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The application of ketimine derivatives of amino acids to peptide synthesis

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THE APPLICATION OF KETIMINE DERIVATIVES

OF AMINO ACIDS TO PEPTIDE SYNTHESIS

A THESIS

submitted in fulfilment of the
requirements for admittance to
the degree of

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by

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SUMMARY

Schiff base condensates of some 2-hydroxyaryl ketones with amino acids have been prepared. The reaction has been shown to proceed without racemization and most of the derivatives could be isolated in their free acid form. Their unusual stability is ascribed to the presence of a strong intramolecular hydrogen bond. The ketimines derived from D-phenylglycine were found to decarboxylate at room temperature.

The N-(2-hydroxyarylmethylene) amino acids have been condensed with amino acid and peptide esters, using standard coupling procedures, to form sterically pure N-protected peptide esters. The N-arylmethylene function can be cleaved from amino groups using 80% acetic acid under conditions which leave the t-butyloxycarbonyl protecting group intact. The potential of ketimine amino protection for peptide synthesis has been demonstrated by the preparation of biologically active peptides which are C-terminal partial sequences of substance P.

The application of the ketimine amino acids to α -amino group protection in solid phase peptide synthesis has also been investigated. However, conditions for removal of the arylmethylene function which would permit its use in conjunction with other side chain protecting groups have not been found in this case. Attachment of the N-(2-hydroxyarylmethylene) amino acids to the resin results

in a marked increase in the stability of the protecting group to the usual methods of acid hydrolysis and since removal of the arylmethylenes function requires the presence of water, this is suggested as being due to the inability of the water molecules to efficiently penetrate the resin beads. Preliminary experiments indicated that under strictly anhydrous conditions the arylmethylenes protecting group could be used as a means of permanent protection of the lysine side chain in solid phase synthesis.

The preparation of tryptophan benzyl ester has been achieved under mild acidic conditions using the arylmethylenes residue as a means of 'fugitive' protection of the α -amino group. Unfortunately the reaction conditions employed led to a sterically impure product.

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INTRODUCTION

Polypeptides and proteins may be considered as polymers of amino acids which are linked together by secondary amide bonds. Proteins possess most diverse biochemical activities and there is no known function of living cells which takes place without their participation.

The early interest in synthetic peptides was directed to their use as substrates for the study of enzyme specificity. Later, peptide synthesis assumed new importance with the discovery that antibiotics, coenzymes and many hormones had a polypeptide structure. Verification of proposed structures of these naturally occurring compounds and a study of their mode of action requires the preparation of peptides with an ordered sequence of amino acids. The physical properties of proteins, such as their conformation in solution, also lend themselves to investigation with the aid of model synthetic peptides. Convenient techniques for the synthesis of peptides are therefore an important prerequisite for the successful study of peptide and protein structure and function¹.

A. CHEMICAL PRINCIPLES OF PEPTIDE SYNTHESIS²⁻⁴

In chemical terminology, the formation of a peptide bond can be described as the acylation of the amino group of one amino acid or peptide by the carboxyl group of a second amino acid or peptide. The simple case of dipeptide formation is shown (1).

Since each amino acid may act either as an acylating agent or as the compound to be acylated, such a reaction clearly cannot proceed unambiguously. The formation of four dipeptides is initially possible and these in turn could undergo further condensations, leading finally to an intractable mixture. The preparation of a desired single product can be achieved if only one amino group and one carboxyl group are available for reaction, while the other amino group and carboxyl group are protected by their conversion to suitable unreactive species. The situation described can be further complicated if trifunctional amino acids are to be used in the condensation. Reactive groups incorporated in the side chains R and R' must also be protected.

In the synthesis of a polypeptide, two broad types of protective groups are therefore required, one of which intermittently protects α -amino and carboxyl functions and liberates them selectively and a second type which permanently blocks other reactive functional groups. All of these protective groups must finally be capable of removal under conditions which do not damage the sensitive peptide product. Most peptides are stable under mild, acidic conditions and it is therefore usual to choose combinations of protective groups which are selectively

removable under acidic conditions.

The most extensively used protecting groups in peptide synthesis are those based on the benzyl and t-butyl residues. For amino protection these residues are used in the form of the benzyloxycarbonyl (Z)⁵ (2) and t-butyloxycarbonyl (Boc)^{6,7} (3) groups and their counterparts for carboxyl protection are the benzyl (Bzl)⁸ (4) and t-butyl (Bu^t)⁹ (5) ester functions.

(2)

(3)

(4)

(5)

Removal of benzyl residues is effected by catalytic hydrogenation (hydrogenolysis) or by treatment with strong acids, such as anhydrous hydrogen bromide in acetic acid or hot trifluoroacetic acid. Cleavage of t-butyl residues is achieved by the use of milder acidic conditions (cold trifluoroacetic acid) and furthermore, the t-butyl group is unaffected by hydrogenation. Since the benzyl and t-butyl residues are each selectively removable in the presence of the other, it is therefore

possible to use one type of residue as a means of intermittent protection while the other type is used to mask side chain functions throughout a peptide synthesis.

The use of a third type of protecting group, more labile to acids than either the benzyl or t-butyl residues, would appear to offer improved yields of peptides by virtue of the minimization of side reactions with acid sensitive functions during the cleavage step. Situations where both benzyl and t-butyl residues, present in a peptide intermediate, need to be retained as the chain is lengthened, in fact necessitate the use of such a group. The 2-nitrophenylsulphenyl (Nps) amino protecting group¹⁰ (6), which can be removed by dilute mineral acid in aprotic solvents at room temperature, exemplifies this third type of residue.

(6)

The extreme acid lability of the nitrophenylsulphenyl group precludes its use for side chain protection in peptide synthesis and it has no carboxyl protecting counterpart of comparable sensitivity to acid.

In fact, this group, and protecting groups of similar acid lability, are specifically designed for the intermittent protection of α -amino functions. A number of urethane blocking groups have been developed for use as acid-labile residues in peptide synthesis. The 2-(p-biphenyl)-isopropylloxycarbonyl (Bpoc) group¹¹ is removed by aqueous acetic acid at room temperature. Of similar acid sensitivity are the 2-phenylisopropylloxycarbonyl (Ppoc) residue¹² and the α,α -dimethyl-3,5-dimethoxybenzylloxycarbonyl (Ddz) protecting group¹³. Another acid-labile blocking group worthy of mention is the triphenylmethyl (trityl) residue, which can be cleaved by dilute acetic acid at room temperature¹⁴. The use of Schiff base and enamine derivatives of amino acids as a means of acid labile α -amino group protection is the subject of later discussion.

The evaluation of potentially useful protecting groups is subject to stringent conditions. Briefly, desirable features are easy introduction with good yields, selective removal under mild conditions which avoid rupture of peptide bonds and the retention of the steric purity of amino acid residues.

The poor acylating properties of the carboxyl group is a second complicating feature of peptide synthesis. The mixing of solutions of an N-protected amino acid (the carboxyl component) and an amino acid ester (the amino component) at room temperature does not

normally lead to peptide bond formation. For coupling to occur, the carboxyl component must be activated by its conversion to a derivative wherein the electrophilic nature of the carbonyl carbon is enhanced, thereby facilitating nucleophilic attack by the amino group. An alternative approach to this problem, involving activation of the amino group, has met with relatively less success¹⁵.

The transformation of an N-protected amino acid to its acid chloride¹⁶ or acid azide¹⁷ are two classical methods which have been employed for carboxyl activation. The former approach (7), pioneered by E. Fischer, has gradually fallen into disfavour for peptide synthesis and now can be regarded as of historical interest only. The high degree of activation ("overactivation") of acid chlorides obviates the risk of racemization and leads to undesired side reactions, such as N-carboxyanhydride formation¹⁸ (8).

The acid azide method for formation of peptide bonds (9), first used by T. Curtius in 1902, remains one of the most important coupling procedures, chiefly because it leads to the least degree of racemization. Unfortunately, the route to the azide from the carboxyl component is indirect. In addition, azides are unstable, and by-products such as amides, isocyanates and urea derivatives are often formed in the reactions. The scope and limitations of this method are the subject of a recent review¹⁹.

Conversion of the N-protected amino acid to a mixed anhydride is another popular method of carboxyl activation. The reaction (10) may take two courses, depending upon the mode of cleavage of the oxygen bridge, but peptide bond formation is favoured by a suitable choice of solvent and R'.

The preferred reagents for anhydride formation are ethyl chloroformate²⁰ and isobutyl chloroformate²¹. Racemization can be a problem unless the coupling reaction is carried out rapidly and at low temperatures after anhydride formation. A comprehensive review of the method has been published by Albertson²².

Another attractive method of peptide bond formation is the aminolysis of activated esters. The technique was pioneered by Wieland²³, who used thiophenyl esters of N-protected amino acids. The introduction of p-nitrophenyl esters²⁴ for this purpose has resulted in the active ester method becoming a most important coupling procedure in peptide synthesis (11). Activation of the carboxyl reaction centre is brought about by the electron attracting properties of the *para* nitro group.

Active esters are less reactive than anhydride activated carboxyl functions and therefore they do not acylate weaker nucleophiles such as free hydroxyl groups in amino acids. One of the main advantages of the active ester method is the possibility of isolation and purification of the reactive species. The p-nitrophenyl esters for example are readily accessible by the reaction of N-protected amino acids and p-nitrophenol using N,N'-dicyclohexylcarbodiimide as dehydrating agent^{25,26}. Other active esters which have been used successfully in peptide bond formation include those of pentachlorophenol²⁷, pentafluorophenol²⁸, N-hydroxysuccinimide²⁹ and N-hydroxypiperidine³⁰.

Coupling reagents may be broadly defined as compounds which, on addition to a mixture of the carboxyl and amino components, lead to the formation of an activated carboxyl species. This reacts *in situ* with the amino component to form the peptide bond. Because they simplify the practical aspects of peptide synthesis, coupling reagents are the most frequently employed method of carboxyl activation.

The reagent, N,N'-dicyclohexylcarbodiimide (DCC) (12) was first introduced to peptide synthesis by Sheehan and Hess³¹ and the mechanism of peptide bond formation has been postulated³² to be shown in Scheme 1.

(15)

SCHEME 1

Addition of the carbodiimide (12) to the N-protected amino acid yields the O-acylisourea (13), which may then react with the amino component to form the

peptide bond. An alternative pathway is visualized if (13) reacts further with the carboxyl component to form the symmetrical anhydride (15). Aminolysis of (15) then leads to the peptide. The main disadvantages associated with the use of DCC are, firstly, the difficulty of complete removal of the N,N'-dicyclohexyl-urea (14) by-product, which is soluble to some extent in most solvents used for the coupling reaction. Reagents, which form water-soluble urea derivatives, such as 1-ethyl-3(3'-dimethylaminopropyl) carbodiimide (EDC) can be used to overcome this problem. Secondly, the O-acylisourea (13) may rearrange by an O→N acyl migration to give the N-acylurea (16). These latter compounds are not acylating agents and are often difficult to separate from the peptide product due to the similarities in solubility and structure. As with the mixed anhydride procedure, the choice of solvent and the temperature of coupling have an important effect on the steric purity of the final product. Non-polar solvents and low reaction temperatures have been found to be most suitable.

The use of nucleophilic hydroxy compounds as

additives in the carbodiimide reaction considerably lowers the dangers of racemization^{3 4} and this technique is gradually gaining favour over the use of carbodiimides alone. These additives, of which N-hydroxysuccinimide^{3 5} (17) and 1-hydroxybenzotriazole^{3 6} (18) are the most commonly used, form active esters from the carboxyl component.

(17)

(18)

The fast formation of these esters, coupled with their rapid consumption by the amino component, results in considerable improvement in the steric purity of the product. In addition, formation of the N-acylurea (16) would be expected to be inhibited by the use of these additives.

1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (19) is an efficient coupling reagent, as demonstrated by Belleau and Malek^{3 7}. The proposed mechanism for peptide bond formation is shown in Scheme 2.

(19)

(20)

(21)

SCHEME 2

The carboxyl component first replaces the 2-ethoxy group of (19) to give (20) which rearranges and eliminates quinoline to form the mixed anhydride (21).

Acylation of the amino component by (21) forms the peptide bond. Coupling with EEDQ shows no detectable racemization by Young's test^{38,39}. Notable advantages of the reagent include the possibility of activating glutamine⁴⁰ asparagine⁴⁰ and hydroxy amino acids³⁷ without side reactions and the formation of easily removed by-products.

The use of coupling reagents in peptide synthesis has been recently reviewed⁴¹.

The biological activity of a peptide or protein is often dependent on steric, as well as chemical purity, so that the stereochemical homogeneity of synthetic peptides is of utmost importance. Because all protein amino acids, except glycine, have at least one asymmetric centre (the α -carbon atom) the possibility of racemization must always be considered and steps must be taken to minimise this possibility. The emphasis which must be placed on this aspect of peptide synthesis is ably illustrated by considering the synthesis of a peptide comprising 100 amino acid units from an L-amino acid containing 0.1 proportion of the D-enantiomer. The probability that all the amino acid residues in the product will be of a single optical configuration is calculated to be effectively zero⁴².

Two mechanisms have been proposed to account for the loss of steric purity during peptide synthesis.

- (a) Under basic conditions, direct proton abstraction from the α -carbon atom⁴³ (22).

- (b) N-acyl amino acids may racemize by the formation of an oxazol-5-one (azlactone)^{44, 45} intermediate (23). Cleavage of the asymmetric carbon-hydrogen bond is facilitated by electron delocalization in the resulting carbanion (24).

In addition, N-protected peptide acids may also racemize by a cyclization mechanism (23) and for this reason peptide chains are normally built up from the carboxyl terminal residue using N-protected amino acids which do not favour azlactone formation.

N-protected peptide acids which contain glycine or proline as the carboxyl terminal residue may, however be activated for coupling as these two amino acids cannot racemize by the azlactonization mechanism. The mechanisms of racemization in peptide synthesis have been the subject of a lengthy review by Goodman and Glaser⁴⁶.

The need to establish the steric purity of intermediates and products during the various steps of a peptide synthesis has resulted in the development of several convenient methods for detection of racemization in the preparation of N-protected amino acids and in peptide coupling.⁴⁸ Classical techniques involve hydrolysis⁴⁷ enzymic digestion^{38,39,49,50} and fractional crystallization⁵¹⁻⁵⁴. Newer procedures make use of gas-liquid chromatographic⁵⁵⁻⁵⁷ or thin-layer chromatographic separation of diastereoisomers or examine the product by nuclear

58-60
magnetic resonance .

B. SOLID PHASE PEPTIDE SYNTHESIS

The basic art of peptide synthesis had remained largely unchanged since Bergmann's introduction⁵ of the benzyloxycarbonyl protecting group until the development of solid phase peptide synthesis by R.B. Merrifield⁶¹. In this technique, the peptide chain is assembled in a stepwise manner while it is covalently anchored to a solid support at one end. At all stages of the synthesis the peptide is therefore insoluble and can be easily separated from excess reactants and by-products. The solid support, a co-polymer of styrene and divinylbenzene, is first chloromethylated. The resulting chloromethyl resin reacts with a salt of the N-protected amino acid to form an ester bond between the amino acid and the resin. The protecting group is then removed and the next amino acid residue is added using one of the conventional procedures for peptide bond formation. This procedure is continued until the peptide is assembled and the required compound is then cleaved from the solid support. The principles of the method are illustrated in Scheme 3.

SCHEME 3

Such a system offers several advantages over classical solution methods of synthesis. Firstly, it is possible to carry out the reaction in a single vessel, thus avoiding the losses involved in repeated transfers of material; secondly the isolation and purification losses involved at each step are avoided and thirdly, the final product may be isolated reasonably pure by the

use of excess reactants to force the individual reaction steps to completion. The success of the solid phase method, in fact, depends upon achieving complete reaction at each step. The failure to achieve this aim, leading to the formation of truncated and failure sequences, which are extremely difficult to separate from the product, is one of the major problems encountered in the solid phase approach. A number of reviews have been published which detail the principles, practical application and limitations of the solid phase method⁶²⁻⁶⁷.

C. SCHIFF BASES AND ENAMINES AS AMINO PROTECTING GROUPS

Primary amines and carbonyl compounds condense, with elimination of water, to form compounds known as imines, anils, azomethines or Schiff bases⁶⁸ (24).

When the carbonyl compound is of aromatic nature, the product is often a yellow crystalline solid and since Schiff bases are readily hydrolyzed by dilute acids, their potential utility for α -amino protection in peptide synthesis is indicated.

Stable aldehyde condensates of α -amino acid salts and esters are well known. Wieland and Schafer⁶⁹ using benzylidene amino acid thiophenyl esters, were first to attempt peptide synthesis with Schiff base amino protection. Later Zervas and colleagues⁷⁰ investigated the application of p-nitrophenyl esters of benzylidene and salicylidene amino acids. In both cases, however, it was shown that the amino component attacked the azomethine carbon atom in preference to the activated carboxyl group.

In contrast to the Schiff base derivatives of α -amino acid salts and esters, those of free amino acids are not readily accessible. Bergmann and Zervas^{71,72} reported the preparation of a benzaldehyde derivative of lysine, later identified⁷³ as N^{ϵ} -benzylidene lysine, whilst Dakin⁷⁴ prepared benzylidene condensates of glycine, alanine and leucine but he considered the products to be polymers. The benzylidene group has been used to protect the ϵ -amino group of lysine during acylation of the α -amino group⁷⁵.

McIntire⁷⁶ investigated the reaction of a number of aromatic aldehydes with amino acids and reported the formation of crystalline condensates using 5-chlorosalicylaldehyde and 2-hydroxy-1-naphthaldehyde. The increased stability of these o-phenolic aldehyde derivatives was attributed to the formation of a strong intramolecular hydrogen bond between the phenolic proton and the imine

nitrogen atom⁷⁷, (25), (26).

(25)

(26)

McIntire's results prompted Sheehan and Grenda⁷⁸ to apply these aldimines to peptide synthesis and some N-terminal valine dipeptide esters were prepared using the DCC coupling procedure. The arylmethylen protecting group was removed by hydrolysis under extremely mild acidic conditions without detectable racemization. The expected compatibility of Schiff base protection of α -amino functions with acid-labile sulphur protecting groups induced Hiskey and Southard⁷⁹ to further study the 5-chlorosalicylidene condensates of amino acids and they concluded that only the derivatives of valine and S-trityl cysteine were useful for peptide synthesis. The azomethine bond in the derivatives of other amino acids proved unstable in the DCC coupling procedure. In addition, they noted that racemization of the S-trityl cysteine condensate could be avoided only if the amino acid was converted to its dicyclohexylammonium salt prior to condensation.

The 2-hydroxy -1-naphthaldehyde derivatives of amino acids have also been subjected to closer investigation by Khosla and colleagues⁸⁰. They found that improved yields and purer compounds could be obtained if dimethylformamide was used in the condensation rather than alcohol, as used by McIntire. A number of pentachlorophenyl active esters of these aldimines were also prepared and promising results were obtained using both the solution and solid phase methods of peptide synthesis. Cleavage of the arylmethyle protecting group in the solid phase method was effected using dilute aqueous hydrochloric acid in dioxane, or better, using benzylamine in dichloromethane.

The use of 1,3-dicarbonyl compounds, such as acetylacetone and benzoylacetone, as potentially acid labile amino protecting groups was proposed by Dane and colleagues⁸¹. Amino acid potassium salts condensed with these reagents affording enamines (27) which could be coupled with amino acid esters using the carbodiimide method, or alternatively, the enamines were converted to cyanomethyl active esters⁸² prior to aminolysis. The protecting groups were removed by a short treatment with dilute hydrochloric acid or acetic acid. The stability of the enamine derivatives was again ascribed to the formation of an intramolecular hydrogen bond.

(27)

Hiskey and Southard⁷⁹ used the benzoylacetone (benzoylmethylvinyl, BMV) protecting group in conjunction with acid sensitive sulphur protecting groups, but they found the lability of the BMV amino acid potassium salts a serious disadvantage to their practical utility and low yields of peptides were obtained on coupling. Later, Southard⁸³ observed that dicyclohexylammonium salts of BMV amino acids had greater stability and he was able to demonstrate⁸⁴ their potential in the solid phase procedure. Removal of the enamine protecting group from the peptide was achieved with dilute hydrochloric acid in aprotic solvent. The application of 1,3-dicarbonyl compounds to amino protection has also been investigated by Balog and colleagues⁸⁵⁻⁸⁹.

Not unrelated to the aforementioned protecting groups was the proposed use by Halpern^{90,91} of dimedone (5,5-dimethylcyclohexane-1,3-dione) (28) for α -amino protection.

(28)

(29)

Dimedone yielded crystalline derivatives (29) with amino acid esters which could be converted to the amino acid derivatives by saponification. The presence of the robust vinylogous amide group in these compounds however, renders them extremely stable to normal methods of acidic hydrolysis. Mild deprotection could be achieved using either bromine water or nitrous acid⁹².

The use of Schiff base condensates derived from amino acids and ketones for peptide synthesis has not been reported in the literature, presumably because such ketimines are considered unstable. However, some crystalline derivatives of o-hydroxy substituted aromatic ketones and α -amino acids have been synthesized by Al-Sayyab and Lawson⁹³ in connection with decarboxylation studies. In view of the expected increased stability of such compounds, compared to the corresponding aldimine derivatives, they would appear potentially useful for peptide synthesis. o-Hydroxy aromatic ketimines should be sufficiently stable to prevent cleavage of the imine bond during coupling, yet labile enough to permit removal of the diarylmethylene protecting species under mild conditions. The aim of the project covered in this thesis was to develop a facile synthetic route to the ketimine condensates of some 2-hydroxybenzophenones (30) and α -amino acids and to investigate the usefulness of these condensates in peptide synthesis, using classical solution methods as well as the solid phase technique.

- (a) $R = C_6H_5$, $R' = Cl$
- (b) $R = C_6H_5$, $R' = CH_3$
- (c) $R = C_6H_5$, $R' = H$
- (d) $R = CH_3$, $R' = H$

(30)

RESULTS AND DISCUSSION

A. PREPARATION OF KETIMINE DERIVATIVES OF AMINO ACIDS

There is a limited literature on the preparation of Schiff base derivatives of amino acids. The approach to aldimines has been to stir a suspension of the amino acid and the aldehyde in methanol⁷⁶ or dimethylformamide⁸⁰ at room temperature, whereas the method⁹³ for ketimine synthesis uses basic conditions under reflux to promote condensation. Both of these approaches are not entirely suitable for the preparation of large amounts of sterically pure amino acid derivatives, which are essential if amino acid condensates are to be used in peptide synthesis. The zwitterionic nature of amino acids renders them poorly soluble in organic solvents, whilst refluxing in the presence of base would certainly increase the possibility of racemization.

Al-Sayyab's method⁹³ for the synthesis of ketimine derivatives has therefore been modified so that the condensation could be carried out under basic conditions at room temperature, thereby minimizing the risk of racemization. The synthetic route is formulated in Scheme 4.

Conversion of amino acids to their tetramethylammonium salts (31) results in a marked increase in the solubility of the amino acids in organic solvents.

(30)

SCHEME 4

The salts (31) could be conveniently condensed with o-hydroxy aromatic ketones (30) in anhydrous methanol and in the presence of 3A molecular sieves to

give the ketimine tetramethylammonium salts (32), a homogeneous solution usually being formed after several days at room temperature. The molecular sieves abstract water selectively in the presence of methanol⁹⁴, thus minimizing the residual water concentration in the reaction mixture and forcing the equilibrium reaction to higher conversion. A binder which is used in the manufacture of molecular sieve sticks may also catalyze the condensation⁹⁵. Unlike the aldimine derivatives of amino acids, which are unstable at pH values below 7⁹⁶ the ketimine derivatives (33) can be isolated by adjusting the pH of an aqueous solution of the salts (32) to a value between 5 and 6.

Of the several 2-hydroxy aromatic ketones investigated for Schiff base formation, 5-chloro-2-hydroxybenzophenone (30a) and 2-hydroxy-5-methylbenzophenone (30b) were found to be the most useful. All the common protein amino acids, except proline, yielded Schiff base condensates from one or the other of these ketones under the described reaction conditions. Whilst most of the ketimines could be isolated as yellow and generally crystalline solids (33a-33w), the derivatives of β -benzyl aspartic acid, γ -benzyl glutamic acid, ϵ -benzyloxycarbonyl lysine, α -t-butyloxycarbonyl lysine, ϵ -t-butyloxycarbonyl lysine, δ -t-butyloxycarbonyl ornithine and ω -nitroarginine were hygroscopic oils or amorphous, hygroscopic solids which decomposed within a few days. These latter seven derivatives could,

however, be conveniently stored by converting them to the dicyclohexylammonium salts (34a-34g).

Difficulty was encountered with the elementary analyses of these salts and only in one instance (the δ -t-butyloxycarbonyl ornithine derivative, 34f) has a satisfactory result been obtained.

Condensation with the ketones was most rapid with the amino acids possessing aromatic side chains, whilst those with aliphatic side chains were slowest to react. The condensation of (30a) with alanine, for example, requires ten to twelve days at room temperature to obtain a homogeneous solution. When stoichiometric amounts of the amino acid and ketone are used, some of the ketimines (33) are isolated in low yield, but this does not pose a practical problem. It is more convenient to employ an excess of the ketone, thus ensuring complete consumption of the amino acid in the condensation. The excess ketone is easily removed and can be readily purified and used again.

As the condensation of the amino acid salt (31) and the ketone (30) proceeded, a red coloration of the methanolic solution was observed to appear and the intensity of this colour increased over several days. With the aromatic amino acids, tryptophan and phenylalanine, which were fastest to react at room temperature (approximately 30 hours), this colour change was particularly marked. In the solid state, several

Schiff bases derived from salicylaldehyde are known to give rise to crystals which change colour from yellow to red upon exposure to light⁹⁷. This involves a change of the enol form of the hydroxy anil (35) to a quinonoid species (36).

Such tautomeric reactions may also take place in solution at room temperature, but the reverse reaction is normally so fast that the colour changes are not perceived by the eye. Under the reaction conditions which have been employed for the preparation of these ketimine derivatives of amino acids, it is therefore assumed that in solution there is some contribution from a quinonoid tautomer of the salt (32). The pure isolated ketimines, however, are unaffected by light, in the solid form or in solution.

Two possible structures may be proposed for the condensates, corresponding to the ketimine (33) and enamine (37) formulations.

P.m.r. Spectrum of
N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) in D₆-d.m.s.o.

FIGURE 1

(33)

(37)

The presence of a singlet for the methylene protons adjacent to the carboxyl function in the p.m.r. spectrum (Figure 1) of the glycine derivative (33a, $R' = C_6H_5$, $R'' = Cl$, $R = H$) indicates the imine structure is preferred to the enamine tautomer. Other spectroscopic evidence also supports the presence of a ketimine structure. The compounds possess ultraviolet absorption maxima near 220 nm ($\log \epsilon$ about 4) which is indicative of azomethine bonds⁹⁸ and in the infrared spectra, a band near 1610 cm^{-1} , although not always resolved from aromatic bands in the same region, has been assigned to absorption by carbon-nitrogen double bonds. This normally appears⁹⁹ in the region $1690\text{--}1635\text{ cm}^{-1}$ but is lowered by conjugation with the aromatic system¹⁰⁰. An absorption band near 1715 cm^{-1} in the infrared spectra due to the carboxyl carbonyl is also present.

Mass spectra of the diarylmethylene protected amino acids give rise to a characteristic peak which is formed by the loss of 44 mass units (CO_2) from the

molecular ion.

(38)

This fragment (the amine or 'A' fragment) (38) can be conveniently used to identify the amino acid. The presence of the amine fragment is similar to that previously reported¹⁰¹ for the condensates of acetylacetone with amino acids and peptides.

The N-(2-hydroxyarylmethylene) amino acids can be safely stored in dry conditions without deterioration and they are stable in aqueous solutions down to pH values about 4 for short periods. The increased stability of these compounds, compared with the corresponding benzophenone analogues, can again be explained by the presence of a strong intramolecular hydrogen bond between the imine nitrogen and the phenolic proton⁷⁷. The p.m.r. spectra of the glycine (33a) and alanine ketimines (33c) substantiate this assertion, as shown by the reluctance of the phenolic proton of these derivatives to exchange with deuterium

TABLE 1 - PHYSICAL CONSTANTS FOR KETIMINE DERIVATIVES OF AMINO ACIDS (33)

Amino Acid Derivative ^a	Yield (%)	m.p. (°C)	[α] _D ^j	m/e ^k	Molecular Formula	Analysis					
						Found	Found	Found	Calc.	Calc.	Calc.
						C	H	N	C	H	N
(33a) 5ClB-Gly-OH ^b	55 ^d	158-160	-	245	C ₁₅ H ₁₂ ClNO ₃	61.8	4.4	4.8	62.2	4.1	4.8
(33b) 5MeB-Gly-OH	65 ^e	154-157	-	225	C ₁₆ H ₁₅ NO ₃	70.8	5.8	5.1	71.3	5.6	5.2
(33c) 5ClB-Ala-OH ^c	71 ^d	180-182	-24°	259	C ₁₆ H ₁₄ ClNO ₃	63.0	4.7	4.5	63.3	4.6	4.6
(33d) 5ClB-D-Ala-OH	62 ^d	180-182	+26°	259	C ₁₆ H ₁₄ ClNO ₃				1		
(33e) 5MeB-Ala-OH	56 ^d	184-186	-52°	239	C ₁₇ H ₁₇ NO ₃	72.0	6.1	4.9	72.1	6.1	4.9
(33f) 5ClB-Val-OH	53 ^d	145-146	-52°	287	C ₁₈ H ₁₈ ClNO ₃	65.3	5.5	4.4	65.2	5.5	4.2
(33g) 5ClB-D-Val-OH	48 ^d	143-145	+49°	287	C ₁₈ H ₁₈ ClNO ₃				1		
(33h) 5MeB-Val-OH	46 ^d	173-174	-46°	267	C ₁₉ H ₂₁ NO ₃	73.2	6.9	4.5	73.3	6.8	4.5
(33i) 5ClB-Ser-OH	62 ^e	152-154	-32°	275	C ₁₆ H ₁₄ ClNO ₄	59.7	4.4	4.6	60.1	4.4	4.4
(33j) 5ClB-(O-Bzl)-Ser-OH	70 ^d	118-120	-40°	365	C ₂₃ H ₂₀ ClNO ₄	67.2	5.0	3.4	67.4	4.9	3.0
(33k) 5ClB-Thr-OH	58 ^e	92-95	-40°	239	C ₁₇ H ₁₆ ClNO ₄	61.4	5.2	4.0	61.2	4.8	4.2
(33l) 5ClB-Trp-OH	72 ^d	160-162	-364°	374	C ₂₄ H ₁₉ ClN ₂ O ₃	68.5	4.6	7.1	68.8	4.6	6.7
(33m) 5ClB-(O-Bzl)-Tyr-OH	47 ^f	73-75	-174°	441	C ₂₉ H ₂₄ ClNO ₄	71.4	5.0	2.4	71.7	5.0	2.9
(33n) 5ClB-(im-Bzl)-His-OH	73 ^d	131-133	-84°	415	C ₂₆ H ₂₂ ClN ₃ O ₃	67.4	4.8	9.4	67.9	4.8	9.1
(33o) 5ClB-Asn-OH	77 ^g	160-161	-48°	302	C ₁₇ H ₁₅ ClN ₂ O ₄	58.7	4.4	8.4	58.9	4.4	9.1
(33p) 5ClB-Gln-OH	46 ^d	106-107	-43°	316	C ₁₈ H ₁₇ ClN ₂ O ₄	60.4	5.0	7.8	59.9	4.8	7.6
(33q) 5MeB-Leu-OH	56 ^d	128-130	-88°	281	C ₂₀ H ₂₃ NO ₃	73.7	7.1	4.6	73.8	7.1	4.3
(33r) 5MeB-D-Leu-OH	45 ^d	128-129	+88°	281	C ₂₀ H ₂₃ NO ₃				1		
(33s) 5MeB-Ile-OH	52 ^d	136-138	-68°	281	C ₂₀ H ₂₃ NO ₃	73.9	7.1	4.6	73.8	7.1	4.3
(33t) 5MeB-Phe-OH	75 ^h	75-80	-240°	315	C ₂₃ H ₂₁ NO ₃	76.7	6.1	3.7	76.9	5.9	3.9
(33u) 5MeB-D-Phe-OH	68 ^h	77-78	+240°	315	C ₂₃ H ₂₁ NO ₃				1		
(33v) 5MeB-(S-Bzl)-Cys-OH	42 ^h	57-59	-136°	361	C ₂₄ H ₂₃ NO ₃ S	71.4	5.6	3.4	71.1	5.7	3.5
(33w) 5MeB-Met-OH	56 ⁱ	119-120	-93°	299	C ₁₉ H ₁₁ NO ₃ S	66.6	6.3	4.0	66.5	6.2	4.1

- a. Abbreviations for amino acids follow IUPAC-IUB recommendations (J. Biol. Chem., 1966, 241, 2491). All amino acids are of L configuration unless otherwise specified. 5ClB = N-[α-phenyl-(5-chloro-2-hydroxybenzylidene)] 5MeB = N-[α-phenyl-(2-hydroxy-5-methylbenzylidene)]. U.v. spectra (anhydrous methanol) λ max. near 220 nm (log ε 4.4). I.r. spectra (Nujol or chloroform) ν max. near 1715 cm⁻¹ (carboxyl).
- b. P.m.r. spectrum (D₆-dimethylsulphoxide) δ 4.1 (s, 2H, -CH₂-). c. P.m.r. spectrum (D₆-dimethylsulphoxide) δ 1.85 (d, 3H, J 5.5Hz, -CH-CH₃), δ 3.9 (q, 1H, J 20Hz and 14Hz, -CH-CH₃).
- d. ethyl acetate-l.p. e. ethyl acetate. f. benzene. g. methanol. h. amorphous solid by evaporation of ether-l.p. solution under reduced pressure at 0°; stored in dessicator at 0° to avoid decomposition.
- i. ether-l.p. j. for measurement: c 0.5 in methanol. k. characteristic fragment is due to loss of CO₂ from M⁺.
- l. not determined.

oxide. Table 1 lists the ketimine derivatives (33) and their physical constants in convenient form.

The reaction of 2-hydroxy aromatic ketones with D-phenylglycine at room temperature did not yield the expected ketimines (39) but instead the decarboxylated benzylamine derivatives (40) were formed.

- (a) $R = C_6H_5, R' = Cl$
- (b) $R = C_6H_5, R' = CH_3$
- (c) $R = C_6H_5, R' = H$
- (d) $R = CH_3, R' = H$

Whilst there is an extensive literature^{102-105,93} on the thermal decomposition of α -amino acids in the presence of ketones, which may result in transamination or decarboxylation of the amino acid derivatives, there appears to be no previous report of a spontaneous decarboxylation of such compounds. The unexpected results obtained with the D-phenylglycine condensates prompted the consideration of a second amino acid which

possessed a structural similarity to phenylglycine and which could possibly undergo a similarly facile decarboxylation. The most obvious feature of phenylglycine (41), when compared with common protein α -amino acids, is the presence of the α -phenyl group.

(41)

(42)

α -Phenylalanine (42) was therefore synthesized¹⁰⁶ as an analogue of phenylglycine and condensation with the ketones (30) was carried out in the usual manner. Ketimine derivatives of the amino acid were formed, but no trace of decarboxylated compounds could be detected in these cases. The feature of phenylglycine which accounts for the spontaneous decarboxylation of the Schiff bases (39) has not as yet been determined, but in view of the biological significance of this reaction¹⁰⁷, further investigation will be warranted.

Racemization of optically active amino acids protected by the diarylmethylene system is not possible through the classical azlactonization mechanism^{44,45} generally proposed for N-acylated amino acids, although

other base catalyzed mechanisms for racemization could be postulated. The optical purity of the ketimines (33) has been demonstrated using gas-liquid chromatographic (g.l.c.) analysis of diastereoisomeric amino acid derivatives. These methods make use of the differing retention times of an LL and DL diastereoisomeric pair on a g.l.c. column. Alanine, recovered after acid hydrolysis of N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-alanine (33c) was converted to N-trifluoroacetyl-L-prolyl-alanine methyl ester⁵² and g.l.c. analysis of this derivative indicated less than 1% of the LD isomer had been formed in the preparation of the Schiff base and its subsequent hydrolysis. Similarly, N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-tryptophan (33l) was hydrolyzed and the recovered tryptophan was esterified with (+)-*sec*-butanol. This ester was converted to its N-trifluoroacetyl derivative and submitted to g.l.c. analysis⁵⁴. Less than 1% of the D isomer of tryptophan was present. In a third experiment phenylalanine was recovered after acid hydrolysis of N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t). The specific rotation of the recovered amino acid was identical to that of the starting material used in the condensation.

TABLE 2 - PHYSICAL CONSTANTS OF KETIMINE PEPTIDE ESTERS (43)

Peptide Derivative ^a	Coupling ^b Method	Base/ Solvent ^c	Yield %	m.p. (°C)	[α] _D ⁱ	m/e ^j	Molecular Formula	Analysis		
								C	H	N
(43a) 5ClB-Gly-Gly-OBzl	A	TEA/CH ₂ Cl ₂	65 ^d	112-114	-	436	C ₂₄ H ₂₁ ClN ₂ O ₄	Found: 65.4 Calc.: 66.0	5.0 4.9	6.3 6.4
(43b) 5ClB-Trp-Gly-OBzl	A	TEA/CH ₂ Cl ₂	85 ^e	70-75	-236 ^o	-	C ₃₃ H ₂₈ ClN ₃ O ₄	Found: 70.0 Calc.: 70.0	5.2 5.0	7.2 7.4
(43c) 5ClB-Trp-Trp-OEt	A	TEA/CH ₂ Cl ₂	91 ^e	88-93	-172 ^o	-	C ₃₇ H ₃₃ ClN ₄ O ₄	Found: 70.1 Calc.: 70.2	5.5 5.3	8.6 8.9
(43d) 5ClB-Gly-Phe-OMe	A	TEA/EtOAc	51 ^f	97-99	-16 ^o	450	C ₂₅ H ₂₃ ClN ₂ O ₄	Found: 66.7 Calc.: 66.6	5.3 5.1	6.3 6.2
(43e) 5ClB-Gln-Gly-OBzl	B	TEA/THF	80 ^g	123-125	-17 ^o	507	C ₂₇ H ₂₆ ClN ₃ O ₅	Found: 63.8 Calc.: 63.8	5.2 5.2	8.2 8.3
(43f) 5ClB-Trp-Glu-(OBzl) ₂	C	TEA/EtOAc	86 ^e	46-52	-180 ^o	-	C ₄₃ H ₃₈ ClN ₃ O ₆	Found: 70.8 Calc.: 70.9	5.5 5.3	5.5 5.8
(43g) 5ClB-Ala-Phe-OMe	A	TEA/EtOAc	83 ^e	75-76	+4 ^o	464	C ₂₆ H ₂₅ ClN ₂ O ₄	Found: 67.0 Calc.: 67.2	5.8 5.4	5.8 6.0
	D	NMM/THF	65 ^e	77-79	+4 ^o	464				
	E	TEA/CH ₂ Cl ₂	72 ^e	76-77	+4 ^o	464				
(43h) 5ClB-Val-Val-OMe	A	TEA/CH ₂ Cl ₂	82 ^e	oil	-	444	C ₂₄ H ₃₀ ClN ₂ O ₄	k		
	D	TEA/THF	70 ^e	oil	-	444				
(43i) 5MeB-Phe-Gly-OBzl	A	TEA/CH ₃ CN	92 ^e	oil	-	506	C ₃₂ H ₃₀ N ₂ O ₄	k		
(43j) 5ClB-Val-Leu-OBzl	B	TEA/THF	87 ^h	oil	-	534	C ₃₁ H ₃₅ ClN ₂ O ₄	k		
(43k) 5ClB-(OBzl)-Tyr- Gly-OBzl	B	NMM/DMF	90 ^h	oil	-	632	C ₃₈ H ₃₃ ClN ₂ O ₅	k		
(43l) 5ClB-(ε-Z)-Lys- Phe-OMe	B	TEA/THF	77 ^h	oil	-	655	C ₃₇ H ₃₈ ClN ₃ O ₆	k		
(43m) 5ClB-Trp-Thr-(ε-Z)- Lys-OBzl	A	TEA/CHCl ₃	69 ^e	68-72	-148 ^o	-	C ₄₉ H ₅₀ ClN ₅ O ₈	Found: 67.7 Calc.: 67.5	6.2 5.8	7.8 8.0

a. I.r. spectra of all compounds (Nujol or chloroform) v max near 3300 and 1680 cm⁻¹ (amide) and 1730 cm⁻¹ (ester). All amino acids are of L-configuration.

b. A = DCC, B = DCC-HOBt, C = EDC, D = EEDQ, E = ONp

c. Abbreviations: TEA = triethylamine, THF = tetrahydrofuran, NMM = N-methylmorpholine, DMF = dimethylformamide.

d. from dichloromethane-ether. e. after chromatography using benzene and benzene-chloroform, then trituration with light petroleum.

f. from ether-light petroleum. g. from ethyl acetate-ether. h. after chromatography using carbon tetrachloride and carbon tetrachloride-ethyl acetate mixtures.

i. for measurement: c 0.5 in methanol. j. molecular ions. k. not determined

B. USE OF KETIMINE DERIVATIVES OF AMINO ACIDS FOR PEPTIDE SYNTHESIS.

The application of the diarylmethylene protected amino acids to the synthesis of peptides has been investigated using various means of carboxyl activation.

Carbodiimide coupling reagents smoothly mediate the condensation of the ketimines (33) with amino acid or peptide esters, yielding the corresponding protected peptides (43) in good yield. Several of these protected peptide derivatives (43a-43m) have been synthesized and characterized by spectroscopic evidence or by elementary analysis (Table 2).

$\text{R}=\text{Cl}, \text{CH}_3$

$\text{Y}=\text{OCH}_3, \text{OC}_2\text{H}_5, \text{OC}_6\text{H}_5, \text{NHCHR}''' \text{COOC}_6\text{H}_5$

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The compounds (43) exhibited both ester (near 1730 cm^{-1}) and amide (near 3300 and 1680 cm^{-1}) bands in their infrared spectra and, with the exception of the N-terminal tryptophan derivatives, molecular ions

were present in their mass spectra. The intense yellow colour of the diarylmethylene protected peptides makes them particularly suitable for isolation by chromatographic means and any by-products derived from DCC and the ketimine amino acids (33) could be readily detected by thin-layer chromatography. For example, the bright yellow N-acylurea by-product (44) formed from DCC and N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) during the synthesis of the dipeptide derivative (43i) was isolated and characterized by comparison with an authentic specimen of (44).

As expected, the use of 1-hydroxybenzotriazole (HOBt)³⁶ in conjunction with DCC greatly inhibited the formation of by-products of type (44) to the extent that they were hardly detectable on thin-layer chromatograms. The DCC-HOBt procedure would therefore seem to be the method of choice for forming peptide bonds from the ketimine amino acids (33).

Not unexpectedly, the problem of removing the last traces of the dicyclohexylurea by-product from the

peptide derivatives (43) was encountered with the DCC coupling procedure and this difficulty could be avoided by the use of EDC³³ which forms a water-soluble urea. The EDC coupling reaction was found to proceed satisfactorily and the protected dipeptide (43f) was obtained in good yield.

Promising results were also obtained with the coupling reagent EEDQ³⁷ which was used to prepare the dipeptide derivatives (43g) and (43h).

The diarylmethylene amino acids (33) also react with *p*-nitrophenol in the presence of DCC²⁶ to yield the corresponding *p*-nitrophenyl active esters (45).

Esters of alanine (45a), valine (45b) and tryptophan (45c) were isolated as yellow crystalline solids and they were characterized by elementary analysis and by spectroscopic methods. Absorption bands in their infrared spectra due to ester⁹⁹ (near 1760 cm⁻¹), imine¹⁰⁰ (near 1610 cm⁻¹) and nitro⁹⁹ (near 1550 and 1350 cm⁻¹) groups were present and the mass spectra of

TABLE 3

PHYSICAL CONSTANTS OF KETIMINE AMINO ACID p-NITROPHENYL (ONp) ESTERS (45)

Ester ^a	Yield	m.p	[α] _D ^d	m/e ^e	Molecular Formula	Found			Calc.		
						C	H	N	C	H	N
(45a) 5ClB-L-Ala-ONp	64 ^b	104-105°	-80°	424	C ₂₂ H ₁₇ ClN ₂ O ₅	62.2	3.9	6.5	62.2	4.0	6.6
(45b) 5ClB-L-Val-ONp	75 ^b	102-104°	-40°	452	C ₂₄ H ₂₁ ClN ₂ O ₅	63.7	4.7	6.0	63.7	4.7	6.2
(45c) 5ClB-L-Trp-ONp	65 ^c	135-139°	-164°	539	C ₃₀ H ₂₂ ClN ₃ O ₅	66.8	4.4	7.6	66.7	4.1	7.8

^a I.r. spectra (Nujol) $\nu_{\max}$ near 1760 (ester), 1610 (imine), 1550 (nitro) and 1350 (nitro) cm⁻¹

^b ether-light petroleum ^c benzene ^d for measurement: c 0.5 in methanol ^e molecular ions

the derivatives (45) gave the appropriate molecular ions as well as a major peak (46) corresponding to the loss of 138 mass units (nitrophenolate ion) (Table 3).

Although the *p*-nitrophenyl esters were obtained crystalline, pure specimens could only be isolated after column chromatography over silica during which they partially decomposed. The preparation of the *p*-nitrophenyl esters of other ketimines (33) yielded impure solids and oils and attempts to purify these compounds by chromatography over silica or alumina columns also resulted in their decomposition.

Peptide synthesis using the *p*-nitrophenyl esters (45) proceeded in low yield under standard reaction conditions²⁴ and most of the active ester starting material could be recovered. The poor results using this method may be due to a steric interference of the bulky protecting group. Condensation could be effected only by refluxing the reaction mixture for relatively long periods (compound 43g).

TABLE 4 - TIME REQUIRED FOR COMPLETE CLEAVAGE OF KETIMINE PROTECTING GROUP FROM PEPTIDES^a

Reagent Temp.	80% v/v Acetic Acid	80% v/v Formic Acid	IN Hydrochloric acid in 90% methanol
	5ClB-Gly- 5MeB-Leu- Boc-Gly- Boc-Leu-	5ClB-Gly- 5MeB-Leu- Boc-peptides	5ClB-Gly- 5MeB-Leu-
20°	16-20 h. 40-48 h. >110 d. ^A -	16-20 h. 40-48 h. ≈2½ h. ^B	10-16 h. 24-30 h.
50°	1½-2 h. 5-6 h. ≈30 d. ≈28 d.	1½-2 h. 5-6 h.	
80°	20-30min. 1½ h. - -	20-30min. 1½ h.	

a. 5ClB-Gly-Gly-OBzl (43a), 5MeB-Leu-Met-OMe (54), Boc-Gly-OEt and Boc-Leu-Met-OMe were used for hydrolysis studies; 0.05 mmol of each compound was dissolved in 0.20 ml of each acid and samples of 1 µl were withdrawn at intervals and examined on t.l.c. using carbon tetrachloride-ethyl acetate (8:2) as solvent system. Detection of compounds is described in the experimental section.

A. See reference 11.

B. H. Kinoshita and H. Kotake, Chemistry Letters, 1974, 631.

The disappointing results obtained with the *p*-nitrophenyl esters (45) prompted a brief study of the potential of pentachlorophenyl active esters of the ketimines (33) with the object of obtaining more stable carboxyl activated derivatives. When *N*-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valine (33f) was reacted with pentachlorophenol in the presence of DCC¹⁰⁸, formation of the active ester was not observed and (33f) was recovered unchanged after normal reaction times. In a second experiment *N*-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) was treated with DCC-pentachlorophenol complex¹⁰⁹. This yielded an oily mixture which contained the active ester as well as the starting materials. Attempts to purify the product by chromatography over silica and alumina columns were unsuccessful and resulted in decomposition of the required ester.

The diarylmethylene protecting group can be removed from protected peptides such as (43) by treatment with acetic acid (80% v/v) or formic acid (80% v/v) or with alcoholic hydrochloric acid (1N, prepared by dilution of concentrated hydrochloric acid 9 times with 90% methanol or 90% ethanol). The rate of cleavage is dependent upon the particular *N*-terminal amino acid residue of (43) and is determined by the degree of steric hindrance caused by the side chain. Table 4 lists the times required to cleave the ketimine protecting group at various temperatures in each acid. The intense

TABLE 5 - PHYSICAL CONSTANTS OF DIKETOPIPERAZINES (52)

Diketopiperazine ^h	Yield	m.p. ^c	[α] _D	m/e ^g	Molecular Formula	Found			Calc.		
						C	H	N	C	H	N
(52a) <i>c</i> -(S-Bzl)-Cys-Ala	44 ^a	207-210°	-4° ^d	264	C ₁₃ H ₁₆ N ₂ O ₂ S	59.2	6.0	10.4	59.1	6.1	10.6
(52b) <i>c</i> -Trp-Gly	59 ^a	293-297° ^A	-23° ^d	243	C ₁₃ H ₁₃ N ₃ O ₂	64.0	5.3	16.8	64.2	5.4	17.3
(52c) <i>c</i> -Met-Gly	56 ^a	210-214°	-10° ^d	188	C ₇ H ₁₂ N ₂ O ₂ S	44.7	6.5	-	44.7	6.4	-
(52d) <i>c</i> -Trp-Trp	66 ^b	265-268°	-81° ^e	-	C ₂₂ H ₂₀ N ₄ O ₂	71.1	5.5	14.5	71.0	5.4	15.0
(52e) <i>c</i> -Leu-Met	62 ^a	236-238°	-52° ^f	244	C ₁₁ H ₂₀ N ₂ O ₂ S	54.3	8.5	11.2	54.1	8.3	11.5

^a methanol-ether ^b *sec*-butanol ^c all compounds melted with decomposition

^d for measurement: c 0.5, dimethylformamide ^e c 1, acetic acid ^f c 0.5, methanol

^g molecular ions ^h all compounds are LL isomers ^A Kopple, K.D. and Ghazarian, H.G., J.Org.Chem.,

1968, 33, 862 report m.p. 299-300.5°. Hofmann, K., and Lande, S., J.Amer.Chem.Soc., 1961, 83, 2286

report m.p. 292-303°, [α]_D²⁸ + 38.2°

yellow colour of the peptide derivatives (43) makes it convenient to follow the progress of their hydrolysis on thin-layer chromatograms. The use of acetic acid (80% v/v) for the hydrolysis of (43) is compatible with the use of the Boc group for the protection of amino acid side chains, since it requires greater than 110 days to cleave the latter group from t-butyloxycarbonyl glycine ethyl ester¹¹. On the other hand, the diarylmethylene protecting group is seen to be more stable to formic acid (80% v/v) than the Boc protecting group. These results illustrate the different mechanisms involved in the cleavage of Schiff base and urethane protecting groups.

The hydrolysis of ketimine dipeptide esters with acetic and formic acid leads to the formation of dipeptide ester acetates and formates which can be readily converted¹¹⁰ to the corresponding diketopiperazines (52) upon heating. This reaction offers a convenient route to these sterically pure cyclic dipeptides (52a-52e) (Table 5). Diketopiperazines are also formed directly from ketimine dipeptide esters by treatment with ammonia at room temperature. This is demonstrated by the preparation of cyclo-L-tryptophyl-L-tryptophan (52d) and of cyclo-L-leucyl-L-methionine (52e) from the protected dipeptides (43c) and (54) respectively.

The arylmethylen protecting group has also been removed with *p*-toluenesulphonic acid monohydrate. Hydrolysis of N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-glycyl-glycine benzyl ester (43a) gave the dipeptide ester tosylate (47d) after four hours at 35°. However, because *p*-toluenesulphonic acid is a solid, the use of greater than equimolar quantities in the hydrolysis creates difficulty in removal of the excess acid from the reaction mixture during the work-up and it is more convenient to use the acids mentioned earlier.

Two methods have been used to demonstrate that peptide bond formation using the coupling reagents DCC, EDC and EEDQ and the subsequent cleavage of the arylmethylen protecting group proceeds without affecting the steric purity of the product. The formation of diketopiperazines from their dipeptide ester salts has been shown¹¹⁰ to proceed without racemization and Westley and colleagues⁵⁷ have demonstrated that many diastereoisomeric pairs of diketopiperazines can be

separated by thin-layer chromatography. The ketimine derivative of L-tryptophyl-L-alanine methyl ester (43q) was therefore prepared by the DCC method. Subsequent acetic acid hydrolysis ^{and heating in sec butanol} converted this compound to the corresponding diketopiperazine, cyclo-tryptophyl-alanine (52f) and steric analysis by thin-layer chromatography detected the LL diastereoisomer only. Similarly, N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-leucyl-L-leucine ethyl ester (43r) prepared using EDC, was converted to cyclo-leucyl-leucine (52g). Only the LL isomer could be detected after thin-layer chromatography.

The g.l.c. analysis of diastereoisomeric N-trifluoroacetyl dipeptide methyl esters ⁵¹ provides another excellent method for the detection of racemization in peptide synthesis. The DCC coupling method was therefore used to synthesize N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valyl-L-valine methyl ester (43h), the amino function was subsequently deblocked and the dipeptide ester was converted to its N-trifluoroacetyl derivative using methyl trifluoroacetate ¹¹¹. Less than 1% of the DL isomer was detected after g.l.c. analysis. Compound (43h) was also prepared using EEDQ coupling reagent and after hydrolysis and derivatization, less than 2% racemization had occurred.

C. THE SYNTHESIS OF SOME N-TERMINAL-SHORTENED
SEQUENCES OF SUBSTANCE P.

Although new protecting groups and coupling reagents are regularly proposed for peptide synthesis, a proper evaluation of their potential is only obtained if they can be demonstrated as useful in the synthesis of biologically active sequences. The steric nature of reactions involving the new protecting group or coupling reagent is easy to follow in such syntheses, since the product is usually compared with sequences isolated from natural sources. The diarylmethylene protected amino acids (33) have therefore been used in the syntheses of some N-terminal-shortened sequences of substance P. The biological activity of these shorter sequences has been previously demonstrated^{112,113}.

Substance P was first detected by von Euler and Gaddum¹¹⁴ in a dried powder obtained from extracts of horse brain and intestine. It possesses a wide range of pharmacological properties, including the ability to cause transient hypotension^{er} and to stimulate salivary secretion and also to bring about the contraction of smooth muscle preparations. In 1970 Chang and Leeman¹¹⁵ isolated a peptide from bovine hypothalamus in pure form and identified this peptide as substance P. Subsequently, in cooperative studies with Niall¹¹⁶ they determined that substance P was an undecapeptide having the sequence (53).

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

1 2 3 4 5 6 7 8 9 10 11

(53)

Treager and colleagues¹¹⁷ synthesized (53) using the solid phase method and employing a benzhydramine support. The synthetic product was found to possess all the chemical and biological properties of the naturally occurring compound. Bayer and Mutter¹¹⁸ have recently synthesized (53) using the liquid phase method^{119,120}.

Conventional syntheses of (53) have been carried out by Yajima's group¹¹² and also by Bergmann and colleagues¹¹³. The former used a fragment condensation strategy, first coupling 8-9 with 10-11 using DCC-HOBt, then adding residues 5, 6 and 7 in a stepwise manner using *p*-nitrophenyl esters. The *p*-methoxybenzyloxycarbonyl^{6,121} protecting groups were removed with trifluoroacetic acid. Sequence 1-2 was then coupled with 3-4 using the pentachlorophenyl ester and 1-4 was coupled with 5-11 using DCC-HOBt. The Z blocking groups on residues 1 and 3 and the nitro group on residue 1 were removed with hydrogen fluoride¹²² to give (53). Bergmann's group coupled residues 1-7 in a stepwise manner to the sequence 8-11. The Bpoc α -amino protecting group was used with residues 2 and 3 and Boc

SCHEME 5

protection was used for other residues and for the lysine side chain. Hydrogen chloride was the deblocking agent. Mixed anhydrides were employed to couple the arginine and glutamine residues whilst other residues were added using trichlorophenyl esters¹³³. Finally, the Boc protecting groups on residues 1 and 3 and the nitro group on residue 1 were cleaved, using hydrogen fluoride, to give (33). Both Yajima and Bergmann also examined the biological activity of carboxyl terminal sequences. The hexapeptide 6-11, heptapeptide 5-11 and octapeptide 4-11 all exhibited higher activity on smooth muscle than (53).

In this project, the ketimine derivatives of amino acids (33) have been used to synthesize the hexapeptide sequences 6-11 in a stepwise manner from the carboxyl terminal methionine. The methods employed are outlined in Scheme 5.

The DCC-HOBt coupling procedure was used throughout, excepting the preparation of the hexapeptide derivative (63) where the EDC procedure was employed. In the initial step, N-[α -phenyl-(2-hydroxy-5-methyl-benzylidene)]-L-leucine (33q) was condensed with L-methionine methyl ester to yield the protected dipeptide (54). Deblocking of (54) with methanolic hydrogen chloride gave the oily ester hydrochloride (55). The amino function of (54) was also deblocked with formic acid to give the peptide ester formate (55a). However, even with careful work-up at low temperatures small

quantities of the diketopiperazine (52e) were formed and preparation of the more stable hydrochloride (55) obviates this problem. Neutralization of (55) and coupling with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) afforded the crystalline tripeptide derivative (56) which after treatment with acetic acid, gave the acetate salt (57). Deblocking of (56) with formic acid gave a product (57a) which was hygroscopic and therefore difficult to handle. Coupling of N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) with the neutralized tripeptide ester acetate (57) yielded the Schiff base (58) and cleavage of (58) with acetic acid gave the required tetrapeptide derivative (59). The formate salt (59a) of this tetrapeptide ester was also characterized. A further coupling of (33t) with the free ester of (59) gave the protected pentapeptide ester (60) and (60) was deblocked with acetic acid to yield (61). The pentapeptide ester acetate (61) was then neutralized and converted to the corresponding amide (62) by the action of ammonia. After chromatographic purification, the pentapeptide amide (62) was condensed with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-glutamine (33p) and the resulting Schiff base peptide amide (63) was hydrolyzed with acetic acid to afford the required hexapeptide amide acetate (64), which was subsequently purified by adsorption chromatography.

SCHEME 6

The tripeptide and tetrapeptide carboxyl terminal sequences of substance P were also synthesized, starting with L-methionine amide, as shown in Scheme 6.

The DCC-HOBt procedure was used for all the coupling reactions. Condensation of N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-leucine (33q) with L-methionine amide gave the dipeptide derivative (65) which could be deblocked with acetic acid to give (66a), as diketopeperazine formation is prohibited. However cleavage with hydrochloric acid was preferred, leading to the known compound (66), which was neutralized and condensed with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) to give the protected tripeptide (67). Deblocking of (67) with acetic acid gave the tripeptide amide (68). After coupling (68) with N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) the resulting tetrapeptide derivative (69) yielded the tetrapeptide amide (70) after hydrolysis of the ketimine protecting group with acetic acid.

The tripeptide (68), tetrapeptide (70), pentapeptide (62) and hexapeptide (64) sequences were assayed for vasodilator, spasmogenic and vasoconstrictor properties using three preparations, viz. the hind limb blood flow of the dog, the isolated guinea pig ileum and the isolated rabbit ear vein¹²³. In all tests, the peptides prepared in this project were found to be

TABLE 6 - MOLAR POTENCY OF C-TERMINAL SEQUENCES OF SUBSTANCE P^a

Peptide	Assay	Dog Femoral Artery		Guinea Pig		Rabbit Ear	
		Blood Flow		Ileum		Vein	
		K(N=4) ^b	S(N=4)	K(N=4)	S(N=6)	K(N=4)	S(N=4)
(64) Hexapeptide		66(8)	65(10)	108(12)	108(14)	43(12)	48(15)
(62) Pentapeptide		5.4(1.4)	4.2(0.8)	2.4(0.4)	2.2(0.2)	1.0(0.3)	0.9(0.2)
(70) Tetrapeptide		c	$2.6 \times 10^{-2}(0.8)$	inactive	inactive	inactive	inactive
(68) Tripeptide		c	$1.1 \times 10^{-3}(0.4)$	inactive	inactive	inactive	inactive

a. Values are relative to natural substance P (100).

b. K refers to compounds synthesized using ketimine amino protection; S refers to reference peptides prepared on a benzhydrylamine resin using Boc amino protection and trifluoroacetic acid for deprotection (See reference 123); N refers to number of tests carried out; bracketed figures are standard errors of the mean.

c. Not determined.

as active as reference peptides prepared by a conventional synthetic method¹²³ and the results were in good agreement with those obtained by both the Yajima and Bergmann groups. Table 6 summarizes the results of the biological tests.

D. KETIMINE PROTECTED AMINO ACIDS IN SOLID PHASE PEPTIDE SYNTHESIS.

The advantages offered by acid-labile amino protecting groups in conventional solution peptide synthesis apply equally to the solid phase method, but their use has been restricted by the unavailability of suitable stable amino acid derivatives. The acid-labile residues which have proved most useful for solid-phase work are the Nps and Bpoc groups⁶⁷ whilst most other easily removable functions have either not been examined in solid phase systems or have been found unpromising after very limited testing.

The first attempts to use Schiff base α -amino protection for solid phase synthesis were made by Merrifield¹²⁴. He employed the 2-hydroxy-1-naphth-aldehyde condensates of amino acids, but without success under the particular coupling conditions used. These same derivatives have been the subject of a more recent investigation by Khosla and colleagues⁸⁰. Their results indicated that the Schiff base condensates could be best

used as their pentachlorophenyl esters for coupling and a simple tripeptide was prepared by the solid phase method. The protecting group was cleaved in one hour by 0.5 M aqueous hydrochloric acid in dioxane or by 7% benzylamine in dichloromethane in thirty minutes. The enamine protecting group, BMV, has also been investigated for solid phase application by Southard and colleagues⁸⁴. A simple tetrapeptide and also a glucagon sequence were successfully synthesized on a benzhydrylamine resin using the DCC coupling procedure. Removal of the BMV blocking group from the N-terminal amino acids was best effected by a thirty minute treatment with 0.4 M aqueous hydrochloric acid in tetrahydrofuran.

A major advantage offered by derivatives such as the 2-hydroxy-1-naphthaldehyde condensates in solid phase synthesis, not possessed by enamine or other acid-labile residues, is the yellow colour which they impart to the resin after coupling. This property permits easy visual monitoring of the deprotection step. The somewhat increased stability of the diarylmethylene amino acids (33) prepared in this project, coupled with their intense yellow colour, suggested that they may be useful for solid phase synthesis.

The ketimine derivatives of amino acids (33) can be readily reacted with chloromethylated peptide resins, yielding the corresponding ketimine amino acyl resins. The ketimine alanyl resin (71) and the ketimine glycyl

resin (72) were prepared using standard esterification procedures, the former by Merrifield's method¹²⁵ and the latter using Loffet's modified technique¹²⁶. The amino acid substitution was comparable to that normally obtained with Boc amino acids under the same conditions and the resins possessed a bright yellow colour. The ketimine condensates (33) could also be coupled with amino acyl resins using the DCC procedure to form the corresponding ketimine dipeptide acyl resins. Trans-esterification of these acyl resins using triethylamine in methanol¹²⁷ affords the protected dipeptide methyl esters.

Unfortunately, a satisfactory method for removal of the ketimine protecting group from the amino or peptide acyl resin has, as yet, not been found. Although the diarylmethylene protecting group is removed with relative ease by weak acids in solution it appears remarkably stabilized when incorporated by the resin. When N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-valyl-alanyl resin (73) was treated with 80% aqueous acetic acid at room temperature, hydrolysis of the imine bond was only just detectable after 24 hours and even after 72 hours the resin still possessed a strong yellow colour. For comparison, N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-valyl-alanine methyl ester (74) was deblocked under similar conditions to those described above. Hydrolysis of the azomethine linkage could be detected almost immediately, and was complete after

35 hours. The poor swelling properties of the resin in acetic acid may account for this observed increase in stability of the diarylmethylene protecting group. The cleavage mechanism involves the participation of water which may have difficulty in penetrating the resin beads in poor swelling solvents.

A number of other reagents have been used in attempts to find a suitable deblocking method for the ketimine protecting group. A 90 minute treatment with dichloromethane-aqueous chloroacetic acid has been used¹¹ to cleave the Bpoc residue in solid phase synthesis. The good swelling properties of the resin in dichloromethane suggested this reagent may be useful in removal of the imine protecting group. N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] glycyl-phenylalanyl resin (75) was used as the test compound and although significant quantities of the protecting group were removed, the yellow colour persisted in the resin even after 9 hours reaction time.

The N-(2-hydroxyarylidene) protecting group was cleaved from the ketimine dipeptide resin (73) using a 4N solution of p-toluenesulphonic acid monohydrate in dioxane. However in view of the long cleavage time (20 hours) and the strong acidic conditions, this reagent does not seem practically useful.

The use of 0.4 N hydrochloric acid in tetrahydrofuran, as used⁸⁴ for removal of the BMV protecting group, cleaved the diarylmethylene residue from N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-glycyl-valyl resin (76) in 2½ hours. However, when phenylalanine was the N-terminal amino acid of the peptide acyl resin (79), removal of the ketimine function required 14 hours using 1N hydrochloric acid in tetrahydrofuran. These results were the most promising obtained, but considering the cleavage time, deprotection for the ketimine protecting group *was not viable with this reagent.*

Transamination employing benzylamine in dichloromethane has also been tested as a means of removing the Schiff base protecting group. The ketimine dipeptide resin (73) was treated with a 0.4 N benzylamine solution, but the transamination reaction was too slow. Increasing the solution strength to 4N resulted in a speedier reaction, but complete discharge of colour from the resin required 10 hours. However, the presence of a peptide on t.l.c. plates suggested that nucleophilic attack by benzylamine at the acyl-resin bond may have also taken place. This thesis was supported when, after treatment of the deblocked resin with triethylamine and methanol, the reaction mixture gave a negative test for the presence of peptide.

The fact that N,N-dimethylhydrazine does not react with esters larger than formates¹²⁸ eliminates the risk of acyl-resin bond cleavage and this prompted a brief investigation of this reagent for removal of the ketimine function. Treatment of N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-glycyl-glycine resin (81) with a 1N solution in dichloromethane had little effect after 48 hours and N,N-dimethylhydrazine is obviously too weak a base to be of use in the cleavage of the diarylmethylene group.

As the removal of Schiff base or enamine protecting groups under acidic conditions requires the presence of water, the diarylmethylene residue should be stable to reagents normally used to cleave the Boc group if anhydrous conditions are employed. This has been demonstrated by coupling N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valine (33f) to glycyl resin, followed by treatment of the resulting ketimine dipeptide resin (83) with 4N hydrogen chloride in dioxane. After base-catalyzed transesterification N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valyl-glycine methyl ester (84) was obtained in good yield. In a second experiment, N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-tryptophan (33l) was coupled to alanyl resin and the transesterified products (43q) both before and after treatment of the resin with 1N hydrogen chloride in acetic acid, were identical. The use of trifluoroacetic acid in dichloromethane in this latter experiment also left the

Schiff base unaffected.

The foregoing results suggested it would be possible to use the diarylmethylene residue to protect the side chain of lysine in solid phase work in conjunction with Boc protection of α -amino functions. To test this hypothesis, N^{α} -t-butyloxycarbonyl- N^{ϵ} -[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-lysine was liberated from its dicyclohexylammonium salt (34d), coupled with glycyl resin and the Boc group was cleaved with trifluoroacetic acid. The resin retained its strong yellow colour and there was no indication of cleavage of the side chain protecting group. A further coupling of the dipeptide resin with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) followed by base-catalyzed transesterification in methanol gave a yellow oil (86) which incorporated two diarylmethylene residues according to its mass spectrum. Hydrolysis of (86) in hydrochloric acid gave the tripeptide derivative glycyl-L-lysyl glycine methyl ester hydrochloride (87)

E. CONCLUDING REMARKS

The N-[α -phenyl-(5-substituted-2-hydroxybenzylidene)] residue offers itself as an extremely useful addition to the many amino protecting groups which are used in peptide synthesis. The N-protected amino acids are synthesized by a simple reaction scheme

from a cheap starting material which may be recovered and purified for further use if required and most derivatives can be obtained in the free acid form. Because they are not N-acyl derivatives, racemization by the formation of oxazol-5-one intermediates is not possible. They are also coloured and ketimine peptide derivatives are thus easily followed by chromatographic means. The ease with which the diarylmethylene protecting group is cleaved from amino groups by dilute acid suggests its use for the introduction of acid sensitive amino acids such as tryptophan, into peptide chains. The mechanism of hydrolysis of the imine bond offers some versatility in the use of the diarylmethylene protecting group and its stability under strictly anhydrous conditions should permit its use, in conjunction with urethane blocking groups, for lysine and ornithine side chain protection in solid phase peptide synthesis.

PREPARATION OF TRYPTOPHAN BENZYL ESTER

The preparation of tryptophan ester derivatives which are suitable for peptide synthesis is complicated by the acid sensitivity of the indole nucleus in the amino acid side chain. The benzyl ester of tryptophan, for example, cannot be prepared by standard esterification procedures due to the formation of degradation products at the acid pH values and high temperatures required for reaction. This particular derivative is normally obtained¹²⁹ by first converting tryptophan to the N-carboxyanhydride. Esterification is then carried out at room temperature using benzyl alcohol-ether saturated with hydrogen chloride.

Maclaren¹³⁰ has proposed a very convenient method for the preparation of amino acid esters which involves the treatment of enamine protected amino acid potassium salts with an alkyl halide in dimethylformamide. This leads to the corresponding enamine amino acid alkyl ester, which is converted by mild acidolysis to the required amino acid ester. The method was primarily designed for the preparation of acid labile ester derivatives, but it was shown that benzyl esters could be obtained by this route. Ester derivatives of tryptophan, however, could not be prepared by this method¹³¹ due to the formation of β -carboline structures.

Maclaren's unsuccessful attempts to prepare esters of tryptophan has prompted a brief investigation into the suitability of the diarylmethylene residue as an alternative "fugitive" N-protecting group in his procedure. Of particular interest was the preparation of tryptophan benzyl ester (Scheme 7).

SCHEME 7

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-tryptophan (331) was converted to its tetramethylammonium salt (88) and esterified using benzyl chloride in dimethylformamide. The resulting N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-tryptophan benzyl ester (89) could be isolated and was a well characterized solid. Removal of the diarylmethylene protecting group was then effected with acetic acid to give the benzyl ester acetate (90) or with formic acid to yield the formate salt (91). However, the effect of the solvent on the salt (88) was to cause complete racemization in the ketimine benzyl ester (89). When the tetramethylammonium salts of several other ketimine amino acid condensates were dissolved in dimethylformamide, these too were found to undergo racemization so that this loss of steric purity was general for amino acids and was not restricted to tryptophan. The use of solvents other than dimethylformamide in the esterification may eliminate racemization but the solubility of the tetramethylammonium salts was found to be a problem in this respect. Application of the potassium salt of (331) to the reaction has not been investigated as yet.

A new route to tryptophan benzyl ester has been recently reported¹³². Thermal decomposition of an N-protected tryptophan benzyl dimethylphenylammonium salt eliminates dimethylaniline forming the N-protected benzyl ester which is deblocked to afford the required compound. No racemization was observed in this method.

EXPERIMENTAL

General

Melting points were determined on a Reichert hot-stage microscope and are uncorrected. All temperatures are expressed in degrees Celsius.

Optical rotations were measured on a Carl Zeiss circle .01° manual polarimeter using a 0.5 dm tube. All measurements were made at 22-25°.

Infrared spectra were recorded on a Perkin-Elmer 357 grating spectrophotometer using sodium chloride cells.

Ultraviolet spectra were recorded on a Hitachi EPS-3T recording instrument using anhydrous methanol as solvent.

P.m.r. spectra were determined using a Jeolco JNM-4H-100 machine with tetramethylsilane as internal reference. Abbreviations s, d, q refer to singlet, doublet and quartet respectively.

Mass spectra were obtained from an E.A.I. Quad 300D low resolution instrument or from a Dupont 21-491B machine.

Gas-liquid chromatographic (g.l.c.) separations were carried out using a Varian 600C gas chromatograph

fitted with a hydrogen flame detector. The column used was an 8 ft. x 1/8 in. O.D. stainless steel tube packed with 5% silicone OV17 on Chromosorb W (silanized) and helium was used as the carrier gas.

Microanalyses were performed by Dr. E. Challen of the University of New South Wales and by the Australian Microanalytical Service, CSIRO, Melbourne.

Column chromatography of peptide derivatives was carried out using Merck silica gel. Thin-layer chromatography (t.l.c.) was performed with Merck silica gel G (.025" layers). Rf values should be taken as a guide only. Preparative layer chromatography was also carried out with Merck silica gel G (0.1" layers). Schiff base derivatives could be visualized directly or by exposure to iodine vapour. Peptide bonds were detected by the hypochlorite - o-tolidine method¹³⁴ and free amino groups were detected with ninhydrin.

Light petroleum refers to the fraction b.p. 60-80°.

Combined organic phases from extracts were dried with anhydrous magnesium sulphate.

Anhydrous solvents were employed in condensation, coupling and recrystallization steps involving the Schiff bases.

Molecular sieve type 3A (1/16" pellets) was manufactured by the Linde Air Products Co. and distributed by BDH Chemicals (Poole, England).

Amino acids and amino acid derivatives used in the condensations were supplied by Fluka (Buchs, Switzerland), Bachem Inc. (Marina Del Rey, California), Sigma Chemical Co. (St. Louis, Missouri) and BDH Chemicals.

The ketones (30a-30d) were obtained from Pfaltz and Bauer (Flushing, New York). *p*-Tolyl benzoate was also supplied by Pfaltz and Bauer.

The Boc amino acyl resins used for solid phase studies were purchased from Schwarz-Mann (Orangeburg, New York). Merrifield peptide resin was supplied by the Pierce Chemical Co. (Rockford, Illinois).

Preparation of Amino Acid Methyl or Ethyl Ester Hydrochlorides.

Methyl esters of alanine, valine and phenylalanine and ethyl esters of tryptophan, tyrosine and leucine were prepared by the method of Brenner and Huber¹³⁵.

To dried methanol or ethanol¹³⁶ (7 ml) cooled in dry ice-acetone was added freshly distilled thionyl

chloride (0.7 ml). The amino acid (1 g) was then refluxed for 45 minutes in the thionyl chloride-alcohol reagent. After evaporation of the solvent, the crystalline residue was twice redissolved in the alcohol and again evaporated and the ester hydrochloride was finally recrystallized from the appropriate alcohol-ether mixture.

Preparation of Amino Acid Benzyl Ester p-Toluenesulphonates

Benzyl esters of glycine, leucine and glutamic acid were prepared according to the procedure of Zervas, Winitz and Greenstein¹³⁷.

A mixture of the amino acid (50 mmol), p-toluenesulphonic acid (50 mmol), benzyl alcohol (10 ml) and benzene-toluene (20 ml, 1:1) were heated under reflux and the liberated water was removed azeotropically by means of a Dean and Stark receiver. When all water was distilled off, the mixture was cooled and ether (200 ml) was added. The ester crystallized after standing in the refrigerator for several hours. Recrystallization was effected from methanol-ether.

Preparation of γ -Benzyl-L-Glutamic Acid¹³⁸

A mixture of glutamic acid (14.7 g, 100 mmol), concentrated hydrochloric acid (250 ml) and benzyl alcohol (100 ml) was heated for 2 hours. After cooling in an ice

bath, ether (800 ml) was added and the white solid was filtered off, then dissolved in water (120 ml). The pH of the solution was adjusted to 6.0-6.5 using lithium hydroxide and after cooling for 2 hours, the product was filtered off, washed with ethanol and ether and recrystallized from boiling water. Yield 4.83 g (20%); m.p. 175-177°; $[\alpha]_D + 20^\circ$ (c 5, methanol) (lit.¹³⁹ m.p. 169-170°; $[\alpha]_D + 18.7^\circ$)

Preparation of 2-Hydroxy-5-methylbenzophenone (30b)¹⁴⁰.

p-Tolyl benzoate (15 g, 70 mmol) and aluminium chloride (12 g, 90 mmol) were mixed thoroughly in a mortar and the mixture was transferred to a round-bottomed flask fitted with a thermometer and an air condenser. The flask was placed in an oil bath at 90° and heated rapidly to 120°, then more slowly until the temperature reached 140°. After 10 min at this temperature, the flask was cooled and the solid material was removed and crushed to a fine powder in a mortar. It was then added, with stirring, to a mixture of 50 g of ice and 30 ml of concentrated hydrochloric acid. The solid product was removed by filtration and washed with copious quantities of water. After recrystallization from ethanol the yield was 10.2 g (68%). A second recrystallization from ethanol gave the analytical specimen, m.p. 80-82° (lit.¹⁴⁰ 83-84°). Found: C, 79.1; H, 5.7. Calculated for $C_{14}H_{12}O_2$: C, 79.2; H, 5.7%.

Preparation of Tetramethylammonium Hydroxide Solution.

A mixture of tetramethylammonium bromide (6.16 g; 40 mmol) and silver oxide (7.00 g; 30 mmol) was stirred for 1 hour in methanol (70 ml), then filtered. The filtrate was diluted to 100 ml with methanol and the correct strength of the base (approximately 0.4 mmol/ml) was then determined by titration of an aliquot with standard sulphuric acid solution using phenolphthalein indicator. The solution keeps satisfactorily when stored at 0° over 3A molecular sieves.

Preparation of Dicyclohexylamine Solution.

Freshly distilled dicyclohexylamine (7.96 ml; 40 mmol) was diluted to 100 ml with methanol and stored over 3A molecular sieves at 0°. The strength of the solution was determined by titration of an aliquot with standard hydrochloric acid using bromocresol green-methyl orange (5:1) mixed indicator¹⁴¹.

General Procedure for the Synthesis of N-[α -Phenyl-(5-substituted-2-hydroxybenzylidene)] Amino Acids (33a-33w).

A mixture of the amino acid (3 mmol), tetramethylammonium hydroxide solution (3 mmol) and the appropriate ketone (30) (3 mmol) was diluted with methanol (20 ml) and kept over a few sticks of type 3A molecular sieve at room temperature until a homogeneous solution resulted (from 1 to 10 days). After removal of the

solvent on a rotary evaporator, water (40 ml) was added and unchanged ketone starting material was filtered off. The pH of the filtrate was then adjusted to 5-6 by careful addition of 20% citric acid solution and the Schiff base was extracted into ethyl acetate-ether (9:1). The dried extract was concentrated and the ketimine was purified using an appropriate solvent system. Table 1 lists the physical constants of the compounds prepared.

For those derivatives kept as dicyclohexylammonium salts (34a-34g) the crude product was twice dissolved in ether, filtered and concentrated, and the residue was immediately dried in a tared flask under vacuum over anhydrous calcium chloride. The free acid was then treated with an equivalent amount of dicyclohexylamine solution (approximately 0.4 mmol/ml methanol) and the solvent was removed on the rotary evaporator. The salt crystallized on standing and recrystallization was effected from a suitable solvent.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]- β -benzyl-L-Aspartic Acid Dicyclohexylammonium salt (34a).

From an ethereal solution, the free acid derivative ($C_{24}H_{20}ClNO_5$) was a hygroscopic oil. ν max (chloroform) 1730 (ester), 1715 (shoulder, acid), 1610 (imine) cm^{-1} ; mass spectrum m/e 393 ('A' fragment).

Preparation of the dicyclohexylammonium salt as described

in the general procedure yielded a yellow crystalline solid but, in spite of several recrystallizations from ethyl acetate, satisfactory analyses could not be obtained for the salt. The yield of the impure derivative was 63%.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]- γ -benzyl-L-Glutamic Acid Dicyclohexylammonium salt (34b).

The free acid derivative ($C_{25}H_{22}ClNO_5$) was obtained as a yellow hygroscopic oil on evaporation of an ethereal solution. ν max (chloroform) 1730 (ester), 1715 (shoulder, acid) and 1610 (imine) cm^{-1} ; mass spectrum m/e 407 ('A' fragment). The dicyclohexylammonium salt was prepared as described in the general procedure and it was recrystallized from methanol-ether. Despite several recrystallizations, satisfactory elementary analyses could not be obtained. The yield of the impure salt was 29%.

N $^{\alpha$ -[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-N $^{\epsilon}$ -benzyloxycarbonyl-L-Lysine Dicyclohexylammonium salt (34c).

Evaporation of an ethereal solution gave the free acid derivative ($C_{27}H_{27}ClN_2O_5$) as a yellow hygroscopic foam. ν max (chloroform) 3450, 1690 (urethane), 1720 (acid) and 1610 (imine) cm^{-1} . Mass spectrum m/e 450 ('A' fragment). Neutralization of the free acid with dicyclohexylamine solution gave the required salt. This

was recrystallized from ethyl acetate but elementary analyses were unsatisfactory. The yield of impure salt was 47%.

N^{α} -t-Butyloxycarbonyl- N^{ϵ} -[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Lysine Dicyclohexylammonium salt (34d).

The free acid derivative ($C_{24}H_{29}ClN_2O_5$) was a hygroscopic foam from ether. ν max (chloroform) 3440, 1695 (urethane), 1720 (acid) and 1610 (imine) cm^{-1} ; mass spectrum m/e 416 ('A' fragment). The dicyclohexylammonium salt was obtained as a yellow solid on evaporation of an ether solution but the analytical results were poor. The yield of the impure product was 54%.

N^{α} -[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]- N^{ϵ} -t-butyloxycarbonyl-L-Lysine Dicyclohexylammonium salt (34e).

Evaporation of an ethereal solution gave the free acid ($C_{24}H_{29}ClN_2O_5$) as a yellow foam. ν max 3450, 1690 (urethane) 1720 (acid) and 1610 (imine) cm^{-1} ; mass spectrum m/e 416 ('A' fragment). The impure dicyclohexylammonium salt was obtained as a yellow solid on evaporating an ether solution. The yield was 64%. Satisfactory analyses were not obtained.

N^α-[α-Phenyl-(5-chloro-2-hydroxybenzylidene)]-N^δ-t-butyl-oxycarbonyl-L-Ornithine Dicyclohexylammonium salt (34f).

From ether the free acid derivative (C₂₃H₂₇ClN₂O₅) was a hygroscopic foam. ν max 3440, 1690 (urethane), 1720 (acid) and 1615 (imine) cm⁻¹; mass spectrum m/e 402 ('A' fragment). After evaporation of the neutralized free acid solution, addition of ether gave the salt which was recrystallized from methanol-ether. Yield 45%; m.p. 170-173°; [α]_D + 22° (c 4, methanol). Found: C, 67.2; H, 8.1; N, 7.0. C₃₅H₅₀ClN₃O₅ requires: C, 66.9, H, 8.0; N, 6.7%.

N^α-[α-Phenyl-(5-chloro-2-hydroxybenzylidene)]-N^ω-Nitroarginine Dicyclohexylammonium salt (34g).

Unreacted nitroarginine was filtered from the reaction mixture and the free acid Schiff base (C₁₉H₂₀N₅O₅Cl) was obtained as an oil after the usual work-up. This oil solidified on rubbing with ether. ν max 1720 (acid) and 1615 (imine) cm⁻¹; mass spectrum m/e 389 ('A' fragment). The yellow solid was observed to be hygroscopic so the free acid derivative was neutralized to give the dicyclohexylammonium salt which crystallized from methanol. Satisfactory analyses could not be obtained. The yield of the impure salt was 36%.

Reaction of D-Phenylglycine with o-Hydroxy Aromatic Ketones (30) - Preparation of Benzylamine Derivatives (40).

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] benzylamine(40a).

D-Phenylglycine (453 mg, 3 mmol), tetramethylammonium hydroxide solution (3 mmol) and 5-chloro-2-hydroxybenzophenone (30a) (699 mg, 3 mmol) were mixed and diluted with methanol (20 ml) and left over 3A molecular sieves at room temperature. After ten days, the solvent was removed and the residue was submitted to column chromatography, with benzene and benzene-ethyl acetate mixtures as eluents. Concentration of the eluate yielded 877 mg (91%) of the benzylamine derivative as a yellow oil, homogeneous on t.l.c. (benzene); mass spectrum m/e 321 (M^+). No carboxyl band was observed in the infrared spectrum (thin film). Condensation of benzylamine with (30a) in methanol and purification as described above gave an identical product.

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)] benzylamine(40b)

D-Phenylglycine (453 mg, 3 mmol) tetramethylammonium hydroxide solution (3 mmol) and 2-hydroxy-5-methylbenzophenone (30b) (636 mg, 3 mmol) were allowed to react and the mixture was worked up as described for compound (40a). Yield 429 mg (48%) as a yellow oil, homogeneous on t.l.c. (benzene); mass spectrum m/e 301 (M^+). No carboxyl band was observed in the infrared spectrum (thin film). Condensation of benzylamine with (30b) in methanol and chromatographic purification as described

previously gave an identical product.

N-[α -Phenyl-(2-hydroxybenzylidene)] benzylamine (40c).

D-Phenylglycine (453 mg, 3 mmol), tetramethylammonium hydroxide solution (3 mmol) and 2-hydroxybenzophenone (30c) (595 mg, 3 mmol) were allowed to react as described for compound (40a) to yield 595 mg (69%) of the product as a yellow crystalline solid, homogeneous on t.l.c. (benzene); m.p. 88-90° from ethanol (lit. ¹⁴² m.p. 78°); mass spectrum m/e 287 (M⁺). Found : C, 83.6; H, 6.0; N, 4.6. Calculated for C₂₀H₁₇NO : C, 83.6; H, 6.0; N, 4.9%.

N-[α -Methyl-(2-hydroxybenzylidene)] benzylamine (40d).

From D-Phenylglycine (453 mg, 3 mmol), tetramethylammonium hydroxide (3 mmol) and 2-hydroxyacetophenone (30d) (419 mg, 3 mmol) was similarly obtained pale yellow crystals homogeneous on t.l.c. (chloroform). Yield 404 mg (60%); m.p. 120° from methanol (lit. ¹⁴² m.p. 125°); mass spectrum m/e 225 (M⁺). Found : C, 80.5; H, 6.7; N, 6.3. Calculated for C₁₅H₁₅NO : C, 80.0; H, 6.7; N, 6.2%.

Reaction of α -Phenylalanine (42) with o-Hydroxy Aromatic Ketones (30).

Preparation of DL- α -Phenylalanine (42).

The compound was prepared by a method which has been described in detail by Steiger¹⁰⁶.

Starting with a mixture of acetophenone (100 mmol), sodium cyanide (110 mmol) and ammonium chloride (100 mmol) the amino acid was obtained in 32% yield with m.p. 285-290^o (sublimation and decomposition) lit.¹⁰⁶ m.p. 265-270^o). Rf 0.49 (n-butanol-acetic acid-water, 4:1:1). Found : C, 65.3; H, 6.7. Calculated for C₉H₁₁NO₂ : C, 65.4; H, 6.7%.

Reaction of (42) with the Ketones (30a-30d).

A mixture of (42) (165 mg, 1 mmol) and the appropriate ketone (30) (1 mmol) was left over 3A molecular sieves in methanol (5 ml) at room temperature. For comparison, 1-phenylethylamine (0.13 ml, 1 mmol) and the appropriate ketone (1 mmol) were similarly condensed. The course of each reaction was followed by t.l.c. (benzene) and over several weeks the only condensation products which could be detected in the reaction of (42) with the ketones did not move from the start of t.l.c. plates. No trace of the decarboxylated derivatives was observed. Condensates of (42) and the ketones were finally worked up as described in the general procedure for the preparation of the ketimine amino acids (33) to give products which possessed carboxyl groups according to their infrared spectra (chloroform).

Steric Analysis of Ketimine Amino Acids

Hydrolysis of N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]
-L-Alanine (33c) and Steric Analysis of Recovered Alanine.

Compound (33c) (152 mg, 0.5 mmol) was dissolved in alcoholic hydrochloric acid (2 ml, 1N) and after standing at room temperature for 10 mins the solvent was removed under reduced pressure. The residue was washed with ether (3 x 5 ml portions), taken up in water (10 ml) and transferred to a Zeokarb 225 (H form) ion exchange column. After elution with 2N ammonia, evaporation of the eluate yielded 44 mg (70%) of the amino acid. The recovered alanine was then refluxed with thionyl chloride-methanol reagent¹³⁵. After evaporation of the solvent, the residue was treated with a solution of N-trifluoroacetyl-L-prolyl chloride in dichloromethane (5 ml) followed by triethylamine (0.14 ml)⁵².

After standing for 1 hour at room temperature, the solution was washed with dilute hydrochloric acid, water, dilute sodium bicarbonate and water. The dried solvent was removed and the oily residue was taken up in ethyl acetate (1 ml) for g.l.c. analysis. DL-Alanine was similarly derivatized to check retention times of the diastereoisomers. G.l.c. analysis indicated less than 2% racemization of the L-alanine had occurred during the condensation and hydrolysis steps.

Hydrolysis of N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]
-L-tryptophan (33l) and Steric Analysis of Recovered
Tryptophan.

Compound (33l) (105 mg, 0.25 mmol) was dissolved in 80% acetic acid (2 ml) and the solution was kept at 80° for 30 min. The reaction mixture was worked up in a similar manner to that described for hydrolysis of the L-alanine derivative (33c) to yield 35 mg (69%) of tryptophan. The recovered amino acid was converted to its N-trifluoroacetyl-(+)-*sec*-butyl ester derivative⁵⁴ for g.l.c. analysis. Less than 1% of D-tryptophan was present.

Hydrolysis of N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]
-L-phenylalanine (33t) - Specific Rotation of Recovered
Phenylalanine.

Compound (33t) (1.08 g, 3 mmol) was treated with alcoholic hydrochloric acid (7 ml, 1N) and left at room temperature overnight. The mixture was thereafter worked up as described for the hydrolysis of the alanine derivative (33c) to give 390 mg (79%) of phenylalanine. From the average of 10 measurements $[\alpha]_D$ for the recovered amino acid was found to be -39° (c 1.8, water). The specific rotation of the phenylalanine starting material used in the preparation of (33t) was also measured under the same conditions and was found to be identical to that of the recovered amino acid.

Synthesis of N-[α -Phenyl-(5-substituted-2-hydroxy-benzylidene)] Peptide Esters (43a-43m).

A. Use of Dicyclohexylcarbodiimide (DCC) Coupling

³¹
Reagent .

The amino acid or peptide ester (1 mmol) was liberated from its salt using either triethylamine (0.14 ml, 1 mmol) or N-methylmorpholine (0.112 ml, 1 mmol). The free ester in an appropriate solvent (Table 2) was then treated with N-[α -phenyl-(5-substituted-2-hydroxy-benzylidene)] amino acid (33) (1 mmol) and after the mixture was cooled to 0°, DCC (206 mg, 1 mmol) was added. After stirring the reaction mixture for 1 hour at 0°, the flask was left overnight in the refrigerator. The urea by-product was then filtered, the solution was evaporated and the residue was taken up in ethyl acetate (30 ml). The reaction mixture was then washed with citric acid solution (20%), water, sodium bicarbonate solution (10%) and water. The dried solution was evaporated to yield the crude product which was purified either by recrystallization or by column chromatography using gradient elution with benzene and benzene-chloroform mixtures or carbon tetrachloride and carbon tetrachloride-ethyl acetate mixtures. Compounds prepared by this procedure are listed in Table 2.

B. Use of 1-Hydroxybenzotriazole-Dicyclohexyl-
carbodiimide (DCC-HOBt) Coupling Procedure³⁶.

Prior to the introduction of DCC, the reaction mixture is treated with HOBt (135 mg, 1 mmol). The procedure is otherwise as outlined in A. Compounds prepared by this method are shown in Table 2.

C. Use of 1-Ethyl-3-(3'-Dimethylaminopropyl)
carbodiimide (EDC) Coupling Reagent³³.

In procedure A, EDC hydrochloride (192 mg, 1 mmol) is substituted for DCC. After standing overnight in the refrigerator, the water-soluble urea by-product was removed in the washing step. Compounds prepared by this procedure are shown in Table 2.

D. Use of 1-Ethoxycarbonyl-2-Ethoxy-1,2-Dihydroquinoline
(EEDQ) Coupling Procedure³⁷.

The amino acid ester salt (1 mmol) in tetrahydrofuran (7 ml) was neutralized with N-methylmorpholine (0.112 ml, 1 mmol) or triethylamine (0.14 ml, 1 mmol) and the N-[α -phenyl-(5-substituted-2-hydroxybenzylidene)] amino acid (33) (1 mmol) followed by EEDQ (247 mg, 1 mmol) was added. After stirring the reaction mixture at room temperature for 5 hours the solvent was evaporated and the residue was dissolved in ethyl acetate (30 ml). The

solution was washed with citric acid solution (20%), water, sodium bicarbonate solution (10%) and water, then dried and concentrated. The product was purified as described in procedure A. Table 2 shows compounds prepared by this method.

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E. Use of p-Nitrophenyl Ester (ONp) Coupling Procedure

(a) Synthesis of N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] amino acid p-Nitrophenyl Esters (45a-45c)²⁶

The N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] amino acid (33, R"=Cl) (1 mmol) and p-nitrophenol (139 mg, 1 mmol) were dissolved in ethyl acetate (5 ml). After cooling the mixture to 0-5°, DCC (206 mg, 1 mmol) was added and the solution was stirred for 2 hours. The urea by-product was then filtered off and the solution was diluted to about 50 ml, then washed with citric acid solution (20%), water, sodium bicarbonate solution (10%) and water. After removal of the dried solvent on a rotary evaporator, purification of the crude reaction mixture which contained unreacted N-protected amino acid and p-nitrophenol was best effected by column chromatography using benzene for elution purposes. The compounds which were characterized are listed in Table 3.

(b) Preparation of N-[α -Phenyl-(5-chloro-2-hydroxy-benzylidene)]-L-Alanyl-L-Phenylalanine Methyl Ester (43g).

L-Phenylalanine methyl ester hydrochloride (216 mg, 1 mmol) in dichloromethane (5 ml) was neutralized with triethylamine (0.14 ml, 1 mmol). N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-alanine p-nitrophenyl ester (45a) (424 mg, 1 mmol) was added and the mixture was refluxed for two days, then cooled and the solvent was evaporated. The residue was taken up in ethyl acetate (50 ml) and the solution was washed with citric acid solution (20%), water, sodium bicarbonate solution (10%) and water. The dried solution was evaporated and the oily residue solidified on trituration with light petroleum. Recrystallization from ether-light petroleum gave the pure product. Physical constants are given in Table 2.

Preparation of N-{N''-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Phenylalanyl}-N, N'-dicyclohexylurea (44).

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) (360 mg, 1 mmol) was dissolved in chloroform (10 ml) and treated with DCC (206 mg, 1 mmol). Triethylamine (0.28 ml, 2 mmol) was added and the mixture was stirred at room temperature for 16 hours. The reaction mixture was filtered, then concentrated and the residue was taken up in ether (30 ml), filtered and after concentrating, the crude product was chromatographed using benzene and benzene-chloroform mixtures as eluents. The fraction with

Rf 0.²⁵~~34~~ (t.l.c.,chloroform) was collected and concentrated. After dissolving the residue in ether and again filtering,the solvent was evaporated and the residue left in the refrigerator. After several days the product crystallized and was collected. Yield 282 mg (50%); m.p. 129-132°; ν max (nujol) 1690, 1650 cm^{-1} ; mass spectrum m/e 565 (M^+). Found : C, 76.9; H, 7.8; N, 7.2. $\text{C}_{36}\text{H}_{43}\text{N}_3\text{O}_3$ requires : C, 76.4; H, 7.7; N, 7.4%.

Isolation and Identification of Compound (44) formed in the Synthesis of Compound (43i).

Using coupling procedure A, compound (43i) was prepared according to Table 2. T.l.c. (chloroform) of the filtered and washed reaction mixture showed two distinct yellow spots with Rf 0.34 (major) and Rf 0.25 (minor). After column chromatography using benzene and benzene chloroform mixtures as eluents, the faster running fraction was identified as compound (43i), formed in 82% yield. Combined fractions of the smaller Rf compound, which contained some of the protected dipeptide, were then submitted to preparative-layer chromatography. After two developments in chloroform-benzene (9:1), the Rf 0.25 fraction was scraped off and then eluted with chloroform. The solvent was evaporated and the oily residue was taken up in ether, the solution was filtered and concentrated and the residue crystallized after standing in the refrigerator

for several days. After collecting the crystals and washing with light petroleum, the compound (8 mg) was found to be identical (t.l.c. behaviour, mixed m.p., infrared and mass spectra) with compound (44).

Attempted Preparation of Pentachlorophenyl Esters of Ketimine Amino Acids.

Attempted Preparation of N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Valine Pentachlorophenyl ester.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valine (33f) (995 mg, 3 mmol) and pentachlorophenol (799 mg, 3 mmol) were suspended in dimethylformamide (10 ml) and DCC (619 mg, 3 mmol) was added. The mixture was stirred at 0-5° for 4 hours and after this time only the starting materials were detected on t.l.c. (benzene). After a further 16 hours at room temperature, no significant reaction had occurred and the synthesis was not pursued.

Attempted Preparation of N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Phenylalanine Pentachlorophenyl Ester.

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) (360 mg, 1 mmol) and DCC-pentachlorophenol complex (1.006 g, 1 mmol) were mixed in dimethylformamide (7 ml) at 0-5° and stirred for 2 hours. After standing the mixture overnight in the refrigerator it was

filtered and t.l.c. (benzene) examination of the filtrate indicated the formation of a product with Rf 0.85. The solvent was evaporated and the residue was dissolved in ethyl acetate (20 ml). The solution was washed with citric acid (20%), water, sodium bicarbonate (10%) and water, the dried organic phase was concentrated and the residue was chromatographed using carbon tetrachloride and carbon tetrachloride-benzene mixtures. The required fractions were pooled and concentrated to give a yellow oil which partially solidified on rubbing with light petroleum but which could not be induced to crystallize. When this material was examined on t.l.c. (benzene) the presence of starting materials was still indicated, suggesting that the ester was decomposing on the silica column. An examination of the infrared spectrum (chloroform) of the mixture showed a sharp absorption band near 1770 cm^{-1} (pentachlorophenyl ester). Despite two further attempts to purify the ester by chromatography over silica and alumina (Merck, neutral), it could not be separated from starting materials.

Removal of the Ketimine Protecting Group (See Table 4).

Preparation of Glycyl-Glycine Benzyl Ester Hydrochloride (47a)

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]
glycyl-glycine benzyl ester (43a) (174 mg, 0.4 mmol) was

dissolved in 1N hydrochloric acid in 90% methanol (2 ml) and the mixture was left at room temperature. T.l.c. examination of the reaction mixture indicated complete cleavage of the protecting group after 10 hours. The crystalline ketone was filtered off and washed with methanol and the filtrate was evaporated to dryness. Benzene (5 ml) was twice added to the residue and evaporated and finally trituration of the residue with ether gave the ester hydrochloride as a white powder which was recrystallized from methanol-ether. Yield 75 mg, (73%); m.p. 155-157° (lit. ¹⁴³ m.p. 160°); Rf 0.25 (chloroform-methanol-acetic acid, 70:10:5). Found : C, 49.9; H, 5.7; N, 10.5. $C_{11}H_{15}ClN_2O_3$ requires : C, 51.1; H, 5.8; N, 10.8%.

A portion of the ester hydrochloride was converted to the N-acetylmethylvinyl derivative ¹⁰¹ as follows: (47a) (about 1 mg) was dissolved in dry methanol (100 µl) and treated with acetylacetone (30 µl), dry ion-exchange beads (AG1-X8, in the bicarbonate form, pH 7-8, Biorad, California) and a few sticks of 3A molecular sieves. After standing overnight, the supernatant liquid was evaporated under reduced pressure and the residue was inserted directly into the mass spectrometer solid probe. Mass spectrum of the N-acetylmethylvinyl derivative m/e 304 (M^+), 112 ('A' fragment).

Preparation of Glycyl-Glycine Benzyl Ester Acetate (47b)

Compound (43a) (218 mg, 0.5 mmol) was dissolved in 80% acetic acid (3 ml) and the solution was left at room temperature. After 16 hours, t.l.c. (chloroform-methanol, 49:1) indicated complete cleavage of the diarylmethylene protecting group and the solution was evaporated to dryness at less than 30° to avoid diketopiperazine formation. Benzene (5 ml) was twice added and the solution was again evaporated. The oily residue was washed with ether (3 x 5 ml) and finally trituration with ether gave the product as a white powder. The derivative was dissolved in methanol (5 ml), the solution was filtered and concentrated and the product was isolated again by addition of ether. Yield 97 mg (70%); m.p. 90-92°. ν max (Nujol) 3240, 1745 and 1685 cm^{-1} ; mass spectrum of N-acetylmethylvinyl derivative m/e 304 (M^+), 112 ('A' fragment); Rf 0.24 (chloroform-methanol-acetic acid, 70:10:5). Analytical results for the compound were poor, probably due to the formation of diketopiperazine when the sample was heated.

Preparation of Glycyl-Glycine Benzyl Ester Formate (47c)

Compound (43a) (174 mg, 0.4 mmol) was dissolved in 80% formic acid (3 ml) and the solution was left at room temperature for 16 hours. T.l.c. (chloroform-methanol, 49:1) indicated that deblocking was complete

and the solution was concentrated at less than 30° to avoid formation of the diketopiperazine. The residue was twice treated with benzene (5 ml) and again evaporated at less than 30° . The oily mixture was washed with ether (4 x 5 ml), but the formate salt could not be induced to crystallize. It was therefore dissolved in methanol (5 ml), the solution was filtered, concentrated and the product was again washed with ether. After drying under vacuum, the yield was 82 mg (77%). ν max (chloroform) 3300, 1745 and 1685 cm^{-1} ; mass spectrum of N-acetylmethylvinyl derivative m/e 304 (M^+), 112 ('A' fragment); R_f 0.26 (chloroform-methanol-acetic acid, 70:10:5).

Preparation of Glycyl-Glycine Benzyl Ester p-toluenesulphonate (47d).

Compound (43a) (218 mg, 0.5 mmol) was refluxed with an ethereal solution of p-toluenesulphonic acid monohydrate (5 ml, 19 mg/ml solution). T.l.c. (chloroform-methanol, 49:1) showed deblocking was complete after 4 hours, when the crystalline, precipitated dipeptide ester tosylate was filtered off and washed with ether. Concentration of the mother liquors and trituration of the residue with ether yielded a second crop of crystals, giving a total yield of 137 mg (70%). Recrystallization from ethanol-ether gave the analytical specimen, m.p. $146-152^{\circ}$; ν max (Nujol) 3300, 1740 and 1680 cm^{-1} . Mass spectrum of N-acetylmethylvinyl derivative, m/e 304 (M^+),

112 ('A' fragment); Rf 0.26 (chloroform-methanol-acetic acid, 70:10:5). Found : C, 54.8; H, 5.5; N, 7.4.

$C_{18}H_{22}N_2O_6S$ requires : C, 54.8; H, 5.6; N, 7.1%.

Preparation of Glycyl-L-Tyrosine (49).

L-Tyrosine ethyl ester hydrochloride (245 mg, 1 mmol) was neutralized with triethylamine (0.14 ml, 1 mmol) in chloroform (7 ml) and the free ester was condensed with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) (289 mg, 1 mmol) using DCC (206 mg, 1 mmol) as described in general coupling procedure A. Column chromatography of the crude reaction mixture using benzene and benzene-chloroform mixtures for elution gave a major fraction which was concentrated. The residue was triturated with light petroleum to give 250 mg (52%) of N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycyl-tyrosine ethyl ester (43n) as a yellow solid (ν max 3420, 1735, 1675 and 1620 cm^{-1} ; mass spectrum m/e 445 (M^+)).

Compound (43n) (250 mg, 0.52 mmol) was dissolved in 1N hydrochloric acid in 90% ethanol (2 ml) and left at room temperature for 10 hours. The reaction mixture was then worked up as described for the preparation of (47a) to give 98 mg (62%) of glycyl-L-tyrosine ethyl ester hydrochloride (48) which on recrystallization from ethanol-ether had m.p. 235-237 $^{\circ}$, $[\alpha]_D +16^{\circ}$ (c 0.5, water)(lit.¹⁴⁴ m.p. 245 $^{\circ}$, $[\alpha]_D +17.1^{\circ}$).

Compound (48) (98 mg, 0.32 mmol) was dissolved in ethanol (5 ml) and 1N sodium hydroxide (2 ml) was added. After standing for 2 hours at room temperature, the solution was concentrated and the residue was taken up in 2N hydrochloric acid (5 ml) and transferred to a Zeokarb 225 (H form) ion exchange column for desalting. After elution with 2N ammonia, concentration of the eluate yielded 45 mg (59%) of glycyl-L-tyrosine. After recrystallization from ethanol-water the compound had m.p. 273-275° (decomposition) and was identical with an authentic specimen of glycyl-L-tyrosine (t.l.c., n-butanol-acetic acid-water, 12:3:5 and infrared spectrum). Found : C, 55.5; H, 6.2; N, 11.3. $C_{11}H_{14}N_2O_4$ requires : C, 55.5; H, 5.9; N, 11.8%.

Preparation of L-Alanyl-L-Valyl-L-Leucine Benzyl Ester Formate (51).

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valyl-L-leucine benzyl ester (43j) (2.67 g, 5 mmol) was dissolved in 80% acetic acid (8 ml) and warmed for 6 hours at 50°. The solution was concentrated at less than 30° and the residue was twice treated with benzene (25 ml) and again evaporated at less than 30°. The oily residue was washed well with ether (5 x 20 ml) and it was then taken up in ethyl acetate (50 ml), the solution was filtered and again concentrated at less than 30°. After washing the residue again with ether, it was dried under vacuum to yield 1.58 g (83%) of oily L-valyl-L-leucine

benzyl ester acetate (50) (Rf 0.35, chloroform-methanol-acetic acid, 70:10:5).

Compound (50) (1.58 g, 4.2 mmol) was immediately dissolved in ethyl acetate (70 ml) and converted to the free ester by neutralization with excess sodium carbonate in iced water (6 ml). The organic phase was washed with iced water (6 x 5 ml), dried and concentrated to about 25 ml and using DCC (887 mg, 4.3 mmol) and HOBT (581 mg, 4.3 mmol), the dipeptide ester was coupled with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-alanine (33c) (1.31 g, 4.3 mmol), according to coupling procedure B, to yield N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-alanyl-L-valyl-L-leucine benzyl ester (43s) (2.06 g, 81%) as yellow crystals from ethyl acetate-ether (mass spectrum m/e 605, M^+).

Compound (43s) (2.06g, 3.4 mmol) was immediately dissolved in 80% formic acid (6 ml) and the solution was warmed at 50° for 6 hours. The solvent was then evaporated and the oily mixture was twice dissolved in benzene (20 ml) and the solution was again evaporated. Ether (40 ml) was added and the oily residue was rubbed to give the tripeptide ester formate (51) as a white solid, which was twice recrystallized from methanol-ether. Yield 1.23 g (83%); m.p. 158-159°; $[\alpha]_D -61^\circ$ (c 4, methanol). Found : C, 60.3; H, 8.0; N, 9.6. $C_{22}H_{35}N_3O_6$ requires : C, 60.4; H, 8.1; N, 9.6%.

Preparation of Diketopiperazines (52) - Deblocking of Ketimine Dipeptide Esters in 80% Acetic Acid.

(a) Cyclo-S-Benzyl-L-Cysteinyl-L-Alanine (52a).

To L-alanine methyl ester hydrochloride (138 mg, 1 mmol) and triethylamine (0.14 ml, 1 mmol) in dichloromethane (7 ml) were added N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-S-benzyl-cysteine (33v) (405 mg, 1 mmol) and DCC (206 mg, 1 mmol). From the reaction mixture after chromatography using benzene and benzene-chloroform mixtures, N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-S-benzyl-cysteinyl-alanine methyl ester (43o) was isolated as an oil, homogeneous on t.l.c. (Rf 0.19, chloroform). Compound (43o) was immediately hydrolyzed by warming in 80% acetic acid (5 ml) at 80° for 40 mins. The solution was evaporated, the residue was washed well with ether (5 x 3 ml portions), then taken up in *sec*-butanol (10 ml)¹¹⁰. After boiling for 2 hours the solution was concentrated to yield (52a) which was recrystallized from methanol-ether. The physical properties are given in Table 5.

(b) Cyclo-L-Tryptophyl-Glycine (52b).

Compound (43b) (283 mg, 0.5 mmol) was hydrolyzed in 80% acetic acid (3 ml) at 80°. After 30 mins the reaction mixture was worked up as described for the preparation of compound (52a) and the crystalline

diketopiperazine was isolated. Recrystallization from methanol-ether gave the pure product with physical properties as listed in Table 5.

(c) Cyclo-L-Methionyl-Glycine (52c).

Glycine benzyl ester tosylate (337 mg, 1 mmol) neutralized with triethylamine (0.14 ml, 1 mmol) in dichloromethane (7 ml) was coupled with N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)] methionine (33w) (343 mg, 1 mmol) using DCC (206 mg, 1 mmol) to afford oily N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-methionyl-glycine benzyl ester (43p) (Rf 0.34, chloroform) after chromatography using benzene and benzene-chloroform mixtures. Compound (43p) was treated with 80% acetic acid (5 ml) and left at room temperature for 48 hours. The reaction mixture was thereafter worked up as described for the previous diketopiperazines. After recrystallization from methanol-ether, compound (52c) was obtained as white crystals. Table 5 gives the physical properties.

(d) Cyclo-L-Tryptophyl-L-Tryptophan (52d).

Compound (43c) (3.00 g, 4.75 mmol) was dissolved in 80% acetic acid (25 ml) and left at room temperature for 48 hours. The solution was then evaporated and work up as described above gave compound (52d) which was recrystallized from *sec*-butanol. The physical data for

the compound are given in Table 5.

(e) - Cyclo-L-Leucyl-L-Methionine (52e)

Compound (54) (470 mg, 1 mmol) was dissolved in 80% acetic acid (5 ml) and warmed at 80° for 2 hours. The reaction mixture was evaporated at less than 30° and the residue was washed with ether (5 x 5 ml) to give the oily dipeptide ester acetate, homogeneous on t.l.c. (chloroform-methanol-acetic acid, 70:30:5). The acetate salt was then converted to the diketopiperazine (52e) as previously described for compound (52a). The physical properties of (52e) are listed in Table 5.

Preparation of Diketopiperazines (52)- Action of Ammonia on Ketimine Dipeptide Esters.

(a) Cyclo-L-Tryptophyl-L-Tryptophan (52d).

Compound (43c) (632 mg, 1 mmol) was treated with ethanol (8 ml) which had been previously saturated with ammonia. The mixture was left for 2 days at room temperature, then evaporated to dryness. Addition of ether (10 ml) to the residue gave the product as a white solid which was filtered off and washed well with ethyl acetate, then ether. The product was recrystallized from *sec*-butanol. Yield 204 mg (55%); m.p. 264-266° (decomposition). $[\alpha]_D -79^\circ$ (c 1, acetic acid). This compound

was identical (t.l.c. in isopropyl ether-chloroform-acetic acid, 6:3:1, Rf 0.27; infrared spectrum; mixed m.p.) with (52d) prepared via the dipeptide ester acetate.

(b) Cyclo-L-Leucyl-L-Methionine (52e).

Ethanol (5 ml) saturated with ammonia was added to compound (54) (470 mg, 1 mmol). After standing at room temperature overnight, the solution was filtered from white crystals which had formed. The filtrate was concentrated and trituration with ether yielded a second crop of crystals. Recrystallization from methanol-ether gave 105 mg (43%); m.p. 236-238° (decomposition); $[\alpha]_D -53^\circ$ (c 0.5, methanol). The compound was identical (t.l.c. in isopropyl ether-chloroform-acetic acid, 6:3:1, Rf 0.48; infrared spectrum; mixed m.p.) with the (52e) prepared via the dipeptide ester acetate.

Steric Analysis of Ketimine Dipeptide Esters (43).

1. Detection of Racemization by Steric Analysis of
⁵⁷Diketopiperazines Prepared by Coupling Procedures A and C.

Steric Analysis of Cyclo-L-Tryptophyl-L-Alanine (52f).

L-Alanine methyl ester hydrochloride (14 mg, 0.1 mmol), triethylamine (.014 ml, 0.1 mmol), N-[α -phenyl-

(5-chloro-2-hydroxybenzylidene)]-L-tryptophan (33l) (42 mg, 0.1 mmol) and DCC (21 mg, 0.1 mmol) were reacted as described in procedure A. The crude ketimine dipeptide ester (43q) was then dissolved in 80% acetic acid (1 ml) and warmed at 80° for 40 mins after which the solution was concentrated and the residue was washed several times by decantation with ether. The residue was then dissolved in *sec*-butanol (1 ml) and boiled for 2 hours. T.l.c. examination of the reaction mixture was carried out in the solvent system chloroform-methanol-acetic acid (14:2:1) to establish the steric purity of the diketopiperazine. The DL diastereoisomer was not detected, indicating that less than 2% racemization had occurred in the coupling and deblocking steps.

Steric Analysis of Cyclo-L-Leucyl-L-Leucine (52g) .

L-Leucine ethyl ester hydrochloride (20 mg, 0.1 mmol), triethylamine (.014 ml, 0.1 mmol), N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-leucine (33q) (33 mg, 0.1 mmol) and EDC (19 mg, 0.1 mmol) were reacted according to coupling procedure C. The crude protected dipeptide (43r) was then hydrolyzed in 80% acetic acid (1 ml) and the resulting dipeptide ester acetate was converted to the diketopiperazine as previously described. The reaction mixture was examined on t.l.c. in the solvent system isopropyl ether-chloroform-acetic acid (6:3:1). The DL diastereoisomer was not detected, indicating less than 2% racemization had occurred throughout the coupling

and deblocking steps.

2. Detection of Racemization by G.L.C. Analysis of
Diastereoisomeric N-Trifluoroacetyl Dipeptide Esters ⁵¹
prepared by Coupling Procedures A and D.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valyl-L-valine methyl ester (43h) (44 mg, 0.1 mmol) prepared via coupling procedure A was dissolved in 1N hydrochloric acid in 90% methanol (0.5 ml). After standing overnight, the solution was concentrated and the residue was twice redissolved in methanol and evaporated. The residue was washed well with ether and the oily hydrochloride salt of the dipeptide ester was taken up in ethyl acetate (0.3 ml). The solution was basified with triethylamine (0.02 ml), methyl trifluoroacetate (0.05 ml) was added and the mixture was left at room temperature for 2 hours. The solution was concentrated, the residue was taken up in ethyl acetate (2 ml) and the mixture was washed with water. The dried solution of the N-trifluoroacetyl-L-valyl-L-valine methyl ester was submitted to g.l.c. analysis. Less than 1% of the DL diastereoisomer was detected.

Compound (43h) prepared by coupling procedure D was similarly converted to the N-trifluoroacetyl derivative. Again, less than 1% of the DL diastereoisomer was present.

Preparation of N-Terminal-Shortened Sequences of
Substance P.

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Leucyl-
L-Methionine Methyl Ester (54).

L-Methionine methyl ester hydrochloride (4.00 g, 20 mmol) was neutralized with N-methylmorpholine (2.24 ml, 20 mmol) in tetrahydrofuran (50 ml) and the free ester was coupled with N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-leucine (33q) (6.52 g, 20 mmol) using HOBt (2.98 g, 22 mmol) and DCC (4.52 g, 22 mmol) according to coupling procedure B. T.l.c. (carbon tetrachloride-ethyl acetate, 8:2) examination of the crude product showed a major spot Rf 0.58 and a trace impurity at Rf 0.65. Column chromatography using gradient elution with carbon tetrachloride and carbon tetrachloride-ethyl acetate mixtures afforded the pure product which was dissolved in ether, filtered and concentrated, then dried under vacuum to yield 8.47 g (90%) of a yellow oil, homogeneous on t.l.c. $\nu_{\max}$ (chloroform) 3320, 1735, 1650 cm^{-1} ; mass spectrum m/e 470 (M^+); Rf 0.33 (chloroform), 0.31 (carbon tetrachloride-ethyl acetate, 9:1), 0.56 (carbon tetrachloride-ethyl acetate, 8:2).

L-Leucyl-L-Methionine Methyl Ester Hydrochloride (55).

Compound (54) (8.47 g, 18 mmol), dissolved in 1N hydrochloric acid in 90% methanol (30 ml), was left at

room temperature for 48 hours. The solvent was removed and after twice evaporating from benzene (20 ml) treatment of the residue with ether (100 ml) gave the oily hydrochloride salt. The mother liquor was decanted, the product was washed several times with ether and the oil was again dissolved in methanol. The solution was filtered, concentrated and addition of ether yielded the pure compound which was dried under vacuum over potassium hydroxide. Yield 4.78 g (85%); ν max (thin film) 1740, 1670 cm^{-1} ; Rf 0.62 (chloroform-methanol-acetic acid, 70:10:5), 0.70 (chloroform-methanol-acetic acid, 70:30:5).

L-Leucyl-L-Methionine Methyl Ester Formate (55a).

Compound (54) (470 mg, 1 mmol) was dissolved in 80% formic acid (3 ml) and warmed at 50° for 6 hours. The mixture was cooled and the solvent was removed on the rotary evaporator at less than 30°. Benzene (10 ml) was twice added and the solution was again evaporated. The residue was washed with ether (4 x 5 ml), then taken up in methanol (10 ml), the solution was filtered and concentrated and the oily product was washed several times with ether. After drying under vacuum, the yield was 193 mg (68%). ν max (thin film) 1740 and 1670 cm^{-1} ; Rf 0.61 (chloroform-methanol-acetic acid, 70:10:5). When the oily formate salt was dissolved in ethyl acetate some insoluble white crystals were observed. These were collected (about 4 mg) and were identified (t.l.c. in isopropyl ether- chloroform-acetic acid, 6:3:1; infrared

spectra; mixed m.p.) as the diketopiperazine (52e)

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] Glycyl-L-Leucyl-L-Methionine Methyl Ester (56).

Compound (55) (4.70 g, 15 mmol) in ethyl acetate (40 ml) was neutralized with N-methylmorpholine (1.68 ml, 15 mmol) and coupling with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) (4.35 g, 15 mmol) using HOBt (2.16 g, 16 mmol) and DCC (3.30 g, 16 mmol) was carried out as described in procedure B. After washing, the crude product crystallized on evaporation of the ethyl acetate solution. The compound was collected, washed with ether and recrystallization from ethyl acetate-ether gave 5.75 g (70%) yield, m.p. 133-137°. $[\alpha]_D -43^\circ$ (c 0.5, methanol); ν max (Nujol) 3280, 1735, 1650, 1640 cm^{-1} ; Rf 0.23 (carbon tetrachloride-ethyl acetate, 7:3), 0.41 (carbon tetrachloride-ethyl acetate, 6:4). Found : C, 59.5; H, 6.4; N, 7.7. $\text{C}_{27}\text{H}_{34}\text{ClN}_3\text{O}_5\text{S}$ requires C, 59.2; H, 6.3; N, 7.7%.

Glycyl-L-Leucyl-L-Methionine Methyl Ester Acetate (57).

Compound (56) (5.48 g, 10 mmol) was deblocked with 80% acetic acid (15 ml) by warming at 50° for 2 hours. Evaporation of the solution gave an oily residue which was concentrated three times from benzene (50 ml). Trituration of the residue with ether afforded a waxy solid which was twice recrystallized from methanol-ether.

Yield 3.66 g (93%); m.p. 96-98°; $[\alpha]_D -43.6^\circ$ (c 5, methanol); ν max (Nujol) 3300, 1735, 1655 cm^{-1} ; Rf 0.69 (chloroform-methanol-acetic acid, 70:30:5). Found : C, 48.8; H, 8.1; N, 10.2. $\text{C}_{16}\text{H}_{31}\text{N}_3\text{O}_6\text{S}$ requires C, 48.8; H, 7.9; N, 10.7%.

Glycyl-L-Leucyl-L-Methionine Methyl Ester Formate (57a).

Compound (56) (548 mg, 1 mmol) was deblocked with 80% formic acid (3 ml) by warming at 50° for 2 hours. The solution was concentrated and after evaporation from benzene (2 x 10 ml) addition of ether (10 ml) to the residue gave an oil. The ether was decanted, the oil was taken up in methanol (10 ml), the solution was filtered and concentrated and the oily residue was again washed with ether. After drying under vacuum the yield was 342 mg (92%) of an amorphous solid which reverted to an oil on exposure to moisture. ν max (chloroform) 1740, 1660 cm^{-1} ; Rf 0.65 (chloroform-methanol-acetic acid, 70:30:5), 0.24 (chloroform-methanol, 9:1)

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Phenyl-alanyl-Glycyl-L-Leucyl-L-Methionine Methyl Ester (58).

Compound (57) (3.54 g, 9 mmol) in ethyl acetate (100 ml) was neutralized by addition of excess sodium carbonate in iced water (10 ml). The organic solution was washed with iced water (6 x 8 ml), dried, concentrated

to about 30 ml and the free ester was then coupled with N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) (3.23 g, 9 mmol) using HOBt (1.35 g, 10 mmol) and DCC (2.06 g, 10 mmol). After the usual work-up (coupling procedure B), the crude compound was chromatographed using carbon tetrachloride and carbon tetrachloride-ethyl acetate mixtures as eluents. The major fractions were pooled and concentrated, the residue was taken up in ether, filtered and evaporation of the solvent gave the clean product as a foamy yellow solid. Yield 5.10 g (84%); m.p. 65-70^o; $[\alpha]_D -159^o$ (c 0.5, methanol); $\nu_{\max}$ (Nujol) 3280, 1735, 1640 cm^{-1} ; Rf 0.15 (carbon tetrachloride-ethyl acetate, 6:4), 0.25 (carbon tetrachloride-ethyl acetate, 1:1). Found : C, 65.4; H, 6.9 ; $\text{C}_{37}\text{H}_{46}\text{N}_4\text{O}_6\text{S}$ requires : C, 65.8; H, 6.9%.

L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Methyl Ester Acetate (59).

Compound (58) (4.72 g, 7 mmol) was deblocked with 80% acetic acid (12 ml) by warming at 50^o for 5 hours and after evaporation of the mixture as described for (57) the crude product was obtained upon addition of ether to the waxy residue. Recrystallization twice from methanol-ether gave the pure product. Yield 3.06 g (81%); m.p. 107-110^o; $[\alpha]_D -19.6^o$ (c 4, methanol); Rf 0.47 (chloroform-methanol-acetic acid, 70:10:5), 0.86 (chloroform-methanol-acetic acid, 70:30:5). Found : C, 55.4; H, 7.6, N, 9.9.

$C_{25}H_{40}N_4O_7S$ requires : C, 55.5; H, 7.5; N, 10.4%.

L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Methyl Ester Formate (59a).

Compound (58) (675 mg, 1 mmol) was dissolved in formic acid and warmed at 50° for 5 hours. After working up the reaction mixture as described for the preparation of (57a), trituration of the oily residue with ether gave the product as a white solid. The compound was recrystallized from methanol-ether. Yield 432 mg (82%); m.p. $105-110^{\circ}$; $[\alpha]_D -37^{\circ}$ (c 4, methanol); Rf 0.50 (chloroform-methanol-acetic acid, 70:10:5).

Found : C, 54.5; H, 7.3; N, 10.3. $C_{24}H_{38}N_4O_7S$ requires : C, 54.7; H, 7.3; N, 10.6%.

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Phenylalanyl-L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Methyl Ester (60).

Compound (59) (2.7 g, 5 mmol) in ethyl acetate (70 ml) was converted to the free ester using sodium carbonate as described previously, and coupling with N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) (1.80 g, 5 mmol) was effected using HOBt (743 mg, 5.5 mmol) and DCC (1.13 g, 5.5 mmol). The crude product in ethyl acetate was washed in the usual way, the dried solution was concentrated and ether (100 ml) was added. The product crystallized on standing overnight

in the refrigerator. After recrystallization from ethyl acetate-ether the yield was 3.25 g (79%); m.p. 104-109^o; $[\alpha]_D$ -135^o (c 4, methanol); ν max (Nujol) 3270, 1730, 1640 cm⁻¹; Rf 0.17 (carbon tetrachloride-ethyl acetate, 1:1), 0.39 (carbon tetrachloride-ethyl acetate, 3:7). Found : C, 67.5; H, 6.7; N, 8.2.

C₄₆H₅₅N₅O₇S requires : C, 67.2; H, 6.7; N, 8.5%.

L-Phenylalanyl-L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Methyl Ester Acetate (61).

Compound (60) (2.88 g, 3.5 mmol) was deblocked with 80% acetic acid (7 ml) in the manner described for compound (59). The crude product was filtered off and purified from methanol-ether. Yield 2.04 g (85%), m.p. 142-145^o. The compound was homogeneous on t.l.c. (Rf 0.51, chloroform-methanol-acetic acid, 70:10:5) and was immediately used in the next step.

L-Phenylalanyl-L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Amide (62).

Compound (61) (1.72 g, 2.5 mmol) was dissolved in methanol (10 ml) and the solution was saturated with ammonia gas at 0^o, then left at room temperature for three days. The solvent was evaporated and the residue was again dissolved in methanol, the solution was filtered and concentrated and the waxy solid was

triturerated with ether. The crude product was collected and chromatographed using chloroform-methanol (9:1) as eluent. Fractions with Rf 0.28 (chloroform-methanol, 9:1) were pooled and the solution was evaporated. The white powder was collected and washed with ethyl acetate, then ether. Yield 1.10 g (74%); m.p. 187-190°; $[\alpha]_D -54^\circ$ (c 1, dimethylformamide); Rf 0.28 (chloroform-methanol, 9:1), 0.82 (n-butanol-pyridine-water-acetic acid, 30:20:24:6). Found : C, 60.4; H, 7.3; N, 13.4 $C_{31}H_{44}N_6O_5S$ requires : C, 60.7; H, 7.2; N, 13.7%. Amino acid analysis : Phe 1.90; Gly 0.98; Leu 1.00; Met 0.98. The results of biological assays are given in Table 6.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Glutaminyl-L-Phenylalanyl-L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Amide (63).

Compound (62) (613 mg, 1 mmol) in dimethylformamide (5 ml) was coupled with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-glutamine (33p) (397 mg, 1.1 mmol) using EDC (210 mg, 1.1 mmol). After removal of the solvent, the oily residue was treated with ethyl acetate and the crude yellow product was collected, dissolved in the minimum amount of dimethylformamide and chromatographed using chloroform-methanol (9:1) for elution. The major fraction (Rf 0.31, chloroform-methanol, 9:1) was collected and concentrated to give a waxy solid. Ethyl acetate was added and the product was filtered off, washed with ethyl acetate, then ether and

dried. Yield 695 mg (73%); m.p. 192-195°; $[\alpha]_D -50^\circ$ (c 1, dimethylformamide); Rf 0.31 (chloroform-methanol, 9:1). Found : C, 61.3; H, 6.5; N, 11.1. $C_{49}H_{49}ClN_8O_8S$ requires : C, 61.6; H, 6.2; N, 11.7%.

L-Glutaminyl-L-Phenylalanyl-L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Amide Acetate (64).

Compound (63) (191 mg, 0.2 mmol) was deblocked with 80% acetic acid (1 ml) by warming at 50° for 4 hours as previously described. Evaporation of the mixture gave a residue which on treatment with ethyl acetate yielded a white powder. This was filtered off and dissolved in a small quantity of dimethylformamide for chromatography using chloroform-methanol-acetic acid (70:10:5) as eluent. Fractions having Rf 0.46 (chloroform-methanol, 9:1) were combined and concentrated to give the clean product which was collected, washed well with ethyl acetate and dried. Yield 127 mg (80%); m.p. 195-200°; $[\alpha]_D -43^\circ$ (c 1, dimethylformamide); Rf 0.90 (n-butanol-pyridine-water-acetic acid, 30:20:24:6), 0.46 (chloroform-methanol, 9:1). Found : C, 57.3; H, 6.8; N, 13.6. $C_{38}H_{56}N_8O_9S$ requires : C, 57.0; H, 7.0; N, 14.0%. The results of biological assays are given in Table 6.

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Leucyl-L-Methionine Amide (65).

L-Methionine amide hydrochloride (1.85 g, 10 mmol) suspended in dimethylformamide (20 ml) was neutralized with N-methylmorpholine (1.23 ml, 10 mmol) and coupled with N-[α -phenyl-(2-hydroxy-5-methyl-, benzylidene)]-L-leucine (33q) (3.58 g, 11 mmol) using HOBt (1.49 g, 11 mmol) and DCC (2.27 g, 11 mmol) as described in coupling procedure B. The crude protected dipeptide was chromatographed using chloroform and chloroform-5% methanol as eluents and fractions with R_f 0.46 (t.l.c., chloroform-methanol, 19:1) were pooled and concentrated. The residue was then dissolved in ether, the solution was filtered and evaporated and the product was dried under vacuum. Yield 4.25 g (93%) of foamy yellow solid which slowly reverted to an oil on standing. ν max (chloroform) 3300, 3190, 1675, 1665 cm^{-1} ; mass spectrum m/e 455 (M^+); R_f 0.78 (chloroform-methanol, 9:1), 0.38 (carbon tetrachloride-ethyl acetate, 1:1).

L-Leucyl-L-Methionine Amide Hydrochloride (66).

Compound (65) (3.525 g, 7.75 mmol) was treated with 1N hydrochloric acid in 90% methanol (12 ml). After standing at room temperature for 48 hours the solution was concentrated and the residue was three times treated with benzene (10 ml) and evaporated. Ether (100 ml)

was added to the residue and the hydrochloride was collected as fine white crystals. The product was recrystallized from methanol-ether. Yield 1.34 g (88%); m.p. 124-125°; $[\alpha]_D +10^\circ$ (c 4, methanol) (lit.¹⁴⁵ m.p. 126-127°, $[\alpha]_D +10^\circ$).

L-Leucyl-L-Methionine Amide Acetate (66a).

Compound (65) (455 mg, 1 mmol) was treated with 80% acetic acid (3 ml) and left at 50° for 5 hours. After working up in the usual manner a waxy residue was obtained. Addition of ether (10 ml) gave a white solid which was collected and recrystallized from methanol-ether. Yield 271 mg (84%); m.p. 122-124°; $[\alpha]_D +5^\circ$ (c 4, methanol); $\nu_{\max}$ (Nujol) 3360, 3300, 1685, 1665 cm^{-1} . Found: C, 48.9; H, 8.5; N, 13.1. $\text{C}_{13}\text{H}_{27}\text{N}_3\text{O}_4\text{S}$ requires : C, 48.6; H, 8.5; N, 13.1%.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] Glycyl-L-Leucyl-L-Methionine Amide (67)

Compound (66) (1.24 g, 6.28 mmol) was suspended in dimethylformamide (13 ml) and neutralized with N-methylmorpholine (0.7 ml, 6.28 mmol). The free amide was coupled with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) (2.00 g, 6.9 mmol) using HOBt (933 mg, 6.9 mmol) and DCC (1.43 g, 6.9 mmol) (coupling procedure B). The washed and dried ethyl acetate solution was evaporated to yield a waxy yellow solid to which

ether (100 ml) was added. The product was filtered off and purified by dissolving in ethyl acetate, filtering and concentrating the solution and further treating the residue with ether. The pure compound was collected and dried to yield 2.45 g (73%), m.p. 144-146°. $[\alpha]_D -36^\circ$ (c 1, methanol); $\nu_{\max}$ (Nujol) 3380, 1665 cm^{-1} ; Rf 0.66 (chloroform-methanol, 9:1), 0.11 (carbon tetrachloride-ethyl acetate, 1:1). Found : C, 58.2; H, 6.5; N, 10.2 $\text{C}_{26}\text{H}_{33}\text{ClN}_4\text{O}_4\text{S}$ requires : C, 58.6; H, 6.3; N, 10.5%.

Glycyl-L-Leucyl-L-Methionine Amide Acetate (68).

Compound (67) (1.066 g, 2 mmol) was warmed at 50° in 80% acetic acid (10 ml) for 2 hours. The mixture was concentrated and the residue was three times dissolved in benzene (10 ml) and the solution was evaporated. To the crystalline residue was added ethyl acetate (20 ml) and the product was then collected and twice recrystallized from methanol-ethyl acetate. Yield 622 mg (82%); m.p. 133-135°; $[\alpha]_D -25^\circ$ (c 1.33, methanol), Rf 0.10 (chloroform-methanol, 9:1), 0.33 (chloroform-methanol-acetic acid 70:30:5). Found : C, 47.3; H, 8.2; N, 14.6. $\text{C}_{15}\text{H}_{30}\text{N}_4\text{O}_5\text{S}$ requires : C, 47.6; H, 7.0; N, 14.8%. Amino acid analysis : Gly 1.01, Leu 1.00, Met 0.82. Table 6 gives the results of biological assays.

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Amide (69).

Compound (68) (379 mg, 1 mmol) in dimethylformamide (5 ml) was neutralized with N-methylmorpholine (0.11 ml, 1 mmol) and coupling with N-[α -phenyl-(2-hydroxy-5-methylbenzylidene)]-L-phenylalanine (33t) (395 mg, 1.1 mmol) using HOBt (149 mg, 1.1 mmol) and DCC (227 mg, 1.1 mmol) was carried out according to procedure B. The reaction mixture was concentrated and the residue was treated with ether (20 ml) to give a waxy yellow solid. After collecting and drying the product, it was redissolved in tetrahydrofuran, the solution was filtered and evaporated and the residue was treated with ethyl acetate (10 ml). The yellow solid was filtered and dried. Yield 505 mg (77%); m.p. 178-180°; $[\alpha]_D -128^\circ$ (c 1, methanol); Rf 0.68 (chloroform-methanol, 9:1), 0.09 (carbon tetrachloride-ethyl acetate, 1:1). Found : C, 65.5; H, 6.8; N, 10.3. $C_{36}H_{45}N_5O_5S$ requires : C, 65.5; H, 6.9; N, 10.6%.

L-Phenylalanyl-Glycyl-L-Leucyl-L-Methionine Amide Acetate (70).

Compound (69) (365 mg, 0.54 mmol) was deblocked by warming in 80% acetic acid (3 ml) for 5 hours. The mixture was then worked up as described for the preparation of compound (68) to give a waxy solid on treating the residue with ethyl acetate (10 ml).

Recrystallization was effected from methanol-ethyl acetate to give 230 mg (81%) of the product, m.p. 146-149°; $[\alpha]_D -16^\circ$ (c 1, methanol); Rf 0.34 (chloroform-methanol, 9:1), 0.67 (chloroform-methanol-acetic acid, 70:30:5) 0.83 (n-butanol-pyridine-water-acetic acid, 30:20:24:6). Found : C, 54.8; H, 7.3; N, 12.9. $C_{24}H_{39}N_5O_6S$ requires : C, 54.8; H, 7.5; N, 13.3%. Amino acid analysis : Phe 1.03, Gly 0.97, Leu 1.00, Met 0.98. Table 6 gives the results of biological assays.

Ketimine Amino Acids in Solid Phase Synthesis.

Attachment of Ketimine Amino Acids to Merrifield Resin.

(a) N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Alanyl Resin¹²⁵ (71).

Merrifield peptide resin (1.0 g, 0.98 meq Cl/g resin) and N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-alanine (33c) (302 mg, 1 mmol) were refluxed in ethyl acetate (5 ml) in the presence of triethylamine (0.14 ml, 1 mmol) for forty hours. The resin was then filtered off and washed with ethyl acetate, ethanol, water, methanol and dichloromethane, each three times. The dried resin was a bright yellow colour. Amino acid analysis indicated a substitution of 0.27 mmol alanine/g resin.

(b) N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]

Glycyl Resin (72)¹²⁶.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]
glycine (33a) (289 mg, 1 mmol) was dissolved in
tetramethylammonium hydroxide solution (1 mmol).
After removal of the solvent the salt was dried overnight
over P_2O_5 , then dissolved in dimethylformamide-dioxane
(10 ml, 1:1). Merrifield resin (1.0 g, 0.98 meq Cl/g resin)
was added and the mixture was heated at 80° for 20 hours.
The filtered resin was washed as described in (a) and
dried. Amino acid analysis indicated the substitution
was 0.30 mmol glycine/g resin.

Preparation of Ketimine Peptide Acyl Resins.

The general procedure adopted was as outlined
in the monograph of Stewart and Young⁶².

- (i) Boc-amino acyl resin (1.00g) was swollen in
dioxane (3 x 10 ml).
- (ii) Removal of the Boc-group - resin shaken for
30 mins in 4N hydrogen chloride in dioxane (10 ml).
- (iii) Dioxane wash (3 x 10 ml).
- (iv) Chloroform wash (3 x 10 ml).
- (v) Neutralization - resin shaken for 10 mins in
triethylamine - chloroform (10 ml, 1:9).

- (vi) Chloroform wash (3 x 10 ml).
- (vii) Dichloromethane wash (3 x 10 ml).
- (viii) Coupling - a 2.5 molar excess of the ketimine amino acid (33) dissolved in dichloromethane (8 ml) was added to the resin and the mixture was shaken for 5 minutes. DCC (2.5 molar excess, 50% w/v in dichloromethane) was then added to the reaction vessel which was shaken for a further 2 hours.
- (ix) Dichloromethane wash (5 x 10 ml).
- (x) Methanol wash (3 x 10 ml).
- (xi) Dichloromethane wash (3 x 10 ml).

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Valyl-L-Alanyl Resin (73).

Boc-alanyl resin (2.0 g, 0.35 mmol alanine/g resin) was deblocked and coupling with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valine (33f) (582 mg, 1.76 mmol) was carried out using DCC as described above.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Valyl-L-Alanine Methyl Ester (74).

A portion (approximately 1/5th) of (73) was removed and suspended in methanol (20 ml). Triethylamine (1.25 ml) was added and the mixture was stirred for 24 hours. The resin was filtered off, the filtrate was

concentrated and the residue was taken up in ethyl acetate (10 ml). After washing with citric acid solution (20%) and water, the dried solution was concentrated. The transesterification product (74) crystallized from ether-light petroleum. ν max (Nujol) 3350, 1740, 1665, 1610 cm^{-1} ; mass spectrum m/e 416 (M^+). R_f 0.33 (chloroform).

Effect of 80% Acetic Acid on (73).

A portion (about 1/5th) of (73) was treated with 80% acetic acid (10 ml). After shaking for 24 hours at room temperature, t.l.c. (chloroform) showed only the slightest trace of ketone in the mother liquor and the colour of the resin was still bright yellow. After 72 hours, the presence of ketone in the solution was no more pronounced and the resin still appeared an intense yellow. For comparison, compound (74) was dissolved in 80% acetic acid (1 ml) and the progress of deblocking was followed by t.l.c. (chloroform). The protecting group was completely removed after 35 hours at room temperature.

Effect of 4N p-Toluenesulphonic Acid Monohydrate on (73).

A further portion (about 1/5th) of (73) was shaken with 4N p-toluenesulphonic acid monohydrate in dioxane (7.64 g in 10 ml) and the progress of reaction was followed by t.l.c. (chloroform). After 3 hours

ketone was detectable in the mother liquor but after 8 hours the resin still retained some yellow colour. After 20 hours shaking, the acid solution was removed and the resin, which was now white, was washed with dioxane. T.l.c. (chloroform) of the solution, however, showed a minor spot, positive to ninhydrin and to peptide detection reagent, which did not move from the start of the plate. This was indicative of some acyl-resin bond cleavage under the strong acidic conditions.

Effect of Benzylamine in dichloromethane on (73).

Another portion (about 1/5th) of (73) was shaken with 0.4 N benzylamine in dichloromethane (10 ml). After 2 hours, the mother liquor was still colourless and no transamination product was detectable on t.l.c. (chloroform). This reagent was drawn off and 4N benzylamine in dichloromethane (10 ml) was added. After 15 minutes, the ketimine derivative of benzylamine (R_f 0.83) was visible on t.l.c. plates (chloroform) as well as a compound (R_f 0.19) positive to both ninhydrin and peptide detection reagent. This latter spot was present in all subsequent t.l.c. samples. The colour of the resin was discharged after 10 hours and the deblocked resin was stirred with triethylamine (0.7 ml) in methanol (10 ml) for 2 hours. A negative result was obtained when this solution was tested for peptides, which indicated complete cleavage of the acyl-resin linkage had occurred

in the benzylamine solution.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] Glycyl-L-Phenylalanyl Resin (75).

Boc-phenylalanyl resin (2.00 g, 0.35 mmol phenylalanine/g resin) was deblocked and coupling with (33a) (508 mg, 1.76 mmol) was effected using DCC as previously described.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] Glycyl-L-Phenylalanine Methyl Ester (43d).

A portion (about $\frac{1}{2}$) of (75) was suspended in methanol (40 ml) containing triethylamine (2.45 ml). After stirring the mixture for ten hours, the resin was filtered off and the filtrate was worked up as described in the preparation of (74). The crude transesterification product was chromatographed using benzene and benzene-chloroform mixtures to give the clean ketimine dipeptide methyl ester (43d) which crystallized from ether-light petroleum. The compound was identical (t.l.c., carbon-tetrachloride-ethyl acetate, 8:2, Rf 0.33; infrared and mass spectra; mixed m.p.) with (43d) prepared according to Table 2.

Effect of Dichloromethane - 75% Chloroacetic Acid on (75).

The remaining portion of (75) was shaken with dichloromethane - 75% chloroacetic acid¹¹ (50:37 w/v, 10 ml). Although significant quantities of the ketone were present in the mother liquor after 6 hours, the resin was still yellow and its colour was not discharged after 9 hours reaction.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] Glycyl-L-Valyl-Resin (76).

Boc-valyl resin (1.00 g, 0.35 mmol valine/g resin) was deblocked and coupling with (33a) (254 mg, 0.88 mmol) was carried out using DCC as previously described.

Effect of 0.4N Hydrochloric Acid in Tetrahydrofuran on (76).

The protected dipeptide acyl resin (76) was shaken with 0.4N hydrochloric acid in tetrahydrofuran⁸⁴ (10 ml). After 30 mins the ketone could be detected in the mother liquor and after 2½ hours the colour had been discharged from the resin. The deblocked resin was washed with tetrahydrofuran (3 x 10 ml) and coupling with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-alanine (33c) (264 mg, 0.88 mmol) using DCC was carried out as previously described to give the ketimine alanyl-glycyl-

valyl resin (77). Transesterification of (77) by stirring the resin in triethylamine (2.45 ml) in methanol (40 ml) for 40 hours, and work-up in the manner described earlier yielded N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-alanyl-glycyl-L-valine methyl ester (78). After chromatography using benzene and benzene-chloroform mixtures, pure (78) was obtained as a yellow oil. This was dried under vacuum. Yield 110 mg (67% based on Boc-valyl resin); mass spectrum m/e 473 (M^+), 258 ('A' fragment); Rf 0.34 (carbon tetrachloride-ethyl acetate, 7:3).

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Phenylalanyl-Glycyl Resin (79).

Boc-glycyl resin (1.00 g, 0.34 mmol glycine/g resin) was deblocked and the glycyl resin was coupled with the ketimine derivative of phenylalanine (33t) (305 mg, 0.85 mmol) using DCC.

N-[α -Phenyl-(2-hydroxy-5-methylbenzylidene)]-L-Phenylalanyl-Glycine Methyl Ester (80).

A portion (about $\frac{1}{2}$) of (79) was transesterified by stirring the resin in triethylamine (1.25 ml) in methanol (20 ml) for 24 hours. The resin was filtered off, the filtrate was taken up in ethyl acetate (10 ml) and washed as previously described. The dried organic

phase was concentrated and the residue was chromatographed using benzene and benzene-chloroform mixtures to give the clean ketimine dipeptide ester (80) as a yellow oil. ν max (chloroform) 3300, 1735, 1660, 1610 cm^{-1} ; mass spectrum m/e 430 (M^+), 314 ('A' fragment) Rf 0.28 (carbon tetrachloride-ethylacetate, 8:2).

Effect of 0.4N Hydrochloric Acid in Tetrahydrofuran on (79).

A portion (about $\frac{1}{4}$) of (79) was suspended in 0.4 N hydrochloric acid in tetrahydrofuran (10 ml). T.l.c. (chloroform) detected the ketone in the mother liquor after 30 mins but after shaking the mixture for 11 hours complete cleavage of the protecting group had still not been effected.

Effect of 1N Hydrochloric Acid in Tetrahydrofuran on (79).

A further portion (about $\frac{1}{4}$) of (79) was shaken with 1N hydrochloric acid in tetrahydrofuran (10 ml). In this instance complete discharge of colour from the resin required 14 hours.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-Glycyl-Glycyl Resin (81).

Boc-glycyl resin (1.00 g, 0.34 mmol glycine/g resin) was deblocked and coupling with (33a) (247 mg,

0.85 mmol) using DCC was carried out as previously described.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)] Glycyl-Glycine Methyl Ester (82).

A portion (about $\frac{1}{4}$) of (81) was transesterified by stirring for 24 hours in triethylamine (0.7 ml) and methanol (10 ml) as has been previously described. After chromatography using benzene and benzene-chloroform mixtures the pure protected dipeptide (83) was isolated crystalline from ethyl acetate-light petroleum. ν max (Nujol) 3280, 1730, 1660, 1610 cm^{-1} ; mass spectrum m/e 360 (M^+), 245 ('A' fragment). R_f 0.34 (carbon tetrachloride-ethyl acetate, 7:3).

Effect of N,N-Dimethylhydrazine in Dichloromethane on (81).

The remainder of (81) was suspended in a 1N solution of N,N-dimethylhydrazine in dichloromethane (10 ml). After shaking for a total of 48 hours at room temperature, a transamination product could not be detected on t.l.c. of the mother liquor and the resin colour appeared unchanged.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Valyl-Glycyl Resin (83).

Boc-glycyl resin (1.00 g, 0.35 mmol glycine/g

resin) was deblocked and coupling of the glycyI resin with (33f) (291 mg, 0.88 mmol) was effected using DCC.

Effect of 4N Hydrogen Chloride in Dioxane on (83).

The resin (83) was suspended in 4N hydrogen chloride in dioxane (10 ml) and shaken for 30 mins. After washing with dioxane (3 x 10 ml) then methanol (3 x 10 ml), methanol (40 ml) containing triethylamine (2.45 ml) was added and the mixture was shaken for 10 hours. The yellow methanolic solution was then separated from the resin and after working up the solution as previously described, N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-valyl-glycine methyl ester (84) was obtained as yellow crystals from ether-light petroleum. Yield 94 mg (67% based on Boc-glycyl resin); m.p. 138-139^o; [α]_D -23^o (c 0.5, methanol); ν max (Nujol) 3280, 1740, 1650, 1605 cm⁻¹; mass spectrum m/e 402 (M⁺). Rf 0.45 (chloroform). Found : C, 63.1; H, 5.9; N, 7.2. C₂₁H₂₃ClN₂O₄ requires : C, 62.6; H, 5.8; N, 7.0%.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Tryptophyl-L-Alanyl Resin (85).

Boc-alanyl resin (1.00 g, 0.35 mmol alanine/g resin) was deblocked and coupling with (331) (367 mg, 0.88 mmol) was carried out using DCC.

N-[α -Phenyl-(5-chloro-2-hydroxybenzylidene)]-L-Tryptophyl-L-Alanine Methyl Ester (43q).

A portion (approximately $\frac{1}{2}$) of (85) was transesterified as previously detailed by stirring the resin for 10 hours in triethylamine (1.25 ml) and methanol (20 ml) to give (43q) as an amorphous yellow solid (70 mg). ν max (Nujol) 3410, 3320, 1740, 1660, 1605 cm^{-1} ; mass spectrum m/e 503 (M^+); R_f 0.30 (chloroform), 0.66 (chloroform-methanol, 99:1).

Effect of 1N Hydrogen Chloride in Acetic Acid on (85).

A further portion (about $\frac{1}{4}$) of (85) was shaken with 1N hydrogen chloride in acetic acid (5 ml) for 30 mins. The resin (85) was then transesterified in the manner described above to give an amorphous yellow solid (24 mg) which was identical to (43q) isolated in the previous experiment.

Effect of 0.1N Trifluoroacetic Acid in Dichloromethane on (85).

The remainder of (85) was suspended in 0.1N trifluoroacetic acid in dichloromethane (5 ml) and the mixture was shaken for 1 hour. T.l.c. (chloroform) gave no indication of cleavage of the protecting group. Transesterification of (85) in the usual way yielded 19 mg of (43q).

Ketimine Protection of the Lysine ϵ -Amino Group

Glycyl-Lysyl-Glycine Methyl Ester Dihydrochloride (87)

Boc-glycyl resin (1.00 g, 0.34 mmol glycine/g resin) was deblocked by shaking the resin in 50% trifluoroacetic acid in dichloromethane¹⁴⁶ (10 ml) for 30 minutes. N^{α} -t-Butyloxycarbonyl- N^{ϵ} -[α -phenyl-(5-chloro-2-hydroxybenzylidene)]-L-lysine dicyclohexylammonium salt (34d) (546 mg, 0.85 mmol) was dissolved in ethyl acetate (10 ml) and the free acid was liberated by careful addition of dilute citric acid solution. The organic phase was washed, dried and concentrated and the free acid was coupled with the deblocked glycyl resin in the usual way. The Boc protecting group was cleaved by a 30 minute treatment with trifluoroacetic acid solution as before and the dipeptide resin was then coupled with N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)] glycine (33a) (246 mg, 0.85 mmol). The protected tripeptide was removed from the resin by transesterification (2.45 ml triethylamine + 30 ml methanol, 24 hour treatment). The yellow solution was filtered from the resin, concentrated, the residue was dissolved in ethyl acetate (10 ml) and the organic phase was washed with citric acid solution and water. After evaporation of the solvent, the residue was chromatographed using carbon tetrachloride and carbon tetrachloride-ethyl acetate mixtures. Concentration of the major fraction gave N-[α -phenyl-(5-chloro-2-hydroxybenzylidene)]glycyl- $\{\epsilon$ -N[α -phenyl-(5-chloro-2-

hydroxybenzylidene)]-L-lysyl-glycine methyl ester (86) as a yellow oil, homogeneous on t.l.c. (Rf 0.15, carbon tetrachloride-ethyl acetate, 6 : 4). After drying under vacuum the yield was 125 mg (52%); ν max (chloroform) 1730, 1670 (broad), 1610 cm^{-1} ; mass spectrum (C.I., isobutane) gave a group of three peaks at m/e 702, 704, 706 with intensity ratios 9 : 6 : 1 (M^+) and a similar group at m/e 643, 645, 647 ($\text{M}^+ - \text{COOMe}$), both indicative of the presence of two Cl atoms in the molecule.

Compound (86) (113 mg, 0.16 mmol) was dissolved in 1N hydrochloric acid in 90% methanol (1.5 ml) and left at room temperature for 8 hours. After removal of the solvent, benzene (2 ml) was twice added and evaporated. Addition of ether (5 ml) to the residue gave an oily product which resisted attempts to solidify. The oil was dissolved in methanol (2 ml), filtered, the solution was concentrated and after washing with ether, the tripeptide ester dihydrochloride was obtained as a foam when the oil was dried under vacuum. The foam reverted to an oil on exposure to air for a short period. Yield 35 mg (63%); ν max (Nujol) 1730, 1665 (broad) cm^{-1} ; mass spectrum of N-acetylmethylvinyl derivative m/e 438 (M^+) 112 ('A' fragment); Rf 0.24 (chloroform-methanol, 9 : 1), 0.60 (chloroform-methanol-acetic acid, 70 : 30 : 5).

Preparation of Tryptophan Benzyl Ester

N-[α -Phenyl-(5-chloro-2-hydroxy-benzylidene)]-DL-Tryptophan
Benzyl Ester (89)

N-[α -Phenyl-(5-chloro-2-hydroxy benzylidene)]
-L-tryptophan (331) (419 mg, 1 mmol), was dissolved in
tetramethylammonium hydroxide solution (1 mmol) and after
evaporation of the solvent, the salt was dried over
 P_2O_5 overnight. The salt was then dissolved in
dimethylformamide (3 ml), benzyl chloride (0.12 ml, 1.1 mmol)
was added and the mixture was stirred at room temperature
for 30 hours. Ethyl acetate (15 ml) was then added and the
solution was washed with sodium bicarbonate (10%) and water.
The dried solution was concentrated to afford the yellow
crystalline product which was recrystallized from ether.
Yield 346 mg (68%); m.p. 134-135°; ν max (Nujol) 3360,
1740, 1610 cm^{-1} ; mass spectrum m/e 508 (M^+); Found:
C, 73.0; H, 4.9; N, 5.4. $C_{31}H_{25}ClN_2O_3$ requires:
C, 73.2; H, 5.0; N, 5.5%. The compound was optically inactive.

DL-Tryptophan Benzyl Ester Acetate (90)

Compound (89) (102 mg, 0.2 mmol) was dissolved in
80% acetic acid (2 ml) and warmed for 2 hours at 80°.
The solution was concentrated and benzene (3 ml) was twice
added and evaporated. Trituration of the residue with ether
gave the required acetate salt as a white powder. After
recrystallization from ethyl acetate the yield was
53 mg (75%); m.p. 108-110° ν max (Nujol) 3400,

1740 cm^{-1} ; mass spectrum of N-acetylmethylvinyl derivative m/e 376 (M^+), 241 ('A' fragment). Found: C, 67.3; H, 6.2; N, 7.6. $\text{C}_{20}\text{H}_{22}\text{N}_2\text{O}_4$ requires: C, 67.8; H, 6.3; N, 7.90%. The compound was optically inactive.

DL-Tryptophan Benzyl Ester Formate (91)

Compound (89) (102 mg, 0.2 mmol) was warmed for 2 hours at 80° in 80% formic acid (2 ml). The faintly red solution was then concentrated and benzene (3 ml) was twice added and evaporated. The residue was rubbed with ether to give a white solid which was recrystallized twice from ethyl acetate. Yield 55 mg (81%); m.p. $149-151^\circ$; ν max (Nujol) 3380, 1740 cm^{-1} ; mass spectrum of N-acetylmethylvinyl derivative m/e 376 (M^+), 241 ('A' fragment); Found: C, 67.3; H, 5.8; N, 8.0. $\text{C}_{19}\text{H}_{20}\text{N}_2\text{O}_4$ requires: C, 67.1; H, 5.9; N, 8.2%. The compound was optically inactive.

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