STUDIES IN PEPTIDE SYNTHESIS

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by

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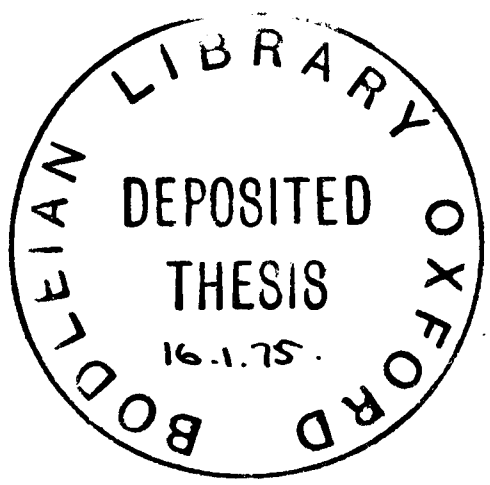

Balliol College and

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To my parents

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ABBREVIATIONS

Abbreviations for amino acids and their use in the formulation of derivatives follow with some exceptions the revised (1971) Recommendations of the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature. I.U.P.A.C. Bulletin, No.23, June 1972.

Other abbreviations which have been used without definition are:

Bpoc	2-(p-Biphenylyl)-isopropyloxycarbonyl
Dcha	Dicyclohexylamine
DMF	N, N-Dimethylformamide
Edta	Ethylenediamine tetra-acetate
I.r.	Infrared
N.m.r.	Nuclear magnetic resonance
ONSu	Succinimido-oxy-
OPic	4-Picolyl ester
Pipoc	Piperidino-oxycarbonyl
T.l.c.	Thin layer chromatography
U.v.	Ultraviolet
BocTF	2,2,2-Trifluoro-N-t-butoxycarbonyl aminoethyl
DCCI	N,N'-Dicyclohexylcarbodi-imide
HOBt	1-Hydroxybenzotriazole
THF	Tetrahydrofuran
Btm	Benzylthiomethyl
DCA	Dichloroacetic acid
CAA	Monochloroacetic acid

All amino acid residues are of the L configuration unless otherwise stated.

CONTENTS

Experimental Section

INSULIN

History

The clinical aspects of diabetes mellitus have been known for a considerable time and the connection between the pancreas, carbohydrate metabolism and the disease was initially realised in 1899 by von Mering and Minkowski.[1] By the removal of the pancreas from dogs it was found that the clinical features of the disease soon appeared after the operation. Experiments were undertaken to isolate this "active principle" in the pancreas and De Meyer was probably the first to isolate an active component which he called "insuline".[2] The first potent extracts were obtained in 1920 by Banting and Best.[3] They demonstrated that by their injection it was possible to obviate the effects of the removal of the pancreas in dogs, and by regular dosage to reinstate to health in cases of diabetes in man.

Abel[4] obtained the first crystalline material from pancreatic extracts which was shown to be proteinaceous in nature and had a greater activity than the extracts. After his initial crystallisation, Abel found that it was not always possible to repeat the process and it was Scott[5] who discovered that the crystals contained zinc and that the addition of zinc ions (or other divalent metal ions such as nickel, cobalt or cadmium) would promote crystallisation.

Preparation and homogeneity

The preparation of a highly-pure insulin for clinical use is nowadays a routine procedure using one of the early industrial extractions due to Romans.[6] The hormone is extracted in acid ethanol-water solution (70-80% ethanol, pH 2.0). The extract is concentrated under vacuum, defatted and adjusted to

pH 8.0 and the precipitate formed is discarded. After further concentration, salting-out with sodium chloride, isoelectric precipitation and crystallisation it is possible to prepare a crystalline material of high biological activity. 4-5 g of crystalline insulin of 24-25 units per mg are usually obtained from about 80 kg of calf pancreas.[7]

Commercial preparations of crystalline insulin from different mammals have been shown to be heterogeneous.[8,9] A thorough study of eleven insulins from different species by polyacrylamide electrophoresis[10] revealed heterogeneity and in most cases a major component with a great number of minor bands. The possibility that the minor components were formed during electrophoresis was eliminated by re-running the main component and obtaining only a single band. The minor fractions showed various biological and immunological activities. Rat insulin showed two major and three minor bands, which was expected since it is known to contain two different B-chains.[11]

The primary structure

A landmark in protein chemistry was achieved when Sanger and his co-workers reported the determination of the complete amino-acid sequence of beef insulin.[12] This breakthrough was not only important in itself but also because the methods developed have been used successfully in determining the sequence of many other proteins. A great number of insulin sequences are now known mainly due to the work of Smith.[11] The major variations are shown in Figure 1.

From the sequence variations of naturally occurring insulins it appears, considering the crystal structure,[13] that there are certain regions of the A- and

The Known Amino Acid Sequences of Insulin[a,b]

	A CHAIN																				
	(1)	(2)	3	4	(5)	(6)	(7)	8	9	10	(11)	12	13	14	15	(16)	17	18	(19)	(20)	(21)	22
Mammals	Gly	Ile	Val	Glu Asp	Gln	Cys	Cys	Thr Ala	Ser Gly	Ile Thr Val	Cys	Ser	Leu	Tyr	Gln	Leu	Gln	Asn	Tyr	Cys	Asn	
Birds								His	Asn	Thr						-						
Fishes			Leu					His	His Lys Arg	Pro		Asn Asp	Lys Ile	Phe	Asp		Gln	Ser Asn				
Guinea pig				Asp				Ala	Gly	Thr		Thr	Arg	His			Gln					
Coypu				Asp				Thr	Asn	Ile			Arg	Asn			Met					Asp

	B CHAIN																											
	0	1	2	3	4	5	(6)	(7)	(8)	9	10	(11)	(12)	13	14	(15)	(16)	17	(18)	(19)	20	21	22	(23)	(24)	25	26	27	28	29	30
Mammals		Phe	Val	Asn Lys	Gln	His	Leu	Cys	Gly	Ser Pro	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys Met	Ala Ser Thr
Birds		Ala	Ala																											
Fishes	Met Ala Val	Ala	Ala Pro	Ala Pro										Asp								Asp						Asn	Ser	Lys (−)	(−)
Guinea pig				Ser	Arg						Asn				Thr			Ser			Gln	Asp	Asp					Ile		Lys	Asp
Coypu		Tyr		Ser		Arg					Gln			Asp	Thr			Ser			Arg	His	Arg			Tyr	Arg	Pro	Asn	Asp	(−)

[a] Circled numbers represent invariant residues.
[b] (−) represents deletions.

Figure 1

B-chains which are strongly conserved and others where mutations are frequent. A core of "invariant" residues is formed in the interior of the molecule comprising of three cystines, leucines -A16, -B6, -B11 and -B15, isoleucine -A2, glycines -B8 and -B23 and valine-B18. The contact residues between the monomers in the dimer form another invariant group: valine-B12, tyrosine-B16 and phenylalanine-B24 and possibly the aromatics B25 and B26. The other invariant residues, glycine-A1, glutamine-A5 and tyrosine-A19 could be involved in the maintenance of tertiary structure as they are situated on the surface of the molecule in its aggregated form.

The variant residues in different species are found on the surface of the

crystalline molecule and because of their accessibility, these residues could determine the immunological properties. Residues on the surface of the molecule can affect its aggregation. Histidine-B10 is important to binding with zinc in the hexamer and is conserved in most insulins, except guinea-pig which does not form a hexamer.

The molecular weight of the insulin monomer was determined to be approximately 6,000 by Sanger[12] but it was known that the protein could undergo association giving aggregates that are a multiple of this value. The first studies carried out at neutral pH estimated a value of 36,000,[14] and since then a large number of papers have been published concerning the molecular weight of insulin. The most significant, however, are the X-ray structural analysis developed by Hodgkin and her coworkers at Oxford.[13] The basic repeating unit in the crystals studied, rhombohedral, is an insulin hexamer in which three dimers in the hexamer are related to one another by a crystallographic threefold axis and the two molecules in each dimer are related to each other by a local twofold axis. In the insulin molecule the two polypeptide chains are held together by two disulphide bridges and by hydrophobic and polar side chain interactions. The stabilization of the hexamer is achieved by predominately non-polar interactions between adjacent dimers and the co-ordination of the molecules to the two zinc ions on the threefold axis. This analysis, at 2.8Å resolution, has helped to correlate the relationship between the structure and biological role of insulin. Figure 2 indicates a spatial arrangement of the dimer viewed perpendicular to the crystal threefold axis. Figure 3 is the suggested arrangement for the connecting peptide in proinsulin, which is seen assembled into a hexamer.

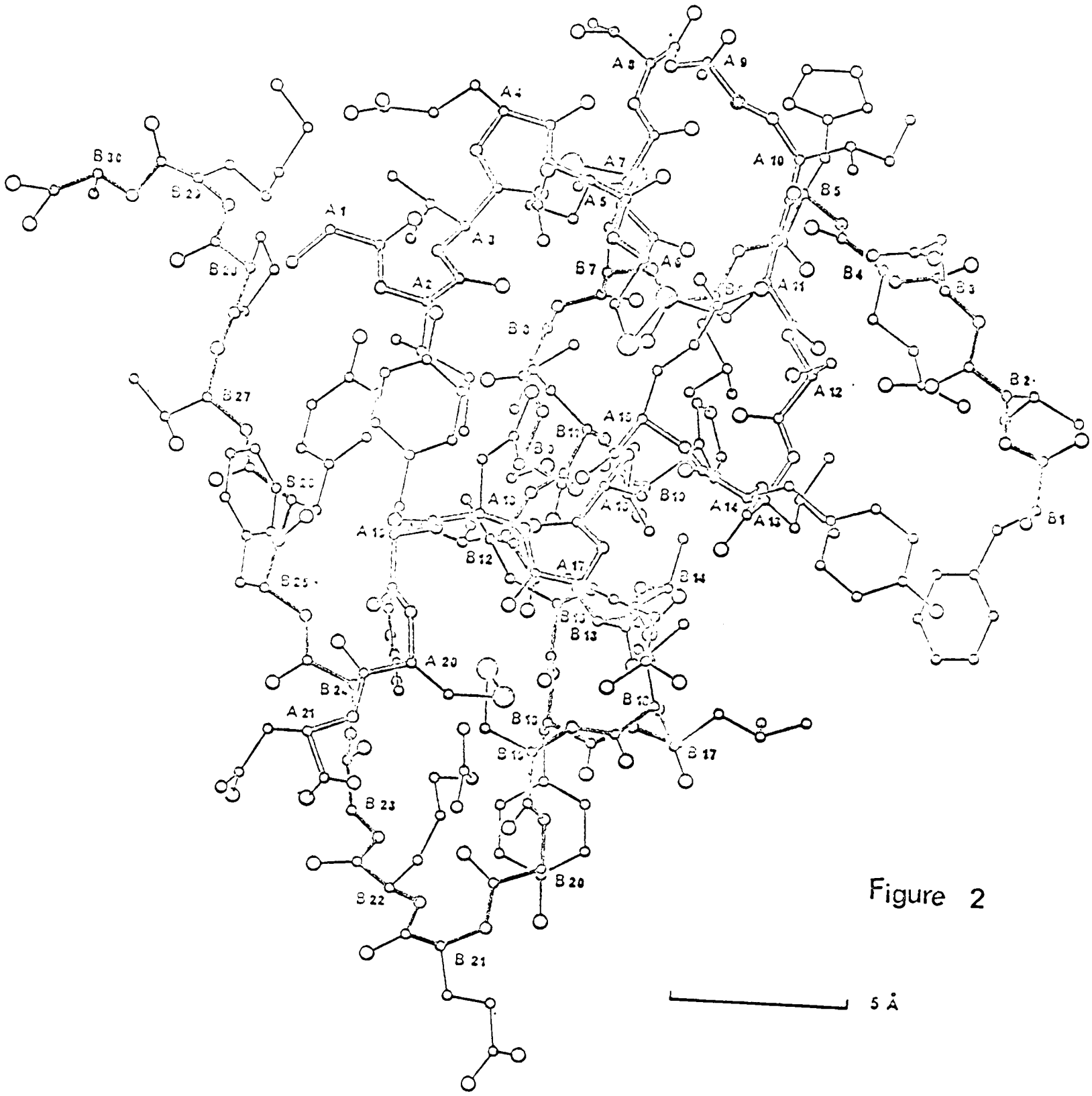


Figure 2

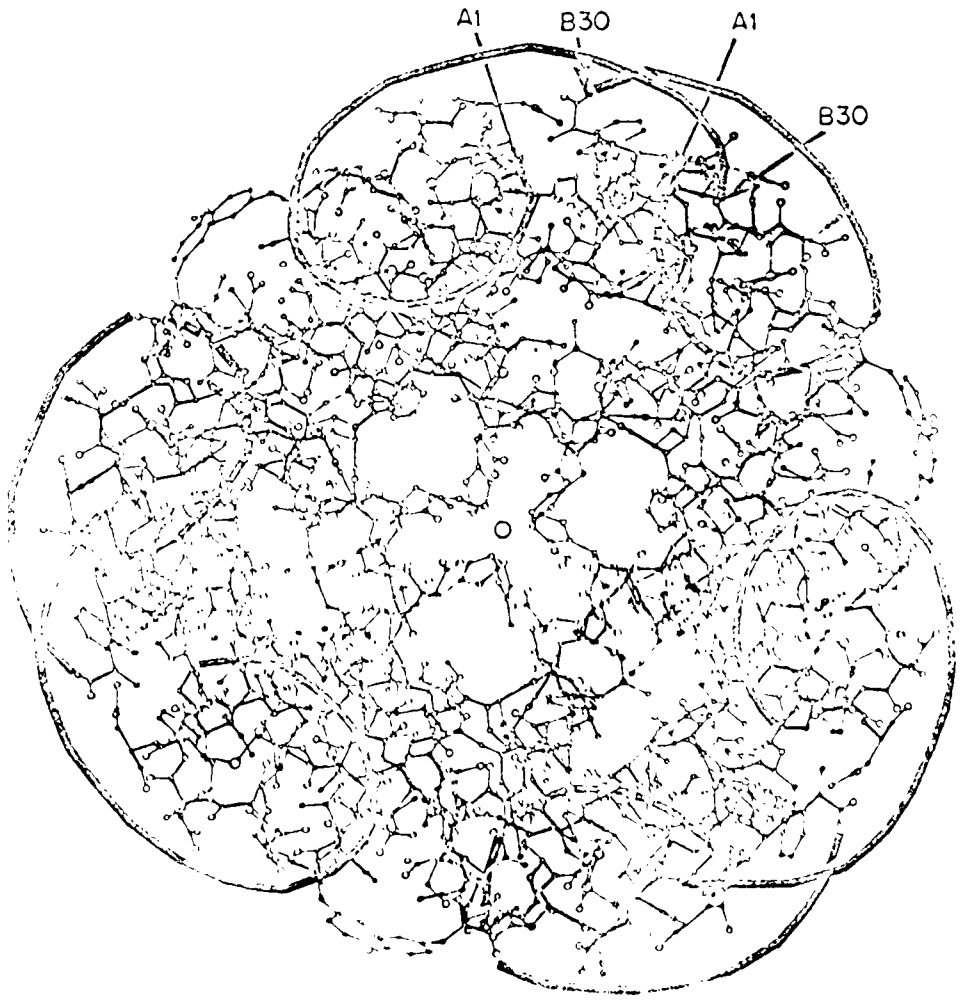


Figure 3

The main parameters affecting the aggregation of the hormone have been studied by Fredericq and others. Investigation into binding on anions,[15] pH,[16,17,18] insulin concentration,[20-22] dependence of zinc ions and other metal divalent ions[19-25] were the main parameters considered.

Proinsulin

Insulin has been shown to be synthesized in vivo as a single-chain precursor,[26] proinsulin, and the sequences of procine,[27] bovine,[28] human,[29] monkey,[30] cod,[31] angler-fish,[32] rat[33] and rabbit[34] proinsulins are now known. Proinsulin is proteolytically cleaved in the beta cell to form the more active double chain of insulin[35] and can be compared to other polypeptide hormones formed from larger, biologically less active precursors by the process of limited intracellular proteolysis, e.g. pancreatic alpha cell,[36] parathyroid[37] and pituitary[38] hormones.

Reduction followed by reoxidation in alkaline buffer results in a 70-80% recovery of the original material.[39] In contrast, reduction-reoxidation of insulin results in a recovery of only 1%.[40] This and other properties[28] suggest that the primary function of proinsulin may be to allow the pairing of cysteine residues necessary to form the tertiary structure of insulin as had been previously proposed.[41] The species variability in the structure of the connecting peptide segment of proinsulin suggests that the biosynthetic requirement is for a single chain precursor. However, there may be a specific necessity for a certain amino acid composition with the location of polar amino acids at the ends of the connecting segment, with relatively non-polar ones in the middle, and the absence of aromatic residues,

histidine and cysteine in the connecting segments, in all species so far investigated.[30]

It has been proposed that the insulin moiety in proinsulin exists in a very similar conformation as insulin itself[42] and that the connecting peptide is in a random coil formation with perhaps two short alpha helix areas.[43] Proinsulin also complexes with zinc to form a hexamer like insulin[42] as in Figure 3. Since proinsulin coprecipitates and cocrystallises with insulin during its commercial preparation the initial method for isolation of proinsulin was by purification of commercially available insulin.[44] Proinsulin and various related proteins have been isolated by means of DEAE-cellulose chromatography and urea-containing buffer[45] and from plasma and other tissues by chromatography on Sephadex G-50[46] and Biogel P-30.[47]

A synthesis of porcine proinsulin has used the strategy of the approach to ribonuclease T_1 and eleven protected fragments covering the entire 84 residues and partially protected derivatives corresponding to the sequences 25-84 and 17-84 have been obtained.[48] Various other fragments have been prepared[49] and one method involves elongation of the A-chain S-sulphonate of bovine insulin.[50] Geiger has recently reported the synthesis of human proinsulin C-peptide and its glutamic acid-9, glutamine-11 analogue.[51]

Chemical Synthesis

Following the pioneering work of du Vigneaud who demonstrated the feasibility of synthesising complex physiological molecules, three independent groups succeeded in preparing insulin, the first protein to be synthesised by

chemical means. Sheep insulin was prepared by German[52] and American[53] groups while the Chinese group[54] prepared beef insulin.

The general strategy was very similar in all three cases. The A- and B-chains were initially prepared and then coupled in the correct order to afford the synthetic chains. Insulin was then prepared by combination of the individual A- and B-chains; the final combination and deprotection of the protected chains proving the most difficult. Initial studies with A- and B-chains obtained by sulphitolysis of natural insulin indicated that only 0.5-5.0% of the theoretical activity was obtained after recombination[40,55,56] and although these later stages were improved recoveries were only in the order of 30-50%.[57] It was demonstrated that the sodium-liquid ammonia method of removing the particular protecting groups of the A- and B-chains was responsible for the poor yields in the blank experiment and also in the recombination of the synthetic A- and B-chains.[56] However, after repeated purification of the recombined synthetic chains, crystalline insulin of high biological activity was obtained by all three groups.

Bovine insulin with specific and total activities comparable to those reported for classical syntheses was synthesised by solid phase methods;[58] but by employing similar protecting groups to the previous methods the same deprotection problems were encountered as well as its inherent difficulties now realised.

Katsoyannis has used fragment condensation to prepare a more suitable protected B-chain which can be deblocked by liquid hydrogen fluoride and the reduced peptide converted to the S-sulphonate by oxidative sulphitolysis.[59]

All the insulin syntheses described so far lack control over the last stage in that the three disulphide bridges are formed simultaneous after vigorous complete

deprotection. Hiskey has outlined plans[60] for a synthesis of ovine insulin which employs greater control over the formation of the disulphide links and final deprotection will be by relatively mild acidolysis. The preparation of the A_{6-13}[60] and A_{14-21}[61] fragments have been reported.

Structure-function studies with chemically modified insulins

It has been suggested that the structure of the zinc insulin hexamer in solution is very similar to the crystal, as determined by X-ray analysis, and that the general three-dimensional structure of at least the dimers may be conserved in all the sequenced insulins.[13] In identifying the amino acids which are essential for its activity, consideration of not only the natural variation of sequences but also the activities of synthetic, chemically modified and enzymatically modified insulins must be made with regard to the crystal structure.

In general, the surface residues of the hexamer are hydrophilic and most are surrounded by solvent in the crystal lattice. These features are also characteristic of enzymes where the results of X-ray analysis have been used extensively to rationalize the solution properties, and they suggest that the structure of insulin will also be similar in solution. This has been confirmed by comparative tritium exchange experiments in crystals and in solution.[62] Results of structure studies in solution can be derived in terms of the examination of the availability of various functional groups, whether buried or exposed, and in relation to the geometry of the molecule.

(a) Modification of amino groups

The reactivity of the amino groups has been studied with a variety of

reagents. In most cases Edman-like reagents show a preferential order of reactivity B1 > A1 >> B29.[63-5] This is in agreement with their positioning in the dimer where the B1 amino group should be freely accessible. There is then the possibility that reaction at the B1 amino group, which is in the area of dimer-dimer contacts in the hexamer, would inhibit any hexamer formation.[66] The A1 amino group is more tightly bound to the structure and removal or modification of this group rather than at the B1 amino group appears to result in greater conformational changes in the insulin molecule.[67]

The ε-amino group of lysine-B29 is about 10Å from the A1 amino group and extends into the solvent; its high pK_a appears to limit its reactivity. The proximity of the A1 and B29 amino groups has led to various bifunctional reagents being cross-linked with these groups. Crystalline beef insulin was reacted with 1.2 equivalents of bis-p-nitrophenyl esters of aliphatic dicarboxylic acids, under dilution conditions in dimethylsulphoxide in the presence of triethylamine to give crystalline derivatives of varying activities.[68] Lindsay has described the preparation of glycine-A1-lysine-B29-succinyl insulin and glycine-A1-hemi-succinyl insulin.[69] This small separation has been supported by immunochemical[67] and spectral studies.[70]

The essential nature of purification and characterisation studies of reaction products was initially realized by Andersen,[117] who reacted insulin with phenylisocyanate, separated the products by reversed partition chromatography. After characterisation (analysis of hydantoins) they proved to be unreacted insulin, monophenylcarbamoyl insulin - 90% substituted at N-terminal glycine and diphenylcarbamoyl insulin substituted at both N-terminal amino groups. The

reported biological activities were 28 IU/mg, 23 IU/mg and 13 IU/mg respectively which indicates that substitution on residue-B1 has not much effect on the biological activity of the hormone but substitution on both N-terminal amino groups decreases the activity by about 50%.

Fluorescent derivatives of insulin were prepared by reaction with fluorescein isothiocyanate[118] and contained between 1.2 and 1.8 bound fluorescein groups per mole of insulin, the primary site being N-terminal phenylalanine. The biological activity, as estimated by hypoglycaemic response produced on fasted rabbits, was approximately 60-80% of unmodified insulin. This reaction was further studied by Bromer[64] who managed to separate mono-, di- and trisubstituted compounds by DEAE-Sephadex chromatography. Monofluoresceinthiocarbamoyl insulin was found to be substituted at one or other of the N-terminal residues, di- and trifluorescein derivatives were substituted at the N-terminal glycine and phenylalanine and, in the triderivative at the ϵ-amino of lysine-B29. The biological activities were found to be mono- 40%, di- 40% and tri- no activity when compared to insulin.

The development of semisynthetic analogues of insulin was probably due to the pioneering work of Levy and Carpenter.[71] By reacting insulin with t-butoxy-carbonyl azide in anhydrous dimethylformamide all the amino groups were protected with the t-butoxycarbonyl group. The product retained a diminished biological activity but on treatment with anhydrous trifluoroacetic acid, the protecting groups were removed and a crystalline insulin of high biological activity was obtained. Triaminoacyl-insulins were also prepared by reacting the p-nitrophenyl ester of t-butoxycarbonyl amino acids with insulin and removing the protecting

t-butoxycarbonyl group with anhydrous trifluoroacetic acid. Derivatives prepared were trialanyl-, triasparaginyl-, trilysyl-, trimethionyl-, and triglutamyl-insulins. The products were thoroughly characterised by amino acid composition, chemical and enzymatic degradation and all possessed about the same biological activity, approximately 40-50% of native insulin.

By using selective Edman degradations a wide variety of shortened and substituted N-terminal chain derivatives have been prepared.[72] Des-phenylalanine-B1-insulin, des-phenylalanine-valine-B1-2-insulin and des-phenylalanine-valine-asparagine-B1-3-insulin all have high blood sugar lowering activities. Glycine-B4 was cyclised to pyruvic acid to give the derivative des-phenylalanine-valine-asparagine-B1-3-pyruvic acid-B4-insulin similarly active. A homogeneous monoiodoinsulin[73] was prepared by reacting A1, B29-bis-t-butoxylcarbonyl-des-phenylalanine-B1-insulin with N-t-butoxylcarbonyl-p-iodophenylalanine 2,4,5-trichlorophenyl ester to yield a derivative with high activity.

(b) Modification of carboxy groups

It has been found that esterification of the carboxylate groups is also consistent with their position on the surface of the molecule. However, two of the groups seem to be involved in interactions with other residues. The carboxylate of glutamate-A4 lies close to lysine-B29 and the A-chain carboxyl terminal is close to the guanidinium group of arginine-B22. Thus these surface interactions may be disturbed on esterification and involve conformational changes in the molecule.[74] The close interaction of asparagine-A21 may also be the reason for limited cleavage of this residue by carboxypeptidase.[75]

Mommaerts and Neurath studied the effect of esterification of the carboxy groups of insulin[76] and conditions were obtained where six moles of methoxyl group per mole of insulin were found. It was concluded that all the carboxy groups of the hormone were completely and exclusively esterified. More drastic conditions gave rise to partial hydrolysis of the amide groups of the molecule. It was claimed that the biological activity was not appreciably altered when up to two-thirds of the carboxy groups were esterified but further incorporation of methoxy groups did.

Esterification in anhydrous hydrogen chloride-methanol[77] gave two main products, an inactive hexamethyl ester of insulin and a fully esterified product containing an N⟶O shift at the bond linking tyrosine-B26 and threonine-B27.

Water-soluble carbodi-imides have been used to modify the carboxy groups[78] and products were obtained in which reaction had taken place at either one or both of the glutamic residues-B13 and -B21 and also at the C-terminal asparagine-A21. Osawa[79] inferred that the partial loss of biological activity was due to substitution of asparagine-A21 which appears to be essential for activity.

(c) Modifications of other functional groups

Studies on structures in solution have indicated that one or more of the tyrosine residues are involved in various forms of interactions which can be correlated to the crystal structure.

Changes in the tyrosine absorption spectra[80] and decreased circular dichroism of the tyrosines are found when the dimers dissociate on dilution or the C-terminal octapeptide is removed by trypsin.[81] This has been suggested to be due to a transfer of B-chain tyrosine residues from a non-polar environment to

aqueous solution.[82] This is exemplified in the case of nitration or iodination where there is a preferred reaction with the A-chain tyrosine residues which are more exposed in the dimer and other higher aggregation states than B-chain tyrosine residues.[67,83,84] Monoiodination of tyrosine-A19 compared to di-iodination of tyrosine-A14 is consistent with the differing environments.[83] The four tyrosines of insulin have been shown to possess the following order of reactivity against tetranitromethane, TyrA14 > A19 > B16 > B26, and with progressive nitration, the reactivities of tyrosines-A14, -A19 and -B16 become very similar whereas tyrosine-B26 maintains a significant lower reactivity. The mononitro-, dinitro- and trinitro-insulins have been isolated[85] and activities measured by immunoassay were 40%, 30% and 18% of insulin respectively. Pure tetranitro-tyrosine insulin was purified by chromatography and retains a biological activity of 12.5 IU/mg and 11.3 IU/mg in the mouse convulsion and immunochemical assays respectively.[119]

Although there is general agreement between studies in solution and the crystal structure small changes of conformation are possible when the species considered is in solution, dimer or hexamer form. Thus a change in the position of tyrosine-A14 may give rise to the reported tyrosyl ionization[86,87] and selective o-cyanolation[88] of residues-A19 and -B16.

It has been suggested that histidines-B5 and -B10 were both essential for biological activity on account of photo-oxidation studies[89,90] but Gattner has found that photo-oxidation of the tyrosines leads to the formation of approximately 30% polymers.[91] Therefore, it is suggested that this method was not the best to study histidine influence on the biological activity of the hormone.

The sulphation of insulin has been reported[92,93] and up to nine products have been isolated by paper electrophoresis.[94] Sulphonation of one threonine, three serines together with sulphonation of a tyrosyl residue, sulphamation of a free amino group and a little p-tyrosyl sulphate is reported. Some have reduced biological activity and have been used to treat resistant diabetics.

Modification at arginine-B22 has been carried out with glyoxal[95,96] where it seems that transamination at amino groups occurs with the formation of the corresponding keto derivative.[97] The biological activity by rat adipose tissue was reported to be 16% of native insulin. Reaction of this glyoxal modified insulin, which had α-keto groups at the α-amino groups, with phenyl glyoxal gave 86% reaction at arginine and the product had 1% activity of insulin. The products were not isolated and the specificity with regard to the imidazole groups of histidine or hydroxyl groups of tyrosine was not considered.

(d) Biological activity of enzymatically degraded insulins

Native insulin is very highly resistant towards hydrolysing enzymes in vitro, the zinc appearing to inhibit the reaction. Leucine aminopeptidase digests only metal-free insulin[98] and insulin is only slowly cleaved by trypsin and chymotrypsin[99] yet the rate of hydrolysis increases rapidly after the breaking of one bond.[100] Insulin is strongly attacked by subtilisin.[101] When insulin is treated with a weak acid, several transformation products are formed and these were identified as desamido insulins.[102] The location of the labile amide bond(s) was achieved by Slobin and Carpenter[103] who suggested that the amide bond of asparagine-A21 is acid labile and that the hydrolysis of the remaining five amide groups of insulin occurs at a much slower rate. There are only two peptide bonds in insulin that are

labile to trypsin, those between arginine-B22 and glycine-B23 and between lysine-B29 and alanine-B30. Nicol[104] showed that tryptic digestion of the hormone afforded desoctapeptide insulin with a reported biological activity of 15% of that of native insulin. The rate of release of alanine-B30 and heptapeptide-B23-29 by trypsin follows essentially the same kinetics but the biological activity of the desoctapeptide insulin was found to be less than 2%[105] and a lower value of approximately 1% has been cited.[106] Through the use of enzymes, trypsin and carboxypeptidase, and acid treatment, Carpenter has reported[107] the preparation of several highly purified fragments lacking residues at the C-terminus of either A- or B- or both chains of insulin.

Figure 4

Biological Activity of Various Bovine Insulin Derivatives

Compound	Biological Activity IU/mg	Type of assay
Desamido-A21-insulin	26	rat diaphragm
"	23	mouse convulsion
Desalanine-B30-des-asparagine-A21-insulin	1.2	" "
"	<1.0	rat diaphragm
"	0.4	immunochemical
Desalanine-B30-desamido-A21-insulin	16	mouse convulsion
"	18	rat diaphragm
"	16	immunochemical
Desalanine-B30-insulin	18	"
Desoctapeptide-insulin	<1	mouse convulsion
"	<1	rat diaphragm
"	<0.4	rabbit blood sugar
"	1.1	immunochemical

The removal of asparagine-A21 has a marked effect on the biological activity; in contrast when the amido group of asparagine-A21 is hydrolysed the activity remains substantially the same as in native insulin. The loss of alanine-B30 decreases the activity by approximately 30% and the removal of the octapeptide nearly abolishes the biological activity of the hormone.

These results suggested that the carboxyl group of asparagine-A21 or aspartic acid and some of the amino acids of the octapeptide are involved in specific interactions which are essential to the biological activity of the hormone. Whether these interactions play the role of maintaining a specific three-dimensional structure and/or are involved in binding to another molecule or membrane is yet to be established. According to Cuatrecasas,[108] there is a possible correlation between binding of insulin to adipose tissue cells and the biological activity of the hormone.

Chemical Synthesis of Analogues

The development of solid phase peptide synthesis has allowed rapid preparation of a variety of A- and B-chains. The method has been extensively reviewed[109] and its limitations with regard to the likely heterogeneity of the initial product and the inherent difficulty of purification must impose severe restrictions on the size of pure peptides which can be prepared. Mainly due to the work of Weitzel[110] over sixty hybrid insulins have been reported. Each synthetic A- or B-chain was hybridised with the corresponding natural chain.[111] However, the biological activity was determined on the prepared analogue without any fractionation and the results obtained must be considered with this in mind.

The greatest problem has been the recombination of A- and B-chains and even the material derived from combining natural A- and B-chains had only 0.5-1.2% of the activity of native insulin. The activity of any analogue prepared is dependent not only on its intrinsic activity but also its actual yield. Thus comparing similar activities of analogues with insulin, it could be assumed that the analogues have a specific activity similar to that of insulin. However, if the synthetic hybrid has little or no activity, then the combination may have been difficult and a poor yield was obtained. Using these hybrids an attempt has been made to give some indication of "essential" residues and those unimportant for activity. Figures 5 and 6 give the relative potencies of hybridised insulins derived from either synthetic A- or B-chain and their corresponding natural chain.

Relative potencies of hybridised insulins derived from synthetic analogues of ovine A-chain and natural B-chain

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	*Biological activity* % Mouse-convulsion assay*	*Rat fat-pad assay*
Gly	Ile	Val	Glu	Gln	Cys	Cys	Ala	Gly	Val	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn	1.0–1.5	1.0–2.0
											Ala										0.8–1.2	0.4–0.9
											Ala							Phe			1.0	0.4
									Leu		Ala										0.6–1.0	
	Leu	Leu							Leu		Ala										inactive	
											Ala										0.05	0.05
											Ala							Phe		Ala	1.0	2.7
											Ala						Ala	Phe		Ala	1.0	4.1
		Leu									Ala										inactive	inactive
	Ala										Ala										inactive	inactive
Ala								Ser													0.2–0.4	0.3
	Gly							Ser													0.1–0.25	0.15
β-Ala											Ala										0.2	0.3
		Ile									Ala										0.1	0.16
				Ala							Ala										0.2–0.4	0.33–0.37
											Ala			Ala							0.1–0.4	0.33–0.37
											Ala		Phe					Phe			1.3	1.0
											Ala		Ala								1.0–1.3	1.4
					Ala				Ala												inactive	inactive
				Glu							Ala										1.5–2.0	2.1
											Ala			Glu							2.5	4.5
	Leu																				0.4	0.3
	Val																				0.6–1.0	0.3–0.7
				Glu							Ala			Glu			Ala	Phe		Ala	2.4	4.0
				Glu							Ala		Ala	Glu			Ala	Phe		Ala	1.0–1.7	0.97
	Val			Glu							Ala		Ala	Glu			Ala	Phe		Ala	0.8	0.54
				Glu					Ala		Ala			Glu			Ala	Phe		Ala	1.8	
				Glu					Ala		Ala		Ala	Glu			Ala	Phe		Ala	3.2	
				Glu							Ala	Ala	Ala	Glu			Ala	Phe		Ala	1.8	

Figure 5

Relative potencies of hybridised insulins derived from synthetic analogues of bovine B-chain and natural A-chain

1	2	3	4	5	6	7	8	9	10	22	23	24	25	26	27	28	29	30	*Biological activity %* *Mouse-convulsion assay*	*Fat-pad assa*
Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala	0.5-1.0	0.4 0.8
		Ala																	0.6-1.1[a]	0.84
			Ala																0.7[a]	0.59
								Ala											0.6[a]	0.45
																			0.6[a]	0.46
			Ala																0.6[a]	0.51
																		Ala	0.5[a]	
																Ala			0.6[a]	
															Ala				1.0[a]	
								Ala							Ala	Ala			1.5[b]	
								Ala							Ala				5.5[c]	
			Ala					Ala							Ala	Ala			1.1[b]	
								Ala							Ala	Ala			0.2[b]	
			Ala					Ala							Ala				5.0[c]	
			Ala					Ala						Ala					2.5[c]	
								Ala							Ala				0.4[c]	
									Ala							Ala			0.7[a]	
				Ala												Ala			0.5[a]	
				Ala					Ala						Ala	Ala			0.5[a]	
				Ala				Ala							Ala	Ala			1.2[d]	
				Ala				Ala	Ala						Ala	Ala			1.3[d]	
				Ala				Ala	Ala						Ala	Ala			1.2[d]	
								Ala	Ala						Ala				5.4[c]	
			Ala					Ala	Ala						Ala				2.4[c]	
			Ala					Ala	Ala						Ala				5.6[c]	
			Ala	Ala				Ala							Ala				2.6[c]	
			Ala	Ala				Ala	Ala						Ala				2.7[c]	
				Ala				Ala	Ala						Ala				2.8[c]	
								Ala		Ala					Ala	Ala			0.1[a]	
								Ala		Orn					Ala				1.0[a]	
								Ala		Ala					Ala				0.1[a]	
								Ala		His					Ala				0.1[a]	
					Ala			Ala	Ala			Ala							1.0[a]	
								Ala	Ala										<0.1[a]	

The activity obtained on hybridisation of each synthetic B-chain with natural A-chain is compared with the activity obtained on hybridisation of natural B-chain with A-chain. In the different series of experiments, the activity obtained with the natural chains was a, 0.5–1.0%; b, 1.2%; c, 5.0%; d, 2.0%; e, 5.5%.

Figure 6

Using synthetic A-chains and natural B-chain, hybrid insulins demonstrated that replacement of tyrosine-A14 or -A19 by phenylalanine resulted in no loss of activity and similarly asparagine-A18 and -A21, serine-A12 and valine-A10 could be replaced by alanine; the side functional groups of these amino acids appearing unimportant for activity. Various residues have been replaced by isofunctional residues but more prolific changes have led to a decrease in activity. Glutamines-A5 and -A15 were exchanged for glutamic acid and isoleucine-A2 was replaced by valine without any reduction of activity. However, if alanine was substituted

at positions A5 or A15, or isoleucine-A2 was replaced by leucine, less active products were obtained. Positions where the "natural" residue appeared irreplaceable were cysteines-A6 and -A11 and the region most sensitive to change was the amino terminal of the A-chain, glycine-A1, isoleucine-A2 and valine-A3.

Considering the X-ray crystal structure, it appears that the hydrophobic residues forming the "invariant core" of the protein are extremely resistant to change. Many of the results confirm chemical and fragment modifications, i.e. replacements of glycine-A1, tyrosines-A14 and -A19 and asparagine-A21. The need of an intrasulphide bridge between residues A6 and A11 is shown although it is known that a sulphur atom can be replaced by a methylene group with some retention of activity.[112] It is claimed that in one analogue where there are eight A-chain replacements there is as much biological activity as the hybrid formed with natural A-chain.

In considering B-chain analogues, it is found that as with fragmented chains, shortened chains lacking residues B1-3 and B28-30 give equally active hybrids as native insulin. Changes involving residues-B4, -B9, -B10 and -B27 were without effect on activity but replacement of histidine-B5, arginine-B22 and tyrosine-B26 by alanine caused activity decreases. The suggestion of histidine-B10 being unimportant is queried with the known role of this residue in intermolecular aggregation.[113]

Fragment condensation has been used to produce shorter A- or B-chains which are then combined with their respective partner in the usual manner. Des-tetrapeptide-B27-30 human insulin[114] has an activity of 11-15 IU/mg by the

mouse convulsion method but destetrapeptide-A1-4 sheep and porcine insulin are biologically inactive.[115]

Further analogues of the B-chain have been studied by Weitzel[116] using conventional or solid phase methods to demonstrate the decrease of biological activity with shorter B-chain length, i.e.

22 27 30	Activity %
-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala	100
-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Ala	100
-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Ala	50
-Cys-Gly-Glu-Arg-Gly-Phe-Ala	30
-Cys-Gly-Glu-Arg-Gly-Ala	10
-Cys-Gly-Glu-Arg-Ala	10
-Cys-Gly-Glu-Ala	<2

Complete synthesis whether by solution or solid phase synthesis is essentially laborious and it is inherently difficult to produce simple chemically modified analogues. Semisynthesis would therefore have marked advantages and this approach is that of the present work which will be discussed in detail in the next chapter.

SEMISYNTHESIS OF PROTEINS

The combination of natural and synthetic peptides would give rise to an alternative method of synthesis for the preparation of polypeptide and protein analogues. This type of semisynthetic approach would be ideal for convenient introduction of amino acid substitutions in enzymes or larger molecules in order to examine the molecular basis for biological action and possible indications of tertiary structure stabilization. Semisynthesis, widely employed in the preparation of natural products, has only recently been applied to peptide and enzyme research.

Several different semisynthetic approaches have been used in peptide synthesis. Single residues can be transformed by specific chemical reactions to yield modified analogues. Semisynthetic products have also been prepared by joining naturally occurring and synthetic peptides by non-covalent interaction, by disulphide bridges and by the formation of new peptide bonds.

Residue modification or transformation

Semisynthesis in peptide chemistry may be accomplished, albeit certain limited conditions, by interconversion of residues in a naturally occurring polypeptide. The amide bonds are therefore neither formed nor broken.

Subtilisin was converted to thiol-subtilisin[169,120] by reacting the N-terminal serine with phenylmethanesulphonyl fluoride followed by thiol acetate to give S-acetyl-thiol-subtilisin; deacetylation yielded thiol-subtilisin, i.e. conversion of a serine protease to a cysteine protease. Similarly the N-terminal serine of adrenocorticotropic hormone was converted to glycine by oxidation with

periodate ion followed by reaction with copper glutamate at pH 6.7.[121]

The residue modification technique is attractive because of the small number of reactions needed to produce the desired product but it is equally evident that in proteins it may be impossible to separate well defined products.

Non-covalent interactions

Combination of a naturally occurring and synthetic peptide has been used to produce semisynthetic enzymes. Bovine pancreatic ribonuclease is cleaved by subtilisin to yield S-peptide, residues 1-20 and S-protein, residues 21-125.[122] After separation, the enzymatically inactive fragments can be recombined in equimolar proportions, without formation of a covalent bond, to give a product with full ribonuclease activity. S-peptides and analogues have been synthesized by two groups. Finn and Hofmann[123] have reported the synthesis of 13 modified S-peptide analogues in which either C- or N-terminal residues were deleated. The capacity, or lack of, these peptides to form an enzymatically active complex with S-protein suggested that residues 8-12, either form part of the catalytic site or allow some conformational change necessary for activity. Histidine-12 was shown to be part of the catalytic site by using the close isoster β-(pyrazoyl-3)-alanine as a replacement and residues 2, 13-15 were important for binding.[124] Studies with proline containing analogues suggested that the first 12 residues of S-peptide were in helical form.[125] This was later confirmed when the crystal structure became known.[126]

Monocovalent interactions of fragments of an extracellular nuclease from Staphylococcus aureus are being studied by Anfinsen.[127] Trypsin cleaves this

nuclease into three fragments: P_1, residues 1-5; P_2, residues 6-47; P_3, residues 48-149. P_2 and P_3 recombine to give the active enzyme. Synthetic P_2 and P_2 analogues have been prepared and tested for their capacity to interact with native P_3 and regenerate enzymatic activity.[128] Methionine-26 and -32 were replaced with norleucine to give a fully active product. Glutamic acid-43 was replaced with glutamine to give a very weakly active product which, however, binds P_3. The initial 12 residues of P_2 can be deleted to give an active peptide, although shorter analogues lose both regenerative capacity and ability to bind to P_3.

The number of other proteins that will specifically reassociate after partial digestion is not known and thus this application of semisynthesis by non-covalent interactions may be quite limited.

Disulphide bond formation

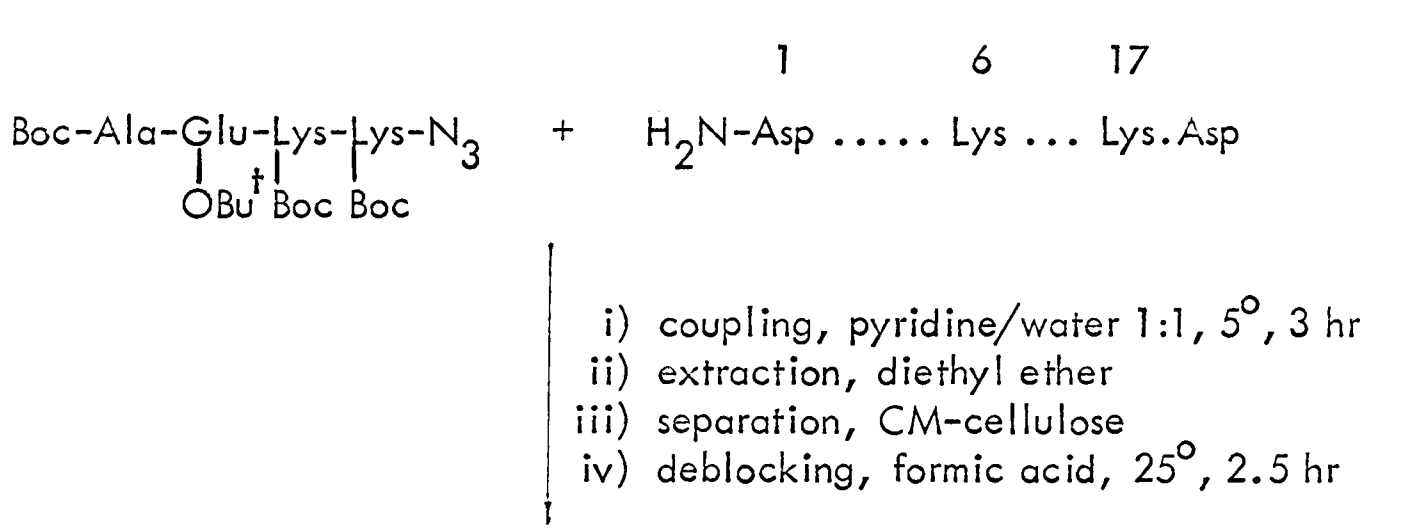
Synthetic and naturally occurring polypeptides can be joined covalently by disulphide bridges to give semisynthetic products; all the studies have involved insulin. Katsoyannis reported four semisynthetic insulins including one unnatural obtained by mixing synthetic and natural ovine and bovine A- and B-chains.[129] The Chinese group also prepared semisynthetic bovine insulin by combining synthetic B-chain and natural A-chain.[130] Yields initially were only 5-10% but the products had the same activity as natural insulin. With insulin A-chain tetra-S-sulphonate as the nucleophilic partner in coupling a number of A-chain analogues extended at the N-terminus have been obtained.[50,131,132] Synthetic insulins prepared using the solid phase technique were initially reported by Merrifield[58]

and Zahn[133] and such as been the development of this method that a great variety of semisynthetic analogues have been produced, as previously mentioned mainly due to the work of Weitzel.[110]

Using 'relays' derived from partial degradation of a protein

Probably the first application of this idea was the preparation of [Lys^{10}]-human β-melanocyte stimulating hormone[134] formed by reaction of a synthetic protected tetrapeptide azide with porcine β-melanocyte stimulating hormone (Figure 7). As the natural peptide contained two lysine residues and therefore three nucleophilic sites the coupling products were separated by virtue of the pK_a differences of the α- and ε-lysine residues by CM-cellulose chromatography.

Figure 7



A general procedure for producing suitably blocked peptides from naturally occurring peptides was suggested in 1968 by Backer and Offord,[135] in which blocked peptides were isolated from hen egg white lysozyme for use as intermediates in peptide synthesis. For general application to peptide synthesis, peptides containing

free α-amino but blocked side chain amino groupsmust be obtained. Enzymatic cleavage of a protein with side chain protection yields peptides with free α-amino groups which can be protected with an independently removable protecting group or else left free for reaction. If the original N-terminal peptide contains lysine it cannot be selectively deprotected and can only be used for synthesis as an N-terminal peptide. However, in peptides containing blocked lysine residues, trypsin will only cleave at arginine. C-terminal carboxy groups must also be free but side chain β- and γ-carboxy groups must be protected, which is achieved by selectively employing trypsin to cleave α-carboxy esters. After enzymatic digestion of an amino-blocked protein with trypsin, the peptide products were isolated and completely esterified with phenyldiazomethane to benzyl esters. C-terminal β-benzyl esters in each case were then preferentially split by trypsin acting as an esterase. Recoveries of histidine were low indicating possible imidazole alkylation by phenyldiazomethane. Blocking of other side chain functions other than amino or carboxy were not discussed extensively.

However, this method has great application in the field of semi-synthetic techniques. Modifications can be made to one protected fragment or a synthetic fragment can be introduced and thus a wide variety of analogues can be prepared by simple condensation steps. The method has been applied to lysozyme[136] and carboxy protection by 1-diazo-2,2-dimethylpropane has been introduced.[137]

Offord has also introduced a method for modifying a single chosen residue in a biological molecule which was applied to insulin.[65] Reaction with phenyl-isothiocyanate under mild conditions not only gave the tri-phenylthiocarbamoyl derivative but also the mono- and di-derivatives. By separating the mono-

phenylthiocarbamoyl form, mainly substituted on phenylalanine-B1, from the others it was possible to obtain a molecule with two free amino groups instead of three. Trifluoroacetylation of these amino groups, subsequent cyclisation and removal of phenylthiocarbamyl-phenylalanine gave des-phenylalanine-B1-insulin with one free amino group. Coupling of various derivatives of phenylalanine, i.e. p-iodo-phenylalanine, at this position (B2) and deprotection of N-acyl protecting groups regenerates a molecule that has the same number of residues as native insulin but contains one specific modification.

Semisynthetic studies on insulin: previous work

During the course of this work two reports were published which concerned the work being undertaken in this thesis and these will be discussed in depth here.

In 1972 Ruttenberg gave brief details of a method for converting porcine insulin into human insulin,[138] i.e. a route converting easily available insulin into the human sequence much needed in the treatment of diabetes. The procedure required complete esterification of porcine insulin with diazomethane, cleavage of the B-chain C-terminal octapeptide with trypsin, protection of α-amino groups with t-butoxycarbonyl azide, coupling a synthetic protected C-terminal human octapeptide, amino group deprotection and finally, saponification of the ester protecting groups. After purification, it was quoted that a typical experiment yielded a total of 2.7 g material starting from 4.0 g porcine insulin and this material had the correct amino acid analysis, biological activity and chromatographic properties associated with human insulin.

However, in many regions of this paper the experimental details are vague

and uncertain; the methods used in esterification with diazomethane and alkaline deprotection were those of Chibnall[139] and these are extremely limited to be of any use. Other practical description is weakly presented or absent.

Obviously with the large possibility of side reactions any independent repitition of this procedure is virtually impossible.

Using methods similar to Ruttenberg, the Shanghai group have investigated the C-terminal sequence of the B-chain with respect to insulin activity.[140,141] Des-octapeptide insulin was obtained from tryptic digestion of natural insulin and coupled with synthetic C-terminal octapeptide to regenerate insulin with high activity after further purification.

Des-hexapeptide insulin was obtained when glycyl-phenylalanine was condensed with des-octapeptide insulin to give an analogue with a specific activity of 25-30% suggesting that residues B23-24 are essential for activity. Other des-hexapeptide analogues have been prepared by replacing glycine-B23 by hydrophobic amino acids; alanine, valine and phenylalanine derivatives were inactive while asparagine and serine derivatives showed slight activity. Interestingly enough replacement using D-amino acids gave active products. D-alanine and D-valine analogues had 5-10% activity while D-phenylalanine and D-serine analogues were 5% and 2% active respectively.

Des-hexapeptide analogues in which phenylalanine-B24 was replaced by arginine, serine and glutamic acid were all inactive. B24 replaced by tyrosine or benzylamine analogues were about 2% active. Des-octapeptide-glycylglycyl-phenylalanine insulin was 2% active but replacement of L-phenylalanine by D-phenylalanine gave a completely inactive analogue. These results seem to suggest

that an aromatic residue at B24 or B25 is essential for activity. Results however were based on analysis of crude products.

Outline of project: concurrent work

The problems associated with the design of analogues of biologically active peptides and proteins and their relationship to structure-activity studies has been the principle concern of peptide synthesis. Although the majority of the work has resulted from experimentation with the chemical modification of the side chains of amino acids, it is extremely difficult, if not impossible, to predict the nature and extent of these modifications with any certainty as has just been demonstrated in the case of insulin. In contrast, the scope of semisynthetic studies allows either by the use of tryptic peptides or synthetic analogues the introduction of specific modifications into biologically active species such as hormones or enzymes.

The object of this thesis is to apply the role of semisynthetic methodology to the case of insulin. The problem is applicable in that tryptic digestion of the hormone removes the C-terminal B-chain octapeptide and hence the replacement of this octapeptide with initially natural and synthetic analogues will provide valuable insight into the octapeptide function in the monomer, dimer and hexamer. The possibility of preparing human insulin from readily available porcine insulin would be of great commercial interest. Thus this thesis investigates the problems of synthesising suitably protected peptide fragments for coupling onto a suitable precursor in the form of a protected des-octapeptide-insulin.

This latter objective, the study of a suitable precursor, is being currently extensively studied in the Department of Molecular Biophysics, University of

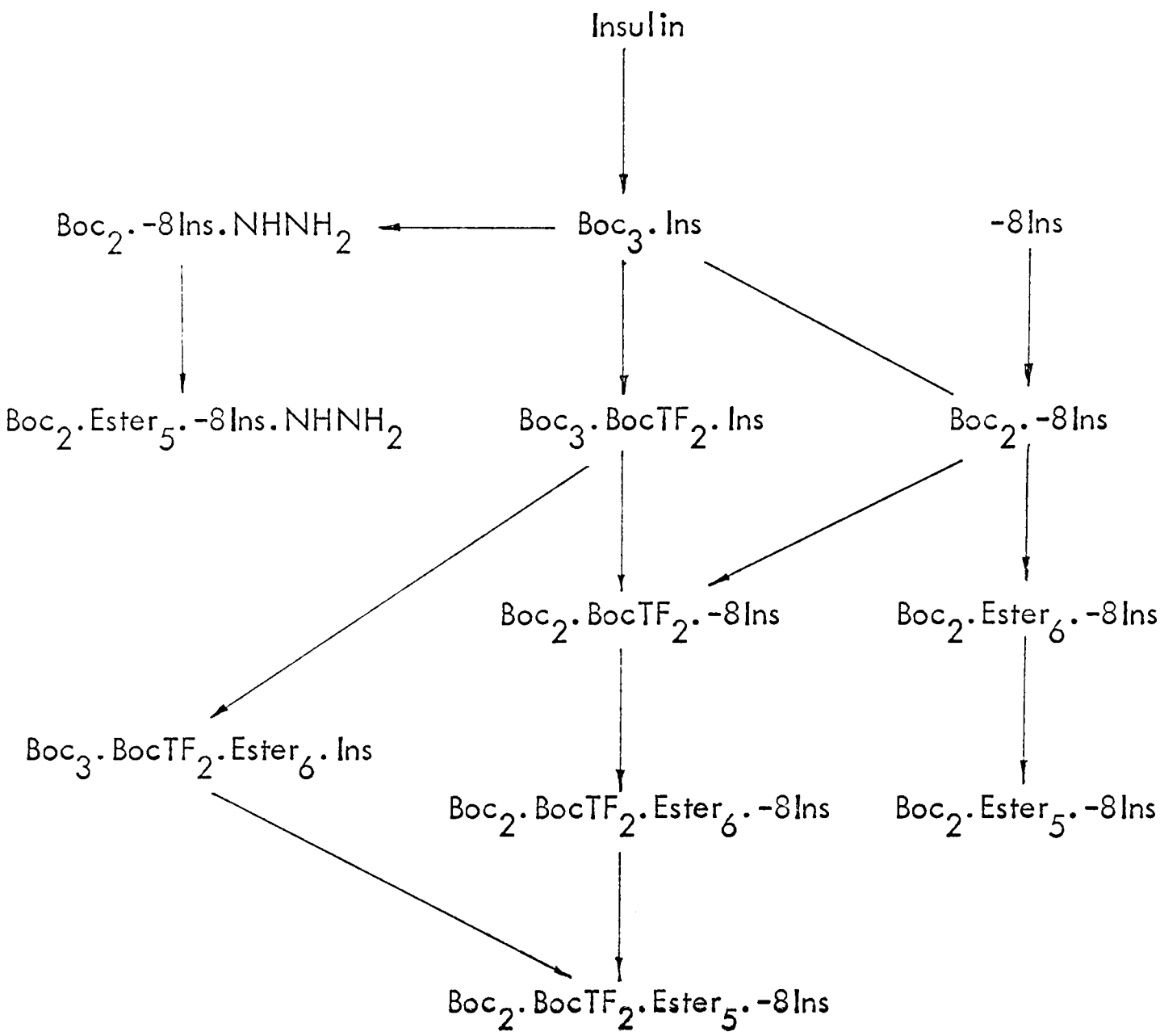
Oxford by Drs. R. E. Offord and H. T. Storey. Their approach and a summary of the possible routes to obtain suitable precursors can now be discussed before the actual work undertaken for this thesis.

The basic requirements for the semi-synthesis of insulin are for reversible amino protection (two α-amino, one ε-amino), reversible carboxy group protection (four γ-carboxy, two α-carboxy), reversible protection of the imidazole groups of the two histidine residues and a viable tryptic cleavage between arginine-B22 and glycine-B23. The three amino groups in the molecule may be successfully protected as the t-butoxycarbonyl (Boc) or benzyloxycarbonyl (Z) derivatives but only the former protecting group may be removed with little or no damage to the rest of hormone molecule. If the t-butoxycarbonyl group is removed by the action of hydrogen bromide in trifluoroacetic acid then a scavenger of anisole must be used if biological activity is to be retained. The two imidazole groups can be reversibly protected as the 2,2,2-trifluoro-N-t-butoxycarbonylaminoethyl (Boc TF)[136,142] derivatives but the insolubility of the product in aqueous or 8M urea media makes quantitation of this step impossible. It is also possible to protect both the imidazole groups and the free amino groups as the Boc TF protected derivatives but some slight denaturation is suggested as the deprotected insulin from this method is not quite so readily crystallised by the Schlichtkrull method[143] as are insulins derived by deprotection of Boc. protected- or Boc. and Boc TF. protected-insulins.

The most difficult problem has been the choice of suitable reversible carboxy protection. The criterion initially applied for successful protection and deprotection of sites in the insulin molecule is that of crystallisability under the

Schlichtkrull conditions. Of the routes under consideration, see Figure 8, in assessing protecting groups, various synthetic pathways allow removal of the C-terminal octapeptide after complete protection. Whereas the other synthetic pathways involve earlier cleavage of the peptide chain to enable any advantage of slight improvement of physical characteristics (such as solubility in semi aqueous media) to be taken. 1-Diazo-2,2-dimethylpropane was employed to produce, after a rearrangement, the t-amyl esters[137] of the insulin carboxy groups. However the presence of a small proportion of neopentyl esters produced by the reagent prior to the rearrangement may have accounted for the very poor yields of insulin after "deprotection" with trifluoroacetic acid. Use of the benzhydryl protection group[144] was then investigated after a successful preparation of the benzhydryl diazoalkane.[145] The low reactivity of the reagent led to long reaction times (overnight with no pH control) and the excess reagent was readily decomposed at the end of the reaction at pH 4. The protecting group could be easily removed by the action of trifluoroacetic acid at room temperature for 40 minutes and the insulin regenerated was found to give some small crystals under the Schlichtkrull conditions. A scavenger of anisole was thought to be beneficial. Great difficulty was experienced in attempts to digest the fully protected insulin derivative with trypsin as the compound was only marginally soluble in dimethyl-formamide/water mixtures in which the activity of trypsin is severely diminished. Acetyl-arginine benzhydryl ester was adequately cleaved by trypsin so that the steric effect of the ester was ruled out as the cause of failure in digestion. Experiments on the tryptic cleavage of benzoylarginine ethyl ester in hydrazine acetate buffers have shown that up to 40% of the product has a hydrazide group

Figure 8



Schematic of Possible Routes to the Precursor to Semi-Synthetic Insulin

-8Ins	des-octapeptide-insulin	$Ester_5$.	carboxy groups except arginine B-22 fully protected.
Boc_2.	α-amino groups protected with t-butoxycarbonyl.	$Ester_6$.	all carboxy groups fully protected.
Boc_3.	α- and ε-amino groups protected with t-butoxy-carbonyl.		
$BocTF_2$.	im-histidines protected with 2,2,2-trifluoro-N-t-butoxycarbonyl amino ethyl.		

incorporated. Synthetic routes were designed to make use of this reaction but after considerable work it was found that denaturation of the protein in the presence of hydrazine acetate buffers could not be prevented. The use of methyl esters of insulin was met with difficulty in finding conditions for removal of the groups which not also give rise to denaturation of the protein. Only partial success was achieved after considerable work.

Favourable results were achieved in the use of p-methoxyphenyl (anisyl) esters[146] which are susceptible to cleavage under the action of trifluoroacetic acid. The solubility of these esters in dimethylformamide/water mixtures is more favourable than that of the benzhydryl esters and some pilot digestions of the protected insulin with trypsin have met with success. Deprotection of Boc and BocTF protected-anisyl insulin with trifluoroacetic acid and subsequent crystallisation has given good crops of crystals and the use of this ester is thus regarded as the most favourable route at the moment.

Peptide Synthesis

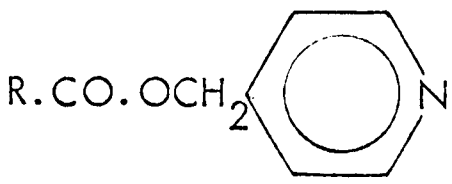
The major strategies of peptide synthesis, stepwise elongation from the C-terminal amino acid or fragment condensation, have been demonstrated with the solid-phase[58] and classical[52-4] syntheses of insulin. The scope and application of these and many other methods have been extensively, critically and constantly reviewed.[147] In view of the success of Young's picolyl ester method[148] in the synthesis of analogues of bradykinin incorporating both the advantages of solid and solution methods, it was decided to approach the synthetic problems using this method of repetitive synthesis.

The Picolyl Ester Method

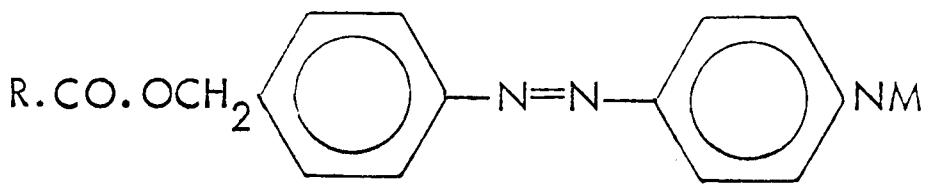
This procedure was introduced by Camble, Garner and Young in 1967[148] and combined important advantages in that peptide formation could proceed in homogeneous solution and then the product could be separated from co-products and excess of reagents by the use of a basic "handle" built into one of the components of the peptide fragment. The advantages over solid-phase synthesis were essentially the mild conditions employed, enabling the progress of the reaction to be followed and establishing its completion; yet because of its homogeneous nature it held the inherent ability of characterisation and possible purification of each intermediate.

The protection of the C-terminal carboxy group is probably the most suitable position for such a "handle" although the imidazole group of histidine has also been used.[149] A basic "handle" is most appropriate for carboxy protection since any components or contaminants would be acidic and simple separation would ensue; any product could be liberated as a free base under mild conditions and transferred into an organic solvent. The use of 4-picolyl esters (1) for carboxy protection was studied and found extremely satisfactory[148,150,151] and recently they have been used for ε-amino protection.[152] Analogous use of p-dimethyl-amino-azobenzyl esters (2) have been examined,[153] the coloured coupled product being absorbed on Sulphoethyl-Sephadex; the tripeptide L-prolyl-L-alanyl-L-phenylalanine was prepared although no further work has been reported. The use of the "handle" principle has been extended to polynucleotide synthesis by Hata.[154] Recently Macrae and Young[155] have used amino acid 4-picolyloxycarbonyl hydrazides (3) in syntheses in which hydrogenolysis gives the hydrazide and this

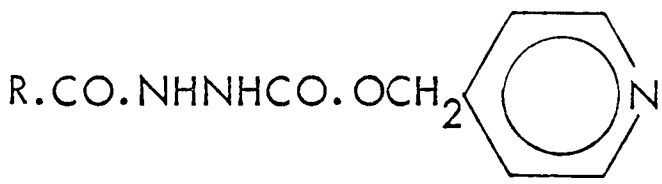
can be coupled via the azide procedure.



(1)



(2)

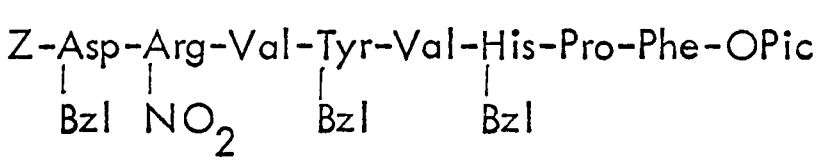


(3)

4-Picolyl esters were originally prepared by reaction of a benzyloxy-carbonylamino acid tetramethylguanidinium salt (or triethylammonium salt) with 4-picolyl chloride but increased yields are obtained by the condensation of benzyloxycarbonylamino or t-butoxycarbonyl amino acids with 4-picolyl alcohol at -5° - 0° with dicyclohexylcarbodi-imide in dichloromethane.[156] In comparison to benzyl esters, they are relatively stable to acid probably because of protonation of the pyridyl nitrogen hinders further proton attack, i.e. after 24 hours in 45% (w/v) hydrogen bromide in acetic acid at room temperature, benzyloxycarbonyl-glycine 4-picolyl ester gave only 4-picolyl ester dihydrobromide, and no glycine could be detected. The benzyloxycarbonyl group can thus be selectively removed in the presence of the 4-picolyl ester and this is the usual procedure for the preparation of amino acid 4-picolyl esters as dihydrobromides. The majority of these are reasonably stable when pure but a small number are hydroscopic. The acylamino acid 4-picolyl esters and amino acid 4-picolyl ester salts prepared so far have been tabulated.[157] A new direct route involving temporary protection of the amino group with ethyl acetoacetate has been suggested by Maclaren.[158]

4-Picolyl esters may be readily cleaved by cold alkali, by hydrogenation in the presence of palladium on charcoal, by sodium in liquid ammonia, and by electrolytic reduction at a mercury cathode under similar mild conditions used for the electrolytic removal of the nitrogroup from nitroarginine and its peptides.[159] In contrast, benzyl esters are not cleaved by electrolytic reduction. The main product of hydrogenolysis of the 4-picolyl residue appears to be 4-methyl-piperidine, although 4-methyl-pyridine has also been detected.[161,162] The action of anhydrous hydrogen fluoride on benzyloxycarbonylglycyl-L-phenylalanine 4-picolyl ester produced only partial cleavage of the 4-picolyl ester group after one hour at room temperature.[162]

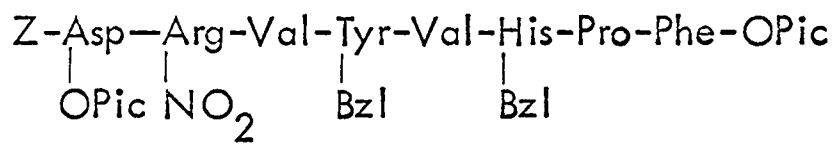
The initial synthesis was of the model peptide used in the initial solid phase development,[58] L-leucyl-L-alanylglycyl-L-valine, and this was reported in 61 and 65% overall yield with regard to the protected peptide.[148,151] The separation of the protected picolyl ester was effected by absorption on Sulphoethyl-Sephadex C-25 (H^+ form) and this required the use of water-miscible solvents. It was found that, as expected, the H^+ form of the ionic resin decomposed t-butoxy-carbonyl groups, but by the saturation of the resin with a very weak base, 3-bromopyridine (pK_a 2.8), which is displaced by the picolyl ester, this was overcome.[150,151] Obviously the acidic phase in which the weakly basic product is separated can be in the form of an acidic solution. In the synthesis of Val^5-angiotensin II (4),[151,163,164] the product of each coupling reaction was extracted



(4)

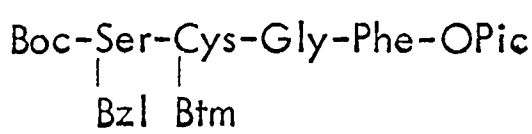
from an organic solvent into aqueous citric acid (avoiding cleavage of t-butoxycarbonyl groups), except in the last stage where the benzyloxycarbonyloctapeptide 4-picolyl ester was insoluble in aqueous citric acid, and it was separated on Sulphoethyl-Sephadex saturated with 3-bromopyridine. The overall yield of the analytically pure protected octapeptide 4-picolyl ester from its C-terminal component was 38%.

As the peptide increases in size the effectiveness of the picolyl group in separation might decrease and the incorporation of additional picolyl groups would be of great advantage. In the synthesis of Val^5-angiotensin II (5), incorporation of the terminal aspartic acid residue as 2,4,5-trichlorophenyl β-4-picolyl benzyloxycarbonyl-L-aspartate gave a derivative soluble in aqueous citric acid.



(5)

The general procedure was then applied to the synthesis of bradykinin and its analogues.[164,165] The disadvantage with this present method is that only aqueous solutions could be used, to swell the Sulphoethyl-Sephadex, and this restricted solvents to only water-miscible ones. After elution, the solvent and base must be evaporated and damage could occur. In the synthesis of the highly lipophilic fragment, t-butoxycarbonyl-O-benzyl-L-seryl-S-benzylthiomethyl-L-cysteinylglycyl-L-phenylalanine 4-picolyl ester (6),[166] partition between ethyl



(6)

acetate and aqueous citric acid was unobtainable, and aqueous potassium hydrogen sulphate was no more effective. The macroreticular sulphonic acid resin Amberlyst-15 was found extremely suitable.[166,167] Similarly with acid-labile protecting groups the resin is first saturated with 3-bromopyridine and this method has been extensively used in recent syntheses of bradykinin.[161,168]

DESCRIPTIVE

The Synthetic Approach

The initial objective was to examine a route to produce a suitable protected insulin C-terminal B-chain octapeptide (7) that would meet the

23 24 25 26 27 28 29 30
H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-OH (7)

requirements defined in the preparation of the protected des-octapeptide-insulin, as outlined in the previous chapter. The intended use of acid-labile protecting groups such as t-butoxycarbonyl and benzhydryl esters for the final protected fragment imposed severe limitations on synthesising the protected octapeptide directly. Thus the synthesis was divided into two parts. The preparation of a fully protected octapeptide by convential strategy and then the production of the required semi-protected peptide containing acid-labile protecting groups by deprotection and reprotection steps.

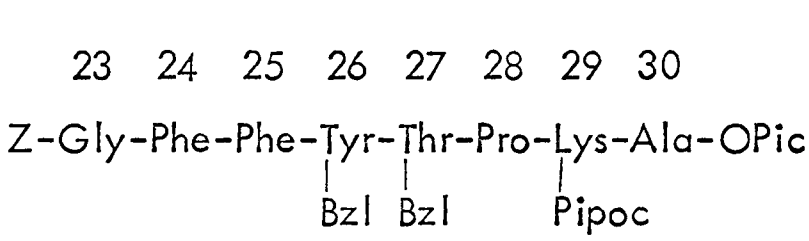
The 4-picolyl ester method was adopted for this synthesis.[148] 4-Picolyl esters are stable to 45% (w/v) hydrogen bromide in acetic acid at room temperature[150] and so may be used in conjunction with the benzyloxycarbonyl group. However, the t-butoxycarbonyl group was thought to be preferable for α-amino protection because of the milder conditions used in its removal, and it was decided to incorporate the majority of the amino acid residues except the ultimate glycine as their t-butoxycarbonyl derivatives.

The piperidino-oxycarbonyl group[170] was used to protect the ε-amino of lysine. It had been previously incorporated as its α-benzyloxycarbonyl derivative but problems associated with the deprotection of this derivative led to the preparation of α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine.

The protection of the alcoholic hydroxyl group of threonine and the phenolic hydroxyl of tyrosine was considered essential to prevent any O-acylation occurring during the coupling reactions. The most successful method of protection, in conjunction with the piperidino-oxycarbonyl and 4-picolyl ester functions, was the use of benzyl ethers which could also be smoothly removed by hydrogenolysis.

At the start of the synthesis the combination of N,N'-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole for coupling reactions[171] had been found extremely advantageous with regard to speed, simplicity of use and above all, free from any racemisation. Thus, the majority of couplings have been performed by this method.

The first goal of the synthesis was the preparation of the protected natural insulin C-terminal B-chain octapeptide (8).



(8)

The protection was designed such that in a series of simple deprotection-reprotection steps it would be possible to produce a semi-protected octapeptide containing only ε-amino and carboxy protection. This peptide, containing only acid-labile groups, could then be coupled to the protected des-octapeptide-insulin bearing similar acid-labile groups. Thus in one mild acidic treatment of the fully prepared protected insulin - two α-amino, one ε-amino, two α-carboxy, four γ-carboxy and two imidazole groups protected - the required completely deprotected insulin would result. A semi-protected peptide of the form (9) where X and Y are acid-labile was required.

H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(X)-Ala-OY (9)

The ε-amino group of lysine could be easily reprotected by t-butoxycarbonyl. The ε-piperidino-oxycarbonyl group can be removed by treatment with sodium dithionite in 50% aqueous acetic acid.[170] The choice of carboxy protection, however, was not so clearly defined and was the most difficult problem encountered in the overall synthesis. The approach to this problem has been outlined in the previous chapter. Initially it was planned to incorporate the t-amyl group[137] for reversible carboxy protection but poor results were obtained. The use of the analogous benzhydryl ester[144] was thought to offer the most advantages and studies have been carried out with this group. Recent studies at the end of the synthesis suggest that p-methoxyphenyl (anisyl) esters have solubility advantages over benzhydryl esters[146] and future work will explore this possibility.

Thus the object of the thesis was extended to product the semi-protected peptide (10) suitable for coupling directly to protected des-octapeptide insulin.

H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Boc)-Ala-$OCHPh_2$ (10)

From the X-ray crystal structure, it is found that there is a two-fold relationship between the molecules in the dimer.[13] This means that the extended C-terminal residues are antiparallel to each other and thus an antiparallel β-pleated sheet structure containing four hydrogen bonds is formed between the monomers. Two of these hydrogen bonds are between the peptides of B24 of one monomer and B26 of the two-fold related monomer; the other two are between their equivalents across the local axis. Figure 9. There are hydrophobic contacts along the local

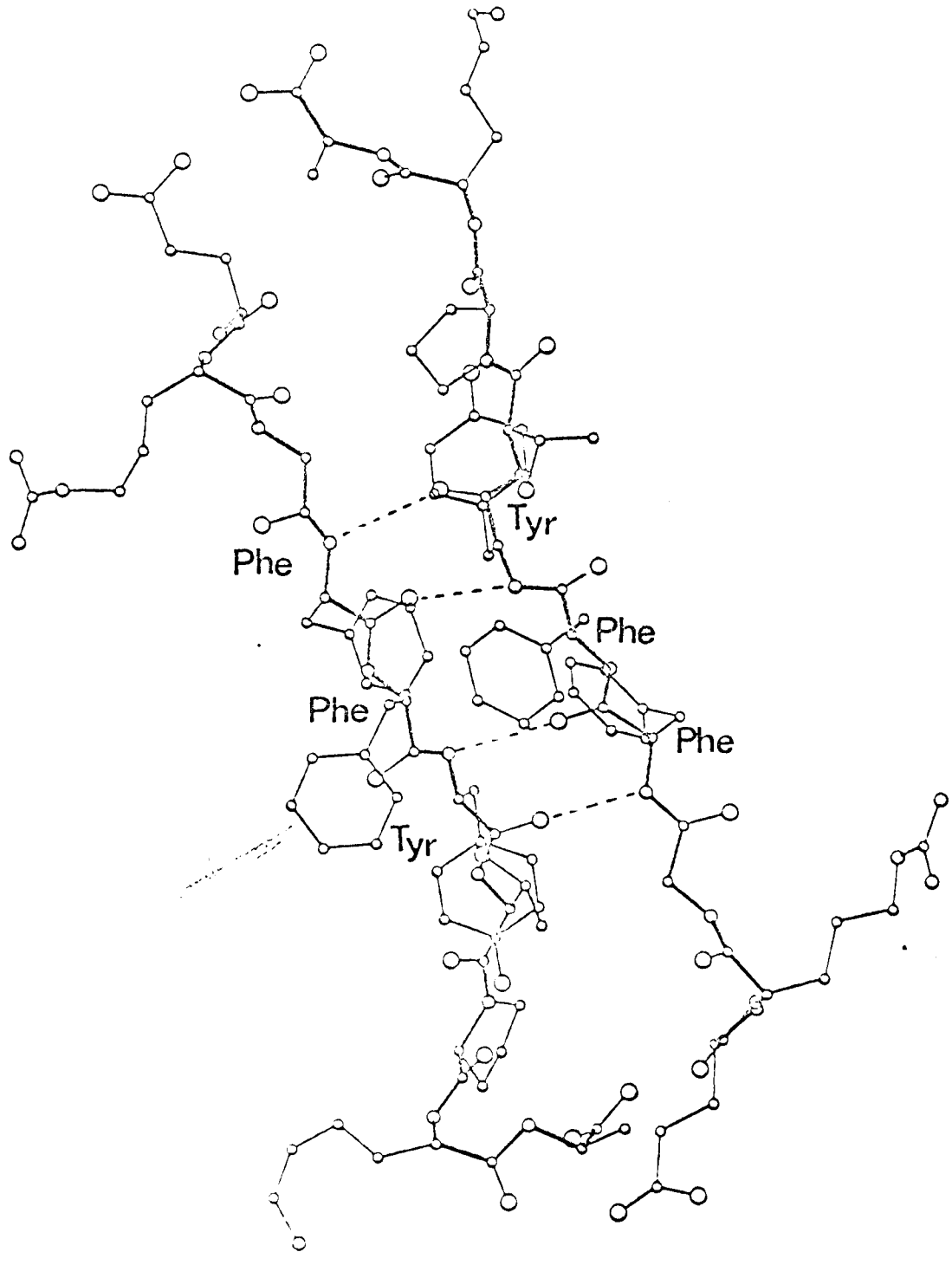


Figure 9

axis involving the equivalent valines-B12, phenylalanines-B24 and -B25. The two phenylalanines-B24 and -B25 are formed together on the surface and partly cover the hydrogen bonds of the β-pleated sheet possibly reinforcing their structural role. Tyrosine-B16 closely approaches tyrosine-B26 and valine-B12. The majority of these residues that are involved in secondary structure contacts, either in α-helix or β-pleated sheet developed between the monomers, belong to the C-terminal B-chain octapeptide. There is considerable evidence for the structure of the dimer in the crystal to be the same in solution and therefore preparation of insulin analogues involving the replacement or modification of phenylalanines-B24 and -B25 would be of great importance in studying the hydrogen bonding system in the dimer and its relationship to the activity of the hormone.

The role of whole side chains, both chemically functional and non-functional, can be examined by their omission. Replacement by glycine is equivalent to elimination of the whole side chain but because its steric properties are so different from those of all other amino acids, the use of alanine, replacing all beyond the β-carbon atom is more satisfactory. This was expected to give a better approximation to the original peptide backbone conformation.

The systematic approaches of Weitzel,[110] in introducing alanine into the A- and B-chains of insulin, and that of the Chinese group[140,141] have already been discussed. It appears as though the aromatic residues, phenylalanine-B24 and -B25 and tyrosine-B26 are essential for activity. It was decided to prepare a series of protected L-alanine octapeptide analogues that could be converted to similarly semi-protected peptides, suitable for direct coupling to protected des-octapeptide-insulin, as the natural peptide (10). The following models were thought suitable.

Z-Gly-Phe-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (11)

Z-Gly-Ala-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (12)

and Z-Gly-Ala-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (13)

These could be prepared in a manner analogous to that of the protected natural octapeptide (8) by the 4-picolyl ester method.

Replacement of one side chain by another may have steric consequences - a change in conformation or conformational freedom of the peptide molecule or an alteration in the topography of the molecular surface - but also results which are chemically functional in the broadest sense. The role of functionality must be extended not only to describe reactivity but encompass charge, hydrogen-bonding capacity and lipophilic or hydrophilic nature.

In an attempt to relate biological results in terms of the hydrogen-bonded nature of the dimer, modifications to the peptide backbone involving substitution on the imino nitrogen were considered. N-Methylation of the amide bonds would reduce the hydrogen-bonding capacity of the monomer and the possibility of dimer formation in the absence of any C-terminal B-chain hydrogen-bonding is uncertain. If dimer formation is impossible then the interesting question is raised as to whether the active species in solution is the monomer or dimer. The replacement of phenylalanines-B24 and -B25 by N-methyl-L-phenylalanine gave rise to the following planned protected octapeptides.

Z-Gly-Phe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (14)

Z-Gly-MePhe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (15)

and Z-Gly-MePhe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (16)

The use of truncated sequences has played an important role in determining sites of critical residues in hormones. This has been applied to insulin in several cases previously reported.[116] In sequences without the multi-functional lysine residue the strategy of the synthesis can be altered and as a consequence the utilisation of the 2-(p-biphenylyl)-isopropyl-oxycarbonyl group with the 4-picolyl ester method was investigated to prepare the following protected tetra- and hexa-peptides.

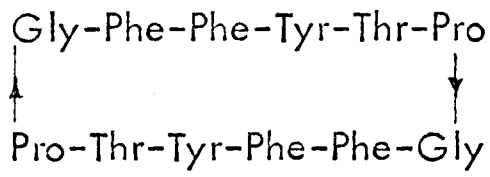
Bpoc-Gly-Phe-Phe-Tyr(Bzl)-OPic (17)

and Bpoc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic (18)

The tetrapeptide (17) contains just those aromatic residues thought to be necessary for activity. While the hexapeptide (18) would serve as a suitable model for the introduction of labelled and fluorescent probes, and heavy atom derivatives by coupling suitable adducts onto the terminal proline residue.

A close examination of the hydrogen-bonded region of the C-terminal B-chain octapeptide in the dimer model (Oxford Enzyme Group) revealed a closely bonded moiety. It was thought possible that a suitable cyclic peptide could be prepared in which the aromatic region was stabilised by intramolecular hydrogen-bonding. The use of prepared protected peptides such as (18) seemed to facilitate

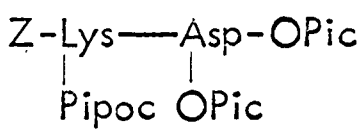
the synthesis of a model such as (19).



(19)

The Preparation of Lysine Derivatives

The piperidino-oxycarbonyl group was first proposed for α- and ε-amino group protection by Stevenson.[170] Two routes for the preparation of α-benzyloxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine were described. α-Benzyloxycarbonyl-L-lysine was reacted with either piperidino succinimido carbonate or piperidino 2,4,5-trichlorophenyl carbonate; the former method was preferred because it gave the easily separable, water soluble co-product, N-hydroxysuccinimide, whereas the 2,4,5-trichlorophenol derived from the latter method proved difficult to remove from the desired product. Nash experienced difficulty in following the proposed method and was unable to prepare the unstable succinimido chloroformate in good yield.[173] However, following the eventual preparation of α-benzyloxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine, from piperidino 2,4,5-trichlorophenyl carbonate, the dipeptide (20) was prepared. Subsequent removal



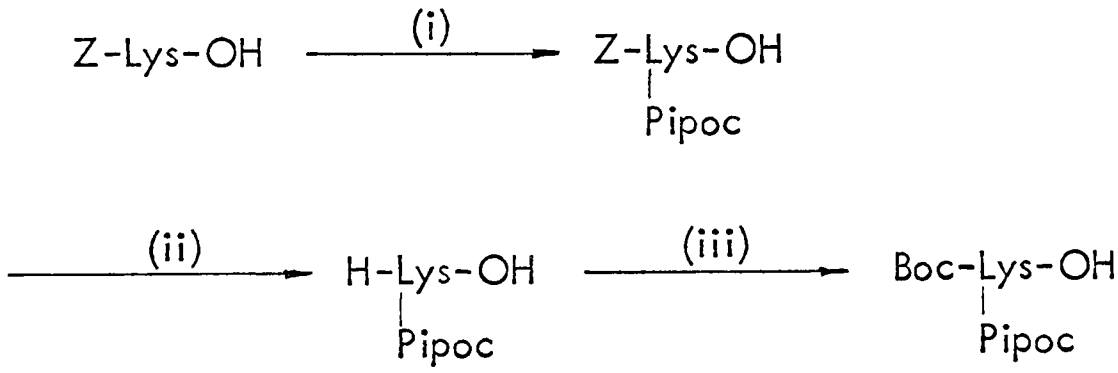
(20)

of the benzyloxycarbonyl group, by treatment with hydrogen bromide in acetic acid, and coupling of the liberated amino component to α-t-butoxycarbonylglycine with N,N'-dicyclohexylcarbodi-imide followed by citric acid isolation, gave rise to a very impure tripeptide which could only be purified by preparative-layer chromato-

graphy. The impurities appeared to arise at the deprotection step.

It was thought necessary therefore to prepare α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine and use this derivative in the projected synthesis in view of the milder acidic conditions employed for the removal of the t-butoxycarbonyl group.

Nash had proposed a route, Figure 10, utilising piperidino 2,4,5-trichlorophenol carbonate and containing the least number of untried stages.[173] This was the situation at the beginning of the present work.



(i) Piperidino-2,4,5-trichlorophenyl carbonate/1,1,3,3-tetramethylguanidine, (ii) HBr/HAc, (iii) Boc azide.

Figure 10

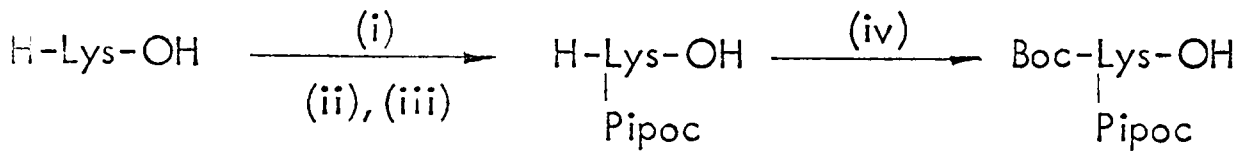
However, the author found that it was not possible to obtain satisfactory yields in all the reaction steps and indeed Nash had experienced isolation problems. α-Benzyloxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine was prepared by reaction of α-benzyloxycarbonyl-L-lysine with piperidino 2,4,5-trichlorophenyl carbonate in the presence of 1,1,3,3-tetramethylguanidine as base, to give a chromatographically pure product. However, using the standard conditions for the removal of the benzyloxycarbonyl group, namely treatment with 45% (w/v) hydrogen bromide in acetic

acid for one hour at room temperature, a minor contaminant thought to be L-lysine was indicated by thin layer chromatography. This did not initially appear to have arisen from the starting material, suggesting that there was some loss of the piperidino-oxycarbonyl group during the acid treatment. This might have explained the impurities found on treating the dipeptide (20) with hydrogen bromide in acetic acid. Nash purified his material by virtue of the solubility differences between ε-piperidino-oxycarbonyl-L-lysine and L-lysine. The impure material being dissolved in a small volume of water, taken to pH 7.0 with sodium hydroxide (4.0M), the white precipitate formed being washed with ethyl acetate and reacted for twenty four hours at pH 9.5 with t-butoxycarbonyl azide. α-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine was obtained as an analytically pure foam and isolated as its cyclohexylammonium salt in 51% yield.

A close examination of the reaction pathway revealed that in the preparation of α-benzyloxycarbonyl-L-lysine by Bevas and Zervas,[174] reaction of benzyl chloroformate with L-lysine via the ε-benzylidene intermediate, a minor amount of L-lysine and ε-benzyloxycarbonyl-L-lysine was detected. Recrystallisation could not remove these impurities and it was thought that the benzylidene derivative was too acid labile. These observations were also reported by Benoiton[175] who claimed that the impurities could be removed by passage of the reaction solution down a lithium hydroxide (0.1M) buffered cupric carbonate-alumina column. However, only low yields were obtained and the product was heavily contaminated with salts.

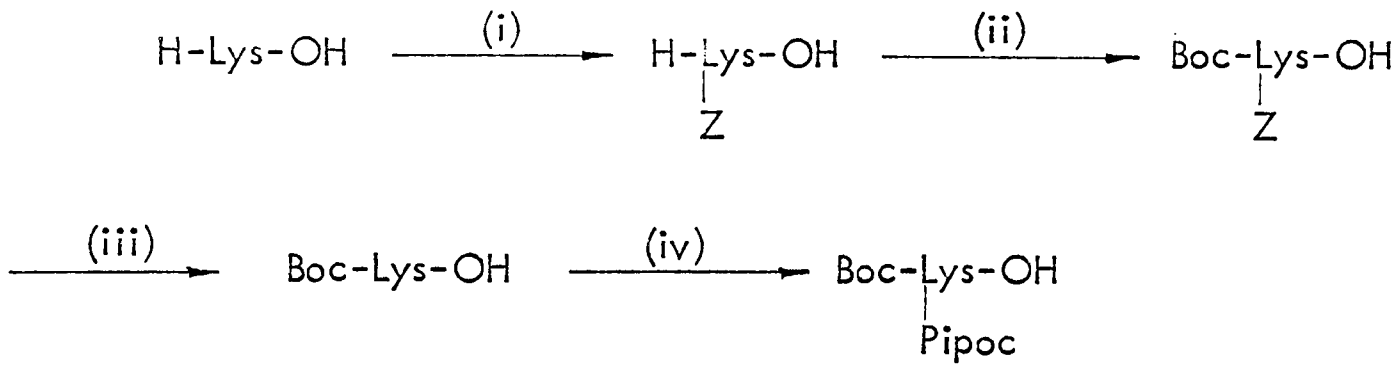
In view of these difficulties two other possible routes were considered.

Figures 11 and 12.



(i) $CuCO_3$, $Cu(OH)_2$, (ii) Piperidino 2,4,5-trichlorophenyl carbonate/1,1,3,3-tetramethylguanidine, (iii) H_2S, (iv) Boc azide.

Figure 11



(i) $CuCO_3$, $Cu(OH)_2$, Z-Cl, EDTA/H_2S, (ii) Boc azide, (iii) H_2/Pd, (iv) Piperidino 2,4,5-trichlorophenyl carbonate/1,1,3,3-tetramethylguanidine.

Figure 12

The route in figure 11 appeared to be straightforward but discouraging results were obtained when trying this method. Possible impurities were L-lysine and ε-piperidino-oxycarbonyl-L-lysine with the resulting purification difficulties. This problem was also found in the analogous preparation of α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-ornithine.[156] The route shown in Figure 12 did seem encouraging and preliminary studies confirmed this.

ε-Benzyloxycarbonyl-L-lysine was prepared via its copper complex.[176] Benzyl chloroformate was reacted with the lysine-copper complex and the cupric ion

was removed with repeated extraction with ethylenediamine tetra-acetic acid disodium salt followed by hydrogen sulphide. The product was recrystallised from 30% aqueous acetic acid to give ε-benzyloxycarbonyl-L-lysine in 55% yield and m.p. 246-8° [lit.[176] m.p. ca. 235°].

ε-Benzyloxycarbonyl-L-lysine was treated with t-butoxycarbonyl azide following the procedure of Schnabel[177] to give α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine as a chromatographically pure oil with correct n.m.r. spectrum in 91% yield. Schnabel reported a 62% yield.

Hydrogenation of the above material in 80% aqueous ethanol with 5% palladium on charcoal gave α-t-butoxycarbonyl-L-lysine in 83.2% yield as a chromatographically pure white solid of m.p. 205-7°, $[\alpha]_D^{21}$ -9.24° (c 1.13 in HAc) [lit.[178] m.p. 204-5°, $[\alpha]_D^{21}$ -10.7° (c 0.85 in HAc)].

2,4,5-Trichlorophenol was converted to its chloroformate by the method suggested by Morley[179] in 96% yield with m.p. 61.5-63.5°. Morley obtained material of m.p. 62-3° in 60% yield after distillation. The chloroformate was directly converted into piperidino 2,4,5-trichlorophenyl carbonate by an adaptation of the method of Stevenson[170] in 93% yield with m.p. 94-7°. Stevenson obtained a 60% yield after recrystallisation from ethyl acetate-petroleum ether with m.p. 97-8°.

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine was prepared by reaction of α-t-butoxycarbonyl-L-lysine with piperidino 2,4,5-trichlorophenyl carbonate in the presence of 1,1,3,3-tetramethylguanidine as base. The product was isolated as its dicyclohexylammonium salt and was recrystallised from propan-2-ol in 68% yield with m.p. 135-6° and satisfactory n.m.r. spectrum and elemental analysis. $[\alpha]_D^{20}$ +8.3° (c 1.07 in MeOH).

The Protected Natural Octapeptide

The synthetic route of the protected natural C-terminal B-chain octapeptide is summarised in Figure 13.

Initially the 4-picolyl derivative of the C-terminal amino acid, namely L-alanine 4-picolyl ester dihydrobromide, was prepared by the method of Gamer.[150] α-Benzyloxycarbonyl-L-alanine 4-picolyl ester, previously prepared by the reaction of α-benzyloxycarbonyl-L-alanine and 4-pyridylmethanol at 0° with N,N'-dicyclohexylcarbodi-imide in dichloromethane,[150] was treated with 45% (w/v) hydrogen bromide in acetic acid for one hour at room temperature. The product was precipitated with diethyl ether, repeatedly washed and recrystallised from aqueous acetone to give L-alanine 4-picolyl ester dihydrobromide in 96.5% yield as white crystals of m.p. 165-7°, $[\alpha]_D^{20}$ +2.03° (c 1 in DMF), chromatographically pure with satisfactory elemental analysis and n.m.r. spectrum.

The dipeptide α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (21) was prepared by coupling L-alanine 4-picolyl ester dihydrobromide and α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine. The N,N'-dicyclohexylcarbodi-imide—1-hydroxybenzotriazole method of König and Geiger[171] was used; in fact this method was adopted for all coupling reactions in the protected octapeptide synthesis. Two approaches are suggested: in the first all the components required to generate the activated carboxy component are mixed with the amino component in one flask - the 'eintopf' procedure; in the second method the acylating components, i.e. N,N'-dicyclohexylcarbodi-imide, 1-hydroxybenzotriazole and the acylating amino acid are stirred in the reaction solvent and the resulting mixture added to the amino component after activation. This second

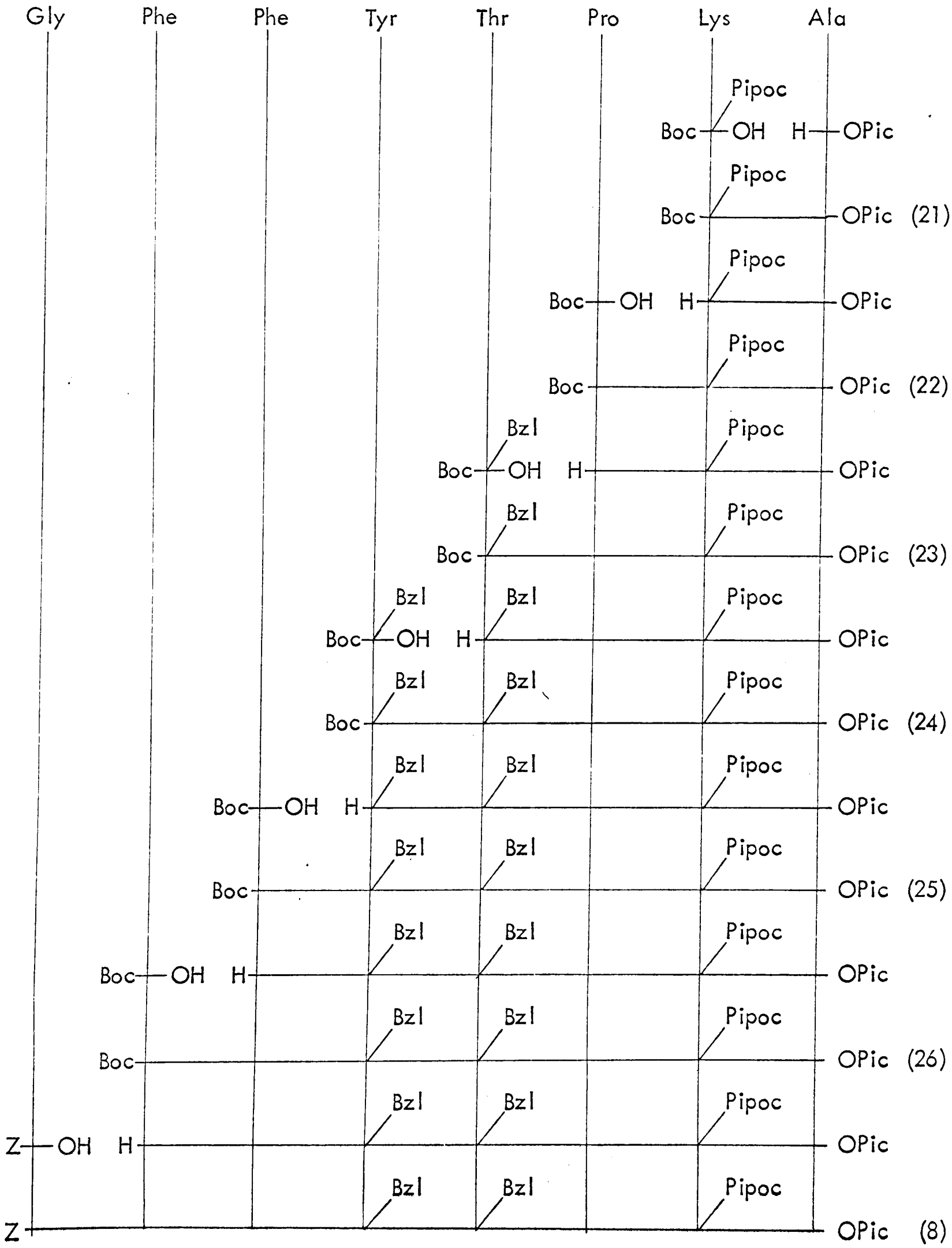

Gly
Phe
Phe
Tyr
Thr
Pro
Lys
Ala
Pipoc
Boc OH H OPic
Pipoc
Boc OPic (21)
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Figure 13

approach was adopted; the pre-activation period was usually thirty minutes to one hour at 0° and a similar period at room temperature. The recommended solvents for this coupling method were tetrahydrofuran and dimethylformamide, in which 1-hydroxybenzotriazole was soluble. The former was preferred being more volatile and it did not interfere with thin layer chromatography as dimethylformamide tended to do.

L-Alanine 4-picolyl ester was liberated from its dihydrobromide salt by dissolving the latter in tetrahydrofuran at 0° with the addition of N,N-di-isopropyl-ethylamine. The acylating mixture (1.2 equivalents) was prepared by stirring α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine, N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole for one hour at 0° and another hour at room temperature and was immediately added to the amino component.

In the preparation of di- and tripeptides the prolonged use of a base such as triethylamine may lead to racemisation and if dioxopiperazine formation is an important side reaction then the use of a sterically hindered amine, N,N-di-iso-propylethylamine was thought advantageous.[172] Triethylamine has been used for latter coupling reactions.

Thin layer chromatography indicated that the reaction was complete after three hours stirring. The peptide was isolated by extraction into citric acid (0.7M) from ethyl acetate-diethyl ether (1:1) followed by neutralisation of the aqueous fraction and re-extraction of the peptide into ethyl acetate. Evaporation gave the protected dipeptide (21) in 95.4% yield as a chromatographically and analytically pure white foam with satisfactory amino acid analysis and n.m.r. spectrum.

The experimental results and physical constants for this and all the protected

natural peptides up to the octapeptide are given for comparison in Figure 14 and amino acid analyses in Figure 15 respectively. A complete general description of the deprotection, coupling and isolation procedures is given at the beginning of the experimental section.

Compound \ Amino acid	Ala	Lys	Pro	Thr	Tyr	Phe	Gly
21	1.00	1.00					
22	1.00	1.03	1.02				
23	1.00	1.04	0.99	0.99			
24	1.00	1.04	1.00	0.99	0.99		
25	1.00	0.98	1.00	0.97	0.95	1.02	
26	1.00	1.03	0.99	0.92	0.94	2.09	
8	1.00	1.01	0.99	0.90	0.96	1.95	1.01

Amino acid analyses of the natural octapeptide intermediates

Figure 15

The conditions for the coupling of α-t-butoxycarbonyl-L-proline and ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester and the isolation of the subsequent tripeptide were very similar to those of the dipeptide which has just been described. The preparation of ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester from the protected dipeptide (21) was affected by excess trifluoroacetic acid at 0° for thirty minutes.

Synthesis of the protected natural octapeptide

Compound	Acylating[a] agent	Yield (%)	Overall yield (%)	$[\alpha]_D^{20}$ (°)[b]	R_F (t.l.c.)	Found (%) C	H	N	Formula	Required (%) C	H	N
21	1.2	95.4[c]		-16.2 (c 1.07)	E4, 0.59; G1, 0.92; G3, 0.93; A2, 0.47	58.31	7.81	12.97	$C_{26}H_{41}N_5O_7$	58.30	7.72	13.08
22	1.45	94.5[c]	90.0	-56.5 (c 0.94)	E4, 0.43; G1, 0.78; G3, 0.72; A2, 0.55	58.05	7.86	12.87	$C_{31}H_{48}N_6O_8$	57.76	7.58	12.85
23	1.2	90.6[c]	81.5	-41.8 (c 1.19)	E4, 0.44; G1, 0.88; G3, 0.76; A2, 0.54	61.42	7.66	11.65	$C_{42}H_{61}N_7O_{10}$	61.20	7.40	11.90
24	1.2	96.4[c]	78.6	-30.7 (c 1.19)	E4, 0.47; G1, 0.95; G3, 0.71; A2, 0.65	64.38	7.20	10.20	$C_{58}H_{76}N_8O_{12}$	64.66	7.11	10.40
25	1.2	90.0[d]	70.6	-32.4 (c 1.05)	E4, 0.61; G1, 0.97; G3, 0.90; A2, 0.69	64.71	6.97	10.16	$C_{67}H_{85}N_9O_{13}.H_2O$	64.75	7.06	10.12
26	1.2	88.1[d]	62.2	-38.6 (c 1.07)	E4, 0.66; G1, 0.87; G3, 0.82; A2, 0.67	65.77	7.12	10.28	$C_{76}H_{94}N_{10}O_{14}.H_2O$	65.60	6.92	10.01
8	1.2	90.0[d]	56.0	-40.3 (c 0.67)	E4, 0.67; G3, 0.97; G4, 0.90; A2, 0.65	64.81	6.57	10.56	$C_{81}H_{95}N_{11}O_{15}$	64.80	6.60	10.28

[a] Molar proportions of the carboxy-component, N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole, relative to the amino-component, used in the last coupling step of the stated compound. [b] Measured in chloroform. [c] Isolated by the citric acid procedure. [d] Isolated by Amberlyst 15 (3 bromopyridinium form).

Figure 14

In preliminary experiments it was found that the ε-piperidino-oxycarbonyl group was slightly heat-sensitive and small amounts of deprotected material had been observed when peptides containing this group had been allowed to reach 30-35° in solution.[173] Therefore, in deprotection steps with trifluoroacetic acid where localised heating could occur, the reaction was carried out at 0° in an ice-bath. Similarly on Amberlyst 15 isolation, the heat evolved on elution of the resin was sometimes sufficient to remove this group and hence elution was carried out at 0-4°.

The fully protected tripeptide, α-t-butoxycarbonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (22) was isolated in 94.5% yield as a chromatographically and analytically pure white foam by the citric acid procedure with satisfactory amino acid analysis and n.m.r. spectrum.

A similar procedure afforded the fully protected tetrapeptide (23) in 90.6% yield as a chromatographically and analytically pure white foam by the citric acid procedure. Satisfactory amino acid analysis and n.m.r. spectrum were obtained.

The citric acid isolation method had been used throughout the synthesis to this point and although the protected pentapeptide (24) was obtained in 96.4% yield as a chromatographically and analytically pure white powder with satisfactory n.m.r. spectrum, the volume of citric acid (0.7M) used for extraction had increased dramatically and it was only after repeated extraction that the peptide was successfully isolated. The cause could be easily seen from the increasing hydrophobic character of the protected peptide.

The alternative method of isolation by the picolyl ester method, which employs the macroreticular resin - Amberlyst 15, was therefore adopted for subsequent

syntheses. A full review of the method is described in the experimental section. However, the important steps were the removal of 1-hydroxybenzotriazole and triethylammonium trifluoroacetate by aqueous extraction, as salts tended to reduce the capacity of the column, the absorption of the protected peptide onto the Amberlyst 15 resin and the removal of non-basic contaminants by washing the column with ethyl acetate (the usual solvent for application of the peptide onto the resin). The acid form of the Amberlyst 15 resin was used but since it was sufficiently acidic to remove α-t-butoxycarbonyl groups, the column was buffered with 3-bromopyridine. The peptide (pK_a 4.5) displaces the 3-bromopyridine (pK_a 2.8) from the resin and is itself later displaced by pyridine (pK_a 5.2). Triethylamine (pK_a 11.0) had been used in earlier syntheses but was found unselective and traces of peptides with free α-amino groups had been eluted[173] whereas pyridine in dimethylformamide was found to be more selective and gave purer products.

The protected hexapeptide (25) was isolated by Amberlyst 15 (3-bromopyridinium form) in 90% yield as a chromatographically and analytically pure white powder with satisfactory amino acid analysis.

In the isolation of the protected heptapeptide (26), the removal of dicyclohexylurea was complicated by the formation of a non-filterable gel which would not solubilise. The gel was evaporated to dryness and dissolved in dimethylformamide. Addition of an equal volume of ethyl acetate at 0° precipitated the dicyclohexylurea. The isolation procedure was continued however applying the peptide to the resin in dichloromethane. The protected heptapeptide (26) was isolated in 88.1% yield as a chromatographically and analytically pure white powder

with satisfactory amino acid analysis.

The increasing tendency in the hydrophobic character of the protected peptide was shown in the last coupling where chloroform was used for application to the Amberlyst 15 (3-bromopyridinium form). Isolation gave the protected octapeptide (8) in 90% yield as a chromatographically and analytically pure white powder with satisfactory amino acid analysis.

The success of the picolyl ester method is clearly demonstrated in Figure 14 which compares the yields of all protected intermediates and gives the overall yield of the protected octapeptide (8) as 56.0% from the 4-picolyl ester dihydrobromide.

The Synthesis of Protected L-Alanine Analogues

The replacement of phenylalanines -B24 and -B25 by L-alanine involved no change in peptide methodology and the synthesis of the protected octapeptide analogues (11-13) was facilitated by the availability of intermediates from the previous synthesis. All couplings were carried out by the N,N'-dicyclohexyl-carbodi-imide/1-hydroxybenzotriazole method in tetrahydrofuran and all the protected peptides were isolated by the Amberlyst 15 (3-bromopyridinium form) procedure.

(1) Synthesis of Z-Gly-Phe-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (11)

The synthetic route is summarised in Figure 16 and the physical characteristics and amino acid analyses of each intermediate are given in Figures 17 and 18 respectively.

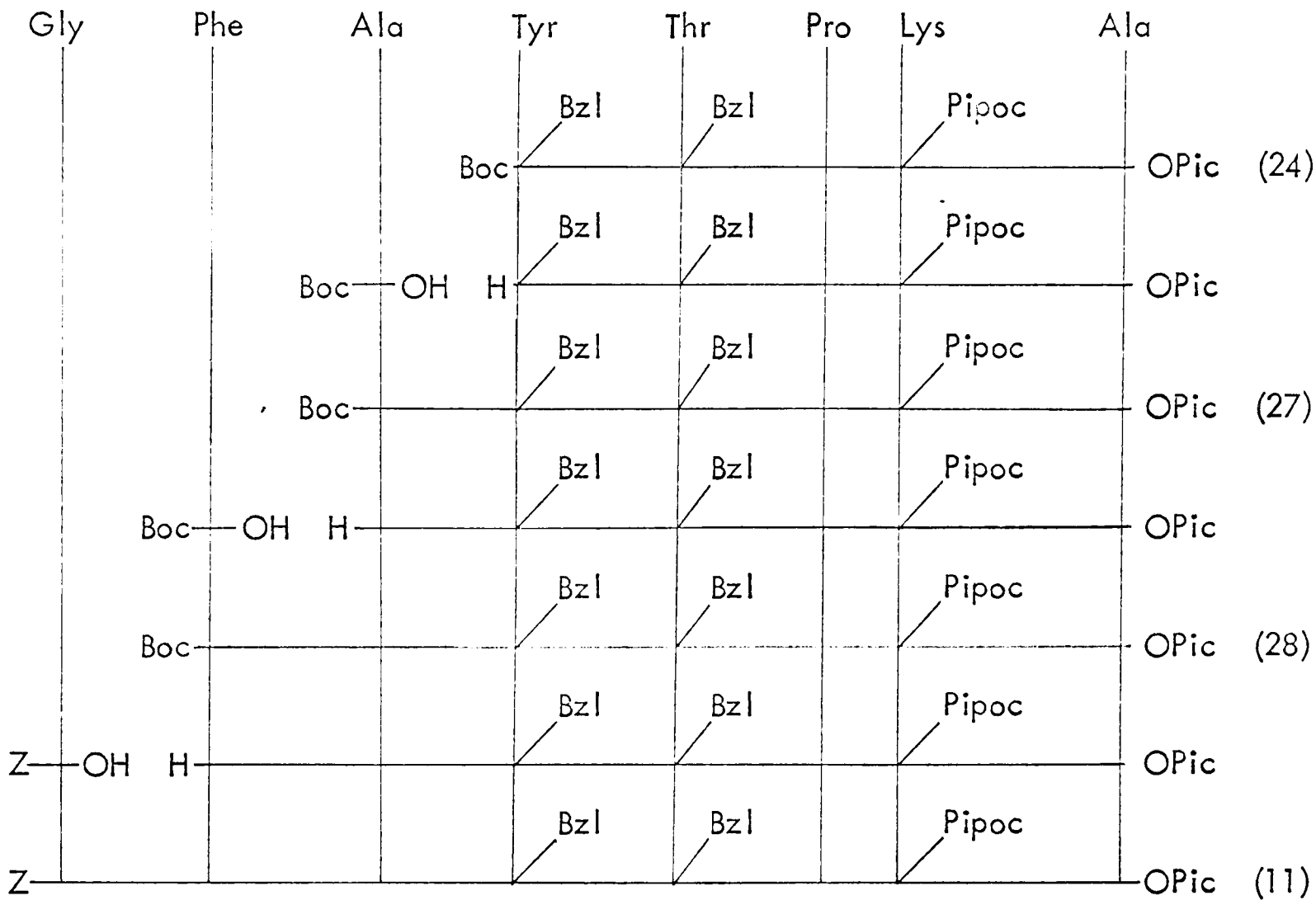


Figure 16

The protected hexapeptide (27) was prepared from the previous intermediate (24) in 87.1% yield as a chromatographically and analytically pure white powder with satisfactory amino acid analysis.

Similar procedures as that of the natural protected octapeptide gave the protected hepta- (28) and octapeptides (11) in 83.5 and 81.3% yields respectively as chromatographically and analytically pure white powders with satisfactory amino acid analyses. The overall yield of the protected octapeptide (11) was 47.6% from the 4-picolyl ester dihydrobromide.

Synthesis of protected L-alanine analogues

Compound	Acylating agent[a]	Yield[c] (%)	Overall yield (%)	$[\alpha]_D^{20}$ (°)[b]	R_F (t.l.c.)	Found (%) C	H	N	Formula	Required (%) C	H	N
27	1.2	87.1	68.5	-36.7 (c 1.02)	E4, 0.66; G3, 0.87; A2, 0.55; N, 0.76	63.51	7.36	10.81	$C_{61}H_{81}N_9O_{13}$	63.80	7.12	10.95
28	1.2	83.5	57.2	-28.3 (c 1.03)	E4, 0.60; G3, 0.77; A2, 0.51; N, 0.92	64.23	7.11	10.82	$C_{70}H_{90}N_{10}O_{14}$	64.40	6.90	10.71
11	1.2	81.3	47.6	-29.1 (c 1.03)	E4, 0.46; G3, 0.87; A2, 0.65; N, 0.90	63.77	6.45	11.18	$C_{75}H_{91}N_{11}O_{15}\cdot H_2O$	63.68	6.63	10.89
29	1.2	84.0	59.4	-46.2 (c 0.95)	E4, 0.65; G3, 0.88; G4, 0.82; A2, 0.53	64.30	7.10	10.71	$C_{70}H_{90}N_{10}O_{14}$	64.40	6.90	10.71
12	1.2	84.1	49.8	-38.5 (c 0.85)	E4, 0.68; G3, 0.86; G4, 0.88; A2, 0.55	63.92	6.64	11.20	$C_{75}H_{91}N_{11}O_{15}\cdot H_2O$	63.68	6.63	10.89
30	1.2	81.2	55.6	-41.3 (c 0.96)	E4, 0.60; G3, 0.74; A2, 0.48; N, 0.90	63.46	7.10	11.26	$C_{64}H_{36}N_{10}O_{14}$	63.03	7.11	11.49
13	1.19	91.8	51.0	-29.3 (c 1.05)	E4, 0.48; G3, 0.66; A2, 0.46; N, 0.85	61.55	6.68	11.61	$C_{69}H_{87}N_{11}O_{15}\cdot 2H_2O$	61.54	6.81	11.44

[a] Molar proportion of the carboxy-component, N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole relative to the amino-component, used in the last step of the stated compound. [b] Measured in chloroform. [c] Isolated by Amberlyst 15 (3-bromopyridinium form).

Figure 17

Compound \ Amino acid	Ala	Lys	Pro	Thr	Tyr	Phe	Gly
27	2.00	1.02	1.01	0.90	0.96		
28	2.00	1.02	0.99	0.92	0.98	1.03	
11	2.00	1.03	0.99	0.93	0.87	1.02	0.99
29	2.00	1.02	0.97	0.97	0.91	1.00	
12	2.00	1.03	0.99	0.93	0.87	1.02	1.00
30	2.98	1.00	1.00	0.98	0.98		
13	2.89	1.00	1.00	0.98	0.98		0.98

Amino acid analyses of protected L-alanine intermediates

Figure 18

(2) Synthesis of Z-Gly-Ala-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (12)

The synthetic route is summarised in Figure 19 and the physical characteristics and amino acid analyses of each intermediate are given in Figures 17 and 18 respectively.

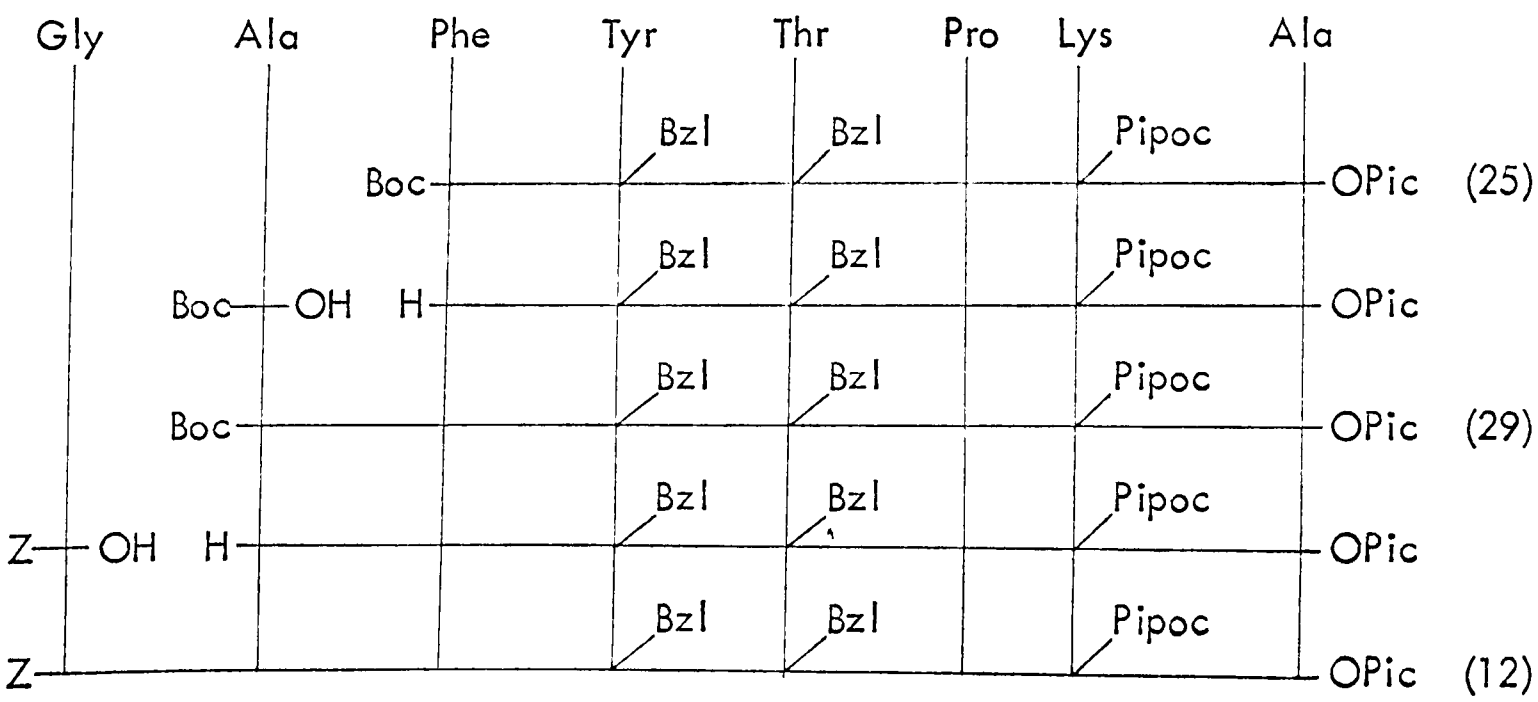


Figure 19

The protected heptapeptide (29) was prepared from the previous intermediate (25) in 84% yield and from that the protected octapeptide (12) in 84.1% yield as chromatographically and analytically pure white powders with satisfactory amino acid analyses.

The overall yield of the protected octapeptide (12) was 49.8% from the 4-picolyl ester dihydrobromide.

(3) Synthesis of Z-Gly-Ala-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (13)

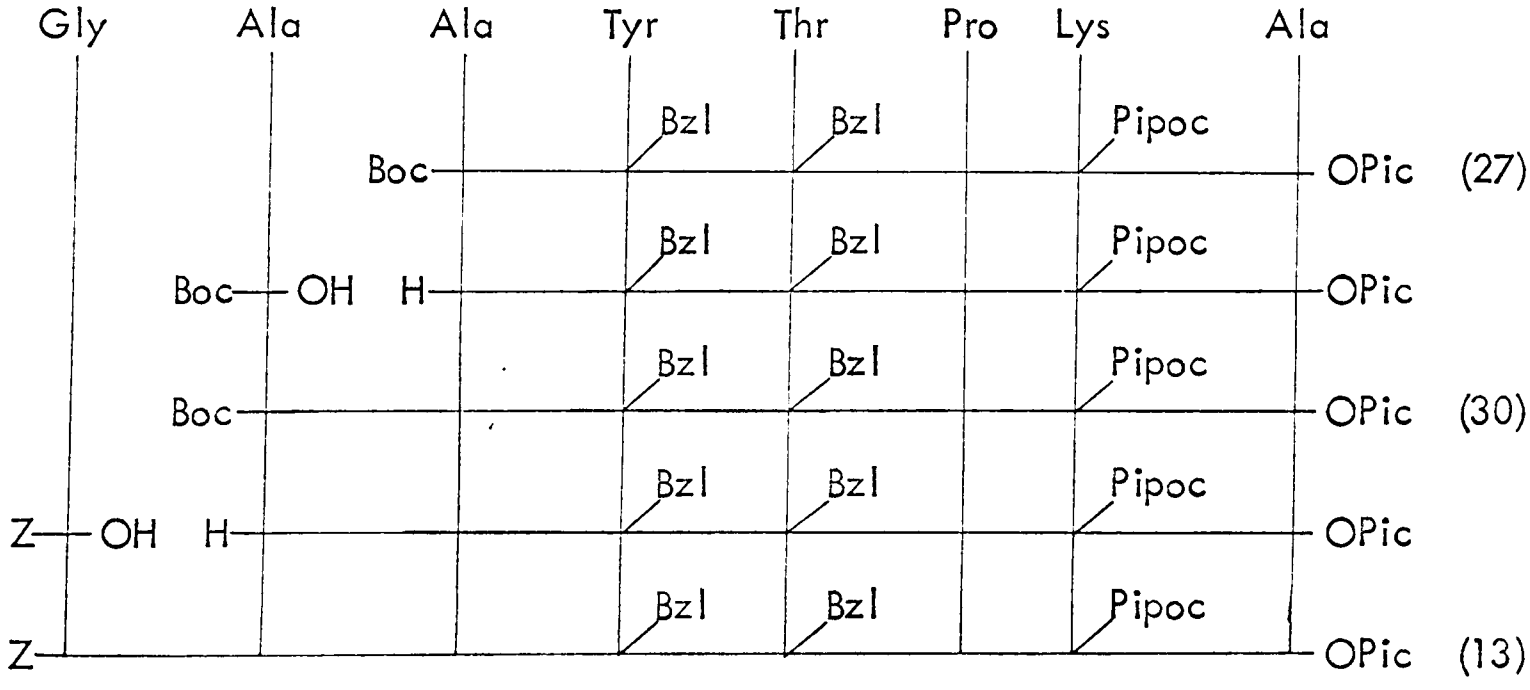


Figure 20

Similarly the protected heptapeptide (30) was prepared from the previous intermediate (27) in 81.2% yield and from that the protected octapeptide (13) in 91.8% yield as chromatographically and analytically pure white powders with

satisfactory amino acid analyses.

The overall yield of the protected octapeptide (13) was 51% from the 4-picolyl ester dihydrobromide.

The Synthesis of N-Methyl-L-phenylalanine Analogues

N-Methyl-amino acids are found in an important group of peptide and depsipeptide antibiotics.[180] N-Methyl-L-isoleucine was found in Enniatin A and N-methyl-L-valine in Enniatin B and actinomycins, similarly N-methyl-L-phenylalanine and p-dimethylamino-N-methyl-L-phenylalanine were found in Staphylomycin. Peptide analogues containing these derivatives have been synthesised and created considerable interest.[181]

It is only recently that detailed investigations have been made into racemisation during acidic deprotection, saponification and peptide bond formation of N-methyl-amino acids, mainly due to Benoiton and co-workers.[182] Optically pure protected N-methyl-amino acids can be prepared by two different methods. Methylation with either sodium hydride and methyl iodide[183] or silver oxide and methyl iodide[184] of N-protected amino acids produced imino nitrogen and carboxy group methylation. Saponification of the N-methyl-N-protected amino acid methyl esters gave the corresponding N-methyl-N-protected amino acids or treatment of the corresponding benzyloxycarbonyl derivative with hydrogen bromide in acetic acid gave the N-methyl-amino acid methyl ester hydrobromide. All derivatives were claimed to be optically pure and free from any unmethylated amino acids. A different approach was suggested by Quitt, Hellerbach and Vogler.[185] N-Benzyl-amino acids were methylated with formic acid and formaldehyde and hydrogenolysis of the product gave optically pure N-methyl amino acids.

The latter procedure was used in this synthesis to prepare N-methyl-L-phenylalanine derivatives. N-Benzyl-L-phenylalanine was prepared by sodium borohydride reduction of the Schiffs base formed between benzaldehyde and L-phenylalanine in 96% yield with m.p. 254-5° [lit.[185] 90% yield, m.p. 255°]. Methylation with formic acid-formaldehyde gave N-benzyl-N-methyl-L-phenylalanine in 96% yield with m.p. 222-3° [lit.[185] yield 94%, m.p. 220-2°]. The optimum time of reaction was found to be two hours. Hydrogenolysis in 90% aqueous acetic acid containing one equivalent of perchloric acid with 5% palladium on charcoal gave N-methyl-L-phenylalanine in 70% yield with m.p. 259-61° [lit.[185] yield 75%, m.p. 260°].

An adaptation of the method of Li and Blake[186] was used to prepare α-t-butoxycarbonyl-N-methyl-L-phenylalanine dicyclohexylammonium salt in 90.1% yield with m.p. 167-70° [lit.[186] yield 52%, m.p. 165-70°].

It had been generally assumed that racemisation would be a far smaller problem than in ordinary peptides, because of the inability of the N-acyl-N-methyl-amino acid derivatives to form oxazolones, and because of their structural similarity to proline derivatives, whose resistance to racemisation is well known. Several recent observations have shown that these assumptions may be incorrect. N-Methyl-L-phenylalanine and L-proline can be racemised by acetic anhydride in acetic acid[187] and partial racemisation of a proline residue during coupling has been reported.[188] Benoiton presents evidence that N-methyl-amino acids are able to racemize to a similar and occasionally greater degree than their primary amino acid counterparts.[182] The extent of racemisation observed in coupling N-protected-L-alanyl-N-methyl-L-leucine with glycine benzyl ester is minimal in the case of the

N-hydroxysuccinimide ester, with N,N'-dicyclohexylcarbodi-imide and N-hydroxy-succinimide, and using N-ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline (in tetrahydrofuran in the absence of salts). However, in most of these coupling reactions a pronounced salt effect was observed. Evidence has now been presented to indicate that the mechanism for racemisation is via a 5-oxo-oxazolium derivative.[182]

There have been no such investigations with N-methyl-L-phenylalanine and as it was hoped to incorporate this residue into protected analogues (14), (15) and (16) it was decided to prepare the model dipeptide (31). α-t-Butoxycarbonyl-

Boc-MePhe-MePhe-OMe (31)

N-methyl-L-phenylalanine was to be coupled with N-methyl-L-phenylalanine methyl ester hydrochloride by various procedures, the product hydrolysed and examined by optical rotation.

N-Methyl-L-phenylalanine was treated with thionyl chloride in absolute methanol to give N-methyl-L-phenylalanine methyl ester hydrochloride in 76% yield and m.p. 85-7° as white crystals with satisfactory elemental analysis and n.m.r. spectrum.

The formation of the dipeptide (31) was not as straightforward as initially envisaged and its sterically hindered nature could be compared to the difficulties experienced in proline to proline couplings. The use of N,N'-dicyclohexylcarbodi-imide alone was completely unsuccessful. A considerable amount of starting material was still present after three days reaction at room temperature and there was no indication of possible dipeptide formation. The use of N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole produced a product thought to be the dipeptide.

However, isolation of the neutral material revealed that the material was N-acyl urea and its structure was confirmed by n.m.r. spectrum and corresponding elemental analysis. An 'eintopf' procedure merely gave the same N-acyl urea. Surprisingly the replacement of 1-hydroxybenzotriazole by N-hydroxysuccinimide did not produce even the urea. Large amounts of starting material were present after several days reaction at room temperature with no new products formed.

However, the addition of the mixed anhydride formed between α-t-butoxy-carbonyl-N-methyl-L-phenylalanine and pivaloyl chloride at -20° to N-methyl-L-phenylalanine methyl ester hydrochloride using N-methylmorpholine as base gave the desired product. The isolated oil had a satisfactory n.m.r. spectrum and elemental analysis and was further characterised by the formation of its hydrazide (32). The dipeptide (31) was hydrolysed in hydrochloric acid in the usual manner and was compared to a similarly treated sample of N-methyl-L-phenylalanine. The optical rotations of the hydrolysed samples were similar. Thus, the incorporation of N-methyl-L-phenylalanine into peptide analogues would be best achieved by the mixed anhydride method.

The replacement of phenylalanines -B24 and -B25 by N-methyl-L-phenyl-alanine involved a change in previous peptide methodology, the use of mixed anhydride couplings, but all the intermediates could be isolated by Amberlyst 15 (3-bromopyridinium form). The synthesis of protected octapeptide analogues (14-16) was facilitated by the availability of intermediates from the previous synthesis.

(1) Synthesis of Z-Gly-Phe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (14)

The synthetic route is summarised in Figure 21 and the physical characteristics

and amino acid analyses for each intermediate are given in Figures 22 and 23 respectively.

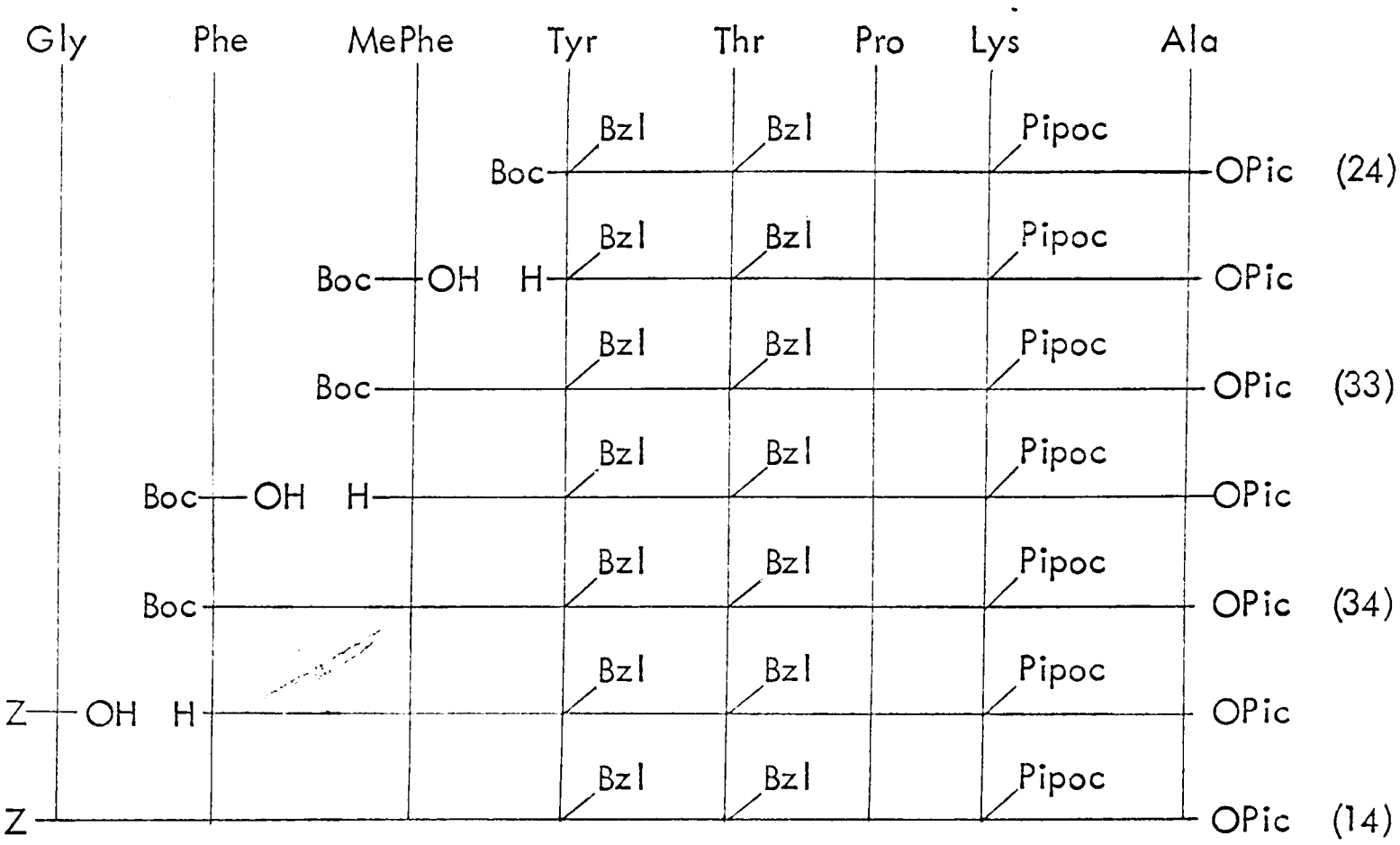


Figure 21

The protected hexapeptide (33) was prepared from the previous intermediate (24) in 85.3% yield, by a mixed anhydride coupling with pivaloyl chloride and N-methylmorpholine as base, as a chromatographically pure white powder with satisfactory elemental and amino acid analysis.

The protected heptapeptide (34) was prepared by coupling with N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole although the reaction was extremely slow and was complete only in 24 hours. An 86% yield was obtained of a chromatographically pure white powder with satisfactory elemental and amino acid analysis.

Synthesis of protected N-methyl-L-phenylalanine analogues

Compound	Acylating[a] agent	Yield[c] (%)	Overall yield (%)	$[\alpha]_D^{20}$ (°)[b]	R_F (t.l.c.)	Found (%) C	H	N	Formula	Required (%) C	H	N
33	1.2	85.3	67.2	-40.6 (c 0.79)	E4, 0.64; G3, 0.74; A2, 0.46; N, 0.86	64.22	7.26	10.20	$C_{68}H_{87}N_9O_{13}.2H_2O$	64.08	7.20	9.89
34	1.2	86.0	57.2	-45.6 (c 0.35)	E4, 0.71; G3, 0.98; A2, 0.60; N, 0.80	65.26	7.23	10.10	$C_{77}H_{96}N_{10}O_{14}.2H_2O$	65.05	7.09	9.85
14	1.1	76.0	43.9	-52.2 (c 0.59)	E4, 0.68; G3, 0.92; A2, 0.62; N, 0.77	65.67	6.85	9.93	$C_{82}H_{97}N_{11}O_{15}.H_2O$	65.89	6.67	10.31
35	1.1	84.5	59.6	-49.2 (c 1.01)	E4, 0.67; G3, 0.86; A2, 0.63; N, 0.94	65.23	6.86	9.89	$C_{72}H_{96}N_{10}O_{14}.2H_2O$	65.05	7.09	9.85
15	1.1	92.7	55.4	-49.0 (c 0.52)	E4, 0.64; G3, 0.77; A2, 0.57; N, 0.95	65.72	7.03	10.66	$C_{82}H_{97}N_{11}O_{15}.H_2O$	65.89	6.67	10.31
36	1.2	82.9	55.8	-63.2 (c 0.6)	E4, 0.66; G3, 0.85; A2, 0.60; N, 0.91	65.18	7.03	10.15	$C_{78}H_{98}N_{10}O_{14}.2H_2O$	65.25	7.16	9.76
16	1.1	82.1	45.8	-52.5 (c 0.13)	E4, 0.65; G3, 0.78; A2, 0.54; N, 0.85	65.24	7.18	9.60	$C_{83}H_{99}N_{11}O_{15}.2H_2O$	65.29	6.80	10.09

[a] Molar proportions of the carboxy-component, N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole relative to the amino-component, used in the last coupling step of the stated compound. [b] Measured in chloroform. [c] Isolated by Amberlyst 15 (3-bromopyridinium form).

Figure 22

Compound \ Amino acid	Ala	Lys	Pro	Thr	Tyr	Phe	MePhe	Gly
33	1.00	1.01	0.99	0.98	0.97	·	1.00	
34	1.00	0.99	0.99	0.99	0.98	0.99	0.98	
14	1.00	1.01	1.02	0.99	0.97	0.97	1.00	0.99
35	1.00	1.01	0.99	0.98	1.00	0.98	0.99	
15	1.00	0.99	0.98	0.99	0.99	0.98	0.99	1.00
36	1.00	1.01	1.03	0.97	0.98		1.99	
16	1.00	1.01	0.98	1.00	0.96		1.97	0.98

Amino acid analyses of protected N-methyl-L-phenylalanine intermediates

Figure 23

The protected octapeptide (14) was obtained in 76% yield and isolated as a chromatographically pure white powder with satisfactory elemental and amino acid analysis.

The overall yield of the protected octapeptide (14) from the 4-picolyl ester dihydrobromide was 43.9%.

(2) Synthesis of Z-Gly-MePhe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (15)

The synthetic route is summarised in Figure 24 and the physical characteristics and amino acid analyses for each intermediate are given in Figures 22 and 23 respectively.

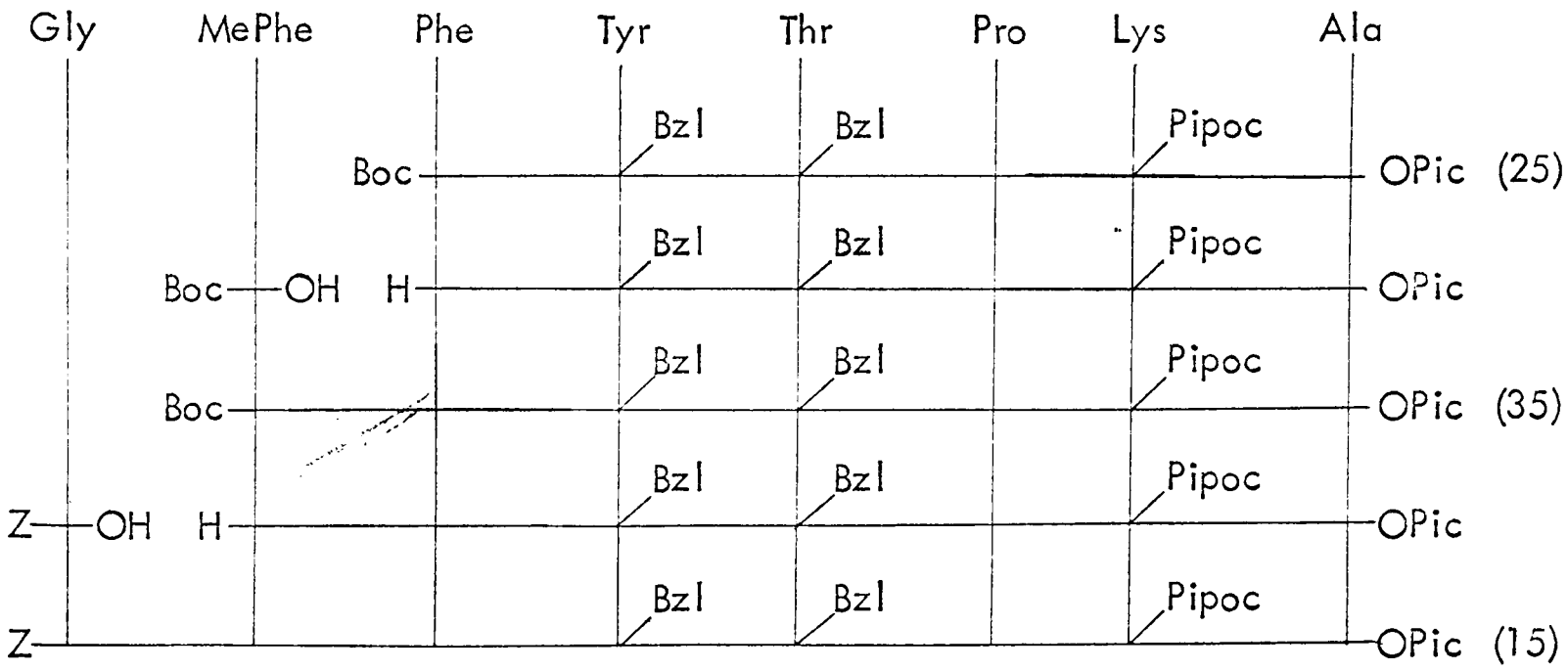


Figure 24

The protected heptapeptide (35) was prepared from the previous intermediate (25) by a similar mixed anhydride coupling as in the previous synthesis, in 84.5% yield as a chromatographically pure white powder with satisfactory elemental and amino acid analysis.

The protected octapeptide (15) was prepared by the N,N'-dicyclohexyl-carbodi-imide—1-hydroxybenzotriazole method in 92.7% yield as a chromatographically pure white powder with satisfactory elemental and amino acid analysis.

The overall yield of the protected octapeptide (15) from the 4-picolyl ester dihydrobromide was 43.9%.

(3) Synthesis of Z-Gly-MePhe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (16)

The synthetic route is summarised in Figure 25 and the physical characteristics and amino acid analyses for each intermediate are given in Figure 22 and 23 respectively.

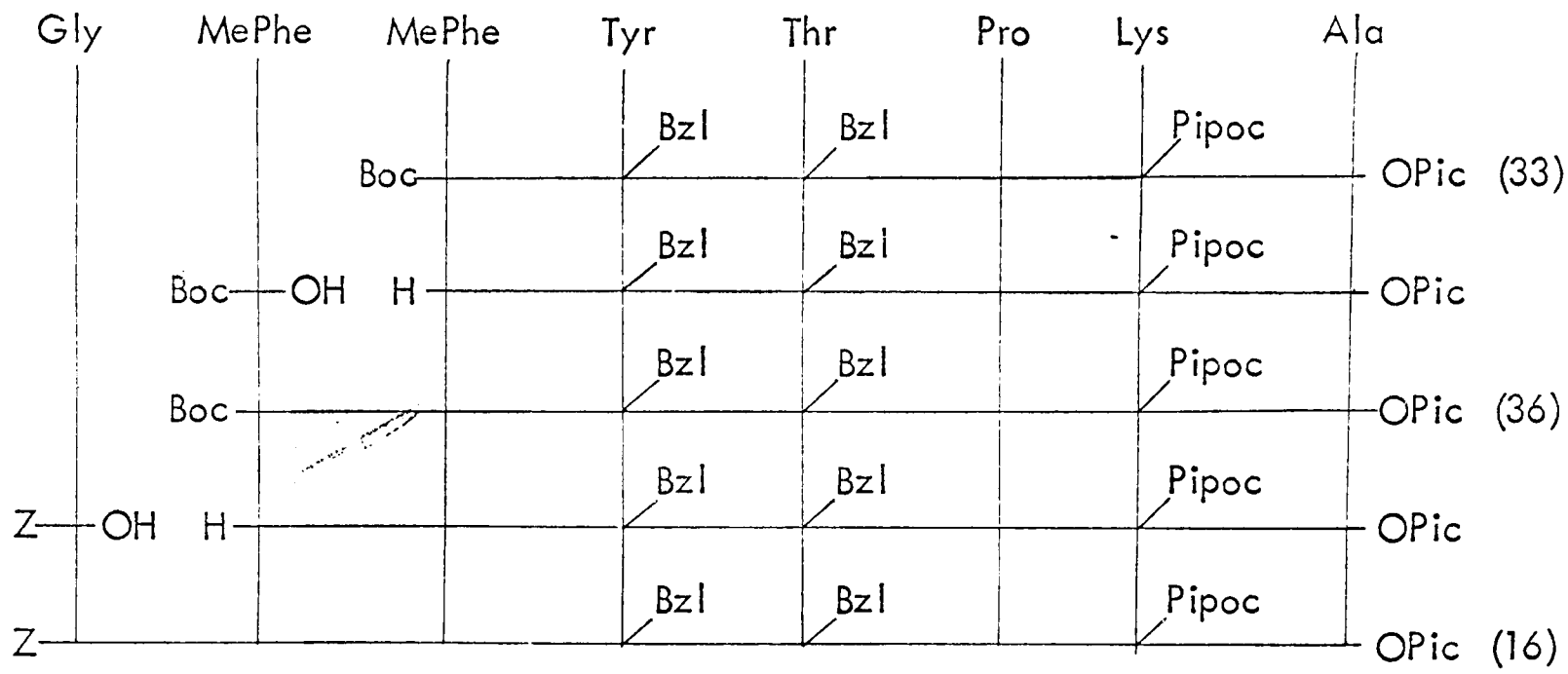


Figure 25

The protected heptapeptide (36) was prepared by a mixed anhydride coupling from hexapeptide (33). The reaction was extremely slow and took two days for completion. Normal isolation by Amberlyst 15 (3-bromopyridinium form) gave a 82.9% yield of a chromatographically pure white powder with satisfactory elemental and amino acid analysis.

The protected octapeptide (16) was prepared in 82.1% yield from (36) by the N,N'-dicyclohexylcarbodi-imide—1-hydroxybenzotriazole method. Isolation by the Amberlyst 15 (3-bromopyridine form) procedure gave the material as a chromatographically pure white powder with satisfactory elemental and amino acid analysis.

The protected octapeptide (16) was prepared in an overall yield of 45.8% from the 4-picolyl ester dihydrobromide.

The Preparation of Truncated Sequences

The 2-(p-biphenylyl)-isopropyloxycarbonyl group[189] had previously not been used with the 4-picolyl ester procedure. Rate studies indicated that it was three

thousand times as acid labile as the t-butoxycarbonyl group.[189] Its versatility is shown in the nearly completed synthesis of lysozyme where it has been used nearly exclusively for α-amino protection in conjunction with t-butyl ether side chain protection.[190]

Preliminary studies with 2-(p-biphenylyl)-isopropyloxycarbonylglycine indicated that it was stable to Amberlyst 15 (3-bromopyridinium form) in dimethyl-formamide for several hours. However, 1-hydroxybenzotriazole was found to decompose the protecting group readily to give 2-biphenyl-propane-2-ol and 2-biphenyl-2-propene. 2-(p-Biphenyl)-isopropyloxycarbonylglycine was introduced as its N-hydroxysuccinimide ester and by a N,N'-dicyclohexylcarbodi-imide coupling of its free acid.

Preparation of protected analogues (17) and (18) would then give the easily prepared semi-protected tetra- and hexapeptides (37) and (38) respectively by routes indicated in Figure 26.

The synthetic route for the preparation of the protected tetrapeptide is summarised in Figure 27 and the physical characteristics for each intermediate are shown in Figure 28.

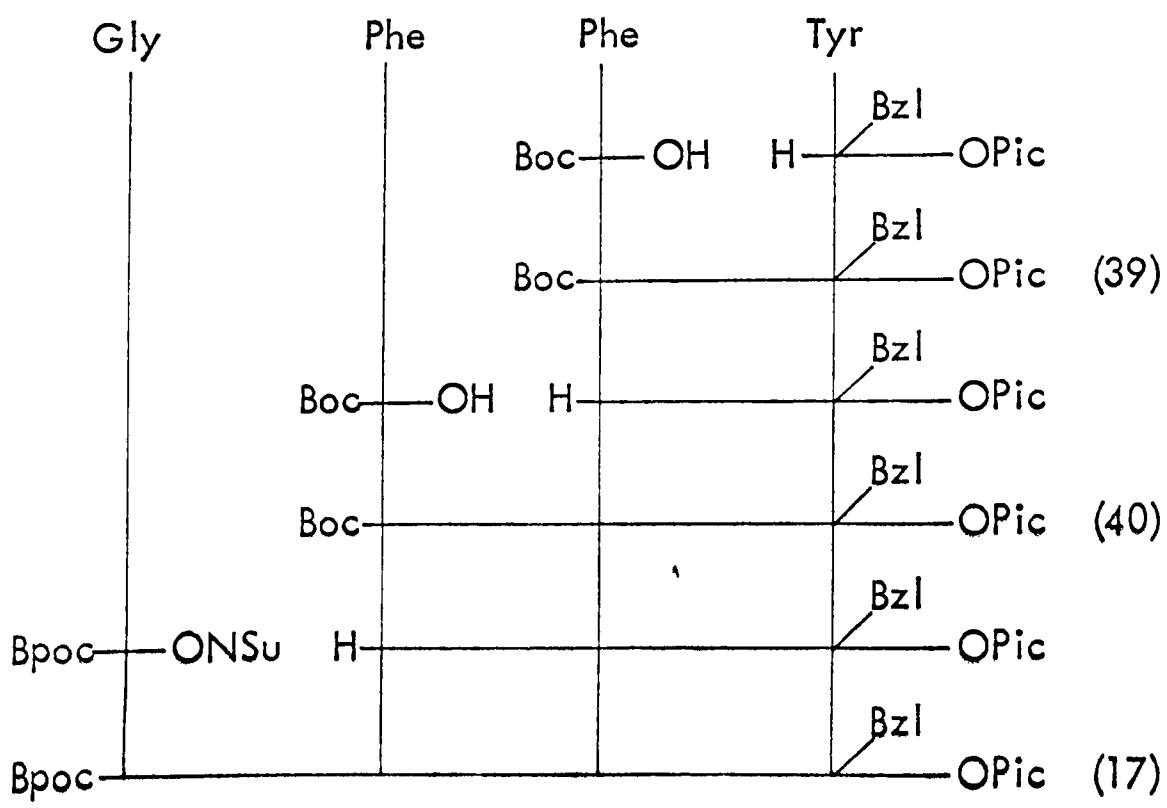
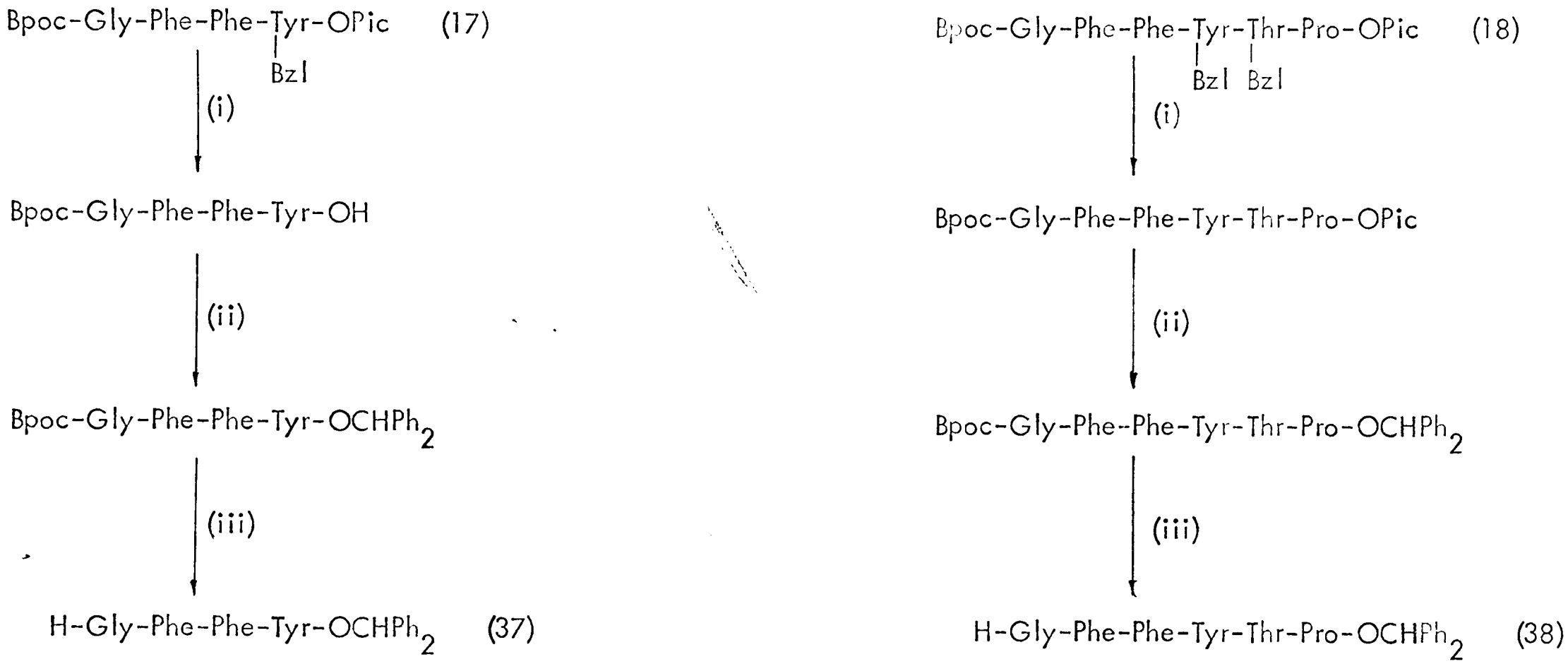


Figure 27



(i) H_2/Pd (ii) Diphenyldiazomethane (iii) 0.15% TFA in dichloromethane

Figure 26

Synthesis of Bpoc-Gly-Phe-Phe-Tyr(Bzl)-OPic

Compound	Acylating agent	Yield[c] (%)	Overall yield (%)	$[\alpha]_D^{20}$ (°)[d]	R_F (t.l.c.)	Found (%) C	H	N	Formula	Required (%) C	H	N
39	1.3[a]	97.5		-12.7 (c 0.97)	E4, 0.64; G3, 0.82; A2, 0.73; G4, 0.80	70.95	6.57	6.92	$C_{36}H_{39}N_3O_6$	70.6	6.56	6.88
40	1.3[a]	83.0	81.0	-26.6 (c 0.91)	E4, 0.82; G3, 0.92; A2, 0.67; N, 0.83	68.44	6.41	7.13	$C_{45}H_{48}N_4O_7.2H_2O$	68.2	6.56	7.08
17	1.05[b]	46.0	37.3	-15.2 (c 0.93)	E4, 0.80; G3, 0.94; A2, 0.75; G1, 0.97	71.55	6.27	7.19	$C_{58}H_{57}N_5O_8.H_2O$	71.7	6.08	7.21

a) Molar proportions of the carboxy-component, N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole, relative to the amino component, used in the last coupling step of the stated compound.

b) Via N-hydroxysuccinimide ester.

c) Isolated by Amberlyst 15 (3-bromopyridinium form).

d) Measured in chloroform.

Figure 28

The 4-picolyl derivative of the C-terminal amino acid, α-t-butoxycarbonyl-O-benzyl-L-tyrosine 4-picolyl ester, was synthesised by reaction of 4-pyridyl-methanol and α-t-butoxycarbonyl-O-benzyl-L-tyrosine at 0° with N,N'-dicyclo-hexylcarbodi-imide in dichloromethane. Attempted purification by partition between citric acid (0.7M) and ethyl acetate and diethyl ether mixtures failed probably because of the lipophilicity of the protected derivative. However a successful isolation was performed on Amberlyst 15 (3-bromopyridinium form). The product was recrystallised from ethyl acetate and petroleum ether in 67% yield as white needle-shaped crystals of m.p. 78-80°, $[\alpha]_D^{20}$ -18.5° (c 1.13 in DMF), chromatographically pure with satisfactory elemental analysis and n.m.r. spectrum.

Treatment of the protected 4-picolyl ester with trifluoroacetic acid gave O-benzyl-L-tyrosine 4-picolyl ester bis trifluoroacetate which was coupled to α-t-butoxycarbonyl-L-phenylalanine using N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole with N,N-di-isopropylethylamine as base. The dipeptide, α-t-butoxycarbonyl-L-phenylalanyl-O-benzyl-L-tyrosine 4-picolyl ester (39) was obtained in 97.5% yield as a chromatographically and analytically pure buff powder by the Amberlyst 15 (3-bromopyridinium form) procedure with a satisfactory n.m.r. spectrum.

The protected tripeptide (40) was prepared in a similar manner from the dipeptide (39) in 83.0% yield as a chromatographically and analytically pure buff powder by the Amberlyst 15 (3-bromopyridinium form) procedure with a satisfactory n.m.r. spectrum.

The protected tetrapeptide (17) was prepared from 2-(p-biphenylyl)-iso-propyloxycarbonylglycine N-hydroxysuccinimide ester and the trifluoracetic acid

treated tripeptide (40). The protected tetrapeptide (17) was isolated by Amberlyst 15 (3-bromopyridinium form) in 46% yield as a chromatographically pure white powder with satisfactory elemental analysis and n.m.r..spectrum.

The overall yield of protected tetrapeptide (17) was 37.3% from the fully protected 4-picolyl ester.

The synthetic routes for the preparation of the protected hexapeptide (18) are given in Figure 29 and the physical characterisations and amino acid analyses are given in Figures 30 and 31 respectively.

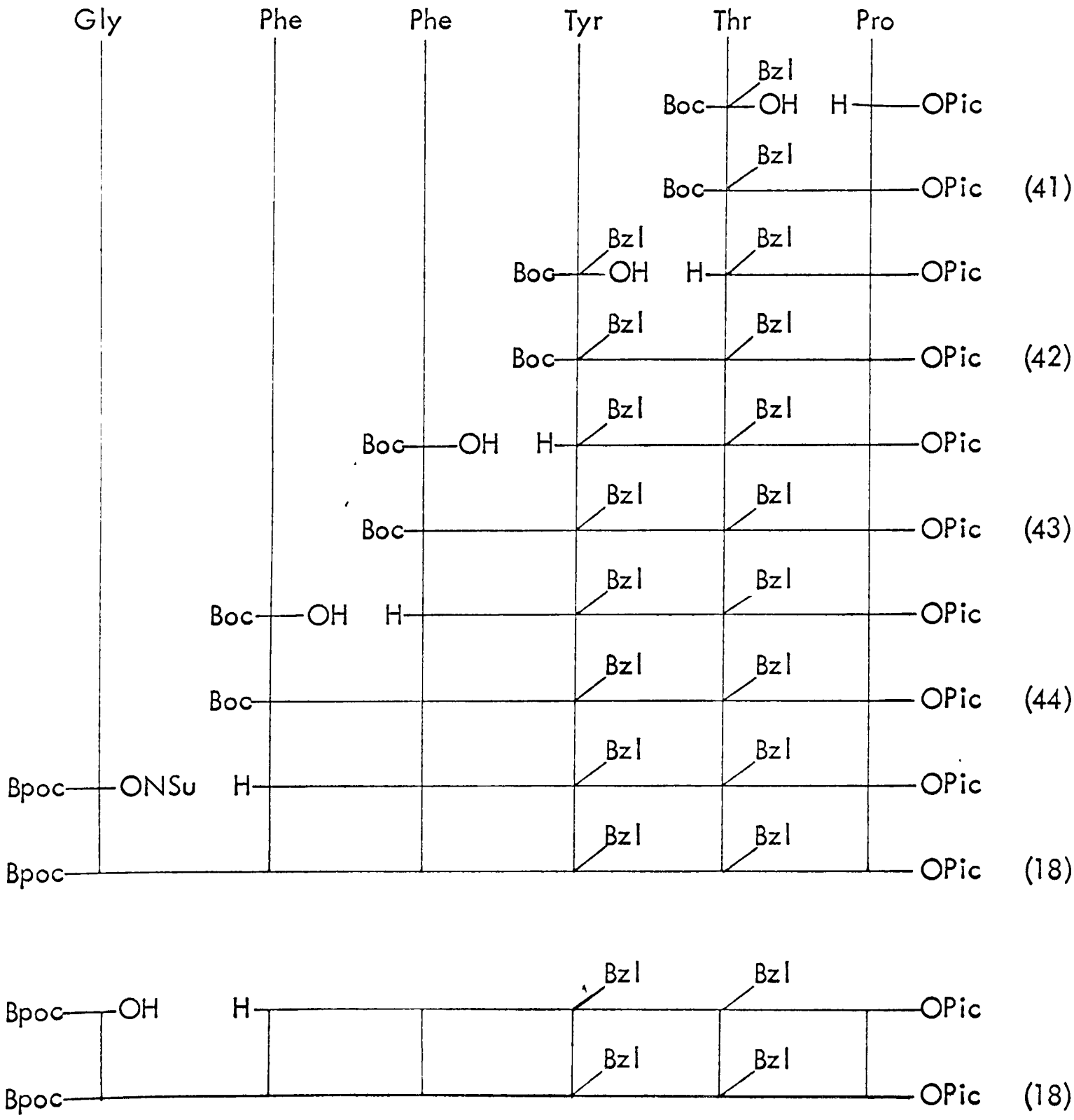


Figure 29

Synthesis of Bpoc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-ProOPic

Compound	Acylating agent	Yield (%)	Overall yield (%)	$[\alpha]_D^{20}$ (°)[e]	R_F (t.l.c.)	Found (%) C	H	N	Formula	Required (%) C	H	N
41	1.2[a]	87.2[c]		-28.2 (c 1.23)	E4, 0.65; G3, 0.86; G1, 0.95; A2, 0.62	64.87	6.93	8.25	$C_{27}H_{35}N_3O_6$	65.17	7.09	8.45
42	1.2[a]	86.6[d]	75.5	-20.0 (c 0.95)	E4, 0.65; G3, 0.90; G1, 0.97; A2, 0.65	67.4	6.71	7.33	$C_{43}H_{50}N_4O_8.H_2O$	67.16	6.82	7.29
43	1.2[a]	91.8[d]	69.4	-19.5 (c 0.99)	E4, 0.57; G3, 0.91; G4, 0.90; A2, 0.68	69.23	6.23	7.58	$C_{52}H_{59}N_5O_9$	69.50	6.62	7.78
44	1.2[a]	88.4[d]	61.2	-29.7 (c 0.41)	E4, 0.63; G3, 0.98; G4, 0.85; A2, 0.68	69.0	6.47	7.78	$C_{61}H_{68}N_6O_{10}.H_2O$	68.8	6.67	7.90
18	1.0[b]	81.0[d]	49.6	-30.6 (c 0.97)	E4, 0.66; G3, 0.82; N, 0.95; A2, 0.7	71.37	6.25	7.75	$C_{74}H_{77}N_7O_{11}$	71.60	6.26	7.91

a) Molar proportions of the carboxy-component, N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole, relative to the amino component, used in the last coupling step of the stated compound.
b) Via N-hydroxysuccinimide ester.
c) Isolated by the citric acid procedure.
d) Isolated by Amberlyst 15 (3-bromopyridinium form).
e) Measured in chloroform.

Figure 30

Compound \ Amino acid	Pro	Thr	Tyr	Phe	Gly
43	1.00	0.99	0.99	1.00	
44	1.00	0.99	1.01	2.03	
18	1.00	1.01	1.00	1.99	1.00

Amino acid analyses of protected hexapeptide intermediates

Figure 31

The synthetic procedure followed exactly that of the preceding syntheses. The dipeptide, α-t-butoxycarbonyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (41) was prepared by coupling α-t-butoxycarbonyl-O-benzyl-L-threonine and L-proline 4-picolyl ester dihydrobromide with N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole using an 'eintopf' procedure. The protected dipeptide (41) was isolated by the citric acid method in 87.2% yield as a chromatographically pure white foam with a satisfactory elemental analysis and n.m.r. spectrum.

The dipeptide (41) was deprotected with trifluoroacetic acid and coupled with α-t-butoxycarbonyl-O-benzyl-L-tyrosine in the usual manner to give the tripeptide (42) in 86.6% yield, isolated by the Amberlyst 15 (3-bromopyridinium form) method as a chromatographically pure white powder with satisfactory elemental analysis and n.m.r. spectrum.

The tetra- and pentapeptides (43) and (44) were prepared in analogous manner in yields of 91.8 and 88.4% respectively, as chromatographically pure white powders with satisfactory elemental and amino acid analyses.

The hexapeptide (18) was prepared by two methods.

The first followed the preparation of the tetrapeptide (43) using 2-(p-biphenylyl)-isopropyloxycarbonylglycine N-hydroxysuccinimide ester and the second was via the free acid and N,N'-dicyclohexylcarbodi-imide. Isolation in both cases was by the Amberlyst 15 (3-bromopyridinium form) procedure and gave yields of 81 and 73% respectively of chromatographically pure white powders with satisfactory elemental and amino acid analyses.

The overall yield of the hexapeptide from the 4-picolyl ester dihydrobromide was 49.6%.

These two syntheses demonstrate the potential use of the extremely acid-labile 2-(p-biphenylyl)-isopropyloxycarbonyl group in conjunction with the 4-picolyl ester procedure. The overall yields for a tetrapeptide and hexapeptide were 37.3 and 49.6% respectively and no problems were encountered with the use of this group.

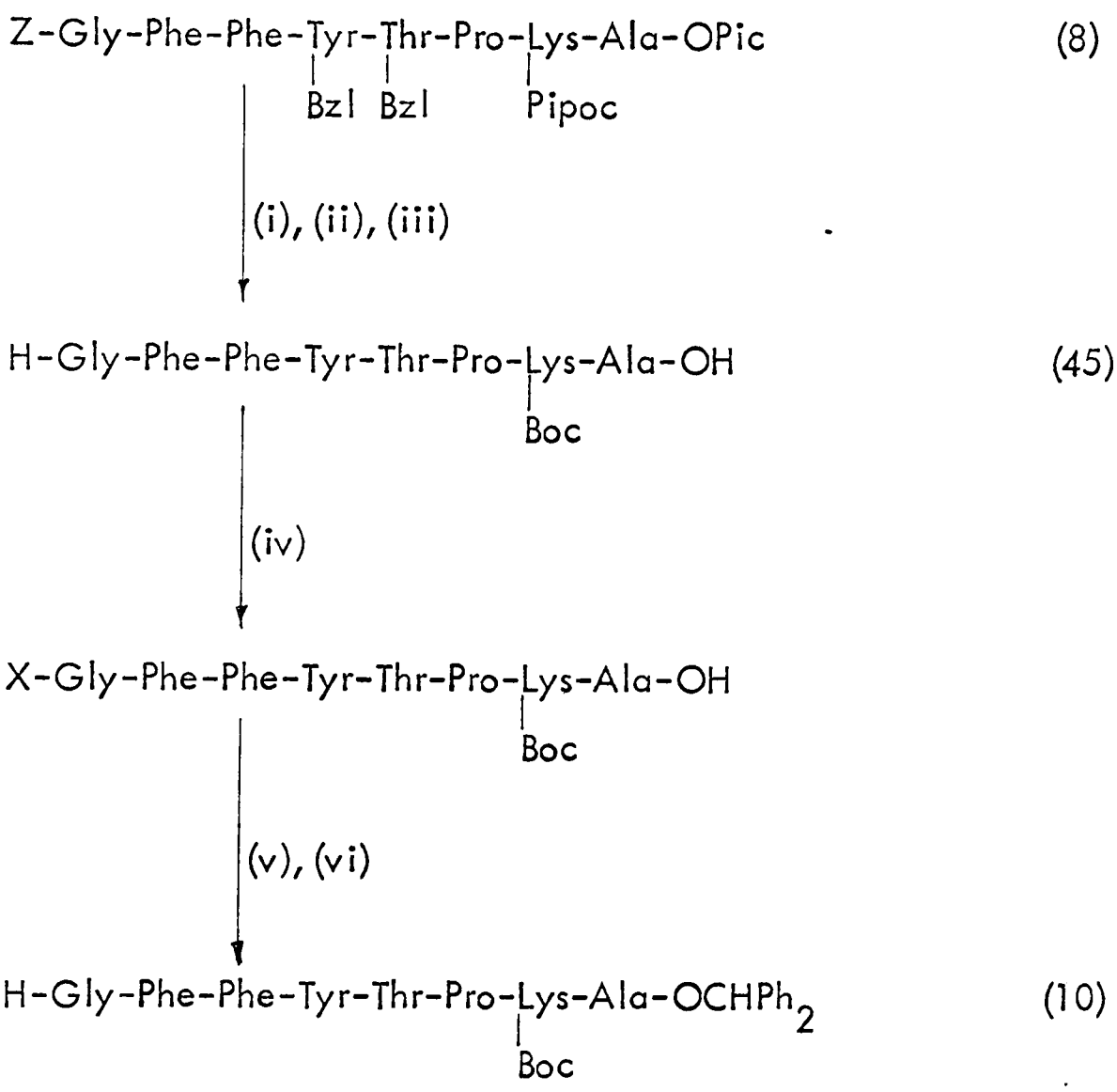
Studies with Diphenyldiazomethane

The projected preparation of the derivative (10) from (8) required a synthetic

$$\text{Z-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Boc)-Ala-OCHPh}_2 \qquad (10)$$

route in the form of Figure 32.

The replacement of piperidino-oxycarbonyl by t-butoxycarbonyl for ε-amino protection followed by hydrogenolysis of the α-benzyloxycarbonyl, benzyl ethers and 4-picolyl ester groups in steps (i), (ii) and (iii) follows known procedures. The most crucial steps would appear to be the reprotection of the α-amino group and following esterification by diphenyldiazomethane and subsequent α-amino deprotection.



(i) Sodium dithionite/50% aqueous HAc (ii) Boc azide

(iii) H_2/Pd (iv) X—α-amino protection

(v) Diphenyldiazomethane (vi) Removal of X.

Figure 32

The planned mild acidic conditions employed for the removal of the t-butoxycarbonyl and benzhydryl ester groups presents a severe constraint of the possible choice of groups for α-amino protection. Two urethane protecting groups were considered that had already been used in the syntheses: 2-(p-biphenylyl)-isopropyloxycarbonyl and piperidino-oxycarbonyl. Both offered the advantages of being directly incorporated into the peptide via their mixed carbonates.' The virtues of each group were examined

using their respective glycine benzhydryl esters as model compounds.

In the original preparation of benzhydryl esters,[144] Hiskey reacted the amino acid derivative with excess diphenyldiazomethane in ethyl acetate for two days at room temperature. Excess diazoalkane was then removed by the addition of concentrated hydrochloric acid. Such vigorous conditions were thought too drastic to be used with protected peptides. A series of organic and inorganic acids were investigated to find milder conditions for hydrolysing excess diphenyldiazomethane. The hydrolysis was observed by the discharge of the intense maroon colour of the diazoalkane by acid to give a colourless solution. The following results were obtained and shown in Figure 33.

Acid	Observation
0.01M Malonic	Colour faded within 3 min
0.01M Tartaric	Gradual hydrolysis, complete within 1 hr
0.7M Citric	" " " " "
Glacial acetic	Complete within 30 sec
50% Aqueous acetic	Complete within 1 min
6M Hydrochloric	Hydrolysis < 10 sec
1M Hydrochloric	Complete within 2 min
0.1M Hydrochloric	Virtually no colour change

Figure 33

The most favourable results were obtained by washing the reaction solution with hydrochloric acid (1.0M) and immediately washing with saturated sodium

hydrogen carbonate solution to remove traces of excess acid.

2-(p-Biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46) was prepared by the above manner. 2-(p-Biphenylyl)-isopropyloxycarbonylglycine was reacted overnight with excess diphenyldiazomethane in ethyl acetate at room temperature. Rapid washing removed excess diazoalkane and the isolated gum was recrystallised from ethyl acetate and petroleum ether in 83.5% yield as needle-shaped crystals of m.p. 113.5-115°, chromatographically pure with satisfactory elemental analysis and n.m.r. spectrum. This compound was examined for its sensitivity to acid by stirring with hydrochloric acid (0.01$\underline{M}$) and only a trace of decomposition, < 5%, was found after 2.5 hours.

α-Piperidino-oxycarbonylglycine benzhydryl ester (47) was prepared from α-piperidino-oxycarbonylglycine in an analogous manner in 72% yield as needle-shaped crystals of m.p. 124-5°, chromatographically pure with satisfactory elemental analysis and n.m.r. spectrum.

The conditions for esterification of protected amino acids and protected peptides were expected to be considerably different. The most probable solvent to be used in the esterification of the protected octapeptide was 85% aqueous dimethylformamide. The stability of diphenyldiazomethane to hydrochloric acid (1.0$\underline{M}$) in these conditions was studied on an autotitrator observing the rate of uptake of acid and colour of the solution. At pH 4.0 there was virtually no hydrolysis. Between pH 3.0 and pH 2.5 the hydrolysis rate increased with the gradual removal of the colour of the solution and the formation of white insoluble material until at pH 2.0 hydrolysis was complete and the solution was colourless.

The decomposition of excess diphenyldiazomethane in the presence of the

model compounds, 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46) and α-piperidino-oxycarbonylglycine benzhydryl ester (47) was investigated in conditions that could be needed for complete solubilisation of the partially protected peptide, i.e. 85% aqueous dimethylformamide.

In the pH range 2.5-4.0, α-piperidino-oxycarbonylglycine benzhydryl ester (47) was completely stable and no decomposition was observed. In the case of 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46) the derivative was stable between pH 3.0 and 4.0 but a small amount of decomposition was observed between pH 2.5-3.0 corresponding to the formation of glycine benzhydryl ester.

The optimum conditions for the removal of the 2-(p-biphenylyl)-isopropyl-oxycarbonyl group in the presence of the benzhydryl ester function was examined with the model, 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46). The decomposition was easily followed by ultraviolet absorption at 254 nm of the liberated 2-biphenyl-propane-2-ol and 2-biphenyl-2-propene. Various acidic reagents were investigated and the results summarised in Figure 34.

Acid	Observation
0.05% TFA in CH_2Cl_2	Very fast removal, < 5 min, trace of glycine after 30 min
0.15% TFA in CH_2Cl_2	Very fast removal, < 30 sec, traces of glycine formed after 30 min
0.5% TFA in CH_2Cl_2 at 0°	Removal in 25-30 min, no trace of any completely deprotected material
6 : 1 (v/v) HAc; DCA	Removal in 30 min
CH_2Cl_2-75% CAA, 50 : 37	Fast removal within 10 min, trace of glycine formed after 30 min

Figure 34

The 2-(p-biphenylyl)-isopropyloxycarbonyl group could be easily removed by various trifluoroacetic acid solutions in dichloromethane although prolonged treatment led to the formation of completely deprotected material. The most successful conditions were met by 0.5% trifluoroacetic acid in dichloromethane at 0°.

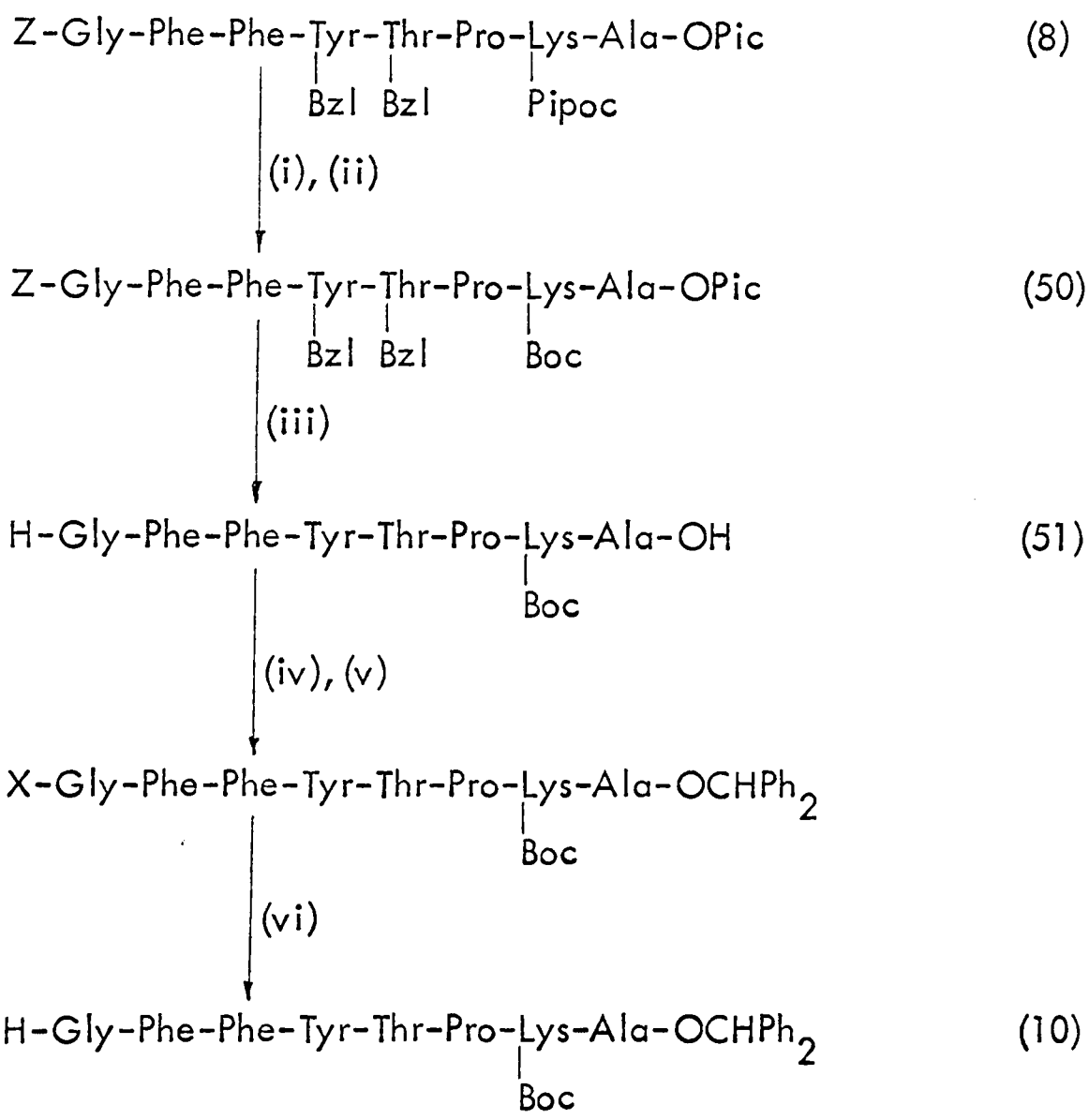
Glycine benzhydryl ester trifluoroacetate (48) was prepared as a chromatographically pure white powder in 89% yield with m.p. 157-160° with satisfactory elemental analysis from (46) by the above procedure.

The piperidino-oxycarbonyl group can be removed under reducing conditions: catalytic hydrogenation, or electrolytic reduction, zinc and acetic acid or with sodium dithionite and acetic acid.[170] The most applicable to the synthetic scheme was the use of sodium dithionite and acetic acid. The model, α-piperidino-oxycarbonylglycine benzhydryl ester (47) was treated with excess sodium dithionite in 50% aqueous acetic acid. Deprotection was complete within 1.25 hours and a sample identical to glycine benzhydryl ester (48) was obtained. Deprotection in 80% aqueous dimethylformamide with excess sodium dithionite was negligible until a trace of 50% aqueous acetic acid was added and then the reaction was slow.

Thus from these model experiments it is shown that both the piperidino-oxycarbonyl and 2-(p-biphenylyl)-isopropyloxycarbonyl protecting groups can be used in conjunction with benzhydryl esters. Both groups could be safely removed in presence of the ester function and the ester could easily be prepared from its diazoalkane in derivatives protected by both these groups. The optimum conditions for deprotection of each group have been ascertained.

Synthesis of the Protected Precursor

The synthesis of the protected natural octapeptide and L-alanine and N-methyl-L-phenylalanine analogues have been described in the previous sections. The preparation of the precursor (10) for coupling to the protected des-octapeptide-insulin was the next objective. This involved the use of the mild acid labile protecting groups, t-butoxycarbonyl and benzhydryl esters as shown in Figure 35.



(i) Sodium dithionite/HAc (ii) Boc azide (iii) H_2/Pd

(iv) α-Amino protection (v) Diphenyldiazomethane

(vi) α-Amino deprotection

Figure 35

The deprotection of the ε-piperidino-oxycarbonyl group and replacement by t-butoxycarbonyl of the lysine residue was examined with the tetrapeptide (23).

Boc-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (23)

Treatment with excess sodium dithionite in 50% aqueous acetic acid completely removed the ε-piperidino-oxycarbonyl group within twenty minutes. The solution was neutralised, evaporated and taken up in dimethylformamide. Overnight reaction with t-butoxycarbonyl azide in the presence of triethylamine gave the protected tetrapeptide (49) in 66.1% yield as a chromatographically and analytically

Boc-Thr(Bzl)-Pro-Lys(Boc)-Ala-OPic (49)

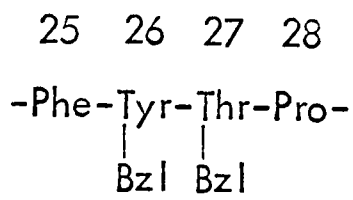
pure white powder. This procedure was repeated for the octapeptide (8) and the reprotected peptide (50) was obtained in 72% yield, chromatographically and analytically pure with the following amino acid analysis. Gly, 1.01; Phe, 1.98; Tyr, 0.98; Thr, 1.00; Pro, 1.00; Lys, 0.99 and Ala, 1.00.

The complete removal of the remaining protecting groups by hydrogenation was found to be not as straightforward as anticipated. Small scale trials were carried out following the reaction by electrophoresis at pH 1.9 and pH 6.5. The use of acidic solvents was initially avoided as prolonged exposure in acidic solution might lead to deprotection of the t-butoxycarbonyl group.

In 80% aqueous dimethylformamide hydrogenolysis was extremely slow and only traces of a possible product was observed. The 4-picolyl group appeared to have been removed on account of the readily detectable 4-methylpyridine on the electrophoretogram. In dimethylformamide containing a trace of acetic acid, the

majority of the product appeared unchanged even after hydrogenation for 64 hours.

The tetrapeptide (23) and pentapeptide (24) were examined as models for the octapeptide as they contained the benzyl and 4-picolyl groups and it was thought that the former existing side by side, i.e.



might be in such a conformation as to prevent complete deprotection. Hydrogenation of (23) in 50% aqueous acetic acid for 24 hours gave a single spot on electrophoresis. Similarly with (24) a single spot was obtained which as well as being ninhydrin positive was also phenolic hydroxyl positive. In both cases there was no additional spots of higher charge attributed to the removal of the t-butoxycarbonyl group which were found when hydrogenation was carried out in 80% aqueous acetic acid. The optimum conditions of hydrogenation of the octapeptide (50) in 50% aqueous acetic acid were studied for 72 hours and the minimum period for complete deprotection was 30 hours. The hydrogenation product was compared with the peptide (51) obtained by tryptic cleavage of protected insulin and was shown to have the same electrophoretic mobilities at pH 1.9 and pH 6.5. The hydrogenation was repeated on a larger scale in 86% yield to give the octapeptide (51), chromatographically and electrophoretically pure with the following amino acid analysis. Gly, 1.01; Phe, 1.97; Tyr, 0.99; Thr, 0.99; Pro, 0.99; Lys, 1.00; Ala, 1.00.

At this stage of the synthesis model studies for the preparation of the des-octapeptide-insulin had not been concluded and a final decision as to whether carboxy protection was to be by benzhydryl or anisyl esters was deferred. However

the direct esterification of the octapeptide (51) had not been previously considered. Treatment of (51) with diphenyldiazomethane for two days at room temperature was successful and no N-alkylated material was observed by electrophoresis and amino acid analysis.

It was concluded that this approach to direct esterification would be suitable for preparation of the desired semi-protected octapeptides. Thus the route indicated in Figure 35 would be applicable without the α-amino protection and could be applied equally well to anisyl ester incorporation.

Cyclisation Studies

The initial investigations into the possibility of preparing a cyclic model peptide based on the B-chain monomer-monomer interactions revolved around model studies. It was soon evident that the simple model proposed (19) would be inadequate because of the severe conditions imposed by the steric nature of the molecule. The use of another amino acid residue as a 'spacer' was found to give the necessary flexibility to the model such that cyclisation might be possible and more importantly that the hydrogen bonds formed in the dimer might also be repeated in the model. The use of a lysine residue between a N-terminal glycine and a C-terminal proline, of a dodecapeptide formed from two Gly-B23 to Pro-B28 hexapeptides, coupled through the ε-amino group and carboxy group produced a flat cyclic structure in which the Van der Waal's distances were such that the possibility of hydrogen bonding was extremely high. The photograph of this model shows the hydrogen-bonded atoms slightly apart, for convenience, and Figure 35 indicates the relevant amino acid residues and the corresponding hydrogen bonding.

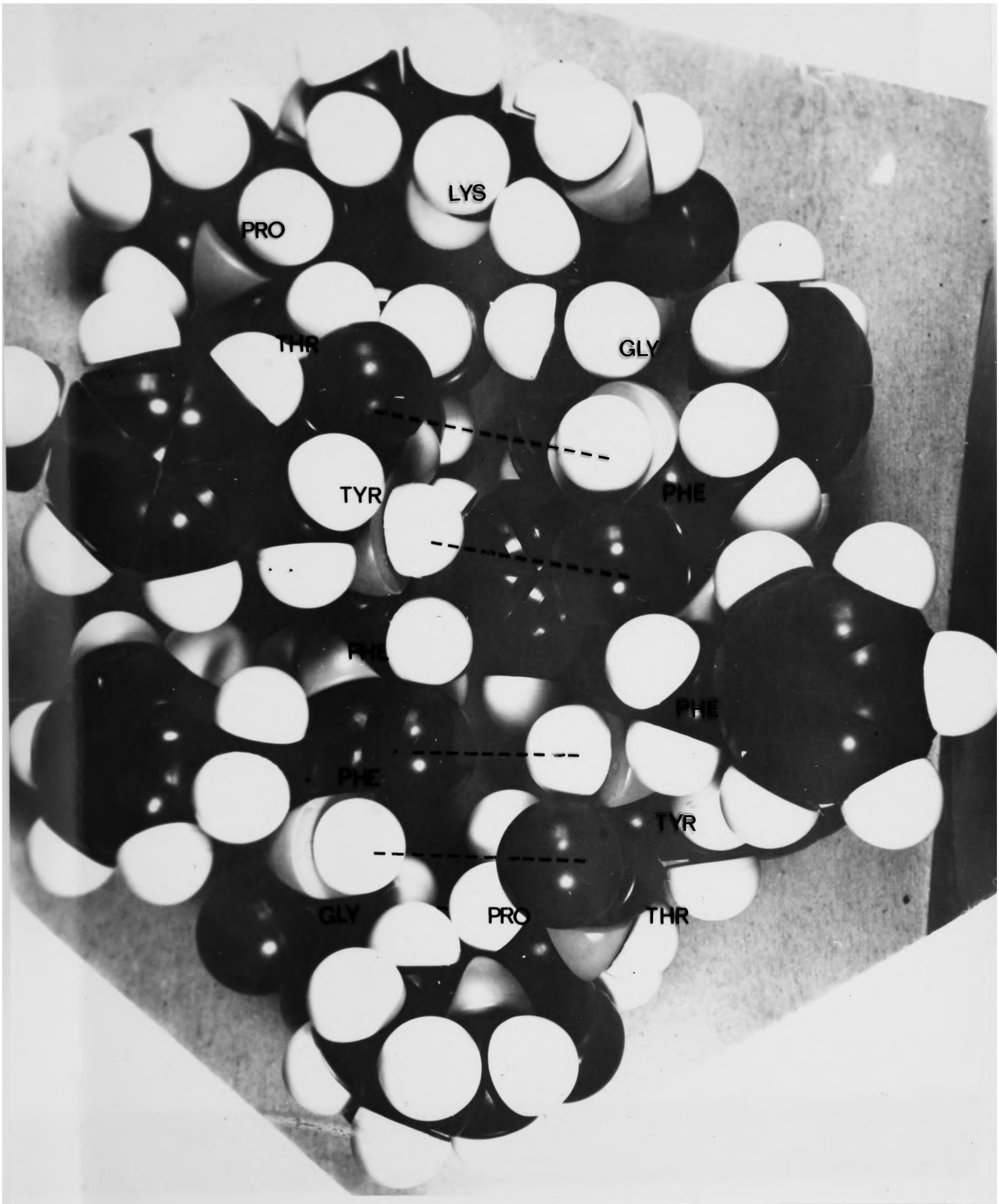


Figure 35

In the synthesis of homomeric heterodetic cyclic peptides there are two alternatives for ring closure: (i) through the formation of an amide or (ii) through the formation of the 'hetero bond'. This latter method has been applied mainly to cyclic disulphides but syntheses have been reported in which the formation of an ester bond through the hydroxyl group of threonine was used.[191] As yet there has been no reported cyclisation through the ε-amino group of lysine. In view of the readily available precursor (24) and protected lysine derivative, t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine, the following hexa- (52) and heptapeptides (53) could be prepared.

Boc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic (52)

Boc-Lys(Pipoc)-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic (53)

Saponification of (53) and trifluoroacetic acid deprotection of (52) would give suitable intermediates for coupling to give the protected tridecapeptide (54), the desired precursor for cyclisation.

Boc-Lys(Pipoc)-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic (54)

The protected heptapeptide (55) was prepared by the synthetic route shown in Figure 36. All couplings were by the N,N'-dicyclohexylcarbodi-imide—1-hydroxybenzotriazole method and isolation carried out by the Amberlyst 15 (3-bromopyridinium form) procedure.

The hexapeptide (52) and heptapeptide (53) were prepared in 94% and 94.1% yield respectively as chromatographically pure white powders with satisfactory

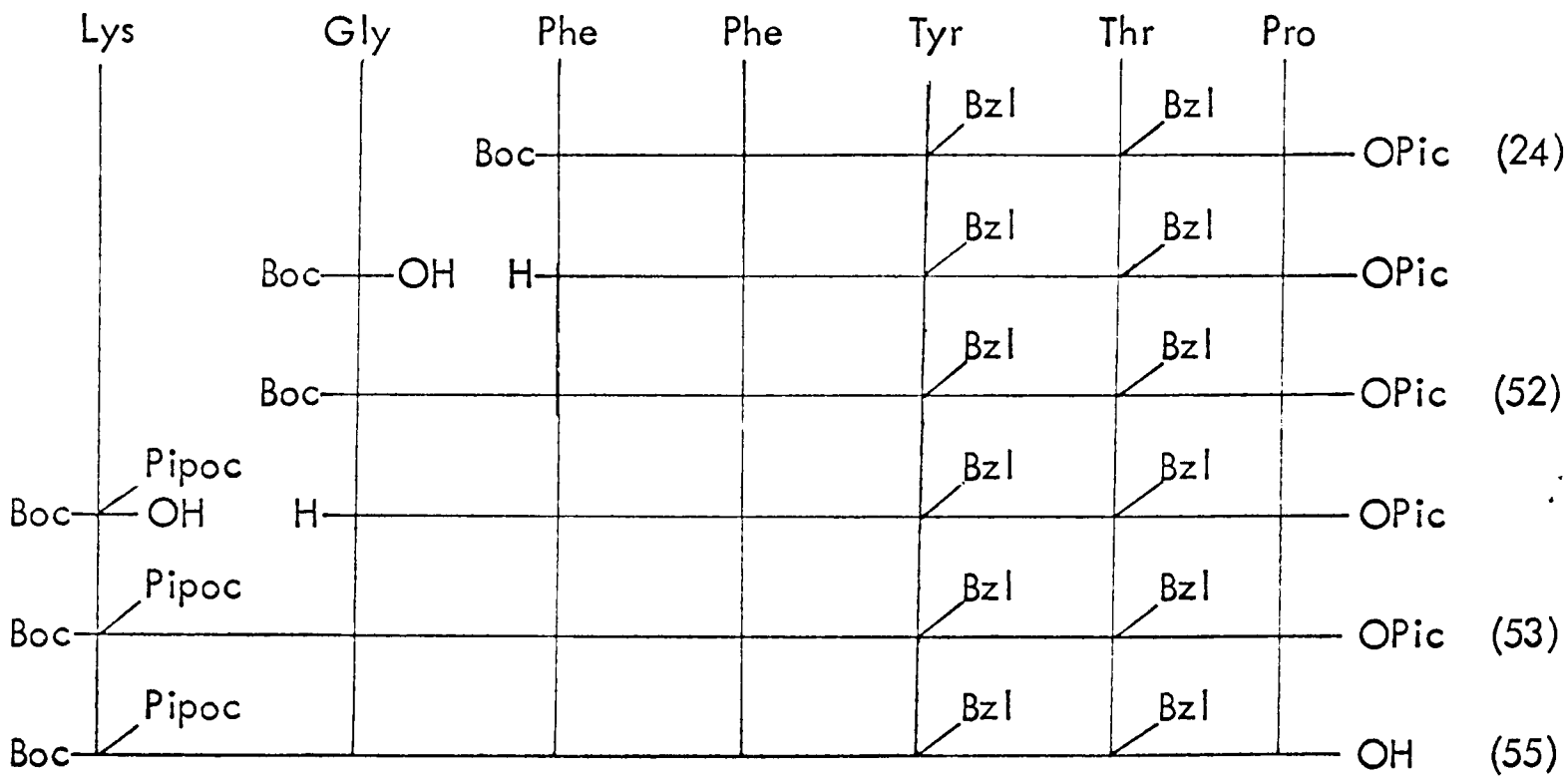


Figure 36

elemental and amino acid analyses.

The saponification of protected peptide 4-picolyl esters was studied with the model tetrapeptide (23). The hydrolysis in aqueous dioxan by a slight excess of

Boc-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic (23)

sodium hydroxide was followed by thin layer chromatography. Reaction was complete within one hour and the addition of citric acid at 0° liberated the free acid into an organic phase. The tetrapeptide (56) was isolated in 86.8% yield as a chromato-

Boc-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OH (56)

graphically pure white powder with satisfactory elemental analysis.

The heptapeptide (55) was prepared in a similar manner as a chromatographically pure white powder with satisfactory elemental analysis and the following amino acid analysis. Lys, 1.02; Gly, 1.00; Phe, 2.02; Tyr, 0.99; Thr, 0.97; Pro, 1.00.

The tridecapeptide (54) would be the largest so far isolated by the 4-picolyl ester method. The heptapeptide (55) was activated in the usual manner though for a longer period with N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole. The hexapeptide (52) was deprotected with trifluoroacetic acid and added to the activated mixture with N,N-di-isopropylethylamine as base. Isolation by Amberlyst 15 (3-bromopyridinium form) after overnight stirring gave the protected tridecapeptide (54) in 72% yield, chromatographically pure with satisfactory elemental analysis. Amino acid analysis gave Lys, 1.00; Gly, 2.02; Phe, 3.99; Tyr, 1.97; Thr, 1.97 and Pro, 2.02.

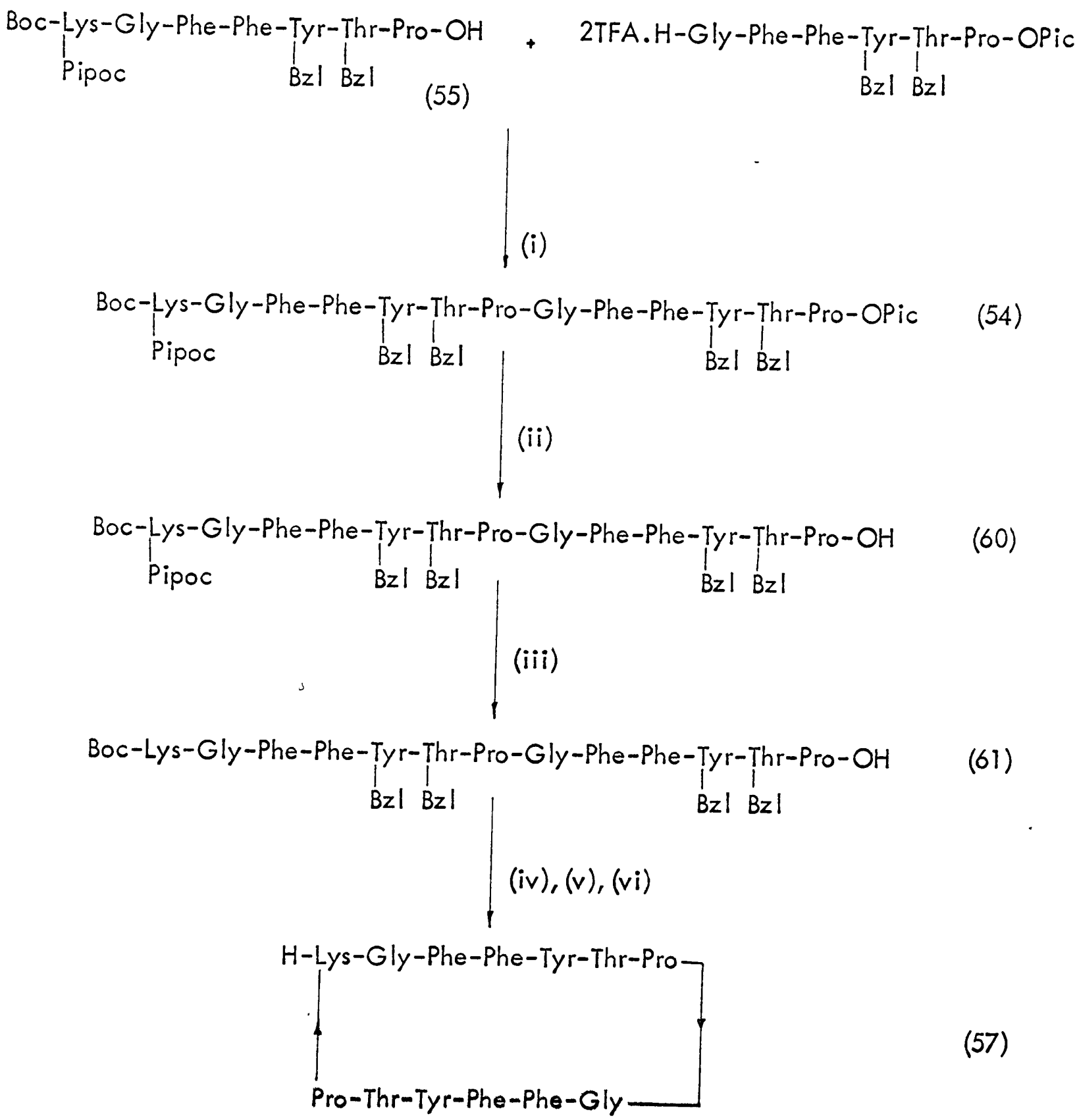
Two routes were considered for cyclisation and these are shown in Figures 37 and 38.

Saponification and then removal of the ε-piperidino-oxycarbonyl group would produce a derivative in a form ready for cyclisation as in Figure 37. Cyclisation followed by hydrogenolysis and trifluoroacetic acid treatment would give the desired product (57). Alternatively, as in Figure 38, the protected hydrazide (58) could be formed. Complete deprotection, followed by an azide coupling and then acidic treatment would give the product (57). The cyclisation reaction could also be performed initially after removal of the ε-piperidino-oxycarbonyl group followed then by complete deprotection.

Both these alternatives were investigated. The model hydrazide (59) was

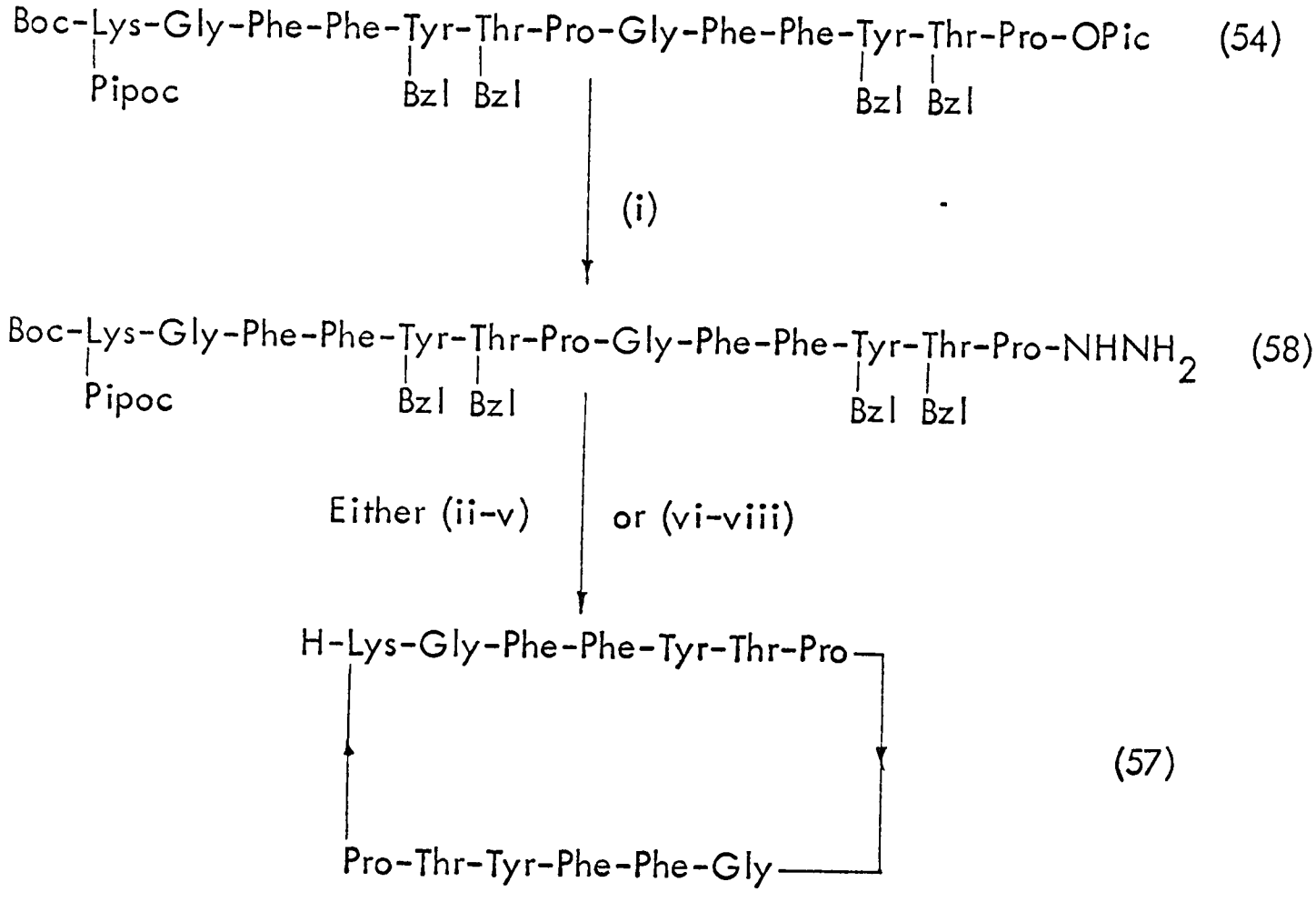
$$\text{Boc-}\underset{\text{Bzl}}{\text{Thr}}\text{-Pro-}\underset{\text{Pipoc}}{\text{Lys}}\text{-Ala-NHNH}_2 \qquad (59)$$

prepared from the tetrapeptide (23) in 86% yield, chromatographically pure and with satisfactory elemental analysis. The tridecapeptide hydrazide (58) was prepared



(i) DCCI/HOBt (ii) NaOH (iii) Sodium dithionite/HAc (iv) Cyclisation reaction (v) H_2/Pd (vi) TFA

Figure 37



(i) NH_2NH_2 (ii) Sodium dithionite/HAc (iii) Azide coupling (iv) H_2/Pd
(v) TFA (vi) H_2/Pd (vii) Azide coupling (viii) TFA

Figure 38

in a similar manner in 75.6% yield as a chromatographically pure white powder with satisfactory elemental analysis. Saponification of the tridecapeptide (54) gave the corresponding free acid (60) in 81.8% yield as a chromatographically pure white powder with satisfactory elemental analysis.

The well-known coupling methods of peptide synthesis have also been applied to cyclisation reactions. The most popular have been the active ester and the N,N'-dicyclohexylcarbodi-imide—N-hydroxysuccinimide methods. The scheme outlined in Figure 37 was adopted as the most straightforward synthesis. Deprotection

of the ε-piperidino-oxycarbonyl group of the tridecapeptide (54) by sodium dithionite in dilute acetic acid afforded the derivative (61) suitable for cyclisation.

Unfortunately, of all the methods attempted for this cyclisation, none were successful in forming the desired amide bond. N,N'-Dicyclohexylcarbodi-imide with either N-hydroxysuccinimide[192] or 1-hydroxybenzotriazole gave the N-acyl urea. In attempted cyclisations with both 2-ethyl-5-phenylisoxazolium-3'-sulphonate[193] and a mixed anhydride procedure[194] only starting material was isolated.

Hydrogenation of the protected tridecapeptide hydrazide (58) in 50% aqueous acetic acid gave material that was inhomogeneous, even after prolonged reaction, by electrophoresis at different pH values.

Thus the attempted preparation of a cyclic peptide containing the B-chain monomer-monomer interactions was unsuccessful. The possible reason for this must be the inability of a suitable conformation to be formed in which cyclisation can take place. The hydrophobic nature of the tridecapeptide, containing four phenyl-alanine, two tyrosine and two proline residues, could be such that correct alignment of the hydrogen-bonding residues is virtually impossible. However, the presence of four benzyl ethers in the protected tridecapeptide could cause sufficient steric hindrance to prevent cyclisation. This seemed unlikely from the model as these protecting functions were all on the outside of a very flat oval structure.

Electrophoresis studies on the prolonged hydrogenolysis of the tridecapeptide hydrazide (58) and acid (55) indicated that the complete removal of the protecting groups might prove difficult. Morley[195] has reported a case where hydrogenation was unable completely to deprotect a peptide containing six benzyl ether groups. It is possible that a similar 'incomplete reduction' occurred with this tridecapeptide.

The Picolyl Ester Method: Conclusions

The majority of this thesis reports the synthesis of a protected C-terminal B-chain insulin octapeptide and a number of analogues by the 4-picolyl ester method. Such has been the recent progress of this method that the synthesis of small peptides of 8-10 residues can be accomplished in high yields in a relatively short period. The syntheses achieved in this work are tabulated in Figure 39 which indicates the overall yields, based on the 4-picolyl ester dihydrobromides, the yield per residue, the coupling method used and the isolation procedure. The overall yields for these protected analogues are between 40 and 58% which is a very good indication of the success of this synthetic procedure. All the protected intermediates were isolated as chromatographically and analytically pure foams or powders with satisfactory amino acid analyses and in the case of the smaller peptides appropriate n.m.r. spectra. The majority of residues were incorporated as their t-butoxycarbonyl derivatives but favourable results were obtained in the isolation of protected peptides containing the 2-(p-biphenylyl)-isopropyloxycarbonyl group. This extremely acid labile protecting group was completely stable to Amberlyst 15 (3-bromopyridinium form) and its success could easily lead to syntheses employing t-butyl ether side chain protection not previously used in conjunction with the 4-picolyl ester method. The majority of couplings were carried out by the N,N'-dicyclohexylcarbodi-imide/1-hydroxybenzotriazole procedure of König and Geiger with complete absence of side products and an average of 90% yield per residue. The largest peptide synthesised so far by the 4-picolyl ester method was the tri-decapeptide (54) coupled by a 7+6 approach in 72% yield and isolated by the Amberlyst 15 (3-bromopyridinium form) procedure.

	PROTECTED PEPTIDE	Overall yield %	Yield/ residue %	Coupling method	Isolation procedure
8	Z-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	56	93.5	1	A + B
11	Z-Gly-Phe-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	47.6	89.8	1	A + B
12	Z-Gly-Ala-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	49.8	92.1	1	A + B
13	Z-Gly-Ala-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	51	92.6	1	A + B
14	Z-Gly-Phe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	43.9	89.3	1 + 3	A + B
15	Z-Gly-MePhe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	55.4	91.5	1 + 3	A + B
16	Z-Gly-MePhe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	45.8	89.6	1 + 3	A + B
17	Bpoc-Gly-Phe-Phe-Tyr(Bzl)-OPic	37.3	75.5	1 + 2	B
18	Bpoc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic	49.6	87	1 + 2	A + B
53	Boc-Lys(Pipoc)-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic	54.1	92	1	B
54	Boc-Lys(Pipoc)-~~[~~-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-~~]~~$_2$-OPic	72		1 (7+6)	B

Coupling Method: 1 DCCI/HOBt
2 Active ester
3 Mixed anhydride

Isolation Procedure A Citric acid
B Amberlyst 15

Figure 39

Thus these results clearly demonstrate the continued success of the 4-picolyl ester method in preparing pure peptides in high yields.

EXPERIMENTAL SECTION

GENERAL INFORMATION

Chromatography

Thin layer chromatograms were run on unbaked Kieselgel $HF_{254/366}$ plates: R_F values refer to the following systems (proportions by volume): n-butanol-acetic acid-water (A2) 10:1:3; toluene, chloroform, ethanol (B) 12:12:1; methanol-chloroform (E4) 1:9; ethyl acetate-pyridine-acetic acid-water mixtures (G1) 60:20:6:11, (G3) 120:20:6:11, (G4) 10:20:6:11; n-butanol-pyridine-acetic acid-water (H) 15:10:3:12; methanol-acetic acid-water (M) 4:2:1; chloroform-methanol-acetic acid (N) 17:2:1.

Spots were detected by ultra-violet illumination, by ninhydrin (1% w/v solution in n-butanol), and by chlorine and starch-iodide[196] (1% starch, 1% potassium iodide, w/v solution in water). When testing for purity, the loading of the plate was 0.7-1.0 mg.

For paper chromatography and electrophoresis the following selective stains were used according to the procedure recommended by Offord.[197]

(i) Cadmium-ninhydrin stain for amino groups

Solution A. 1% w/v ninhydrin in acetone, analytical reagent grade. Solution B. Cadmium acetate (1 g) dissolved in glacial acetic acid (100 ml) and water (50 ml). The chromatogram was dipped in a mixture of 100 volumes of A and 15 volumes of B and dried. Various colours are obtained depending to some extent on the N-terminal and sometimes the penultimate residues, i.e.

glycine, proline, cysteine - yellow

threonine, serine - yellow-orange

asparagine - orange

others - red.

Valine and isoleucine are slow to react and take between 18-24 hours for the colour (red) to reach its maximum intensity.

(ii) α-Nitroso-β-naphthol stain for tyrosine

Solution A. α-Nitroso-β-naphthol dissolved in acetone to give a 1% solution. Solution B. Fuming nitric acid added to acetone to give a 10% solution. The chromatogram was dipped in solution A, dried, dipped in solution B and after superficial drying (7-10 minutes) was warmed over a heating element. Tyrosine stained red while tryptophan gave a brown colour.

(iii) Hydrazide reagent[198]

Solution A. Ferric chloride (0.3M) in acetic acid (0.1M). Solution B. Potassium ferricyanide (0.2M) in acetic acid (0.1M). The chromatogram was sprayed with a mixture of equal volumes of solutions A and B. Hydrazides gave an intense blue colour.

High voltage electrophoresis was performed on Whatman chromatography paper (Nos. 1 and 3MC) in glass tanks containing White Spirit 100 (Esso Ltd.) essentially as described by Michl.[199] The usual voltage gradient employed was 60-80 v/cm depending upon the size of paper used. The buffer compositions of the systems used were:

pH 6.5 Pyridine-acetic acid-water (25:1:225 v/v).

pH 1.9 2% Formic acid + 8% acetic acid in water (v/v).

Measurement of electrophoretic mobilities was facilitated by the presence of a marker mixture spotted on the origin prior to running. The composition was as follows:

amino acids - lysine, histidine, arginine, aspartic acid.

dyes - ε-dinitrophenyl-lysine, xylene cyanol FF (FF), orange G, acid Fuchsin and Methyl green.

Physical measurements

Melting points were determined with a Kofler hot-stage apparatus; they are uncorrected.

Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter (1 dm. cell).

Infra-red spectra were recorded on a Unicam SP1000 spectrophotometer.

Ultra-violet spectra were determined on a Unicam SP800A spectrophotometer.

Nuclear magnetic resonance spectra were recorded by Mrs. E. Richards on Perkin-Elmer R10 60 MHz or R14 100 MHz spectrometers.

Elemental analysis was carried out by Dr. F. B. Strauss.

Amino acid analyses were carried out on a Jeol JLC-5AH automatic analyser. Normally 2×10^{-4} mg of peptide was hydrolysed in hydrochloric acid (6M, 1.5-2.0 ml) for 18-24 hours at 110° in vacuum. The residue obtained upon evaporation was dissolved in hydrochloric acid (0.01M) to give solutions containing 50-100 nanomoles per ml and were submitted for analysis.

The colour constant for N-methyl-L-phenylalanine was determined from a known quantity (200 nanomole ml^{-1}) of unhydrolysed prepared sample.

Solutions in organic solvents were dried with magnesium sulphate or sodium sulphate.

Evaporations were carried out at room temperature on a rotary evaporator with either a water or oil pump.

Solvents and reagents

Diethyl ether was stored over calcium chloride and filtered before use.

Dichloromethane and chloroform were passed through a column of alumina (grade H) before use to remove traces of formaldehyde, ethanol, etc.

Tetrahydrofuran and dioxan were passed through a column of alumina (grade H) before use and were tested for absence of peroxides by the starch-iodide reaction. For use with Amberlyst-15 and coupling reactions, dimethylformamide must be free from dimethylamine and formic acid. It was stirred overnight with solid potassium carbonate (to neutralise formic acid), filtered, refluxed over phthalic acid (5% by weight) for one hour, and distilled under nitrogen using a fractionating column at 45°/15 mm, discarding the initial 5-10% of the distillate. The remainder was checked for amines by the fluorodinitrobenzene method of Stewart and Young,[200] using their acceptability limits.

Triethylamine, N,N-di-isopropylethylamine and N-methylmorpholine were stored over potassium hydroxide and redistilled, rejecting the initial 5% distillate, b.p.s 89.7-89.9°, 30.2-30.5°/15 mm and 115-116° respectively.

Thionyl chloride was distilled from triphenyl phosphite (5% v/v), b.p. 78.8°.

Pivaloyl chloride was distilled and the initial 5% distillate rejected, b.p. 105-6°.

3-Bromopyridine was detected for traces of pyridine by thin layer chromatography [R_F's (E4) 0.63 and 0.50 respectively]. Any pyridine was removed by fractional distillation (pyridine, b.p. 32°/13 mm; 3-bromopyridine, b.p. 58.8°/13 mm).

Amberlyst-15 resin was supplied in the H^+ form. It was prepared for use by adding the resin (V ml) to an equal volume of dimethylformamide (standard reagent) and leaving for several days to extract coloured products. It was then poured into a column, drained and washed with 10V ml each of methanol, deionised water, sodium hydroxide (1.0M), water, methanol, sulphuric acid (0.5 M), water and finally methanol. Each batch was titrated by adding an excess (25 ml) of sodium hydroxide (1.0M) to 5 ml resin, stirring for 30 minutes, when 5 ml portions were back-titrated

with hydrochloric acid (1.0M). The resin usually had a capacity of 1.75-1.85 milliequivalents per ml. When required for use, the resin (V ml) was washed with ethyl acetate (3V ml) and saturated overnight with 3-bromopyridine in ethyl acetate (V ml, 20% v/v). Excess 3-bromopyridine was detected by thin layer chromatography (E4, ultra-violet light), and washed off the column with ethyl acetate (2V ml) and recovered by fractional distillation. The resin was then washed with the peptide application solvent.

t-Butoxycarbonyl azide was prepared from the corresponding hydrazide by the method of Carpino[201] and redistilled at 17-20°/0.1 mm pressure. Yield 90-95%.

1-Hydroxybenzotriazole was prepared by the method of König and Geiger.[171] m.p. 156-8°, yield 67% (lit.[171] m.p. 157°, yield 73%).

N-Hydroxypiperidine was prepared by the method of Sabel[202] by Mr. N. N. Petter. m.p. 34-7°, yield 78% (lit.[202] m.p. ~ 40°, yield 62%).

Diphenyldiazomethane was prepared by the method of Miller.[203] m.p. 30°, yield 90%, $\nu_{max.}$ (CCl_4) 2025 cm^{-1} (lit.[203] m.p. 29-32°, yield 89%).

The following α-t-butoxycarbonyl amino acid derivatives were prepared by the method of Schnabel[177] by Mr. N. N. Petter.

α-t-Butoxycarbonyl-L-alanine m.p. 81-3°, yield 86% (lit.[177] 80-2°, yield 96%).

α-t-Butoxycarbonylglycine m.p. 89-90°, yield 85% (lit.[177] 88.5-90°, yield 90%).

α-t-Butoxycarbonyl-L-phenylalanine m.p. 85-8°, yield 96% (lit.[177] 84-6°, 91%).

α-t-Butoxycarbonyl-L-proline m.p. 134-6°, yield 60% (lit.[177] 136-7°, 96%).

α-t-Butoxycarbonyl-O-benzyl-L-threonine was prepared by the method of Mizoguchi[204] et al., m.p. 113-4°, yield 66% (lit.[204] m.p. 115-6°).

α-t-Butoxycarbonyl-O-benzyl-L-tyrosine was prepared by the method of Morley.

m.p. 110-2°, yield 58% (lit.[205] m.p. 112-3°, yield 75%).

α-Benzyloxycarbonylglycine was prepared by the method of De Tar[206] et al. by Mr. N. N. Petter, m.p. 120°, yield 80% (lit.[206] m.p. 120°, yield 89%).

L-Proline 4-picolyl ester dihydrobromide was prepared by the method of Camble,[150] m.p. 161-2°, yield 86% (lit.[150] m.p. 158-162°, yield 88%).

α-Benzyloxycarbonyl-L-alanine 4-picolyl ester was prepared by the method of Camble,[150] m.p. 111-113°, yield 54% (lit.[150] m.p. 111-112.5°, yield 64%).

Piperidino-oxycarbonylglycine was prepared by the method of Stevenson,[170] m.p. 118-119°, yield 75% (lit.[170] m.p. 119-20°, yield 80%).

2-(p-Biphenylyl)-isopropyloxycarbonylglycine N-hydroxysuccinimide ester was a generous gift from Dr. J. H. Jones[207] [m.p. 131-4°, $\nu_{max.}$ 1829, 1794, 1746 cm^{-1}, 1725 cm^{-1} shoulder ($CHCl_3$). τ ($CDCl_3$) 2.3-2.8 (9H, complex, aromatic biphenyl), 4.68 (1H, t, J 3 Hz, urethane NH), 5.78 (2H, q, J 3 Hz, CH_2), 7.26 (4H, s, $OC.CH_2.CH_2.CO$), 8.20 (6H, s, $CH_3.C.CH_3$). $C_{22}H_{22}N_2O_6$ requires C, 64.05; H, 5.5; N, 6.9%. Found: C, 64.4; H, 5.4; N, 6.8%].

Each operation of a generalised coupling reaction will now be outlined in greater detail than is practical for each individual coupling step.

Removal of α-amino protection: "deprotection step"

t-Butoxycarbonyl has normally been used for α-amino protection and was removed by treatment with trifluoroacetic acid. The protected peptide was dissolved in refrigerated anhydrous trifluoroacetic acid (2-5 ml per mmole) and deprotection confirmed by thin layer chromatography (G1 and G3). After 15-30 minutes evaporation of the clear solution at 0.5-1.0 mm usually gave a clear gum which on trituration with diethyl ether, to remove traces of trifluoroacetic acid, afforded the

white trifluoroacetate salt. This was not characterised but used immediately for the next stage.

Liberation of the amino component

When the coupling reaction was to be carried out in dimethylformamide, the salt of the amino component was dissolved in this solvent, excess (approximately four equivalents) triethylamine was added with cooling. After 10-15 minutes the excess triethylamine was removed at room temperature by rotary evaporation although prolonged evaporation was avoided to prevent loss of triethylamine from the triethyl-ammonium trifluoroacetate, regenerating the salt of the amino component. For coupling reactions in other solvents, usually tetrahydrofuran, it was found convenient to dissolve or suspend the salt cooled in chloroform and add the excess triethylamine; when solution was complete the solution was evaporated to dryness and the residual amino component taken up in the required solvent.

For coupling reactions involving amino acid esters or dipeptide esters in which dioxopiperazine formation was an important side reaction, the salt was dissolved in the coupling solvent and the acylating agent and finally a slight excess of the hindered amine, N,N-di-isopropylethylamine, was added.

Coupling reactions

In the majority of cases, N,N'-dicyclohexylcarbodi-imide and 1-hydroxy-benzotriazole have been used to facilitate the coupling reaction. Tetrahydrofuran and dimethylformamide were exclusively used as solvents; the former was preferred because of its greater volatility.

The acylating amino acid, N,N'-dicyclohexylcarbodi-imide and 1-hydroxy-benzotriazole were used in 1.05-1.25 molar proportions although in the case of

highly hindered couplings, 1.5 molar proportions have been used. The "preactivation procedure" was used, the active ester was formed after one hour at 0^{o} and a similar period at room temperature before the amino component was added. Active ester and mixed anhydride couplings have also been used successfully. The pH should be between four and five at this stage. An exceptionally low pH usually indicated that insufficient triethylamine had been used and this was overcome by the cautious addition of N,N-di-isopropylethylamine to the required pH. A high pH suggested incomplete removal of triethylamine and was corrected by removing the excess base by evaporation or by adding 1-hydroxybenzotriazole. The mixture was stirred at room temperature and the reaction followed by thin layer chromatography (E4 and G3) and the plates ($HF_{254/366}$) examined by ultra-violet light and ninhydrin. The amino component was readily observed by its immediate reaction with ninhydrin at room temperature to give a distinctive yellow colour. α-t-Butoxycarbonyl amino acids and peptides usually gave a purple colour with ninhydrin on heating to 100^{o} in an oven. The reaction was continued until thin layer chromatography revealed no amino component (sensitivity 0.1-0.2%) usually within one hour of addition of the amino component.

Isolation: (i) Citric acid

The citric acid procedure was found to be extremely convenient and gave high yields but its application was limited to cases in which the protected 4-picolyl ester (i) partitions successfully between aqueous citric acid and a water immiscible organic solvent, and (ii) could be extracted from the basified layer into an organic solvent. In some cases it was possible to purify hexapeptides by this method though many protected peptides were too lipophilic.

In this method an equal volume of analar ethyl acetate was added to the coupling mixture which was stirred for one hour at 0°. The dicyclohexylurea and 1-hydroxybenzotriazole were filtered, washed with more ethyl acetate and the combined filtrate was evaporated to give an oil or foam. The residue was taken up in a water-immiscible solvent, usually diethyl ether or ethyl acetate and repeatedly extracted with equal volumes of citric acid (0.7M) until no product remained in the organic layer (thin layer chromatography E4 and G3). The combined aqueous fractions were then basified with solid sodium hydrogen carbonate to pH 8.5 and the product was extracted into ethyl acetate (occasionally dichloromethane or chloroform). The final organic solutions were washed with brine (sodium citrate is slightly soluble in wet ethyl acetate), dried and evaporation gave the protected peptide in yields of 85-98%.

(ii) Amberlyst-15 Method

The isolation on Amberlyst-15 (saturated with 3-bromopyridine) was advantageous in that it could be applied to every coupling reaction so far examined and where the citric acid method was inappropriate this method was extremely satisfactory. Yields were, perhaps slightly lower than the citric acid methods, in general 80-95%.

The exchange of the picolyl ester for 3-bromopyridine on the resin appeared to be rather slow, and although it was convenient to use columns of resin, the procedure was essentially batch-wise. The preferred solvent for application to the column was ethyl acetate but dichloromethane, chloroform and dimethylformamide have been used. The dicyclohexylurea and 1-hydroxybenzotriazole were removed as before in the citric acid procedure and the oil or foam formed on evaporation was

taken up in ethyl acetate if possible, or dichloromethane, chloroform or mixtures thereof, and washed with saturated sodium hydrogen carbonate, to remove traces of 1-hydroxybenzotriazole, water, to remove salts which reduce the capacity of the column, brine and dried. The solution could then be applied to the Amberlyst-15 (3-bromopyridinium form, 20% v/v, approximately 15 ml prepared resin per 1 milli-equivalent of protected peptide picolyl ester). If the product was not soluble in any of these organic solvents, the washings could be carried out on the residue from evaporation; the final residue was dried at 1 mm pressure and then taken up in dimethylformamide for application to the column.

The product was applied to the column in a minimum volume and rocked for about one hour until thin layer chromatography (E4 and G3) indicated complete absorption. Contaminants were washed off by means of the application solvent which was continued until no more contaminant was detected in the eluate by thin layer chromatography which also detected any loss of product. The application to the resin and subsequent washes have now been automated and batches of resin (15-100 ml) have been successfully treated.[156] The product was then eluted by a mixture of refrigerated pyridine (analar) in dimethylformamide (40% v/v), to prevent any removal of protecting groups by localised liberation of heat, such as piperidino-oxycarbonyl. The eluate was evaporated to dryness at 1 mm pressure and the residue was triturated with diethyl ether, or precipitated from an organic solvent by diethyl ether or 40/60 petroleum ether, to remove traces of 3-bromopyridine. 3-Bromo-pyridine could be extracted from highly lipophilic products by water. The products obtained were normally pure by elemental and amino acid analysis.

2,4,5-Trichlorophenyl chloroformate

This was prepared by the method of Morley.[179] 2,4,5-Trichlorophenol (125 g, 0.63 mole) was partly dissolved in toluene (250 ml) to form a thick slurry and was added to a mechanically stirred solution of phosgene (100 g, 72 ml, 1.0 mole) in toluene (50 ml) at -30°. N,N-Dimethylaniline (77.5 g, 0.64 mole) was added at such a rate that the reaction temperature was maintained between 5-10°. As the base was added, trichlorophenol first dissolved to give a clear solution, then dimethylaniline hydrochloride precipitated. The mixture was stirred at room temperature overnight. Ice-water (100 g) was added to the reaction mixture, cooled in a methanol-solid carbon dioxide bath, and stirred cautiously. The mixture was filtered from a small quantity of bis-(2,4,5-trichlorophenyl) carbonate. The organic layer in the filtrate was separated, washed with brine (75 ml) and dried and evaporated to yield crude, solid 2,4,5-trichlorophenyl chloroformate (158 g, 96%) of m.p. 61.5-63.5° and $\nu_{max.}$ (CCl_4) 1790 cm^{-1}. Bis-(2,4,5-trichlorophenyl) carbonate had m.p. 169-172° and $\nu_{max.}$ (CCl_4) 1795 cm^{-1}.

Piperidino 2,4,5-trichlorophenyl carbonate

To a stirred solution of crude 2,4,5-trichlorophenyl chloroformate (52 g, 0.2 mole) in dichloromethane (200 ml) cooled to -10° was added dropwise a solution of 1-hydroxypiperidine (20.2 g, 0.2 mole) in dichloromethane (60 ml). N,N-Dimethylaniline (25 ml, 24.2 g, 0.2 mole) was added dropwise to the mixture keeping the reaction temperature between -15° and -10° with stirring for a few minutes. The solution was immediately washed with hydrochloric acid (2M, 200 ml) and filtered from a small quantity of precipitate. The organic layer was washed with saturated sodium hydrogen carbonate (2 x 100 ml), brine (2 x 100 ml), dried and

evaporated to give the mixed carbonate (60.5 g, 93%) as a white solid of m.p. 94-97° and $\nu_{max.}$ (CCl_4) 1795 cm^{-1}. $C_{12}H_{12}NO_3Cl_3$ requires C, 44.4; H, 3.73; N, 4.32; Cl, 32.77%. Found: C, 44.67; H, 3.98; N, 4.31; Cl, 32.91%.

Stevenson[170] obtained a yield of 60% after recrystallisation from ethyl acetate-petroleum ether with m.p. 97-8°, and $\nu_{max.}$ (CCl_4) 1790 cm^{-1}.

ε-Benzyloxycarbonyl-L-lysine

L-lysine hydrochloride (36.6 g, 0.2 mole) was dissolved in water (90 ml) and warmed until the solution was just boiling. Copper sulphate pentahydrate (26.0 g, 0.14 mole) was added giving an immediate dark blue colour. Concentrated sodium carbonate solution was added to neutralise the sulphuric acid produced and the dark blue solution was filtered from a trace of precipitate. The solution was brought to pH 10.0 with sodium hydroxide (2M) and cooled to 0° in an ice/salt bath. Benzyl chloroformate (40 ml, 0.25 mole) was added slowly but with vigorous stirring over 1 hour maintaining the solution at pH 10.0 and 0°. Stirring was continued for an additional hour at room temperature. The light-blue complex was filtered and washed with ice-cold water followed by ice-cold ethanol.

The light-blue complex was boiled with an aqueous solution of ethylene-diamine-tetra-acetic acid disodium salt (93 g, 0.25 mole) until the colour of the solution disappeared. The solution was cooled, filtered and the process repeated with fresh reagent leaving a white solid. Any last trace of copper ion was removed by passing hydrogen sulphide through a boiling 30% aqueous acetic acid solution containing the product while maintaining vigorous stirring. The hot solution was quickly filtered to remove copper sulphide and the crude product crystallised on cooling. ε-Benzyloxycarbonyl-L-lysine (35 g, 55%) was recrystallised from the

minimum quantity of 30% aqueous acetic acid and washed with ice-cold water. m.p. 246-8°, R_F (G4) 0.67; R_F (M) 0.75; R_F (A2) 0.35. $[\alpha]_D^{20}$ +15.8° (c 2.0 in 2M HCl) [lit.[176] m.p. ca. 235°, $[\alpha]_D^{25}$ +14.0 (c 2.86 in dil.HCl)].

α-t-Butoxycarbonyl-ε-benzyloxycarbonyl-L-lysine

ε-Benzyloxycarbonyl-L-lysine (17.3 g, 0.062 mole) was dissolved in 50% aqueous dioxan (100 ml) at 30-35° and brought to pH 10.2 with sodium hydroxide (4M) on a "pH stat". t-Butoxycarbonyl azide (12 g, 0.08 mole) was added and the mixture stirred at 30° and pH 10.2 for 24 hours, when t.l.c. (E4, G3) indicated that the reaction was complete. The reaction mixture was extracted with diethyl ether (3 x 50 ml), cooled to 0° and acidified to pH 3.5 with solid citric acid. The product was extracted with ethyl acetate (4 x 75 ml) and the combined organic phases washed with water (2 x 100 ml), brine (2 x 150 ml), dried and evaporated to give α-t-butoxycarbonyl-ε-benzyloxycarbonyl-L-lysine (21.3 g, 91%) as a chromatographically pure colourless oil. R_F (G3) 0.85, R_F (G1) 0.90 [lit.[178] yield 62%].

α-t-Butoxycarbonyl-L-lysine

α-t-Butoxycarbonyl-ε-benzyloxycarbonyl-L-lysine (55 g, 0.145 mole) was dissolved in 80% aqueous ethanol (250 ml) with 5% palladium on charcoal (5 g). Hydrogenation at atmospheric pressure was carried out for 5 hours when t.l.c. (G3) indicated that the reaction was complete. The mixture was filtered through Celite which was washed with ethanol (50 ml), 50% aqueous ethanol (50 ml) and water (50 ml). The filtrate and washings were evaporated to dryness giving a white foam. The foam was taken up in warm ethanol (200 ml), filtered hot and allowed to

crystallise. The white solid formed on standing at -20° was filtered, washed with diethyl ether to give α-t-butoxycarbonyl-L-lysine (29.1 g, 83.2%) of m.p. 205-7°, R_F (A2) 0.36; R_F (G1) 0.11. $[\alpha]_D^{21}$ -9.24° (c 1.13 in HAc); $[\alpha]_D^{22}$ +22.0° (c 1.11 in MeOH). $C_{11}H_{22}O_4N_2$ requires C, 54.64; H, 9.00; N, 11.37%. Found: C, 54.65; H, 9.01; N, 11.28% [lit.[208] m.p. 204-5°, 201.5-203°, $[\alpha]_D^{21}$ -10.7° (c 0.85 in HAc)].

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine dicyclohexylammonium salt

α-t-Butoxycarbonyl-L-lysine (23.5 g, 0.0955 mole) was suspended in dimethylformamide (100 ml) and 1,1,3,3-tetramethylguanidine (12.5 ml, 0.0955 mole) was added dropwise. Piperidino 2,4,5-trichlorophenyl carbonate (45.1 g, 0.143 mole) was added slowly over 1.5 hours to the solution. T.l.c. (E4, G3) indicated that the reaction was complete after stirring for 4 hours at room temperature. The solution was evaporated and the residue taken up in chloroform (200 ml). The solution was washed with citric acid (0.7M, 3 x 200 ml), brine (2 x 100 ml) and evaporated to give a light brown oil which was taken up in sodium hydroxide (0.6M, 300 ml) and washed with dichloromethane (3 x 200 ml). The aqueous layer was acidified at 0° with solid citric acid to pH 3.5 and the liberated oil extracted with ethyl acetate (3 x 200 ml). The combined extracts were washed with water (2 x 50 ml), brine (2 x 50 ml) and dried. T.l.c. (E4, G3) detected a single ninhydrin spot contaminated by a trace of 2,4,5-trichlorophenol. Evaporation gave the crude product as a white foam which was taken up in diethyl ether (75 ml); dicyclohexylamine (19 ml, 0.0955 mole) in diethyl ether (50 ml) was added. Cooling overnight at -20° gave a white precipitate which was recrystallised from propan-2-ol to give α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine dicyclohexylammonium salt (35.9g, 68%)

as a chromatographically pure white powder of m.p. 135-6°, R_F (A2) 0.68; R_F (G1) 0.92; R_F (G3) 0.80; R_F (N) 0.67, $[\alpha]_{589}^{20}$ +8.3° (c 1.07 in MeOH). $C_{29}H_{54}N_4O_6$ requires C, 62.80; H, 9.75; N, 9.90%. Found: C, 63.03; H, 9.88; N, 9.75. τ ($CDCl_3$) -0.3 (1H, s, $CO_2\underline{H}$), 3.2 (1H, t, pipoc $N\underline{H}$), 4.7 (1H, complex, t.Boc $N\underline{H}$), 5.7 (1H, complex, NH.$C\underline{H}$.CO_2H), 6.6-6.9 (4H, complex, piperidino 2- and 6- protons), 7.1-7.5 (2H, complex, .$C\underline{H}_2$.NH.CO.O), 7.9-9.0 (12H, complex, piperidino 3-, 4-, 5-protons; lysyl NH.$(C\underline{H}_2)_3$.CH), 8.5 (9H, s, $(C\underline{H}_3)_3$C.).

Synthesis of the protected natural octapeptide

L-Alanine 4-picolyl ester dihydrobromide

This was prepared in an analogous manner to that of other 4-picolyl ester dihydrobromides.[150] α-Benzyloxycarbonyl-L-alanine 4-picolyl ester (9.0 g, 2.86 mmole) was dissolved in glacial acetic acid (15 ml) and a solution of hydrogen bromide in acetic acid (45 ml; 45% w/v) was added slowly with extensive stirring. After 1 hour, diethyl ether (200 ml) was added and the precipitate was collected as a white sticky solid, washed with more diethyl ether (3 x 200 ml) and recrystallised from aqueous acetone giving the 4-picolyl ester dihydrobromide (9.07 g, 96.5%) as white crystals of m.p. 165-7°, R_F (G4) 0.73; R_F (H) 0.45, solvents (E4), (G1), (G3) and (A2) gave streak R_F 0.20-0.30, $[\alpha]_{589}^{20}$ +2.03° (c 1 in Me_2NCHO). $C_9H_{14}N_2O_2Br_2$ requires C, 31.6; H, 4.1; N, 8.2; Br, 46.8%. Found: C, 31.48; H, 3.91; N, 7.97; Br, 46.49%. τ (D_2O) 1.25 (2H, d, pyridyl 2- and 6-protons), 1.90 (2H, d, pyridyl 3- and 5-protons), 4.35 (2H, s, pyridyl .$C\underline{H}_2O$), 5.32 (1H, q, NH_3^+.$C\underline{H}$.(CH_3).CO) and 8.30 (3H, d, NH_3^+.CH($C\underline{H}_3$).CO).

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (21)

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine dicyclohexylammonium salt (13.2 g, 24 mmole) was partitioned between citric acid (0.7M, 250 ml) and ethyl acetate (250 ml). The aqueous layer was re-extracted with ethyl acetate (2 x 100 ml) and the combined organic fractions washed with water (2 x 50 ml), brine (2 x 50 ml) and dried. Evaporation gave the free acid as a chromatographically pure oil (8.95 g, 24 mmole).

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine (8.95 g, 24 mmole) and 1-hydroxybenzotriazole (3.67 g, 24 mmole) were dissolved in tetrahydrofuran (50 ml). N,N'-Dicyclohexylcarbodi-imide (4.95 g, 24 mmole) was added and the mixture stirred at 0° for 1 hour and room temperature for 1 hour.

L-Alanine 4-picolyl ester dihydrobromide (6.84 g, 20 mmole) was suspended in chloroform (50 ml) at 0°, N,N-di-isopropylethylamine (7 ml, 40 mmole) was added; the solution was evaporated to an oil and taken up in tetrahydrofuran (20 ml) and added to the acylating mixture. T.l.c. (E4, G3) indicated that the reaction was complete within 3 hours. Isolation by the citric acid procedure gave the protected dipeptide (21) (9.86 g, 95.4%) as a chromatographically pure, white foam of m.p. 29-30°, R_F (E4) 0.59; R_F(G1) 0.92; R_F (G3) 0.93; R_F (A2) 0.47; R_F (H) 0.79, $[\alpha]_{589}^{20}$ -16.2°; $[\alpha]_{578}^{20}$ -16.5°; $[\alpha]_{546}^{20}$ -18.4°; $[\alpha]_{436}^{20}$ -31.7°; $[\alpha]_{365}^{20}$ -52.8° (c 1.07 in $CHCl_3$). $C_{26}H_{41}N_5O_7$ requires C, 58.30; H, 7.72; N, 13.08%. Found: C, 58.31; H, 7.81; N, 12.97%. Amino-acid analysis gave Lys, 1.00; Ala, 1.00. Based on Ala = 1.00. τ ($CDCl_3$) 1.41 (2H, d, pyridyl 2- and 6-protons), 2.76 (2H, d, pyridyl 3- and 5-protons), 3.06 (1H, peptide NH), 3.30 (1H, complex, piperidino-oxycarbonyl NH), 4.76 (1H, urethane NH), 4.81 (2H, s, pyridyl.CH_2.O), 5.38 (1H,

complex, NH.C<u>H</u>.$(CH_2)_4$), 5.90 (1H, quartet, J 8 c/sec, NH.C<u>H</u>.CH_3), 6.80 4H, complex, piperidino 2- and 6-protons), 7.31 (2H, complex, lysyl.C<u>H</u>$_2$.NH.CO.), 8.0-8.9 (12H, complex, piperidino 3-, 4- and 5-protons, lysyl $CH_2(C\underline{H}_2)_3$.CH), 8.50 (3H, d, J 8 c/sec, CH.C<u>H</u>$_3$), 8.56 (9H, s, $(C\underline{H}_3)_3$C.).

<u>α-t-Butoxycarbonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester</u> (22)

α-t-Butoxycarbonyl-L-proline (6.76 g, 24.4 mmole) was dissolved in tetrahydrofuran (50 ml) and 1-hydroxybenzotriazole (3.81 g, 24.4 mmole) followed by N,N'-dicyclohexylcarbodi-imide (5.02 g, 24.4 mmole) was added. This acylating mixture was stirred at 0° for 1 hour and 1 hour at room temperature.

The dipeptide (21) (8.82 g, 17 mmole) was shaken in trifluoroacetic acid (45 ml, 600 mmole) for 20 minutes in an ice-bath and evaporated to a clear oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (20 ml) and added to the previously activated mixture. The solution was brought to pH 8 with N,N-di-isopropylethylamine (12 ml, 69 mmole) and stirred at room temperature. T.l.c. (E4, G3) indicated that the reaction was complete within 1 hour. Extraction by the citric acid procedure gave the <u>protected tripeptide</u> (22) (9.77 g, 94.5%) as a chromatographically pure white foam of m.p. 45-47°, R_F (E4) 0.43; R_F (G1) 0.78; R_F (G3) 0.72; R_F (G4) 0.82; R_F (A2) 0.55, $[\alpha]^{20}_{589}$ -65.0°; $[\alpha]^{20}_{578}$ -68.4°; $[\alpha]^{20}_{546}$ -78.0°; $[\alpha]^{20}_{436}$ -140.5°; $[\alpha]^{20}_{365}$ -233.0° (c 0.94 in $CHCl_3$). $C_{31}H_{48}N_6O_6$ requires C, 57.76; H, 7.58; N, 12.85%. Found: C, 58.05; H, 7.96; N, 12.87%. Amino-acid analysis gave Pro, 1.02; Lys, 1.03; Ala, 1.00. Based on Ala = 1.00. τ ($CDCl_3$) 1.41 (2H, d, pyridyl 2- and 6-protons), 2.75 (2H, d, pyridyl 3- and 5-protons), 3.05 (1H, peptide, N<u>H</u>), 3.24 (1H, complex,

piperidino-oxycarbonyl NH), 4.80 (2H, s, pyridyl.CH_2.O), 5.40 (1H, complex, NH.CH.$(CH_2)_4$), 5.80 (2H, complex, NH.CH.CO,; NH.CH.CH_3), 6.5 (2H, complex, prolyl N.CH_2.CH_2), 6.82 (4H, complex, piperidino 2- and 6-protons), 7.30 (2H, complex, lysyl .CH_2.NH.CO.), 8.0-8.9 (16H, complex, piperidino 3-, 4- and 5-protons; lysyl $(CH_2)_3$.CH.; prolyl .$(CH_2)_2$.CH), 8.50 (3H, d, J 8 c/sec, CH.CH_3), 8.56 (9H, s, $(CH_3)_3$.C).

α-t-Butoxycarbonyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (23)

α-t-Butoxycarbonyl-O-benzyl-L-threonine (5.56 g, 18 mmole) and 1-hydroxybenzotriazole (2.84 g, 18 mmole) were dissolved in tetrahydrofuran (50 ml). N,N'-Dicyclohexylcarbodi-imide (3.71 g, 18 mmole) was added and the acylating mixture stirred at 0° for 1 hour and room temperature for 1 hour.

The tripeptide (22) (9.5 g, 15 mmole) was shaken with trifluoroacetic acid (40 ml, 520 mmole) for 20 minutes at 0° and evaporated to give a colourless oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (25 ml) and added to the acylating mixture. N,N-di-isopropylethylamine (5.35 ml, 30 mmole) was added to bring the solution to pH 8 which was stirred for 1 hour at room temperature when t.l.c. (E4, G3) indicated that the reaction was complete. Isolation as before by the citric acid procedure gave the protected tetrapeptide (23) (11.1 g, 90.6%) as a chromatographically pure, white foam of m.p. 57-59°, R_F (E4) 0.44; R_F (G1) 0.88; R_F (G3) 0.76; R_F (G4) 0.80; R_F (A2) 0.52, $[\alpha]_{589}^{20}$ -41.8°; $[\alpha]_{578}^{20}$ -44.5°; $[\alpha]_{546}^{20}$ -50.4°; $[\alpha]_{436}^{20}$ -90.6°; $[\alpha]_{365}^{20}$ -152.5° (c 1.13 in $CHCl_3$). $C_{42}H_{61}N_7O_{10}$ requires C, 61.2; H, 7.40; N, 11.90%. Found: C, 61.42; H, 7.66; N, 11.65%. Amino-acid analysis gave Thr, 0.99; Pro, 0.99; Lys, 1.04; Ala, 1.00. Based on Ala = 1.00.

α-t-Butoxycarbonyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (24)

α-t-Butoxycarbonyl-O-benzyl-L-tyrosine (4.02 g, 10.8 mmole) and 1-hydroxybenzotriazole (1.65 g, 10.8 mmole) were dissolved in tetrahydrofuran (20 ml). N,N'-Dicyclohexylcarbodi-imide (2.23 g, 10.8 mmole) was added and the mixture stirred at 0° for 1 hour and at room temperature for 2 hours.

The tetrapeptide (23) (7.40 g, 9.0 mmole) was shaken in trifluoroacetic acid (40 ml, 520 mmole) for 20 minutes at 0° and the solution was evaporated to give a slightly yellow oil. Trituration with 30-40° petroleum ether gave a white solid which was dissolved in chloroform (50 ml). The solution was cooled to 0° and triethylamine (6.0 ml, 44 mmole) added; evaporation gave a yellow oil which was dissolved in tetrahydrofuran (25 ml) and added to the acylating mixture. T.l.c. (E4, G3) indicated that the reaction was complete within 1 hour and isolation by the citric acid procedure gave the protected pentapeptide (24) (9.84 g, 96.4%) as a chromatographically pure, white powder of m.p. 72-74°, R_F (E4) 0.47; R_F (G1) 0.95; R_F (G3) 0.73; R_F (A2) 0.65, $[\alpha]_{589}^{20}$ -30.7°; $[\alpha]_{578}^{20}$ -31.6°; $[\alpha]_{546}^{20}$ -36.0°; $[\alpha]_{436}^{20}$ -65.1°; $[\alpha]_{365}^{20}$ -108.5° (c 1.19 in $CHCl_3$). $C_{58}H_{76}N_8O_{12}$ requires C, 64.66; H, 7.11; N, 10.40%. Found: C, 64.38; H, 7.20; N, 10.20%. Amino-acid analysis gave Tyr, 0.99; Thr, 0.99; Pro, 1.00; Lys, 1.04; Ala, 1.00. Based on Ala = 1.00.

α-t-Butoxycarbonyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (25)

α-t-Butoxycarbonyl-L-phenylalanine (0.636 g, 2.4 mmole) and 1-hydroxybenzotriazole (0.367 g, 2.4 mmole) were dissolved in tetrahydrofuran (15 ml).

N,N'-Dicyclohexylcarbodi-imide (0.494 g, 2.4 mmole) was added and the mixture stirred at 0° for 1 hour and 1.5 hours at room temperature.

The pentapeptide (24) (2.152 g, 2.0 mmole) was shaken in trifluoroacetic acid (10 ml, 130 mmole) for 15 minutes at 0° and evaporated to give an oil. Trituration with diethyl ether gave a white solid which was dissolved in chloroform (20 ml). The solution was cooled to 0° and triethylamine (1.12 ml, 8.0 mmole) added; evaporation gave a semi-solid which was dissolved in tetrahydrofuran (5 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete after 25 minutes. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected hexapeptide (25) (2.20 g, 90%) as a chromatographically pure, white powder of m.p. 99-100°, R_F (E4) 0.61; R_F (G1) 0.97; R_F (G3) 0.90; R_F (A2) 0.69, $[\alpha]_{589}^{20}$ -32.4°; $[\alpha]_{578}^{20}$ -34.2°; $[\alpha]_{546}^{20}$ -38.9°; $[\alpha]_{436}^{20}$ -68.7°; $[\alpha]_{365}^{20}$ -114.0° (c 1.05 in $CHCl_3$). $C_{67}H_{85}N_9O_{13}.H_2O$ requires C, 64.75; H, 7.06; N, 10.12%. Found: C, 64.71; H, 6.97; N, 10.16%. Amino-acid analysis gave Phe, 1.02; Tyr, 0.95; Thr, 0.97; Pro, 1.00; Lys, 0.98; Ala, 1.00. Based on Ala = 1.00.

α-t-Butoxycarbonyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (26)

α-t-Butoxycarbonyl-L-phenylalanine (0.318 g, 1.2 mmole) and 1-hydroxybenzotriazole (0.172 g, 1.2 mmole) were dissolved in tetrahydrofuran (10 ml). N,N'-Dicyclohexylcarbodi-imide (0.247 g, 1.2 mmole) was added and the mixture stirred at 0° for 1 hour, and 2.5 hours at room temperature.

The hexapeptide (25) (1.224 g, 1.0 mmole) was shaken in trifluoroacetic acid (5 ml, 65 mmole) for 20 minutes at 0° and evaporated to give a yellow oil.

Trituration with diethyl ether gave a white solid which was dissolved in chloroform (10 ml). The solution was cooled to 0° and triethylamine (0.55 ml, 4.0 mmole) added; evaporation gave a solid which was dissolved in tetrahydrofuran (5 ml) and added to the activated mixture. The solution was stirred at room temperature for 1 hour. T.l.c. (E4, G3) indicated that the reaction was complete after 20 minutes. Ethyl acetate (15 ml analar) was added and the solution stirred at 0° for 1 hour. A non-filterable gel was formed which did not go into solution when more ethyl acetate was added. The gel was evaporated to dryness and dimethylformamide (50 ml) and ethyl acetate (50 ml analar) added. This mixture was stirred at 0° for 1 hour. The dicyclohexylurea was filtered, washed with cooled ethyl acetate and the filtrate evaporated to an oil. The oil was taken up in ethyl acetate (50 ml) and washed with saturated sodium hydrogen carbonate (3 x 25 ml), water (2 x 25 ml), brine (2 x 25 ml) and dried overnight. A solid gel was formed which was evaporated until dryness and the heptapeptide taken up in dichloromethane (50 ml) and filtered. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (26) (1.207 g, 88.1%) as a chromatographically pure, white powder of m.p. 129-131°, R_F (E4) 0.66; R_F (G1) 0.87; R_F (G3) 0.87; R_F (A2) 0.67, $[\alpha]_{589}^{20}$ -38.6°; $[\alpha]_{578}^{20}$ -40.5°; $[\alpha]_{546}^{20}$ -45.2°; $[\alpha]_{436}^{20}$ -81.6°; $[\alpha]_{365}^{20}$ -137.8° (c 1.07 in $CHCl_3$). $C_{76}H_{94}N_{10}O_{14}.H_2O$ requires C, 65.60; H, 6.92; N, 10.01%. Found: C, 65.77; H, 7.12; N, 10.28%. Amino-acid analysis gave Phe, 2.09; Tyr, 0.94; Thr, 0.92; Pro, 0.99; Lys, 1.03; Ala, 1.00. Based on Ala = 1.00.

α-Benzyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (8)

α-Benzyloxycarbonylglycine (126 mg, 0.6 mmole) and 1-hydroxybenzotriazole

(92 mg, 0.6 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-Dicyclohexyl-carbodi-imide (124 mg, 0.6 mmole) was added and the mixture stirred for 1.5 hours at 0° and 1 hour at room temperature.

The heptapeptide (26) (686 mg, 0.5 mmole) was shaken in trifluoroacetic acid (2 ml, 26 mmole) for 20 minutes at 0° and evaporated to give a thick oil. Trituration with diethyl ether gave a white solid which was dissolved in chloroform (10 ml). The solution was cooled to 0° and triethylamine (0.275 ml, 2 mmole) added; evaporation gave a solid which was dissolved in tetrahydrofuran (5 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete within 20 minutes and the product was isolated by the Amberlyst 15 (3-bromopyridinium form) procedure to give the protected octapeptide (8) (652 mg, 90%) as a chromatographically pure, white powder of m.p. 114-116°, R_F (E4) 0.67; R_F (G3) 0.97; R_F (G4) 0.90; R_F (A2) 0.65, $[\alpha]_{589}^{20}$ -40.3°; $[\alpha]_{576}^{20}$ -42.40°; $[\alpha]_{546}^{20}$ -48.7°; $[\alpha]_{436}^{20}$ -87.6°; $[\alpha]_{365}^{20}$ -147.0° (c 0.67 in $CHCl_3$). $C_{81}H_{95}N_{11}O_{15}$ requires C, 64.80; H, 6.60; N, 10.28%. Found: C, 64.81; H, 6.57; N, 10.56%. Amino-acid analysis gave Gly, 1.01; Phe, 1.95; Tyr, 0.96; Thr, 0.90; Pro, 0.99; Lys, 1.01; Ala, 1.00. Based on Ala = 1.00.

Synthesis of protected L-alanine analogues

α-t-Butoxycarbonyl-L-alanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (27)

α-t-Butoxycarbonyl-L-alanine (0.454 g, 2.4 mmole) and 1-hydroxybenzotriazole (0.336 g, 2.4 mmole) were dissolved in tetrahydrofuran (15 ml). N,N'-Dicyclohexylcarbodi-imide (0.494 g, 2.4 mmole) was added and the mixture stirred

at 0° for 45 minutes and room temperature for 1.5 hours.

The pentapeptide (24) (2.152 g, 2.0 mmole) was shaken in trifluoroacetic acid (10 ml, 130 mmole) for 20 minutes at 0° and evaporated to give an oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (10 ml). The solution was cooled to 0° and triethylamine (1.1 ml, 8 mmole) added; evaporation gave a colourless oil which was taken up in tetrahydrofuran (10 ml) and added to the activated mixture. The reaction was complete after overnight stirring at room temperature and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected hexapeptide (27) (2.0 g, 87.1%) as a chromatographically pure, white powder of m.p. 87-90°, R_F (E4) 0.66; R_F (G3) 0.87; R_F (A2) 0.55; R_F (N) 0.76, $[\alpha]_{589}^{20}$ -36.7°; $[\alpha]_{578}^{20}$ -38.3°; $[\alpha]_{546}^{20}$ -44.0°; $[\alpha]_{436}^{20}$ -78.7°; $[\alpha]_{365}^{20}$ -132.6° (c 1.02 in $CHCl_3$). $C_{61}H_{81}N_9O_{13}$ requires C, 63.80; H, 7.12; N, 10.95%. Found: C, 63.51; H, 7.36; N, 10.81%. Amino-acid analysis gave Ala, 2.00; Tyr, 0.96; Thr, 0.90; Pro, 1.01; Lys, 1.02. Average value taken.

α-t-Butoxycarbonyl-L-phenylalanyl-L-alanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (28)

α-t-Butoxycarbonyl-L-phenylalanine (0.271 g, 1.02 mmole) and 1-hydroxybenzotriazole (0.157 g, 1.02 mmole) were dissolved in tetrahydrofuran (10 ml). N,N'-Dicyclohexylcarbodi-imide (0.210 g, 1.02 mmole) was added and the mixture stirred at 0° for 1 hour and at room temperature for 1.5 hours.

The hexapeptide (27) (0.976 g, 0.85 mmole) was shaken in trifluoroacetic acid (3 ml, 39 mmole) for 20 minutes at 0° and evaporated to give an oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (10 ml).

The solution was cooled to 0° and triethylamine (0.47 ml, 3.3 mmole) added; evaporation gave a colourless oil which was taken up in tetrahydrofuran (5 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete within one hour and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (28) (0.927 g, 83.5%) as a chromatographically pure, white powder of m.p. 128-9°, R_F (E4) 0.60; R_F (G3) 0.77; R_F (G4) 0.89; R_F (A2) 0.51; R_F (N) 0.92, $[\alpha]_{589}^{20}$ -28.3°; $[\alpha]_{578}^{20}$ -32.2°; $[\alpha]_{546}^{20}$ -36.8°; $[\alpha]_{436}^{20}$ -65.4°; $[\alpha]_{365}^{20}$ -110.0° (c 1.03 in $CHCl_3$). $C_{70}H_{90}N_{10}O_{14}$ requires C, 64.4; H, 6.90; N, 10.71%. Found: C, 64.23; H, 7.11; N, 10.71%. Amino-acid analysis gave Ala, 2.00; Phe, 1.03; Tyr, 0.98; Thr, 0.92; Pro, 0.99; Lys, 1.02. Average value taken.

α-Benzyloxycarbonylglycyl-L-phenylalanyl-L-alanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (11)

α-Benzyloxycarbonylglycine (163 mg, 0.78 mmole) and 1-hydroxybenzotriazole (119 mg, 0.78 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-Dicyclohexylcarbodi-imide (161 mg, 0.78 mmole) was added and the mixture stirred for 1 hour at 0° and 2 hours at room temperature.

The heptapeptide (28) (870 mg, 0.65 mmole) was shaken in trifluoroacetic acid (2 ml, 26 mmole) for 20 minutes at 0° and evaporated to give a yellowish oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml). The solution was cooled to 0° and triethylamine (0.36 ml, 2.6 mmole) added; evaporation gave a yellow oil which was taken up in tetrahydrofuran (5 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction

was complete within 15 minutes and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected octapeptide (11) (0.71 g, 81.3%) as a chromatographically pure, white powder of m.p. 108-9°, R_F (E4) 0.46; R_F (G3) 0.87; R_F (G4) 0.95; R_F (A2) 0.65; R_F (N) 0.90, $[\alpha]_{589}^{20}$ -29.1°; $[\alpha]_{578}^{20}$ -30.3°; $[\alpha]_{546}^{20}$ -34.6°; $[\alpha]_{436}^{20}$ -62.3°; $[\alpha]_{365}^{20}$ -104.8° (c 1.03 in $CHCl_3$). $C_{75}H_{91}N_{11}O_{15}.H_2O$ requires C, 63.68; H, 6.63; N, 10.89%. Found: C, 63.77; H, 6.45; N, 11.18%. Amino-acid analysis gave Gly, 0.99; Phe, 1.02; Tyr, 0.87; Thr, 0.93; Pro, 0.99; Lys, 1.03; Ala, 2.00. Average value taken.

α-t-Butoxycarbonyl-L-alanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (29)

α-t-Butoxycarbonyl-L-alanine (182 mg, 0.96 mmole) and 1-hydroxybenzotriazole (147 mg, 0.96 mmole) were dissolved in tetrahydrofuran (10 ml). N,N'-Dicyclohexylcarbodi-imide (198 mg, 0.96 mmole) was added and the mixture stirred at 0° for 1 hour and room temperature for 1 hour.

The hexapeptide (25) (978 mg, 0.8 mmole) was shaken in trifluoroacetic acid (3 ml, 39 mmole) for 20 minutes at 0° and the solution was evaporated to give a colourless oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml). The solution was cooled to 0° and triethylamine (0.45 ml, 3.2 mmole) added; evaporation gave a clear oil which was dissolved in tetrahydrofuran (5 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete within 40 minutes and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (29) (1.10 g, 84%) as a chromatographically pure white powder of m.p. 104-6°, R_F (E4) 0.65; R_F (G3) 0.88; R_F (G4) 0.82; R_F (A2) 0.53, $[\alpha]_{589}^{20}$ -46.2°;

$[\alpha]_{578}^{20}$ -47.9°; $[\alpha]_{536}^{20}$ -53.6°; $[\alpha]_{436}^{20}$ -96.5°; $[\alpha]_{365}^{20}$ -162.8° (c 0.95 in $CHCl_3$). $C_{70}H_{90}N_{10}O_{14}$ requires C, 64.40; H, 6.90; N, 10.71%. Found: C, 64.30; H, 7.10; N, 10.71%. Amino-acid analysis gave Ala, 2.00; Phe, 1.00; Tyr, 0.91; Thr, 0.97; Pro, 0.97; Lys, 1.02. Average value taken.

α-Benzyloxycarbonylglycyl-L-alanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (12)

α-Benzyloxycarbonylglycine (125.5 mg, 0.6 mmole) and 1-hydroxybenzotriazole (92 mg, 0.6 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-Dicyclohexylcarbodi-imide (124 mg, 0.6 mmole) was added and the mixture stirred at 0° for 1 hour and 1.5 hours at room temperature.

The heptapeptide (29) (650 mg, 0.5 mmole) was shaken in trifluoroacetic acid (5 ml, .65 mmole) for 20 minutes at 0° and evaporated to give an oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml). The solution was cooled to 0° and triethylamine (0.275 ml, 2.0 mmole) added; evaporation gave a clear oil which was dissolved in tetrahydrofuran (5 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete within 1 hour and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected octapeptide (12) (590 mg, 84.1%) as a chromatographically pure white powder of m.p. 107-110°, R_F (E4) 0.68; R_F (G3) 0.86; R_F (G4) 0.88; R_F (A2) 0.55, $[\alpha]_{589}^{20}$ -38.5°; $[\alpha]_{578}^{20}$ -40.6°; $[\alpha]_{546}^{20}$ -47.5°; $[\alpha]_{436}^{20}$ -84.5°; $[\alpha]_{365}^{20}$ -143.0° (c 0.85 in $CHCl_3$). $C_{75}H_{91}N_{11}O_{15}.H_2O$ requires C, 63.68; H, 6.63; N, 10.89%. Found: C, 63.92; H, 6.64; N, 11.20%. Amino-acid analysis gave Gly, 1.00; Phe, 1.02; Tyr, 0.87; Thr, 0.93; Pro, 0.99; Lys, 1.03; Ala, 2.00. Average value taken.

α-t-Butoxycarbonyl-L-alanyl-L-alanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (30)

α-t-Butoxycarbonyl-L-alanine (193 mg, 1.02 mmole) and 1-hydroxybenzotriazole (157 mg, 1.02 mmole) were dissolved in tetrahydrofuran (10 ml). N,N'-Dicyclohexylcarbodi-imide (210 mg, 1.02 mmole) was added and the mixture stirred at 0° for 1 hour and at room temperature for 1 hour.

The hexapeptide (30) (0.976 g, 0.85 mmole) was shaken in trifluoroacetic acid (3 ml, 39 mmole) for 20 minutes at 0° and evaporated to give a solid gum. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (10 ml). The solution was cooled to 0° and triethylamine (0.47 ml, 3.3 mmole) added; evaporation gave a colourless oil which was taken up in tetrahydrofuran (10 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete within 1 hour and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (30) (0.85 g, 81.2%) as a chromatographically pure, white powder of m.p. 102-3°, R_F (E4) 0.60; R_F (G3) 0.74; R_F (G4) 0.87; R_F (A2) 0.48; R_F (N) 0.90, $[\alpha]_{589}^{20}$ -41.3°; $[\alpha]_{578}^{20}$ -44.7°; $[\alpha]_{546}^{20}$ -51.0°; $[\alpha]_{436}^{20}$ -91.4°; $[\alpha]_{365}^{20}$ -154.1° (c 0.96 in $CHCl_3$). $C_{64}H_{86}N_{10}O_{14}$ requires C, 63.03; H, 7.11; N, 11.49%. Found: C, 63.46; H, 7.10; N, 11.57%. Amino-acid analysis gave Ala, 2.98; Tyr, 0.98; Thr, 0.98; Pro, 1.00; Lys, 1.00. Average value taken.

α-Benzyloxycarbonylglycyl-L-alanyl-L-alanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (13)

α-Benzyloxycarbonylglycine (163 mg, 0.68 mmole) and 1-hydroxybenzotriazole (104 mg, 0.68 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-

Dicyclohexylcarbodi-imide (161 mg, 0.68 mmole) was added and the mixture stirred for 40 minutes at 0° and 1 hour at room temperature.

The heptapeptide (30) (800 mg, 0.57 mmole) was shaken in trifluoroacetic acid (2 ml, 26 mmole) for 20 minutes at 0° and evaporated to a solid gum. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml). The solution was cooled to 0° and triethylamine (0.36 ml, 2.28 mmole) added; evaporation gave a colourless oil which was taken up in tetrahydrofuran (10 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete after 10 minutes and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected octapeptide (13) (690 mg, 91.8%) as a chromatographically pure, white powder of m.p. 107-110°, R_F (E4) 0.48; R_F (G3) 0.66; R_F (A2) 0.46; R_F (N) 0.85, $[\alpha]_{598}^{20}$ -29.3°; $[\alpha]_{576}^{20}$ -30.8°; $[\alpha]_{546}^{20}$ -34.8°; $[\alpha]_{436}^{20}$ -62.7°; $[\alpha]_{365}^{20}$ -107.0° (c 1.05 in $CHCl_3$). $C_{69}H_{87}N_{11}O_{15}.2H_2O$ requires C, 61.54; H, 6.81; N, 11.44%. Found: C, 61.55; H, 6.68; N, 11.61%. Amino-acid analysis gave Gly, 0.98; Ala, 2.89; Tyr, 0.98; Thr, 0.98; Pro, 1.00; Lys, 1.00. Average value taken.

Synthesis of N-methyl-L-phenylalanine analogues

N-Benzyl-L-phenylalanine

This was prepared by the method of Quitt,[185] involving reduction with sodium borohydride of the Schiff's base formed between benzaldehyde and L-phenylalanine. m.p. 254-5°, yield 96%, $[\alpha]_{589}^{20}$ +27.2° (c 1.02 in 6M HCl/HAc, 1:1) [lit.[185] m.p. 255°, yield 90%, $[\alpha]_{589}^{22}$ +26.9° (c 1 in 6M HCl/HAc)].

N-Benzyl-N-methyl-L-phenylalanine

The method used was an adaptation of that of Quitt.[185] N-Benzyl-L-phenylalanine (26.5 g, 0.1 mole) was refluxed with formic acid (33.6 ml, 0.9 mole) and formaldehyde (30.0 ml, 0.9 mole) for 4 hours. The solution was evaporated under vacuum to yield a white solid which was recrystallised from water containing a trace of acetic acid, to assist solubilisation. The white crystals were filtered and dried. m.p. 222-3°, yield (28.4 g, 96%), $[\alpha]_{589}^{24}$ +21.9° (c 0.99 in 6M HCl/HAc, 1:1) [lit.[185] m.p. 220-2°, yield 94%, $[\alpha]_{589}^{24}$ +20.0° (c 1 in 6M HCl/HAc, 1:1)].

The reaction was repeated varying the time of reaction and the minimum period for optimum reaction was 2 hours

N-Methyl-L-phenylalanine

The preparation from N-benzyl-N-methyl-L-phenylalanine by Quitt[185] was used. m.p. 259-61°, yield 70%, $[\alpha]_{589}^{21}$ +25.6° (c 1.02 in 6M HCl), +49.7° (c 1.0 in MNaOH) [lit.[185] m.p. 260°, yield 75%, $[\alpha]_{589}^{21}$ +26.6° (c 1.0 in 6M HCl), +49.3° (c 1.0 in M NaOH)].

α-t-Butoxycarbonyl-N-methyl-L-phenylalanine dicyclohexylammonium salt

The method used was an adaptation of that reported by Blake and Li.[186] N-Methyl-L-phenylalanine (2.5 g, 1.4 mmole) was suspended in 60% aqueous dioxan (5 ml) and adjusted to pH 10.5 with sodium hydroxide (4M). t-Butoxycarbonyl azide (2.4 ml, 1.7 mmole) was added in small portions with stirring and kept at constant pH by autotitrator overnight. T.l.c. (G3) indicated that the reaction was then complete. Water was added to bring the total volume to 20 ml; the solution was washed with diethyl ether (3 x 20 ml), cooled to 0° and brought to pH 3.5 with solid

citric acid. The product was extracted into ethyl acetate (2 x 20 ml), washed with water (2 x 20 ml), brine (2 x 20 ml) and dried. Evaporation gave a colourless oil which was taken up in diethyl ether (25 ml) and dicyclohexylamine (2.56 g, 1.4 mmole) added. Overnight at 4° gave a white precipitate which was filtered and dried to give α-t-butoxycarbonyl-N-methyl-L-phenylalanine dicyclohexylammonium salt (5.32 g, 90.1%) as a white powder of m.p. 167-70°, $[\alpha]_{589}^{21}$ -24.2° (c 0.99 in MeOH). $C_{17}H_{44}N_2O_4$ requires C, 70.4; H, 9.63; N, 6.08%. Found: C, 70.46; H, 9.54; N, 6.20% [lit.[186] yield 52%, m.p. 165-70°, $[\alpha]_{21}^{D}$ -25.0° (c 1.0 in MeOH)].

N-Methyl-L-phenylalanine methyl ester hydrochloride

N-Methyl-L-phenylalanine (1.54 g, 0.86 mmole) was added to a solution of thionyl chloride (0.70 ml, 0.946 mmole) and absolute methanol (10 ml) cooled to -8°. The mixture was refluxed for 5 hours when t.l.c. (N, G1) indicated that the reaction was complete. Evaporation gave a colourless oil which was recrystallised from methanol to give N-methyl-L-phenylalanine methyl ester hydrochloride (1.51 g, 76%) as white crystals of m.p. 85-7°, R_F (N) 0.52; R_F (G1) 0.48, $[\alpha]_{589}^{20}$ +59.1°; $[\alpha]_{578}^{20}$ +61.6°; $[\alpha]_{546}^{20}$ +70.9°; $[\alpha]_{436}^{20}$ +129.8°; $[\alpha]_{365}^{20}$ +218.8° (c 1.01 in $CHCl_3$). $C_{11}H_{16}NO_2Cl$ requires C, 57.51; H, 7.02; N, 6.10; Cl, 15.44%. Found: C, 57.21; H, 7.12; N, 6.25; Cl, 15.26%. τ ($CDCl_3$) 2.69 (5H, s, $C_6\underline{H}_5.CH_2$), 5.9 (2H, c, $N\underline{H}_2^+.CH_3$), 6.32 (3H, s, $.OC\underline{H}_3$), 6.5 (3H, complex, $NH_2^+.C\underline{H}.CO$; $CH.C\underline{H}_2$), 7.23 (3H, s, $C\underline{H}_3.NH_2^+$.).

α-t-Butoxycarbonyl-N-methyl-L-phenylalanyl-N-methyl-L-phenylalanine methyl ester (31)

α-t-Butoxycarbonyl-N-methyl-L-phenylalanine (180 mg, 1.05 mmole)

previously liberated from its dicyclohexylammonium salt (480 mg, 1.05 mmole) was dissolved in tetrahydrofuran (5 ml) and cooled to -20° (acetone/solid carbon dioxide). N-Methylmorpholine (0.118 ml, 1.05 mmole) and pivaloyl chloride (0.105 ml, 1.05 mmole) were added maintaining the temperature between -15 and -20°. After 10 minutes N-methylmorpholine (0.111 ml, 1.0 mmole) was added to N-methyl-L-phenylalanine methyl ester hydrochloride (231 mg, 1.0 mmole) in tetrahydrofuran (5 ml) at -10° and the mixture immediately added to the previously activated acylating component. Stirring was continued at room temperature for 24 hours when t.l.c. (E4, G3) indicated complete reaction. The solution was evaporated, taken up in ethyl acetate (15 ml) and washed with citric acid (0.7M, 2 x 10 ml), water (5 ml), saturated sodium hydrogen carbonate (2 x 10 ml), water (5 ml), brine (5 ml) and dried. Evaporation gave the protected dipeptide (31) (400 mg, 87.6%) as a colourless oil, R_F (E4) 0.78; R_F (G3) 0.95; R_F (N) 0.95, $[\alpha]_{589}^{20}$ -30.1° (c 1.01 in $CHCl_3$). $C_{26}H_{34}N_2O_5.H_2O$ requires C, 66.1; H, 7.2; N, 5.92%. Found: C, 66.5; H, 7.10; N, 5.71%. τ ($CDCl_3$) 2.76 (10H, s, aromatic), 4.81 and 5.12 (2H, c, N.CH.CO), 6.30 (3H, s, CO.OCH_3), 6.5-7.0 (4H, complex, CH.CH_2.C_6H_5), 7.0-7.6 (6H, complex, N.CH_3), 8.6-8.7 (9H, s, $(CH_3)_3$.C.O).

An aliquot of the protected dipeptide (31) (100 mg) was hydrolysed in vacuum for 24 hours at 110° in hydrochloric acid (6M, 1.5 ml). The sample was evaporated and dried to give a white solid identical to that of similarly treated N-methyl-L-phenylalanine. $[\alpha]_{589}^{20}$ +24.7° (c 0.81 in 6M HCl), +48.1° (c 0.69 in M NaOH) [lit. $[\alpha]_{589}^{21}$ +26.6° (c 1.0 in 6MHCl), +49.3° (c 1.0 in M NaOH)].

α-t-Butoxycarbonyl-N-methyl-L-phenylalanyl-N-methyl-L-phenylalanine hydrazide (32)

Excess hydrazine hydrate (0.0314 ml, 0.6 mmole) was added to the protected dipeptide (31) (50 mg, 0.091 mmole) in methanol (5 ml). T.l.c. (E4, G3) indicated that the reaction was complete after overnight stirring at room temperature. Evaporation gave a colourless gum, trituration with diethyl ether gave a white solid which was filtered, washed with cold methanol and dried to yield the protected dipeptide hydrazide (32) (46 mg, 92%) as a white powder of m.p. 135-6°, R_F (E4) 0.44; R_F (G3) 0.61, $[\alpha]_{589}^{20}$ -28.7°; $[\alpha]_{578}^{20}$ -29.9°; $[\alpha]_{546}^{20}$ -34.8°; $[\alpha]_{436}^{20}$ -64.0°; $[\alpha]_{365}^{20}$ -111.2° (c 0.58 in MeOH). $C_{25}H_{34}N_4O_4$ requires C, 66.05; H, 7.54; N, 12.33%. Found: C, 65.78; H, 7.39; N, 12.34%.

Attempted preparations of α-t-butoxycarbonyl-N-methyl-L-phenylalanyl-N-methyl-L-phenylalanine methyl ester (31)

(i) N,N'-Dicyclohexylcarbodi-imide

α-t-Butoxycarbonyl-N-methyl-L-phenylalanine (184 mg, 0.662 mmole) and N,N'-dicyclohexylcarbodi-imide (142 mg, 0.662 mmole) were stirred at 0° for 1 hour and room temperature for 1 hour in tetrahydrofuran (5 ml). N-Methyl-L-phenylalanine methyl ester hydrochloride (154 mg, 0.662 mmole) was added and the mixture brought to pH 8.5 with N,N-di-isopropylethylamine (0.156 ml, 0.662 mmole). After stirring overnight at room temperature t.l.c. (E4, G1, G3, N) indicated considerable amounts of starting materials and after 3 days there was no significant change in its composition.

(ii) N,N'-Dicyclohexylcarbodi-imide/1-hydroxybenzotriazole

α-t-Butoxycarbonyl-N-methyl-L-phenylalanine (189 mg, 0.675 mmole) and

1-hydroxybenzotriazole (96 mg, 0.675 mmole) were dissolved in tetrahydrofuran (3 ml). N,N'-Dicyclohexylcarbodi-imide (146 mg, 0.675 mmole) was added and the mixture stirred at 0° for 1 hour and room temperature for 1 hour. N-Methyl-L-phenylalanine methyl ester hydrochloride (156 mg, 0.675 mmole) and N,N-di-isopropylethylamine (0.159 ml, 0.675 mmole) were added in tetrahydrofuran (5 ml). Stirring for 2 days at room temperature gave rise to an addition compound identified by t.l.c. with high R_F values in solvents E4 and G3. Ethyl acetate (5 ml analar) was added and the mixture stirred at 0° for 1 hour. A small amount of fine white precipitate, probably dicyclohexylurea, was filtered off, and the filtrate evaporated to an oil, which was taken up in ethyl acetate (15 ml) and washed with citric acid (0.7M, 2 x 10 ml), water (5 ml), saturated sodium hydrogen carbonate (2 x 10 ml), water (5 ml), brine (5 ml) and dried. The white solid, m.p. 131-4°, obtained on evaporation was examined by n.m.r. and contained additional signals in the 7.9-8.5 τ region and the expected $-OCH_3$ signal at 6.30 τ was absent. This strongly suggested that the isolated product was α-t-butoxycarbonyl-N-methyl-L-phenylalanine N-acyl dicyclohexylurea. $C_{28}H_{43}N_3O_5$ requires C, 69.51; H, 8.94; N, 8.68%. Found: C, 69.77; H, 8.74; N, 8.71%. τ ($CDCl_3$) 2.81 (5H, s, aromatic), 4.8 (1H, s, CO.NH.C_6H_{11}), 6.21 (1H, s, N.CH.CO), 6.85 (2H, complex, CH.CH_2.C_6H_5), 7.15 (3H, s, N.CH_3), 7.9-8.5 (22H, complex, .N.C_6H_{11}), 8.6 (9H, s, $(CH_3)_3$.C).

(iii) N,N'-Dicyclohexylcarbodi-imide/N-hydroxybenzotriazole - 'eintopf' method

Method (ii) was repeated albeit the addition of N,N'-dicyclohexylcarbodi-imide was delayed and added to the one-flask mixture of α-t-butoxycarbonyl-N-methyl-L-phenylalanine, N-methyl-L-phenylalanine methyl ester hydrochloride and

1-hydroxybenzotriazole in tetrahydrofuran at pH 6-7 and 0°. A product similar to that obtained in (ii) was formed.

(iv) N,N'-Dicyclohexylcarbodi-imide/N-hydroxysuccinimide

Method (ii) was repeated but replacing 1-hydroxybenzotriazole by N-hydroxysuccinimide. T.l.c. (E4, G3) indicated that stirring for several days produced only a small amount of possible product and large amounts of starting material still remained.

α-t-Butoxycarbonyl-N-methyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (33)

α-t-Butoxycarbonyl-N-methyl-L-phenylalanine (0.667 g, 2.4 mmole) previously liberated from its dicyclohexylammonium salt (1.12 g, 2.4 mmole) was dissolved in tetrahydrofuran (5 ml) and cooled to -20° (acetone/solid carbon dioxide). N-Methylmorpholine (0.268 ml, 2.4 mmole) and pivaloyl chloride (0.24 ml, 2.4 mmole) were added maintaining the temperature between -15 and -20° forming a fine white precipitate.

Meanwhile the pentapeptide (24) (2.152 g, 2 mmole) was shaken in trifluoroacetic acid (20 ml, 260 mmole) for 20 minutes at 0° and the solution was evaporated to give a colourless oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (20 ml), N-methylmorpholine (0.446 ml, 4 mmole) was added and the mixture cooled to -20°. The mixture was added to the previously prepared acylating component. T.l.c. (E4, G3) after 10 minutes indicated that the reaction was complete which was then evaporated to a white solid. The solid was only partially soluble in ethyl acetate and was therefore taken up in dichloromethane (100 ml). Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave

the protected hexapeptide (33) (2.105 g, 85.3%) as a chromatographically pure white powder of m.p. 71-4°, R_F (E4) 0.64; R_F (G3) 0.74; R_F (A2) 0.46; R_F (N) 0.86, $[\alpha]_{589}^{20}$ -40.6°; $[\alpha]_{578}^{20}$ -42.1°; $[\alpha]_{546}^{20}$ -48.6°; $[\alpha]_{436}^{20}$ -89.0°; $[\alpha]_{365}^{20}$ -152.5° (c 0.79 in $CHCl_3$). $C_{68}H_{87}N_9O_{13}.2H_2O$ requires C, 64.08; H, 7.20; N, 9.89%. Found: C, 64.22; H, 7.26; N, 10.20%. Amino acid analysis gave MePhe, 1.00; Tyr, 0.97; Thr, 0.98; Pro, 0.99; Lys, 1.01; Ala, 1.00. Based on Ala = 1.00.

α-t-Butoxycarbonyl-L-phenylalanyl-N-methyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (34)

α-t-Butoxycarbonyl-L-phenylalanine (127 mg, 0.48 mmole) and 1-hydroxy-benzotriazole (73.5 mg, 0.48 mmole) were dissolved in tetrahydrofuran (10 ml). N,N'-Dicyclohexylcarbodi-imide (99 mg, 0.48 mmole) was added and the mixture stirred at 0° for 1 hour and room temperature for 2 hours.

The hexapeptide (33) (495 mg, 0.4 mmole) was shaken in trifluoroacetic acid (5 ml, 65 mmole) for 20 minutes at 0° and the solution was evaporated to give a colourless oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml). The solution was cooled to 0° and triethylamine (0.222 ml, 1.6 mmole) added; evaporation gave a clear oil which was dissolved in tetrahydrofuran (5 ml) and added to the previously activated mixture. T.l.c. (E4, G3) indicated that the reaction proceeded slowly and it was over 24 hours before no amino component could be detected. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (34) (441 mg, 86%) as a chromatographically pure, white powder of m.p. 82-5°, R_F (E4) 0.71; R_F (G3) 0.98; R_F (N) 0.80;

R_F (A2) 0.60, $[\alpha]_{589}^{20}$ -45.6°; $[\alpha]_{578}^{20}$ -47.0°; $[\alpha]_{546}^{20}$ -53.4°; $[\alpha]_{436}^{20}$ -98.0°; $[\alpha]_{365}^{20}$ -171.8° (c 0.35 in $CHCl_3$). $C_{77}H_{96}N_{10}O_{14}.2H_2O$ requires C, 65.05; H, 7.09; N, 9.85%. Found: C, 65.26; H, 7.23; N, 10.10%. Amino acid analysis gave Phe, 0.99; MePhe, 0.98; Tyr, 0.98; Thr, 0.99; Pro, 0.99; Lys, 0.99; Ala, 1.00. Based on Ala = 1.00.

α-Benzyloxycarbonylglycyl-L-phenylalanyl-N-methyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (14)

α-Benzyloxycarbonylglycine (69 mg, 0.33 mmole) and 1-hydroxybenzotriazole (50.5 mg, 0.33 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-Dicyclohexyl-carbodi-imide (68 mg, 0.33 mmole) was added and the mixture stirred at 0° for 1 hour and room temperature for 2 hours.

The heptapeptide (34) (415 mg, 0.3 mmole) was shaken in trifluoroacetic acid (2 ml, 26 mmole) for 20 minutes at 0° to give a yellow oil which was evaporated to give a gum. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml). The solution was cooled to 0° and triethylamine (0.165 ml, 1.2 mmole) added; evaporation gave a clear oil which was dissolved in tetrahydro-furan (5 ml) and added to the previously activated mixture. T.l.c. (E4, G3) after 10 minutes indicated that the reaction was complete; isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected octapeptide (14) (338 mg, 76%) as a chromatographically pure, white powder of m.p. 75-80°, R_F (E4) 0.68; R_F (G3) 0.92; R_F (A2) 0.62; R_F (N) 0.77, $[\alpha]_{589}^{20}$ -52.2°; $[\alpha]_{578}^{20}$ -54.7°; $[\alpha]_{546}^{20}$ -63.0°; $[\alpha]_{436}^{20}$ -114.5°; $[\alpha]_{365}^{20}$ -198.0° (c 0.59 in $CHCl_3$). $C_{82}H_{97}N_{11}O_{15}.2H_2O$ requires C, 65.89; H, 6.67; N, 10.31%. Found: C, 65.67;

H, 6.85; N, 9.93%. Amino acid analysis gave Gly, 0.99; Phe, 0.97; MePhe, 1.00; Tyr, 0.97; Thr, 0.99; Pro, 1.02; Lys, 1.01; Ala, 1.00. Based on Ala = 1.00.

α-t-Butoxycarbonyl-N-methyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (35)

α-t-Butoxycarbonyl-N-methyl-L-phenylalanine (0.182 g, 0.716 mmole) previously liberated from its dicyclohexylammonium salt (0.333 g, 0.716 mmole) was dissolved in tetrahydrofuran (5 ml) and cooled to -20° (acetone/solid carbon dioxide). N-Methylmorpholine (0.08 ml, 0.716 mmole) and pivaloyl chloride (0.088 ml, 0.716 mmole) were added maintaining the temperature between -15° and -20°.

Meanwhile the heptapeptide (25) (0.80 g, 0.651 mmole) was shaken in trifluoroacetic acid (10 ml, 130 mmole) for 20 minutes at 0° and the solution was evaporated to give a colourless oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml), N-methylmorpholine (0.16 ml, 1.32 mmole) was added and the mixture cooled to -20°. Addition of this mixture to the simultaneously prepared acylating component gave an immediate white precipitate. T.l.c. (E4, G3) indicated that the reaction was complete after stirring overnight at room temperature. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (35) (761.5 mg, 84.5%) as a chromatographically pure white powder of m.p. 92-5°, R_F (E4) 0.67; R_F (G3) 0.86; R_F (A2) 0.63; R_F (N) 0.94, $[\alpha]_{589}^{20}$ -49.2°; $[\alpha]_{578}^{20}$ -51.6°; $[\alpha]_{546}^{20}$ -59.3°; $[\alpha]_{436}^{20}$ -108.0°; $[\alpha]_{365}^{20}$ -186.1° (c 1.01 in $CHCl_3$). $C_{77}H_{96}N_{10}O_{14}.2H_2O$ requires C, 65.05; H, 7.09; N, 9.85%. Found: C, 65.23; H, 6.86; N, 9.89%. Amino acid analysis

gave MePhe, 0.99; Phe, 0.98; Tyr, 1.00; Thr, 0.98; Pro, 0.99; Lys, 1.01; Ala, 1.00. Based on Ala = 1.00.

α-Benzyloxycarbonylglycyl-N-methyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (15)

α-Benzyloxycarbonylglycine (115 mg, 0.55 mmole) and 1-hydroxybenzotriazole (84 mg, 0.55 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-Dicyclohexylcarbodi-imide (113 mg, 0.55 mmole) was added and the mixture stirred at 0° for 1 hour and 1.5 hours at room temperature.

The heptapeptide (35) (692 mg, 0.5 mmole) was shaken in trifluoroacetic acid (5 ml, 65 mmole) for 20 minutes at 0° and evaporated to give a yellowish oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml). The solution was cooled to 0° and triethylamine (0.35 ml, 2.0 mmole) added; evaporation gave a colourless oil which was taken up in tetrahydrofuran (10 ml) and added to the activated mixture. The pH was corrected to 6.5 by addition of 1-hydrobenzotriazole. After stirring overnight at room temperature t.l.c. (E4, G3) indicated that the reaction was complete and isolation by the Amberlyst 15 (3-bromo-pyridinium form) procedure gave the protected octapeptide (15) (741 mg, 92.7%) as a chromatographically pure, buff solid of m.p. 95-8°, R_F (E4) 0.64; R_F (G3) 0.77; R_F (A2) 0.57; R_F (N) 0.95, $[\alpha]_{589}^{20}$ -49.0°; $[\alpha]_{578}^{20}$ -51.3°; $[\alpha]_{546}^{20}$ -59.0°; $[\alpha]_{436}^{20}$ -108.1°; $[\alpha]_{365}^{20}$ -186.5° (c 0.518 in $CHCl_3$). $C_{82}H_{97}N_{11}O_{15}.H_2O$ requires C, 65.89; H, 6.67; N, 10.31%. Found: C, 65.72; H, 7.03; N, 10.66%. Amino acid analysis gave Gly, 1.00; MePhe, 0.99; Phe, 0.98; Tyr, 0.99; Thr, 0.99; Pro, 0.98; Lys, 0.99; Ala, 1.00. Based on Ala = 1.00.

α-t-Butoxycarbonyl-N-methyl-L-phenylalanyl-N-methyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (36)

α-t-Butoxycarbonyl-N-methyl-L-phenylalanine (134 mg, 0.48 mmole) previously liberated from its dicyclohexylammonium salt (225 mg, 0.48 mmole) was dissolved in tetrahydrofuran (5 ml) and cooled to -20° (acetone/solid carbon dioxide). N-Methylmorpholine (0.0535 ml, 0.48 mmole) and pivaloyl chloride (0.0575 ml, 0.48 mmole) were added maintaining the temperature between -15 and -20° forming a fine white precipitate.

Meanwhile the hexapeptide (36) (500 mg, 0.4 mmole) was shaken in trifluoroacetic acid (5 ml, 65 mmole) for 20 minutes at 0° and the solution was evaporated to give a clear oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml), N-methylmorpholine (0.0894 ml, 0.8 mmole) was added and the mixture cooled to -20°. The mixture was added to the previously prepared acylating component. T.l.c. (E4, G3, N) indicated that the rate of coupling was extremely slow but was complete after stirring at room temperature for 2 days. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (36) (447 mg, 82.9%) as a chromatographically pure white powder of m.p. 78-82°, R_F (E4) 0.66; R_F (G3) 0.85; R_F (A2) 0.60; R_F (N) 0.91, $[\alpha]_{589}^{20}$ -63.2°; $[\alpha]_{578}^{20}$ -66.1°; $[\alpha]_{546}^{20}$ -76.0°; $[\alpha]_{436}^{20}$ -139.0°; $[\alpha]_{365}^{20}$ -239.5° (c 0.6 in $CHCl_3$). $C_{78}H_{98}N_{10}O_{14}.2H_2O$ requires C, 65.25; H, 7.16; N, 9.76%. Found: C, 65.18; H, 7.03; N, 10.15%. Amino acid analysis gave MePhe, 1.99; Tyr, 0.98; Thr, 0.97; Pro, 1.03; Lys, 1.01; Ala, 1.00. Based on Ala = 1.00.

α-Benzyloxycarbonylglycyl-N-methyl-L-phenylalanyl-N-methyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (16)

α-Benzyloxycarbonylglycine (39.5 mg, 0.189 mmole) and 1-hydroxybenzotriazole (29 mg, 0.189 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-Dicyclohexylcarbodi-imide (39 mg, 0.139 mmole) was added and the mixture stirred at 0° for 1 hour and 1.5 hours at room temperature.

The heptapeptide (36) (240 mg, 0.172 mmole) was shaken in trifluoroacetic acid (5 ml, 65 mmole) for 30 minutes at 0° to give a yellow solution which was evaporated to a gum. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (5 ml) and triethylamine (0.096 ml, 0.688 mmole) added. Evaporation gave a clear oil which was dissolved in tetrahydrofuran (5 ml) and added to the activated mixture. After stirring overnight at room temperature, t.l.c. (E4, G3) indicated complete reaction. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected octapeptide (16) (210 mg, 82.1%) as a chromatographically pure buff powder of R_F (E4) 0.65; R_F (G3) 0.78; R_F (A2) 0.54; R_F (N) 0.85, $[\alpha]_{589}^{20}$ -52.5°; $[\alpha]_{578}^{20}$ -57.6°; $[\alpha]_{546}^{20}$ -63.0°; $[\alpha]_{436}^{20}$ -116.2°; $[\alpha]_{365}^{20}$ -200.5° (c 0.13 in $CHCl_3$). $C_{83}H_{99}N_{11}O_{15}.2H_2O$ requires C, 65.29; H, 6.80; N, 10.09%. Found: C, 65.24; H, 7.18; N, 9.60%. Amino acid analysis gave Gly, 0.98; MePhe, 1.97; Tyr, 0.96; Thr, 1.00; Pro, 0.98; Lys, 1.01; Ala, 1.00. Based on Ala = 1.00.

Preparation of Truncated Sequences

(1) Bpoc-Gly-Phe-Phe-Tyr(Bzl)-OPic (17)

α-t-Butoxycarbonyl-O-benzyl-L-tyrosine 4-picolyl ester

N,N'-Dicyclohexylcarbodi-imide (5.56 g, 2.7 mmole) was slowly added to a solution of α-t-butoxycarbonyl-O-benzyl-L-tyrosine (10 g, 2.7 mmole) and 4-pyridyl-methanol (2.18 g, 2.0 mmole) in dichloromethane (80 ml) in an ice/salt bath over 2 hours. The reaction was stirred overnight when t.l.c. (E4) indicated that all the 4-pyridyl-methanol had reacted. The solution was stirred at 0° with ethyl acetate (80 ml), dicyclohexylurea was filtered off and washed with more cooled ethyl acetate and the combined filtrates evaporated to give a yellow-brown oil.

Attempted partition between citric acid (0.7M) and diethyl ether and ethyl acetate mixtures failed probably because of the lipophilicity of the product. The oil was taken up in ethyl acetate (100 ml), washed with saturated sodium hydrogen carbonate (2 x 100 ml), brine (2 x 100 ml) and dried. The solution was applied to Amberlyst 15 (300 ml, 3-bromopyridinium form). T.l.c. (E4, G3) indicated that the product had been completely absorbed within 1 hour. The column was washed with ethyl acetate (500 ml) and the product eluted with pyridine (0.5M) in ethyl acetate (500 ml). Evaporation of the eluate gave a light oil which on trituration with 60/80 petroleum ether gave a white solid. Recrystallisation from a mixture of 60/80 petroleum ether and ethyl acetate gave the protected 4-picolyl ester (6.20 g, 67%) as white needle-shaped crystals of m.p. 78-80°, R_F (E4) 0.72; R_F (G1) 0.98; R_F (G3) 0.93; R_F (A2) 0.74; R_F (M) 0.86, $[\alpha]_D^{20}$ -18.5° (c 1.13 in Me_2NCHO).

$C_{27}H_{30}N_2O_5$ requires C, 70.12; H, 6.5; N, 6.06%. Found: C, 70.25; H, 6.56; N, 6.11%. τ ($CDCl_3$) 1.38 (2H, d, pyridyl 2- and 6-protons), 2.60 (5H, s, $C_6\underline{H}_5.CH_2$.), 2.85 (2H, d, pyridyl 3- and 5-protons), 3.05 (4H, complex, $.CH_2.C_6\underline{H}_4.OCH_2$. and 1H, peptide, N$\underline{H}$), 4.86 (2H, s, pyridyl .$C\underline{H}_2$.O), 4.95 (2H, s, phenyl .$C\underline{H}_2$.O), 5.35 (1H, complex, NH.C$\underline{H}$.CO.), 6.90 (2H, d, J = 8 c/sec, CH.$C\underline{H}_2.C_6H_4$.) and 8.56 (9H, s, $(C\underline{H}_3)_3$.C.).

<u>α-t-Butoxycarbonyl-L-phenylalanyl-O-benzyl-L-tyrosine 4-picolyl ester</u> (39)

α-t-Butoxycarbonyl-L-phenylalanine (3.44 g, 13 mmole) and 1-hydroxybenzotriazole (2.05 g, 13 mmole) were dissolved in dimethylformamide (15 ml). N,N'-Dicyclohexylcarbodi-imide (2.68 g, 13 mmole) was added and the mixture stirred at 0° for 1 hour and room temperature for 1 hour.

α-t-Butoxycarbonyl-O-benzyl-L-tyrosine 4-picolyl ester (4.62 g, 10 mmole) was shaken in trifluoroacetic acid (15 ml, 215 mmole) for 20 minutes at 0°. Evaporation gave a yellow oil which on trituration with diethyl ether gave a white solid. This was taken up in dimethylformamide (10 ml) and added to the activated mixture. N,N-Di-isopropylethylamine (3.5 ml, 20 mmole) was added to the mixture and the reaction stirred overnight. T.l.c. (E4, G3) indicated complete reaction and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the <u>protected dipeptide</u> (39) (7.69 g, 97.5%) as a chromatographically pure buff powder of m.p. 147-8°, R_F (E4) 0.64; R_F (G3) 0.82; R_F (A2) 0.73; R_F (G4) 0.80, $[\alpha]_{589}^{20}$ -12.7°; $[\alpha]_{578}^{20}$ -11.5°; $[\alpha]_{546}^{20}$ -12.5°; $[\alpha]_{436}^{20}$ -19.0°; $[\alpha]_{365}^{20}$ -59.5° (c 0.97 in $CHCl_3$). $C_{36}H_{39}N_3O_6$ requires C, 70.6; H, 6.56; N, 6.88%. Found: C, 70.95; H, 6.57; N, 6.92%. τ ($CDCl_3$) 1.41 (2H, d, pyridyl 2- and 6-protons), 2.5-2.8 (14H, complex, aromatic protons), 2.85 (2H, d, pyridyl 3- and 5-protons),

3.65 (2H, complex, peptide NH), 4.9 (2H, s, pyridyl.CH_2), 5.0 (2H, s, $C_6H_5.CH_2.O$), 5.6 (2H, complex, NHCH.CO.), 6.9 (4H, t, $CH.CH_2.C_6H_5$, $CH.CH_2.C_6H_4$.), 8.6 (9H, s, $(CH_3)_3.C$).

α-t-Butoxycarbonyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosine 4-picolyl ester (40)

α-t-Butoxycarbonyl-L-phenylalanine (2.0 g, 7.8 mmole) and 1-hydroxybenzotriazole (1.18 g, 7.8 mmole) were dissolved in dimethylformamide (20 ml). N,N'-Dicyclohexylcarbodi-imide (1.62 g, 7.8 mmole) was added and the mixture stirred for 1 hour at 0° and 1 hour at room temperature.

The dipeptide (39) (3.65 g, 6 mmole) was shaken in trifluoroacetic acid (15 ml, 195 mmole) for 30 minutes at 0° and evaporated to give a gum which on trituration with diethyl ether gave a white solid. This was dissolved in dimethylformamide (5 ml) and N,N-di-isopropylethylamine (2.4 ml, 12 mmole) was added. The solution was added to the activated mixture and stirred overnight. T.l.c. (E4, G3) indicated complete reaction and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected tripeptide (40) (3.95 g, 83.0%) as a chromatographically pure buff powder of m.p. 148-148.5° and R_F (E4) 0.82; R_F (G3) 0.92; R_F (A2) 0.67; R_F (N) 0.83, $[\alpha]^{20}_{589}$ -26.6°; $[\alpha]^{20}_{578}$ -18.1°; $[\alpha]^{20}_{546}$ -19.5°; $[\alpha]^{20}_{436}$ -33.2° (c 0.91 in $CHCl_3$). $C_{45}H_{48}N_4O_7.2H_2O$ requires C, 68.2; H, 6.56; N, 7.08%. Found: C, 68.44; H, 6.41; N, 7.13%.

2-(p-Biphenylyl)-isopropyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosine 4-picolyl ester (17)

The tripeptide (40) (792 mg, 1.00 mmole) was shaken in trifluoroacetic acid

(4 ml, 52 mmole) for 20 minutes to give a clear oil. Trituration with diethyl ether afforded a white solid which was dissolved in dimethylformamide (1 ml) and cooled to 0°. 2-(p-Biphenylyl)-isopropyloxycarbonylglycine-N-hydroxysuccinimide ester (430 mg, 1.05 mmole) and triethylamine (0.278 ml, 2 mmole) were added and the mixture stirred at room temperature. T.l.c. (E4, G3) indicated that the reaction was complete within 40 minutes. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected tetrapeptide (17) (435 mg, 46%) as a chromatographically pure white powder of m.p. 83-4° and R_F (E4) 0.80; R_F (G3) 0.94; R_F (A2) 0.75; R_F (G1) 0.97, $[\alpha]_{589}^{20}$ -15.2°; $[\alpha]_{578}^{20}$ -15.1°; $[\alpha]_{546}^{20}$ -16.6°; $[\alpha]_{436}^{20}$ -23.8°; $[\alpha]_{365}^{20}$ -73.8° (c 0.93 in $CHCl_3$). $C_{58}H_{57}N_5O_8.H_2O$ requires C, 71.7; H, 6.08; N, 7.21%. Found: C, 71.55; H, 6.27; N, 7.19%.

(2) Bpoc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic (18)

α-t-Butoxycarbonyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (41)

α-t-Butoxycarbonyl-O-benzyl-L-threonine (6.5 g, 21.0 mmole) and 1-hydroxybenzotriazole (3.21 g, 21.0 mmole) were dissolved in tetrahydrofuran (50 ml). N,N'-Dicyclohexylcarbodi-imide (4.32 g, 21.0 mmole) was added and the mixture stirred for 40 minutes at 0° and 1 hour at room temperature.

L-Proline 4-picolyl ester dihydrobromide (6.54 g, 17.5 mmole) was dissolved in a mixture of tetrahydrofuran (50 ml) and dimethylformamide (25 ml) and added to the activated mixture. N,N-di-isopropylethylamine (6.15 ml, 35 mmole) was added to the mixture at 0°. T.l.c. (E4, G3) indicated that the reaction was complete after 3 hours. Isolation by the citric acid procedure gave the protected dipeptide (41)

(7.69 g, 87.2%) as a chromatographically pure, white foam of m.p. 28-30°, R_F (E4) 0.65; R_F (G3) 0.86; R_F (G1) 0.95; R_F (A2) 0.62, $[\alpha]_{589}^{20}$ -28.2°; $[\alpha]_{578}^{20}$ -29.3°; $[\alpha]_{546}^{20}$ -33.2°; $[\alpha]_{436}^{20}$ -56.2°; $[\alpha]_{365}^{20}$ -87.0° (c 1.23 in $CHCl_3$). $C_{27}H_{35}N_3O_6$ requires C, 65.17; H, 7.09; N, 8.45%. Found: C, 64.87; H, 6.93; N, 8.25%. τ ($CDCl_3$) 1.45 (2H, d, pyridyl 2- and 6-protons), 2.72 (5H, s, $C_6\underline{H}_5.CH_2.$), 2.75 (2H, d, pyridyl 3- and 5-protons), 4.58 (1H, complex, t.butyl N$\underline{H}$), 4.85 (2H, s, pyridyl. $C\underline{H}_2.$), 5.48 (5H, s, $C_6H_5.C\underline{H}_2.O$), 5.6 (1H, complex, $CH_3.C\underline{H}.$), 6.3 (3H, complex, $N.C\underline{H}_2.(CH_2)_2$; $CH_2.C\underline{H}.CO$), 8.0 (4H, complex, $CH_2.(C\underline{H}_2)_2.CH$), 8.55 (9H, s, $(C\underline{H}_3)_3.C.$), 8.8 (3H, d, J 8 c/sec, $.C\underline{H}_3.CH$).

α-t-Butoxycarbonyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (42)

α-t-Butoxycarbonyl-O-benzyl-L-tyrosine (4.45 g, 12.0 mmole) and 1-hydroxybenzotriazole (1.84 g, 12.0 mmole) were dissolved in tetrahydrofuran (25 ml). N,N'-Dicyclohexylcarbodi-imide (2.47 g, 12.0 mmole) was added and the mixture stirred for 1 hour at 0° and 1 hour at room temperature.

The dipeptide (41) (4.97 g, 10.0 mmole) was shaken in trifluoroacetic acid (20 ml, 260 mmole) for 20 minutes at 0° and evaporated to a yellow oil. Trituration with diethyl ether gave a white solid which was taken up in tetrahydrofuran (20 ml) and added to the activated mixture. The solution was brought to pH 8 with N,N-di-idopropylethylamine (3.5 ml, 20 mmole) and stirred at room temperature. T.l.c. (E4, G3) indicated that there was still amino component present after 30 minutes but the reaction was complete after 3 hours. Isolation by the Amberlyst 15 (3-bromo-pyridinium form) procedure gave the protected tripeptide (42) (6.49 g, 86.6%) as a

chromatographically pure, white powder of m.p. 48-50°, R_F (E4) 0.65; R_F (G1) 0.97; R_F (G3) 0.90; R_F (A2) 0.65, $[\alpha]_{589}^{20}$ -20.0°; $[\alpha]_{578}^{20}$ -21.7°; $[\alpha]_{546}^{20}$ -24.3°; $[\alpha]_{436}^{20}$ -39.5°; $[\alpha]_{365}^{20}$ -60.4° (c 0.95 in $CHCl_3$). $C_{43}H_{50}N_4O_8.H_2O$ requires C, 67.16; H, 6.82; N, 7.29%. Found: C, 67.4; H, 6.71; N, 7.33%.

α-t-Butoxycarbonyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (43)

α-t-Butoxycarbonyl-L-phenylalanine (1.58 g, 6.0 mmole) and 1-hydroxybenzotriazole (0.918 g, 6.0 mmole) were dissolved in tetrahydrofuran (50 ml). N,N'-Dicyclocarbodi-imide (1.24 g, 6.0 mmole) was added and the mixture stirred at 0° for 1 hour and 2.5 hours at room temperature.

The tripeptide (42) (3.75 g, 5.0 mmole) was shaken in trifluoroacetic acid (15 ml, 195 mmole) for 20 minutes at 0° and evaporated to give a dark oil. Trituration with diethyl ether gave a white solid which was taken up in chloroform (20 ml). The solution was cooled to 0° and triethylamine (3.6 ml, 20 mmole) added; evaporation gave a colourless oil which was taken up in tetrahydrofuran (10 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete after 1 hour and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected tetrapeptide (43) (4.13 g, 91.8%) as a chromatographically pure, white powder of m.p. 90-2°, R_F (E4) 0.57; R_F (G3) 0.91; R_F (G4) 0.90; R_F (A2) 0.68, $[\alpha]_{589}^{20}$ -19.5°; $[\alpha]_{578}^{20}$ -20.1°; $[\alpha]_{546}^{20}$ -22.9°; $[\alpha]_{436}^{20}$ -36.9°; $[\alpha]_{365}^{20}$ -57.0° (c 0.99 in $CHCl_3$). $C_{52}H_{59}N_5O_9$ requires C, 69.50; H, 6.62; N, 7.78%. Found: C, 69.23; H, 6.23; N, 7.58%. Amino-acid analysis gave Phe, 1.00; Tyr, 0.99; Thr, 0.99; Pro, 1.00. Based on Pro = 1.00.

α-t-Butoxycarbonyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (44)

α-t-Butoxycarbonyl-L-phenylalanine (1.43 g, 5.4 mmole) and 1-hydroxybenzotriazole (0.826 g, 5.4 mmole) were dissolved in tetrahydrofuran (30 ml). N,N'-Dicyclocarbodi-imide (1.11 g, 5.4 mmole) was added and the mixture stirred at 0° for 1 hour and at room temperature for 1.5 hours.

The tetrapeptide (43) (4.05 g, 4.5 mmole) was shaken in trifluoroacetic acid (15 ml, 195 mmole) for 20 minutes at 0° and evaporated to give a yellow oil. Trituration with diethyl ether gave a white solid which was taken up in chloroform (30 ml). The solution was cooled to 0° and triethylamine (2.46 ml, 18 mmole) added; evaporation gave a colourless oil which was taken up in tetrahydrofuran (10 ml) and added to the activated mixture. T.l.c. (E4, G3) indicated that the reaction was complete within 30 minutes and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected pentapeptide (44) (4.15 g, 88.4%) as a chromatographically pure, white powder of m.p. 138-40°, R_F (E4) 0.63; R_F (G3) 0.98; R_F (G4) 0.85; R_F (A2) 0.68, $[\alpha]_{589}^{20}$ -29.7°; $[\alpha]_{578}^{20}$ -31.2°; $[\alpha]_{546}^{20}$ -35.0°; $[\alpha]_{436}^{20}$ -61.0°; $[\alpha]_{365}^{20}$ -96.5° (c 0.41 in $CHCl_3$). $C_{61}H_{68}N_6O_{10}.H_2O$ requires C, 68.80; H, 6.67; N, 7.90%. Found: C, 69.0; H, 6.47; N, 7.78%. Amino-acid analysis gave Phe, 2.03; Tyr, 1.01; Thr, 0.99; Pro, 1.00. Based on Pro = 1.00.

2-(p-Biphenylyl)-isopropyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (18)

The pentapeptide (44) (1.063 g, 1 mmole) was shaken in trifluoroacetic acid (5 ml, 65 mmole) for 20 minutes at 0°. Evaporation gave a clear oil which on

trituration with diethyl ether gave a white solid. This was taken up in chloroform (10 ml), cooled to 0° and triethylamine (0.55 ml, 4 mmole) added; evaporation gave a slightly coloured oil which was taken up in tetrahydrofuran (10 ml) and added to 2-(p-biphenylyl)-isopropyloxycarbonylglycine N-hydroxy-succinimide ester (0.43 g, 1 mmole) in tetrahydrofuran (10 ml) at 0°. T.l.c. (E4, G3) indicated that the reaction was complete after 2 hours and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected hexapeptide (18) (0.992 g, 81%) as a chromatographically pure white powder of m.p. 153-4° and R_F (E4) 0.66; R_F (G3) 0.82; R_F (N) 0.95; R_F (A2) 0.70, $[\alpha]_{589}^{20}$ -30.6°; $[\alpha]_{578}^{20}$ -33.3°; $[\alpha]_{546}^{20}$ -36.9°; $[\alpha]_{436}^{20}$ -64.4°; $[\alpha]_{365}^{20}$ -104.2° (c 0.97 in $CHCl_3$). $C_{74}H_{77}N_7O_{11}$ requires C, 71.60; H, 6.26; N, 7.91%. Found: C, 71.37; H, 6.25; N, 7.75%. Amino-acid analysis gave Gly, 1.00; Phe, 1.99; Tyr, 1.00; Thr, 1.01; Pro, 1.00. Based on Pro = 1.00.

The protected hexapeptide (18) was also prepared in the following manner. 2-(p-Biphenylyl)-isopropyloxycarbonylglycine was liberated from its dicyclohexyl-ammonium salt (0.545 g, 1.1 mmole) by partitioning between diethyl ethyl (50 ml) and citric acid (1.0M, 100 ml) at 0°. The organic layer was washed with water (2 x 50 ml), brine (50 ml) and dried. T.l.c. (R_F (E4) 0.31; R_F (G3) 0.81) indicated that the protected amino acid had suffered no decomposition on liberation. Evaporation gave a white solid which was taken up in tetrahydrofuran (10 ml), N,N'-dicyclo-hexylcarbodi-imide (0.227 g, 1.1 mmole) was added and the mixture stirred at 0° for 1 hour.

The pentapeptide (44) (1.063 g, 1 mmole) was shaken in trifluoroacetic acid for 20 minutes at 0° and evaporated to give an oil. Trituration with diethyl ether

gave a white solid which was dissolved in chloroform at 0°. Triethylamine (0.55 ml, 4 mmole) was added and the solution evaporated to give an oil which was taken up in tetrahydrofuran (10 ml) and added to the previously activated mixture. T.l.c. (E4, G3) indicated that the reaction was extremely slow but was completed within 2 days. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected hexapeptide (18) (0.91 g, 73%) with similar characteristics as before.

Studies with Diphenyldiazomethane

In view of the projected difficulties thought to arise in the decomposition of excess diphenyldiazomethane used in the projected esterification of protected peptides, a series of organic and inorganic acids were investigated as possible replacements for concentrated hydrochloric acid used in the original reference.[144]

A solution of diphenyldiazomethane in ethyl acetate (1 mmole ml^{-1}) was stirred with acid (1 ml) and the disappearance of the deep maroon colour noted with time.

(i) Malonic acid (0.01M). Colour gradually faded and was completely gone in 3 minutes.

(ii) Tartaric acid (0.01M). No colour change within 5 minutes but gradual hydrolysis gave a colourless solution within 1 hour.

(iii) Citric acid (0.7M). No immediate colour change. Gradual hydrolysis within 1 hour.

(iv) Glacial acetic acid. Complete decolourisation within 30 secs.

(v) 50% Aqueous acetic acid. Complete decolourisation within 1 minute.

(vi) Hydrochloric acid (6M). Complete decolourisation < 10 secs.

(vii) Hydrochloric acid (1.0M). Complete decolourisation in 2 minutes.

(viii) Hydrochloric acid (0.1M). Only very slow colour change.

Thus probably the most convenient and mildest conditions are satisfied by shaking with hydrochloric acid (1.0M) and immediately washing the solution with water to remove traces of acid. This has been successfully demonstrated in the preparation of 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46).

The stability of (46) was investigated to its reaction with very dilute acid for a prolonged period. A solution of (46) (20 mg, 0.04 mmole) in chloroform (2 ml) was vigorously shaken with hydrochloric acid (0.01M, 2 ml) and studied by t.l.c. (E4, B). A trace of decomposition was observed after 5 minutes but this did not increase after 2.5 hours and it was only after 2 days that complete decomposition was obtained.

The stability of diphenyldiazomethane to acid was studied by titrating aliquots (100 λ) in 85% aqueous dimethylformamide with hydrochloric acid (1.0M) on an autotitrator and observing the rate of uptake of acid and colour of solution. The original solution is a deep maroon colour.

pH 4.0 Virtually no hydrolysis.

pH 3.0 Slow uptake of acid with the appearance of traces of white insoluble material.

pH 2.5 Slow hydrolysis, colour almost removed indicating little diphenyldiazomethane remaining, increase of insoluble material.

pH 2.0 Complete hydrolysis; solution colourless.

The decomposition of excess diphenyldiazomethane in conditions that would be used in projected reactions, i.e. protected octapeptides, was investigated using

piperidino-oxycarbonylglycine and 2-(biphenylyl)-isopropyloxycarbonylglycine benzhydryl esters (47) and (46) respectively. It was thought that 85% aqueous dimethylformamide would be needed for complete solubilisation of partially protected peptides such as (51).

85% Aqueous dimethylformamide (1.5 ml) was brought to pH 2.5 with hydrochloric acid (1.0M) on an autoburette. Piperidino-oxycarbonylglycine benzhydryl ester in dimethylformamide (100 λ, 95 mg ml^{-1}) and diphenyldiazomethane in dimethylformamide (100 λ, 500 mg ml^{-1}) were added and the excess diphenyldiazomethane decomposed by the addition of hydrochloric acid (1.0M) from an autoburette. When hydrolysis was complete the solution was brought to pH 6-7 with sodium hydroxide (1.0M) and investigated by t.l.c. (E4, B). This was repeated at pH 3.0 and 4.0. Control experiments were carried out with diphenyldiazomethane and (47) separately.

Over the pH range 2.5-4.0 it was found that piperidino-oxycarbonylglycine benzhydryl ester (47) was completely stable and that the additional spots observed by t.l.c. could be assigned to decomposition products of the diphenyldiazomethane.

This procedure was repeated with 2-(biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (44) and it was found that at pH 2.5-3.0 a small amount of decomposition had occurred with the liberation of 2-biphenyl-propan-2-ol and 2-biphenyl-2-propene and formation of glycine benzhydryl ester of approximately 5%.

2-(p-Biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46)

2-(p-Biphenylyl)-isopropyloxycarbonylglycine (313 mg, 1.0 mmole) was liberated from its dicyclohexylammonium salt (494 mg, 1.0 mmole) by partition between citric acid (0.7M, 50 ml) and ethyl acetate (2 x 25 ml) at 0°. The combined

organic fractions were washed with water (25 ml), brine (25 ml) and dried at 4°. Evaporation gave a white solid which was taken up in ethyl acetate (10 ml). Diphenyldiazomethane (234 mg, 1.2 mmole) in ethyl acetate (5 ml) was added and the mixture stirred overnight when the original dark red solution had become almost colourless. T.l.c. (E4, N) indicated complete reaction. Concentrated hydrochloric acid was added dropwise until the solution became colourless. It was then immediately washed with water (3 x 25 ml), saturated sodium hydrogen carbonate (3 x 25 ml), water (3 x 25 ml), brine (3 x 25 ml) and dried. Evaporation gave a colourless gum which was recrystallised from diethyl ether and 30-40° petroleum ether to give 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46) (400 mg, 83.5%) as long white needle crystals of m.p. 113.5-115°, R_F (E4) 0.87; R_F (G3) 0.98; R_F (A2) 0.72; R_F (N) 0.85; R_F (B) 0.66. $C_{31}H_{29}NO_4$ requires C, 77.64; H, 6.10; N, 2.92%. Found: C, 77.65; H, 6.16; N, 2.94%. τ ($CDCl_3$) 2.52-2.68 (19H, complex, aromatic), 3.08 (1H, s, $(C_6H_5)_2.C\underline{H}$), 4.8 (1H, $N\underline{H}.CH_2$), 6.0 (2H, d, $NH.C\underline{H}_2.CO.$), 8.32 (6H, s, $(C\underline{H}_3)_2.C.$).

In another preparation it was found possible to remove excess diphenyldiazomethane by washing the organic layers with portions of hydrochloric acid (1.0M). This milder method proved more satisfactory.

Removal of the 2-(p-biphenylyl)-isopropyloxycarbonyl group

The compound 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46) (1 ml, 6 mg ml^{-1} in CH_2Cl_2) was treated in various acidic solvents to ascertain the optimum conditions for removal of the 2-(p-biphenylyl)-isopropyloxycarbonyl group in the presence of the benzhydryl ester group. The reaction was easily followed by absorbance at 254 nm of the liberated 2-biphenyl-propan-2-ol

and 2-biphenyl-2-propene R_F (E4) 0.83 and 0.64, R_F (B) 0.65, and 0.18 respectively.

(i) 0.05% trifluoroacetic acid in dichloromethane (1 ml)

Very fast removal, within 5 minutes, formation of traces of glycine after 30 minutes.

(ii) 0.15% trifluoroacetic acid in dichloromethane (1 ml)

Very fast removal,. within 30 seconds, traces of glycine after 30 minutes.

(iii) 6:1 (v/v) acetic acid; dichloroacetic acid (1 ml)

Fast reaction, completion within 30 minutes.

(iv) Dichloromethane-75% chloroacetic acid, 50:37 (1 ml)

Fast reaction, completion within 10 minutes, traces of possibly glycine formed after 30 minutes.

(v) 0.5% trifluoroacetic acid in dichloromethane (1 ml), model compound solution (2 ml). 0°.

Removal in 25-30 minutes, no traces of any completely deprotected material.

Thus it is apparent that the 2-(p-biphenylyl)-isopropyloxycarbonyl group could be readily removed in the presence of the benzhydryl ester group although any prolonged treatment caused some ester decomposition. It was concluded that the most satisfactory conditions were met by employing 0.5% trifluoroacetic acid in dichloromethane at 0°.

Glycine benzhydryl ester trifluoroacetate (48)

To a cooled stirred solution of 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46) (205.5 mg, 0.428 mmole) in dichloromethane (0.5 ml) was added 0.5% trifluoroacetic acid in dichloromethane (1 ml). T.l.c. (E4, B)

indicated a slow reaction but deprotection was complete within one hour. The solution was evaporated and triturated with diethyl ether to give glycine benzhydryl ester trifluoroacetate (48) (130 mg, 89%) as a white powder of m.p. 157-160° and R_F (E4) 0.25 streak, R_F (B) 0.15 streak. $C_{17}H_{16}NO_4F_3$ requires C, 57.43; H, 4.54; N, 3.94; F, 16.04%. Found: C, 57.15; H, 4.68; N, 4.11; F, 16.18%.

Piperidino-oxycarbonylglycine benzhydryl ester (47)

Piperidino-oxycarbonylglycine (185 mg, 0.915 mmole) was dissolved in a mixture of ethyl acetate (10 ml) and chloroform (2 ml). Diphenyldiazomethane (215 mg, 1.15 mmole) in ethyl acetate (5 ml) was added and the mixture stirred overnight at room temperature. The deep maroon colour of the solution gradually disappeared and t.l.c. (E4, G3) indicated complete reaction. The solution was washed rapidly with hydrochloric acid (1.0M, 2 x 3 ml), water (2 x 5 ml), saturated sodium hydrogen carbonate (2 x 5 ml), water (2 x 5 ml), brine (2 x 5 ml) and dried. Evaporation gave a white solid which was recrystallised from ethyl acetate/30-40° petroleum ether to give piperidino-oxycarbonylglycine benzhydryl ester (47) (240.8 mg, 72.0%) as white needle crystals of m.p. 124-5°. $C_{21}H_{24}N_2O_4$ requires C, 68.5; H, 6.57; N, 7.63%. Found: C, 68.15; H, 6.47; N, 7.63%.

τ ($CDCl_3$) 2.58 (10H, complex, aromatic), 3.10 (1H, s, CO.CH), 4.8 (1H, s, NH), 5.85 (2H, d, NH.CH_2.CO), 7.0 (4H, d, CH_2.N.CH_2), 8.3 (c, 6H, CH_2.$(CH_2)_3$.CH_2).

Removal of the piperidino-oxycarbonyl group

The compound piperidino-oxycarbonylglycine benzhydryl ester (47) was investigated to determine optimum conditions for the removal of the piperidino-

oxycarbonyl group in the presence of the benzhydryl ester group. T.l.c. (E4, B) indicated formation of liberated piperidine R_F (E4 and B) 0.0.

(i) 80% aqueous dimethylformamide

Piperidino-oxycarbonylglycine benzhydryl ester (47) (30 mg, 0.0815 mmole) was dissolved in 80% aqueous dimethylformamide (1 ml). Excess sodium dithionite (50 mg, 0.26 mmole) was added. The solution turned gradually yellow but after 5 hours only slight deprotection had taken place. A trace of 50% aqueous acetic acid was added and stirring continued overnight when complete removal had occurred.

(ii) 50% aqueous acetic acid

Piperidino-oxycarbonylglycine benzhydryl ester (47) (30 mg, 0.0815 mmole) was dissolved in 50% aqueous acetic acid (0.5 ml). Excess sodium dithionite (50 mg, 0.26 mmole) was added with immediate evolution of sulphur dioxide and formation of a yellow solution. T.l.c. (E4, B) indicated complete deprotection within 75 minutes giving a product identical to the sample obtained from the deprotection of 2-(p-biphenylyl)-isopropyloxycarbonylglycine benzhydryl ester (46).

Studies of the Protected Precursor

α-t-Butoxycarbonyl-O-benzyl-L-threonyl-L-prolyl-ε-t-butoxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (49)

The tetrapeptide (23) (141 mg, 0.157 mmole) was dissolved in 50% aqueous acetic acid (0.5 ml), sodium dithionite (50 mg, 0.26 mmole) was added and the mixture stirred for 20 minutes at room temperature. The solution turned immediately orange and finally gave a clear solution. T.l.c. (E4, G3) indicated complete removal of the ε-piperidino-oxycarbonyl group. The solution was neutralised with

sodium hydrogen carbonate and evaporated to give a white solid which was taken up in dimethylformamide (5 ml). Triethylamine (0.0217 ml, 0.471 mmole) and excess t-butoxycarbonyl azide (0.026 ml, 0.184 mmole) were added and the mixture stirred at room temperature overnight. Evaporation produced a white solid which was triturated with 40-60° petroleum ether, to remove any excess t-butoxycarbonyl azide and cooled in an ice-salt bath. Citric acid (0.7M, 10 ml) was added and the product extracted into ethyl acetate (2 x 10 ml) with solid sodium hydrogen carbonate. The combined organic phases were washed with water (2 x 10 ml), brine (2 x 10 ml) and dried. Evaporation gave a colourless gum which on standing under 40-60° petroleum ether and trituration gave the protected tetrapeptide (49) (89.1 mg, 66.1%) as a chromatographically pure white powder of m.p. 55-7°, R_F (E4) 0.60; R_F (G3) 0.81; R_F (A2) 0.48, $[\alpha]_{589}^{20}$ -29.7°; $[\alpha]_{578}^{20}$ -31.1°; $[\alpha]_{546}^{20}$ -34.1°; $[\alpha]_{436}^{20}$ -64.5°; $[\alpha]_{365}^{20}$ -109.0° (c 1.03 in $CHCl_3$). $C_{41}H_{60}N_6O_{10}.H_2O$ requires C, 60.42; H, 7.67; N, 10.31%. Found: C, 60.94; H, 7.72; N, 10.04%.

α-Benzyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolyl-ε-t-butoxycarbonyl-L-lysyl-L-alanine 4-picolyl ester (50)

The octapeptide (8) (200 mg, 0.14 mmole) was dissolved in 50% aqueous acetic acid (2 ml). Excess sodium dithionite (50 mg, 0.26 mmole) was added and the mixture stirred at room temperature for 20 minutes. T.l.c. (E4, G3) indicated complete removal of the ε-piperidino-oxycarbonyl group; the solution was neutralised with sodium hydrogen carbonate, evaporated to a white solid, triturated with water and dried. This was taken up in dimethylformamide (10 ml) and triethylamine added until pH 9.8. Excess t-butoxycarbonyl azide (0.4 ml, 0.28 mmole) was added and

the mixture stirred overnight at room temperature. Evaporation gave a yellow oil which was triturated with diethyl ether, to remove excess t-butoxycarbonyl azide, and was taken up in chloroform (10 ml) and washed with water (10 ml), saturated sodium hydrogen carbonate (2 x 10 ml), brine (2 x 10 ml) and dried. The solution was applied to Amberlyst 15 (4 ml, 3-bromopyridinium form). T.l.c. (E4, G3) indicated that the peptide had been completely absorbed within 1 hour. The column was washed with chloroform (100 ml) and eluted with 40% pyridine in dimethyl-formamide (100 ml cooled overnight). Evaporation of the eluate gave an oil which on trituration with diethyl ether gave the protected octapeptide (50) (145 mg, 72%) as a chromatographically pure, white powder of m.p. 114-21°, R_F (E4) 0.66; R_F (G3) 0.82; R_F (A2) 0.54; R_F (N) 0.75, $[\alpha]_{589}^{20}$ -32.6°; $[\alpha]_{578}^{20}$ -33.9°; $[\alpha]_{546}^{20}$ -38.9°; $[\alpha]_{436}^{20}$ -69.4°; $[\alpha]_{365}^{20}$ -116.3° (c 1 in $CHCl_3$). $C_{81}H_{94}N_{10}O_{15}.2H_2O$ requires C, 65.2; H, 6.66; N, 9.51%. Found: C, 65.60; H, 6.55; N, 9.44%. Amino acid analysis gave Gly, 1.01; Phe, 1.98; Tyr, 0.98; Thr, 1.00; Pro, 1.00; Lys, 0.99; Ala, 1.00. Based on Ala = 1.00.

Hydrogenation studies

(i) 80% aqueous dimethylformamide

The octapeptide (50) (10 mg) was hydrogenated in 80% aqueous dimethyl-formamide (1 ml) with 5% palladium on charcoal. After 32 hours, electrophoresis at pH 1.9 and pH 6.5 indicated a heavy base line spot and only traces of a possible product. There was however an intense spot corresponding to 4-methyl-piperidine, the reduction product of the 4-picolyl ester.

(ii) Dimethylformamide containing a trace of acetic acid

Hydrogenation of the octapeptide (50) (5 mg) with 5% palladium on charcoal

gave a product that remained as a base line spot on similar electrophoresis even after 64 hours.

(iii) 50% Aqueous acetic acid

The tetrapeptide (23) (28 mg) in 50% aqueous acetic acid (2.8 ml) was hydrogenated with 10% palladium on charcoal (10 mg). Electrophoresis after 27 hours indicated complete reaction. $E_{Arg}^{pH\,1.9}$ 0.43; $E_{Arg}^{pH\,6.5}$ 0.01. Ninhydrin positive, initially yellow turning red due to a free ε-amino group.

(iv) The pentapeptide (24) (31 mg) in 50% aqueous acetic acid (3 ml) was hydrogenated with palladium black (10 mg) for 72 hours. The reaction was complete after 27 hours and no further change took place. $E_{Arg}^{pH\,1.9}$ 0.34; $E_{Arg}^{pH\,6.5}$ 0.02. Ninhydrin positive, initially yellow turning red as in (iii), and also tyrosine positive. It was found that the α-nitroso-β-naphthol reagent is positive only for a free phenolic hydroxyl group.

(v) 80% Aqueous acetic acid

The octapeptide (50) (10 mg) in 80% aqueous acetic acid (3 ml) was hydrogenated with 10% palladium on charcoal (10 mg) for 18 hours. Electrophoresis, pH 1.9 and pH 6.5, indicated that the previous base line spot had disappeared and was replaced with spots, $E_{Arg}^{pH\,1.9}$ 0.26 and $E_{Arg}^{pH\,1.9}$ 0.45, both yellow with ninhydrin but the latter turning red rapidly indicating a free ε-amino group and hence t-butoxycarbonyl cleavage. This latter spot increased in intensity after further hydrogenation.

(vi) 50% Aqueous acetic acid

The octapeptide (50) (20 mg) in 50% aqueous acetic acid (2 ml) was hydrogenated with 10% palladium on charcoal (10 mg) for 30 hours. Electrophoresis

gave a single ninhydrin and tyrosine positive spot, $E^{pH\,1.9}_{Arg}$ 0.27 and $E^{pH\,6.5}_{Arg}$ 0.01.

(vii) 50% Aqueous acetic acid

The alanine analogue (13) (20 mg) was hydrogenated as in (vi). Electrophoresis gave a single ninhydrin and tyrosine spot, $E^{pH\,1.9}_{Arg}$ 0.55 and $E^{pH\,6.5}_{Arg}$ 0.30.

Glycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-ε-t-butoxycarbonyl-L-lysyl-L-alanine (51)

The protected octapeptide (50) (62.1 mg, 0.042 mmole) was dissolved in 50% aqueous acetic acid (6 ml) and hydrogenated for 30 hours with 10% palladium on charcoal (10 mg). The solution was filtered through Celite and washed with more 50% aqueous acetic acid (10 ml); evaporation gave the octapeptide (51) (40 mg, 86%) as a chromatographically pure white powder, R_F (G4) 0.80; R_F (H) 0.64, $E^{pH\,1.9}_{Arg}$ 0.30 and $E^{pH\,6.5}_{Arg}$ 0.01, identical to a sample of the octapeptide (51) obtained from a trypsin cleavage of the fully protected insulin (Dr. H. T. Storey).

$C_{52}H_{71}N_9O_{13}.CH_3COOH.H_2O$ requires C, 58.5; H, 6.96; N, 11.35%. Found: C, 58.67; H, 6.67; N, 11.74%. Amino acid analysis gave Gly, 1.01; Phe, 1.97; Tyr, 0.98; Thr, 0.99; Pro, 0.99; Lys, 0.99; Ala, 1.00. Based on Ala = 1.00.

Glycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-ε-t-butoxycarbonyl-L-lysyl-L-alanine benzhydryl ester (10)

The octapeptide (51) (0.35 mg) was dissolved in dimethylformamide (0.5 ml). Excess diphenyldiazomethane (100 λ, 100 mg ml^{-1} in dimethylformamide) was added and the mixture stirred for 48 hours at room temperature. The maroon colour was still present and excess diphenyldiazomethane was decomposed with hydrochloric

acid (0.1M) to give a colourless solution. Evaporation and trituration with diethyl ether gave a white solid. Electrophoresis before and after trifluoroacetic acid treatment gave single spots at pH 1.9 and pH 6.5. Amino acid analysis gave Gly, 0.98; Phe, 1.97; Tyr, 0.97; Thr, 0.96; Pro, 1.00; Lys, 0.99; Ala, 1.00. Based on Ala = 1.00. Thus there was no evidence for N-alkylation. $E_{Arg}^{pH\,1.9}$ 0.30; $E_{Arg}^{pH\,6.5}$ 0.02. Trifluoroacetic acid treated: $E_{Arg}^{pH\,1.9}$ 0.38; $E_{Arg}^{pH\,6.5}$ 0.02.

Cyclisation Studies

α-t-Butoxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (52)

α-t-Butoxycarbonylglycine (0.96 g, 5.5 mmole) and 1-hydroxybenzotriazole (0.84 g, 5.5 mmole) were dissolved in tetrahydrofuran (25 ml). N,N'-Dicyclocarbodi-imide (1.13 g, 5.5 mmole) was added and the mixture stirred at 0° for 1 hour and at room temperature for 1.5 hours.

The pentapeptide (44) (5.24 g, 5.0 mmole) was shaken in trifluoroacetic acid (30 ml, 390 mmole) at 0° for 20 minutes and evaporated to give a brownish oil. Trituration with diethyl ether gave a white solid which was taken up in tetrahydrofuran (20 ml) and added to the acylating mixture. N,N-Di-isopropylethylamine (1.36 ml, 10 mmole) was added dropwise to the activated mixture to pH 8. T.l.c. (E4, G3) indicated that the reaction was complete within 15 minutes and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected hexapeptide (52) (5.23 g, 94%) as a chromatographically pure, white powder of m.p. 162-3°, R_F (E4) 0.65; R_F (G3) 0.77; R_F (A2) 0.65, $[\alpha]_{589}^{20}$ -38.4°; $[\alpha]_{578}^{20}$ -40.0°; $[\alpha]_{546}^{20}$ -45.6°; $[\alpha]_{436}^{20}$ -78.90°; $[\alpha]_{365}^{20}$ -127.0° (c 0.62 in $CHCl_3$).

$C_{64}H_{71}N_7O_{11}.2H_2O$ requires C, 66.70; H, 6.52; N, 8.51%. Found: C, 66.54; H, 6.76; N, 8.54%. Amino-acid analysis gave Gly, 1.00; Phe, 1.99; Tyr, 1.00; Thr, 1.01; Pro, 1.00. Based on Pro = 1.00.

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (53)

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine (0.465 g, 1.25 mmole liberated from the dicyclohexylammonium salt) and 1-hydroxybenzotriazole (0.197 g, 1.25 mmole) were dissolved in tetrahydrofuran (20 ml). N,N'-Dicyclohexylcarbodiimide (0.258 g, 1.25 mmole) was added and the mixture stirred for 1.5 hours at 0° and 1 hour at room temperature.

The hexapeptide (52) (1.14 g, 1.0 mmole) was shaken in trifluoroacetic acid (5 ml, 65 mmole) for 20 minutes at 0° and evaporated to give a yellowish oil. Trituration with diethyl ether gave a white solid which was dissolved in tetrahydrofuran (10 ml) and added to the acylating mixture. N,N-Di-isopropylethylamine (0.34 ml, 2.0 mmole) was added dropwise to the activated mixture to pH 8. T.l.c. (E4, G3) indicated that the reaction was complete within 1 hour and isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected heptapeptide (53) (0.896 g, 94.1%) as a chromatographically pure white powder of m.p. 160-2°, R_F (E4) 0.58; R_F (G3) 0.71; R_F (A2) 0.57, $[\alpha]_{589}^{20}$ -31.4°; $[\alpha]_{578}^{20}$ -32.6°; $[\alpha]_{546}^{20}$ -37.5°; $[\alpha]_{436}^{20}$ -64.8°; $[\alpha]_{365}^{20}$ -104.0° (c 1.01 in $CHCl_3$). $C_{75}H_{92}N_{10}O_{14}.H_2O$ requires C, 65.48; H, 6.89; N, 10.18%. Found: C, 65.21; H, 6.99; N, 10.53%. Amino acid analysis gave Lys, 1.00; Gly, 1.00; Phe, 2.02; Tyr, 0.98; Thr, 1.00; Pro, 1.00. Based on Pro = 1.00.

α-t-Butoxycarbonyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine (56)

The tetrapeptide (23) (80.1 mg, 0.0972 mmole) was dissolved in dioxan (1 ml). Sodium hydroxide (1.32 ml, 0.1M, 0.132 mmole) was added and the mixture stirred at room temperature for 1 hour. Water (10 ml) was added and the pH brought to 3.5 by the addition of solid citric acid. The free acid was extracted into ethyl acetate (2 x 5 ml) and the combined organic fractions were washed with water (5 ml), brine (5 ml) and dried. Evaporation gave a colourless oil which on trituration with diethyl ether gave the protected tetrapeptide (56) (60.9 mg, 86.8%) as a chromatographically pure white powder of m.p. 69-71°, R_F(E4) 0.24 streak; R_F (G3) 0.45; R_F (A2) 0.60; R_F (N) 0.85, $[\alpha]_{589}^{20}$ -31.8°; $[\alpha]_{578}^{20}$ -32.3°; $[\alpha]_{546}^{20}$ -36.6°; $[\alpha]_{436}^{20}$ -66.1°; $[\alpha]_{365}^{20}$ -114.1° (c 1.0 in $CHCl_3$). $C_{36}H_{56}N_6O_{10}$ requires C, 58.99; H, 7.70; N, 11.47%. Found: C, 58.72; H, 8.08; N, 11.19%.

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline (55)

The heptapeptide (53) (642.1 mg, 0.474 mmole) was dissolved in dimethylformamide (1 ml). Excess sodium hydroxide (1.0M, 0.57 ml, 0.57 mmole) was added and the solution stirred for 1.5 hours. T.l.c. (E4, G3) indicated that complete hydrolysis had occurred. The solution was cooled to 0°, water (20 ml) was added and brought to pH 3.5 by the addition of solid citric acid. The product was extracted into chloroform (2 x 10 ml) and the combined fractions washed with water (3 x 10 ml), brine (2 x 10 ml) and dried. Evaporation gave the protected heptapeptide (55) (570 mg, 94.2%) as a chromatographically pure white powder of m.p. 182-4°, R_F (E4) 0.18 streak; R_F (G3) 0.79; R_F (N) 0.95; R_F (A2) 0.83, $[\alpha]_{589}^{20}$ -22.8°;

$[\alpha]_{578}^{20}$ -23.6°; $[\alpha]_{546}^{20}$ -29.7°; $[\alpha]_{436}^{20}$ -44.8°; $[\alpha]_{365}^{20}$ -71.9° (c 0.26 in $CHCl_3$). $C_{69}H_{87}N_9O_{14}.H_2O$ requires C, 64.52; H, 6.98; N, 9.82%. Found: C, 64.47; H, 7.30; N, 10.00%. Amino acid analysis gave Lys, 1.02; Gly, 1.00; Phe, 2.02; Tyr, 0.99; Thr, 0.97; Pro, 1.00. Based on Pro = 1.00.

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline 4-picolyl ester (54)

The heptapeptide (55) (481 mg, 0.352 mmole) and 1-hydroxybenzotriazole (53.8 mg, 0.352 mmole) were dissolved in tetrahydrofuran (5 ml). N,N'-Dicyclohexylcarbodi-imide (72.5 mg, 0.352 mmole) was added and the mixture stirred at 0° for 2 hours and room temperature for 1 hour when t.l.c. (E4, G3) indicated that the active ester had formed.

The hexapeptide (52) (357 mg, 0.32 mmole) was shaken in trifluoroacetic acid (2 ml, 26 mmole) for 20 minutes at 0° and evaporated to give a yellow oil which on trituration with diethyl ether gave a white solid. This was taken up in tetrahydrofuran (5 ml), N,N-di-isopropylethylamine (0.111 ml, 0.64 mmole) was added to a cooled solution and the mixture added to the previously activated heptapeptide. T.l.c. (E4, G3) after stirring overnight indicated that the reaction was complete. Isolation by the Amberlyst 15 (3-bromopyridinium form) procedure gave the protected tridecapeptide (54) (602 mg, 72.0%) as a chromatographically pure white powder, R_F (E4) 0.66; R_F (G3) 0.98; R_F (N) 0.91; R_F (A2) 0.67, $[\alpha]_{589}^{20}$ -31.8°; $[\alpha]_{578}^{20}$ -34.3°; $[\alpha]_{546}^{20}$ -48.4°; $[\alpha]_{436}^{20}$ -65.8°; $[\alpha]_{365}^{20}$ -104.5° (c 0.51 in $CHCl_3$). $C_{127}H_{148}N_{16}O_{22}$ requires C, 67.77; H, 6.63; N, 9.96%. Found: C, 67.48;

H, 6.68; N, 10.21%. Amino acid analysis gave Lys, 1.00; Gly, 2.02; Phe, 3.99; Tyr, 1.97; Thr, 1.97; Pro, 2.02. Based on Lys = 1.00.

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline (60)

The tridecapeptide (54) (30 mg, 0.0134 mmole) was dissolved in 50% aqueous dioxan (1 ml). Excess sodium hydroxide (1 ml, 0.1 mmole) was added and the mixture stirred for 1 hour when t.l.c. (E4, G3) indicated complete hydrolysis. The solution was diluted with water (10 ml), cooled to 0° and brought to pH 3.5 by the addition of solid citric acid. The product was extracted into chloroform (2 x 10 ml) and the combined organic fractions washed with water (3 x 10 ml), brine (2 x 10 ml) and dried. Evaporation gave the protected tridecapeptide (60) (23.8 mg, 81.1%) as a chromatographically pure white solid of m.p. 176-80°, R_F (E4) 0.20 streak; R_F (G3) 0.80, $[\alpha]_{589}^{20}$ -22.6°; $[\alpha]_{578}^{20}$ -21.6°; $[\alpha]_{546}^{20}$ -23.8°; $[\alpha]_{436}^{20}$ -41.7°; $[\alpha]_{365}^{20}$ -82.1° (c 0.69 in $CHCl_3$). $C_{121}H_{143}N_{15}O_{22}.2H_2O$ requires C, 66.22; H, 6.71; N, 9.58%. Found: C, 65.73; H, 7.11; N, 9.77%.

Hydrogenation of (60) (10 mg) in 50% aqueous acetic acid (1 ml) for 48 hours gave a mixture of products, 2-3 spots on electrophoresis at pH 1.9 and pH 6.5. Continued hydrogenation did not resolve this situation.

α-t-Butoxycarbonyl-O-benzyl-L-threonyl-L-prolyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine hydrazide (59)

The tetrapeptide (23) (50 mg, 0.0608 mmole) was dissolved in methanol (1 ml). An excess of hydrazine hydrate (0.0314 ml, 0.6 mmole) was added and the mixture

stirred at room temperature. The reaction was followed by t.l.c. (E4, G3) and the product was detected by hydrazide spray. A fine white precipitate formed and the reaction was complete within 1 hour. The product was filtered, washed with cold methanol to yield the protected tetrapeptide hydrazide (59) (39 mg, 86.0%) as a chromatographically pure white powder of m.p. 120-1°, R_F (E4) 0.46; R_F (G3) 0.66; R_F (N) 0.62, $[\alpha]_{589}^{20}$ -29.3°; $[\alpha]_{578}^{20}$ -30.8°; $[\alpha]_{546}^{20}$ -34.8°; $[\alpha]_{436}^{20}$ -60.6°; $[\alpha]_{365}^{20}$ -97.4° (c 0.33 in $CHCl_3$). $C_{36}H_{58}N_8O_9.H_2O$ requires C, 56.52; H, 7.90; N, 14.65%. Found: C, 56.50; H, 7.61; N, 14.98%.

α-t-Butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline hydrazide (58)

The protected tridecapeptide (54) (33.6 mg, 0.015 mmole) was dissolved in methanol (1 ml). Excess hydrazine hydrate (0.2 ml, 42 mmole) was added and the mixture stirred overnight at room temperature. T.l.c. (E4, G3, hydrazine reagent) indicated that the reaction was complete. The solution was evaporated to a white solid, triturated with diethyl ether and dried to give the protected tridecapeptide hydrazide (58) (25 mg, 75.6%) as a chromatographically pure white powder of m.p. 183-6°, R_F (E4) 0.60; R_F (G3) 0.91, $[\alpha]_{589}^{20}$ -25.1° (c 0.71 in $CHCl_3$). $C_{121}H_{145}N_{17}O_{21}.2H_2O$ requires C, 65.77; H, 6.79; N, 10.78%. Found: C, 66.10; H, 6.40; N, 10.59%.

Hydrogenation of (58) (10 mg) in 50% aqueous acetic acid (1 ml) with 5% palladium on charcoal (10 mg) for 48 hours gave a mixture of products, 3 main spots on electrophoresis pH 1.9 and pH 6.5. Continued hydrogenolysis failed to improve this situation.

Attempted cyclisation of N-α-t-butoxycarbonyl-L-lysylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-prolylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-O-benzyl-L-threonyl-L-proline (61)

The protected tridecapeptide (60) (225 mg, 0.1 mmole) was dissolved in 20% aqueous acetic acid (0.5 ml) and excess sodium dithionite (50 mg) was added. The solution turned orange and then cleared after 20 minutes. T.l.c. (E4, G3) indicated complete removal of the ε-piperidino-oxycarbonyl group. The solution was neutralised with sodium hydrogen carbonate and evaporated to dryness and triturated with water. The deprotected material (61) was dried at high vacuum.

(i) N,N'-Dicyclohexylcarbodi-imide—1-hydroxybenzotriazole

The deprotected tridecapeptide (61) (50 mg, 0.022 mmole) was dissolved in dimethylformamide (20 ml). N,N'-Dicyclohexylcarbodi-imide (25 mg, 0.022 mmol 1-hydroxybenzotriazole (20 mg, 0.022 mmole) and triethylamine (0.03 ml, 0.022 mmole) were added and the mixture stirred at room temperature for several days. T.l.c. (E4, G3) strongly suggested that a large amount of starting product and urea was present. There was no indication of any formation of other products.

(ii) N,N'-Dicyclohexylcarbodi-imide—N-hydroxysuccinimide

The deprotected tridecapeptide (61) (50 mg, 0.022 mmole) was dissolved in dimethylformamide (35 ml) and N-hydroxysuccinimide (25 mg) added. The solution was stirred for 1.5 hours at 0°. N,N'-Dicyclohexylcarbodi-imide (30 mg) was added and the solution neutralised with N,N-di-idopropylethylamine and stirred for 2 days at room temperature. The mixture was evaporated and taken up in chloroform (25 ml) and washed with water (20 ml), saturated sodium hydrogen carbonate (3 x 20 ml), brine (2 x 20 ml) and dried. T.l.c. (E4, G3, A2) indicated

that no cyclic product with high R_F values had formed and that only starting material and urea were present.

(iii) 2-Ethyl-5-phenylisoxazolium-3'-sulphonate

The method of Blaha and Rudinger[193] was followed. The deprotected tridecapeptide (61) (50 mg, 0.022 mmole) was dissolved in dimethylformamide (35 ml) at 0° and stirred with 2-ethyl-5-phenylisoxazolium-3'-sulphate (25 mg) for 2 hours. N,N'-Di-isopropylethylamine (0.031 ml, 0.022 mmole) was added and the solution diluted with dimethylformamide (10 ml). After 60 hours the mixture was isolated as in (ii) and no cyclic product could be detected by t.l.c. (E4, G3, A2). Large amounts of starting material were still present.

(iv) Mixed anhydride[194]

The protected tridecapeptide (61) (50 mg, 0.022 mmole) was dissolved in tetrahydrofuran (30 ml) and cooled to -20°. s-Butyl chloroformate (0.25 ml) was added and the mixture stirred at -10° for 20 minutes; the solution was neutralised with N,N-di-isopropylethylamine and diluted with tetrahydrofuran (20 ml). The mixture was stirred for 60 hours at room temperature and isolated as in (ii). T.l.c. (E4, G3, A2) only detected starting materials without any indication of the formation of a cyclic product.

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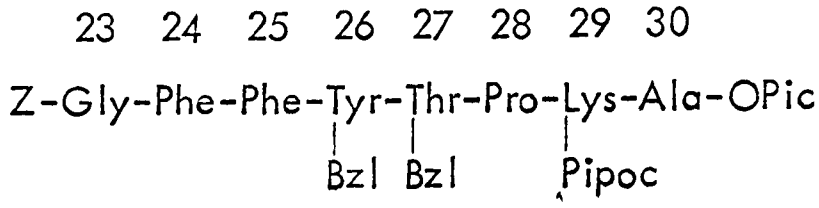
ABSTRACT

The preparation of polypeptide and protein analogues by conventional or fragment condensation synthesis is often a complex and time-consuming procedure. The combination of protected natural peptide fragments, isolated by enzymatic cleavage, and synthetic peptides could give these analogues more rapidly and conveniently than before. A semisynthetic approach is therefore an alternative approach for effective introduction of amino acid substitutions in enzymes and proteins. Insulin offers an excellent opportunity in which this approach can be investigated as tryptic cleavage removes the B-chain C-terminal octapeptide to give des-octapeptide insulin. This thesis reports the synthesis of protected B-chain C-terminal peptides that could be coupled to a similarly protected des-octapeptide insulin. Complete deprotection would then yield a semisynthetic insulin.

The intended use of acid labile groups such as t-butoxycarbonyl and benzhydryl esters for the final protected fragments imposed severe restrictions on planning and synthesising the protected fragments. The syntheses were therefore devised such that fully protected peptides could be prepared by conventional strategy and then converted to a much milder protected form suitable for coupling.

The 4-picolyl ester method[1] was used to synthesise the fully protected natural octapeptide (1) using the t-butoxycarbonyl group for α-amino protection,



(1)

except for the ultimate glycine residue which was incorporated as its benzyloxy-carbonyl derivative, piperidino-oxycarbonyl for ε-amino lysine protection and benzyl ethers for threonine and tyrosine hydroxyl group protection. The use of these groups was such that by a series of deprotection-reprotection steps the mildly protected derivative (2) could be prepared and this would be in a form ready for coupling. The synthetic route for the preparation of (1) is outlined in Figure 1.

$$\text{H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Boc)-Ala-OCHPh}_2 \qquad (2)$$

The combination of N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole for coupling reactions was employed throughout the synthesis due to its advantages with regard to speed, simplicity of use and absence of racemisation.[2]

The piperidino-oxycarbonyl group had been proposed for α- and ε-protection by Stevenson[3] and was introduced by either piperidino succinimido carbonate or piperidino 2,4,5-trichlorophenyl carbonate. Nash[4] found the latter preferable and obtained α-benzyloxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine which was converted to α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine by treatment with hydrogen bromide in acetic acid and the t-butoxycarbonyl azide. This method proved unsatisfactory because of low yields and impurities thought to be L-lysine and ε-benzyloxycarbonyl-L-lysine arising at each stage. A new synthetic route was proposed as in Figure 2. Treatment of ε-benzyloxycarbonyl-L-lysine with t-butoxycarbonyl azide gave α-t-butoxycarbonyl-ε-benzyloxycarbonyl-L-lysine in 91% yield. Hydrogenation in 80% aqueous ethanol gave α-t-butoxycarbonyl-L-lysine in 83.2% yield which was reacted with piperidino 2,4,5-trichlorophenyl

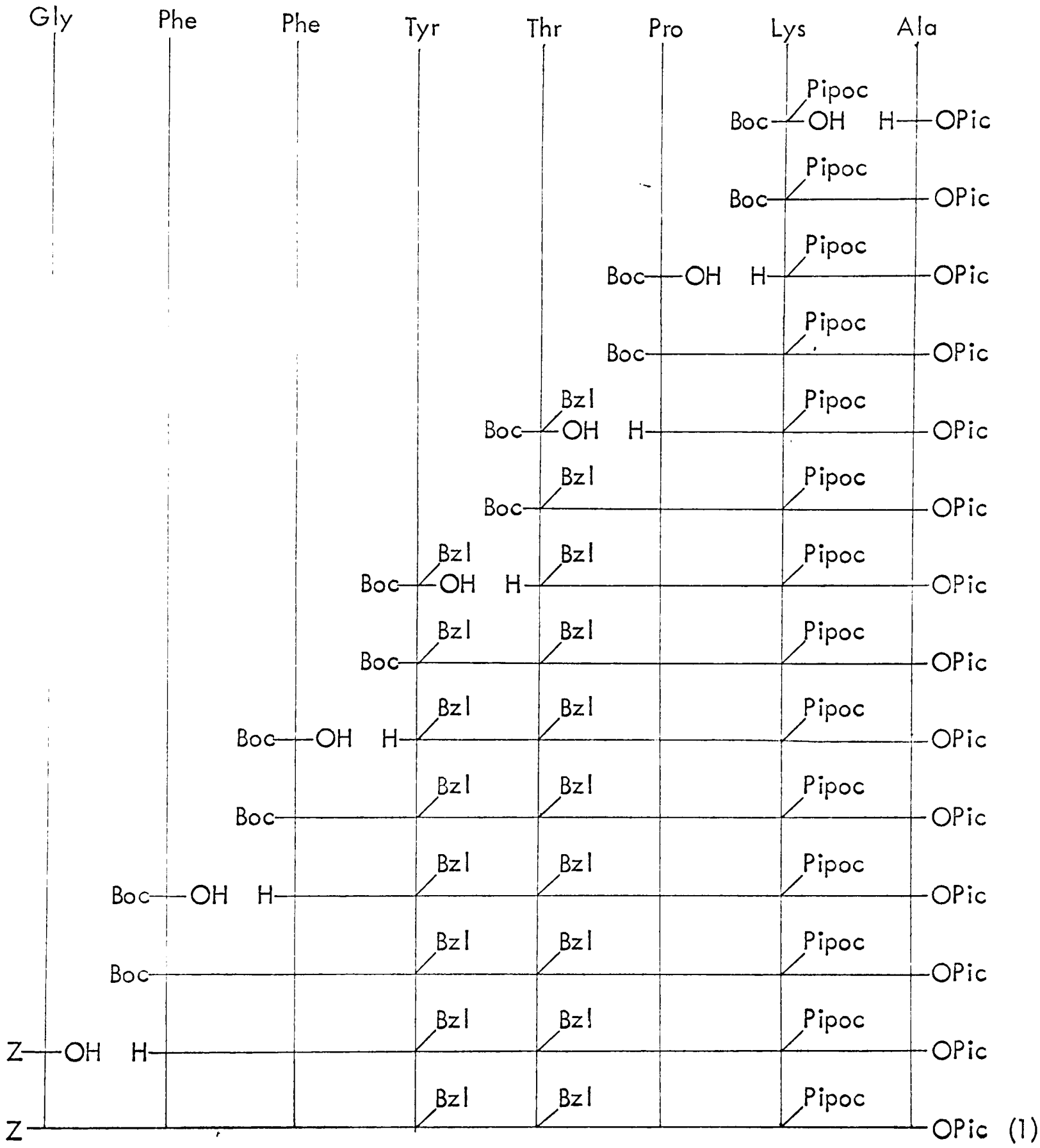

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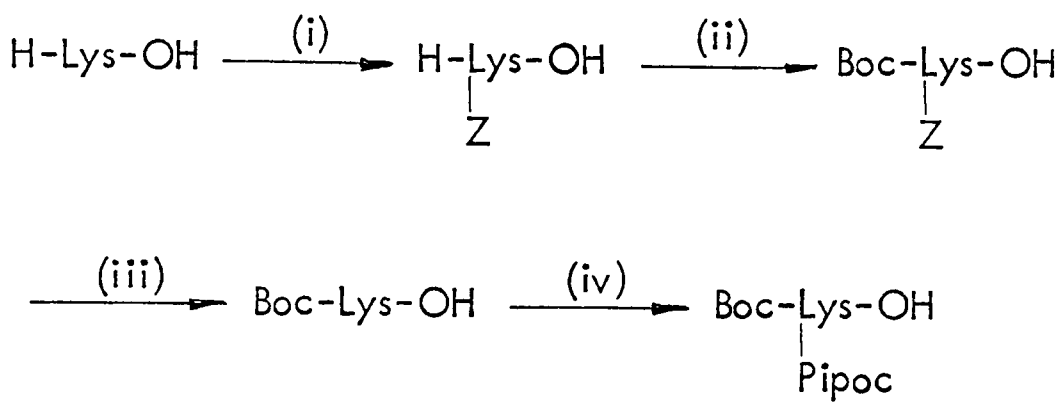
Figure 1

carbonate to give α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine, which was isolated as its dicyclohexylammonium salt in 68% yield.



(i) $CuCO_3$, $Cu(OH)_2$, Z-Cl, EDTA/H_2S (ii) Boc azide

(iii) H_2/Pd (iv) Piperidino 2,4,5-trichlorophenyl carbonate/ 1,1,3,3-tetramethyl-guanidine.

Figure 2

The C-terminal derivative L-alanine 4-picolyl ester dihydrobromide was prepared by the method of Garner[5] in 96.5% yield. The dipeptide, α-t-butoxy-carbonyl-ε-piperidino-oxycarbonyl-L-lysyl-L-alanine 4-picolyl ester, was prepared by coupling α-t-butoxycarbonyl-ε-piperidino-oxycarbonyl-L-lysine and L-alanine 4-picolyl ester dihydrobromide with N,N'-dicyclohexylcarbodi-imide and 1-hydroxy-benzotriazole. Isolation by extraction into citric acid (0.7M) gave the dipeptide in 95.4% yield, chromatographically and analytically pure with satisfactory amino acid analysis and n.m.r. spectrum. The dipeptide was deprotected with trifluoro-acetic acid and coupled to α-t-butoxycarbonyl-L-proline in a similar manner as before. Repetition of the process gave the protected pentapeptide although extraction with citric acid was increasingly difficult because of the hydrophobic nature of the

peptide and isolation of the hexa-, hepta- and octapeptides was carried out using the Amberlyst 15 (3-bromopyridinium form) procedure. All protected intermediates were prepared in high yields, >90%, chromatographically and analytically pure with satisfactory amino acid analyses. The overall yield of the protected octapeptide from the 4-picolyl ester dihydrobromide was 56%.

From the X-ray analysis of insulin and chemical and biological studies,[6] it is known that the aromatic residues phenylalanine -B24 and -B25 are essential for activity of the hormone and play an active role in the hydrogen-bonding capacity of the monomer in the dimer. Using the 4-picolyl ester method and synthetic schemes similar to Figure 1, analogues involving the replacement or modification of residues B24 and B25 were considered.

The most straightforward replacement was of alanine for phenylalanine; the analogues (3-5) were prepared exactly as before by the 4-picolyl ester method in high yields.

In an attempt to relate biological results in terms of the hydrogen-bonded nature of the dimer, modification of the B-chain imino nitrogens was considered. N-Methylation would reduce the ability of the monomer to form hydrogen bonds and this could be accomplished by the replacement of phenylalanine by N-methyl-L-phenylalanine. Only recently has there been any detailed studies involving peptide bond formation and the risk of racemisation with N-methyl-amino acids;[7] there had been no such investigation with N-methyl-L-phenylalanine. It was decided to prepare the dipeptide (6) to ascertain the optimum method for coupling without racemisation. N-Methyl-L-phenylalanine was prepared by the method of

Boc-MePhe-MePhe-OMe (6)

Quitt[8] and α-t-butoxycarbonyl-N-methyl-L-phenylalanine was prepared by an adaptation of the method of Blake and Li.[9] N-Methyl-L-phenylalanine methyl ester hydrochloride was formed by reaction of the acid and thionyl chloride in methanol at -10° in 76% yield. The preparation of the dipeptide (6) was not as simple as initially thought. The use of N,N'-dicyclohexylcarbodi-imide was completely unsuccessful, whereas N,N'-dicyclohexylcarbodi-imide and 1-hydroxy-benzotriazole in either a normal or an 'eintopf' procedure gave mainly the N-acyl urea which was characterised. Surprisingly the analogous use of N-hydroxy-succinimide gave no product at all. However, the use of a mixed anhydride procedure gave the dipeptide (6). Hydrolysis of the product gave material which had the same characteristics as N-methyl-L-phenylalanine. The dipeptide (6) was also characterised as its hydrazide. Thus the incorporation of N-methyl-L-phenyl-alanine was carried out by a mixed anhydride procedure to give the protected analogues (7-9) which were isolated as before by the Amberlyst 15 (3-bromopyridinium form) procedure.

The 2-(p-biphenylyl)-isopropyloxycarbonyl group, not previously used with the 4-picolyl ester method, was investigated for its suitability in this type of synthetic approach and although it is extremely acid labile it did not undergo any cleavage on Amberlyst 15 (3-bromopyridinium form). Two truncated derivatives (10 and 11) were prepared using the 2-(p-biphenylyl)-isopropyloxycarbonyl group for the α-amino protection of the terminal glycine residue in similar high yields as before. Such was the success of this derivative it is thought possible that in future work it could be used in conjunction with t-butyl ether side group protection and 4-picolyl esters.

	PROTECTED PEPTIDE	Overall Yield %	Yield/ residue %	Isolation Procedure
1	Z-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	56	93.5	A + B
3	Z-Gly-Phe-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	47.6	89.8	A + B
4	Z-Gly-Ala-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	49.8	92.1	A + B
5	Z-Gly-Ala-Ala-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	51	92.6	A + B
7	Z-Gly-Phe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	43.9	89.3	A + B
8	Z-Gly-MePhe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	55.4	91.5	A + B
9	Z-Gly-MePhe-MePhe-Tyr(Bzl)-Thr(Bzl)-Pro-Lys(Pipoc)-Ala-OPic	45.8	89.6	A + B
10	Bpoc-Gly-Phe-Phe-Tyr(Bzl)-OPic	37.3	75.5	B
11	Bpoc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic	49.6	87	B
19	Boc-Lys(Pipoc)-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro-OPic	54.1	92	B
22	Boc-Lys(Pipoc)-[-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-Pro$\frac{1}{2}$-OPic	72		B

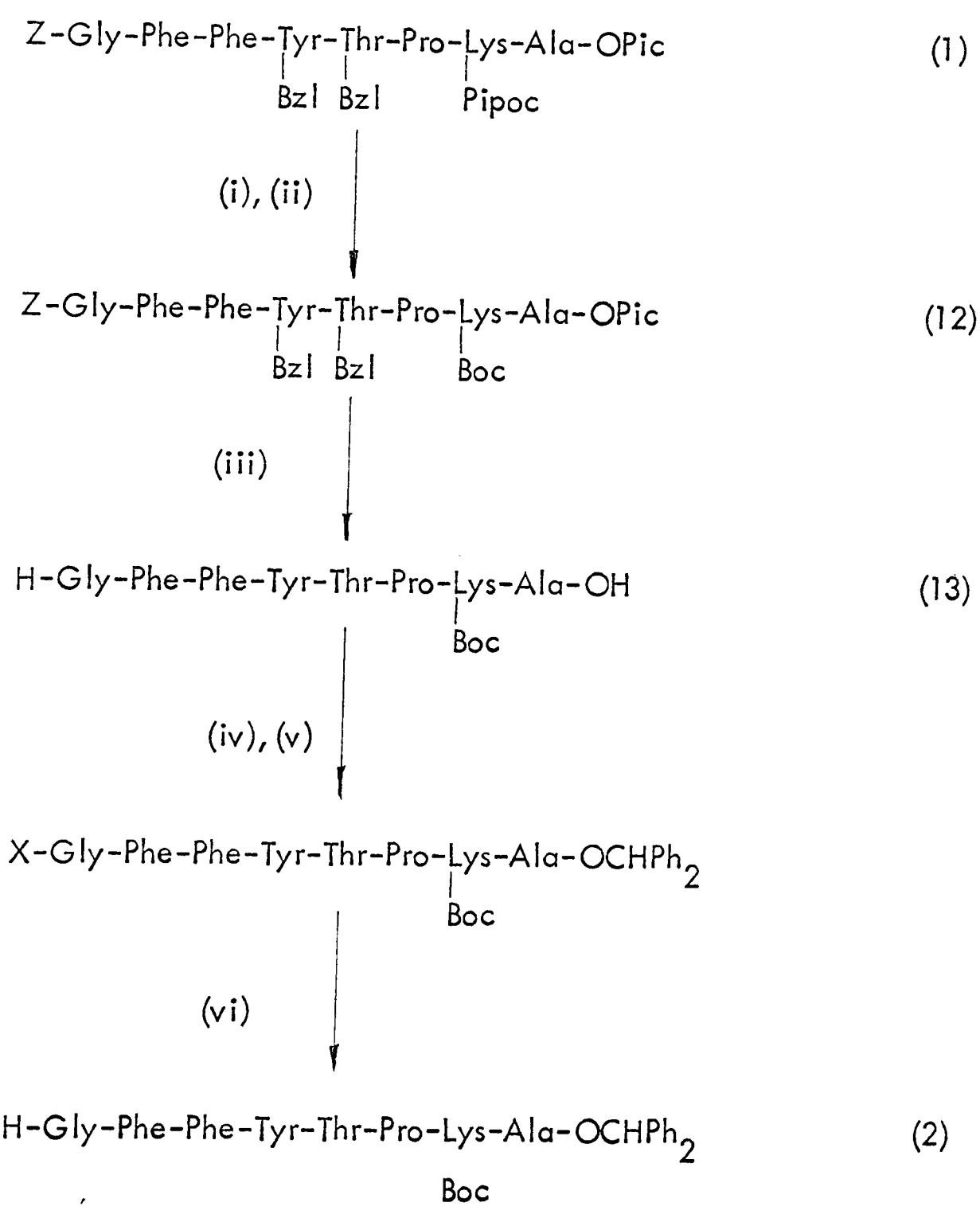
A Citric acid

B Amberlyst 15 (3-bromopyridinium form)

Figure 3

The success of the 4-picolyl ester method is outlined in Figure 3 which indicates the overall yields, yield per residue and isolation procedure of all the peptide derivatives prepared.

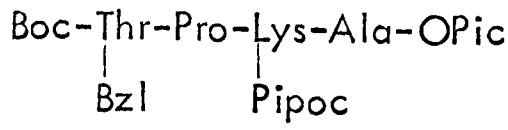
The preparation of the mildly protected natural octapeptide (2) for coupling to a protected des-octapeptide-insulin anticipated a synthetic route as in Figure 4.



(i) Sodium dithionite/HAc (ii) Boc azide (iii) H_2/Pd (iv) X α-amino protection (v) Diphenyldiazomethane (vi) -X α-amino deprotection.

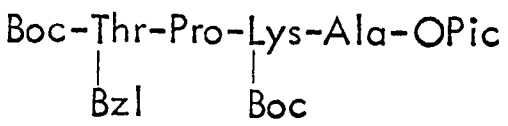
Figure 4

The ε-piperidino-oxycarbonyl group could be cleaved under reducing conditions. Using the model (14), treatment with sodium dithionite in 50% aqueous acetic acid



(14)

cleaved the group within twenty minutes and reaction with t-butoxycarbonyl azide with triethylamine as base gave the derivative (15). This procedure was satisfactory



(15)

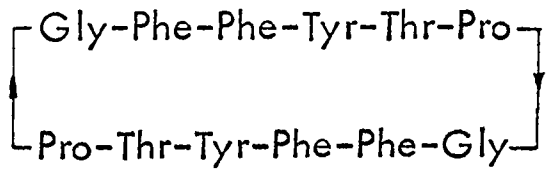
repeated on the protected octapeptide (1) to give the derivative (12).

The synthesis required the utilisation of benzhydryl esters for carboxy group protection. Two urethane protecting groups were considered for α-amino protection, 2-(p-biphenylyl)-isopropyloxycarbonyl and α-piperidino-oxycarbonyl, and these were examined using their respective glycine benzhydryl esters as model compounds. Both were found satisfactory and their removal in the presence of the ester function could be achieved in 0.5% trifluoroacetic acid in dichloromethane at 0° and with sodium dithionite in 50% aqueous acetic acid respectively. Conditions for the removal of excess diazoalkane were found in which neither group was affected; rapid washing with hydrochloric acid (1.0M) being the easiest treatment.

At this point of the synthesis model studies for the preparation of the protected des-octapeptide-insulin had not been concluded and it was undecided as to whether carboxy protection was to be by benzhydryl or the analogous anisyl ester. However, hydrogenation of (12) in 50% aqueous acetic acid had afforded

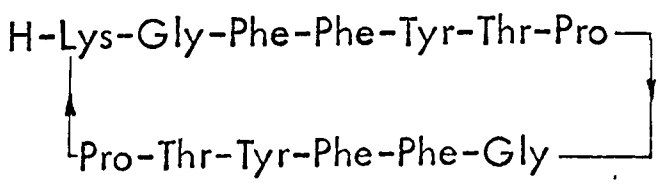
the precursor (13) after extensive model studies to obtain conditions in which complete hydrogenation occurred. The direct esterification of the octapeptide (13) with diphenyldiazomethane proved successful and no N-alkylated material was obtained by electrophoresis and amino acid analysis. It was concluded that the further step envisaged in Figure 4, of α-amino protection before esterification could be omitted and that the direct esterification would also be applied to the introduction of anisyl esters if they were considered more satisfactory.

The C-terminal B-chain octapeptide is at the interface between the monomers in the dimer and is involved in extensive hydrogen-bonding. The possibility that this region could have biological activity was investigated by the attempted preparation of cyclic peptides in which the aromatic region was stabilised by intramolecular hydrogen-bonding. Previous prepared intermediates suggested the synthesis of a model such as (16). Model studies revealed that a 'spacer' of lysine was needed



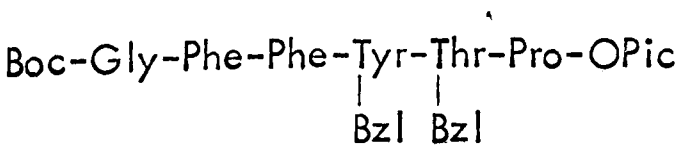
(16)

to give a satisfactory model structure in which the possibility of hydrogen bonding was extremely high as in (17). The hexa- and heptapeptides (18 and 19) were



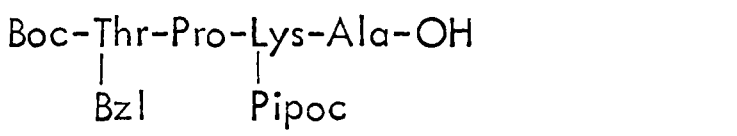
(17)

prepared by the 4-picolyl ester method as before. Saponification of (19) was

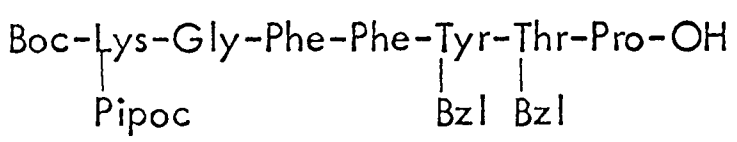


(18)

studied using the model (15) in aqueous dioxan with excess sodium hydroxide and (20) was prepared in 86.8% yield. This was repeated with (19) to give the heptapeptide (21). A 7+6 coupling with N,N'-dicyclohexylcarbodi-imide and



(20)



(21)

1-hydroxybenzotriazole gave the protected tridecapeptide (22) in 72% yield - the largest peptide so far isolated by the 4-picolyl ester method. The ε-piperidinooxycarbonyl group was removed by sodium dithionite in aqueous acetic acid and the 4-picolyl ester by saponification but attempted cyclisation with N,N'-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole or N-hydroxysuccinimide, Woodward's Reagent K and a mixed anhydride coupling were unsuccessful.

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