A value of 369.1903 was obtained while *N*(α)-3-bis(*t*-butoxycarbonyl)-L-histidine methyl ester (34) required a mass of 369.1900. Brown *et al.*[49] found the product (34) was unstable while Hodges[67] reported the product to be stable for up to nine months. The product (34) synthesised in the course of this work

was also found to be stable on storage as evidenced by repeating the melting point, NMR and optical rotation measurements.



Scheme 21

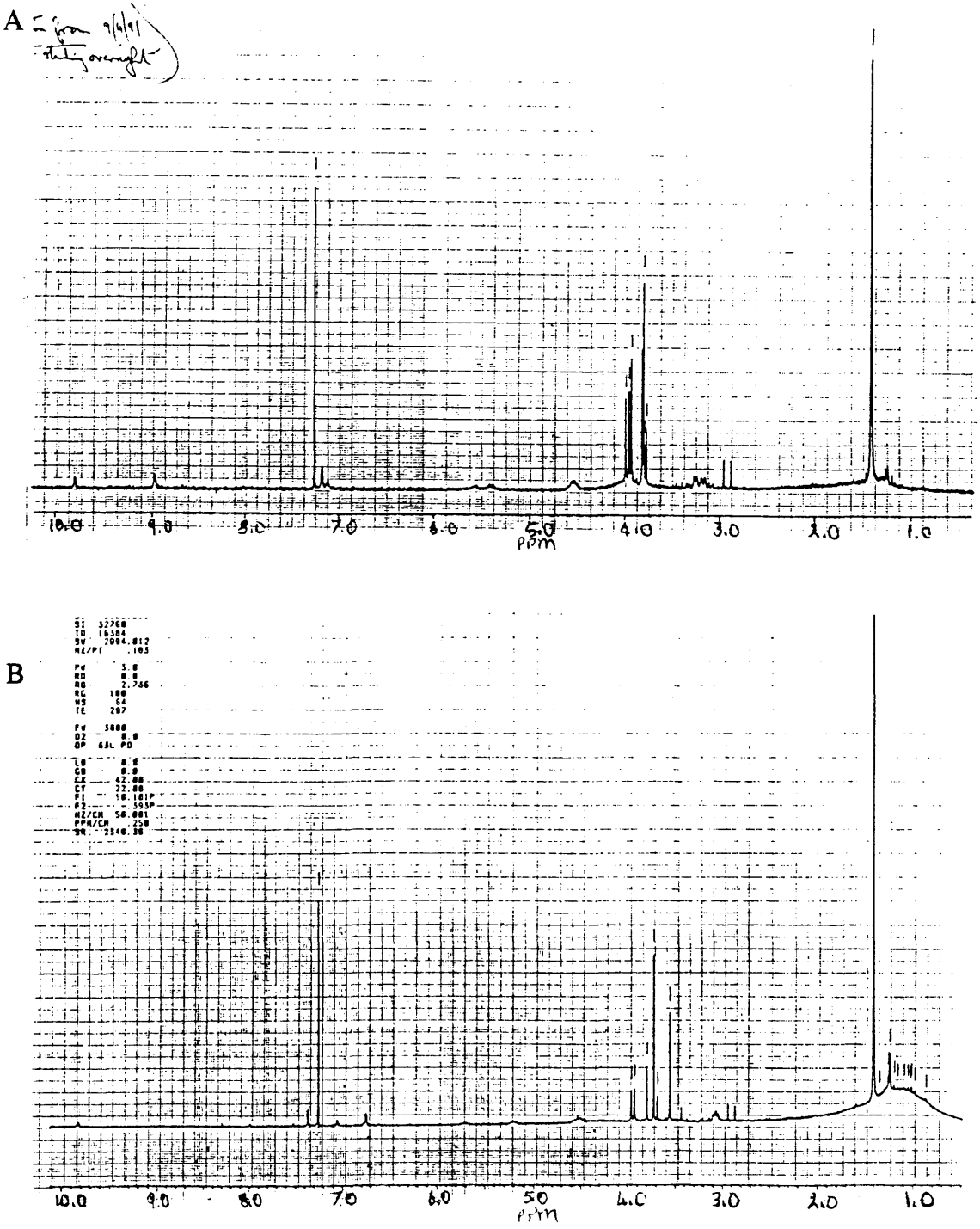
The next stage was then to find a methylating agent for the methylation of the 1-position of (34). Two reagents were used, the first was trimethyloxonium hexachloroantimonate. When this reagent was used a black tar like substance was obtained which

was insoluble in a number of organic solvents as well as water. No product could be isolated from this reaction mixture.

The second reagent tried was methyl iodide. The procedure initially involved using methyl iodide as the solvent as well as the methylating agent, but even after 24 hours very little reaction had taken place. Acetonitrile was then used as the solvent with methyl iodide in excess, but again very little product was obtained. When ethyl acetate was the solvent, the reaction between methyl iodide and the protected L-histidine derivative took place but a mixture of compounds was obtained. *N,N*-Dimethyl formamide (DMF) was found to be the best solvent for a cleaner and faster reaction, although there was still a mixture of compounds present at the end of the reaction.

On looking at the mixture of reaction products, the most obvious explanation was that the *t*-butoxycarbonyl group was removed from the 3-position of (34) before the methylation took place. This would result in a mixture of the 1- (35) and 3-methyl isomers. Proton NMR spectroscopy proved that this was not the case. The region where the *N*-Me signals occurred contained too many singlets to be only a mixture of the two isomers (Figure 26). When some base was added to the mixture in D_2O, one set of imidazole protons shifted while the other set did not. If the mixture contained the two isomers then both sets of the imidazole protons would be expected to shift. This led to the conclusion that the mixture contained the 1-methyl derivative and the 1,3-dimethyl derivative. This mixture was separated by extracting a solution of the mixture in chloroform or ethyl acetate with aqueous ammonia.

Figure 26 A. ^1H NMR spectrum of the mixture of monomethylated (35) and dimethylated salt products obtained on methylation of *N*(α),3-bis(*t*-butoxycarbonyl)-L-histidine methyl ester (34). B. Proton NMR spectrum obtained upon addition of base to the mixture.(35) and the 1,3-dimethylated salt.



The desired, crystalline *N*(α)-*t*-butoxycarbonyl-1-methyl-L-histidine methyl ester (35) was obtained on addition of ethyl acetate to the oil after evaporation of the dried organic portion. The yields of the product (35) obtained were low, around 40%. It would appear that the *t*-butoxycarbonyl group lowers the nucleophilicity of the ring to such an extent that the monomethylated compound is more nucleophilic towards the methyl iodide than is the protected compound. This was probably also the mixture obtained when ethyl acetate was the solvent. The result is that more of the dimethylated salt product was obtained since the reaction was left for longer in ethyl acetate.

N(α)-*t*-Butoxycarbonyl-1-methyl-L-histidine methyl ester (35) was obtained as a crystalline solid with m.p. 108-110 ^{o}C and $[\alpha]_D^{20}$ +27.9^{o} (*c*, 0.47 in $CHCl_3$). The ^{1}H NMR spectrum showed one peak at δ 1.42 which integrated as nine protons and two singlets at δ 3.61 and δ 3.75 which each integrated as three protons. Nuclear Overhauser Enhancement (NOE) experiments were carried out to determine two things; to distinguish between the *O*-Me and the *N*-Me signals of (35) and to determine which ring nitrogen had been methylated. Irradiation of the proton at C-2 on the imidazole ring of (35) gave a NOE of 4.9% on the singlet at δ 3.61 while irradiation of the singlet at δ 3.61 produced a NOE of 7.5% on the signal due to the proton at C-2. Irradiations of the proton at C-4 and the singlet at δ 3.75 gave no Nuclear Overhauser Enhancements. It can be concluded from these experiments that the 1-position of the ring is methylated and the *N*-Me signal resonates at a higher field than the *O*-Me signal. The ^{13}C NMR spectrum of (35) showed a signal due to the *N*-CH_3 carbon at δ34.0. In the IR spectrum of (35), the ester was seen at 1750

cm^{-1}, the urethane showed as a signal at 1715 cm^{-1} and a signal due to the *N*-Me could be seen at 2960 cm^{-1}. The mass spectrum of (35) contained peaks due to the parent ion as well as a peak with *m/z* 95 common to L-histidine derivatives methylated on the imidazole ring. An accurate mass measurement of 283.1530 was obtained while *N*(α)-*t*-butoxycarbonyl-1-methyl-L-histidine methyl ester (35) required a mass of 283.1532.

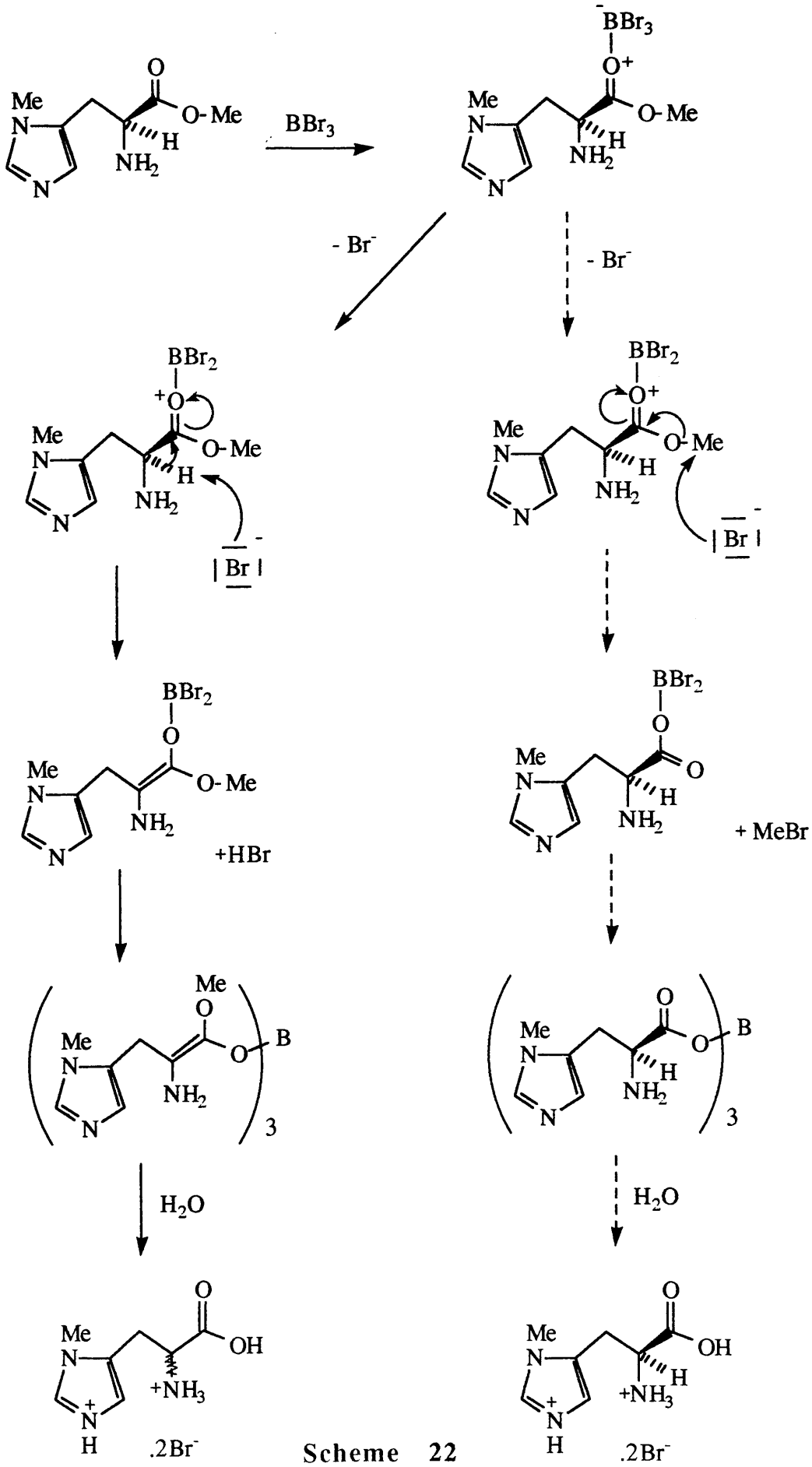
Beyerman and his associates[74] had heated their protected derivative (Scheme 20) in 6M hydrochloric acid for nine hours with no repercussions on the optical activity of the compound. It was therefore assumed that this method could also be safely used to hydrolyse the carbamate and the ester of (35). The reaction was monitored by TLC and the removal of the protecting groups was found to be complete in three hours. The 1-methylhistidine (33) dihydrochloride obtained, however, was found to be completely racemic by optical rotation measurements. The conditions for acid hydrolysis were varied. The mildest conditions used were to stir the compound in 6M hydrochloric acid at room temperature for twenty four hours. Each time the product had an optical rotation value of zero.

Felix reported the use of boron tribromide to effect the cleavage of methyl esters as well as carbamates within two hours.[61] This method had been successful for a number of protected amino acids including *N*(α)-carbobenzoxy-L-histidine. It was reported that this method had no detectable, detrimental effects on the optical purity of the products. The procedure reported by Felix was tried for the deprotection of *N*(α)-*t*-butoxycarbonyl-1-methyl-L-histidine methyl ester (35). After two hours the product of the reaction after work-up was

examined by proton NMR spectroscopy. The carbamate was indeed cleaved, but the methyl ester was intact. This procedure was tried again and the course of the reaction was monitored by TLC. The methyl ester was entirely removed only after four days. The product at the end of this time did not exhibit any optical activity. This implied that, since the methyl ester was so resilient to cleavage, it was easier for the bromide ion to abstract the α-proton than to attack the methyl group (Scheme 22).

The important feature for the conservation of optical purity in the final deprotection stage (Scheme 21) seems to be the order in which the protecting groups are removed. Beyerman and his associates[74] used an amide to protect the α-amino function (Scheme 20). In their case the methyl ester is likely to be hydrolysed first and then the amide. In both the procedures used in this work (Scheme 21) to deprotect the *N*(α)-*t*-butoxycarbonyl-1-methyl-L-histidine methyl ester (35), the carbamate is removed first. The next choice would then be to remove the methyl ester selectively in the presence of the carbamate. It was thought feasible to try base hydrolysis of the ester since carbamates are supposed to be stable under basic conditions. In practice the carbamate was also removed on treatment with 1M sodium hydroxide for ten minutes and the product was once again racemic. The carbamate was probably hydrolysed upon addition of acid in the work up.

No other methods of deprotecting compound (35) were attempted hence 1-methylhistidine (33) dihydrochloride was isolated as a racemic product on hydrolysis of (35) with 6M hydrochloric acid.



Scheme 22

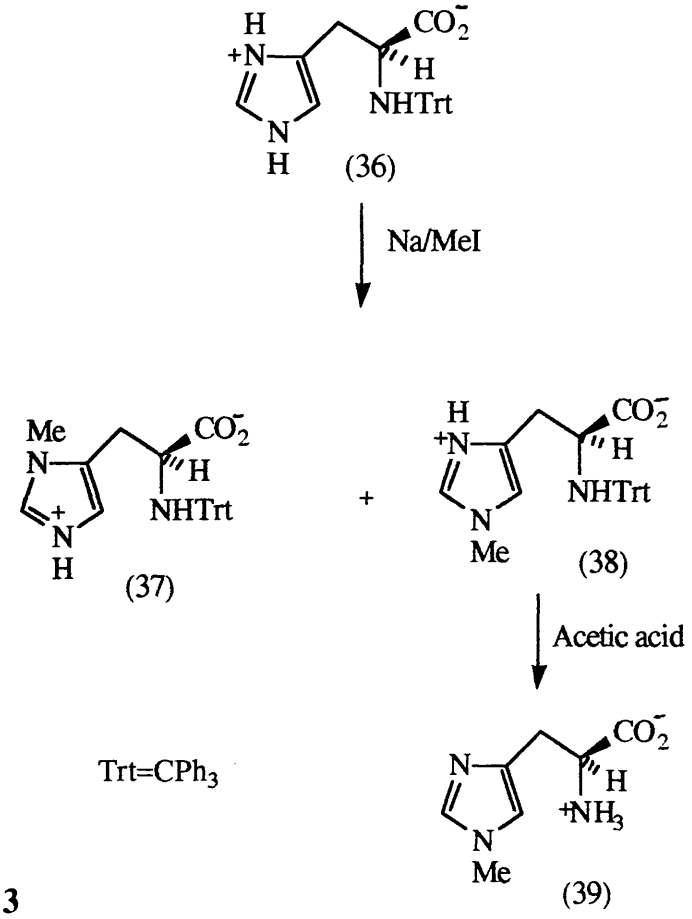
The racemic product obtained was a hygroscopic solid. The *N*-Me signal could be seen in the proton NMR spectrum for (33) as a singlet at δ 3.86 which integrated as three protons. It resonated at δ 34.7 in the ^{13}C NMR spectrum. In the IR spectrum of (33), the NH stretch could be seen at 3420 cm^{-1} and a group of signals typical of a carboxylic acid were seen in the region 2700-3040 cm^{-1}. The *N*-Me was seen at 2680 cm^{-1} and a signal due to the carboxylate anion could be seen at 1595 cm^{-1}. The mass spectrum showed the parent ion as well as a peak due to the ion with *m/z* 95, typical of an *N*-methylated histidine derivative. An accurate mass measurement of 169.0845 was obtained while 1-methylhistidine (33) required a mass of 169.0851.

It should be possible for the racemic 1-methylhistidine to be acylated with acetic anhydride and the acylated product to be resolved by the action of Acylase I. This should lead to 1-methyl-L-histidine and *N*-acetyl-1-methyl-D-histidine. Unfortunately, time did not allow for this resolution to be carried out.

5.2 3-Methyl-L-Histidine (39) Dihydrochloride

There have been two synthetic routes to 3-methyl-L-histidine (39) reported in the literature. Barlos *et al.* reported a two stage synthesis starting from *N*(α)-trityl-L-histidine (36) (Scheme 23).[152] These workers reacted this compound with methyl iodide in the presence of sodium to yield a mixture of 1-(37) and 3-methyl-*N*(α)-trityl-L-histidine (38). 3-Methyl-*N*(α)-trityl-L-histidine (38) crystallised preferentially from the reaction mixture. The trityl group was removed upon treatment with acid.

There were various problems with this route. Barlos *et al.*[152] used commercially available *N*(α)-trityl-L-histidine (36), but this was not available in the course of this work.



Scheme 23

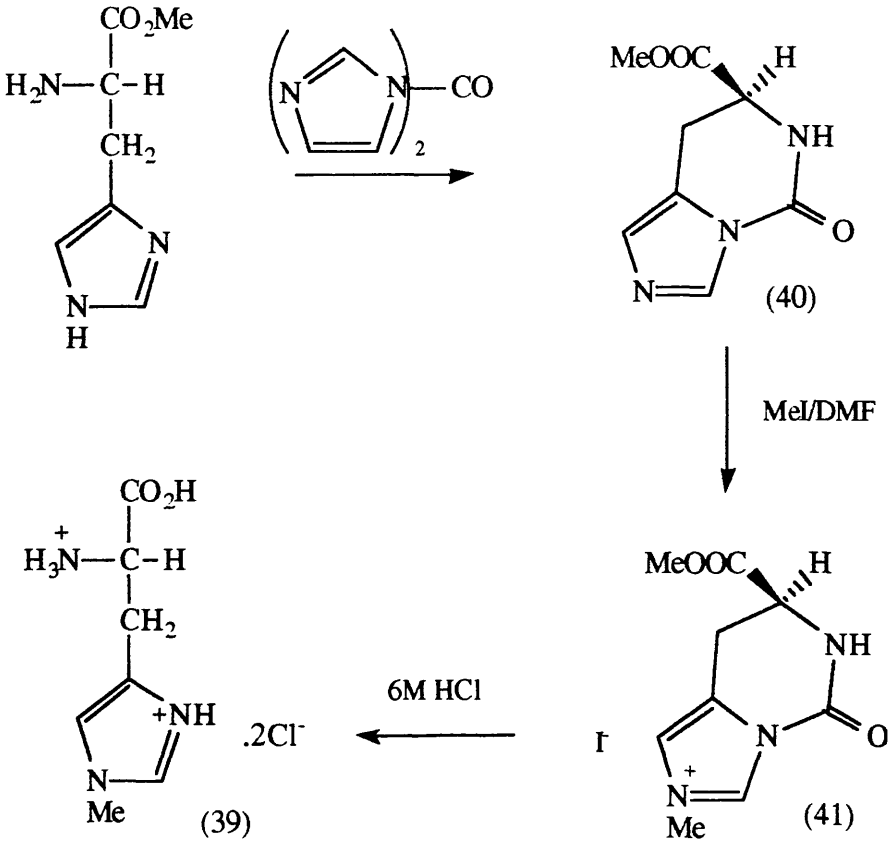
A search of the literature revealed that this compound is not very accessible. Barlos *et al.* have published a route to tritylated L-histidine derivatives.[53] They managed to synthesise a mixture of *N*(α),3-ditrityl-L-histidine and 3-trityl-L-histidine but not *N*(α)-trityl-L-histidine (36). The reason for this is probably because the *N*(α)-trityl derivative is less stable than the 3-trityl derivative.[54] Since the starting material was not available either commercially or by synthesis, this was not a viable route. It would not have been attractive even if the starting material was available since

the presence of sodium in the reaction mixture was likely to induce racemisation. Also a mixture was obtained which would have meant lower yields of the desired product and potential difficulties with purification.

The synthesis of 3-methyl-L-histidine (39) dihydrochloride was reported by Noordam *et al.*[153] They synthesised the methyl ester of L-histidine and went on to react it with *N,N'*-carbonyldiimidazole to form the cyclic derivative (7*S*)-5,6,7,8-tetrahydro-7-(methoxycarbonyl)-5-oxoimidazo[1,5-*c*]pyrimidine (40) (Scheme 24). This reaction formed a ring between the *N*(α)-atom and the 1-position; thus the 3-position was rendered the only site available for alkylation. Noordam *et al.* used methyl iodide to methylate the 3-position and hydrolysed the methylated salt (41) with acid to afford 3-methyl-L-histidine (39) dihydrochloride. On converting the salt they obtained into its free base, the 3-methyl-L-histidine (39) obtained had comparable optical activity to commercially available 3-methyl-L-histidine (39). This reaction sequence appeared to be straightforward, hence it was employed as the chosen route to 3-methyl-L-histidine (39) dihydrochloride in the course of this work.

Commercially available L-histidine methyl ester was used as the starting material in this work. This was reacted with *N,N'*-carbonyldiimidazole as described by Noordam *et al.*[153] The product (40) had m.p. 155-157 °C and $[\alpha]_D^{20}$ +58.4° (*c*, 0.9 in methanol) compared to the literature values[153] of m.p. 156-157 °C and $[\alpha]_D^{25}$ +59° (*c*, 1.2 in methanol). In the proton NMR spectrum the ABX system could be clearly seen as well as a singlet at δ 3.80, integrating as three protons, due to the *O*-Me. Noordam *et al.*[153] assigned the carbon of the O<u>C</u>H$_3$ as the higher field signal as

compared to the signal due to $\underline{C}HCH_2$. The ^{13}C NMR spectrum of (40) obtained in the course of this work showed the $\underline{C}HCH_2$ at δ 53.4 while the $O\underline{C}H_3$ resonated at δ 54.1. The ester was seen in the IR spectrum at 1755 cm^{-1} and the carbonyl of the urea was seen at 1715 cm^{-1}. An accurate mass measurement of 195.0620 was obtained while the product (40) required a value of 195.0643.



Scheme 24

The next step was to methylate (+)-(7S)-5,6,7,8-tetrahydro-7-(methoxycarbonyl)-5-oxoimidazo[1,5-c]pyrimidine (40). This was achieved on following the reported procedure. The product (41) was obtained in crystalline form with m.p. 160-162 °C and $[\alpha]_D^{20}$ +43.4° (*c*, 1.0 in water), compared to the literature values[153]

of m.p. 162 °C (dec) and $[\alpha]_D^{25}$ +46.0° (*c*, 0.7 in water). The *N*-CH_3 was seen in the proton NMR spectrum as a singlet at δ 3.87, integrating as three protons. It resonated in the ^{13}C NMR spectrum at δ 40.0. The IR spectrum showed a stretch due to the ester at 1760 cm^{-1} and the carbonyl of the urea was seen at 1700 cm^{-1}. The mass spectrum contained peaks due to the parent ion and the ion with *m/z* 95, typical of *N*-methylated histidine derivatives. A value of 210.0881 was obtained from an accurate mass measurement, while (41) required a mass of 210.0879.

Noordam *et al.*[153] went on to treat the *N*-methylated derivative (41) with 6M hydrochloric acid to obtain 3-methyl-L-histidine (39) dihydrochloride. The compound was isolated as a hygroscopic solid in 84% yield. Contrary to the results obtained by Noordam *et al.*,[153] the product (39) was racemic. The *N*-Me signal was seen in the proton NMR spectrum at δ 3.59 and in the ^{13}C NMR spectrum at δ 36.2. NOE experiments were carried out to confirm that the 3-methyl isomer had been synthesised. When the proton at C-2 of (39) was irradiated, an NOE of 1.7% was observed in the signal due to the protons of the *N*-Me group. When the proton at C-4 was irradiated, an NOE of 2.3% was seen in this signal. Irradiation of the *N*-Me protons of (39) produced an NOE of 1.5% in the signal due to the proton at C-2, and an NOE of 1.8% in the signal due to the proton at C-4. These results confirmed that 3-methylhistidine had been synthesised. The IR spectrum of (39) showed peaks due to *N*-CH_3 stretch at 2940 cm^{-1}, the carboxylic acid stretches in the region 2500-2800 cm^{-1}, and the carboxylate anion at around 1630 cm^{-1}. The mass spectrum of (39) showed the parent ion peak and the peak with *m/z* 95, typical of *N*-methylated histidine derivatives. An accurate mass

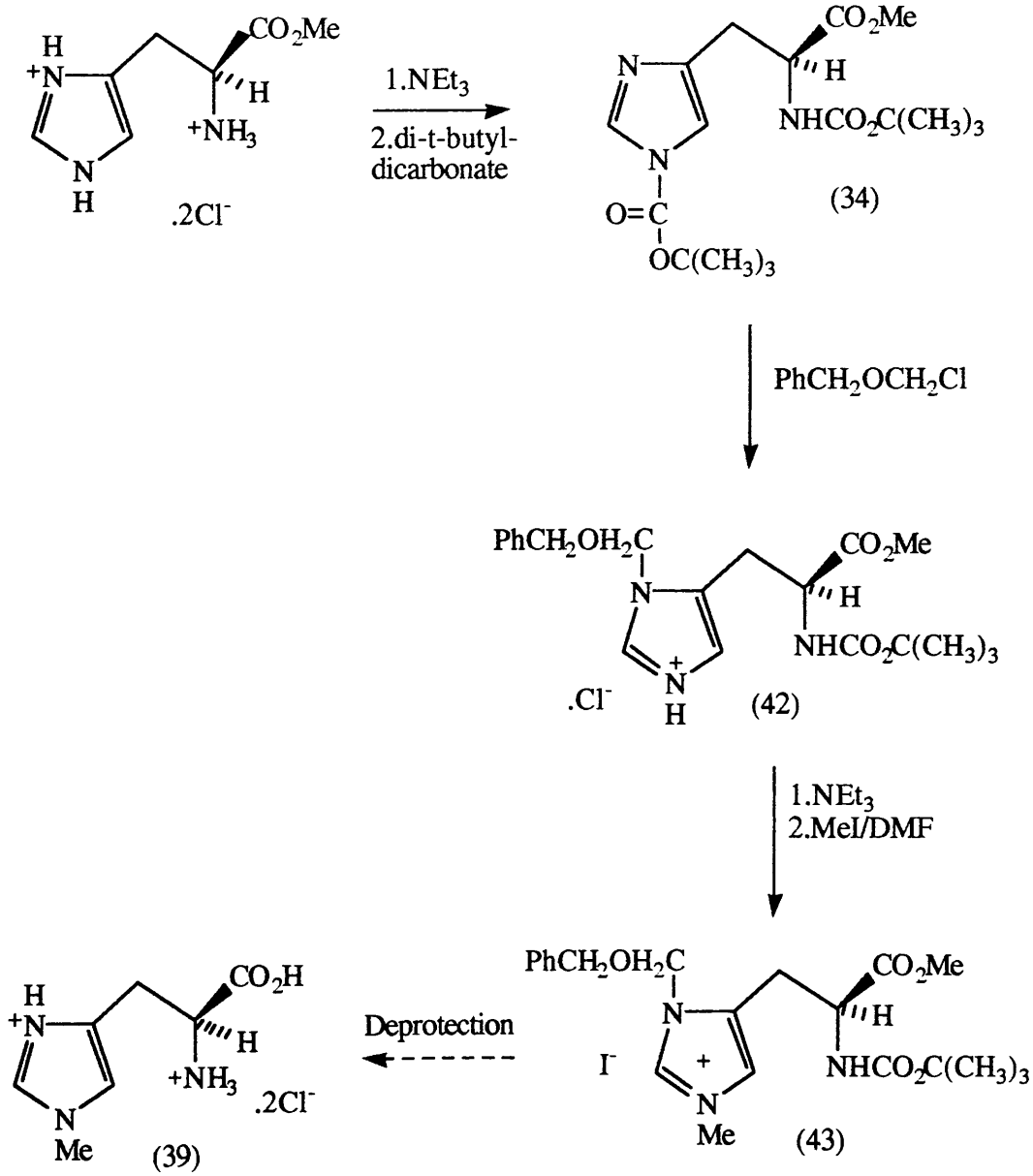
measurement of (39) gave a value of 169.0848, while $C_7H_{11}N_3O_2$ required a mass of 169.0851.

The route to 3-methylhistidine (39) dihydrochloride described is not generally applicable in this work since the α-nitrogen atom of the L-histidine residue requires to be underivatised for the reaction with *N,N'*-carbonyldiimidazole. The route also failed to produce an optically active product. For these reasons, a second route to 3-methyl-L-histidine was investigated with a view to extending it to the synthesis of the dipeptide derivatives.

Brown *et al.*[49] reacted *N*(α),3-bis(*t*-butoxycarbonyl)-L-histidine methyl ester (34) with benzylchloromethyl ether to protect the 1-position of the imidazole ring of L-histidine. This reaction was used in the course of this work to allow methylation to take place at the 3-position on the ring (Scheme 25).

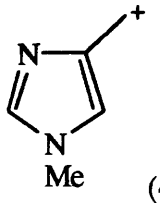
N(α),3-bis(*t*-Butoxycarbonyl)-L-histidine methyl ester (34) was obtained as described in Section 5.1. This compound was reacted with benzylchloromethyl ether as described by Brown *et al.*[49] 1-Benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-L-histidine methyl ester hydrochloride (42) was obtained in crystalline form with m.p. 140-142 °C and $[\alpha]_D^{20}$ -16.9° (*c*, 1.0 in methanol) compared to the literature values[49] of m.p. 152 °C and $[\alpha]_D^{25}$ -19.1° (*c*, 1.0 in methanol). The benzoxymethyl group was seen in the proton NMR spectrum as an AB system at δ 4.69, integrating as two protons, a singlet at δ 5.82, integrating as two protons and a singlet at δ 7.29, integrating as five protons. The lower field imidazole proton was shifted downfield to δ 9.78. The signal due to $O\underline{C}H_2N$ was seen in the ^{13}C NMR spectrum at δ 72.8 and that due to $Ph\underline{C}H_2O$ was seen at δ 77.1. The signals due to the benzyl group were present in the

aromatic region of the spectrum. In the IR spectrum of (42), stretching due to the C-O of the ether could be seen at 1100 cm^{-1} as well as the urethane at 1740 cm^{-1} and the ester at 1745 cm^{-1}. An accurate mass measurement of (42) gave the mass as 389.1934, while 1-benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-L-histidine methyl ester required 389.1950.



Scheme 25

The next stage was to methylate the compound (42). It was first treated in DMF with one equivalent of triethylamine to liberate the free base form. Methyl iodide was then used as the methylating agent. The methyl ester (43) of 1-benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-3-methyl-L-histidinium iodide was isolated at the end of the reaction as a crystalline solid with m.p. 134-136 °C and $[\alpha]_D^{20}$ -13.7° (*c*, 0.93 in ethanol). The *N*-Me signal was seen in the proton NMR spectrum at δ 3.96. This was distinguished from the *O*-Me signal once Nuclear Overhauser Enhancement (NOE) experiments had been carried out. These experiments were also used to confirm the location of the methyl group on the 3-position (43). When the proton at C-2 of the imidazole ring in (43) was irradiated an NOE of 2.5% was observed in the signal at δ 3.96. When the proton at C-4 was irradiated an NOE of 2.7% was observed in this signal. When that signal at δ 3.96 was irradiated an NOE of 1.7% was observed in the signal due to the proton at C-2 and an enhancement of 6.1% was observed in the signal due to the proton at C-4. Since irradiation of both the imidazole protons induced an NOE in the signal due to the methyl protons then the methyl group must be in the 3-position of (43). The *N*-Me signal could be seen in the ^{13}C NMR spectrum at δ 37.5. The IR spectrum of (43) showed a stretch due to NH at 3390 cm^{-1}, the ester at 1740 cm^{-1}, the urethane at 1710 cm^{-1} and the ether at 1100 cm^{-1}. The mass spectrum of (43) showed peaks due to the loss of the benzyloxymethyl group and the loss of the t-butyloxycarbonyl group. There was also a peak due to the methyl imidazolyl ion (44).



(44)

The final stage was to remove the protecting groups to yield 3-methyl-L-histidine (39). The first step in the deprotection sequence was to remove the methyl ester from (43). The fastest method of doing this was base hydrolysis. There was a fear that this would cause some racemisation of the L-histidine residue but there are several cases reported in the literature where sodium hydroxide has been used to hydrolyse methyl ester derivatives of L-histidine. In each case the product was reported to be optically active. 1-Benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-3-methyl-L-histidinium iodide was isolated as an oil. When an optical rotation measurement was made, the acid was racemic. The ester (43) was then treated with 6M hydrochloric acid for three hours in an attempt to generate the acid with some optical activity. Unfortunately the product from this reaction was also racemic. It was evident, in each case, from the proton and ^{13}C NMR spectra that the methyl ester had been completely hydrolysed.

The next stage was then to remove the benzoxymethyl group by reduction with hydrogen in the presence of a catalyst. The procedure published by Brown *et al.*[49] was followed to effect the removal of this protecting group. Even after forty eight hours at atmospheric pressure no reaction was observed and the protected derivative was recovered each time. Catalytic transfer hydrogenolysis was also attempted with ammonium formate as the hydrogen source. Again no reaction was observed. A third

method of removing the benzoxymethyl group by reduction with hydrogen in the presence of a catalyst was attempted, this time under pressure. The reaction was monitored by removing aliquots and examining them using proton NMR spectroscopy. It appeared that the imidazole ring was being reduced as well as the benzoxymethyl group. Thus the integration of the proton NMR signals showed the disappearance of both the imidazole protons and the benzoxymethyl protons compared to the other signals. The only explanation for the failure of the benzoxymethyl group to be removed cleanly is the positive charge on the imidazole ring which will probably render the ring more susceptible to reduction.

The only method remaining for the removal of the benzoxymethyl group was by acidolysis. This method was used by Colombo *et al.*[46] but no data are available for the product they obtained. Firstly the methyl ester (43) was hydrolysed as before by the action of sodium hydroxide. The acid derivative extracted from the reaction mixture was subsequently treated with 6M hydrochloric acid. After 24 hours of heating, the mixture was examined by NMR spectroscopy. The proton NMR spectrum was very complex. A singlet could be seen at δ 2.55, two singlets at δ 3.55 and δ 3.67 and what appeared to be three sets of imidazole protons. The product was extracted with triethylamine in chloroform to convert everything into the free base form and the proton NMR spectrum was run again. It was clearer now that the mixture was composed essentially of two compounds, most likely the 1- and 3-methyl isomers of histidine. The ^{13}C NMR spectrum showed two *N*-Me signals as well as two sets of signals for the histidine nucleus. This added weight to the idea that a mixture of the two isomers had formed. TLC also indicated that the mixture

contained the two isomers. No explanation for this occurrence has been found.

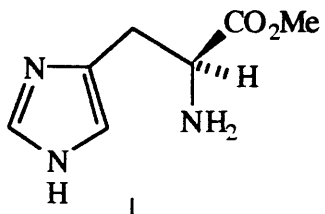
5.3 Dipeptides Containing 1-Methylhistidine

A general route to the synthesis of 1-methylglycyl-L-histidine (50), 1-methyl-L-carnosine (anserine) (3), and 1-methyl-L-homocarnosine (51) was required. The general idea was to protect the amino function of the second amino acid, couple this to L-histidine methyl ester, protect selectively the 3-position of the imidazole ring, methylate the 1-position and finally deprotect to obtain the product as shown in Scheme 26.

The first stage was to find a suitable protecting group for the amino function of the second amino acid. The carbobenzoxy (CBZ)-group was chosen, since it should be stable under the conditions employed during the peptide coupling step, and it could also be selectively removed in the presence of other protecting groups.

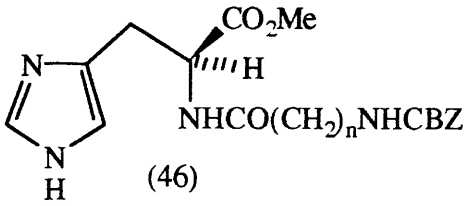
Thus CBZ-glycine, CBZ-β-alanine and CBZ-4-aminobutyric acid were synthesised under Schotten-Baumann conditions.[48] The yields of the products (45) were always in excess of 70% and the three derivatives were obtained as crystalline solids. The compounds showed a number of common features in the different spectra obtained.

Scheme 26



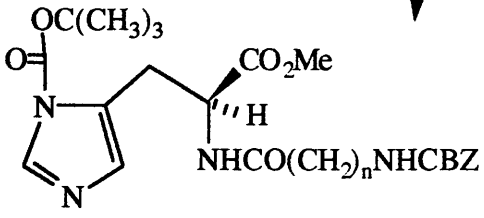
CBZ-NH(CH$_2$)$_n$CO$_2$H

(45)

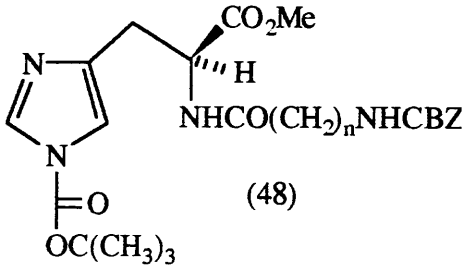


(46)

di-t-butyl dicarbonate/
DABCO

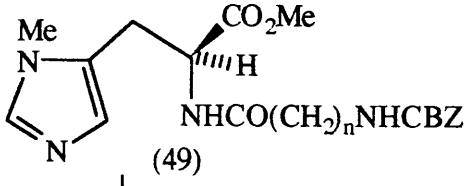


(47)



(48)

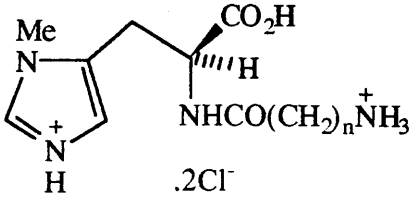
MeI/DMF



(49)

deprotection

	n
(50)	1
(3)	2
(51)	3



.2Cl$^-$

The proton NMR spectra for each compound (45) showed a singlet which integrated as five protons, around δ 7.5 and a singlet, integrating as two protons, at around δ 5.2. These signals were due to the CBZ-group. The CH_2 of the CBZ-group was seen at around δ 68.0 in each of the ^{13}C NMR spectra. The signals due to the phenyl ring of the CBZ-group resonated in the aromatic region as expected. The quaternary carbon of the urethane was seen at around δ 159.0 for each of the derivatives. The IR spectra for compounds (45) showed a stretch due to NH at around 3340 cm^{-1} in each case, as well as signals due to a carboxylic acid in the region 2900-3100 cm^{-1}. The urethane was seen at around 1700 cm^{-1} and the carboxylate at 1550 cm^{-1} in the spectrum of each of the CBZ derivatives (45).

The mass spectra of (45) showed a common peak with *m/z* 91 which corresponded to the benzyl ion and the parent ion was seen in all three cases. An accurate mass measurement was made for each compound. A mass of 209.0682 was measured for CBZ-glycine (45, n=1), while this compound required a mass of 209.0688. CBZ-β-alanine (45, n=2) required a value of 223.0845, this was also the mass measured. CBZ-4-aminobutyric acid (45, n=3) required a mass of 237.1001, the value obtained was 237.0998.

The next stage was to couple these CBZ-protected amino acids (45) to L-histidine methyl ester. L-Histidine methyl ester was liberated from the commercially available dihydrochloride by the addition of exactly two equivalents of triethylamine to a suspension of the salt in methanol. The mixed anhydride method of peptide coupling was employed since it had been successful in the past. Again isobutyl chloroformate was used to generate the

mixed anhydride with the protected second amino acid in the activation stage. The coupling reaction was then allowed to proceed. The product (46) in each case was isolated as a solid in yields in excess of 70%. The spectra obtained for each dipeptide derivative (46) showed a number of common features.

In the proton NMR spectrum for each CBZ-dipeptide methyl ester (46), the signals common to the histidine residue were clearly seen. That is, the two imidazole protons in the aromatic region and the ABX system due to $-CHCH_2-$. These were in addition to the signals due to the CBZ-group discussed previously and a singlet at around δ 3.87, integrating as three protons, due to the methyl ester. The ^{13}C NMR spectra showed signals due to the CBZ-group, the histidine residue, a quartet at around δ 53.1 due to the methyl ester, and two singlets at around δ 174.0 due to the ester and the amide carbonyls.

The IR spectra of compounds (46) had a number of common features. A signal due to NH stretching was seen at around 3320 cm^{-1}, while the ester was observed at around 1750 cm^{-1}. The signal due to the carbonyl of the urethane occurred at around 1700 cm^{-1} and that of the amide could be seen at around 1650 cm^{-1}. The mass spectra of (46) all contained the relevant parent ion peak as well as a peak due to the ion *m/z* 81, common to histidine derivatives and a peak due to the benzyl group.

N-Carbobenzoxyglycyl-L-histidine methyl ester (46, n=1) was isolated as a solid in a yield of 67%. The product had m.p. 89-91 °C and $[\alpha]_D^{20}$ -2.3° (*c*, 1.0 in 83% aqueous ethanol). The glycyl residue was seen as a singlet at δ 3.99, integrating as two protons, in the proton NMR spectrum and a signal at δ 45.0 in the ^{13}C NMR spectrum. An accurate mass measurement of 360.1422 was

obtained while the calculated value for $C_{17}H_{20}N_4O_5$ was 360.1434. No reference to this derivative could be found in the literature.

N-Carbobenzoxy-L-carnosine methyl ester (46, n=2) was obtained in 72% yield when CBZ-β-alanine was coupled to L-histidine methyl ester. The product had m.p. 92-94 °C and $[\alpha]_D^{20}$ -4.4° (*c*, 1.0 in 83% aqueous ethanol). The β-alanyl residue was identified in the proton NMR spectrum as two triplets at δ 2.60 and δ 3.54, each integrating as two protons. The two signals due to this residue were seen at δ 37.1 and δ 38.6 in the ^{13}C NMR spectrum. An accurate mass of 374.1589 was measured while $C_{18}H_{22}N_4O_5$ required a value of 374.1590. Again no reference to this compound could be found in the literature.

N-CBZ-4-aminobutyric acid (45, n=3) was coupled to L-histidine methyl ester to give *N*-carbobenzoxy-L-homocarnosine methyl ester (46, n=3) in a yield of 78%. The product had m.p. 103-105 °C and $[\alpha]_D^{20}$ -7.0° (*c*, 1.0 in 83% aqueous ethanol). The proton NMR spectrum showed a multiplet at δ 1.92 and triplets at δ 2.41 and δ 3.32, each integrating as two protons. These signals were due to the 4-aminobutyryl residue. The ^{13}C NMR spectrum showed three signals due to this residue at δ 30.2, δ 34.2 and δ 41.4. An accurate mass measurement was obtained. A value of 388.1745 was measured while $C_{19}H_{24}N_4O_5$ required *M*, 388.1747. No reference to this compound was found in the literature.

The next stage was to protect the 3-position of the imidazole ring in compounds (46) in order to allow methylation of the 1-position (Scheme 26). A number of protecting groups were considered. The trityl group was the first choice for protecting the 3-position since it would be stable during the methylation reaction and could be removed under mild acidic conditions. The

most important reason, though, was that only the 3-trityl isomer would be formed since steric hindrance would prevent the 1-trityl isomer from forming. Trityl chloride was reacted with *N*-carbobenzoxy-L-homocarnosine methyl ester in the presence of triethylamine. The identity of the isolated product was confirmed using proton NMR spectroscopy. *N*-Carbobenzoxy-3-trityl-L-homocarnosine was then treated with methyl iodide. The reaction was allowed to proceed for up to forty eight hours but the maximum yield of product obtained was 17%. This was judged by integration of the signals in the proton NMR spectra. The reaction was probably failing to produce good yields due to the steric congestion around the imidazole ring caused by the trityl group. This was most likely preventing the approach of the electrophile and hence hindering the reaction from taking place.

The acetyl group was the next choice for protecting the 3-position of the imidazole ring in (46). It is known that *N*-acyl imidazole derivatives are unstable but the use of the acetyl protecting group for the imidazole ring has been reported.[154] This group was considered since the derivatives would be easy to prepare and again the group was thought to locate specifically at the 3-position of the ring. *N*-Carbobenzoxy-L-homocarnosine methyl ester (46, n=3) was reacted with acetic anhydride using the procedure of Jones *et al.*[154] Attempts to purify the product from acetic acid at the end of the reaction resulted in the partial decomposition of the 3-acetyl product. It was decided then not to isolate the 3-acetyl derivative but to react it immediately with methyl iodide. The reaction proceeded well and a good yield of methylated product was isolated (67%). Unfortunately the desired *N*-carbobenzoxy-1-methyl-L-homocarnosine methyl ester was

contaminated with about 10% of the 3-isomer as determined by integration of the imidazole signals in the proton NMR spectrum. Several attempts were made to purify the desired product by chromatography but these failed. It was then decided to abandon this route since the aim had been to synthesise a single isomer without the need for difficult purification procedures.

The search for a successful protecting group for the 3-position continued. The *t*-butoxycarbonyl (*t*-BOC) group was chosen as the next candidate. It has been reported that the reaction of di-*t*-butyl dicarbonate with the imidazole ring of an L-histidine derivative produced both the 1- and 3-*t*-BOC derivatives.[154] Because these isomers had been easily separated using chromatography, it was decided to apply these methods to this work.

The first procedure used to introduce the *t*-BOC-group onto the imidazole ring was that used by Jones and his co-workers.[154] It involved mixing di-*t*-butyl dicarbonate with the L-histidine derivatives (46) in dichloromethane and leaving the solution overnight. On examination of the oil obtained on removal of the solvent, proton NMR spectroscopy showed that little reaction had taken place. The reaction mixture was left for a longer time but still very little reaction occurred. The situation did not change even when the amount of di-*t*-butyl dicarbonate was increased or the solvent was changed to methanol. When diazobicyclo[2.2.2]octane (DABCO) was added to the reaction mixture, the reaction proceeded and all of the starting material disappeared. DABCO is a non nucleophilic base and probably enhances the nucleophilicity of the imidazole ring by abstracting a proton. The imidazole ring would then be more reactive towards

the reagent. The two isomers (47) and (48) were formed as expected. The mixtures were separated using flash column chromatography in the first instance. Better separation was later achieved when gravity column chromatography was used. Thus both the 1- (47) and 3-*t*-butoxycarbonyl-*N*-carbobenzoxy-dipeptide methyl esters (48) were isolated isocratically with ethyl acetate as the eluant. Ethyl acetate was also the solvent used to develop TLC plates. The compounds (47) were used in the synthesis of the dipeptides containing 3-methylhistidine and will be discussed in Section 5.4.

The derivatives (48) had a number of common features in their spectra. The *t*-butyl group was seen in the proton NMR spectra as a singlet at around δ 1.60, integrating as nine protons. The *t*-butyl group appeared as a quartet at δ 28.5 and a singlet at around δ 87.0 in the ^{13}C NMR spectrum. The urethane carbonyl was seen at around δ 147.0. The IR spectra of (48) showed the ester stretch at around 1755 cm^{-1}, the urethane stretches at around 1730 cm^{-1} and the amide stretch between 1650 and 1700 cm^{-1}. The mass spectra of (48) showed, in each case, the parent ion, a peak due to the loss of the *t*-BOC-group and the peak with *m/z* 81, typical of histidine derivatives.

3-*t*-Butoxycarbonyl-*N*(α)-carbobenzoxyglycyl-L-histidine methyl ester (48, n=1), isolated as an oil in 44% yield, had R_F 0.2. The product was optically active with $[\alpha]_D^{20}$ +12° (*c*, 0.9 in $CHCl_3$). An accurate mass measurement gave a value of 460.1936. The value calculated for $C_{22}H_{28}N_4O_7$ was 460.1958.

3-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-carnosine methyl ester (48, n=2) (R_F 0.23) was obtained in 53% yield with $[\alpha]_D^{20}$ +17.1° (*c*, 0.96 in $CHCl_3$). A value of 474.2101 was obtained when

the accurate mass was measured. The value calculated for $C_{23}H_{30}N_4O_7$ was 474.21114.

3-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-homocarnosine methyl ester (48, n=3) was isolated in 63% yield. The product had R_F 0.25 and $[\alpha]_D^{20}$ +14.6° (*c*, 1.1 in $CHCl_3$). An accurate mass measurement of 488.2282 was obtained while the value calculated for $C_{24}H_{32}N_4O_7$ was 488.2271.

The next stage in the sequence was to methylate the 1-position of compounds (48). Methyl iodide was employed as the methylating agent since it had been used successfully in other applications. The procedure used for the methylation of *N*(α),3-bis(*t*-BOC)-L-histidine methyl ester (34) discussed in Section 5.1 was followed. Again mixtures of the monomethylated and the dimethylated salt products were obtained. The desired *N*-CBZ-1-methyldipeptide methyl esters (49) were isolated by extracting a solution of the product in chloroform with aqueous ammonia to remove the dimethylated salt.

The 1-methyl derivatives (49) shared a number of common features in their spectra. Each of the proton NMR spectra showed a peak due to the methyl ester in the region δ 3.40-3.60, integrating as three protons. The AB part of the ABX system due to -$CHCH_2$- was observed as a broad doublet in each of the proton NMR spectra. These spectra also showed a broad singlet due to NH in the region of δ 5.0-6.0. The *N*-CH_3 was seen as a quartet in the ^{13}C NMR spectra at around δ 32.0-33.0. In the IR spectra a stretch due to *N*-CH_3 was observed in the region 2950-2985 cm^{-1}. The mass spectra contained the parent ions as well as peaks due to the loss of the amino side chains. The mass spectra also showed peaks due to the benzyl ion and the peak with *m/z* 95, common to *N*-

methylated histidine derivatives. No reference to any of these compounds could be found in the literature.

N(α)-Carbobenzoxyglycyl-1-methyl-L-histidine methyl ester (49, n=1) was isolated as an oil in 65% yield. The product had $[\alpha]_D^{20}$ -3.8° (*c*, 1.1 in ethanol). The proton NMR spectrum showed the signal due to the $-CH_2-$ of the glycyl residue as an AB system, this was probably due to steric interactions. An accurate mass measurement was made. A value of 374.1592 was obtained while the calculated mass for $C_{18}H_{22}N_4O_5$ was 374.1590.

N-Carbobenzoxy-1-methyl-L-carnosine methyl ester (49, n=2) was obtained in 58% yield. The oil was optically active, with $[\alpha]_D^{20}$ -4.0° (*c*, 0.7 in ethanol). A value of 388.1752 was obtained from an accurate mass measurement. The calculated value for $C_{19}H_{24}N_4O_5$ was 388.1747.

N-Carbobenzoxy-1-methyl-L-homocarnosine methyl ester (49, n=3) was obtained in 56% yield as an oil. The product had $[\alpha]_D^{20}$ -5.8° (*c*, 0.8 in ethanol). An accurate mass measurement of 402.1879 was obtained while the calculated value for $C_{20}H_{26}N_4O_5$ was 402.1903.

The final stage in this reaction sequence was to deprotect the terminal amino function and the acid function in compounds (49). A number of methods were investigated in an effort to obtain optically active products. Each of the methods used was applied to the deprotection of *N*-carbobenzoxy-1-methyl-L-carnosine methyl ester (49, n=2) as a model compound in order that they could be compared.

The first three methods involved the removal of the methyl ester and the CBZ-group using a one step procedure. The compound (49, n=2) was treated with 6M hydrochloric acid.

Hydrolysis of the ester and the CBZ-group was complete in around six hours when the solution was heated at 90 °C. On examination of the 1-methylcarnosine (3) dihydrochloride obtained at the end of the reaction, the compound was found to be racemic by optical rotation measurements. The protected compound was then treated with 6M hydrochloric acid at room temperature. The complete removal of the ester and the urethane took 24 hours at this temperature and the product (3) was still not optically active.

The second method was to treat the protected compound (49, n=2) with boron tribromide.[61] The same observations made in Section 5.1 were noted, that is, the removal of the CBZ-group was complete within two hours but the methyl ester was only completely removed after a number of days. The product (3) obtained from this reaction was also racemic by optical rotation measurements (*cf.* Scheme 22).

The third method was to dissolve the protected compound (49, n=2) in 6M hydrochloric acid, treat the solution with 10% palladium on carbon and subject the mixture to hydrogenolysis. The removal of the CBZ-group was effected before the hydrolysis of the methyl ester was complete. The hydrogenolysis was stopped in order to prevent reduction of the imidazole ring and the remaining methyl ester was removed on heating the acidic solution. Once again the 1-methylcarnosine (3) dihydrochloride was racemic at the end of the reaction.

The next deprotection sequences attempted, involved the selective removal of the ester of (49, n=2) followed by the removal of the CBZ-group. To remove the ester, a methanolic solution of *N*-carbobenzoxy-1-methyl-L-carnosine methyl ester was treated with 1M sodium hydroxide. The reaction was

complete in ten minutes. When an optical rotation measurement of the *N*-carbobenzoxy-1-methylcarnosine obtained was made, the value was 0°. This method was then not suitable for removal of the methyl ester since it induced racemisation. The second method for the selective removal of the methyl ester was to hydrolyse the ester (49, n=2) with 6M hydrochloric acid for two hours. The isolated product was shown to have the CBZ-group intact on examination of the NMR spectra. When an optical rotation was measured, the value was -3.5° (*c*, 1.7 in methanol). The product then possessed some optical activity.

The next stage was to find a suitable method to remove the CBZ-group. Both of the methods tried involved the removal of this group by hydrogenolysis. Catalytic transfer hydrogenolysis was the first procedure used. Ammonium formate was the hydrogen donor and cleavage was achieved in one hour. The product isolated from the reaction mixture was not, however, optically active. A methanolic solution of *N*-carbobenzoxy-1-methyl-L-carnosine was then treated with 10% palladium on carbon and hydrogenated at atmospheric pressure. Once again the deprotected product (3) obtained at the end of the reaction was racemic.

None of the methods employed produced an optically active product hence the deprotection of *N*(α)-carbobenzoxyglycyl-1-methyl-L-histidine methyl ester (49, n=1) and *N*-carbobenzoxy-L-homocarnosine methyl ester (49, n=3) was achieved upon the treatment of these compounds with 6M hydrochloric acid at 90 °C. This was the most straight forward method and the highest yielding deprotection reaction. The three 1-methylhistidine dipeptide derivatives, (3), (50) and (51), showed the signals

typical of a histidine derivative in the proton NMR spectra. That is, the two imidazole protons in the aromatic region and the ABX system due to -$CHCH_2$-. The signals typical of a histidine derivative were also present in the ^{13}C NMR spectra. The mass spectra all contained the parent ion and the ion with *m/z* 95, typical of *N*-methylhistidine derivatives.

1-Methylglycylhistidine (50) dihydrochloride was isolated as a hygroscopic solid in 89% yield. The product was not optically active. The proton NMR spectrum contained the *N*-Me signal as a singlet integrating as three protons at δ 3.73. The singlet due to the -CH_2- of the glycyl residue resonated in the same region. The ^{13}C NMR spectrum of (50) showed the *N*-Me signal at δ 34.7, the -CH_2- of the glycyl residue at δ 41.2, the carbonyl of the amide at δ 169.9 and that of the acid at δ 171.3. NOE experiments were carried out to confirm that the methyl group was located in the 1-position. When the proton at C-2 of (50) was irradiated an NOE of 2.5% was observed in the singlet due to the *N*-Me protons. No NOE was observed when the proton at C-4 was irradiated. When the singlet due to *N*-Me protons was irradiated, an NOE of 5% was observed in the signal due to the proton at C-2 but no enhancement was observed in the signal due to proton at C-4. The results of these experiments led to the conclusion that the imidazole ring is indeed methylated in the 1-position. The amide was seen in the IR spectrum at 1740 cm^{-1}, while the acid was seen at 1630 cm^{-1}. An accurate mass measurement of (50) was made. A value of 226.1067 was obtained while $C_9H_{14}N_4O_3$ required a mass of 226.1066.

1-Methylcarnosine (3) dihydrochloride was isolated as a hygroscopic solid in 86% yield. When an optical rotation

measurement was made, the value was 0°. The proton NMR spectrum showed the *N*-Me signal as a singlet, integrating as three protons at δ 3.67. The signals due to the β-alanyl residue were seen as two triplets, each integrating as two protons, at δ 2.54 and δ 3.04. The ^{13}C NMR spectrum showed signals due to the carbons of the β-alanyl residue at δ 32.7 and δ 36.4. The carbon of the *N*-Me group resonated at δ 34.0, while the signal due to the carbonyl of the amide was seen at δ 172.2 and that due to the acid at δ 172.5. NOE experiments were run to determine the substitution pattern on the imidazole ring of (3). When the proton at C-2 was irradiated an NOE of 7.1% was observed in the signal due to the *N*-Me group. When the proton at C-4 of (3) was irradiated no significant enhancement was observed in the other signals. When the protons of the *N*-Me group were irradiated an NOE of 15.5% was observed in the signal due to the proton at C-2, while no significant enhancement was observed in the signal due to the proton at C-4. These results confirmed that the imidazole ring is methylated in the 1-position. The amide was seen in the IR spectrum at 1660 cm^{-1}, while the acid was observed at 1735 cm^{-1}. An accurate mass measurement of (3) gave a value of 240.1223. $C_{10}H_{16}N_4O_3$ required a measurement of 240.1222.

1-Methylhomocarnosine (51) dihydrochloride was obtained as a hygroscopic solid in 87% yield. The product obtained was racemic by optical activity measurements. The proton NMR spectrum showed a signal, integrating as three protons, due to the *N*-Me group at δ 3.68. A multiplet at δ 1.74 and two triplets at δ 2.31 and δ 2.84, all integrating as two protons, were due to the $-CH_2CH_2CH_2-$ of the 4-aminobutyryl residue. The signals due to this residue were seen in the ^{13}C spectrum at δ 23.1, δ 31.7 and δ

39.9. The carbon of the *N*-Me group resonated at δ 34.6, while a signal due to the amide carbonyl was seen at δ 171.2 and that due to the acid was observed at δ 178.0. NOE experiments were used to establish which imidazole isomer had been synthesised. When the proton at C-2 (51) was irradiated, an NOE of 4.8% was observed in the signal due to the *N*-Me protons. When the proton at C-4 was irradiated, no enhancements were observed. When the protons of the *N*-Me group were irradiated, an NOE of 4.8% was observed in the signal due to the proton at C-2, while no enhancement was observed in the signal due to the proton at C-4. The IR spectrum of (51) contained a signal due to the amide at 1610 cm^{-1} and a signal due to the acid at 1730 cm^{-1}. A value of 254.1377 was obtained from an accurate mass measurement, while $C_{11}H_{18}N_4O_3$ required a value of 254.1379.

1-Methylglycylhistidine (50), 1-methylcarnosine (3) and 1-methylhomocarnosine (51) were, therefore, obtained as their dihydrochloride salts as pure isomers. Unfortunately, none of the compounds synthesised possessed any optical activity. It remains to be proved at which step in the synthesis, racemisation of these compounds was occurring. The product of each step in the sequence would have to be examined to determine the optical purity of the intermediate. This would undoubtedly require the deprotection of at least one of the carboxyl or amino functions which in itself may induce racemisation. It seems highly likely that most of the racemisation takes place in the final deprotection steps since the conditions required to remove the methyl ester are known to be sufficient to cause racemisation of susceptible α-amino acid residues.[47,68] If time had allowed, a different protecting group would have been chosen for the carboxyl

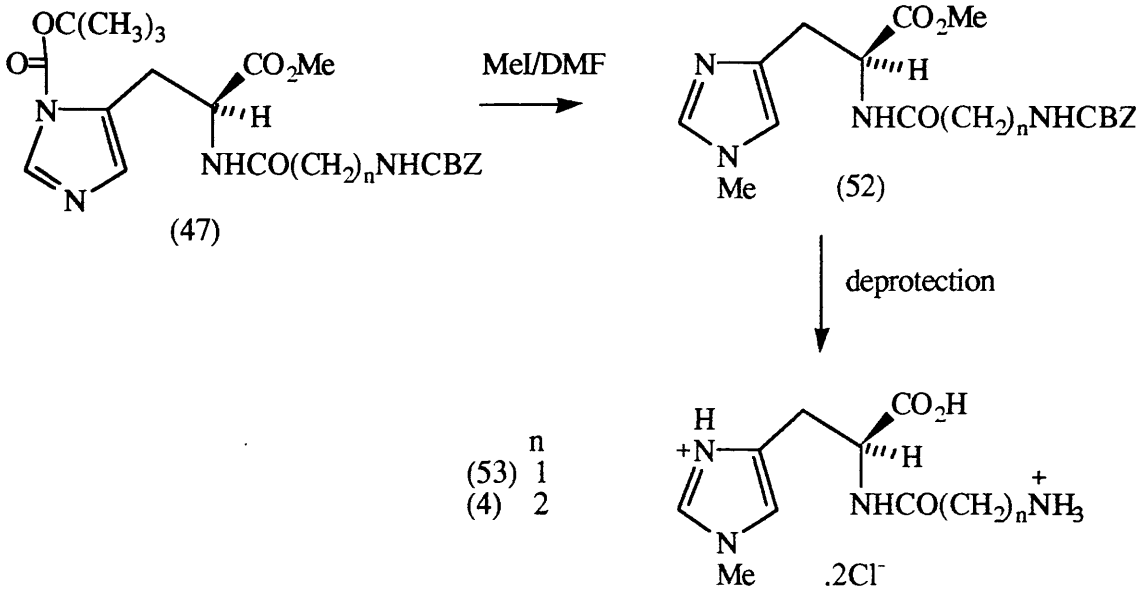
function of L-histidine. This protecting group would have been stable under the conditions of the reaction scheme but should be more labile in the final deprotection step. For example the carboxyl function could have been protected as the *t*-butyl ester which is more labile to acid than is the methyl ester.

5.4 Synthesis of Dipeptides Containing 3-Methyl-L-histidine

It has already been established that 3-methylcarnosine (balenine) (4), occurs in muscle tissue. The other two compounds of interest are 3-methylglycylhistidine (53) and 3-methylhomocarnosine. These three compounds are not available commercially and although balenine has been isolated from natural sources, no synthetic route to these compounds has been found in the literature.

The route to the dipeptides containing 1-methylhistidine was discussed in Section 5.3. The route (Scheme 26) involved protecting the 3-position of the imidazole ring of each of the derivatives with the *t*-butoxycarbonyl (*t*-BOC) group. The 1-*t*-BOC isomers (47) were also formed in this reaction and could be isolated by chromatography. Both 1-*t*-butoxycarbonyl-*N*(α)-carbobenzoxyglycyl-L-histidine methyl ester (47, n=1) and 1-*t*-butoxycarbonyl-*N*-carbobenzoxy-L-carnosine methyl ester (47, n=2) were isolated in sufficient yield to be used in the synthesis of the 3-methyl derivatives (Scheme 27). 1-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-homocarnosine methyl ester (47, n=3) was only

formed in very small amounts and could not be isolated in pure form.



Scheme 27

1-*t*-Butoxycarbonyl-*N*(α)-carbobenzoxyglycyl-L-histidine methyl ester (47, n=1) was isolated as an oil in 37% yield. The product was optically active with $[\alpha]_D^{20}$ -29.3° (*c*, 0.98 in $CHCl_3$). The product was identified on examination of the spectra obtained. The *t*-butyl peak was seen as a singlet, integrating as nine protons, at δ 1.58 in the proton NMR spectrum. The $-CH_2-$ of the glycyl residue was seen as an AB system, integrating as two protons, at δ 4.20. The signals typical of a histidine methyl ester derivative and a CBZ derivative were also in evidence. The ^{13}C NMR spectrum of (47, n=1) showed peaks due to the *t*-butyl group at δ 28.5 and δ 85.1. The carbon of the $-CH_2-$ of the glycyl residue, resonated at δ 49.3. The carbonyl of the *t*-BOC-group was observed at δ 148.9, while that of the CBZ-group could be seen at δ 152.0. The IR spectrum showed peaks due to the ester at 1820

cm^{-1}, the urethanes at 1760 cm^{-1} and the amide at 1745 cm^{-1}. The mass spectrum of (47, n=1) showed peaks due to the loss of the *t*-BOC-group and the ion with *m/z* 81, common to histidine derivatives.

1-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-carnosine methyl ester (47, n=2) was isolated as an oil in 27% yield. An optical rotation measurement gave $[\alpha]_D^{20}$ -10.7° (*c*, 1.0 in $CHCl_3$). The proton NMR spectrum of (47, n=2) showed the *t*-butyl peak as a singlet, integrating as nine protons, at δ 1.57. The β-alanyl residue was seen as two triplets at δ 2.53 and δ 3.93, each integrating as two protons. The ^{13}C NMR spectrum of (47, n=2) contained peaks due to the *t*-butyl group at δ 28.5 and δ 86.4. The carbonyl of the *t*-BOC-group resonated at δ 152.3, while that of the CBZ-group was seen at δ 154.2. The ester was observed in the IR spectrum at 1790 cm^{-1}, while the urethanes were seen at 1755 cm^{-1} and the amide at 1680 cm^{-1}. The mass spectrum of (47, n=2) contained the parent ion as well as peaks due to the loss of the urethanes and the ion with *m/z* 81, typical of histidine derivatives. An accurate mass measurement gave a value of 474.2122, while $C_{23}H_{30}N_4O_7$ required a measurement of 474.2114.

Methylation of protected compounds (47) was achieved using methyl iodide. The method was the same as that described in Sections 5.1 and 5.3 and a mixture of the 3-methyl and the 1,3-dimethyl derivatives was obtained. The 3-methyl compounds (52) were isolated on treatment with aqueous ammonia as previously described.

N(α)-Carbobenzoxyglycyl-3-methyl-L-histidine methyl ester (52, n=1) was obtained in 48% yield. The oil was optically active with $[\alpha]_D^{20}$ -18° (*c*, 1.3 in $CHCl_3$). The proton NMR spectrum

showed the signal due to the protons of the *N*-Me group as a singlet, integrating as three protons, at δ 4.65. This group was seen in the ^{13}C NMR spectrum at δ 37.5. The IR spectrum of (52, n=1) contained signals due to *N*-CH_3 at 2990 cm^{-1}, the ester and urethane at 1750 cm^{-1} and the amide at 1730 cm^{-1}. The mass spectrum showed the parent ion peak as well as a peak due to the methylated imidazole ion with *m/z* 95. An accurate mass measurement of (52, n=1) gave a value of 374.1589, while $C_{18}H_{22}N_4O_5$ required a measurement of 374.1590.

N-Carbobenzoxy-3-methyl-L-carnosine methyl ester (52, n=2) was obtained in 54% yield. An optical rotation measurement gave $[\alpha]_D^{20}$ -3.2° (*c*, 0.9 in $CHCl_3$). The proton NMR spectrum showed the two singlets due to the *N*-Me and the methyl ester protons were very close in chemical shift at δ 3.72. In the ^{13}C NMR spectrum, the peak due to the *N*-Me was seen at δ 33.5. The IR spectrum showed stretches due to *N*-CH_3 at 3000 cm^{-1}, the ester at 1790 cm^{-1}, the urethane at 1750 cm^{-1} and the amide at 1690 cm^{-1}. The mass spectrum contained peaks due to the parent ion and the ion with *m/z* 95, typical of *N*-methylated histidine derivatives. A value of 388.1738 was obtained when an accurate mass measurement of (52, n=2) was made. $C_{19}H_{24}N_4O_5$ required a value of 388.1747.

The final stage involved the removal of the protecting groups from compounds (52). The same methods as those discussed in Section 5.3 were used and again the products (4) and (53) of each method were racemic. It was decided then to use acid hydrolysis for the removal of both the methyl ester and the CBZ-groups in order to obtain the best yields of products.

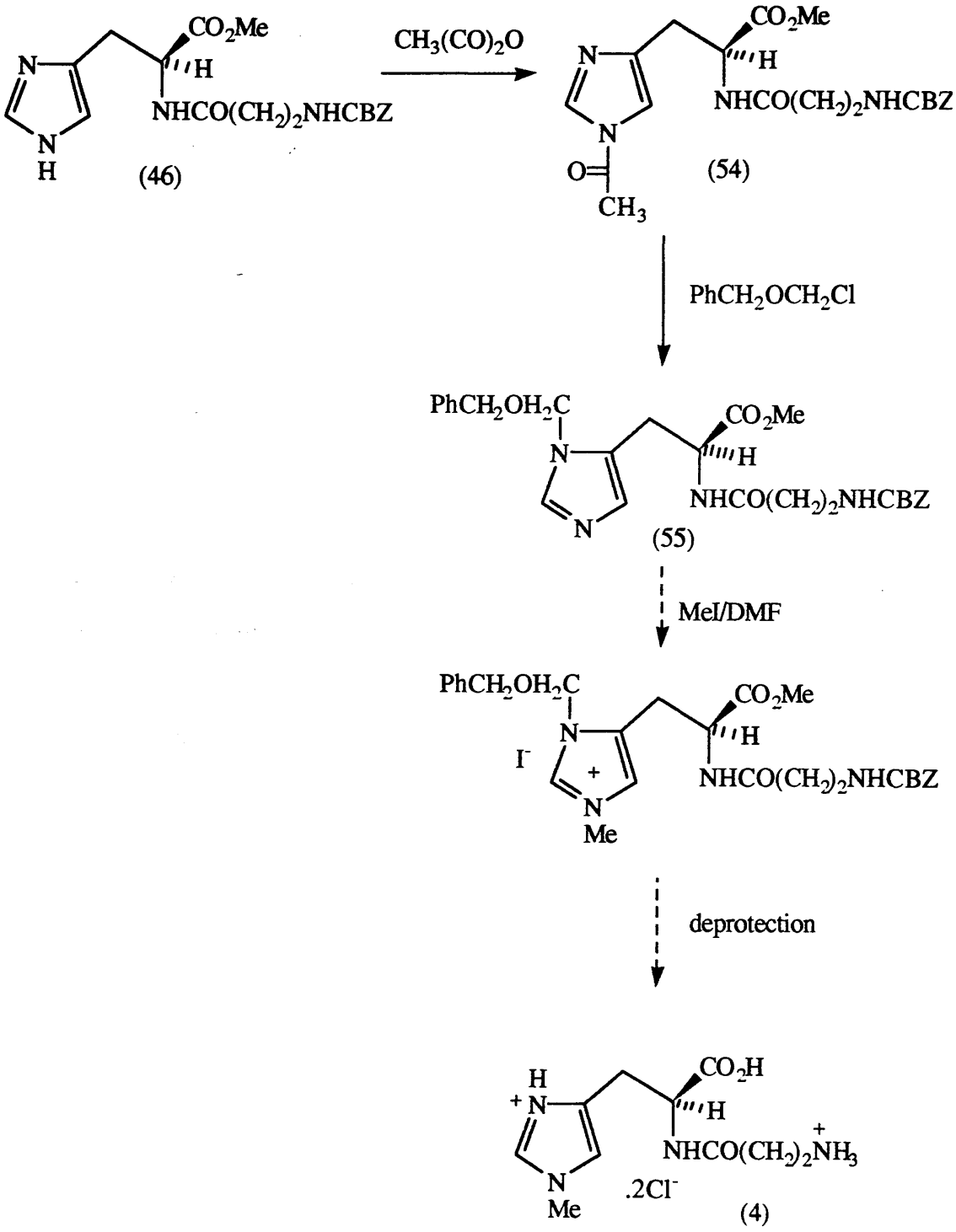
3-Methylglycylhistidine (53) dihydrochloride was obtained as a hygroscopic solid in 79% yield. The proton NMR spectrum showed the signal due to the *N*-Me protons as a singlet, integrating as three protons, at δ 3.68. This signal was seen in the ^{13}C NMR spectrum of (53) at δ 31.9. NOE experiments were used to determine which of the *N*-methyl isomers had been synthesised. When the proton at C-2 of (53) was irradiated an NOE of 2% was observed in the signal due to the *N*-Me protons. When the proton at C-4 was irradiated an NOE of 2.4% was observed in that signal. When the protons of the *N*-Me group were irradiated an NOE of 1.7% was observed in the signal due to the proton at C-2 and an NOE of 1.5% was observed in the signal due to the proton at C-4. These results led to the conclusion that 3-methylglycylhistidine (53) dihydrochloride had been synthesised. The IR spectrum contained stretches due to *N*-CH_3 at 3110 cm^{-1}, the amide at 1610 cm^{-1} and the acid at 1720 cm^{-1}. The mass spectrum of (53) showed peaks due to the parent ion and the ion with *m/z* 95. An accurate mass measurement gave a value of 226.1071 while $C_9H_{14}N_4O_3$ required a value of 226.1066.

3-Methylcarnosine (4) dihydrochloride was isolated as a hygroscopic solid in 81% yield. The proton NMR spectrum of (4) showed the peak due to the *N*-Me group as a singlet integrating as three protons, at δ 3.71. The signal due to this group was seen in the ^{13}C NMR spectrum at δ 34.6. NOE experiments were used to determine the substitution pattern of the imidazole ring. When the proton at C-2 of (4) was irradiated an NOE of 15% was observed in the signal due to the *N*-Me protons. When the proton at C-4 was irradiated, an NOE of 4% was observed in this signal. Irradiation of the protons of the *N*-Me group produced a NOE of

11% in the signals due to the protons at C-2 and C-4. These results indicated that 3-methylcarnosine (4) dihydrochloride had been synthesised. The IR spectrum showed a stretch due to the amide at 1725 cm^{-1} and the acid at 1610 cm^{-1}. The mass spectrum of (4) contained peaks due to the parent ion and the ion with *m/z* 95, typical of *N*-methylhistidine derivatives. An accurate mass measurement gave a value of 240.1211 while $C_{10}H_{16}N_4O_3$ required a value of 240.1129.

Since the 1-*t*-BOC derivatives used in the synthetic route to produce these dipeptides containing 3-methylhistidine were essentially by-products in the route to the dipeptides containing 1-methylhistidine, the amount of material obtained was very small. The route was not therefore useful in producing large quantities of the dipeptides containing 3-methylhistidine. It was also not possible to obtain 3-methylhomocarnosine by this route. For these reasons, an alternative route (Scheme 28) was investigated. *N*-Carbobenzoxy-L-carnosine methyl ester (46, n=2) was the starting material for this route. The synthesis of this compound was discussed in Section 5.3.

N-Carbobenzoxy-L-carnosine methyl ester (46, n=2) was reacted with acetic anhydride to produce 3-acetyl-*N*-carbobenzoxy-carnosine methyl ester (54). Most of the acetic acid was removed but the compound was not isolated due to it being unstable. Instead it was reacted straight away with benzylchloromethyl ether. 1-Benzoxymethyl-*N*-carbobenzoxy-L-carnosine methyl ester (55) was isolated as an oil in 86% yield. The product was optically active with $[\alpha]_D^{20}$ -6.0° (*c*, 1.6 in methanol). The proton NMR spectrum of (55) showed three singlets at δ 4.68, δ 5.01 and δ 5.69, each integrating as two



Scheme 28

protons. These were due to the CH_2 protons of the CBZ- and the benzyloxymethyl-groups. The spectrum also showed a complex signal in the aromatic region, integrating as eleven protons. The IR spectrum of (55) showed a stretch due to the ester at 1740

cm^{-1}, the urethane at 1715 cm^{-1} and the amide at 1670 cm^{-1}. The mass spectrum of (55) showed the peak with *m/z* 81, typical of histidine derivatives.

The next stage was to methylate the 3-position of (55). Methyl iodide was once again the reagent of choice. The reaction was monitored over 48 hours but little change could be seen by TLC. The reaction mixture was examined by proton NMR spectroscopy at the end of this time. It could be seen that very little methylated product had been formed This may have been due to steric hindrance around the imidazole nucleus. This was therefore not going to be a viable route for the synthesis of dipeptides containing 3-methylhistidine.

5.5 Conclusions

The work to produce individual *N*-methylated L-histidine derivatives was being run concurrently. This meant that any problems encountered were shared between groups of compounds. For example all of the compounds were protected by the methyl ester and hence problems with conserving optical activity were encountered for all of the derivatives. If time had allowed, other protecting groups for the carboxyl function would have been investigated. For example the *t*-butyl ester or silyl esters would be more labile at the end of the reaction sequence.

Clearly a new route is required for the synthesis of the dipeptides containing 3-methylhistidine and in particular 3-methylhomocarnosine. Different protecting groups would need to be investigated. A lot of work would be required to find a suitable combination for the protection of the nitrogens in the

imidazole ring. It is obvious from the results obtained in the synthesis of 3-methylhistidine that the benzoxymethyl group is wholly unsuitable for protecting the 1-position of these L-histidine derivatives prior to methylation. It seems that it is impossible to remove it cleanly to leave the desired 3-methyl derivative.

1-Methylhistidine (33), 3-methylhistidine (39), 1-methylglycylhistidine (50), 3-methylglycylhistidine (53), 1-methylcarnosine (3), 3-methylcarnosine (4) and 1-methylhomocarnosine (51) were synthesised as their dihydrochlorides to produce chemically pure isomers. Unfortunately none of the compounds were optically active. This was true even for the 3-methylhistidine (39) obtained using the procedure of Noordam *et al.*[153] These workers claimed to synthesise material which was comparable in optical activity to that of the natural compound. That was not the case with the compound produced in the course of this work.

It should be possible for the racemic *N*-methyl derivatives to be acylated with acetic anhydride and the *N*-acylated product to be resolved by the action of Acylase I. This should lead to 1-methyl-L-amino acid or dipeptide and *N*-acetyl-1-methyl-D-amino acid or dipeptide. Unfortunately, time did not allow for this resolution to be carried out.

5.6 Testing of *N*-Methylated Histidine Derivatives

The testing of the *N*-methylated histidine derivatives synthesised during the course of this work is still being undertaken. The results are not available at this time.

Chapter 6
Experimental

6.1 General

Infra red spectra were recorded on a Perkin Elmer 580 spectrophotometer. Nuclear magnetic resonance spectra were obtained with a Perkin Elmer R32 spectrometer operating at 90 MHz, or a Bruker WP200-SY or AM200-SY spectrometer operating at 200 MHz (δ_H) and 50 MHz (δ_C). Spectra were run in D_2O with *t*-butanol as the internal standard at 1.30 ppm (δ_H) and 31.6 ppm (δ_C) unless otherwise specified. The multiplicities of the ^{13}C NMR spectra were determined using DEPT spectra with pulse angles of $\theta=90^o$ and $\theta=135^o$. Equilibrium Nuclear Overhauser Enhancement measurements were performed by the difference method at 200 MHz. The samples were dissolved in either $CDCl_3$ or D_2O for these measurements. Mass spectra were recorded using an A.E.I. MS12 or 902 spectrometer.

All melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with an Optical Activity Ltd. AA-10 polarimeter in a 1 dm cell.

TLC was carried out on Merck Kieselgel 60 F_{254} plastic sheets of 2mm thickness. Ninhydrin or iodine were used as the visualising agents. TLC solvent system 1: chloroform/methanol/ammonia (80:19:1); solvent system 2: ethyl acetate; solvent system 3: chloroform/methanol/water/ammonia (25:22:5:5). Chromatographic purification was carried out by dry column chromatography using Kieselgel 60 (Merck, 70-230 mesh).

HPLC was carried out using a reverse phase Silica C18 column and a solvent system of water and methanol (55:45).

Triethylamine was purified by distillation from potassium hydroxide and stored over potassium hydroxide pellets. N,N-Dimethylformamide (DMF) was purified by distillation from calcium hydride and stored over 4A sieves. Organic solutions were dried over anhydrous magnesium sulphate.

6.2 Experimental to Chapter 4

6.2.1 *N*-Acylation of Amino Acids

General procedure 1

The amino acid (10 mmol), was dissolved in water (3 ml). The solution was stirred at room temperature while acetic or propionic anhydride (20 mmol) was added. Stirring was continued for 15 min then the mixture was concentrated under reduced pressure at temperatures below 40 ^{o}C to yield a glassy residue.

General procedure 2

The amino acid (10 mmol) was dissolved in glacial acetic or propionic acid (3 ml). Acetic or propionic anhydride (20 mmol) was added and the mixture was heated at reflux for 1 h. At the end of this time, water (1 ml) was added and the solution was concentrated under reduced pressure to yield a glassy residue.

N-Acetyl-L-histidine (23)

L-Histidine (1.55 g, 10 mmol) was acylated with acetic anhydride as described in general procedure 1. The resultant glassy residue solidified on storing in a desiccator containing potassium

hydroxide pellets and phosphorus pentoxide under vacuum. Recrystallisation from water on the addition of acetone yielded *N*-acetyl-L-histidine (23) (1.69 g, 86%); m.p. 154 °C; $[\alpha]_D^{20}$ +39.5° (*c*, 0.9 in H_2O), lit.,[118] m.p. 169 °C (dec.), $[\alpha]_D^{20}$ +44.7° (*c*, 1.0 in H_2O); υ_{max} (KBr disc) 3350, 3140, 1660, 1590 and 1550 cm^{-1}; δ_H 2.05 (3H, s), 3.09-3.38 (2H, AB part of an ABX system), 4.52-4.59 (1H, X part of an ABX system), 7.33 (1H, s) and 8.66 (1H, s); δ_C 23.8 (q, $\underline{C}H_3CO$), 29.3 (t, $\underline{C}H_2CH$), 56.1 (d, $CH_2\underline{C}H$), 118.1 (d, C-4), 131.7 (s, C-5), 135.1 (d, C-2), 175.4 (s, $\underline{C}OCH_3$) and 178.5 (s, $\underline{C}O_2H$); *m/z* 197 (M^+, 16.3%), 138 (25%), 110 (56%) and 81 (100%). Found: M^+, 197.0798. $C_8H_{11}N_3O_3$ required *M*, 197.0800.

N-*Propionyl-L-histidine (24)*

L-Histidine (1.55 g, 10 mmol) was acylated according to general procedure 1 using propionic anhydride as the acylating agent. The resultant glassy residue was stored in a vacuum desiccator containing potassium hydroxide pellets and phosphorus pentoxide. The solid obtained was recrystallised from ethanol to yield *N*-propionyl-L-histidine (24) (1.75 g, 83%); m.p. 118-120 °C; $[\alpha]_D^{20}$ +29.9° (*c*, 1.2 in H_2O); υ_{max} (KBr disc) 3400, 3110, 1640 and 1580 cm^{-1}; δ_H 1.09 (3H, t), 2.30 (2H, q), 3.06-3.38 (2H, AB part of an ABX system), 4.53-4.60 (1H, X part of an ABX system), 7.32 (1H, s) and 8.63 (1H, s); δ_C 11.4 (q, $CH_2\underline{C}H_3$), 29.5 (t, $\underline{C}H_2CH$), 31.0 (t, $\underline{C}H_2CH_3$), 55.8 (d, $CH_2\underline{C}H$), 118.7 (d, C-4), 131.8 (s, C-5), 135.0 (d, C-2), 178.5 (s, $\underline{C}OCH_2$) and 179.3 (s, $\underline{C}O_2H$); *m/z* 211 (M^+, 2.6%), 165 (14%), 137 (26%), 120 (56%) and 81 (100%). Found: M^+, 211.0962. Calcd. for $C_9H_{13}N_3O_3$; *M*, 211.0957.

N-*Acetylglycine*

Glycine (7.5 g, 0.1 mol) was dissolved in water (30 ml) according to the method described by Herbst and Shemin.[142] Acetic anhydride (20.4 g, 0.2 mol) was added dropwise with stirring. After 20 min the solution was left to stand overnight at 0 °C to allow crystallisation to take place. The product was collected by filtration and recrystallised from water to yield *N*-acetylglycine (10.3 g, 88%); m.p. 207-208 °C, lit.,[142] 88-92%, m.p. 207-208 °C; υ_{max} (KBr disc) 3350, 1720, 1580 and 1550 cm^{-1}; δ_H 2.11 (3H, s) and 4.04 (2H, s); δ_C 23.6 (q, $CO\underline{C}H_3$), 43.1 (t, $\underline{C}H_2NH$), 175.5 (s, $\underline{C}OCH_3$) and 176.7 (s, $\underline{C}O_2H$); *m/z* 117 (M^+, 10.4%) and 72 (100%). Found: M^+, 117.0424. $C_4H_7NO_3$ required *M*, 117.0426.

N-*Propionylglycine*

Glycine (3.8 g, 0.05 mol) was acylated by the method described in general procedure 1 using propionic anhydride as the reagent. The solid produced on concentration of the solution was crystallised from methanol to yield *N*-propionylglycine (5.44 g, 83%); m.p. 124-126 °C; υ_{max} (KBr disc) 3350, 1705, 1600 and 1550 cm^{-1}; δ_H 1.18 (3H, t), 2.38 (2H, q) and 4.03 (2H, s); δ_C 11.1 (q, $\underline{C}H_3CH_2$), 30.6 (t, $CH_3\underline{C}H_2$), 43.0 (t, CH_2NH), 175.5 (s, $\underline{C}OCH_2$) and 180.3 (s, $\underline{C}O_2H$); *m/z* 131 (M^+, 43.4%), 84 (54%) and 57 (100%). Found: M^+, 131.0583. Calcd. for $C_5H_9NO_3$; *M*, 131.0582.

N-*Acetyl-β-alanine*

β-Alanine (8.9 g, 0.1 mol) was acylated with acetic anhydride in acetic acid using general procedure 2. A glassy residue was obtained on concentration of the solvent. This was dried under vacuum in the presence of potassium hydroxide to produce a solid

which was crystallised from acetone to yield *N*-acetyl-β-alanine (10.3 g, 79%); m.p. 79-81 °C, lit.,[147] 80-82 °C; υ_{max} (KBr disc) 3350, 1710, 1620 and 1565 cm^{-1}; δ_H 2.03 (3H, s), 2.65 (2H, t) and 3.49 (2H, s); δ_C 23.8 (q, $CO\underline{C}H_3$), 35.4 (t, $\underline{C}H_2CO_2H$), 37.1 (t, $\underline{C}H_2NH$), 176.1 (s, $\underline{C}OCH_3$) and 178.1 (s, $\underline{C}O_2H$); *m/z* 131 (M^+, 26%) and 88 (100%). Found: M^+, 131.0589. $C_5H_9NO_3$ required *M*, 131.0582.

N-*Propionyl*-β-*alanine*

β-Alanine (4.45 g, 0.05 mol) was acylated with propionic anhydride in propionic acid as described in general procedure 2. The resultant oil was stored in a vacuum desiccator containing potassium hydroxide to remove any residual traces of acid. The product, *N*-propionyl-β-alanine, was isolated as an oil (7.25 g, 71%); υ_{max} ($CHCl_3$ solution) 3460, 3020, 1740, 1670, 1640 and 1530 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.63 ppm) 0.88 (3H, t), 2.01 (2H, q), 2.38 (2H, t) and 3.23 (2H, t); δ_C (dioxan was the external standard at 67.8 ppm) 10.7 (q, $CH_2\underline{C}H_3$), 30.2 (t, $\underline{C}H_2CH_3$), 34.6 (t, $\underline{C}H_2CO_2$), 36.1 (t, $\underline{C}H_2NH$), 177.2 (s, $NH\underline{C}O$) and 179.1 (s, $\underline{C}O_2H$); *m/z* 145 (M^+, 41%), 99 (89%) and 88 (89%). Found: M^+, 145.0738. Calcd. for $C_6H_{11}NO_3$; *M*, 145.0739.

N-*Acetyl*-L-*alanine*

L-Alanine (4.45 g, 0.05 mol) was acylated with acetic anhydride in water according to general procedure 1. The resultant oil was stored in a vacuum desiccator containing potassium hydroxide for several h. The oil was subsequently crystallised from ethyl acetate to yield *N*-acetyl-L-alanine (3.8 g, 58%); m.p. 123-125 °C; $[\alpha]_D^{20}$ -58° (*c*, 1.3 in H_2O), lit.,[143] m.p. 125 °C, $[\alpha]_D$ -66.2° (*c*, 1.0 in H_2O); υ_{max} (KBr disc) 3320, 1710, 1620 and 1560 cm^{-1}; δ_H 1.47

(3H, d), 2.08 (3H, s) and 4.39 (1H, q); δ_C 18.1 (q, CH<u>C</u>H$_3$), 23.5(q, CO<u>C</u>H$_3$), 50.6 (d, <u>C</u>HCH$_3$), 175.9 (s, <u>C</u>OCH$_3$) and 178.7 (s, <u>C</u>O$_2$H); *m/z* 131 (M^+, 6.7%) and 86 (100%). Found: M^+ 131.0580. $C_5H_9NO_3$ required *M*, 131.0582.

N-*Acetyl-D-alanine*

D-Alanine (2.22 g, 25 mmol) was acylated exactly in the manner described previously for the acylation of L-alanine. The product was crystallised also from ethyl acetate to yield *N*-acetyl-D-alanine (1.75 g, 53%); m.p. 124-126 °C; $[\alpha]_D^{20}$ +56.6° (c, 1.4 in H_2O), lit.,[143] m.p. 125 °C, $[\alpha]_D^{20}$ +66.2° (*c*, 1.0 in H_2O); υ_{max} (KBr disc) 3280, 3095, 2980, 1710, 1705, 1610 and 1560 cm^{-1}; δ_H 1.47 (3H, d), 2.08 (3H, s) and 4.38 (1H, q); δ_C 18.1 (q, CH<u>C</u>H$_3$), 23.5 (q, CO<u>C</u>H$_3$), 50.7 (d, <u>C</u>HCH$_3$), 176.1 (s, <u>C</u>OCH$_3$) and 178.8 (s, <u>C</u>O$_2$H); *m/z* 131 (M^+, 5.5%) and 86 (100%). Found: M^+, 131.0583. $C_5H_9NO_3$ required *M*, 131.0582.

N-*Acetyl-4-aminobutyric acid*

4-Aminobutyric acid (8.24 g, 0.08 mol) was acylated using acetic anhydride in acetic acid according to general procedure 2. The solid residue obtained on concentration of the solvent was recrystallised from acetone to yield *N*-acetyl-4-aminobutyric acid (9.49 g, 82%); m.p. 123-125 °C; υ_{max} (KBr disc) 3360, 1720, 1595 and 1550 cm^{-1}; δ_H 1.86 (2H, m), 2.03 (3H, s), 2.47 (2H, t) and 3.27 (2H, t); δ_C 23.8 (q, CO<u>C</u>H$_3$), 25.8 (t, <u>C</u>H$_2$CH$_2$NH), 33.1 (t, <u>C</u>H$_2$CO$_2$H), 40.5 (t, <u>C</u>H$_2$NH), 176.1 (s, <u>C</u>OCH$_3$) and 180.0 (s, <u>C</u>O$_2$H); *m/z* 145 (M^+, 19%), 102 (100%), 86 (98%) and 72 (74%). Found: M^+, 145.0746. Calcd. for $C_6H_{11}NO_3$; *M*, 145.0739.

N-*Propionyl-4-aminobutyric acid*

4-Aminobutyric acid (8.24 g, 80 mmol) was acylated by using propionic anhydride in propionic acid as described in general procedure 2. The resultant oil was crystallised from acetone to yield *N*-propionyl-4-aminobutyric acid (9.86 g, 78%); m.p. 100-102 °C; υ_{max} (KBr disc) 3355, 1700, 1605 and 1550 cm^{-1}; δ_H 1.16 (3H, t), 1.86 (2H, m), 3.29 (2H, q), 2.46 (2H, t) and 3.28 (2H, t); δ_C 12.1 (q, $CH_2\underline{C}H_3$), 26.4 (t, $\underline{C}H_2CH_2NH$), 32.2 (t, $\underline{C}H_2CH_3$), 33.6 (t, $\underline{C}H_2CO_2H$), 40.9 (t, $\underline{C}H_2NH$), 180.40 (s, $\underline{C}OCH_2$) and 180.43 (s, $\underline{C}O_2H$); *m/z* 159 (M^+, 15.4%), 112 (26.3%), 102 (38.3%), 100 (20%) and 86 (43%). Found: M^+, 159.0886. Calcd. for $C_7H_{13}NO_3$; *M*, 159.0895.

6.2.2 *N*-Acyldipeptides

L-Histidine benzyl ester ditosylate (25)

A suspension of L-histidine free base (1.55 g, 1 mmol) and *p*-toluenesulphonic acid monohydrate (4.42 g, 1 mmol) in chloroform (10 ml) was heated at reflux together with benzyl alchohol (10 ml). The water produced in the course of the reaction was collected in a Dean and Stark separator designed for use with solvents denser than water. The removal of water was more efficient when the separator was filled with silica gel and anhydrous sodium carbonate. After 24 h, the mixture was cooled, filtered, and the solvent was removed under reduced pressure. The residue obtained was triturated with diethyl ether to yield L-histidine benzyl ester ditosylate (25) as a white hygroscopic solid. Yield 45-70%; m.p. 140-146 °C; $[\alpha]_D^{20}$ -2.4° (*c*, 1.0 in H_2O), lit.,[77] 93%, m.p. 138-146 °C, $[\alpha]_D^{20}$ -4.13° (*c*, 0.94 in H_2O), lit.,[75] 85-90%, m.p. 146-149 °C, $[\alpha]_D^{30}$ -2.4° (*c*, 4.7 in H_2O); υ_{max} (KBr disc) 3120

and 1730 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.50 ppm) 2.54 (6H, s), 3.51-3.65 (2H, AB part of an ABX system), 4.53-4.63 (1H, X part of an ABX system), 4.78 (2H, s), 7.40-7.94 (14H, aromatic H) and 8.99 (1H, s); δ_C (CD_3OD; CD_2HOD was the internal standard at 49.7 ppm) 21.6 (q, $Ph\underline{C}H_3$), 26.7 (t, $CH\underline{C}H_2$), 53.1 (d, $\underline{C}HCH_2$), 73.4 (t, $\underline{C}H_2Ph$), 119.9 (d, C-4), 127.2-130.2 (aromatic), 136.0 (s, Ph), 142.2 (d, C-2), 143.4 (s, C-5) and 170.4 (s, $\underline{C}O_2CH_2$); *m/z* 155 (2.3%), 110 (8%), 91 (100%) and 81 (45%).

General procedure 3

Mixed Anhydride Coupling of N-*Acylamino Acids to the Benzyl Ester of* L-*Histidine*

L-Histidine benzyl ester ditosylate (25) (10 mmol) was dissolved in dichloromethane containing triethylamine (20 mmol). Triethylammonium tosylate was removed on filtration and the solution of the free base was stored at 0 °C until it was required. The *N*-acylamino acid (15 mmol) was dissolved in dichloromethane (25 ml) containing triethylamine (15 mmol) and the solution was cooled to -5 °C with the aid of an ice/methanol bath. Ethyl chloroformate or isobutyl chloroformate (15 mmol) was added to the cooled solution with stirring. Stirring was continued for 5 min. At the end of this time the mixture was filtered and the filtrate was added to the cooled solution containing L-histidine benzyl ester. The resulting mixture was stored at 0 °C until TLC (solvent system 1) showed the reaction to be complete. The organic solution was washed twice with an equivalent volume of sodium bicarbonate solution (1M). The organic layer was dried, filtered and concentrated under reduced pressure at room temperature to yield an oil.

N-*Propionyl-L-histidine benzyl ester*

L-Histidine benzyl ester ditosylate (25) (1.15 g, 1.9 mmol) was reacted with propionic acid (0.28 g, 3.8 mmol) according to general procedure 3 to yield the product as an oil. This oil was crystallised from chloroform. *N*-Propionyl-L-histidine benzyl ester was isolated on filtration (0.35 g, 62%); m.p. 124-125 °C; $[\alpha]_D^{20}$ -17.0° (*c*, 0.32 in ethanol); υ_{max} (KBr disc) 3300, 1740, 1660 and 1535 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.5 ppm) 1.25 (3H, t), 2.39 (2H, q), 3.12-3.37 (2H, AB part of an ABX system), 4.87-4.94 (1H, X part of an ABX system), 5.31 (2H, s), 6.99 (1H, s), 7.50 (5H, s) and 7.82 (1H, s); δ_C (CD_3OD; CD_2HOD was the internal standard at 49.3 ppm) 10.6 (q, $\underline{C}H_3CH_2$), 30.1 (t, $\underline{C}H_2CH$), 30.2 (t, $CH_3\underline{C}H_2$), 54.4 (d, $CH_2\underline{C}H$), 68.2 (t, $Ph\underline{C}H_2$), 118.3 (d, C-4), 129.5 (d, Ph), 129.6 (d, Ph), 129.8 (d, Ph), 134.6 (s, C-5), 136.6 (d, C-2), 137.4 (s, Ph), 173.1 (s, $NH\underline{C}O$) and 177.3 (s, $\underline{C}O_2CH_2$); *m/z* 301 (M^+, 20%), 110 (79%), 91 (81%) and 81 (60%). Found: M^+ 301.1423. Calcd. for $C_{16}H_{19}N_3O_3$; *M*, 301.1426.

A number of *N*-acylamino acids were coupled to L-histidine benzyl ester. All of the products showed the benzyl ester present in the proton and ^{13}C NMR spectra. These derivatives also showed similarities in their IR spectra to the data quoted for *N*-propionyl-L-histidine benzyl ester. The accurate mass data collected for these compounds are listed below:

(A). The value obtained for *N*-acetylglycyl-L-histidine benzyl ester was 344.1479, while the mass calculated for $C_{17}H_{20}N_4O_4$ was 344.1485.

(B). A value of 358.1628 was obtained for *N*-propionylglycyl-L-histidine benzyl ester. $C_{18}H_{22}N_4O_4$ required a measurement of 358.1641

(C). The mass measured for *N*-acetyl-L-carnosine benzyl ester was 358.1618, this compound required a value of 358.1641.

(D). A measurement of 372.1807 was obtained for *N*-propionyl-L-carnosine benzyl ester. $C_{19}H_{24}N_4O_4$ required a mass of 372.1798.

(E). The mass measured for *N*-acetyl-L-homocarnosine benzyl ester was 372.1794, while this compound required a value of 372.1798.

(F). A value of 386.1948 was obtained for *N*-propionyl-L-homocarnosine. This compound required a mass of 386.1954.

General procedure 4

Mixed Anhydride Coupling of N-*Acylamino Acids to L-Histidine Protected as a Metal Salt.*

L-Histidine (2.4 g, 15 mmol) was dissolved in sufficient lithium or potassium hydroxide solution (0.33M) to produce a pH of 9.6. This solution was stored at 0 ^{o}C until it was required. The second *N*-acylated amino acid (18 mmol) was dissolved in the minimum amount of ethyleneglycol dimethylether, DMF or acetonitrile containing triethylamine (1.8 g, 18 mmol). The resultant solution was cooled to -5 ^{o}C with the aid of an ice/methanol bath. Isobutylchloroformate (2.45 g, 18 mmol) was added dropwise with stirring and the mixture was stirred for a further 3 min. The triethylammonium chloride formed was removed by filtration and the filtrate was added with vigorous stirring to the solution containing L-histidine. The stirring was continued for a few min to allow the evolution of carbon dioxide to take place. The

mixture was then stored at 0 °C for 2 h. Once the reaction was complete the solution was acidified to pH 5 on addition of hydrochloric acid (1M). It was then concentrated at room temperature and under reduced pressure to yield the product as a glassy residue.

N-*Acetylglycyl-L-histidine (17)*

N-Acetylglycine (2.11 g, 18 mmol) was coupled to L-histidine (2.4 g, 15 mmol) using general procedure 4. Lithium hydroxide was the aqueous base and ethyleneglycol dimethyl ether was the organic solvent. The resultant residue was triturated with isopropanol (50 ml) and the mixture was then heated. Enough water (approx. 12 ml) was added to the mixture to dissolve the oil which was formed on heating the solution at reflux. Unreacted L-histidine crystallised out on cooling. This was removed by filtration and isopropanol was added dropwise to the filtrate until it turned cloudy. Crystallisation of the product subsequently occurred. The product was recrystallised from water on the addition of isopropanol until the flame test for the presence of lithium was negative. *N*-Acetylglycyl-L-histidine (17) was obtained as a white solid (2.74 g, 72%); m.p. 140-142 °C; $[\alpha]_D^{20}$ +31.1° (*c*, 1.1 in H_2O); υ_{max} (KBr disc) 3400, 3300, 1640 and 1570 cm^{-1}; δ_H 2.10 (3H, s), 3.13-3.40 (2H, AB part of an ABX system), 3.92 (2H, s), 4.54-4.61 (1H, X part of an ABX system), 7.30 (1H, s) and 8.64 (1H, s); δ_C 23.7 (q, $CO\underline{C}H_3$), 29.2 (t, $CH\underline{C}H_2$), 44.5 (t, $\underline{C}H_2NH$), 55.9 (d, $\underline{C}HCH_2$) 118.8 (d, C-4), 131.6 (s, C-5), 135.1 (d, C-2), 172.9 (s, $\underline{C}OCH_3$), 176.6 (s, $\underline{C}OCH_2$) and 178.1 (s, $\underline{C}O_2H$); *m/z* 254 (M^+, 1.9%), 81 (100%) and 72 (86%). Found: M^+, 254.1021. $C_{10}H_{14}N_4O_4$ required *M*, 254.1015.

N-*Propionylglycyl-L-histidine (20)*

N-Propionylglycine (2.36 g, 8 mmol) was coupled to L-histidine (2.4 g, 15 mmol) using general procedure 4 with acetonitrile as the organic solvent and potassium hydroxide as the aqueous base. The product was extracted from the residue at the end of the reaction into boiling ethanol. The ethanol solution was filtered and allowed to stand until the product crystallised out. Recrystallisation from an ethanol/water mixture produced *N*-propionylglycyl-L-histidine (20) (2.57 g, 64%); m.p. 200-202 °C, $[\alpha]_D^{20}$ +22.8° (*c*, 0.94 in H_2O); υ_{max} (KBr disc) 3300, 1640 and 1545 cm^{-1}; δ_H 1.17 (3H, t), 2.37 (2H, q), 3.11-3.40 (2H, AB part of an ABX system), 3.93 (2H, s), 4.55-4.62 (1H, X part of an ABX system), 7.30 (1H, s) and 8.65 (1H, s); δ_C 11.3 (q, $CH_2\underline{C}H_3$), 29.1 (t, $\underline{C}H_2CH$), 30.8 (t, $\underline{C}H_2CH_3$), 44.5 (t, $\underline{C}H_2NH$), 55.7 (d, $CH_2\underline{C}H$), 118.8 (d, C-4), 131.4 (s, C-5), 135.1 (d, C-2), 173.0 (s, $\underline{C}OCH_2CH_3$), 177.9 (s, $\underline{C}OCH_2NH$) and 180.7 (s, $\underline{C}O_2H$); *m/z* 268 (M^+, 2.9%), 137 (31%), 86 (44%) and 81 (100%). Found: M^+, 268.1172. Calcd. for $C_{16}H_{11}N_4O_4$; *M*, 268.1172.

N-*Acetyl-L-carnosine (18)*

Method 1

L-Carnosine (2) (2.26 g, 10 mmol) was acylated with acetic anhydride in water by the method described in general procedure 1. The glassy residue was stored in a vacuum desiccator containing potassium hydroxide pellets and phosphorus pentoxide until a white solid was obtained. The solid was recrystallised from methanol to yield *N*-acetyl-L-carnosine (18) (2.14 g, 80%); m.p. 158-160 °C; $[\alpha]_D^{20}$ +14.4° (*c*, 1.0 in H_2O).

Method 2

N-Acetyl-β-alanine (1.57 g, 12 mmol) was coupled to L-histidine (1.59 g, 10 mmol) by the method described in general procedure 4 with potassium hydroxide as the aqueous base and acetonitrile as the organic solvent. The white solid produced on removal of the solvent was heated in methanol. The hot methanol solution was filtered and the filtrate allowed to stand until crystallisation of the product took place. Recrystallisation from methanol yielded *N*-acetyl-L-carnosine (18) (1.58 g, 59%); m.p. 158-160 °C; $[\alpha]_D^{20}$ +20.1° (*c*, 1.14 in H_2O); υ_{max} (KBr disc) 3450, 3320, 1630, 1590 and 1520 cm^{-1}; δ_H (D_2O; acetone was the internal standard at 2.20 ppm) 1.91 (3H, s), 2.44 (2H, t), 2.99-3.28 (2H, AB part of an ABX system), 3.35 (2H, t), 4.46-4.52 (1H, X part of an ABX system), 7.24 (1H, s) and 8.58 (1H, s); δ_C (D_2O; dioxan was the external standard at 67.8 ppm) 21.6 (q, $CO\underline{C}H_3$), 27.3 (t, $\underline{C}H_2CH$), 35.1 (t, $CO\underline{C}H_2$), 35.9 (t, $\underline{C}H_2NH$), 54.0 (d, $CH_2\underline{C}H$), 116.7 (d, C-4), 129.8 (s, C-5), 133.2 (d, C-2), 173.4 (s, $\underline{C}OCH_3$), 174.1 (s, $\underline{C}OCH_2$) and 176.4 (s, $\underline{C}O_2H$); *m/z* 268 (M^+, 1.0%), 110 (29%), 88 (33%), 82 (100%) and 81 (70%). Found: M^+, 296.1479. $C_{11}H_{16}N_4O_4$ required *M*, 268.1172.

N-*Propionyl-L-carnosine (21)*

Method 1

L-Carnosine (2) (2.26 g, 10 mmol) was acylated with propionic anhydride as described in general procedure 1 with water as the solvent. The resultant oil was triturated with acetone then dried in a vacuum desiccator containing potassium hydroxide pellets and phosphorus pentoxide until a white solid formed. The product was recrystallised from ethanol to yield *N*-propionyl-L-

carnosine (21) (2.0 g, 71%); m.p. 194-195 °C; $[\alpha]_D^{20}$ +14.6° (*c*, 1.2 in H_2O).

Method 2

N-Propionyl-β-alanine (1.74 g, 12 mmol) was coupled to L-histidine (1.59 g, 10 mmol) according to the method desribed in general procedure 4. Acetonitrile was the organic solvent and potassium hydroxide was the aqueous base. At the end of the reaction the oil was heated in ethanol and filtered. The filtrate was allowed to stand until the product crystallised. *N*-Propionyl-L-carnosine (21) was isolated as a white solid (1.5 g, 53%); m.p. 194-195 °C; $[\alpha]_D^{20}$ +15.5° (*c*, 1.2 in H_2O); υ_{max} (KBr disc) 3310, 3285, 1660, 1630, 1570 and 1545 cm^{-1}; δ_H 1.12 (3H, t), 2.25 (2H, q), 2.53 (2H, t), 3.07-3.36 (2H, AB part of an ABX system), 3.45 (2H, t), 4.52-4.58 (1H, X part of an ABX system), 7.32 (1H, s) and 8.64 (1H, s); δ_C 11.5 (q, $CH_2\underline{C}H_3$), 29.3 (t, $CH\underline{C}H_2$), 31.0 (t, $\underline{C}H_2CH_3$), 37.1 (t, $\underline{C}H_2CO$), 37.7 (t, $NH\underline{C}H_2$), 56.0 (d, $\underline{C}HCH_2$), 118.6 (d, C-4), 131.8 (s, C-5), 135.2 (d, C-2), 175.3 (s, $\underline{C}OCH_2CH_3$), 178.4 (s, $\underline{C}OCH_2CH_2$) and 179.8 (s, $\underline{C}O_2H$); *m/z* 282 (M^+, 1.5%), 137 (28%), 109 (63%) and 81 (100%). Found: M^+, 282.1326. Calcd. for $C_{12}H_{18}N_4O_4$; *M*, 282.1328.

N-*Acetyl-L-homocarnosine (19)*

N-Acetyl-4-aminobutyric acid (1.74 g, 12 mmol) was coupled to L-histidine (1.59 g, 10 mmol) according to general procedure 4. DMF was the organic solvent and lithium hydroxide was the aqueous base. The resultant glassy residue was triturated with isopropanol (50 ml) which was subsequently decanted off. Ethanol (15 ml) and water (1 ml) were added and the mixture was

heated until a clear solution was obtained. Isopropanol was added on cooling to produce a turbid solution from which the product crystallised. The crystalline solid was washed with 1% water in acetone to remove residual lithium chloride and was then recrystallised from ethanol to yield *N*-acetyl-L-homocarnosine (19) (1.95 g, 69%); m.p. 120 °C; $[\alpha]_D^{20}$ +14.7° (*c*, 0.8 in H_2O); υ_{max} (KBr disc) 3320, 3280, 3100, 1630 and 1540 cm^{-1}; δ_H 1.77 (2H, t), 2.02 (3H, s), 2.33 (2H, t), 3.06-3.38 (2H, AB part of an ABX system), 3.16 (2H, t), 4.56-4.60 (1H, X part of an ABX system), 7.32 (1H, s) and 8.64 (1H, s); δ_C 23.8 (q, $CO\underline{C}H_3$), 26.6 (t, $\underline{C}H_2CH$), 29.4 (t, $CH_2\underline{C}H_2CH_2$), 34.9 (t, $CO\underline{C}H_2$), 40.6 (t, $\underline{C}H_2NH$), 55.9 (d, $CH_2\underline{C}H$), 118.7 (d, C-4), 131.8 (s, C-5), 135.1 (d, C-2), 176.1 (s, $\underline{C}OCH_3$), 177.4 (s, $\underline{C}OCH_2$), 178.5 (s, $\underline{C}O_2H$); *m/z* 282 (M^+, 0.2%), 86 (89%) and 81 (90%). Found: M^+, 282.1332. Calcd. for $C_{12}H_{18}N_4O_4$; *M*, 282.1328.

N-*Propionyl-L-homocarnosine (22)*

N-Propionyl-4-aminobutyric acid (3.18 g, 20 mmol) was coupled to L-histidine (2.3 g, 15 mmol) according to the method described in general procedure 4. Acetonitrile was the organic solvent and potassium hydroxide was the aqueous base. The residual oil was dissolved in the minimum amount of hot isopropanol. The solution was cooled and filtered. Acetone was added to the filtrate to initiate crystallisation of the product. *N*-Propionyl-L-homocarnosine (22) was obtained on filtration as a white hygroscopic solid (2.71 g, 61%); m.p. 114-116 °C; $[\alpha]_D^{20}$ +10.3° (*c*, 1.3 in H_2O); υ_{max} (KBr disc) 3420, 3280, 1640 and 1550 cm^{-1}; δ_H (D_2O; dioxan was the external standard at 3.53 ppm) 0.89 (3H, t), 1.51 (2H, m), 2.02 (2H, q), 2.08 (2H, t), 2.80-3.12 (2H, AB part of

an ABX system), 2.92 (2H, t), 4.28-4.35 (1H, X part of an ABX system), 7.06 (1H, s) and 8.38 (1H, s); δ_C (D_2O; dioxan was the external reference at 67.8 ppm) 10.7 (q, $CH_2\underline{C}H_3$), 25.8 (t, $CH\underline{C}H_2$), 28.6 (t, $CH_2\underline{C}H_2CH_2$), 30.3 (t, $\underline{C}H_2CH_3$), 34.0 (t, $CO\underline{C}H_2CH_2$), 39.6 (t, $\underline{C}H_2NH$), 55.0 (d, $\underline{C}HCH_2$), 117.8 (d, C-4), 130.9 (s, C-5), 134.3 (d, C-2), 176.4 (s, $\underline{C}OCH_2CH_3$), 177.6 (s, $\underline{C}OCH_2CH_2$) and 179.1 (s, $\underline{C}O_2H$); *m/z* 296 (M^+, 1.8%), 120 (93%) and 81 (100%). Found: M^+, 296.1479. Calcd. for $C_{13}H_{20}N_4O_4$; *M*, 296.1485.

N-*Acetyl-L-alanyl-L-histidine (28)*

N-Acetyl-L-alanine (1.31 g, 10 mmol) was coupled to L-histidine (1.22 g, 7.9 mmol) using general procedure 4. DMF was the organic solvent and lithium hydroxide was the aqueous base. The residue obtained at the end of the reaction was triturated with isopropanol (20 ml). The mixture was heated and water (3 ml) was added, methanol was then added dropwise until a clear solution resulted. The cooled solution was filtered, the filtrate was concentrated, and the residue was crystallised from ethanol to yield *N*-acetyl-L-alanyl-L-histidine (28) (1.26 g, 59%); m.p. 150-152 °C; $[\alpha]_D^{20}$ -32.8° (*c*, 0.9 in 6M HCl); υ_{max} (KBr disc) 3450, 3280, 1645 and 1540 cm^{-1}; δ_H 1.39 (3H, d), 2.08 (3H, s), 3.11-3.39 (2H, AB part of an ABX system), 4.30 (1H, q), 4.51-4.57 (1H, X part of an ABX system), 7.32 (1H, s) and 8.64 (1H, s); δ_C 18.4 (q, $CH\underline{C}H_3$), 23.5 (q, $CO\underline{C}H_3$), 29.2 (t, $\underline{C}H_2CH$), 51.7 (d, $\underline{C}HCH_3$), 55.9 (d, $CH_2\underline{C}H$), 118.8 (d, C-4), 131.4 (s, C-5), 135.1 (d, C-2), 175.9 (s, $\underline{C}OCH_3$), 176.5 (s, $\underline{C}OCHCH_3$) and 180.0 (s, $\underline{C}O_2H$); *m/z* 268 (M^+, 1.4%), 164 (24%), 137 (78%), 120 (100%), 109 (31%) and 81 (91%). Found: M^+, 268.1168. Calcd. for $C_{11}H_{16}N_4O_4$; *M*, 268.1172.

N-*acetyl-D-alanyl-L-histidine (29)*

N-Acetyl-D-alanine (1.97 g, 15 mmol) was coupled to L-histidine (1.55 g, 10 mmol) using general procedure 4. DMF was the organic solvent and potassium hydroxide was the aqueous base. The residue obtained was heated in ethanol and the solution was cooled and filtered. The ethanol solution was left at room temperature until the product crystallised. *N*-Acetyl-D-alanyl-L-histidine (29) was obtained as a white crystalline solid (1.56 g, 58%); m.p. 133-135 °C; $[\alpha]_D^{20}$ -42.1° (*c*, 0.8 in 6M HCl); υ_{max} (KBr disc) 3280, 1640, 1565 and 1540 cm^{-1}; δ_H 1.39 (3H, d), 2.06 (3H, s), 3.11-3.39 (2H, AB part of an ABX system), 4.31 (1H, q), 4.51-4.57 (1H, X part of an ABX system), 7.32 (1H, s) and 8.64 (1H, s); δ_C 18.4 (q, $CH\underline{C}H_3$), 23.5 (q, $CO\underline{C}H_3$), 29.2 (t, $CH\underline{C}H_2$), 51.8 (d, $\underline{C}HCH_3$), 55.9 (d, $\underline{C}HCH_2$), 118.8 (d, C-4), 131.4 (s, C-5), 135.1 (d, C-2), 175.9 (s, $\underline{C}OCH_3$), 176.6 (s, $\underline{C}OCH$) and 177.9 (s, $\underline{C}O_2H$); *m/z* 268 (M^+, 1.9%), 164 (22%), 137 (75%), 109 (34%) and 81 (85%). Found: M^+, 268.1170. Calcd. for $C_{11}H_{16}N_4O_4$; *M*, 268.1172.

6.2.3 Procedures Used For the Determination of Optical Purity

Coupling of (R)-α-Methylbenzylamine to N-*Acyldipeptides*

N-ethyl-*N*'-dimethylaminopropylcarbodiimide (39.5 mg, 0.2 mmol) was added to a suspension of the *N*-acyldipeptide (0.2 mmol) in pyridine (3 ml). *(R)*-α-Methylbenzylamine (25.8 µl, 0.2 mmol) was added and the mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure and the resultant oil was extracted with ethyl acetate (3 x 3 ml). The combined organic extracts were washed

with water (2 ml), dried and concentrated under reduced pressure to yield the diastereoisomeric products. Each product was dissolved in methanol (2 ml) and applied to the HPLC column. The peaks were detected by a UV spectrometer at 254 nm.

Europium(III)-(R)-propane-1,2-diaminetetra-acetate

Concentrated hydrochloric acid (6 mmol) was dropped into a suspension of europium(III) oxide (0.35 g, 1 mmol) in water (5 ml). The mixture was heated at reflux with stirring until a clear solution was obtained. More acid was added to effect dissolution if required. Potassium carbonate solution (1M) was added dropwise to precipitate the europium(III) carbonate. The solid was collected on filtration and dried. The dried solid was then added gradually to a warm solution of (*R*)-propane-1,2-diaminetetra-acetate (0.6 g, 2 mmol) in water (8 ml). The solution was adjusted to pH 9 upon the addition of potassium carbonate solution (1M). The solution was filtered and the filtrate was concentrated under reduced pressure to yield the product, europium(III)-(*R*)-propane-1,2-diaminetetra-acetate. This was used according to the procedure published by Kabuto and Sasaki.[136,137]

Reaction of N-*Acetylhistidine with Brucine*

Brucine (0.43 g, 1 mmol) was added to a solution of acetyl-DL-histidine (0.2 g, 1 mmol) in ethanol (6 ml). The solution was heated for 10 min and then concentrated under reduced pressure to yield a syrup. The product crystallised from water.

Reaction of N-*Acylamino Acids and Peptides with Acylase I*

The *N*-acylamino acid or peptide (0.1 mmol) was dissolved in D_2O (1 ml). The solution was adjusted to pD 8 upon addition of sodium deuteroxide (1M). Hog Kidney Acylase I (5 mg, 1700 U/mg) was added and the solution was incubated at 38 °C for 24 h.

6.3 Experimental to Chapter 5

6.3.1 Synthesis of 1-Methylhistidine (33)

Method 1[74]

N(α)-*Benzoyl-L-histidine (30)*

L-Histidine monohydrochloride (6.3 g, 33 mmol) was dissolved in sodium hydroxide solution (2M; 30 ml). The solution was ice cooled and stirred while benzoyl chloride (4.64 ml) was added simultaneously with sodium hydroxide solution (2M; 65 ml) over a period of 1.5 h. At the end of this time the solution was acidified with the addition of hydrochloric acid (5M; 21 ml) and finally glacial acetic acid (17.6M; 0.29 ml). The *N*(α)-benzoyl-L-histidine precipitated immediately, solidifying into little rods. The reaction mixture was left at 0 °C for 1 h and then it was filtered. The solid was collected and washed with ice cold water and diethyl ether. The product was dried at 100 °C. Recrystallisation from boiling water yielded pure *N*(α)-benzoyl-L-histidine (30) (5.75 g, 87%); m.p. 246-247 °C; $[\alpha]_D^{20}$ -44.0° (*c*, 1.0 in 2M HCl); lit.,[74] m.p. 247 °C; $[\alpha]_D^{20}$ -46.6° (*c*, 6.4 in 2M HCl); υ_{max} (KBr disc) 3475, 3440, 3280, 3120, 1630 and 1580 cm^{-1}; δ_H (D_2O/DCl; *t*-butanol was the internal standard at 1.3 ppm) 3.33-3.57 (2H, AB part of an ABX system), 4.96-5.04 (X part of an ABX system), 7.37 (1H, s), 7.42-

7.75 (5H, s) and 8.67 (1H, s); δ_C (D_2O/DCl; *t*-butanol was the internal standard at 31.6 ppm) 28.0 (t, $CH\underline{C}H_2$), 54.2 (d, $\underline{C}HCH_2$), 119.2 (d, C-4), 129.2 (d, Ph), 130.8 (d, Ph), 134.0 (s, C-5), 134.7 (d, C-2), 135.2 (s, Ph), 172.3 (s, $NH\underline{C}O$) and 175.1 (s, $\underline{C}O_2H$); *m/z* 259 (M^+, 4%), 110 (14%), 105 (100%) and 81 (100%). Found: M^+ 259.0956. Calcd. for $C_{13}H_{13}N_3O_3$; *M*, 259.0957.

N(α)-*Benzoyl-L-histidine methyl ester (31)*

N(α)-Benzoyl-L-histidine (30) (2.7 g, 10.4 mmol) was suspended in methanol (27 ml). The mixture was saturated with HCl gas at room temperature. The resulting solution was heated at reflux for 2 min and the solvent was subsequently removed under reduced pressure. The residue was dissolved in absolute ethanol and once again the solution was concentrated. The oil was stored in a vacuum desiccator containing potassium hydroxide pellets until no traces of the acid remained. The residue was then dissolved in hot water (20 ml), the solution was cooled and treated with ammonia (0.88) until there was a permanent odour of the amine. *N*(α)-Benzoyl-L-histidine methyl ester (31) crystallised into prisms. These were collected on filtration, washed with water and dried at 100 °C (1.9 g, 67%); m.p. 158-160 °C; $[\alpha]_D^{20}$ -27.5°, (*c*, 0.8 in 6M HCl), lit.,[74] m.p. 159-160 °C; υ_{max} (KBr disc) 3280, 1750, 1660 and 1530 cm^{-1}; δ_H (D_2O/DCl; HOD was the internal standard at 4.67 ppm) 1.39-1.65 (2H, AB part of an ABX system), 1.87 (3H, s), 3.05-3.15 (1H, X part of an ABX system), 5.43-5.80 (6H, aromatic H) and 6.73 (1H, s); δ_C (D_2O/DCl; dioxan was the external standard at 67.8 ppm) 26.0 (t, $CH\underline{C}H_2$), 52.2 (q, $CO_2\underline{C}H_3$), 53.5 (d, $\underline{C}HCH_2$), 117.2 (d, C-4), 127.2 (d, Ph), 128.7 (d, Ph), 128.8 (d, Ph), 131.9 (s, Ph), 132.8 (d, C-2), 133.2 (s, C-5), 170.6 (s, $NH\underline{C}O$) and

171.9 (s, $\underline{C}O_2CH_3$); *m/z* 273 (M^+, 11%), 152 (26%), 105 (100%) and 81 (74%). Found M^+ 273.1106, $C_{14}H_{15}N_3O_3$ required; *M*, 273.1113.

Attempted synthesis of N(α),3-*dibenzoyl-L-histidine methyl ester (32)*

Benzoyl chloride (0.75 g, 5.4 mmol) in tetrahydrofuran (THF) (3.8 ml) was added to a stirred mixture of *N*(α)-benzoyl-L-histidine methyl ester (31) (1.4 g, 5 mmol) and dicyclohexylamine (0.94 g, 5.2 mmol) in THF (38 ml) over a period of 1.5 h. Stirring was continued for 30 min and the solution was then filtered. The solid was washed with THF (3 ml) and the combined THF solutions were concentrated under reduced pressure to give an oily residue.

Method 2

N(α),3-*bis*(t-*Butoxycarbonyl)-L-histidine methyl ester (34)*

L-Histidine methyl ester dihydrochloride (2.42 g, 0.01 mol) was dissolved in methanol (8 ml) containing triethylamine (2.02 g, 0.02 mol). Di-*t*-butyl dicarbonate (4.8 g, 0.02 mol) was added with stirring. Stirring was continued for 20 h. A white precipitate formed during the course of the reaction. The solvent was concentrated, and the residue was taken up in acetone and filtered. The filtrate was evaporated under reduced pressure. Chloroform (10 ml) was added and the mixture was extracted with 10% citric acid (2 x 2 ml). The organic layer was further extracted with water (2 ml). After drying, the organic layer was concentrated to yield an oil. The oil was crystallised from petroleum ether (b.p. 40-60 °C) to yield *N*(α),3-bis(*t*-butoxycarbonyl)-L-histidine methyl ester (34) (3.21 g, 87%); m.p.

88-90 °C; $[\alpha]_D^{20}$ +25.9° (*c*, 0.9 in CCl_4); lit.,[67] m.p. 85-88 °C, $[\alpha]_D^{20}$ +19.9° (*c*, 1.16 in $CHCl_3$); lit.,[49] m.p. 96 °C, $[\alpha]_D^{20}$ + 25.6° (*c*, 1.0 in CCl_4); υ_{max} ($CHCl_3$ solution) 3440, 2980, 1750, 1710, 1500, 1480 and 1380 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.21 (9H, s), 1.40 (9H, s), 2.83 (2H, AB part of an ABX system), 3.51 (3H, s), 4.30-4.40 (1H, X part of an ABX system), 5.74 (1H, d), 6.96 (1H, s) and 7.78 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 28.0 (q, $C(\underline{C}H_3)_3$), 28.5 (q, $C(\underline{C}H_3)_3$), 30.4 (t, $CH\underline{C}H_2$), 52.4 (d, $\underline{C}HCH_2$), 53.4 (q, $CO_2\underline{C}H_3$), 79.7 (s, $\underline{C}(CH_3)_3$), 85.7 (s, $\underline{C}(CH_3)_3$), 114.7 (d, C-4), 137.1 (d, C-2), 138.9 (s, C-5), 147.0 (s, $\underline{C}O_2C(CH_3)_3$), 155.7 (s, $\underline{C}O_2C(CH_3)_3$) and 172.5 (s, $\underline{C}O_2CH_3$); *m/z* 369 (M^+, 3.8%), 313 (22%), 196 (16%), 152 (77%), 110 (22%) and 81 (68%). Found: M^+ 369.1903, $C_{17}H_{27}N_3O_6$ required; *M*, 369.1900.

Attempted N-*Methylation of the Imidazole Ring of* L-*Histidine Derivatives Using Trimethyloxonium Hexachloroantimonate*

The protected L-histidine derivative (5 mmol) was dissolved in anhydrous nitromethane (10 ml). Trimethyloxonium hexachloroantimonate (6 mmol) in anhydrous nitromethane (5 ml) was added at room temperature with vigorous stirring. Stirring was continued for four h. The solution was then concentrated under reduced pressure to yield a black tar like substance which was insoluble in water and various organic solvents.

General procedure 5

N-*Methylation of the Imidazole Ring of* L-*Histidine Derivatives*

The L-histidine derivative (5.0 mmol) was dissolved in DMF (3 ml) and methyl iodide (1.42 g, 10 mmol) was added. The reaction mixture was heated at reflux (45 °C) until TLC (solvent system 1) showed the reaction to be complete. Water (2 ml) was added and the solution was concentrated under reduced pressure. The DMF was removed on azeotroping with further volumes of water. The oil was dissolved in ethyl acetate or chloroform (5 ml) and the solution was extracted with aqueous ammonia until the organic portion was colourless. The organic layer was dried, filtered and concentrated under reduced pressure.

N(α)-t-*Butoxycarbonyl*-1-*methyl*-L-*histidine methyl ester (35)*

N(α),3-bis(*t*-Butoxycarbonyl)-L-histidine methyl ester (34) (1.7 g, 5 mmol) was methylated according to general procedure 5. Diethyl ether was added to the oil obtained on concentration of the ethyl acetate and crystallisation took place to yield *N*(α)-*t*-butoxycarbonyl-1-methyl-L-histidine methyl ester (35) as a white crystalline solid (0.57 g, 40%); m.p. 108-110 °C; $[\alpha]_D^{20}$ +27.9° (*c*, 0.47 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3450, 3040, 2995, 2980, 1750, 1710, 1500 and 1440 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.42 (9H, s), 2.95-3.22 (2H, AB part of an ABX system), 3.61 (3H, s), 3.75 (3H, s), 4.48-4.58 (1H, X part of an ABX system), 5.25 (1H, NH), 6.71 (1H, s) and 7.62 (1H, s); δ_C (D_2O; dioxan was the external standard at 67.8 ppm) 26.2 (t, $CH\underline{C}H_2$), 28.3 (q, $C(\underline{C}H_3)_3$), 34.0 (q, $N\underline{C}H_3$), 52.9 (d, $\underline{C}HCH_2$), 54.0 (q, $O\underline{C}H_3$), 82.5 (s, $\underline{C}(CH_3)_3$), 119.0 (d, C-4), 131.4 (s, C-5), 136.0 (d, C-2), 157.9 (s, $\underline{C}O_2C(CH_3)_3$) and 173.4 (s, $\underline{C}O_2CH_3$); *m/z* 283 (M^+, 2.1%),

227 (17%), 210 (18%) and 95 (100%). Found M^+ 283.1530. Calcd. for $C_{13}H_{21}N_3O_4$; *M*, 283.1532.

General procedure 6

Deprotection of Methyl Ester and Carbamate Derivatives by Acid Hydrolysis

The protected derivative (1 mmol) was dissolved in a minimum amount of methanol. Hydrochloric acid (6M; 15 ml) was added and the solution was heated at 90 °C until TLC (Solvent system 1) showed the deprotection to be complete. The solution was then concentrated under reduced pressure to yield the free amino acid or peptide as the acid salt.

General procedure 7

Cleavage of Methyl Esters and Carbamates with Boron Tribromide[61]

The protected histidine derivative (2.0 mmol) was dissolved in dry methylene chloride (25 ml) and cooled to -10 °C with the aid of an ice/methanol bath. 1M Boron tribromide in methylene chloride (1 mmol per atom which is complexed) was added and the mixture was stirred at -10 °C for a further h. Stirring was continued at room temperature for several d until TLC (solvent system 1) showed complete cleavage of the ester had been effected. Water (50 ml) was added with care and the layers were separated. The organic portion was washed with water (3 x 25 ml). The combined aqueous layers were concentrated under reduced pressure. The solid obtained was dissolved in methanol and concentrated several times.

1-Methylhistidine (33) dihydrochloride

1-Methyl-*N*(α)-*t*-butoxycarbonyl-L-histidine methyl ester (35) (0.242 g, 0.86 mmol) was dissolved in a small amount of methanol and treated with hydrochloric acid (6M, 12 ml) according to general procedure 6. The reaction was complete in 3 h. 1-Methylhistidine (33) dihydrochloride was isolated as a hygroscopic foam. The product was crystallised from ethanol on the addition of diethyl ether. (0.138 g, 95%); m.p. 214-218 °C, lit.,[74] m.p. 216-219 °C (dec), R_F 0.61 (TLC solvent system 3); υ_{max} (KBr disc) 3420, 2940, 2680, 2500 and 1595 cm^{-1}; δ_H 3.28 (2H, AB part of an ABX system), 3.86 (3H, s), 4.01-4.08 (1H, X part of an ABX system), 7.32 (1H, s) and 8.44 (1H, s); δ_C 26.6 (t, $CH\underline{C}H_2$), 34.7 (q, $N\underline{C}H_3$), 55.1 (d, $\underline{C}HCH_2$), 122.9 (d, C-4), 130.3 (s, C-5), 138.9 (d, C-2) and 174.8 (s, $\underline{C}O_2H$); *m/z* 169 (M^+, 1%), 124 (4%) and 95 (100%). Found: M^+, 169.0845. $C_7H_{11}N_3O_2$ required *M*, 169.0851.

1-Methylhistidine (33) dihydrobromide

1-Methyl-*N*(α)-*t*-butoxycarbonyl-L-histidine methyl ester (35) (0.24 g, 0.85 mmol) was dissolved in methylene chloride (22 ml). The solution was treated with boron tribromide according to the method described in general procedure 7. The oil obtained on concentration of the methanolic solution was dissolved in a small amount of water and boiled with activated charcoal. The charcoal was removed upon filtration of the mixture through celite. The aqueous solution was then concentrated to yield an oil. All attempts to crystallise the oil failed. The oil had identical proton and ^{13}C NMR, IR and mass spectra to the product (33) obtained as the dihydrochloride salt.

6.3.2 3-Methylhistidine (39)

Method 1[153]

(+)-(7S)-5,6,7,8-*Tetrahydro*-7-(*methoxycarbonyl*)-5-*oxoimidazo*-[1,5-c]*pyrimidine (40)*

A mixture of L-histidine methyl ester dihydrochloride (1.5 g, 6.2 mmol) and *N,N'*-carbonyldiimidazole (1.0 g, 6.2 mmol) in DMF (31 ml) was stirred at 60 °C for 3 h. At the end of this time, the solvent was removed under reduced pressure, yielding an oil. The oil was dissolved in sodium bicarbonate solution (1M; 62 ml) and the aqueous solution was extracted with chloroform (8 x 12 ml). The combined organic extracts were dried, filtered and concentrated under reduced pressure to yield a solid. The product (40) was crystallised from methanol (1.1 g, 94%); m.p. 155-157 °C; $[\alpha]_D^{20}$ +58.4° (*c*, 0.9 in methanol), lit.,[153] m.p. 156-157 °C, $[\alpha]_D^{25}$ +59.0° (*c*, 1.2 in methanol); υ_{max} (KBr disc) 3120, 1755, 1715 and 1410 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 3.07-3.43 (2H, AB part of an ABX system), 3.80 (3H, s), 4.32-4.40 (1H, X part of an ABX system), 6.44 (1H, NH), 6.87 (1H, s) and 8.14 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 23.8 (t, $CH\underline{C}H_2$), 53.4 (d, $\underline{C}HCH_2$), 54.1 (q, $O\underline{C}H_3$), 124.5 (s, C-5), 127.1 (d, C-4), 135.9 (d, C-2), 148.4 (s, $N\underline{C}ONH$) and 170.2 (s, $\underline{C}O_2CH_3$); *m/z* 195 (M^+, 27%), 152 (39%) and 81 (100%). Found: M^+, 195.0620. $C_8H_9N_3O_3$ required *M*, 195.0643.

(+)-(7S)-5,6,7,8-*Tetrahydro*-7-(*methoxycarbonyl*)-2-*methyl*-5-*oxoimidazo*[1,5-c]*pyrimidinium iodide (41)*

Methyl iodide (3.3 g, 23.5 mmol) was added to a solution of (+)-(7S)-5,6,7,8-tetrahydro-7-(methoxycarbonyl)-5-oxoimidazo[1,5-

c]pyrimidine (40) (0.29 g, 1.5 mmol) in DMF (3 ml). The mixture was heated at reflux for 2.5 h. It was then cooled to room temperature and diethyl ether (15 ml) was added. The oil crystallised on standing at 0 °C overnight. The product (41) was recrystallised from methanol (0.47 g, 93%); m.p. 160-162 °C; $[\alpha]_D^{20}$ +43.4°, (*c*, 1.0 in H_2O), lit.,[153] m.p. 162 °C (dec.), $[\alpha]_D^{25}$ +46.0° (*c*, 0.7 in H_2O); υ_{max} (KBr disc) 3095, 1760 and 1700 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.67 ppm) 3.38-3.41 (2H, AB part of an ABX system), 3.67 (3H, s), 3.87 (3H, s), 4.62-4.67 (1H, X part of an ABX system), 7.32 (1H, s) and 9.26 (1H, s); δ_C (D_2O; dioxan was the external standard at 67.8 ppm) 22.6 (t, $CH\underline{C}H_2$), 38.0 (q, $N\underline{C}H_3$), 53.1 (d, $\underline{C}HCH_2$), 55.0 (q, $O\underline{C}H_3$), 121.9 (d, C-4), 129.5 (s, C-5), 136.4 (d, C-2), 146.8 (s, $N\underline{C}ONH$) and 172.4 (s, $\underline{C}O_2CH_3$); *m/z* 210 (M^+, 8%), and 95 (32%). Found: M^+, 210.0881. $C_9H_{12}N_3O_3$ required *M*, 210.0879.

3-Methylhistidine (39) dihydrochloride

(+)-(7S)-5,6,7,8-Tetrahydro-7-(methoxycarbonyl)-2-methyl-5-oxoimidazo[1,5-c]pyrimidinium iodide (41) (0.4 g, 1.2 mmol) was dissolved in hydrochloric acid (6M; 5 ml). The solution was heated at reflux for 8 h. The solution was concentrated under reduced pressure to yield 3-methylhistidine (39) dihydrochloride as a glassy solid. The product was crystallised from ethanol upon the addition of diethyl ether (0.23 g, 79%); m.p. 254-256 °C, lit.,[153] m.p. 258-259 °C (dec.), $[\alpha]_D^{25}$ +8.2° (*c*, 2.7 in H_2O); R_F 0.57 (TLC solvent system 3); υ_{max} (KBr disc) 2940, 2500-2800 and 1630 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.67 ppm) 2.97-3.13 (2H, AB part of an ABX system), 3.59 (3H, s), 4.16-4.22 (1H, X part of an ABX system), 7.07 (1H, s) and 8.05 (1H, s); δ_C (D_2O; dioxan

was the external standard at 67.8 ppm) 27.6 (t, CH$\underline{C}H_2$), 36.2 (q, N$\underline{C}H_3$), 54.9 (d, $\underline{C}HCH_2$), 122.4 (d, C-4), 130.4 (s, C-5), 137.3 (d, C-2) and 173.9 (s, $\underline{C}O_2H$); *m/z* 169 (M^+, 1.9%) and 95 (43%). Found: M^+, 169.0848. $C_7H_{11}N_3O_2$ required *M*, 169.0851.

Method 2

*1-Benzoxymethyl-*N(α)-t-*butoxycarbonyl-L-histidine methyl ester hydrochloride (42).*[49]

Benzyl chloromethyl ether (0.33 ml, 2.37 mmol) was added to a solution of *N*(α),3-bis(*t*-butoxycarbonyl)-L-histidine methyl ester (34) (0.58 g, 1.5 mmol) in dichloromethane (3.6 ml) and the mixture was set aside overnight. The solution was concentrated and the residue was dissolved in methanol (0.6 ml). Diethyl ether (7.2 ml) was added to produce a slightly turbid solution from which 1-benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-L-histidine methyl ester hydrochloride (42) crystallised (0.52 g, 82%); m.p. 138-140 °C; $[\alpha]_D^{20}$ -16° (*c*, 1.0 in methanol), lit.,[49] m.p. 152 °C, $[\alpha]_D^{20}$ -19.1° (*c*, 1.0 in methanol); υ_{max} (KBr disc) 3300, 3100, 3000, 1745, 1720 and 1370 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.38 (9H, s), 3.02-3.40 (2H, AB part of an ABX system), 3.72 (3H, s), 4.48-4.62 (1H, X part of an ABX system), 4.68 (2H, d), 5.82 (2H, s), 7.18 (1H, s), 7.29 (5H, s) and 9.78 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 27.3 (t, $\underline{C}H_2CH$), 28.8 (q, C($\underline{C}H_3)_3$), 52.9 (d, $CH_2\underline{C}H$), 53.7 (q, O$\underline{C}H_3$), 72.8 (t, O$\underline{C}H_2$N), 77.1 (t, Ph$\underline{C}H_2$O), 81.5 (s, $\underline{C}(CH_3)_3$), 119.2 (d, C-4), 128.8 (d, Ph), 129.2 (d, Ph), 129.3 (d, Ph), 130.6 (s, C-5), 136.2 (s, Ph), 136.9 (d, C-2),155.8 (s, NH$\underline{C}O_2$) and 171.6 (s, $\underline{C}O_2$Me);

m/z 389 (M^+, 9%), 202 (26%) and 91 (100%). Found: M^+, 389.1934. $C_{20}H_{27}N_3O_5$ required *M*, 389.1950.

*1-Benzoxymethyl-*N(α)-t-*butoxycarbonyl-3-methyl-L-histidinium methyl ester iodide (43)*

1-Benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-L-histidine methyl ester hydrochloride (42) (0.21 g, 2 mmol) was dissolved in DMF (2 ml) containing triethylamine (0.20 g, 2 mmol). After a few min stirring, triethylammonium chloride was removed by filtration. Methyl iodide (4.41 g, 31 mmol) was added to the filtrate and the solution was heated at reflux (45 °C) for 3 h. The reaction mixture was concentrated and the residue was dissolved in acetone. The product crystallised on addition of diethyl ether (0.95 g, 89%); m.p.134-136 °C; $[\alpha]_D^{20}$ -13.7° (*c*, 0.9 in methanol); υ_{max} (KBr disc) 3390, 3090, 2970, 1735, 1710, 1510 and 1500 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.44 (9H, s), 3.10-3.45 (2H, AB part of an ABX system), 3.80 (3H, s), 3.96 (3H, s), 4.50-4.60 (1H, X part of an ABX system), 4.85 (2H, s), 5.1 (br s, NH), 5.89 (2H, s), 7.17 (1H, s), 7.35 (5H, m) and 10.24 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 27.5 (t, $CH\underline{C}H_2$), 28.8 (q, $C(\underline{C}H_3)_3$), 37.5 (q, $N\underline{C}H_3$), 52.9 (d, $\underline{C}HCH_2$), 53.8 (q, $O\underline{C}H_3$), 73.3 (t, $O\underline{C}H_2N$), 77.5 (t, $Ph\underline{C}H_2$), 81.4 (s, $\underline{C}(CH_3)_3$), 122.6 (d, C-4), 128.9 (d, Ph), 129.0 (d, Ph), 129.3 (d, Ph), 132.1 (s, Ph), 136.4 (s, C-5), 138.4 (d, C-2), 156.1 (s, $\underline{C}O_2C(CH_3)_3$) and 171.5 (s, $\underline{C}O_2CH_3$); *m/z* 283 (3.4%), 226 (4.5%), 183 (1.2%), 166 (26.5%), 124 (15%) and 95 (32%).

General Procedure 8

Base Hydrolysis of Methyl Ester Derivatives

The methyl ester derivative (5 mmol) was dissolved in methanol (15 ml). Sodium hydroxide solution (1M; 5.7 ml) was added and the mixture was stirred at room temperature for 10 min. The solution was then adjusted to pH 4 upon the dropwise addition of hydrochloric acid (1M). The solution was then extracted with chloroform (3 x 10 ml). The combined chloroform extracts were dried, filtered and concentrated at reduced pressure to yield the acid.

*1-Benzoxymethyl-3-methyl-*N*(α)-t-butoxycarbonyl-histidinium iodide*

1-Benzoxymethyl-3-methyl-N(α)-*t*-butoxycarbonyl-histidinium methyl ester iodide (43) (2.65 g, 5 mmol) was treated with sodium hydroxide according to the method described in general procedure 8. Concentration of the organic solution yielded 1-benzoxymethyl-3-methyl-*N*(α)-*t*-butoxycarbonyl histidinium iodide as an oil (2.05 g, 79%); δ_H (D_2O; acetone was the internal standard at 2.28 ppm) 1.30 (9H, s), 3.37-3.62 (2H, AB part of an ABX system), 3.87 (3H, s), 4.32-4.40 (1H, s), 4.81 (2H, s), 5.78 (2H, s), 7.45 (6H, aromatic) and 8.85 (1H, s); δ_C (D_2O; acetone was the internal standard at 32.3 ppm) 26.1 (t, $CH\underline{C}H_2$), 31.6 (q, $C(\underline{C}H_3)_3$), 37.9 (q, $N\underline{C}H_3$), 53.7 (d, $\underline{C}HCH_2$), 72.0 (s, $\underline{C}(CH_3)_3$), 74.5 (t, $O\underline{C}H_2N$), 78.3 (t, $Ph\underline{C}H_2$), 125.6 (d, C-4), 130.4 (d, Ph), 130.6 (s, Ph), 130.8 (d, Ph), 130.9 (d, Ph), 137.5 (s, C-5), 139.6 (d, C-2), 156.7 (s, $\underline{C}O_2C(CH_3)_3$) and 172.6 (s, $\underline{C}O_2H$).

Attempts to Remove the Benzoxymethyl group

(A).1-Benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-3-methyl histidinium iodide (2.0 g, 4 mmol) was dissolved in methanol (50 ml). Ammonium formate (1.6 g, 25 mmol) and palladium on carbon (10%; 3 g) was added and the mixture was heated at reflux. Aliquots were removed for TLC. No reaction was observed after 24 h. Starting material was recovered on filtration and evaporation of the solvent.

(B).1-Benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-3-methyl histidinium iodide (2.0 g, 4 mmol) was dissolved in 80% aqueous acetic acid (25 ml). Palladium on carbon (10%; 100 mg) was added and the suspension was hydrogenated at atmospheric pressure. Even after 48 h only starting material was recovered.

(C).1-Benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-3-methyl histidinium iodide (2.0 g, 4 mmol) was dissolved in 80% aqueous acetic acid (25 ml). Palladium on carbon (10%; 100 mg) was added and the suspension was hydrogenated under pressure (2 atm.).

(D).1-Benzoxymethyl-*N*(α)-*t*-butoxycarbonyl-3-methyl histidinium iodide (340 mg, 0.68 mmol) was dissolved in methanol (5 ml). Hydrochloric acid (6M; 10 ml) was added and the solution was heated at reflux for 24 h. The mixture was then cooled and filtered. The filtrate was concentrated under reduced pressure to yield an oil.

6.3.3 Dipeptides Containing 1-Methylhistidine

General procedure 9

N-*Carbobenzoxy-protected Amino Acids*

The amino acid (22 mmol) was dissolved in sodium hydroxide solution (2M; 11.1 ml) and the mixture was cooled to 0 °C. With good stirring and cooling, benzyl chloroformate (4.1 g, 24 mmol) was added dropwise over 45 min simultaneously with sodium hydroxide solution (4M; 5.6 ml). Stirring was continued for a further 30 min. The reaction mixture was washed several times with diethyl ether (5 ml) to remove unreacted benzyl chloroformate. The aqueous solution was acidified to Congo Red on addition of hydrochloric acid (6M).

General procedure 10

Mixed Anhydride Coupling of N-*Carbobenzoxy-protected Amino Acids to* L-*Histidine Methyl Ester*

L-Histidine methyl ester dihydrochloride (1.21 g, 5 mmol) was dissolved in methanol (10 ml) containing triethylamine (1.01 g, 10 mmol). The solution was stored at 0 °C until it was required. The *N*-carbobenzoxy-protected amino acid (6 mmol) was dissolved in acetonitrile (15 ml) containing triethylamine (0.61 g, 6 mmol). This solution was cooled to -5 °C with the aid of an ice/methanol bath. Isobutyl chloroformate (0.82 g, 6 mmol) was added to the cooled solution with stirring. Stirring was continued for 5 min. At the end of this time the mixture was added to the cooled solution containing L-histidine methyl ester with vigorous stirring. The combined mixture was set aside for 2 h at 0 °C. The solvent was then removed under reduced pressure to yield an oily residue.

The residue was partitioned between ethyl acetate (20 ml) and water (10 ml). The aqueous layer was extracted with ethyl acetate (2 x 10 ml) and the combined organic fractions were dried and concentrated.

N-*Carbobenzoxy-3-tritylhomocarnosine methyl ester*

Triphenylmethylchloride (1.4 g, 5 mmol) was dissolved in toluene (5 ml) containing triethylamine (0.52 g, 5 mmol). *N*-Carbobenzoxyhomocarnosine methyl ester (1.9 g, 5 mmol) was powdered and added to the solution. The suspension was heated at reflux for 1 h. At the end of this time, the mixture was filtered, and the filtrate was washed with water (5 ml), dried and concentrated. The residue was treated several times with diethyl ether (10 ml). Each time the solvent was removed on evaporation under reduced pressure until the product was obtained as a crispy foam (1.7 g, 54%); δ_H (90 MHz) ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm)1.9 (2H, m), 2.4 (2H, t), 3.1-3.3 (4H, m), 3.6 (3H, s), 5.0 (1H, m), 7.0-7.5 (22H, m).

Attempted N-*methylation of* N-*carbobenzoxy-3-trityl homocarnosine methyl ester*

N-Carbobenzoxy-3-tritylhomocarnosine methyl ester (0.14 g, 0.2 mmol) was treated with methyl iodide according to the method described in general procedure 5. The reaction was monitored by TLC (solvent system 1). Even after 48 h, very little product had formed.

*3-Acetyl-*N*-carbobenzoxy-homocarnosine methyl ester*

N-Carbobenzoxy-L-homocarnosine methyl ester (0.2 g, 0.5 mmol) was added to acetic anhydride (0.6 ml) and the mixture was stirred at room temperature for 10 min. The solvent was then removed under reduced pressure at temperatures below 40 °C to yield the product as an oil (0.14 g, 64%); $[\alpha]_D^{20}$ +12.3° (*c*, 0.8 in $CHCl_3$); δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.84 (2H, m), 2.32 (2H, t), 2.50 (3H, s), 3.13 (2H, t), 3.16-3.23 (2H, AB part of an ABX system), 3.69 (3H, s), 4.85-4.89 (1H, X part of an ABX system), 5.06 (2H, s), 7.28 (1H, s), 7.34 (5H, s) and 8.31 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 23.2 (q, $CO\underline{C}H_3$), 26.0 (t, $CH\underline{C}H_2$), 29.6 (t, $CH_2\underline{C}H_2CH_2$), 33.9 (t, $CO\underline{C}H_2$), 40.6 (t, $\underline{C}H_2NH$), 52.5 (d, $\underline{C}HCH_2$), 53.3 (q, $CO_2\underline{C}H_3$), 67.3 (t, $\underline{C}H_2Ph$), 116.4 (d, C-4), 128.8 (d, Ph), 129.1 (d, Ph), 129.2 (d, Ph), 134.5 (s, C-5), 137.0 (d, C-2), 137.3 (s, Ph), 157.5 (s, $\underline{C}O_2CH_2$), 172.1 (s, $NH\underline{C}OCH_2$) and 173.2 (s, $\underline{C}O_2CH_3$).

Attempted synthesis of N*-carbobenzoxy-1-methylhomocarnosine methyl ester*

3-Acetyl-*N*-carbobenzoxyhomocarnosine methyl ester (50 mg, 0.1 mmol) was treated with methyl iodide according to the method described in general procedure 5. On examination of the mixture at the end of the reaction both the 1- and 3-isomers had been formed.

General procedure 11

Introduction of an N-t-*Butoxycarbonyl Group onto the Imidazole Ring of* N-*carbobenzoxy-protected Dipeptides Containing* L-*Histidine*

The *N*-carbobenzoxy-protected dipeptide (2.6 mmol) was dissolved in methylene chloride (20 ml) containing diazobicyclo[2.2.2]octane (DABCO) (0.29 g, 2.6 mmol). Di-*t*-butyl dicarbonate (1.29 g, 3.5 mmol) was added with stirring. Stirring was continued at room temperature for several h. The progress of the reaction was monitored by TLC (solvent system 2). Two isomers were formed during the course of the reaction. These were separated in two batches using positive pressure column chromatography. The column dimensions were 20 cm by 1.5 cm. Ethyl acetate was the solvent used to elute the column.

General Procedure 12

Removal of CBZ-Group

(A). The *N*-CBZ-protected derivative (5 mmol) was dissolved in methanol (5 ml). Ammonium formate (0.15 g, 2.4 mmol) and palladium on carbon (10%; 0.17 g) were added. The mixture was heated at reflux until TLC (solvent system 1) showed cleavage of the CBZ-group was complete. The mixture was filtered and concentrated to yield the free dipeptide.

(B). The *N*-CBZ-protected dipeptide derivative (5 mmol) was dissolved in methanol (10 ml) or hydrochloric acid (10 ml). Palladium on carbon (10%; 50 mg) was added and the solution was hydrogenated until cleavage of the CBZ-group was complete. The mixture was filtered through celite and the filtrate was

concentrated under reduced pressure to yield the dipeptide derivative.

(C). The *N*-CBZ-protected dipeptide derivative (5 mmol) was dissolved in a small amount of methanol. Hydrochloric acid (6M; 15 ml) was added and the mixture was heated at reflux for 6 h or until TLC (solvent system 1) indicated cleavage was complete. The solution was then concentrated under reduced pressure to yield the dipeptide derivative as a dihydrochloride salt.

N-*Carbobenzoxyglycine (45, n=1)*

Glycine (1.65 g, 22 mmol) was derivatised using general procedure 9. On acidification the product separated as an oil. On storing at 0 °C for several h crystallisation of the oil occurred. Recrystallisation from methanol containing a few drops of water yielded *N*-carbobenzoxyglycine (45, n=1) (3.36 g, 73%); m.p. 123-125 °C; υ_{max} (KBr disc) 3340, 1730, 1710, 1695, 1680 and 1540 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.5 ppm) 4.03 (2H, s), 5.27 (2H, s), 7.51 (5H, m); δ_C (CD_3OD; CD_2HOD was the internal standard at 49 ppm) 43.4 (t, $NH\underline{C}H_2$), 68.0 (t, $\underline{C}H_2Ph$), 129.0 (d, Ph), 129.3 (d, Ph), 129.7 (d, Ph), 138.2 (s, Ph), 159.3 (s, $NH\underline{C}O$) and 174.0 (s, $\underline{C}O_2H$); *m/z* 209 (M^+, 16%), 108 (100%) and 91 (86%). Found: M^+, 209.0682. Calcd. for $C_{10}H_{11}NO_4$; *M*, 209.0688.

N(α)-*Carbobenzoxyglycyl-L-histidine methyl ester (46, n=1)*

N-Carbobenzoxyglycine (45, n=1) (1.56 g, 7 mmol) was coupled to L-histidine methyl ester according to general procedure 10. The residue obtained was extracted with hot diethyl ether. It was subsequently crystallised from chloroform on the addition of

diethyl ether to yield *N*(α)-carbobenzoxyglycyl-L-histidine methyl ester (46, n=1) (1.21 g, 67%); m.p. 89-91 °C; $[\alpha]_D^{20}$ -2.3° (*c*, 1.0 in 83% aqueous ethanol); υ_{max} (KBr disc) 3320, 1745, 1700, 1660 and 1435 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.5 ppm) 3.16-3.38 (AB part of an ABX system), 3.86 (3H, s), 3.99 (2H, s), 4.87-4.93 (X part of an ABX system), 5.28 (2H, s), 7.11 (1H, s), 7.49 (5H, s) and 7.88 (1H, s); δ_C (CD_3OD; CD_2HOD was the internal standard at 49.3 ppm) 30.0 (t, $CH\underline{C}H_2$), 45.0 (t, $\underline{C}H_2NH$), 53.2 (q, $CO_2\underline{C}H_3$), 54.1 (d, $\underline{C}HCH_2$), 68.1 (t, $\underline{C}H_2Ph$), 118.5 (d, C-4), 129.1 (d, Ph), 129.3 (d, Ph), 129.8 (d, Ph), 134.1 (s, C-5), 136.5 (d, C-2), 138.3 (s, Ph), 159.2 (s, $NH\underline{C}O_2CH_2$), 172.4 (s, $NH\underline{C}OCH_2$) and 173.2 (s, $\underline{C}O_2CH_3$); *m/z* 360 (M^+, 3.4%), 152 (51%), 108 (100%), 91 (79%) and 81 (80%). Found: M^+, 360.1422. Calcd. for $C_{17}H_{20}N_4O_5$; *M*, 360.1434.

1-Methylglycylhistidine (50)

3-t-*Butoxycarbonyl*-N(α)-*carbobenzoxyglycyl*-L-*histidine methyl ester (48, n=1)*

General procedure 11 was followed to react *N*(α)-carbobenzoxyglycyl-*L*-histidine methyl ester (46, n=1) (1.79 g, 5 mmol) with di-*t*-butyl dicarbonate. The fractions containing the more polar product, R_F 0.2 (TLC system 2), were pooled and the solvent was concentrated to yield the product (48, n=1) as a colourless oil (1.0 g, 44%); $[\alpha]_D^{20}$ +12° (*c*, 0.9 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3450, 3040, 1760, 1740, 1690 and 1510 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.60 (9H, s), 3.07-3.15 (2H, AB part of an ABX system), 3.70 (3H, s), 3.93 (2H, m), 4.81-4.87 (1H, X part of an ABX system), 5.11 (2H, s), 5.61 (1H, br

s), 7.19 (1H, s), 7.33 (5H, s) and 8.08 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 28.5 (q, $C(\underline{C}H_3)_3$), 29.4 (t, $CH\underline{C}H_2$), 45.1 (t, $\underline{C}H_2NH$), 52.6 (q, $CO_2\underline{C}H_3$), 53.3 (d, $\underline{C}HCH_2$), 67.7 (t, $\underline{C}H_2Ph$), 87.6 (s, $\underline{C}(CH_3)_3$), 115.8 (d, C-4), 128.7 (d, Ph), 128.8 (d, Ph), 129.2 (d, Ph), 136.9 (s, Ph), 137.1 (d, C-2), 137.7 (s, C-5), 146.7 (s, $\underline{C}OC(CH_3)_3$), 157.1 (s, $\underline{C}OCH_2Ph$), 169.7 (s, $NH\underline{C}OCH_2$) and 171.8 (s, $\underline{C}O_2CH_3$); *m/z* 460 (M^+, 0.3%), 152 (38%), 108 (79%), 91 (100%) and 81 (50%). Found: M^+, 460.1936. Calcd. for $C_{22}H_{28}N_4O_7$; *M*, 460.1958.

N(α)-*Carbobenzoxyglycyl*-1-*methyl*-L-*histidine methyl ester (49, n=1)*

3-t-Butoxycarbonyl-*N*(α)-carbobenzoxyglycyl-L-histidine methyl ester (48, n=1) (0.87 g, 1.9 mmol) was methylated according to general procedure 5. The *N*-methylated product (49, n=1) was obtained as an oil (0.46 g, 65%); $[\alpha]_D^{20}$ -3.8° (*c*, 1.1 in ethanol); υ_{max} ($CHCl_3$ solution) 3010, 2950, 1720, 1680 and 1500 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 3.09-3.12 (2H, AB part of an ABX system), 3.55 (3H, s), 3.72 (3H, s), 3.85 (2H, d), 4.72-4.86 (X part of an ABX system), 5.08 (2H, s), 5.95 (1H, NH), 6.85 (1H, s), 7.31 (5H, s) and 7.68 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 26.7 (t, $CH\underline{C}H_2$), 32.2 (q, $N\underline{C}H_3$), 44.8 (t, $\underline{C}H_2NH$), 52.2 (d, $\underline{C}HCH_2$), 53.3 (q, $CO_2\underline{C}H_3$), 67.7 (t, $\underline{C}H_2Ph$), 127.3 (d, C-4), 127.6 (s, Ph), 128.6 (d, Ph), 128.8 (d, Ph), 129.1 (d, Ph), 136.8 (s, C-5), 138.5 (d, C-2), 157.3 (s, $\underline{C}O_2CH_2Ph$), 170.2 (s, $NH\underline{C}OCH_2$) and 172.0 (s, $\underline{C}O_2CH_3$); *m/z* 374 (M^+, 23%), 166 (90%), 108 (29%), 95(100%) and 91 (87%). Found: M^+, 374.1592. Calcd. for $C_{18}H_{22}N_4O_5$; *M*, 374.1590.

1-*Methylglycylhistidine (50) dihydrochloride*

N(α)-Carbobenzoxyglycyl-1-methyl-L-histidine methyl ester (49, n=1) (0.5 g, 1.3 mmol) was treated with hydrochloric acid according to general procedure 6. The residue obtained at the end of the reaction was dissolved in ethanol several times. Each time the solution was concentrated under reduced pressure. 1-Methylglycylhistidine (50) dihydrochloride was obtained as a hygroscopic solid (260 mg, 89%); R_F 0.57 (TLC solvent system 3); υ_{max} (KBr disc) 3400, 3060, 1740, 1680, 1550 and 1430 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.67 ppm) 3.15-3.37 (2H, AB part of an ABX system), 3.72 (3H, s), 3.73 (2H, s), 4.21-4.28 (1H, X part of an ABX system), 7.32 (1H, s) and 8.57 (1H, s); δ_C (D_2O; dioxan was the external standard at 67.8 ppm) 24.8 (t, $CH\underline{C}H_2$), 34.7 (q, $N\underline{C}H_3$), 41.2 (t, $\underline{C}H_2NH$), 52.3 (d, $\underline{C}HCH_2$), 120.1 (d, C-4), 129.4 (s, C-5), 137.2 (d, C-2), 169.9 (s, $NH\underline{C}O$) and 171.3 (s, $\underline{C}O_2H$); *m/z* 226 (M^+, 2.5%) and 95 (100%). Found: M^+, 226.1067. Calcd. for $C_9H_{14}N_4O_3$; *M*, 226.1066.

N-*Carbobenzoxy-β-alanine (45, n=2)*

β-Alanine (1.96 g, 22 mmol) was reacted with benzyl chloroformate as described in general procedure 9. On acidification the product precipitated as a white solid. Recrystallisation from methanol containing a few drops of water yielded *N*-carbobenzoxy-β-alanine (45, n=2) (3.97 g, 81%); m.p. 106-108 °C; υ_{max} (KBr disc) 3340, 1690, 1680 and 1535 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.5 ppm) 2.69 (2H, t), 3.56 (2H, t), 5.25 (2H, s) and 7.51 (5H, s); δ_C (CD_3OD; CD_2HOD was the internal standard at 49.3 ppm) 35.5 (t, $NHCH_2\underline{C}H_2$), 38.0 (t, $NH\underline{C}H_2CH_2$), 67.7 (t, $\underline{C}H_2PH$), 129.0 (d, Ph), 129.2 (d, Ph), 129.7 (d,

Ph), 138.5 (s, Ph), 159.0 (s, NH<u>C</u>O) and 175.8 (s, <u>C</u>O_2H); *m/z* 223 (M^+, 9%), 108 (100%) and 91 (81%). Found: M^+, 223.0845. Calcd. for $C_{11}H_{13}NO_4$; *M*, 223.0845.

N-*Carbobenzoxy-L-carnosine methyl ester (46, n=2)*

Using general procedure 10, *N*-carbobenzoxy-β-alanine (45, n=2) (1.34 g, 6 mmol) was coupled to L-histidine methyl ester. The resultant residue was heated with diethyl ether. The diethyl ether was subsequently decanted off. The oil was then crystallised from ethanol on the addition of diethyl ether to yield *N*-carbobenzoxy-L-carnosine methyl ester (46, n=2) (1.35 g, 72%); m.p. 92-94 °C; $[\alpha]_D^{20}$ -4.4° (*c*, 1.0 in 83% aqueous ethanol); υ_{max} (KBr disc) 3340, 1750, 1695, 1660, 1645 and 1530 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.5 ppm) 2.60 (2H, t), 3.12-3.34 (2H, AB part of an ABX system), 3.54 (2H, t), 3.62-3.72 (1H, s), 5.25 (2H, s), 7.06 (1H, s), 7.51 (5H, s) and 7.78 (1H, s); δ_C (CD_3OD; CD_2HOD was the internal standard at 49.3 ppm) 30.3 (t, CH<u>C</u>H_2), 37.1 (t, CO<u>C</u>H_2), 38.6 (t, <u>C</u>H_2NH), 53.1 (q, CO_2<u>C</u>H_3), 54.3 (d, <u>C</u>HCH_2), 67.7 (t, <u>C</u>H_2Ph), 118.3 (d, C-4), 129.1 (d, Ph), 129.3 (d, Ph), 129.8 (d, Ph), 135.0 (s, C-5), 136.6 (d, C-2), 138.5 (s, Ph), 159.0 (s, NH<u>C</u>O_2CH_2), 173.7 (s, NH<u>C</u>OCH_2) and 174.0 (s, <u>C</u>O_2CH_3); *m/z* 374 (M^+, 12%), 152 (58%), 108 (93%), 91 (100%) and 81 (36%). Found: M^+, 374.1587. Calcd. for $C_{18}H_{22}N_4O_5$; *M*, 374.1590.

1-Methylcarnosine (3)

3-t-*Butoxycarbonyl*-N-*carbobenzoxy*-L-*carnosine methyl ester (48, n=2)*

Di-*t*-butyl dicarbonate was reacted with *N*-carbobenzoxy-*L*-carnosine methyl ester (46, n=2) (1.9 g, 5 mmol) according to general procedure 11. The fractions containing the more polar product, R_F 2.3 (TLC solvent system 2), were pooled and the solvent was removed under reduced pressure. *3-t*-Butoxycarbonyl-*N*-carbobenzoxy-*L*-carnosine methyl ester (48, n=2) was isolated as a colourless oil (1.26 g, 53%); $[\alpha]_D^{20}$ +17.1° (*c*, 0.96 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3325, 2982, 2951, 1755, 1722, 1672 and 1531 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.59 (9H, s), 2.47 (2H, t), 3.02-3.13 (2H, AB part of an ABX system), 3.49 (2H, t), 3.70 (3H, s), 4.74-4.84 (1H, X part of an ABX system), 5.09 (2H, s), 5.96 (1H, NH), 7.16 (1H, s), 7.32 (5H, s) and 8.05 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 28.5 (q, $C(\underline{C}H_3)_3$), 29.5 (t, $CH\underline{C}H_2$), 37.0 (t, $CO\underline{C}H_2$), 38.0 (t, $\underline{C}H_2NH$), 52.9 (q, $CO_2\underline{C}H_3$), 53.3 (d, $\underline{C}HCH_2$), 67.1 (t, $\underline{C}H_2Ph$), 86.5 (s, $\underline{C}(CH_3)_3$), 115.6 (d, C-4), 128.6 (d, Ph), 128.7 (d, Ph), 129.2 (d, Ph), 137.3 (s, C-5), 137.8 (d, C-2), 139.0 (s, Ph), 147.3 (s, $NH\underline{C}O_2C(CH_3)_3$), 157.2 (s, $NH\underline{C}O_2CH_2Ph$), 172.1 (s, $NH\underline{C}OCH_2$) and 172.3 (s, $\underline{C}O_2CH_3$); *m/z* 474 (M^+, 4.5%), 374 (24%), 168 (19%), 108 (35%), 91 (100%) and 81 (56%). Found: M^+, 474.2101. Calcd. for $C_{23}H_{30}N_4O_7$; *M*, 474.2114.

N-*Carbobenzoxy*-1-*methyl*-L-*carnosine methyl ester (49 n=2)*

3-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-carnosine methyl ester (48, n=2) (1.2 g, 2.5 mmol) was methylated according to the

method described in general procedure 5. The oil obtained at the end of the reaction was dissolved in ethyl acetate (3 ml) and this solution was extracted with aqueous ammonia. The organic protion was dried, filtered and evaporated to yield *N*-Carbobenzoxy-1-methyl-L-carnosine methyl ester (49, n=2) as an oil. (0.5 g, 58%); $[\alpha]_D^{20}$ -4.0° (*c*, 0.7 in ethanol); υ_{max} ($CHCl_3$ solution) 3040, 2985, 1740, 1680 and 1510 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 2.45 (2H, t), 3.09-3.13 (2H, AB part of an ABX system), 3.41 (2H, t), 3.61 (3H, s), 3.73 (3H, s), 4.73-4.83 (1H, X part of an ABX system), 5.07 (2H, s), 5.51 (1H, NH), 6.93 (1H, s), 7.33 (5H, s) and 7.79 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 26.8 (t, $CH\underline{C}H_2$), 33.2 (q, $N\underline{C}H_3$), 36.4 (t, $CO\underline{C}H_2$), 37.7 (t, $\underline{C}H_2NH$), 51.9 (d, $\underline{C}HCH_2$), 53.5 (q, $CO_2\underline{C}H_3$), 67.3 (t, $\underline{C}H_2Ph$), 125.5 (d, C-4), 128.3 (s, C-5), 128.8 (d, Ph), 129.0 (d, Ph), 129.2 (d, Ph), 137.2 (s, Ph), 137.8 (d, C-2), 157.1 (s, $NH\underline{C}O_2CH_2Ph$), 171.8 (s, $NH\underline{C}OCH_2$) and 172.2 (s, $\underline{C}O_2CH_3$); *m/z* 388 (M^+, 14%), 329 (17%), 166 (54%), 108 (45%), 95 (100%) and 91 (62%). Found: M^+, 388.1752. Calcd. for $C_{19}H_{24}N_4O_5$; *M*, 388.1747.

1-*Methylcarnosine (3) dihydrochloride*

N-Carbobenzoxy-1-methyl-L-carnosine methyl ester (49, n=2) (0.4 g, 1 mmol) was treated with hydrochloric acid (6M) according to general procedure 6. The residue obtained at the end of the reaction was treated with ethanol and the solution was concentrated several times to yield 1-methylcarnosine (3) dihydrochloride as a hygroscopic solid (0.2 g, 86%); R_F 0.56 (TLC solvent system 3); υ_{max} (KBr disc) 3420, 3040. 1740, 1660 and 1550 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.67 ppm) 2.54 (2H, t), 2.99-3.25 (2H, AB part of an ABX system), 3.04 (2H,

t), 3.67 (3H, X part of an ABX system), 7.14 (1H, s) and 8.47 (1H, s); δ_C 26.6 (t, CH$\underline{C}$H$_2$), 32.7 (t, CO$\underline{C}$H$_2$), 34.0 (q, N$\underline{C}$H$_3$), 36.4 (t, $\underline{C}$H$_2$NH), 54.0 (d, $\underline{C}$HCH$_2$), 118.2 (d, C-4), 132.0 (s, C-5), 135.8 (d, C-2), 172.5 (s, NH$\underline{C}$OCH$_2$) and 177.2 (s, $\underline{C}$O$_2$H); *m/z* 240 (M^+, 1.4%) and 95 (100%). Found: M^+, 240.1223. $C_{10}H_{16}N_4O_3$ required *M*, 240.1222.

N-*Carbobenzoxy-4-aminobutyric acid (45, n=3)*

General procedure 9 was used to derivatise 4-aminobutyric acid (2.27 g, 22 mmol). On acidification the product separated as an oil. On storing at 0 °C for several h the product crystallised. Recrystallisation from methanol containing a few drops of water yielded *N*-carbobenzoxy-4-aminobutyric acid (45, n=3) (3.97 g, 76%); m.p. 61-62 °C; υ_{max} (KBr disc) 3340, 1690, 1555 and 1455 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.5 ppm) 1.96 (2H, m), 2.50 (2H, t), 3.34 (2H, t), 5.24 (2H, s) and 7.50 (5H, m); δ_C (CD_3OD; CD_2HOD was the internal standard at 49.3 ppm) 26.5 (t, NHCH$_2\underline{C}$H$_2$CH$_2$), 32.3 (t, NHCH$_2$CH$_2\underline{C}$H$_2$), 41.4 (t, NH$\underline{C}$H$_2$CH$_2$CH$_2$), 67.6 (t, $\underline{C}$H$_2$Ph), 129.0 (d, Ph), 129.2 (d, Ph), 129.7 (d, Ph), 138.6 (s, Ph), 159.1 (s, NH$\underline{C}$O) and 177.3 (s, $\underline{C}$O$_2$H); *m/z* 237 (M^+, 4%), 112 (4%), 108 (100%) and 91 (89%). Found: M^+, 237.0998. Calcd. for $C_{12}H_{15}NO_4$; *M*, 237.1001.

N-*Carbobenzoxy-L-homocarnosine methyl ester (46, n=3)*

N-Carbobenzoxy-4-aminobutyric acid (45, n=3) (1.42 g, 6 mmol) was coupled to L-histidine methyl ester using general procedure 10. The oil was heated with diethyl ether. The diethyl ether was decanted off and the oil was dissolved in ethanol. A crystalline solid was obtained on the addition of diethyl ether to this solution

to yield *N*-carbobenzoxy-L-homocarnosine methyl ester (46, n=3) (1.52 g, 78%); m.p. 103-105 °C; $[\alpha]_D^{20}$ -7.0° (*c*, 1.0 in 83% aqueous ethanol); υ_{max} (KBr disc) 3340, 1750, 1695, 1660, 1645 and 1530 cm^{-1}; δ_H (CD_3OD; CD_2HOD was the internal standard at 3.5 ppm) 1.92 (2H, m), 2.41 (2H, t), 3.10-3.35 (2H, AB part of an ABX system), 3.32 (2H, t), 3.89 (3H, s), 4.83-4.90 (1H, X part of an ABX system), 5.25 (2H, s), 7.07 (1H, s), 7.52 (5H, s) and 7.82 (1H, s); δ_C (CD_3OD; CD_2HOD was the internal standard at 49.3 ppm) 27.4 (t, $CH\underline{C}H_2$), 30.2 (t, $CH_2\underline{C}H_2CH_2$), 34.2 (t, $CO\underline{C}H_2$), 41.4 (t, $\underline{C}H_2NH$), 53.0 (q, $CO_2\underline{C}H_3$), 54.2 (d, $\underline{C}HCH_2$), 67.7 (t, $\underline{C}H_2Ph$), 118.4 (d, C-4), 129.1 (d, Ph), 129.2 (d, Ph), 129.7 (d, Ph), 134.7 (s, C-5), 136.6 (d, C-2), 138.7 (s, Ph), 159.2 (s, $NH\underline{C}O_2CH_2$), 173.7 (s, $NH\underline{C}OCH_2$) and 175.7 (s, $\underline{C}O_2CH_3$); *m/z* 388 (M^+, 13%), 152 (23%), 110 (19%), 108 (25%), 91 (100%) and 81 (47%). Found: M^+, 388.1745. Calcd. for $C_{19}H_{24}N_4O_5$; *M*, 388.1747.

1-Methylhomocarnosine (51)

3-t-*Butoxycarbonyl*-N-*carbobenzoxy-L-homocarnosine methyl ester (48, n=3)*

N-Carbobenzoxy-L-homocarnosine methyl ester (46, n=3) (1.2 g, 3.1 mmol) was treated with di-*t*-butyl dicarbonate according to general procedure 11. The fractions containing the more polar product R_F 0.25 (TLC solvent system 2) were pooled. The combined solution was concentrated under reduced pressure to yield 3-*t*-butoxycarbonyl-*N*-carbobenzoxy-L-homocarnosine methyl ester (48, n=3) as an oil (0.95 g, 63%); $[\alpha]_D^{20}$ +14.6° (*c*, 1.1 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3325, 2980, 2951, 1755, 1658, 1539 and 1390 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at

7.25 ppm) 1.58 (9H, s), 1.86 (2H, m), 2.29 (2H, t), 3.02 (2H, t), 3.09-3.39 (2H, AB part of an ABX system), 3.68 (3H, s), 4.80-4.90 (1H, X part of an ABX system), 5.08 (2H, s), 5.56 (1H, NH), 7.11 (1H, s), 7.34 (5H, s) and 7.95 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 26.1 (t, $CH\underline{C}H_2$), 28.5 (q, $C(\underline{C}H_3)_3$), 30.1 (t, $CH_2\underline{C}H_2CH_2$), 34.1 (t, $\underline{C}H_2CH_2CH_2$), 40.7 (t, $CH_2CH_2\underline{C}H_2$), 52.7 (d, $\underline{C}HCH_2$), 53.1 (q, $O\underline{C}H_3$), 67.2 (t, $\underline{C}H_2Ph$), 86.5 (s, $\underline{C}(CH_3)_3$), 115.3 (d, C-4), 128.7 (d, Ph), 128.9 (d, Ph), 129.1 (d, Ph), 137.3 (s, C-5), 137.7 (d, C-2), 139.1 (s, Ph), 147.4 (s, $\underline{C}O_2C(CH_3)_3$), 157.3 (s, $\underline{C}O_2CH_2Ph$), 172.4 (s, $NH\underline{C}OCH_2$) and 173.0 (s, $\underline{C}O_2CH_3$); *m/z* 488 (M^+, 0.2%), 388 (10%), 225 (9%), 108 (65%), 95 (54%) and 91 (100%). Found: M^+, 488.2282. Calcd. for $C_{24}H_{32}N_4O_7$; *M*, 488.2271.

N-*Carbobenzoxy-1-methyl-L-homocarnosine methyl ester (49, n=3)*

3-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-homocarnosine methyl ester (48, n=3) (0.8 g, 1.6 mmol) was treated with methyl iodide according to the method described in general procedure 5. The residue at the end of the reaction was dissolved in ethyl acetate (2 ml) and extracted with aqueous ammonia. The organic portion was dried, filtered and concentrated to yield *N*-carbobenzoxy-1-methyl-L-homocarnosine methyl ester (49, n=3) as an oil (0.36 g, 56%); $[\alpha]_D^{20}$ -5.8° (*c*, 0.8 in ethanol); υ_{max} (KBr disc) 3280, 3040, 2960, 1740, 1710, 1660, 1525 and 1450 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.76 (2H, m), 2.20 (2H, t), 3.04-3.19 (2H, AB part of an ABX system), 3.59 (3H, s), 3.72 (3H, s), 4.73-4.84 (1H, X part of an ABX system), 5.05 (2H, s), 6.91 (1H, s), 7.31 (5H, s) and 7.68 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 26.6 (t, $CH_2\underline{C}H_2CH_2$), 27.0 (t, $CH\underline{C}H_2$) 32.6 (q,

$N\underline{C}H_3$), 33.8 (t, $\underline{C}H_2CH_2CH_2$), 40.7 (t, $CH_2CH_2\underline{C}H_2$), 51.9 (d, $\underline{C}HCH_2$), 53.4 (q, $O\underline{C}H_3$), 67.3 (t, $\underline{C}H_2Ph$), 127.0 (d, C-4), 128.7 (d, Ph), 128.8 (d, Ph), 129.2 (d, Ph), 132.0 (s, C-5), 137.2 (s, Ph), 137.3 (d, C-2), 157.6 (s, $NH\underline{C}O_2CH_2Ph$), 172.1 (s, $NH\underline{C}OCH_2$) and 173.5 (s, $\underline{C}O_2CH_3$); *m/z* 402 (M^+, 2%), 166 (4.4%), 108 (12%) 95 (14%) and 91 (100%). Found: M^+, 402.1879. Calcd. for $C_{20}H_{26}N_4O_5$; *M*, 402.1903.

1-Methylhomocarnosine (51) dihydrochloride

N-Carbobenzoxy-1-methyl-L-homocarnosine methyl ester (49, n=3) (0.32 g, 0.8 mmol) was treated with 6M hydrochloric acid according to general procedure 6. The residue at the end of the reaction was treated several times with ethanol and concentrated under reduced pressure. 1-Methylhomocarnosine (51) dihydrochloride was obtained as a hygroscopic solid (0.18 g, 87%); R_F 0.51 (TLC solvent system 3); υ_{max} (KBr disc) 3420, 3040, 1730 and 1610 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.67 ppm) 1.76 (2H, m), 2.30 (2H, t), 2.84 (2H, t), 3.16-3.39 (2H, AB part of an ABX system), 3.68 (3H, s), 4.22-4.29 (1H, X part of an ABX system), 7.29 (1H, s) and 8.54 (1H, s); δ_C (D_2O; dioxan was the external standard at 67.8 ppm) 23.1 (t, $CH_2\underline{C}H_2CH_2$), 24.9 (t, $CH\underline{C}H_2$), 31.7 (t, $CO\underline{C}H_2$), 34.6 (q, $N\underline{C}H_3$), 39.9 (t, $\underline{C}H_2NH$), 52.3 (d, $\underline{C}HCH_2$), 120.1 (d, C-4), 129.3 (s, C-5), 137.1 (d, C-2), 171.2 (s, $NH\underline{C}OCH_2$) and 178.0 (s, $\underline{C}O_2H$); *m/z* 254 (M^+, 1.2%) and 95 (100%). Found: M^+, 254.1377. Calcd. for $C_{11}H_{18}N_4O_3$; *M*, 254.1379.

6.3.4 Dipeptides Containing 3-Methylhistidine

3-Methylglycylhistidine (53)

1-t-*Butoxycarbonyl*-N(α)-*carbobenzoxyglycyl*-L-*histidine methyl ester (47, n=1)*

N(α)-Carbobenzoxyglycyl-*L*-histidine methyl ester (46, n=1) (1.79 g, 5 mmol) was reacted with di-*t*-butyl dicarbonate as described in general procedure 11. Those fractions containing the less polar product, R_F 0.46 (TLC solvent system 2), were collected together and the solvent was removed under reduced pressure. 1-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-histidine methyl ester (47, n=1) was isolated as a colourless oil (0.85 g, 37%); $[\alpha]_D^{20}$ -29.3° (*c*, 0.98 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3040, 3000, 1820, 1760 and 1745 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.58 (9H, s), 3.32-3.54 (2H, AB part of an ABX system), 3.76 (3H, s), 4.20 (2H, d), 4.66 (2H, s), 5.03-5.14 (1H, X part of an ABX system), 7.17 (1H, s), 7.32 (5H, m) and 7.98 (1H, d); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 26.6 (t, $CH\underline{C}H_2$), 28.5 (q, $C(\underline{C}H_3)_3$), 49.3 (t, $\underline{C}H_2NH$), 53.1 d, $\underline{C}HCH_2$), 53.7 (q, $CO_2\underline{C}H_3$), 65.8 (t, $\underline{C}H_2Ph$), 85.1 (s, $\underline{C}(CH_3)_3$), 115.6 (d, C-4), 127.6 (d, Ph), 128.1 (d, Ph), 129.15 (d, Ph), 137.4 (d, C-2), 138.5 (s, C-5), 147.2 (s, Ph), 148.9 (s, $\underline{C}O_2C(CH_3)_3$), 152.0 (s, $\underline{C}O_2CH_2Ph$), 167.9 (s, $NH\underline{C}OCH_2$) and 168.9 (s, $\underline{C}O_2CH_3$); *m/z* 460 (M^+, 1.2%), 252 (30%), 193 (33%), 152 (100%), 91 (28%) and 81 (40%). Found: M^+, 460.1963. Calcd. for $C_{22}H_{28}N_4O_7$; *M*, 460.1958.

N(α)-*Carbobenzoxyglycyl-3-methyl-L-histidine methyl ester (52, n=1)*

1-*t*-Butoxycarbonyl-*N*-carbobenzoxy-L-histidine methyl ester (47, n=1) (0.8 g, 1.7 mmol) was methylated according to the method set out in general procedure 5. The oil obtained at the end of the reaction was dissolved in chloroform (3 ml) and extracted with aqueous ammonia. The organic portion was dried, filtered and concentrated under reduced pressure to yield *N*(α)-carbobenzoxyglycyl-3-methyl-L-histidine methyl ester (52, n=1) as an oil (0.3 g, 48%) $[\alpha]_D^{20}$ -18.0° (*c*, 1.3 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3020, 2990, 1750, 1730 and 1220 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 3.87-3.94 (2H, AB part of an ABX system), 4.65 (3H, s), 4.67 (3H, s), 5.07 (2H, s), 5.71 (1H, NH), 7.23 (1H, s), 7.32 (5H, s) and 7.97 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 28.5 (t, $CH\underline{C}H_2$), 37.5 (q, $N\underline{C}H_3$), 43.2 (t, $\underline{C}H_2NH$), 53.2 (q, $O\underline{C}H_3$), 53.7 (d, $\underline{C}HCH_2$), 67.6 (t, $\underline{C}H_2Ph$), 117.9 (d, C-4), 127.7 (d, Ph), 128.6 (d, Ph), 129.0 (s, Ph), 129.1 (d, Ph), 134.1 (s, C-5), 136.8 (d, C-2), 157.2 (s, $\underline{C}O_2CH_2Ph$), 172.3 (s, $NH\underline{C}OCH_2$) and 173.4 (s, $\underline{C}O_2CH_3$); *m/z* 374 (M^+, 2%), 283 (14%), 166 (73%), 108 (20%), 95 (75%) and 91 (71%). Found: M^+, 374.1589. Calcd. for $C_{18}H_{22}N_4O_5$; *M*, 374.1590.

3-*Methylglycylhistidine (53) dihydrochloride*

N(α)-Carbobenzoxyglycyl-3-methyl-L-histidine methyl ester (52, n=1) (0.25g, 0.7 mmol) was treated with 6M hydrochloric acid according to general procedure 6. The residue obtained at the end of the reaction was treated several times with ethanol and each time the solution was concentrated. 1-Methylglycylhistidine dihydrochloride was obtained as a hygroscopic solid (0.12 g, 79%);

R_F 0.63 (TLC solvent system 3); υ_{max} (thin film) 3400, 3110, 1720, 1610 and 1490 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.67 ppm) 3.18-3.56 (2H, AB part of an ABX system), 3.68 (3H, s), 3.78 (2H, s), 4.00-4.08 (1H, X part of an ABX system), 7.18 (1H, s) and 8.47 (1H, s); δ_C (D_2O; dioxan was the external standard at 67.8 ppm) 26.6 (t, CH<u>C</u>H_2), 31.9 (q, N<u>C</u>H_3), 41.2 (t, <u>C</u>H_2NH), 54.5 (d, <u>C</u>HCH_2), 114.0 (d, C-4), 127.7 (s, C-5), 130.4 (d, C-2), 169.8 (s, NH<u>C</u>O) and 171.2 (s, <u>C</u>O_2H); *m/z* 226 (M^+, 0.4%) and 95 (100%). Found: M^+, 226.1071. Calcd. for $C_9H_{14}N_4O_3$; *M*, 226.1066.

<u>3-Methylcarnosine (4)</u>

1-t-*Butoxycarbonyl*-N-*carbobenzoxy*-L-*carnosine methyl ester (47, n=2)*

N-Carbobenzoxy-L-carnosine methyl ester (46, n=2) (1.9 g, 5 mmol) was treated with di-*t*-butyl dicarbonate according to general procedure 11. The fractions containing the less polar product, R_F 0.32 (TLC solvent system 2) were combined and the solvent was concentrated to yield 1-*t*-butoxycarbonyl-L-carnosine methyl ester (47, n=2) as a colourless oil (0.64 g, 27%); $[\alpha]_D^{20}$ -10.7° (*c*, 1.0 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3440, 3020, 3000, 1755, 1680 and 1400 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 1.57 (9H, s), 2.53 (2H, t), 2.90-3.12 (2H, AB part of an ABX system), 3.66 (2H, s), 3.93 (2H, t), 4.72-4.82 (1H, X part of an ABX system), 5.19 (2H, s), 7.10 (1H, s), 7.32 (5H, m) and 7.93 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 28.5 (q, C(<u>C</u>H_3)$_3$), 30.0 (t, CH<u>C</u>H_2), 36.2 (t, CO<u>C</u>H_2), 43.5 (t, <u>C</u>H_2NH), 52.5 (d, <u>C</u>HCH_2), 53.0 (q, CO_2<u>C</u>H_3), 69.0 (t, <u>C</u>H_2Ph), 86.4 (s, <u>C</u>(CH_3)$_3$), 115.2 (d, C-4), 128.8 (d, Ph), 128.9 (d, Ph), 129.1 (d, Ph),

136.1 (s, C-5), 137.5 (d, C-2), 139.1 (s, Ph), 152.3 (s, $\underline{C}O_2C(CH_3)_3$), 154.2 (s, $\underline{C}O_2CH_2Ph$), 170.6 (s, $NH\underline{C}OCH_2$) and 172.2 (s, $\underline{C}O_2CH_3$); *m/z* 474 (M^+, 6.4%), 374 (17%), 168 (17%), 152 (50%), 108 (36%), 91 (100%) and 81 (32%). Found: M^+, 474.2122. Calcd. for $C_{23}H_{30}N_4O_7$; *M*, 474.2114.

N-*Carbobenzoxy-3-methyl-L-carnosine methyl ester (52, n=2)*

1-*t*-Butoxycarbonyl-L-carnosine methyl ester (47, n=2) (0.6 g, 1.3 mmol) was treated with methyl iodide according to the method described in general procedure 5. The residue at the end of the reaction was dissolved in chloroform (3 ml) and extracted with aqueous ammonia. The organic portion was dried, filtered and concentrated under reduced pressure to yield *N*-carbobenzoxy-3-methyl-L-carnosine methyl ester (52, n=2) as an oil (0.27 g, 54%); $[\alpha]_D^{20}$ -3.2° (*c*, 0.9 in $CHCl_3$); υ_{max} ($CHCl_3$ solution) 3440, 3040, 3000, 1790, 1750, 1690 and 1510 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 2.58 (2H, t), 3.02-3.20 (2H, AB part of an ABX system), 3.71 (3H, s), 3.73 (3H, s), 3.91 (2H, t), 4.71-4.81 (1H, X part of an ABX system), 7.04 (1H, s), 7.33 (5H, m) and 8.23 (1H, s); δ_C ($CDCl_3$; $CHCl_3$ was the internal standard at 77.7 ppm) 26.6 (t, $CH\underline{C}H_2$), 33.5 (q, $N\underline{C}H_3$), 35.9 (t, $CO\underline{C}H_2$), 43.4 (t, $\underline{C}H_2NH$), 51.9 (d, $\underline{C}HCH_2$), 53.5 (q, $O\underline{C}H_3$), 69.2 (t, $\underline{C}H_2Ph$), 122.8 (d, C-4), 128.9 (d, Ph), 129.0 (d, Ph), 129.2 (d, Ph), 129.5 (s, C-5), 136.1 (d, C-2), 137.0 (s, Ph), 154.4 (s, $NH\underline{C}O_2CH_2Ph$), 171.4 (s, $NH\underline{C}OCH_2$) and 171.7 (s, $\underline{C}O_2CH_3$); *m/z* 388 (M^+, 3%), 298 (4%), 280 (13%), 166 (100%), 108 (38%), 95 (78%) and 91 (61%). Found: M^+, 388.1738. Calcd. for $C_{19}H_{24}N_4O_5$; *M*, 388.1747.

3-Methylcarnosine (4) dihydrochloride

N-Carbobenzoxy-3-methyl-L-carnosine methyl ester (52, n=2) (0.23 g, 0.6 mmol) was treated with 6M hydrochloric acid according to general procedure 6. The residue obtained at the end of the reaction was dissolved several times in ethanol. Each time the solution was concentrated under reduced pressure to yield 3-methylcarnosine (4) dihydrochloride as a hygroscopic solid (0.12 g, 81%); R_F 0.60 (TLC solvent system 3); υ_{max} (thin film) 3380, 1730, 1610 and 1560 cm^{-1}; δ_H (D_2O; HOD was the internal standard at 4.67 ppm) 2.63 (2H, t), 3.13 (2H, t), 3.17-3.41 (2H, AB part of an ABX system), 3.71 (3H, s), 4.22-4.29 (1H, X part of an ABX system), 7.31 (1H, s) and 8.56 (1H, s); δ_C (D_2O; dioxan was the external standard at 67.8 ppm) 24.9 (t, $CH\underline{C}H_2$), 32.0 (t, $CO\underline{C}H_2$), 34.6 (q, $N\underline{C}H_3$), 36.2 (t, $\underline{C}H_2NH$), 52.4 (d, $\underline{C}HCH_2$), 120.0 (d, C-4), 129.4 (s, C-5), 137.1 (d, C-2), 171.3 (s, $NH\underline{C}OCH_2$) and 175.5 (s, $\underline{C}O_2H$), *m/z* 240 (M^+, 0.1%) and 95 (100%). Found: M^+, 240.1211. Calcd. for $C_{10}H_{16}N_4O_3$; *M*, 240.1129.

1-Benzoxymethyl-N-carbobenzoxy-L-carnosine methyl ester (55)

N-Carbobenzoxy-L-carnosine methyl ester (46, n=2) (1.1 g, 3 mmol) was added to acetic anhydride (3.5 ml). The mixture was stirred at room temperature for 10 min. The solution was then concentrated under reduced pressure to yield 3-acetyl-*N*-carbobenzoxy-L-carnosine methyl ester (54). This was not isolated but dissolved in dichloromethane (4 ml). Benzylchloromethyl ether was added dropwise to this solution with stirring. The mixture was stirred overnight at room temperature. The solution was then extracted with aqueous ammonia. The organic portion was dried, filtered and evaporated

to yield 1-benzoxymethyl-*N*-carbobenzoxy-L-carnosine methyl ester (55) as an oil (1.3 g, 86%); $[\alpha]_D^{20}$ -6.0° (*c*, 1.6 in methanol); υ_{max} ($CHCl_3$ solution) 3000, 1740, 1715, 1670 and 1500 cm^{-1}; δ_H ($CDCl_3$; $CHCl_3$ was the internal standard at 7.25 ppm) 2.50 (2H, t), 3.25 (3H, t), 3.27-3.44 (2H, AB part of an ABX system), 3.65 (3H, s), 4.61-4.63 (1H, X part of an ABX system), 4.68 (2H, s), 5.01 (2H, s), 5.69 (2H, s), 7.27 (11H, m) and 9.37 (1H, s); *m/z* 168 (25%) and 81 (58%).

Attempted synthesis of 1-*benzoxymethyl*-N-*carbobenzoxy-3-methyl-L-carnosine methyl ester*

1-Benzoxymethyl-*N*-carbobenzoxy-L-carnosine methyl ester (55) (1.27 g, 2.6 mmol) was dissolved in DMF (5 ml). Methyl iodide (1.45 g, 10.2 mmol) was added and the solution was heated at reflux (45 °C). The reaction was monitored by TLC over a period of 48 h. Very little product was formed, the reaction mixture consisted mainly of starting material.

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