# SYNTHESIS OF PEPTIDES VIA COBALT(III) CHELATES OF AMINO ACID METHYL ESTERS

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Chemistry at Massey University.

DEREK ROBIN KNIGHTON

1979

## ACKNOWLEDGEMENTS

The author is indebted to the Australian National University for a vacation scholarship (1976–1977) which enabled him to work in the laboratory of Dr. D. A. Buckingham and, thus, learn techniques for the preparation and purification of cobalt(III) chelated amino acid and peptide complexes. The continued assistance of Dr. C. R. Clark and Prof. D. A. Buckingham has been of invaluable support throughout this project.

The author gratefully acknowledges the encouragement and advice of his supervisor, Dr. W. S. Hancock, whose experience in peptide chemistry has been invaluable.

The amino acid and mass spectral analyses of many samples by Dr. G. G. Midwinter and Prof. R. Hodges respectively is acknowledged.

The skillful and understanding typing of Mrs. S. Coates is especially appreciated.

The accurate reproduction of diagrams by Mrs. M. V. Knighton is also gratefully acknowledged.

## SUMMARY

A method for synthesising peptides via cobalt(III) chelated amino acid esters has been further developed.

The yields at each stage of the syntheses of two tetrapeptides, Ala-Gly-Phe-Phe-OBzl and Leu-Ala-Gly-Gly-Oet, and of three tripeptides, Gly-Phe-Phe-OBzl, Ala-Gly-Gly-OEt and Pro-Gly-Gly-Oet, have been investigated quantitatively. Moderate overall yields were obtained.

Side reactions, which occurred during coupling reactions using cobalt(III) chelated proline methyl ester, were investigated by analysis of reaction products and by qualitative analysis of reaction products by cation exchange chromatography.

The solid phase synthesis of a few peptides were attempted but low yields were recorded. The acidity of reaction solutions was at least partially responsible for lowered yields.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

# CHAPTER 1

## INTRODUCTION

### PEPTIDE SYNTHESIS - THE NEED FOR FURTHER METHODS

The rapid development of the field of peptide synthesis provides an ever increasing number of techniques available to the peptide chemist (1, 2, 11). Each technique which has been developed is subject to particular advantages and disadvantages. For example, one of the most widely used methods, solid phase peptide synthesis (3), has several associated problems. These are: diketopiperazine formation (4, 5), lability of the ester linkage to the solid phase (6), slower coupling reactions compared with solution phase syntheses (12), the occurrence of deletion peptides which are difficult to separate from the product (7), chain termination caused by steric hindrance from the resin (8) or the conformation of the peptide (9) and the side reactions which occur during the addition of glutamine and asparagine residues when N,N'-dicyclohexylcarbodiimide (DCC) is used as the coupling reagent (10). Since no one technique has overcome all of the problems associated with peptide synthesis there is a need for continued research in this area. The number of amino acids and their diverse properties results in an enormous variety of possible synthetic peptides. With a large variety of techniques at his disposal the peptide chemist is better equipped to solve particular problems associated with the synthesis of these peptides. This thesis describes the effective use of one more synthesis technique.

## THE STRATEGY USED IN PEPTIDE SYNTHESIS

Current methods of peptide synthesis involve the sequential addition of amino acids from the carboxy to the amino terminal. To ensure only the amino group of the growing peptide chain, $NH_2$-Peptide, is available for reaction, the amino group on the amino acid must first be protected (with group X). The carbonyl on the amino acid is then activated (with group Y) and the protected and activated amino acid is added to the growing peptide chain resulting in a specific condensation reaction. The amino protection is removed and the peptide is then free to be used in another cycle as shown in Figure 1.1.

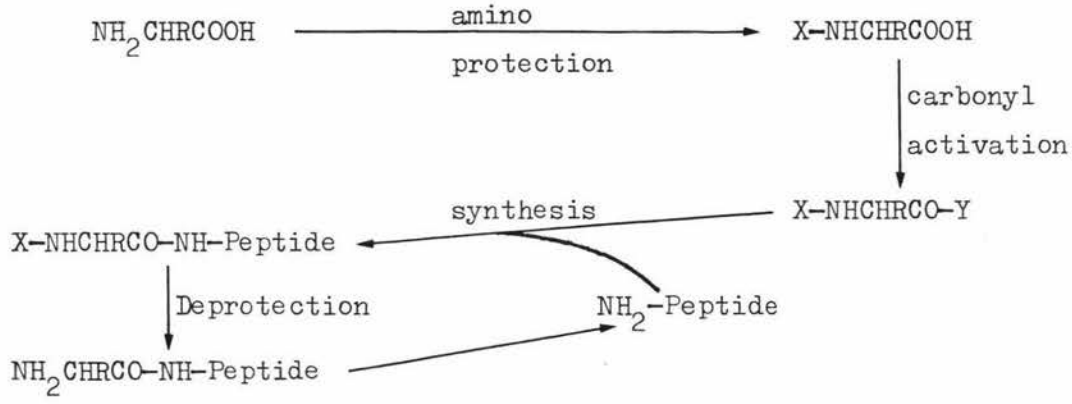



Figure 1.1. General Strategy used in Peptide Synthesis.

The strategy shown in Figure 1.1 is not complicated by side chain protection and deprotection.

The method of peptide synthesis described in this thesis involves a cobalt(III) ion both protecting the amino terminus of the amino acid ester by the co-ordination of its lone pair of electrons and activating the amino acid ester carbonyl carbon towards attack by the growing peptide's amino group.

## THE THEORY OF ACTIVATED ESTERS

The aminolysis of cobalt(III) chelated amino acid esters belongs to a large class of carbonyl activation used in peptide synthesis. This class, activated and active esters, includes the aminolysis of p-nitrophenol, thiophenyl, p-nitrothiophenyl and cyanomethyl esters. The rates of aminolysis of amino acid esters are increased by the enhancement of the electrophillic character of the ester carbonyl carbon (13). This can be achieved in two ways: by the use of electron withdrawing substituents on the alcohol moiety, as in the examples above, Figure 1.2A, or by co-ordinating the carbonyl oxygen atom to a positively charged metal ion thus polarising the C=O bonding, as in cobalt(III) chelated amino acid esters, Figure 1.2B.

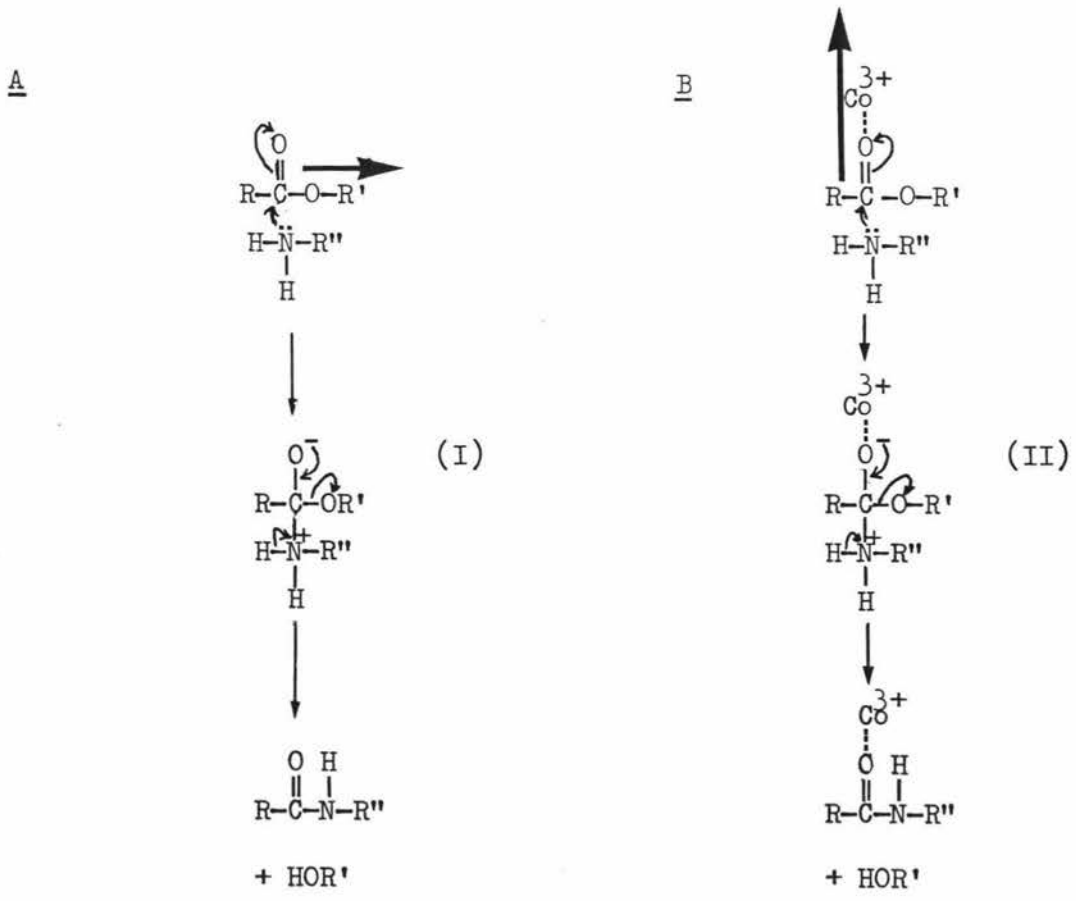



Figure 1.2. Comparison of two methods of carbonyl activation.

The large arrows show the direction in which electrons are attracted by induction. In both activation methods the formation of the tetrahedral amino-carbinol intermediates, I and II, by the nucleophillic attack of the amine is facilitated by the positive character of the carbonyl carbon atom (13-15).

The stability of the tetrahedral amino-carbinol intermediates is very different for the two activation methods. Electron withdrawing substituents on the alcohol moiety tend to destabilise the intermediate and, therefore, this species does not accumulate in detectable concentrations in the aminolysis of organic esters (16). In contrast, co-ordination, as in II, stabilises the intermediate with respect to loss of alcohol. Dekkers (17) has observed the accumulation of this species and has obtained rate data for the formation and breakdown of the tetrahedral intermediate.

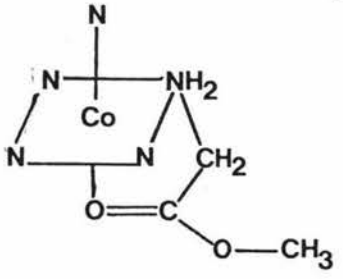
## COBALT(III) COMPLEXES AS BLOCKING GROUPS IN PEPTIDE SYNTHESIS

Cobalt(III) complexes have been used very effectively as blocking groups in the synthesis of peptides where DCC has been used as the carbonyl activating reagent. The $Co^{3+}(NH_3)_5$ moiety has been used as a carboxylate protecting group where the growing peptide chain was co-ordinated through its carboxy oxygen only (18), while the $Co^{3+}(en)_2$ moiety has been used as an amino protecting group for dipeptides during coupling of these peptides to amino acids bound to insoluable supports (19). The N terminal amino acid was chelated through the amino nitrogen and amide oxygen in the later example. The complexes were found to be stable under the conditions used for peptide synthesis.

## THE AMINOLYSIS OF COBALT(III) CHELATED AMINO ACID ESTERS

The method of peptide synthesis described by this thesis involves the simultaneous amino protection and carbonyl activation of chelated amino acid esters by the same cobalt(III) centre, III.

III *

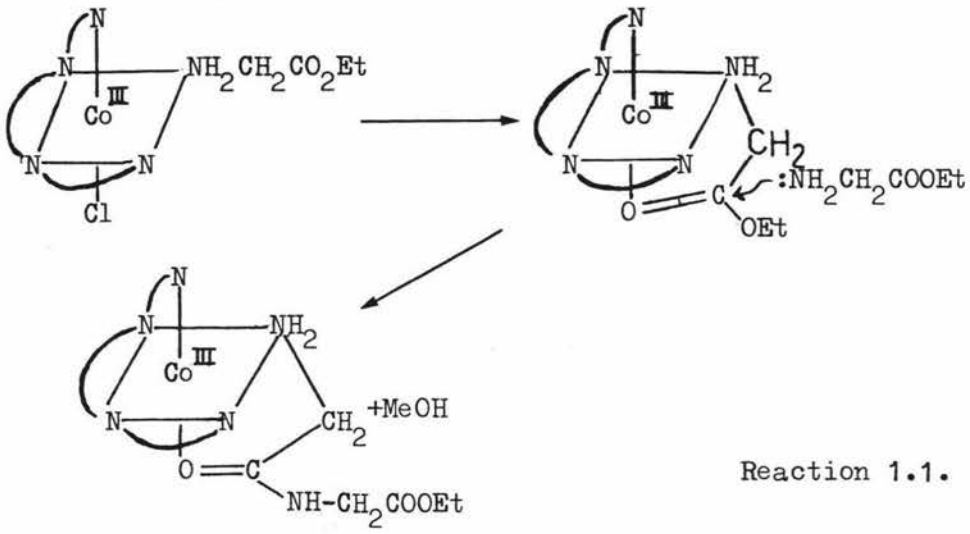



The usefulness of this method has been shown in a number of studies.

In 1967 Buckingham, et al (20), reported the rapid synthesis of a peptide bond within the co-ordination sphere of the cobalt(III) centre. The reaction occurred between $\alpha$ or $\beta$-$[Co(trien)(TBP)_2]^{3+}$ and a 2fold excess of Gly-OEt at room temperature, in non aqueous solution, and resulted in the formation of $\beta_2$-$[Co(trien)Gly\text{-}Gly\text{-}OEt]^{3+}$ in approximately 80% yield. The same product was obtained when $\beta_2$-$[Co(trien)(Gly\text{-}OEt)Cl](ClO_4)_2$ was treated with Gly-OEt using sulpholane or dimethylformamide as solvent. The product was identified by $^1H$ NMR, infrared and visible spectra and confirmed by an Xray structural study. $^{14}C$ labeling of glycine showed that no significant exchange of co-ordinated Gly-OEt occurred during the synthesis and confirmed that the amino acid initially co-ordinated to the metal was the amino terminal amino acid in the peptide. The reactions were proposed to take place via the chelated amino acid ester, Reaction 1.1.

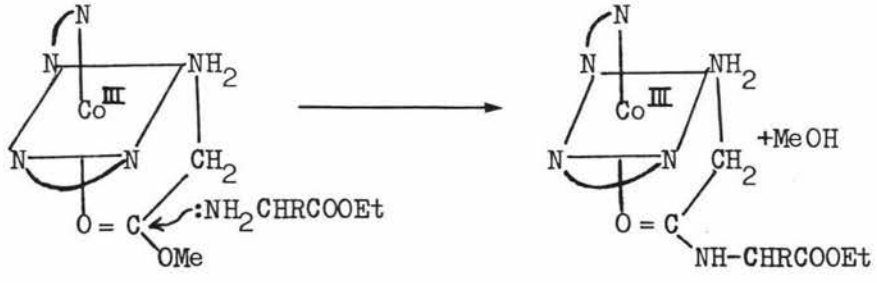
* Where N represents co-ordinated amines.

Collman and Kimura (22) independently reported very similar reactions using the $Co(trien)^{3+}$ moiety.



Reaction 1.1.

This was confirmed by the isolation and characterisation of the chelated amino acid ester (21), $[Co(en)_2Gly\text{-}OMe](ClO_4)_3$, which was found to be reactive to nucleophiles and readily formed dipeptides when treated with amino acid ethyl esters. Reaction 1.2.



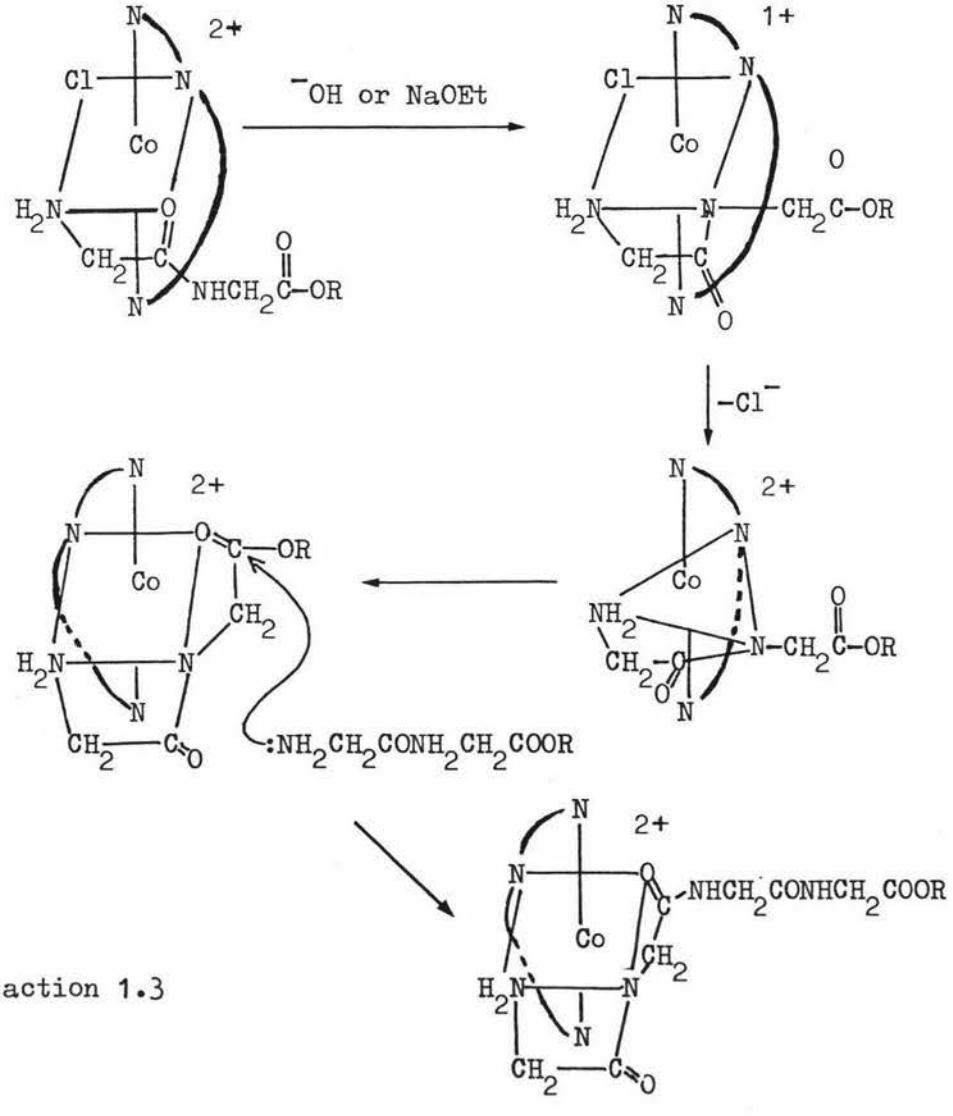
Reaction 1.2.

The chelated ester was found to be very easily hydrolysed in the presence of water to yield $[Co(en)_2Gly]^{2+}$ and methanol.

The application of the three-site moiety $Co(dien)^{3+}$ to peptide bond formation by Wu and Busch (23) demonstrated the versatility of Co(III) as an activator of the ester carbonyl group.* The treatment

* dien = diethylenetriamine

of $Co(dien)X_3$ (X = Cl,$NO_2$) with Gly-OEt and Gly-Gly-OEt yielded $[Co(dien)Gly\text{-}Gly\text{-}OEt]^{3+}$ and $[Co(dien)Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ respectively. Formation of the peptide bond was found to occur via the chelated amino acid or peptide ester. The formation of the tetrapeptide ester required the initial chelation of the amino terminal glycine in Gly-Gly-OEt, followed by loss of the amide proton and isomerisation of the cobalt-amide linkage from the O bonded to the N bonded form. Reaction 1.3 is consistent with the observed facts.




Reaction 1.3

Reaction 1.3 offers the possibility of protecting the amino terminal and activating the carboxy terminal of dipeptides in a general reaction scheme involving the formation of polypeptides from dipeptides.

Dekkers (15) has used $Co^{3+}(en)_2$ chelated amino acid esters to prepare a large number of small peptides which were characterised by $^1H$ NMR spectroscopy. The $[Co(en)_2aa\text{-}OR]^{3+}$ complexes were treated with a 10 molar excess of the amino acid ester in non aqueous solvent. Dekkers states that yields of $[Co(en)_2peptide\text{-}OR]^{3+}$ were usually greater than 70% but did not say how the yields were ascertained. Due to the hygroscopic properties of the peptide no satisfactory elemental analyses were obtained. The free peptides were not isolated or characterised.

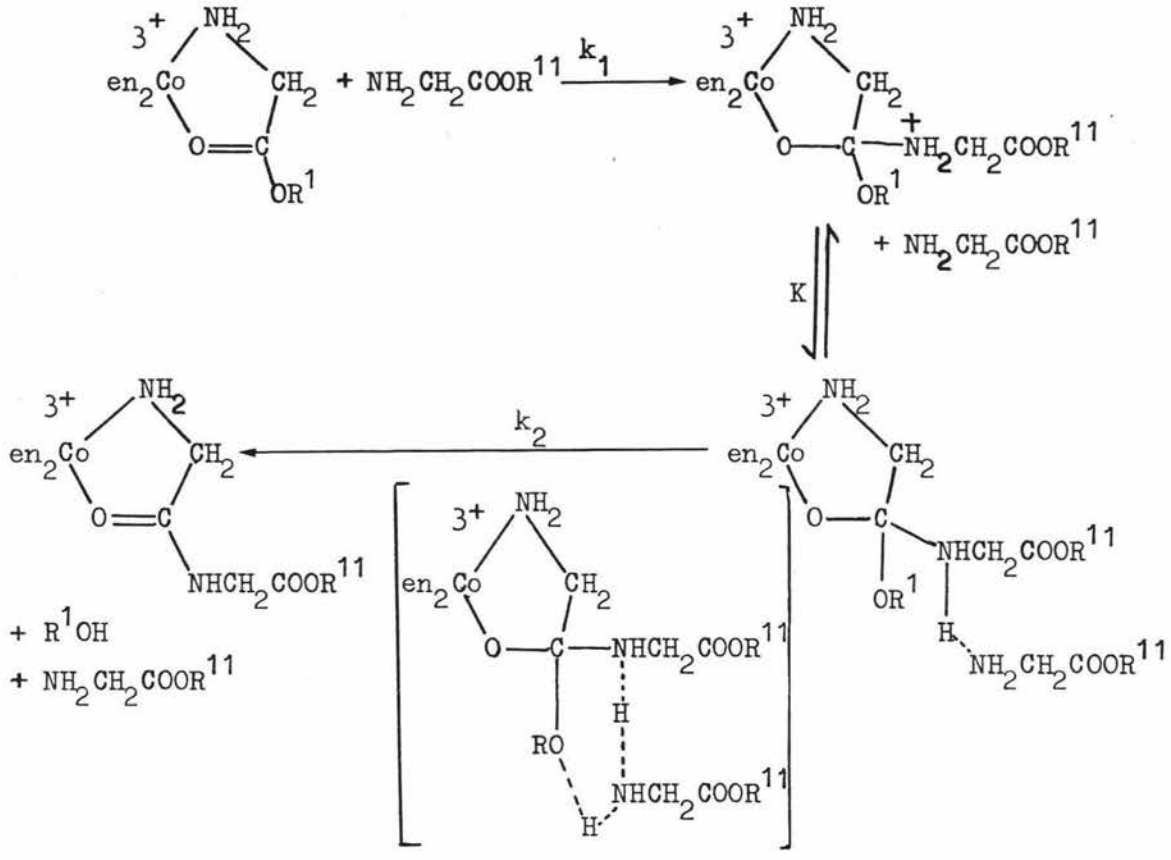
Dekkers observed the formation of a long lived intermediate in the reaction of $[Co(en)_2GlyOCH(CH_3)_2]^{3+}$ with Gly-OEt (24). The orange complex turned red on addition of the amino acid ester then slowly faded to orange. This two stage process was clearly observed at 450nm with an immediate increase in absorbance at this wavelength (maximum after 40 seconds, dimethylsulphoxide, 25°C) followed by a slower decrease in absorbance. Absorbance at $1630cm^{-1}$, that part of the infrared spectrum corresponding to co-ordinated carbonyl vibrations, showed an immediate decrease in absorbance followed by a slower increase. Analysis of rate data for these two processes showed the first process followed the rate law

$$v\ [Co^{3+}(en)_2Gly\text{-}OCH(CH_3)_2] = k_1\ [Co^{3+}(en)_2Gly\text{-}OCH(CH_3)_2][Gly\text{-}OEt]$$

while the second process followed the rate law

$$v\ [\text{tetrahedral amino-carbinol intermediate}] = \frac{Kk_2\ [\text{tetrahedral intermediate}][Gly\text{-}OEt]}{1+K[Gly\text{-}OEt]}$$

The rate laws are consistent with the following mechanism:



Dekkers observed only a small effect due to addition of protonated amine on the rate of amino-carbinol intermediate disappearance and reasoned that the mechanism could not entail loss of the nitrogen proton on the amino-carbinol intermediate followed by attack of protonated amine at $OR^1$ and simultaneous loss of $R^1OH$.

Dekker also observed a decrease in rates of both processes with increase in conductivity in dimethylsulphoxide. The rate of decomposition of the tetrahedral intermediate was not increased above a concentration of base of 1M.

Dekkers observed that different chiralities of the Co(III) moieties give rise to the selective formation of peptides. For example, the tetrahedral intermediate for $[Co(en)_2Gly\text{-}Ala]^{2+}$ was formed three times faster than for $[Co(en)_2Gly\text{-}Ala]^{2+}$. It was reasoned that this difference came from non bonded interactions between hydrogen atoms of the adjacent ethylenediamine chelate and the incoming amino acid (25). The decay of the tetrahedral intermediate did not appear to be stereospecific.

Some peptides have been synthesised by Bentley using the aminolysis of cobalt(III) chelated amino acid esters. Bentley reported the successful attachment of $[Co(en)_2Gly\text{-}OMe]^{3+}$ to insulin, glucagon and Leu-Tyr-Val-Arg-Phe-Ala using a 100fold excess of the chelated ester in dimethylsulphoxide for 10 minutes (no yields given(26)).

Bentley also reported high coupling yields (96%) of $[Co(en)_2Gly\text{-}OMe]^{3+}$ onto amino acids bound to insoluble polymer supports in dry dimethylsulphoxide (27).

CHOICE OF THE COBALT(III) MOIETY.

Several $Co^{III}(N)_4$ systems have been evaluated as possible amino terminal protecting and carbonyl activating groups for peptide bond formation. The $[Co(trien)aa]^{3+}$ complexes of almost all the amino acids have been prepared but Dekkers encountered difficulties in esterifying these complexes and also encountered difficulties due to the presence of stereo- and diastereoisomers (there are four chiral centres per complex)(28).

The $[Co(trien)aa]^{2+}$ (trien = 2,2',2"-triaminotriethylamine) complexes of some amino acids have been prepared, however, the synthesis of the trien ligand is lengthy and thus the $Co(en)_2^{3+}$ and $Co(trien)^{3+}$ moieties are more accessible (28).

The $[Co(NH_3)_4aa]^{2+}$ complexes of several amino acids have been prepared but Dekkers reports low yields of the above complexes and low solubility of the related complex in organic solvents compared to the $[Co(trien)aa]^{2+}$ and $[Co(en)_2aa]^{2+}$ complexes.

The choice of the $Co(en)_2^{3+}$ for the protection of the amino terminus and activation of the carboxy terminus of amino acid esters was, therefore, made on the basis of work carried out by Dekkers since this moiety involved none of the problems above.

CHOICE OF CONDENSATION REACTION SOLVENT.

The solvent used to dissolve the active complex $[Co(en)_2aa\text{-}OMe](CF_3SO_3)_3$ and the growing peptide chain in the condensation step must be able to be dried, be unreactive towards the chelated amino acid ester and it must dissolve both reactants. Dimethyl formamide, acetonitrile, N-dimethylacetamide, acetone, methanol and dimethyl sulphoxide all allow condensation to occur but some solvents showed more desirable characteristics than others. $[Co(en)_2GlyOMe](ClO_4)_3$ was not soluble enough in dimethyl formamide or acetone and when acetonitrile or N-dimethylacetamide was used as the solvent intense brown solutions formed and the product dipeptide complex was slowly decomposed (29). Dekkers also recorded reaction times in various solvents (30), Table 1.1.

Table 1.1. Rate of Peptide[a] Formation in Various Non Aqueous Solvents. *

| Solvent | Reaction Time[b] |
|---|---|
| Methanol | 1 sec |
| Acetone | 10 sec |
| DMSO | 30 sec |
| Acetonitrile | 25 sec |

a $[Co^{3+}(en)_2Gly\text{-}OMe]$ $2.5\times10^{-3}$M, Gly-OEt = $2.0\times10^{-2}$M.

b $10t^{\frac{1}{2}}$ for addition of amino acid ester to chelated ester.

The solvent of preference is Methanol (which can be dried adequately by distillation or storing over molecular sieves) since it gives no side reactions and gives a fast rate. If the peptide or amino acid ester would not dissolve in methanol, dimethylsulphoxide was used since it, too, gives no side reactions but would appear to give rise to slower coupling reactions.

HYDROLYSIS AND RACEMISATION -

FACTORS WHICH MAY OPERATE TO DECREASE YIELDS.

A good synthesis method should, at each step of the synthesis, satisfy the following criteria.

a). Produce a good yield of peptide bond.

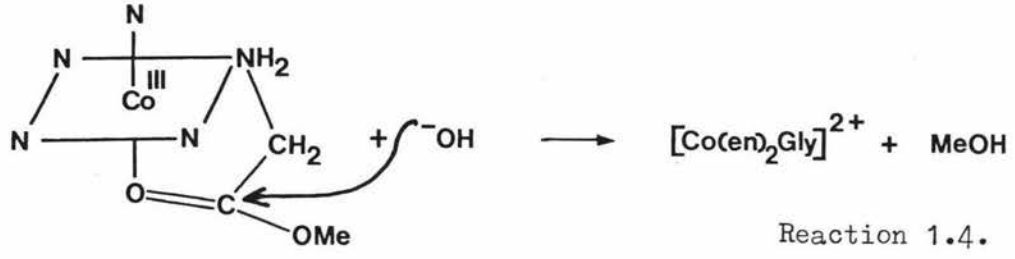
b). React without loss of optical purity of the amino acids.

c). Result in a peptide derivative which is easily prepared for the next step in the synthesis.

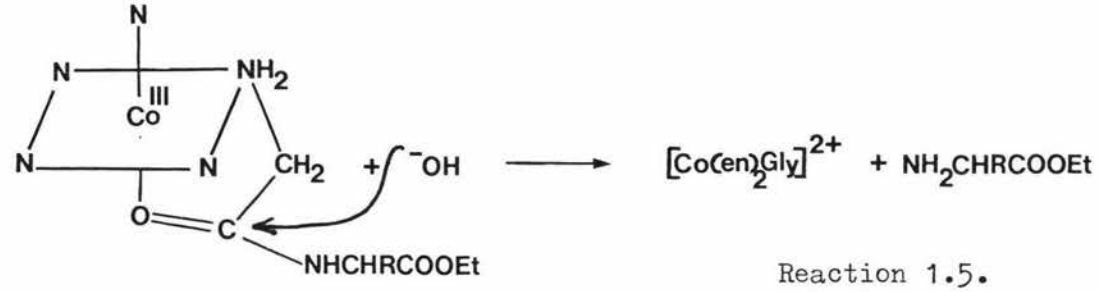
Compliance with criterion a) will be decreased if the peptide bond is hydrolysed to any extent.

* Reproduced from Table 3.5, Ref 15.

Just as amino acid esters are activated towards aminolysis by co-ordination through the carbonyl oxygen, so are amino acid esters and amides activated to hydrolysis by co-ordination of the carbonyl oxygen to the cobalt(III) ion, Reactions 1.4 and 1.5.




Reaction 1.4.




Reaction 1.5.

Rate enhancements of $10^4$–$10^6$ have been found for hydrolysis of chelated peptides compared with the unchelated species (31).

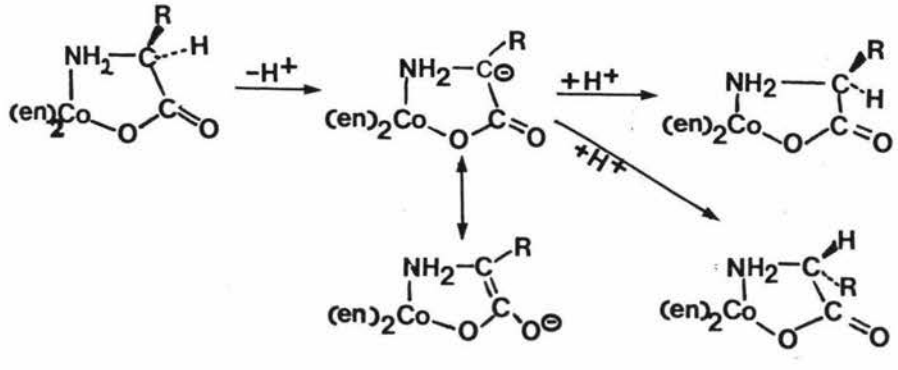
Much work has been done on elucidating the mechanisms of hydrolysis (32–36). Reactions of the type Reaction 1.5 have been used as an N terminal peptide sequencing technique (37–39). To decrease the rate of hydrolysis of chelated peptides the pH of aqueous solutions must be kept slightly acid.

Any racemisation which occurs lowers the yield of desired peptide. Racemisation of only 2% per coupling for a 50 residue peptide results in 36% of the optically pure peptide being formed.

Initial work by Dekkers (28) tested the possible racemisation during peptide bond formation of $\Lambda$-$[Co(en)_2Ala\text{-}OMe]^{3+}$ with Gly-OEt in methanol after approximately one half life for the condensation,

(approximately 1 minute). After quenching with acid the products were isolated as $[Co(en)_2Ala]^{2+}$ and $\Lambda$-$[Co(en)_2Ala\text{-}Gly\text{-}OEt]^{3+}$. An upper limit to racemisation of 2% for the l-alanine in the dipeptide complex was established polarimetrically at 546nm. The rotation was compared with optically pure $\Lambda$-$[Co(en)_2Ala\text{-}Gly\text{-}OEt]^{3+}$ and found to be the same within experimental error. $^1H$ NMR spectroscopy of the dipeptide complex set the maximum of racemisation at 5%. Analysis of hydrolysed samples of Ala-Gly-OEt from the experiment above and from an analytical grade source with D-amino acid oxidase showed the upper limit of racemisation was 0.7% but this value is low compared to the racemisation caused by the hydrolysis (8.2%). Dekkers concluded that less than 0.7% racemisation occurred under the experimental conditions used for the coupling reaction and that the rate of racemisation was approximately 4000 times slower than the formation of the peptide bond.

Chelation of amino acids to Co(III) centre increases the rate of racemisation of these amino acids. This occurs through a planar carbanion intermediate which is stabilised by co-ordination through the oxygen of the carboxy group (44) as in Reaction 1.6.




Reaction 1.6

This effective increase in acidity of the $\alpha$ methylene hydrogens upon chelation is shown by the disappearance of the peaks caused by these hydrogens on $^1H$ NMR when the complex $[Co(en)_2Gly]^{2+}$ was treated with basic $D_2O$ (45). Dekkers (28) found complete racemisation of $\Lambda$-$[Co(en)_2Ala-Gly-OEt]^{3+}$ prepared *in situ* from $\Lambda$-$[Co(en)_2Ala-OMe]^{3+}$ and Gly-OEt (0.1M) after 25 hours in methanol (28).

The danger of racemisation occurring during the aminolysis of chelated amino acid esters is decreased by short coupling times.

## SCOPE OF THESIS

This thesis is a quantitative investigation into the yields of synthetic peptides using the techniques described in previous sections. Chaper 2 describes the preparation of the activated esters $[Co(en)_2aa-OMe]^{3+}$. Chapter 3 relates problems associated with solid phase synthesis of peptides and the use of methyl fluorosulphonate for preparing the activated esters. Chapter 4 is a quantitative approach to synthesis of peptides larger than two residues in solution phase.

# CHAPTER 2.

## PREPARATION OF THE AMINO ACID AND AMINO ACID METHYL ESTER COMPLEXES.

### SECTION A: PREPARATION OF $[Co(en)_2aa]^{2+}$ COMPLEXES.

The $[Co(en)_2aa]^{2+}$ complexes were prepared by a modified method of Meisenheimer (40).

Materials.

The amino acids used were of the L configuration (with the exception of glycine). Sigma grade amino acids were used. The methanol and acetone used were laboratory reagents used without further purification. All other chemicals used were of laboratory reagent grade.

The amino acid complexes were prepared via the following scheme:

$$[Co(en)_2CO_3]Cl \rightarrow trans\text{-}[Co(en)_2Br_2]Br \rightarrow [Co(en)_2aa]I_2$$

Preparation of Carbonatobis(ethylenediamine)cobalt(III) Chloride 1.5 Hydrate.

$[Co(en)_2CO_3]Cl.\ 1.5\ H_2O$

Carbon dioxide was bubbled into a mixture of ethylenediamine (anhydrous, 105$cm^3$) and water (123$cm^3$) for three hours in an ice bath. The cold solution was then treated with cobalt(II) chloride hexahydrate (195g, 0.82mol) in water (170$cm^3$). The solution turned red-violet. The carbon dioxide addition and stirring were continued during the preparation. Hydrogen peroxide (30%,200$cm^3$, 3.9mol) was then added slowly over two hours during which time the temperature rose to 35°C. The solution was heated to 75°C with a heating mantel then replaced

into the ice bath. When the temperature had decreased to 20°C the cooling was discontinued and lithium hydroxide monohydrate (34.4g, 0.82 mol) was added. The flow rate of carbon dioxide was increased and the temperature of the solution rose to 35°C. The solution was left for one hour during which time the product crystalised. Methanol ($500cm^3$) was added rapidly and the mixture was cooled to approximately -5°C by the addition of dry ice. The precipitated solid was filtered and washed with 50% ethanol ($100cm^3$). The product was recrystalised from hot water ($100cm^3$), washed with methanol, acetone and air-dried then dried in a vacuum oven at 40°C for ten hours. Yield 137g, 56%.

Elemental Analysis calculated for $Co(C_5H_{16}N_4O_3)Cl$:C,21.87; H,5.87; N,20.41. Found: C,21.88; H,5.91; N,20.35.

Preparation of Carbonatobis (ethylenediamine)cobalt(III) Bromide.

The carbonato chloride complex prepared previously was dissolved in a minimum of water and an excess of sodium bromide added. The solution was cooled to 0°C and ethanol added to precipitate the more insoluble bromide complex. This was filtered and washed with ethanol and acetone, then air-dried. Yield 92%.

Preparation of trans-Dibromobis(ethylenediamine)cobalt(III) Bromide.

HBr (47%, $200cm^3$) at 70°C was treated with $[Co(en)_2CO_3]Br$ (50g) over 15 minutes. The mixture was maintained at 70°C for one hour then cooled to 2°C. The product was filtered from the mixture then washed with ethanol.

The green crystals were added to ethanol ($200cm^3$) and stirred for one hour to remove HBr from the crystals. If necessary the mixture

was filtered and the crystals were added to fresh ethanol so that the pH of the ethanol was between 5 and 7. The product was washed with ethanol and acetone, then air-dried. Yield 60g, 98%.

GENERAL PREPARATION OF AMINO ACIDOBIS(ETHYLENEDIAMINE)COBALT(III) IODIDE.

$[Co(en)_2aa]I_2$

Amino acid (0.02mol) and $LiOH.H_2O$ (0.84g, 0.02 mol) were dissolved in water ($10cm^3$). Trans-$[Co(en)_2Br_2]Br$ (8.4g, 0.02 mol) was slurried with methanol ($100cm^3$) and the slurry added to the solution. The green dibromo complex immediately turned purple. The mixture was refluxed for 30 minutes during which time it became coloured orange-red and some solid precipitated. The mixture was diluted to 2 litres with water and the complexes from the reaction mixture were sorbed onto Dowex 50W x 2 cation exchange resin (chloride form, 30 x 5cm). The complexes were eluted with HCl(1N) until good separation of the coloured species were obtained, then the eluant was changed to 2N and finally 3N HCl to collect the orange-red $[Co(en)_2aa]^{2+}$ species. The eluant containing this species was reduced to dryness under vacuum at 50°C. The solid was dissolved in a minimum of water and reduced to dryness under the same conditions. This treatment was repeated until no HCl vapour could be smelt above the crystals (usually 3 times). The orange-red crystals were dissolved in a minimum of water (approximately $40cm^3$) and the pH corrected to neutrality with NaOH (concentrated). NaI (6g, 0.04 mol) was dissolved in the solution which was cooled to 3°C for several hours. The crystals were filtered, washed with methanol and acetone, then air-dried. Before being methylated the complexes were dried under oil pump vacuum at 60°C for at least 5 hours.

For the yields and elemental analyses of the individual complexes see below. In all preparations the $[Co(en)_2aa]^{2+}$ species was orange.

$[Co(en)_2Ala]I_2$

Yield 53%

Analysis calculated for $Co(C_7H_{22}N_5O_2)I_2$: C,16.14; H,4.26; N,13.44.

Found: C,15.9; H,4.3; N,12.9.

$[Co(en)_2Gly]I_2$

Yield 66%.

Analysis calculated for $Co(C_6H_{20}N_5O_2)I_2$: C,14.21; H,3.98; N,13.82.

Found: C,14.1; H,4.2; N,13.1.

$[Co(en)_2Leu]BrI$ *

Yield 45%

Analysis Calculated for $Co(C_{10}H_{28}N_5O_2)BrI$: C,23.27; H,5.49; N,13.57.

Found: C,23.4; H,5.5; N,13.6.

$[Co(en)_2Phe]I_2$ (see discussion).

Yield 70%

Analysis calculated for $Co(C_{13}H_{26}N_5O_2)I_2$: C,26.15; H,4.39; N,11.73.

Found: C,26.2; H,4.2; N,11.8.

$[Co(en)_2Pro]I_2$

Yield 50%

Analysis calculated for $Co(C_9H_{24}N_5O_2)I_2$: C,19.76; H,4.42; N,12.80.

Found: C,19.0; H,4.4; N,13.0.

$[Co(en)_2Val]I_2$

Yield 42%

Analysis calculated for $Co(C_9H_{26}N_5O_2)I_2$: C,19.69; H,4.77; N,12.76.

Found: C,19.9; H,5.2; N,12.8.

* The addition of only an equimolar amount of NaI resulted in the precipitation of the complex with only one Iodide counter ion.

Discussion.

The preparation of $[Co(en)_2Phe]I_2$ was identical to the others except that isolation of the species was carried out on a Sephadex SP-$C_{25}$ cation exchange column eluting with hydrochloric acid (0.1N - 0.3N). The phenylalanine interacts with Dowex resins resulting in very long retention times and a diffuse band. This makes such resins unsuitable for purifying $[Co(en)_2Phe]^{2+}$ ions.

TABLE 2.1

Absorption Spectra of $[Co(en)_2aa]^{2+}$ ions in $H_2O$ at 25° *

| Complex** | $\lambda_{max}$ (nm) | $\varepsilon_{max}$ | $\lambda_{max}$ | $\varepsilon_{max}$ | % Yield |
|---|---|---|---|---|---|
| $[Co(en)_2Gly]I_2$ | 487 | 97 | 347 | 107 | 80 |
| $[Co(en)_2Ala]I_2$ | 487 | 109 | 348 | 117 | 75 |
| $[Co(en)_2Val]I_2$ | 487 | 101 | 348 | 110 | 80 |
| $[Co(en)_2Leu]I_2$ | 486 | 109 | 347 | 130 | 75 |
| $[Co(en)_2Ile]I_2$ | 486 | 110 | 347 | 132 | 78 |
| $[Co(en)_2Thr]I_2$ | 486 | 102 | 347 | 110 | 70 |
| $[Co(en)_2Ser]I_2$ | 486 | 118 | 345 | 135 | 68 |
| $[Co(en)_2Lys]I_2$ | 487 | 101 | 348 | 120 | 76 |
| $[Co(en)_2Gln]I_2$ | 486 | 94 | 347 | 133 | 65 |
| $[Co(en)_2Asn]I_2$ | 486 | 103 | 347 | 113 | 50 |
| $[Co(en)_2Met]I_2$ | 486 | 102 | 346 | 134 | 70 |
| $[Co(en)_2Phe]I_2$ | 487 | 103 | 346 | 128 | 82 |
| $[Co(en)_2Tyr]I_2$ | 490 | 108 | 340 | 187 | 72 |
| $[Co(en)_2Trp]I_2$ | 486 | 103 | 340 | 298 | 65 |
| $[Co(en)_2Pro]I_2$ | 488 | 103 | 346 | 125 | 85 |

* Reproduced from Reference (15), p.36.

** Abbreviationsused for amino acids are listed in Appendix II.

The $[Co(en)_2aa]^{2+}$ ions for aa = Gly,Ala,Val,Leu,Ile,Thr,Ser,Lys, Gln,Asn,Met,Phe,Tyr,Trp and Pro have been prepared and characterised previously by Dekkers (15). The absorbtion spectra for each of these complexes is listed in Table 2.1 together with the % yield of the complex.

Since the above work was completed several other $[Co(en)_2aa]^{2+}$ complexes of side chain protected amino acids have been prepared.

aa = Thr(OBzl), Ser(OBzl), Tyr(OBzl), Lys(z), Asp(OBzl), Glu(OBzl) and nitro-arginine. The active esters, $[Co(en)_2aa\text{-}OMe](CF_3SO_3)_3$, of these side chain protected amino acids have been prepared and characterised (46).

## SECTION B: PREPARATION OF $[Co(en)_2aa\text{-}OMe]^{3+}$ COMPLEXES.

WARNING: The Methylating Reagents used in this section are extremely hazardous. Small quantities absorbed through the skin or inhaled as vapour are LETHAL.

Materials:

Methyl fluorosulphonate (Aldrich) and trifluoromethanesulphonic acid (Eastman) were used directly without purification. Dimethyl sulphate (BDH, 99% pure) was distilled under vacuum before use. The trimethyl phosphate (Koch-light Laboratories Ltd.) was of reagent grade.

Drying of solvents:

Ether (reagent grade) was dried by standing over anhydrous $CaCl_2$ pellets for at least two days. The ether was decanted off and type 4A molecular sieves were added at least 24 hours before the ether was required and the mixture occasionally shaken. Methanol (A.R. grade) was dried by distillating twice under anhydrous conditions.

Because of the rapid hydrolysis which occurs in the presence of water all reactions were carried out under anhydrous conditions.

Preparation of Methyl Trifluoromethanesulphonate ($CF_3SO_3CH_3$).

Trifluoromethanesulphonic acid ($100cm^3$, 1.1 mol) was added to dimethylsulphate ($100cm^3$, 1.05 mol) and the mixture refluxed using a $25cm^3$ spiral packed column. The product was collected by distillation under anhydrous conditions (bp 97°C). As the distillation proceeded the contents of the reaction vessel darkened. The product was stored in a glass stoppered conical flask in a fume hood. Yield 107g, 97%.

Preparation of $[Co(en)_2aa\text{-}OMe](CF_3SO_3)_3$ Complexes.

$[Co(en)_2aa]I_2$ (10g) was added to trimethylphosphate (50g). Methyl trifluoromethanesulphonate (16g) was added resulting in a solution. The solution was stirred for 15 minutes and then a few drops of the reaction mixture were subjected to the $NH_3$/MeOH test (see at the end of this section). If $[Co(en)_2aa]^{2+}$ was identified by the test more methylating agent (5g) was added. If no $[Co(en)_2aa]^{2+}$ was identified the product was isolated as follows. The reaction mixture was poured slowly into rapidly stirred ether ($800cm^3$). The product oiled out and was dissolved in methanol (approximately $2cm^3$). The solution was slowly poured into rapidly stirred ether ($600cm^3$) and the product oiled out again. The oil was dissolved in a minimum of methanol and poured slowly into rapidly stirred ether ($1200cm^3$) and the product crystalised as a fine orange powder. The precipitate was filtered from the solution on a No.4 sintered glass filter and washed with ether. The product was dried under vacuum at room temperature then stored in a desiccator over $P_2O_5$. The prepared complexes were extremely hygroscopic. Difficulties were encountered in the final

crystalisation step. The formation of unfilterable fine precipitates and oiling out of product at the third precipitation lowered the yields obtained.

Yields.

$[Co(en)_2Ala\text{-}OMe](CF_3SO_3)$ 84%; $[Co(en)_2Gly\text{-}OMe](CF_3SO_3)_3$ 30%;
$[Co(en)_2Leu\text{-}OMe](CF_3SO_3)$ 48%; $[Co(en)_2Pro\text{-}OMe](CF_3SO_3)_3$ 22%;
$[Co(en)_2Val\text{-}OMe](CF_3SO_3)$ 29%.

Much better yields have been obtained by other workers in this field. Analytical grade ether might be expected to improve yields.

Preparation of $[Co(en)_2aa\text{-}OMe](FSO_3)_3$ Complexes.

The preparation of these complexes was accomplished in exactly the same way as for the $(CF_3SO_3)$ analog except that $FSO_3CH_3$ was used as the methylating agent, also in an 8fold excess.

Yields.

$[Co(en)_2Ala\text{-}OMe](FSO_3)_3$ 77% $[Co(en)_2Phe\text{-}OMe](FSO_3)_3$ 58%
$[Co(en)_2Pro\text{-}OMe](FSO_3)_3$ 62%

Test for Chelated Amino Acid Esters - $NH_3$/MeOH Test.

A small amount (approximately $0.1cm^3$) of the reaction mixture or isolated $[Co(en)_2aa\text{-}OMe](CF_3SO_3)_3$ salt was dissolved in dry methanol (3A molecular sieves, approximately $1cm^3$). Methanol saturated with ammonia (10 drops) was added, and the solution was shaken then immediately quenched with acetic acid ($0.5cm^3$, glacial). The solution was diluted to $20cm^3$ with water, then sorbed onto a column of Sephadex SP-$C_{25}$ cation exchange resin. The $[Co(en)_2aa]^{2+}$ species resulting from unesterified complex was eluted with 0.2M pyridinium acetate or $NaClO_4$. The esterified complex reacted with ammonia to yield $[Co(en)_2aa\text{-}NH_2]^{3+}$ which was eluted with 0.3M pyridinium acetate or $NaClO_4$. The ratio of the $2^+$ and $3^+$ orange-red bands was used as direct evidence of the ratio of esterified and unesterified complex in the

reaction mixture.

Discussion.

A number of methods for esterifying the $[Co(en)_2aa]^{2+}$ complexes have been attempted. These are summarised in Table 2.2.

TABLE 2.2

Esterification methods for $[Co(en)_2Gly]^{2+}$

| Reagent | Solvent | Conditions | Source | Yield[a] and Comments |
|---|---|---|---|---|
| $PCl_3$ | MeOH | c | (28) | 95% |
| HCl | MeOH | c 1.5M in HCl | (28) | 85% |
| $CH_3COCl$ | MeOH | c | (28) | 50% |
| $SOCl_2$ | MeOH | c | (28) | 40% |
| Triethyloxonium fluoroborate | $CH_2Cl_2$/DMSO | c | (28) | b |
| $BF_3$ | MeOH | c | (28) | b |
| 2,2 -dimethyl-oxypropane | $CH_2Cl_2$/DMSO | c | (28) | b |
| triethylortho-formate | $CH_2Cl_2$/DMSO | c | (28) | b |
| $CH_3COCl$ | - | refluxed | (67) | b |
| $CH_3COCl$ | $(CH_3)_3PO$ | refluxed | (67) | b, blue complex formed |
| $(CF_3CO)_2O$ | $(CH_3)_3PO$ | d | (67) | b |
| Carbonyldiimidazole | DMF | d | (67) | b |
| $CH_3COCl$ | DMF | d | (67) | b |
| $CH_3COCl$ | DMF | refluxed | (67) | b,decomposition |
| ethylchloroformate | - | d | (67) | b |
| ethylchloroformate | $(CH_3)_3PO$ | d | (67) | b |
| $SOCl_2$ | - | d | (67) | b |
| $CF_3SO_3CH_3$ | $(CH_3)_3PO$ | 20°C 30min. | This study | 100% |
| $FSO_3CH_3$ | $(CH_3)_3PO$ | 20°C 30min. | This study | 100% |
| $FSO_3H$ | Me H | 20°C | (50) | 95% |

a. Yields were estimated from ratio of $2^+$ to $3^+$ species after cation exchange chromatography of the reaction mixture treated with Gly-OEt or ammonia.

b. Little or no chelated ester product.

c. 20°C, 18 hours.

d. 20°C, 6 hours.

The esterification method used preferentially in this study was treatment of the chelated amino acid with an 8 fold excess of $CF_3SO_3CH_3$. This reagent effectively added a methyl group directly to the unco-ordinated oxygen.

The $FSO_3H$/MeOH method was used by Friar to obtain high yields of the chelated esters of phenylalanine and proline. These reacted rapidly in the presence of excess $NH_3$/MeOH but did not couple effectively with equimolar amounts of amino acid esters (50). This suggests that acid was precipitated along with the chelated esters which produced very acid reaction conditions. These conditions do not favour aminolysis reactions.

A similar situation is thought to have occurred in the preparation of chelated amino acid esters for solid-phase peptide synthesis (Chapter 3), due to $FSO_3H$ present as a contaminant in the $FSO_3CH_3$ reagent. These chelated esters reacted rapidly with excess ammonia but were largely unreactive to amino acids bound to insoluble polymers. The methanolic coupling solutions were acid (pH of approximately 2) for all syntheses which were checked.

The use of the reagent $CF_3SO_3CH_3$ did not appear to result in acid precipitating with the chelated esters and no problems associated with the solubility of the chelated amino acid esters were encountered.

Another method, used by Dekkers (28), to obtain the $[Co(en)_2aa\text{-}OMe]^{3+}$ complexes was the chelation of the monodentate amino acid esters (<u>cis</u>- $Co(en)_2X(aa\text{-}OR)\ X_2$, X=Cl,Br) as shown in Reaction 2.1.

Reaction 2.1. $[Co(en)_2Br(aa\text{-}OR)]^{2+} + Ag^+ \longrightarrow [Co(en)_2aa\text{-}OR]^{3+} + AgBr(s)$

This method had only limited application since the monodentate esters were difficult to isolate and yields were poor. Good yields of the monodentate ester were only obtained in the case of

glycine. Furthermore, conversion of the monodentate ester to the chelated ester required carefully controlled anhydrous conditions and purification of the final product was wasteful. The mechanism of similar reactions have been studied and are thought to occur through a 5 co-ordinate intermediate (47-49).

The chelated esters of all of the common amino acids or their side chain protected forms have been prepared with the exceptions of methionine, cysteine, glutamine, asparagine, tryptophan, histidine and hydroxyproline (46).

# CHAPTER 3

## SOLID PHASE PEPTIDE SYNTHESIS VIA COBALT(III) CHELATES OF AMINO ACID METHYL ESTERS

For all syntheses attempted on solid phase, the synthesis strategy is outlined in Figure 3.1.

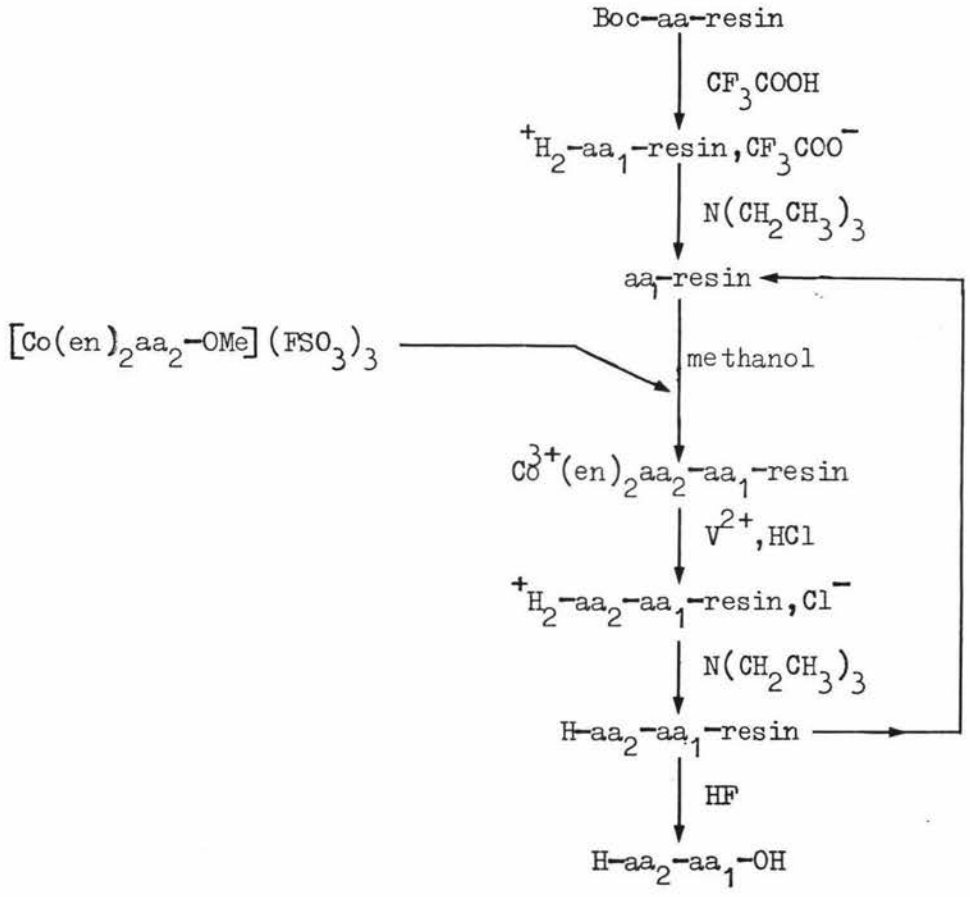



Figure 3.1. Synthesis strategy for solid phase peptide synthesis using Cobalt(III) chelated amino acid esters.

Solid Phase Techniques.

All syntheses were performed using a Schwarz/Mann automatic peptide synthesiser in the manual mode of operation. The reaction vessel was maintained under a dry nitrogen atmosphere and shaken vigorously for the stated times.

Additions of cobalt(III) chelated active esters solutions and of $V^{2+}$ solutions were accomplished by quickly adding the solution through a port at the top of the reaction vessel.

Boc-amino acid-polymers were prepared from chloromethylated polystyrene resins by the method of Stewart and Young (42).

The preparation of the active ester complexes is given in the preceding chapter.

The methanol solvent used was A.R. grade stored over type 3A molecular sieves. The trifluoroacetic acid was freshly distilled from $CrO_3$. The triethylamine was A.R. grade used without further purification. Dichloromethane was laboratory grade distilled and passed through a silica column.

The preparation of methanolic $V^{2+}$ is as follows: dry HCl was bubbled through dry methanol until the solution was 1M HCl by weight. $VOSO_4.H_2O$ was added to this solution to make the final concentration of $VO^{2+}$ 1M. Zinc amalgam (2%, $10cm^3/50cm^3$ solution) (see Chapter 4 for preparation), was added to the blue solution and the mixture left in a stoppered bottle. The formation of the violet $V^{2+}$ species indicated the reagent was ready for use. For preparation of aqueous $V^{2+}$ solutions the $VOSO_4.H_2O$ salt was dissolved in 1M HCl.

Detection of free amines on the aminoacyl resins was done on small quantities of the resin using the bromocresol purple test (43).

Hydrolysis of peptide-resin samples was carried out in $H_2O$:propionic acid (1:5) which was 3M in p-toluenesulphonic acid and contained 2% anisole. Approximately 10mg of the resin was placed in a teflon sealed hydrolysis tube and the tube evacuated, then heated at 130°C for 3 hours. The insoluble polymer was then filtered off and the sample diluted and neutralised to $10cm^3$ at pH=7. A sample (200µl) was loaded onto the automatic amino acid analyser.

## SECTION A: PREPARATION OF $Co^{3+}(en)_2$Phe-Leu-MERRIFIELD POLYMER

Materials.

The Boc-Leu-Polymer was prepared from a Merrifield type 2% cross-linked polystyrene resin. This resin contained 203µmol Boc-Leu/g resin.

Method.

The Boc-Leu-Polymer (3.0g) was treated with trifluoroacetic acid (50% v/v in dichloromethane,$20cm^3$) for 10 minutes, followed by another treatment with trifluoroacetic acid (25% v/v in dichloromethane,$20cm^3$) for 30 minutes, to remove the amino-blocking group (t-butyloxycarbonyl). The resin was then washed three times with each of: dichloromethane, triethylamine (20% v/v in dichloromethane), and dichloromethane ($20cm^3$/wash). A bromocresol purple test carried out on the resin at this stage was strongly positive showing that the t-butyloxycarbonyl group had been removed.

The washed resin was preswollen in methanol (5 minutes), then $[Co(en)_2Phe\text{-}OMe](FSO_3)_3$ (0.5g, $7.6 \times 10^{-4}$mol, 1.2 fold excess)

dissolved in methanol ($10cm^3$) was added for each of three separate treatments lasting for 15 minutes, 2 hours and 3 hours respectively. Bromocresol purple tests carried out on the resin after each treatment became progressively less positive, while the resin became coloured a light orange.

No attempt was made to quantify the yield of peptide at this stage. The resulting resin was used directly in sections B and C of this chapter.

## SECTION B: ATTEMPTED PREPARATION OF Pro-Phe-Leu-MERRIFIELD POLYMER

### Materials.

The resin prepared in Section A was used directly in this experiment.

### Method and Results.

The resin (0.6g) was treated twice with an acidic methanol solution of $VSO_4$ (1M $VSO_4$, 1M HCl, $2cm^3$) for 15 minutes. This treatment resulted in a green coloured resin. The resin was washed three times with each of: trifluoroacetic acid (25% v/v in dichloromethane), dichloromethane, triethylamine (10% v/v in dichloromethane), and dichloromethane, ($10cm^3$ of each). This series of washes removed most of the green species from the resin leaving only a faint green colouration. A bromocresol purple test of this resin was very positive for free amine.

The resin was then treated with $[Co(en)_2Pro\text{-}OMe](FSO_3)_3$ (0.2g, $3.4 \times 10^{-4}$mol, 2.7 fold excess) in methanol ($10cm^3$) for 10 hours. The treatment was repeated twice with fresh activated ester for 3 hours and 10 hours, respectively. The resin was coloured light

orange at this stage. A bromocresol purple test was still positive for free amine but the test had decreased in intensity.

The light orange resin was treated twice with a methanol solution of $VSO_4$ (1M $VSO_4$, 1M HCl, $2cm^3$) for 5 minutes and was coloured green after these treatments. The resin was washed three times with water ($10cm^3$) for 5 minutes, then 3 times with methanol ($10cm^3$) for 5 minutes. These washes rapidly removed all traces of the green colour. A bromocresol purple test of this resin was only slightly positive for free amine but, after the resin had been washed three times with $10cm^3$ of each of the following: dichloromethane, triethylamine (10% v/v in dichloromethane), and dichloromethane, a bromocresol purple test was very positive for free amine. A small amount of the resin (10.8mg) was hydrolysed (see Solid Phase Techniques) and an amino acid analysis of the hydrolysate revealed that the ratio of amino acids present was proline - 0.00, leucine - 1.00 and phenylalanine - 0.04. No trace of proline was observed.

## SECTION C: ATTEMPTED PREPARATION OF Phe-Phe-Leu-MERRIFIELD POLYMER

### Materials.

The resin prepared in Section A was used directly in this experiment.

### Method and Results.

The resin (2.2g) was treated with a methanol solution of $VSO_4$ (1M $VSO_4$, 1M HCl, $2.5cm^3$) for 5 minutes. After this time the predominant species present was the intense violet $V^{2+}$ ion.

The resin was filtered, then washed with $10cm^3$ of each of dichloromethane, methanol and trifluoroacetate acid (50%, 1 hour). These solvents failed to remove the green-grey colour from the resin. The resin was then washed for 5 minutes with each of water, triethylamine (10% v/v in dichloromethane), water and methanol. These solvents removed all traces of colour from the resin. A bromocresol purple test on the reduced resin indicated a large proportion of free amine. The bulk of the resin was freeze-dried for 12 hours to remove all traces of water.

The dried peptide resin was shaken with $[Co(en)_2Phe\text{-}OMe](FSO_3)_3$ (0.3g, $4.6 \times 10^{-4}$, an equimolar amount) dissolved in methanol ($10cm^3$) for each of four treatments lasting 15 minuthes, 15 minutes, 12 hours and 40 minutes, respectively. After this treatment the resin was coloured a light fawn/pink and a bromocresol purple test on the resin was negative for free amine groups.

The removal of the cobalt(III) complex from the resin was brought about by treatment with an acidic methanol solution of $VSO_4$ (1M $VSO_4$, 1M HCl, $2cm^3$) for 2 minutes. The colour of the reducing solution changed from an intense violet to green during this time. The treatment was repeated using fresh reagent (2 minutes). The reducing solution remained violet during this time. A water wash was again necessary to remove the green compounds from the resin. A bromocresol purple test of the resin was positive for a large amount of free amine. A small portion of the resin (10.5mg) was hydrolysed and the hydrolysate analysed for amino acid content. The ratio of amino acids was leucine - 1.00, phenylalanine - 0.08. A trace of isoleucine was also observed.

## SECTION D: ATTEMPTED PREPARATION OF Ala-Phe-GRAFTED POLYMER

### Materials.

Resin used was polypropylethylene powder to which had been grafted a thin layer of polystyrene (28.7% of total weight) chloromethylated to 0.66 meq/g resin. The Boc-Phe-Grafted Polymer was prepared by the method of Young and Stewart (42).

### Method and Results.

An amino acid analysis of a hydrolysed sample of the resin revealed that the substitution of Boc-Phe had occurred to a total of 0.205 meq/g Boc-Phe-polymer. The Boc-Phe-Grafted polymer (0.271g) was treated with trifluoroacetic acid (25% v/v in dichloromethane, $20cm^3$) for 10 minutes to remove the amino protecting Boc group. The resulting Phe-Grafted polymer was washed 3 times with each of dichloromethane, triethylamine (10% v/v in dichloromethane) and dichloromethane ($20cm^3$ of each).

The washed resin was treated with $[Co(en)_2Ala\text{-}OMe](FSO_3)_3$ (0.14g, $2.6 \times 10^{-4}$mol, 5 fold excess) dissolved in methanol ($3cm^3$) for 2 hours. After this time the resin was coloured a light pink-orange but washing the resin for 5 minutes with methanol removed all traces of colour. A further treatment with $[Co(en)_2Ala\text{-}OMe](FSO_3)_3$ (0.2g, $3.7 \times 10^{-4}$mol, 6.7 fold excess) dissolved in methanol ($5cm^3$), for 27 hours resulted in a pink coloured resin. Tests with ammonia/methanol (see Chapter 2, Preparation of Active Esters - Techniques) on small quantities of reaction mixture filtrate showed that the ratio of $[Co(en)_2Phe\text{-}OMe]^{3+}$ to $[Co(en)_2Phe]^{2+}$ was 70:30 after 4 hours and 10:90 after 24 hours. The pH of the reaction mixture was measured after 27 hours and was found to have a pH of approximately 2.

A sample of the pink coloured resin was washed with methanol ($10cm^3$) which did not completely remove the colour. The resin was treated with aqueous acidic $VSO_4$ (0.5M $MSO_4$, 1M HCl) for 5 minutes. The filtered resin was not coloured green as previous reduction steps in Sections A, B and C. An amino acid analysis of the hydrolysed resin revealed that only a slight trace of alanine was present, less than 0.1% of the amount of phenylalanine.

TABLE 3.1

SUMMARY OF RESULTS

| Chelated Ester* | Nucleophile | Total Reaction Time (Hrs) | Excess of Active Ester‡ | Yield |
|---|---|---|---|---|
| $[Co(en)_2Phe\text{-}OMe](FSO_3)_3$ | Leu-Merrifield polymer | 5.25 | 1.2x3 | 4% |
| $[Co(en)_2Pro\text{-}OMe](FSO_3)_3$ | 4% Phe-Leu-Merrifield Polymer + 96% Leu " " " | 23 | 2.7x3 | 0% |
| $[Co(en)_2Phe\text{-}OMe](FSO_3)_3$ | 4% Phe-Leu-Merrifield Polymer + 96% Leu " " " | 13 | 1.0x4 | 4% |
| $[Co(en)_2Ala\text{-}OMe](FSO_3)_3$ | Phe-grafted polymer | 29 | 5x2 | <0.1% |

* The chelated esters were dissolved in methanol.

‡ x2 indicates the resin had two treatments of this excess.

Discussion.

Reactions of active esters of Boc-Amino acids with amino acyl esters to form peptide bonds occur at much slower rates when the amino acyl moiety is esterified to an insoluble polymer support (12). For example, Boc-Leu-PCP reacts with Gly-$OBu^t$ over 200 times faster than with Gly-O-Polymer, due to the steric hindrance caused by the polymeric support.

The rates of reaction of cobalt(III) activated amino acid esters with amino acyl esters are also expected to be slower for steric reasons, when the nucleophilic amino acyl moiety is bound to an insoluble polymer compared with a soluble ester as has been observed by Bentley (41). The reactions of $[Co(en)_2Gly\text{-}OMe]^{3+}$ and $[Co(en)_2Ala\text{-}OMe]$ with amino acyl polymer supports (glycine, alanine, phenylalanine, valine) where the active esters are in 100 molar excess, required 10 minutes reaction time to yield 47% - 91% peptide bond depending on the steric hindrance of the amino acyl amino acid. The same reactions occurred within seconds (above 95% yield of peptide-complex) when amino acyl ethyl esters were used (30, 46).

The stability of $Co^{3+}(en)_2$ peptide-polymeric support species to hydrolysis by water has been established (19). The loss of orange-pink colour from resins after synthesis attempts when washed with water must, therefore, be attributed to cobalt(III) species which have not reacted with amino acyl groups, i.e. have not been bound to the resin.

In this study the positive bromocresol purple test carried out on the deprotected Leu-resin shows that the free amine of the leucine was produced. Analysis of the resin from Section B revealed that the yield of Phe-Leu-resin in Section A was 4% which supports the weak orange colouration of the resin.

The next coupling with proline in Section B failed to produce any proline bound to the resin. The reason for this is not clear but the bromocresol purple test indicates that free amino groups were present. The further coupling of the resin from Section A with the active ester of Phe yielded a further 4% of the bound amino acid. This

coupling may have occurred on the free amino groups on the resin in general or on the less plentiful (4%) but more sterically favoured dipeptide–resin free amine. This study did not distinguish between these possibilities.

The very low yield of peptide in Section D indicates that the use of a less sterically hindered resin does not increase the yield. The problem of poor yields in these solid phase preparations, therefore, lies with the species in solution. The low pH (1–2) of the reaction mixtures tested would result in the full protonation of the free amino groups (pK = 8–10) necessary for coupling.

The low pH of the solutions of the $[Co(en)_2aa\text{–}OMe](FSO_3)_3$ salts was thought to be caused by acid which precipitated with the active ester during the preparation of the active esters from the reaction of $FSO_3CH_3$ with the $[Co(en)aa]^{2+}$ complexes (see Chapter 2). It was noticed in preparing the $[Co(en)_2aa\text{–}OMe](FSO_3)_3$ complexes, that with freshly opened $FSO_3CH_3$ an 8 fold excess drove the reaction to completion but after a few weeks in a glass stoppered container up to 16 fold excesses of the reagent were necessary. This would be consistant with the hydrolysis of the reagent as in Reaction 3.1 with atmospheric water.

$$FSO_3CH_3 + H_2O \longrightarrow FSO_3H + CH_3OH \qquad 3.1$$

The same sensitivity to hydrolysis was not observed with $CF_3SO_3CH_3$, and the methanolic solutions of $[Co(en)_2aa\text{–}OMe](CF_3SO_3)_3$ isolated from this reagent were not acidic (pH of approximately 7).

When $[Co(en)_2Phe\text{–}OMe](FSO_3)_3$ or $[Co(en)_2Pro\text{–}OMe](FSO_3)_3$ was added to water and the resulting solution was titrated with sodium hydroxide, two mole equivalents of base were required to neutralise the solution. One of the protons came from the reaction of the active ester with water (Reaction 3.2) while the other source must have been precipitated in a 1:1 mole ratio with the active esters

during their preparation.

$$[Co(en)_2Phe\text{-}OMe]^{3+} + H_2O \longrightarrow [Co(en)_2Phe]^{2+} + MeOH + H^+ \qquad 3.2$$

Reaction 3.2 may have contributed to the acidity of the reaction mixtures to some extent but the methanol was very dry (3A molecular seives) and this contribution was expected to be slight.

Difficulties with solubility in methanol of $[Co(en)_2Ala\text{-}OMe](FSO_3)_3$, $[Co(en)_2Phe\text{-}OMe](FSO_3)_3$ and $[Co(en)_2Pro\text{-}OMe](FSO_3)_3$ were encountered. Batches which were readily soluble in methanol immediately after preparation were almost completely insoluble after 2 months of standing in closed containers and evacuated in a desiccator over $P_2O_5$. When the mixture of complex in methanol was tested with ammonia/methanol the predominant species was shown to be $[Co(en)_2aa\text{-}OMe]^{3+}$. No such tendency has been observed with the $(CF_3SO_3^-)$ series. Clearly the $(CF_3SO_3^-)$ salts have definite advantages over the $(FSO_3^-)$ salts in peptide synthesis via Cobalt(III) chelated amino acid esters.

Experiments using $[Co(en)_2aa\text{-}OMe](CF_3SO_3)_3$ salts to prepare peptides using solid phase techniques would confirm if the low yields were caused by the acidity of the reaction mixtures.

$[Co(en)_2aa\text{-}OMe](FSO_3)_3$ salts may be viable as intermediates in peptide synthesis if they could be isolated acid free or titrated to neutrality, with triethylamine in an autotitrator, in situ.

The large decrease in the positiveness of the bromocresol purple test during a coupling reaction may be due to the acidity of the coupling reactions. The bromocresol purple test should be used only after a triethylamine wash of the resin to give a true indication of the proportion of unreacted amino groups.

The colouration of resins may be due, to some extent, to Co and V species which are merely trapped in the polymer. Water and methanol washes remove these species but not those bound to the resin by peptide

bonds (19). The absorbtion of coloured chromium complexes to the resin has been observed in another study (27).

Conclusions.

It appears, under the conditions used, that the reagent $FSO_3CH_3$ is not effective in preparing neutral salts of cobalt(III) chelated amino acid esters. The reagent $CF_3SO_3CH_3$ is a better alternative for preparing these esters from the cobalt(III) chelated amino acid.

The use of vanadous solutions to reduce cobalt(III) species attached to insoluble polystyrene polymers causes the colouration of the resin with green vanadium species which are removed only be aqueous solutions. The use of another reducing agent for Cobalt(III) in solid phase peptide synthesis is, therefore, indicated.

# CHAPTER 4

## SOLUTION PHASE PEPTIDE SYNTHESIS VIA COBALT(III) CHELATES OF AMINO ACID METHYL ESTERS

This chapter describes the solution phase synthesis of two tetrapeptides, three tripeptides and the attempted synthesis of a further two tripeptides. These peptides were prepared by successive chain elongation through the aminolysis of cobalt(III) chelated amino acid methyl esters by the amino terminus of the growing peptide sequence.

### Materials and Methods Used Throughout Chapter.

All amino acids used were of the L configuration with the exception of glycine. The preparation of all cobalt(III) chelated amino acid methyl esters used here has been given in Chapter 2 of this thesis. All water used was distilled and deionised. Pyridinium Acetate was prepared by mixing equimolar amounts of pyridine (A.R. grade) and acetic acid (A.R. grade) and diluting to the required concentration.

### Ion Exchange Columns.

Unless otherwise stated, "analytical column" in the text refers to SP-C25-120 mesh Sephadex, $Cl^-$ form (10cm x 1cm) and "preparative column" refers to V.D.C. viscose $SP^{10}$ 50-100 mesh pyridinium form (9cm x 6.5cm diameter).

Zinc Amalgam (2%).

Zinc (granulated, 3g) was shaken for a short time in hydrochloric acid (1N) to which a trace of copper sulphate had been added. When the surface of the metal had been thoroughly freed from oxide an aqueous solution of mercuric chloride was added and the mixture shaken until the zinc was well covered with a deposit of mercury. The metal was washed with water and clean mercury ($10cm^3$) was added. The mixture was covered with hydrochloric acid (1N) and left for at least 3 hours. The amalgam was prepared fresh for each reduction as a precipitate formed on top of the amalgam several days after preparation. The amalgam was added to the cobalt(III) complexes at pH 1 and the progress of the reduction followed by the colour change from deep orange to light purple. The pH of the solution above the amalgam was checked throughout the reduction. When no orange was visible the reduction was complete.

Gel Filtration.

All gel filtration steps were performed on the same column. The resin used was Bio-Gel P-2, minus 400 mesh, polyacrylamide gel from Bio-Rad (70 x 2.5cm). Solvent flowed under gravity from a reservoir 10cm above the column. The column was coupled to two Uvicord L.K.B Bromma 8300 u.v. spectrophotometers with 100 µl flow-through cells monitoring the u.v. absorbtion of the eluant at 254nm and 280nm and to a type CDM2e Radiometer Copenhagen flow-through conductivity meter. The eluant was collected using an L.K.B. UtroRac 7000 fraction collector set to count 80 drops into each tube. Conductivity and u.v. elution profiles were recorded

on an L.K.B. Chopper Bar Recorder 6520. The eluant was acetic acid (2N) and the complete system was run at 3°C. Fractions of $3.6cm^3$ were collected from the column. To test for free amino groups in the fractions, quantities of each fraction tested were concentrated on a spot on an alumina thin layer chromatography plate and eluted with a solvent. The spots were then visualised by spraying with ninhydrin solution and heating.

Hydrolysis of Peptides.

All hydrolyses were performed in 6N hydrochloric acid in evacuated teflon sealed hydrolysis tubes for 22 hours at 110°C.

Freeze Drying of Samples.

Freeze drying of samples was accomplished by freezing the solution in a shell around the inside of a round bottomed flask then evaporating to dryness under oil pump vacuum using traps immersed in methanol at -40°C to trap the evaporated solvents.

Thin Layer Chromatography.

Analar grade solvents were used throughout. The mixed solvents used are listed below:

solvent 2; n-butanol:pyridine:acetic acid:water, 15:10:3:12 (v/v)

solvent 3; acetic acid:methanol, 9:1 (v/v)

solvent 4; n-butanol:acetic acid:water, 4:2:1 (v/v)

solvent 5; n-butanol:pyridine:water, 2:2:1 (v/v)

solvent 6; ethyl acetate:pyridine:acetic acid:water, 5:5:1:3 (v/v)

The thin layers used were Eastman chromatogram sheets with poly(ethyleneterepthalate) flexible backing.

Silica; 100 micron thick coating of silica gel with polyvinylacid as binder ("silica u.v." denotes the presence of a fluorescent indicator).

Alumina; 100 micron thick coating of alumina gel with polyvinylalcohol as binder.

None of these plates were activated before use.

The plates were spotted with samples dissolved in solvent and the solvent evaporated by heating with a hand-held drier. The plates were then developed in small jars containing approximately 0.5cm of solvent and heated to dry the solvent from the layer. Then the plates were sprayed with a ninhydrin solution and allowed to air dry. Heating of the plates with the hand-held drier caused the reaction with ninhydrin to occur and visible spots appeared on the plates. Rf's were calculated from the distances, origin to solvent front and origin to centre of spot.

The ninhydrin was puriss A.R. grade from Koch-Light Laboratories Ltd. The ninhydrin solution was 0.15g of ninhydrin dissolved in Acetone ($20cm^3$).

High Pressure Liquid Chromatography.

A Waters high pressure liquid chromatography system was used which included two M-6000 solvent delivery units, an M-660 solvent programmer and a U6K universal liquid chromatograph injector coupled to a Cecil 212 variable wavelength u.v. monitor with an 8 µl flow-through cell. The µ-Bondapak-$C_{18}$ column (10µm, 300mm x 4mm) was

also from Waters Associates. Sample injections were made with a Pressure-Lok Liquid Syringe, series B-110, from Precision Sampling Corp. Filtration of solvents was carried out using a 0.5 μm Millipore filter on a Pyrex filter holder (Millipore Corp.). Peptide samples were not filtered. All bulk solvents were degassed separately for the following times: water for at least 30 minutes and acetonitrile for 1.0 minutes. After a gradient had been run the base line was allowed to equilibrate before the next injection of sample. All peptide samples were dissolved in 0.1% phosphoric acid.

All solvents used were Analar grade. Water was glass distilled and deionised. The acetonitrile, supplied by Fisher Scientific Co., was further purified by the method of Walter and Ramaley (60). Orthophosphoric acid was from May & Baker Ltd.

The flow rate used was 1.5$cm^3$/minute and was maintained by a pressure of 3000 p.s.i. All tests were at room temperature (approximately 22°C).

Mass Spectroscopy.

All mass measurements were made with an A.E.I. MS902 mass spectrometer with a resolving power of one thousand. The bombarding electron energy was 70eV. Samples were introduced on a direct insertion probe at temperatures just high enough to vaporize the sample.

## SECTION A: THE PREPARATION OF Gly-Phe-Phe-OBzl

Materials.

The dimethylsulphoxide was A.R. grade used without further purification. Benzyl phenylalanylphenylalanate triflouroacetate salt was prepared as described in Appendix I.

Preparation of (Benzyl glycylphenylalanylphenylalanate)bis(ethylenediamine)cobalt(III) Acetate.

$[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl](CH_3COO)_3$.

Method.

$^+H_2\text{-}Phe\text{-}Phe\text{-}OBzl, CF_3COO^-$ (0.25g, $6.25 \times 10^{-4}$mol) was dissolved in dimethylsulphoxide ($1.5cm^3$) and enough triethylamine added (60mg, $5.95 \times 10^{-4}$mol), to free 95% of the peptide amino group from its triflouroacetate salt. $[Co(en)_2Gly\text{-}OMe](CF_3SO_3)_3$ (0.42g, $5.88 \times 10^{-4}$mol) was dissolved in the solution and the reaction stirred throughout under anhydrous conditions. After 3 minutes and after 30 minutes, approximately $0.1cm^3$ of the reaction mixture was removed, diluted with water ($3cm^3$), sorbed onto two analytical cation exchange columns and the reaction products eluted, Figure 4.1. The bulk of the dark red solution was quenched with acetic acid ($1cm^3$) after 30 minutes of reaction time and diluted with water to $100cm^3$. Unreacted $^+H_2\text{-}Phe\text{-}Phe\text{-}OBzl, CH_3COO^-$ precipitated as a white solid immediately following the dilution and was filtered from the solution. The filtrate was sorbed onto a preparative cation exchange column, washed with water ($50cm^3$) and then eluted with pyridinium acetate (0.2M). When the two bands were well separated the concentration of eluant was increased to 1.0M and the slower eluted species was collected in $800cm^3$ and evaporated to dryness under reduced pressure at 40°C. The product was

then dissolved in water ($10cm^3$), freeze-dried, and weighed.

Results.

The elution profiles of the reaction mixture on the analytical columns show that the proportions of $[Co(en)_2Gly]^{2+}$ and of $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ are 25% and 75% at 3 minutes and 20% and 80% at 30 minutes. The proportion of each species was estimated to the nearest 5% by the intensity of their colour on the columns. Both species were orange.

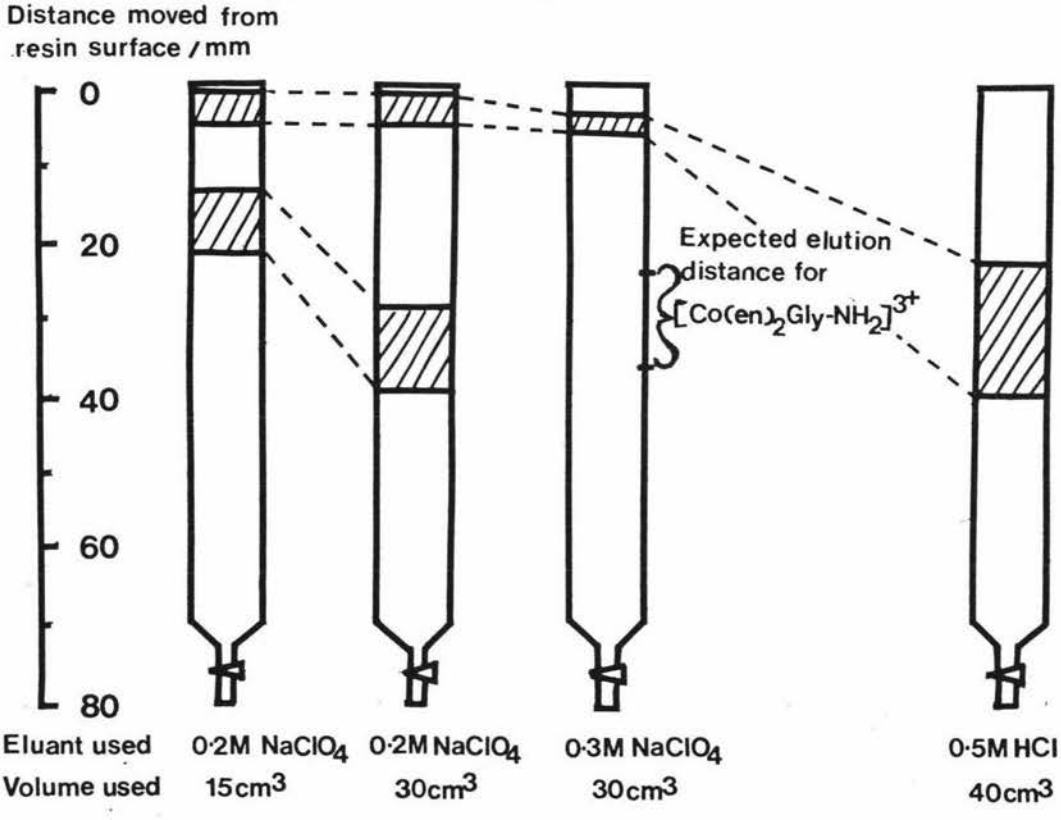



Figure 4.1. Elution profile of reaction products of $Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl^{3+}$ preparation on SP-$C_{25}$-120 Sephadex, $Cl^-$ form, (100mm x 10mm).

No other bands were visible except in the 30 minute elution profile which showed a very weak orange band under the $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ band. The first species eluted was identified as $[Co(en)_2Gly]^{2+}$ by its colour and mobility on the cation exchange

column compared to an authentic sample of $[Co(en)_2Gly]I_2$. The second species eluted was identified as $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ by its colour and mobility on the cation exchange column relative to $[Co(en)_2Gly]^{2+}$, its rapid reduction with zinc amalgam and the identification of ethylenediamine and Gly-Phe-Phe-OBzl in the reduced complex mixture by thin layer chromatography.

The product was a pink-orange crystalline solid. Yield of $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl](CH_3COO)_3$ = 120mg, $1.47 \times 10^{-4}$mol. Based on $[Co(en)_2Gly\text{-}OMe](CF_3SO_3)_3$ reactant and allowing for the material removed for the analytical columns, the yield was 28%.

Reduction of $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl](CH_3COO)_3$ and the Isolation of the Free Peptide.

Method.

A portion of the freeze-dried complex $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl](CH_3COO)_3$ (57mg, $6.97 \times 10^{-5}$mol) was dissolved in water and the pH adjusted to 0.8 with hydrochloric acid (concentrated, 5 drops). Zinc amalgam (2%, $10cm^3$) was added and the mixture stirred throughout. The reduction was complete in 5 minutes and was accompanied by a slight increase in pH to 0.9. Acetic acid ($1cm^3$) was added to make the solution approximately 2N in acetic acid then the cloudy solution was filtered and the filtrate ($9cm^3$) was loaded onto the gel filtration column. The column was eluted with acetic acid (2N) and every fifth fraction tested for ninhydrin reactivity (see Materials and Methods section). The amount of precipitate filtered from the solution before gel filtration was minimal (approximately 1mg) and this was not expected to affect the yield of peptide.

Results.

Two ninhydrin reactive species were detected, Figure 4.2. The first eluted species was ethylenediamine which was identified by thin layer chromatography with standards. Ethylenediamine reacts with ninhydrin on an alumina thin layer to form an intense purple spot with Rf = 0.17 in solvent 6.

The second eluted ninhydrin reactive species was identified as Gly-Phe-Phe-OBzl.

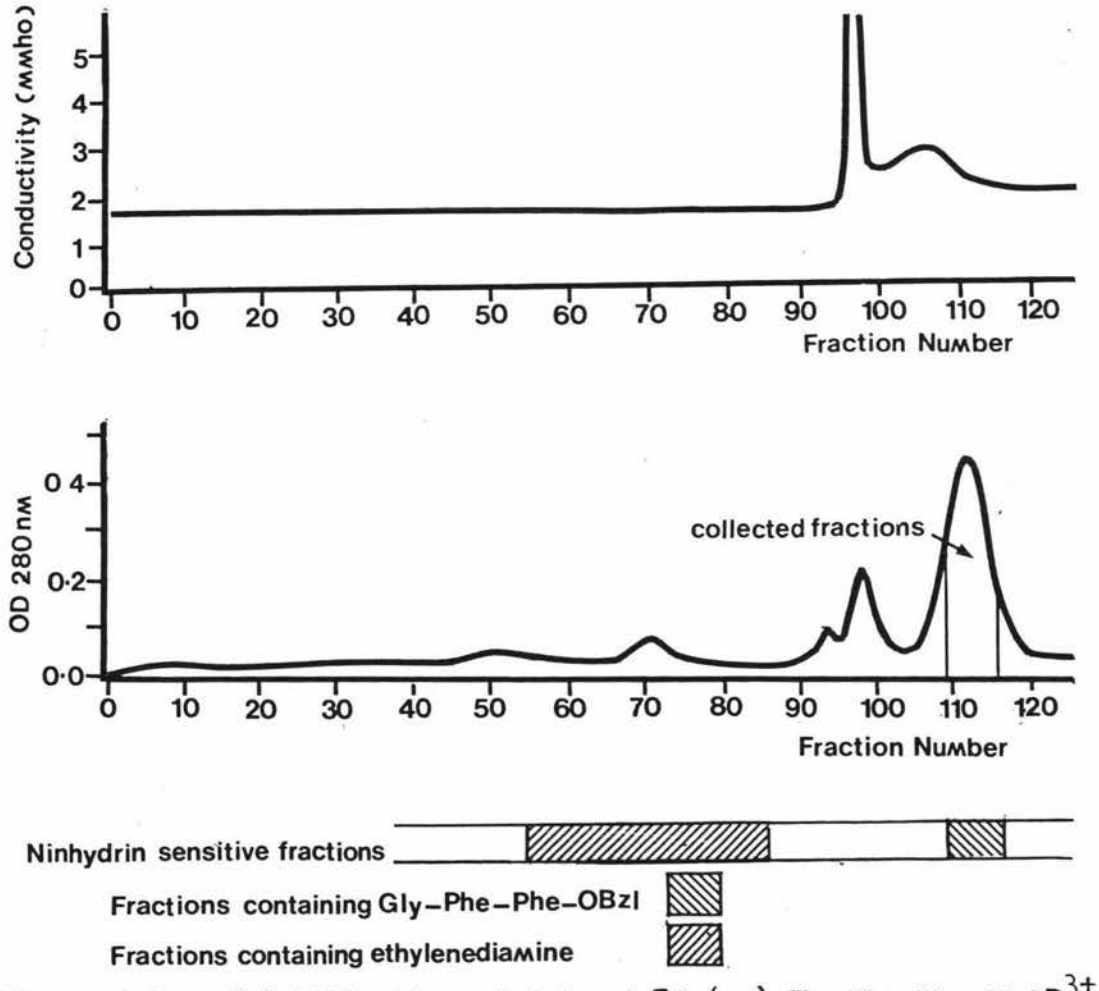



Figure 4.2. Gel Filtration of Reduced $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$

Fractions 110 to 116 inclusive were freeze-dried to a fluffy white-pale green powder. Yield = 30.0mg, $5.77 \times 10^{-5}$mol, i.e. 83%. Amino acid analysis of the hydrolysate of a weighed amount of peptide suggests the yield is slightly lower due to non-volatile

salts in the freeze-dried material. Yield based on moles of glycine in the hydrolysate = 24.6mg, $4.73 \times 10^{-5}$mol, i.e. 67%.

Amino acid analysis of the hydrolysed peptide gives the following ratio of amino acids: glycine 1.00, phenylalanine 2.01. Mass spectroscopy of the peptide identifies the molecular ion and expected fragmentation ions, Table 4.1 and Figure 4.3.

| m/e | Molecular Formula | Structural Designation |
|---|---|---|
| 459 | $C_{27}H_{29}O_4N_3$ | $[H\text{-}Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{+\cdot}$ |
| 387 | $C_{25}H_{25}O_3N$ | $[C_7H_7CH_2CO\text{-}Phe\text{-}OBzl]^{+\cdot}$ |
| 386 | $C_{25}H_{24}O_3N$ | $C_7H_7\overset{+}{C}HCO\text{-}Phe\text{-}OBzl$ |
| 368 | $C_{20}H_{22}O_4N_3$ | loss of one $C_7H_7$ group |
| 294 | $C_{18}H_{18}O_2N_2$ | $[\boxed{\text{-Phe-Phe}}]^{+\cdot}$ |
| 205 | $C_{11}H_{13}O_2N_2$ | $H\text{-}Gly\text{-}NHCH(C_7H_7)C{\equiv}O^{+}$ |
| 177 | $C_{10}H_{13}ON_2$ | $H\text{-}Gly\text{-}\overset{+}{N}H{=}CH(C_7H_7)$ |
| 131 | $C_9H_7O$ | $C_7H_7CH_2C{\equiv}O^{+}$ |
| 120 | $C_8H_{10}N$ | $C_6H_5\text{-}CH_2CH{=}\overset{+}{N}H_2$ |
| 108 | $C_7H_8O$ | $[C_6H_5\text{-}CH_2OH]^{+\cdot}$ |
| 107 | $C_7H_7O$ | $C_6H_5\text{-}CH_2\text{-}\overset{+}{O}$ |
| 91 | $C_7H_7$ | $C_6H_5\text{-}\overset{+}{C}H_2$ |

Table 4.1. High Resolution Data of the Mass Spectrum of Gly-Phe-Phe-OBzl.

A phthalate derivative fragment ion, $C_8H_5O_3$, was identified in the spectrum.

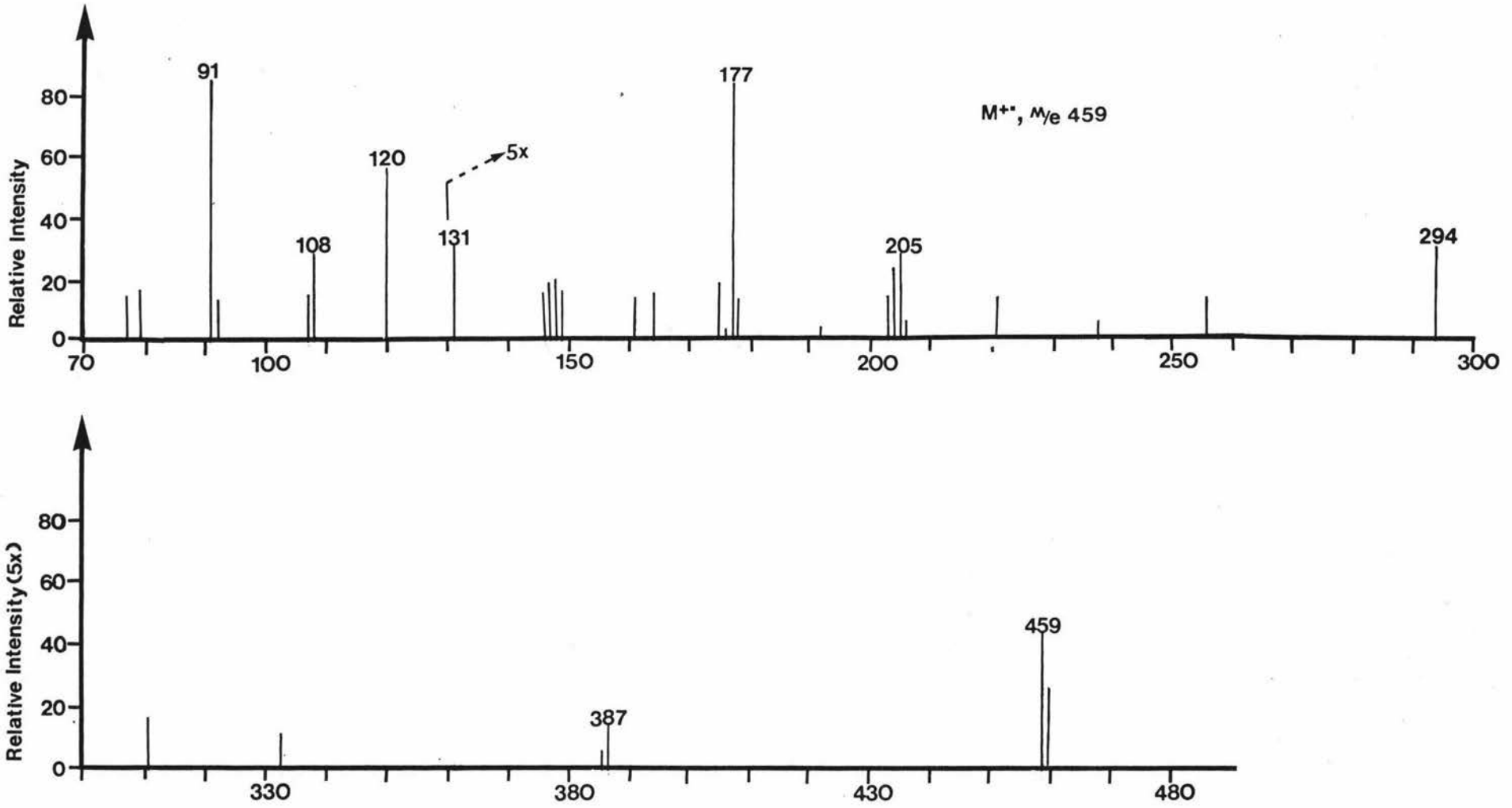



Figure 4.3. The relative intensity of major ions in the mass spectrum of Gly-Phe-Phe-OBzl.

Thin layer chromatography of the peptide was carried out in several solvents with glycine and $^{+}H_2$-Phe-Phe-OBzl,$CF_3COO^{-}$ as standards, Table 4.2.

| Solvent | Thin Layer | Gly-Phe-Phe-OBzl | Phe-Phe-OBzl | Glycine |
|---|---|---|---|---|
| Methanol | silica | 0.57 | 0.59 | 0.07 |
| Ethanol | silica | 0.74 | 0.82 | 0 |
| Chloroform | silica | 0.06 | 0.64 | 0 |
| Acetone | silica | 0.84 | 0.88 | 0 |
| Solvent 6 | alumina | 0.95 | - | - |

Table 4.2. Rf's of Gly-Phe-Phe-OBzl on thin layers.

Gly-Phe-Phe-OBzl reacted with ninhydrin to give a yellow spot on the thin layer. Phe-Phe-OBzl reacted to give a yellow spot which turned orange after 5 minutes of standing at room temperature and glycine reacted to give an orange spot which turned red-brown on standing at room temperature. A trace of impurity was visible as a purple spot after the reaction with ninhydrin. The impurity had Rf = 0 in acetone and chloroform and Rf = 0.03 in methanol and did not correspond to either glycine or Phe-Phe-OBzl on the silica thin layers.

Analysis by HPLC at 254nm monitors only those compounds with aromatic groups. Analysis at this wavelength shows an essentially pure product eluted at 13.8 minutes with a minor aromatic impurity at 15.6 minutes, Figure 4.4A. HPLC analysis at high loadings monitoring at 230nm shows that the peptide has three impurities, Figure 4.4B. Acetic acid and salts are eluted with the breakthrough

volume at 2.3 minutes. A trace of an impurity is eluted at 13.1 minutes and a minor impurity eluted at 18.8 minutes corresponded to the aromatic impurity found in the 254nm runs. Control gradients at both wavelengths did not contain peaks other than a gentle rise in absorbtion with higher acetonitrile concentration.

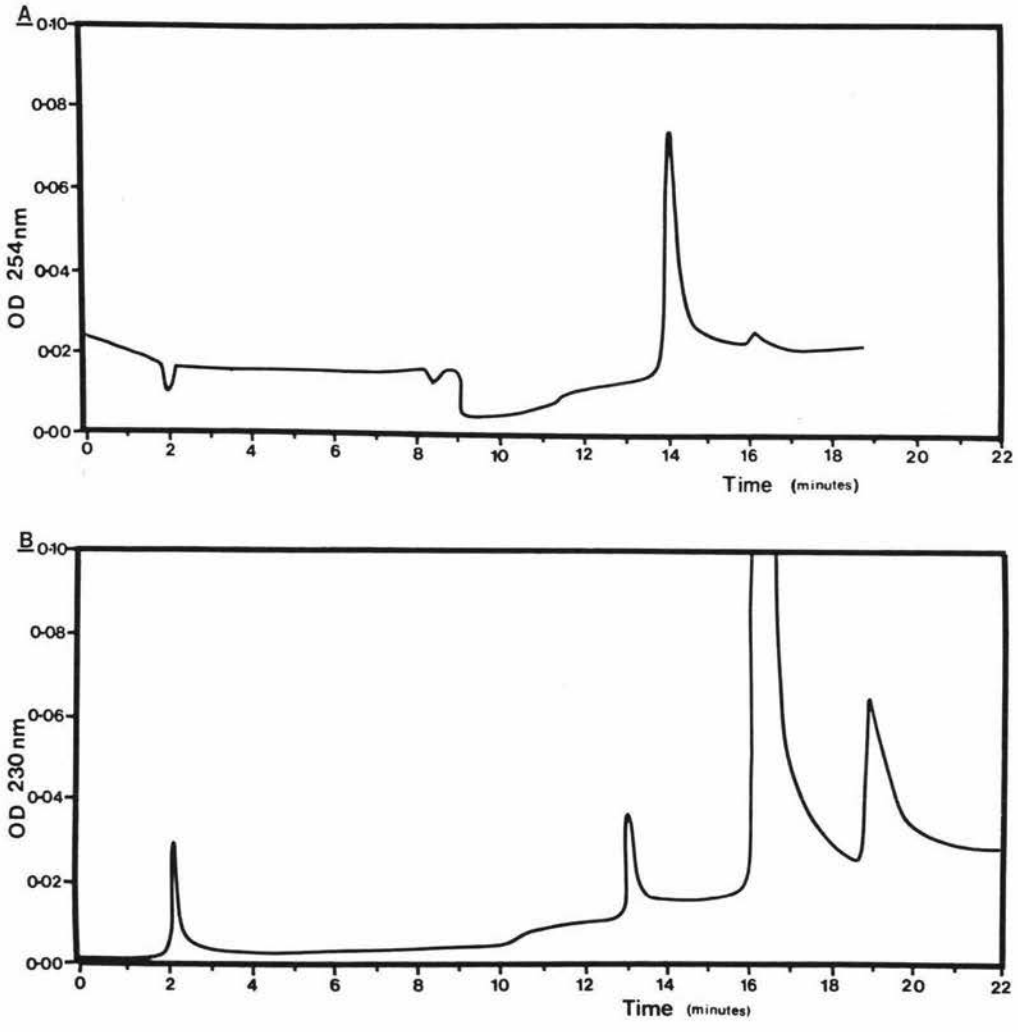



Figure 4.4. Analysis of Gly-Phe-Phe-OBzl product by HPLC. In A, 20µg of peptide was injected and analysed at 254nm. The sample was analysed by elution with 0.1% phosphoric acid for four minutes followed by a ten minute linear gradient of 0 to 75% acetonitrile with 0.1% phosphoric acid. In B, 50µg of peptide was injected and analysed at 230nm. The sample was analysed by elution with 0.1% phosphoric acid for five minutes followed by a fifteen minute linear gradient of 0 to 75% acetonitrile with 0.1% phosphoric acid.

Discussion.

Dipeptide esters cannot be stored as free amines for long periods because they can undergo rapid intramolecular aminolysis to form the very stable diketopiperazine (4-5). For this reason the free amine of $^{+}H_2$-Phe-Phe-OBzl,$CF_3COO^-$ was prepared <u>in situ</u> by the addition of triethylamine to the reaction mixture.

The reaction between $[Co(en)_2Gly-OMe]^{3+}$ and H-Phe-Phe-OBzl proceeds very quickly as can be seen from the column analysis. After 3 minutes 75% of the visible cobalt species were the form of product. Very little reaction took place after 3 minutes however. At 30 minutes only an estimated 5% more of the reactants had reacted.

From the analytical column results a yield of 80% for $[Co(en)_2Gly-Phe-Phe-OBzl](CH_3COO)_3$ was expected. The actual yield was almost a third of this at 28%. This loss may be due in part to hydrolysis of the Gly-Phe bond giving rise to the diffuse orange band which appeared to originate from the $[Co(en)_2$ Gly-Phe-Phe-OBzl$]^{3+}$ band in the column analyses of the 30 minute reaction time sample. Decomposition of this band was not seen on the preparative column but the darker colour of this resin may have hidden this effect. The rapid hydrolysis of $[Co(en)_2$ Phe-Phe-OBzl$]^{3+}$ while being eluted from a cation exchange resin at pH 5 has been observed by Friar (50). This phenomenon led to very low yields of peptide.

The $[Co(en)_2Gly-Phe-Phe-OBzl]^{3+}$ ion is very slow moving on the analytical columns when compared with $[Co(en)_2Gly-NH_2]^{3+}$, Figure 4.1. This is probably caused by adsorbtion of the aromatic moiety to the bed material. Adsorbtion of aromatic compounds to cross-linked dextran gels has been observed by Gelotte (52).

Isolation of the free peptide by gel filtration reveals that the polyacrylamide resin adsorbs the Gly-Phe-Phe-OBzl to such an extent that the peptide is eluted after the salt peak. The elution of the largest molecule last is contrary to the molecular sieving principle of the column, but adsorbtion of other aromatic compounds to this polyacrylamide resin has been observed (53).

The peptide is essentially homogeneous as shown by thin layer chromatography and HPLC. Mass spectroscopy and amino acid analysis confirm the structure of the tripeptide. The minor impurity eluted after the peptide on the HPLC runs is probably the phthalate derivative seen in the mass spectrograph since it absorbs at 254nm. Phthalate derivatives are found in most organic solvents where they are leached from plastics in contact with them. Thus, any preparation which entails the concentrating of organic solvents usually contain phthalate derivatives as an impurity.

SECTION B: THE PREPARATION OF Ala-Gly-Phe-Phe-OBzl.

Materials.

The acetate salt of Gly-Phe-Phe-OBzl was prepared in Section A.

The cation exchange column used was SP-$C_{25}$-120 mesh Sephadex, $Cl^-$form, (8cm x 1cm diameter).

Preparation of (Benzyl alanylglycylphenylalanylphenylalanate)bis (ethylenediamine)cobalt(III) Acetate.

$[Co(en)_2Ala\text{-}Gly\text{-}Phe\text{-}Phe\text{-}OBzl](CH_3COO)_3$.

Method.

$^+H_2$-Gly-Phe-Phe-OBzl,$CH_3COO^-$ (10.2mg, allowing for salts in peptide = $1.58 \times 10^{-5}$mol) was dissolved in dimethylsulphoxide ($0.17cm^3$) to which had been added enough triethylamine (2.0mg, $1.96 \times 10^{-5}$mol) to just free the amino group of the peptide from its acetate salt. $[Co(en)_2Ala\text{-}OMe](CF_3SO_3)_3$(38mg,$5.27 \times 10^{-5}$mol), a 2.7 fold excess, was dissolved in the solution and the reaction stirred throughout under anhydrous conditions. After 20 minutes the red solution was quenched with acetic acid (10 drops) and diluted to $5cm^3$ with water. The resulting solution was sorbed onto the cation exchange column and elution begun with 0.2M pyridinium acetate. When the two bands were well separated the eluant was changed to 1.0M pyridinium acetate and the slower moving band was collected in $50cm^3$ of eluant. This band was decreased in volume to $10cm^3$ under reduced pressure then freeze-dried. Yield of $[Co(en)_2Ala\text{-}Gly\text{-}Phe\text{-}Phe\text{-}OBzl](CH_3COO)_3$ = 12.4mg, $1.4 \times 10^{-5}$mol, i.e. 88% based on Gly-Phe-Phe-OBzl reactant.

Results.

Two orange coloured bands were observed on the cation exchange column (Figure 4.5). The first and second bands eluted represented 80% and 20% of the visible reaction products respectively. The first species eluted was identified as $[Co(en)_2Ala]^{2+}$ by colour and by the observed retention volume on the cation exchange column compared with an authentic sample of $[Co(en)_2Ala]I_2$. The second species eluted was identified as $[Co(en)_2Ala\text{-}Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ by colour, ease of reduction of the complex and the subsequent identification of the ligands by thin layer chromatography and amino acid analysis.

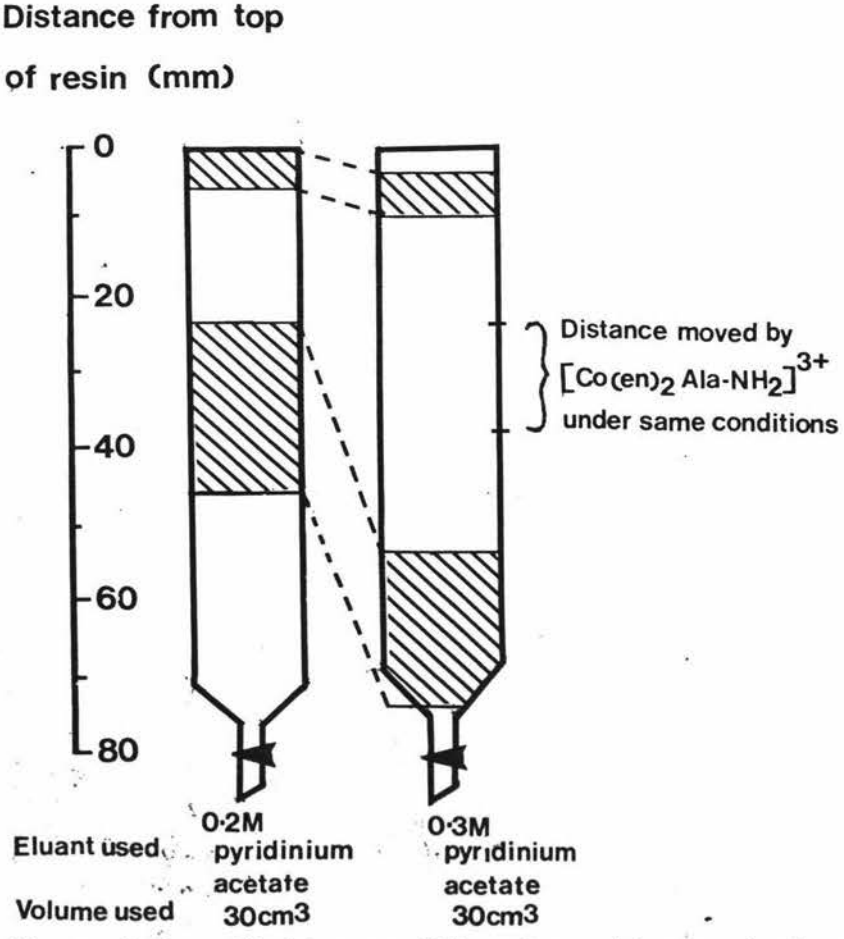



Figure 4.5. Elution profile of reaction products of $[Co(en)_2Ala\text{-}Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ preparation on SP-C25-120 Sephadex, $Cl^-$ form.

Reduction of $[Co(en)_2Ala-Gly-Phe-Phe-OBzl](CH_3COO)_3$ and the Isolation of the Free Peptide.

Method.

The freeze-dried complex $[Co(en)_2Ala-Gly-Phe-Phe-OBzl](CH_3COO)_3$ (12.4mg, $1.4 \times 10^{-5}$mol) was dissolved in water ($1cm^3$) and the pH of the solution adjusted to 1.0 with hydrochloric acid (concentrated, 4 drops). Zinc amalgam (2%, $5cm^3$) was added and the reaction stirred for 5 minutes after which time the reduction was complete. A white compound was precipitated during the reduction. The reduction solution and the precipitate were decanted from the amalgam and acetic acid ($1cm^3$) was added to bring the concentration of acetic acid to approximately 2N. The precipitate dissolved in this solution and the clear solution ($5cm^3$) was loaded onto the gel filtration column and eluted with 2N acetic acid. Fractions of $3.6cm^3$ were collected and every fifth fraction was tested by thin layer chromatography for reactivity with ninhydrin.

Results.

Only one ninhydrin reactive species was detected. This was found in fractions 50 to 70 and was identified as ethylenediamine by its purple ninhydrin reaction, its elution volume on the gel filtration column and by thin layer chromatography using alumina plates and solvent 6 (Appendix I) Rf = 0.16. All fractions from 31 to 100 excluding those containing ethylene diamine were combined and freeze-dried. Flow-through spectrophotometer cells monitoring at 280nm and 254nm did not show significant peaks on the elution profile, Figure 4.6.

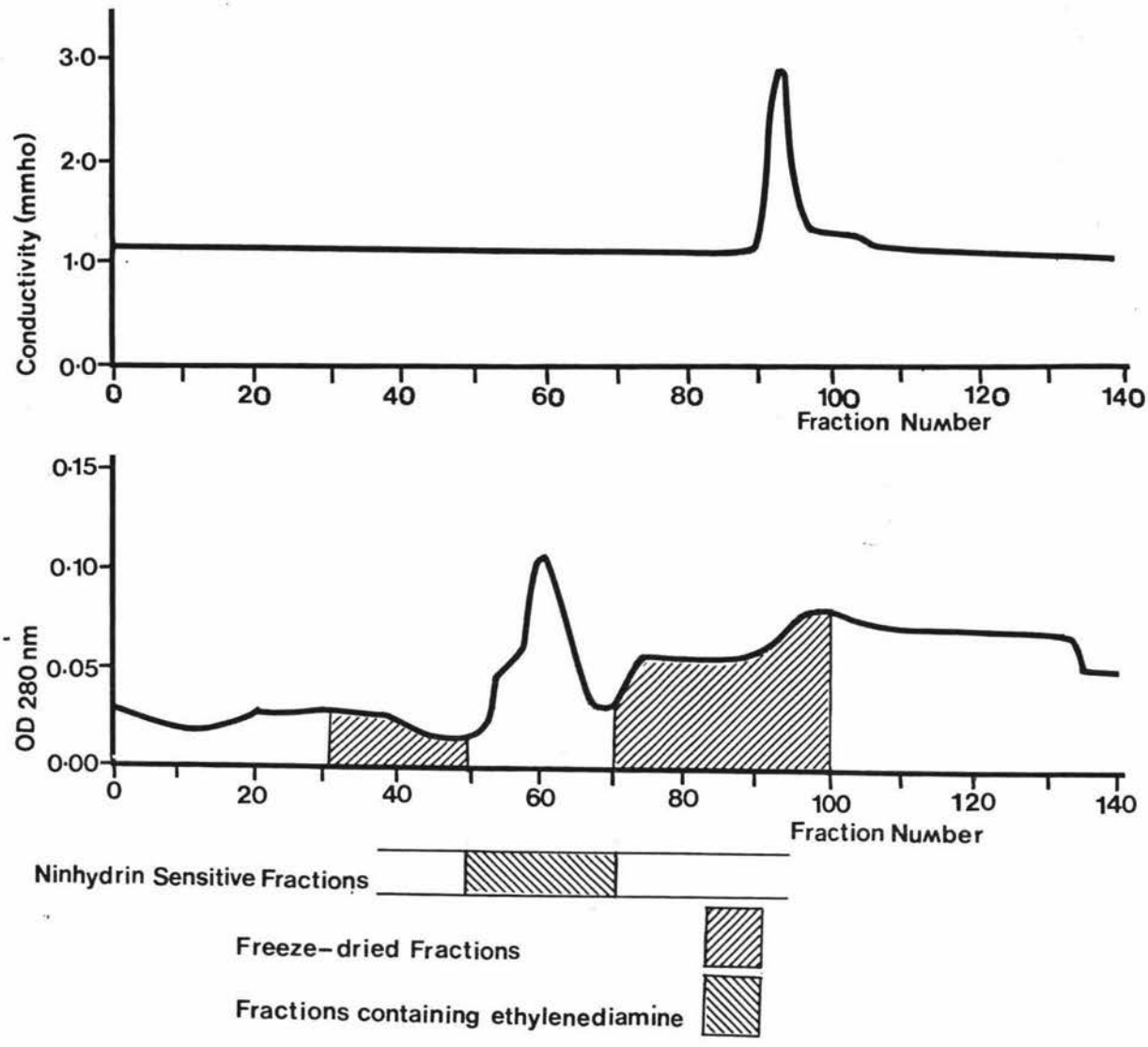



Figure 4.6. Gel Filtration of Reduced $[Co(en)_2Ala\text{-}Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$.

The freeze-dried material contained a compound which reacted with ninhydrin to give a yellow spot on a thin layer chromatography plate. An amino acid analysis of this material shows the following amino acid composition: alanine 1.04, glycine 1.00 and phenylalanine 1.96. From the amount of each amino acid present it was possible to calculate the percentage of the freeze-dried material which is the peptide

$^{+}H_2$-Ala-Gly-Phe-Phe-OBzl,$CH_3COO^-$, i.e. 36%. The peptide was characterised by TLC as in Table 4.3. An impurity identified by TLC as a visible pink compound, probably the reduced cobalt species in the salt peak, was included in the freeze-dried fraction.

| Solvent | $^{+}H_2$-Ala-Gly-Phe-Phe-OBzl, $CH_3COO^-$ | $^{+}H_2$-Gly-Phe-Phe-OBzl, $CH_3COO^-$ |
|---|---|---|
| Solvent 3 | 0.22 | 0.36 |
| Solvent 5 | 0.84 | 0.87 |
| Methanol | 0.62 | 0.57 |
| Ethyl acetate | 0 | 0.04 |
| Acetone | 0.55 | 0.84 |
| Chloroform | 0 | 0.06 |

Table 4.3. Rf's of Ala-Gly-Phe-Phe-OBzl and Standard 5 on Silica Thin Layer Plates.

Ala-Gly-Phe-Phe-OBzl reacted with ninhydrin on the thin layer to form a yellow compound which turns orange after standing for 15 minutes at room temperature and Gly-Phe-Phe-OBzl forms a stable yellow compound under the same conditions. Yield of freeze-dried material = 7.2mg but this includes much salt, the entire salt peak in fact. Yield of $^{+}H_2$-Ala-Gly-Phe-Phe-OBzl,$CH_3COO^-$ based on the amino acid analysis is 2.6mg,4.36 x $10^{-6}$mol, i.e. 31%.

Discussion.

This successful preparation marks a complete cycle of peptide synthesis via cobalt(III) chelates of amino acid methyl esters. It shows unequivocally that the scheme used can be successfully applied to peptides longer than two residues in length.

The yield of $[Co(en)_2Ala-Gly-Phe-Phe-OBzl](CH_3COO)_3$ in this preparation is very good compared to that of $[Co(en)_2$ Gly-Phe-Phe-OBzl$](CH_3COO)_3$ in Section A. This can probably be attributed to the greater stability of the tetrapeptide complex to hydrolysis since no disintegration of the $[Co(en)_2$ Ala-Gly-Phe-Phe-OBzl$]^{3+}$ band was observed.

After gel filtration of the reduced complex fractions containing Ala-Gly-Phe-Phe-OBzl could not be identified by spotting on an alumina thin layer and reacting with ninhydrin. No sharp peaks on the 280nm elution profile were visible except one corresponding to ethylenediamine. It is most likely, therefore, that the product was distributed over a large number of fractions and its dilute form prevented a visible reaction with ninhydrin. The low yield for isolation of peptide in this preparation may be attributable partially to the loss of approximately one third of the product due to difficulties in exactly locating the fractions containing the peptide. A chromogenic ester instead of the benzyl ester would be of great assistance in visualising the peptide in situations like this.

The peptide was characterised by thin layer chromatography which showed an essentially homogeneous product containing some cobalt(II) species eluted in the salt peak. Amino acid analysis verifies that the correct ratios of alanine, glycine and phenylalanine are present. Upon reaction with ninhydrin the peptide initially forms a yellow compound which is qualitative evidence of the presence of the ester. Phe-Phe-OBzl, Gly-Phe-Phe-OBzl, Gly-Gly-OEt, Ala-Gly-Gly-OEt and Leu-Ala-Gly-Gly-OEt all initially stain yellow when reacted with ninhydrin on thin layers. The benzyl ester survives the conditions of synthesis and purification in Section A and therefore should survive these same conditions in this preparation.

## SECTION C: PREPARATION OF Ala-Gly-Gly-OEt.

Materials.

$^{+}H_2$-Gly-Gly-OEt,$Cl^{-}$ was purchased from Sigma. The methanol used was A.R. grade, distilled twice under anhydrous conditions.

The ion exchange of the Ala-Gly-Gly-OEt and ethylene-diamine mixture was done on an SP-C25-120mesh Sephadex, pyridinium form, 8 x 1cm column with a linear 0.05M to 0.20M pyridinium acetate gradient..

Preparation of (Ethyl alanylglycylglycinate)bis(ethylene-diamine)cobalt(III) Acetate.

$[Co(en)_2Ala-Gly-Gly-OEt](CH_3COO)_3$.

Method.

$^{+}H_2$-Gly-Gly-OEt,$Cl^{-}$ (0.20g,$1.01 \times 10^{-3}$mol) was dissolved in methanol ($1.0cm^3$) and triethylamine (0.101g,$1.00 \times 10^{-3}$mol). $[Co(en)_2Ala-OMe](CF_3SO_3)_3$ (1.46g,$2.00 \times 10^{-3}$mol) was added and the solution stirred under anhydrous conditions at a pH of 8.6. After 10 minutes the reaction was quenched with acetic acid ($0.5cm^3$) and the solution made up to $200cm^3$ with water. A small portion of this solution ($10cm^3$) was sorbed onto an analytical cation exchange column and eluted with 0.2M pyridinium acetate. When the two bands were well separated the eluant was changed to 1.0M pyridinium acetate and the second band eluted was collected in 1litre of the eluant. This band was evaporated to dryness under reduced pressure at 40°C, then dissolved in water and evaporated to dryness

three times under reduced pressure at 40°C. The product was not weighed at this stage.

Results.

Two bands of approximately equal intensity were found on the analytical cation exchange column, Figure 4.7. The first species eluted was identified as the orange-red $[Co(en)_2Ala]^{2+}$ ion by its colour and mobility on the column compared to the mobility of an authentic sample of $[Co(en)_2Ala]I_2$. The second species eluted was identified as the $[Co(en)_2Ala\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ ion by its colour, its rapid reduction with zinc amalgam and the consequent analysis of its ligands by thin layer chromatography, amino acid analysis and mass spectroscopy.

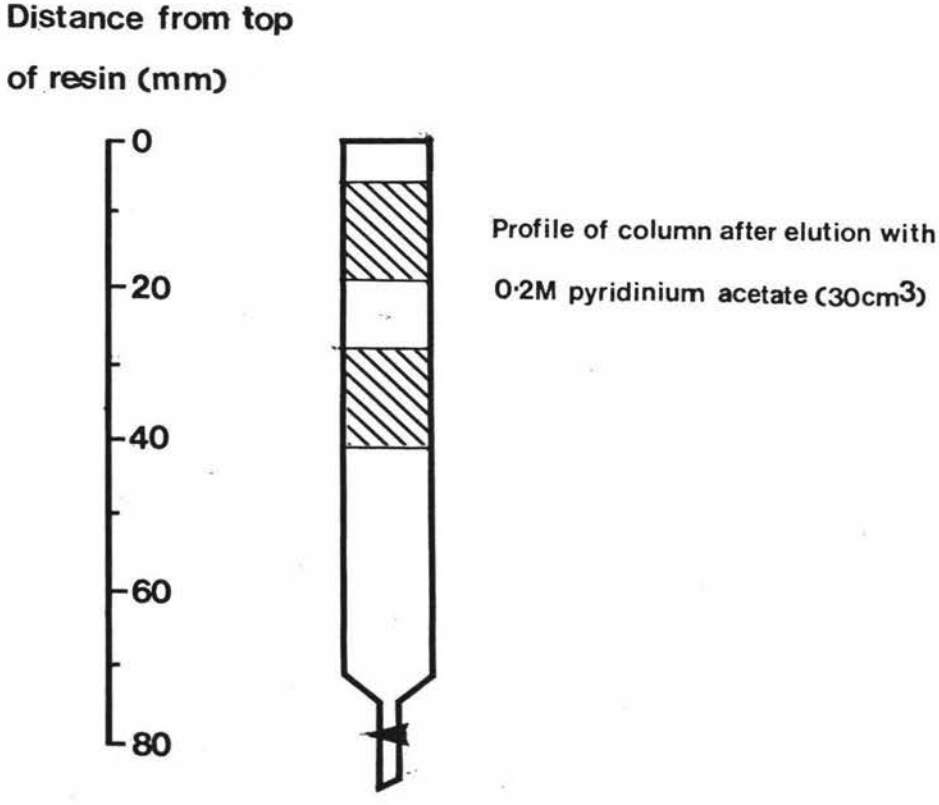



Figure 4.7. The profile of the ion exchange column showing the reaction products of the $[Co(en)_2Ala\text{-}OMe]^{3+}$ reaction with Gly-Gly-OEt.

Reduction of $[Co(en)_2Ala-Gly-Gly-OEt](CH_3COO)_3$ and the Isolation of the Free Peptide.

Method.

All of the dried $[Co(en)_2Ala-Gly-Gly-OEt](CH_3COO)_3$ was dissolved in water ($5cm^3$) and the pH of the solution adjusted to 1.0 with concentrated hydrochloric acid (10 drops). 2% zinc amalgam ($10cm^3$) was added and the mixture stirred. After 5 minutes the pH of the solution was 5.3 and the intensity of the orange colour of the solution had ceased to decrease. The addition of concentrated hydrochloric acid (2 drops) decreased the pH of the solution to 4.9 and the reduction was complete in 15 seconds. The reduced solution ($7cm^3$) was loaded onto the gel filtration column and eluted with 2N acetic acid. The fractions containing the tripeptide ester were pooled and freeze-dried. There was a major contaminant of ethylenediamine in the product which could not be removed by a further gel filtration step in the same system. The fractions containing the tripeptide ester were evaporated to a viscous oil under reduced pressure at 40°C and dissolved in water ($50cm^3$). This solution (pH 5, conductivity = 5 mmoh) was sorbed onto a cation exchange column and eluted with 0.05M pyridinium acetate ($50cm^3$) followed by a linear 0.05M to 0.20M pyridinium acetate gradient. Fractions of $7.2cm^3$ were collected from the column. The fractions containing tripeptide ester were pooled and freeze-dried. Yield of $^+H_2$-Ala-Gly-Gly-OEt,$CH_3COO^-$ (m.wt. = 291.3) = 104mg, $3.51 \times 10^{-4}$ mol. Yield, based on amino acid analysis results = $2.82 \times 10^{-4}$ mol. Therefore, the true yield based on the Gly-Gly-OEt reactant and allowing for the analytical column = 30%.

Results.

The two ninhydrin sensitive products, ethylenediamine and Ala-Gly-Gly-OEt could not be separated by gel filtration, Figure 4.8. These two products were separated by cation exchange chromatography (Figure 4.9).

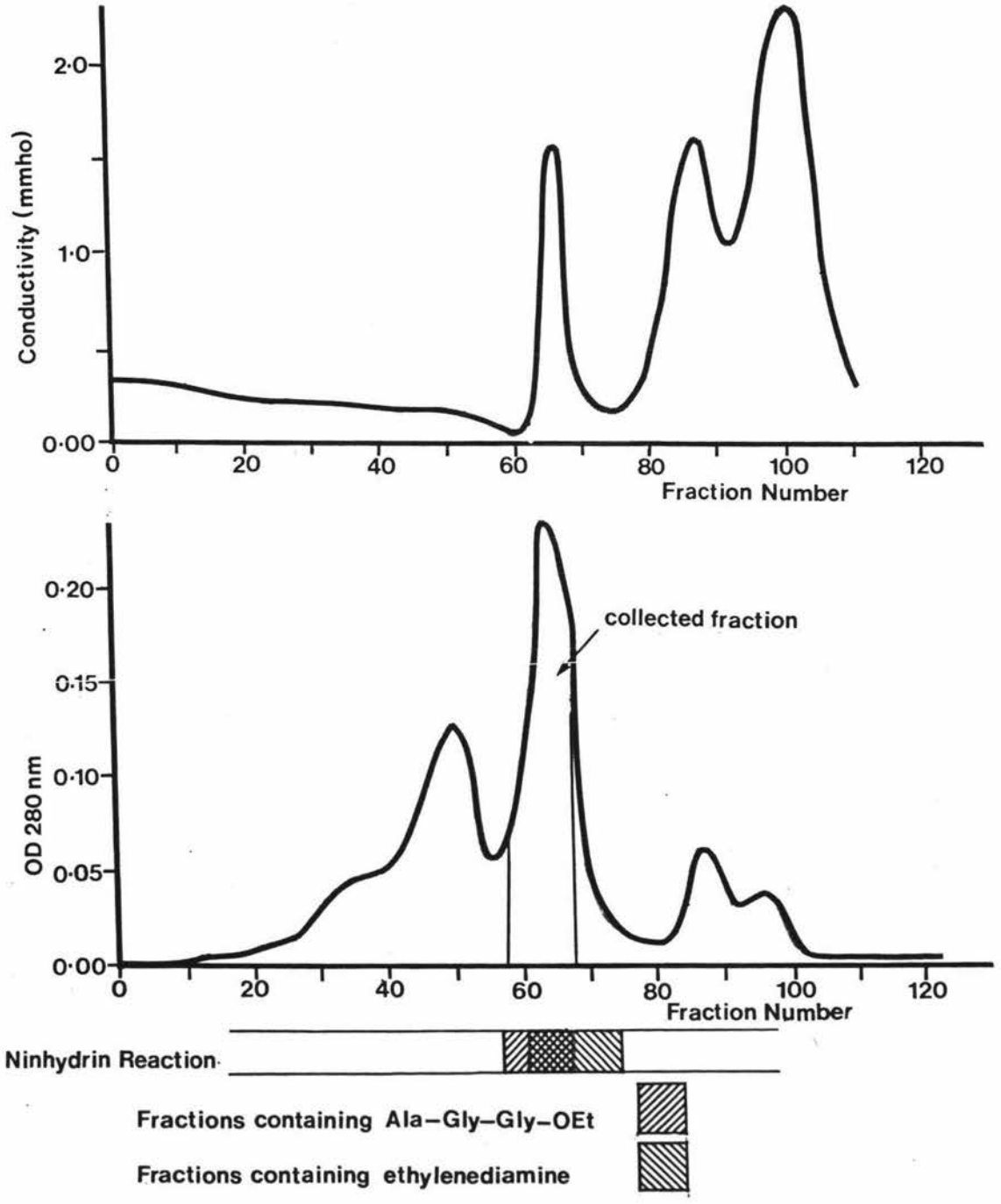



Figure 4.8. Gel Filtration of Reduced $Co(en)_2Ala\text{-}Gly\text{-}Gly\text{-}OEt^{3+}$.

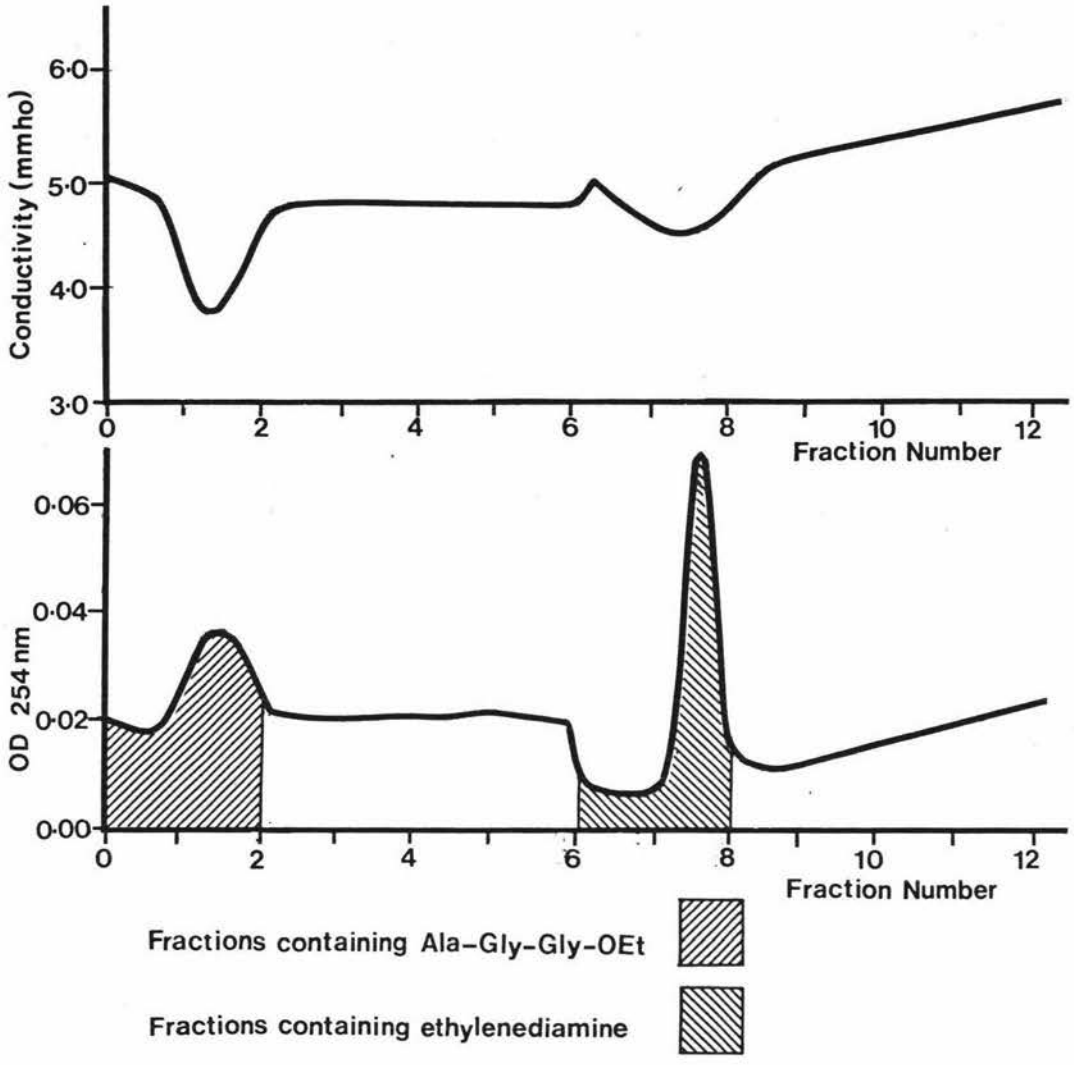



Figure 4.9. Separation of Ala-Gly-Gly-OEt from Ethylenediamine by cation exchange chromatography.

The fractions containing the tripeptide ester were pooled and freeze-dried. An amino acid analysis of an hydrolysate of the peptide revealed the amino acid ratio as: alanine 1.09, glycine 2.00. The amino acid analysis revealed that only 79% by weight of the freeze-dried material was $^{+}H_2$-Ala-Gly-Gly-OEt, $CH_3COO^{-}$. Characterisation of the tripeptide ester by thin

layer chromatography (Table 4.4) showed it to react with ninhydrin to form an orange-yellow spot which turned an orange-purple colour on further heating. $^{+}H_2$-Gly-Gly-OEt,Cl and L-alanine develop as yellow and purple spots respectively when sprayed with ninhydrin solution on the silica thin layers and heated to 120°C.

| Solvent | $^{+}H_2$-Ala-Gly-Gly-OEt, $CH_3COO^{-}$ | $^{+}H_2$-Gly-Gly-OEt, $Cl^{-}$ | Alanine |
|---|---|---|---|
| Solvent 2 | 0.60 | 0.59 | 0.13 |
| Solvent 4 | 0.14 | 0.19 | 0 |
| Solvent 5 | 0.54 | 0.50 | 0.10 |
| Solvent 6 | 0.70 | 0.67 | 0.16 |
| Methanol | 0.46 | 0.43 | 0.13 |
| Ethanol | 0.06 | 0.02 | 0 |

Table 4.4. Rf's of Ala-Gly-Gly-OEt on Silica Thin Layers.

Traces of two impurities in the freeze-dried material were observed by thin layer chromatography. One impurity co-chromatographed with and gave a similar ninhydrin reaction to alanine. The other developed as a mauve spot when reacted with ninhydrin and had Rf's of 0.13 and 0.24 in Solvent 4 and methanol respectively.

Mass spectroscopy of the $^{+}H_2$-Ala-Gly-Gly-OEt,$CH_3COO^{-}$ reveals an intense peak for the molecular ion $[Ala\text{-}Gly\text{-}Gly\text{-}OEt]^{+\cdot}$, and many of the expected fragment ions, Figure 4.10.

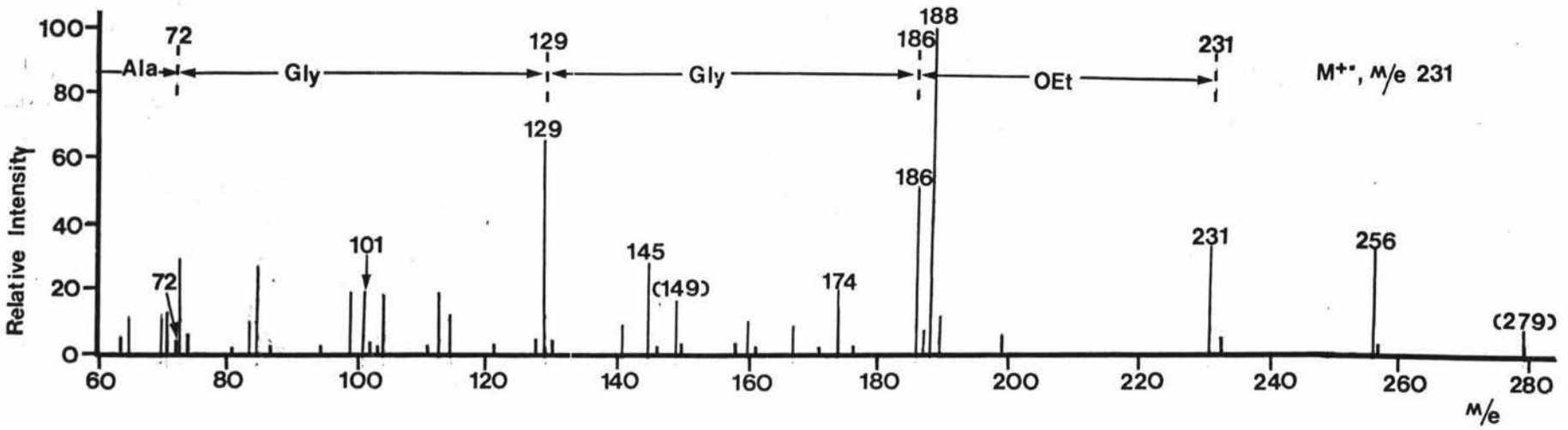



Figure 4.10. Mass Spectrum of Synthetic Ala-Gly-Gly-OEt.

High Resolution Data:

| | | | |
|---|---|---|---|
| m/e 231 = $C_9H_{17}N_3O_4$ | which is | $[NH_2CH(CH_3)CO\text{-}NHCH_2CO\text{-}NHCH_2CO\text{-}OCH_2CH_3]^{+\cdot}$ |
| m/e 188 = $C_8H_{16}N_2O_3$ | which is | $[\overline{NHCH_2(CH_3)}\quad\overline{CH_2}CO\text{-}NHCH_2CO\text{-}OCH_2CH_3]^{+\cdot}$ |
| m/e 186 = $C_7H_{12}N_3O_3$ | which is | $NH_2CH(CH_3)CO\text{-}NHCH_2CO\text{-}NHCH_2C{\equiv}O^{+}$ |
| m/e 145 = $C_6H_9N_2O_2$ | which is | $[CH_3CO\text{-}NHCH_2CO\text{-}OCH_2CH_3]^{+\cdot}$ |
| m/e 129 = $C_5H_9N_2O_2$ | which is | $NH_2CH(CH_3)CO\text{-}NHCH_2C{\equiv}O^{+}$ |
| m/e 101 = $C_4H_9N_2O$ | which is | $NH_2CH(CH_3)CO\text{-}\overset{+}{N}H{=}CH_2$ |
| m/e 72 = $C_3H_6NO$ | which is | $NH_2CH(CH_3)C{\equiv}O^{+}$ |

HPLC analysis of a 0.1% phosphoric acid solution of the freeze-dried peptide shows the peptide is eluted after 3.8 minutes, Figure 4.11A. The impurities eluted at 2.4 and 5.8 minutes are related to the acetic acid content of the sample, c.f. Figure 4.11B.

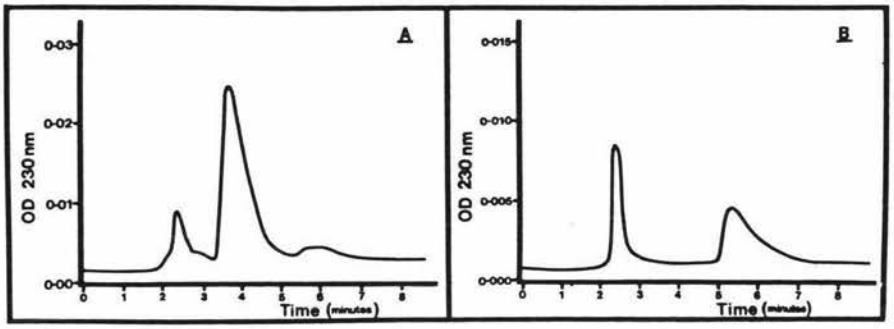



Figure 4.11. The analysis of Ala-Gly-Gly-OEt product by HPLC. In A 44μg of the tripeptide was injected and in B 50μg of acetic acid was injected. Both A and B were analysed with 0.1% phosphoric acid at 230nm.

A 0-75% acetonitrile gradient run after the elution of the peaks in Figure 4.11A confirms that no impurities with longer retention times are present.

Discussion.

The preparation of the $[Co(en)_2Ala\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ species proceeded in good yield since the $[Co(en)_2Ala]^{2+}$ and $[Co(en)_2Ala\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ bands appear to have equal intensity on the analytical column and $[Co(en)_2Ala\text{-}OMe]^{3+}$ was used in a twofold excess. The separation of these species was straight forward with no other bands being present.

Reduction of the isolated peptide complex proceeded rapidly in acid solution. The unco-ordinated peptide and ethylenediamine were difficult to separate because they co-chromatographed on the gel filtration column. This separation was easily achieved by ion exchange chromatography. The overall yield of peptide was 30% but the use of four column purification steps has caused some losses. Ion exchange chromatography may be a superior technique for isolating small peptides, e.g. Ala-Gly-Gly-OEt, from ethylenediamine.

The amino acid analysis of the hydrolysed peptide indicates the correct ratio of amino acids; $alanine_1$, $glycine_2$ and indicates that 79% of the freeze-dried material is $^{+}H_2\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt, CH_3COO^{-}$.

Thin layer chromatography of the peptide material establishes that the peptide is essentially pure with only traces of two ninhydrin reactive compounds present.

The mass spectrum of the peptide clearly establishes the structure as being Ala-Gly-Gly-OEt. The fragment ions at m/e72, m/e129 and m/e186 are identified by high resolution data as the N terminal peptide fragments, alanine, Ala-Gly and Ala-Gly-Gly respectively. Typically mass spectra of underivatised peptides

are complex and difficult to analyse*. Low vapour pressures of the underivatised peptides usually mean low intensities of the high m/e fragment peaks and the underivatised groups of the peptides lead to rearrangements (54). One such rearrangement observed in the mass spectrum of Ala-Gly-Gly-OEt is the transition m/e 231 — m/e 188 which is confirmed by a metastable peak. The rearrangement consists the loss of CHNO from the middle of the peptide and produces the largest peak in the spectrum. The mass spectrum of the protected peptide Acetyl-D,L-Ala-Gly-Gly-OEt does not show a similar loss of CHNO from the molecular ion, i.e. an M-43 peak (55). It is likely, therefore, that the free amino group in Ala-Gly-Gly-OEt is involved in a cyclic transition state resulting in the non terminal loss of CHNO and that this process is unfavourable in the N-acetylated derivative. It seems likely that a similar rearrangement occurs in the formation of the m/e 174 fragment. High resolution data indicates this fragment is due to the loss of $CH_2$-CO-NH from the middle of the peptide. The peaks at m/e 279 and m/e 149 were identified by high resolution data as fragment ions of a phthalate plasticiser commonly found in organic solvents. The intense peak at m/e 256 was identified by high resolution data as $C_{11}H_{18}N_3O_4$ but could not be related to other peaks in the spectrum.

* Permethylated peptides and $LiAlH_4$ reduced peptides produce less complex mass spectra.

HPLC analysis of the freeze-dried peptide confirms the purity of the peptide established by thin layer chromatography. The elution profile shows a mixture of the peptide and acetic acid with a shoulder on the 2.4 minute peak indicating the elution of salts with the breakthrough volume. The elution of no further peaks with a 0-75% acetonitrile gradient indicates that the peptide is free from non-polar contaminants.

SECTION D: PREPARATION OF Leu-Ala-Gly-Gly-OEt.

Materials.

$^{+}H_2$-Ala-Gly-Gly-OEt,$CH_3COO^-$ , prepared in Section D, was used without further purification.

The dimethylsulphoxide was A.R. grade used without further purification. An SP-C25-120 mesh Sephadex column, 18cm x 1cm, pyridinium form, was used to separate the cobalt complexes.

Preparation of (Ethyl leucylalanylglycylglycinate)bis(ethylenediamine)cobalt(III) Acetate.

$[Co(en)_2Leu\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt](CH_3COO)_3$.

Method.

$^{+}H_2$-Ala-Gly-Gly-OEt,$CH_3COO^-$ (26.0mg, allowing for salts in peptide = $7.05 \times 10^{-5}$mol) was dissolved in dimethylsulphoxide ($0.33cm^3$) to which had been added enough triethylamine (9.5mg) to just neutralise the N terminus of the tripeptide. $[Co(en)_2$ Leu-OMe$](CF_3SO_3)_3$ (0.15g, $1.95 \times 10^{-4}$mol) was added and the solution stirred under anhydrous conditions. After 10 minutes the solution was tested by thin layer chromatography (Silica/ Methanol) for unreacted Ala-Gly-Gly-OEt. An estimated 5µl of reaction mixture was spotted on the thin layer for each test. A large amount of this peptide remained in the solution. $[Co(en)_2Leu\text{-}OMe](CF_3SO_3)_3$ (0.30g, $3.90 \times 10^{-4}$mol) was added and after 10 minutes the solution was tested for unreacted Ala-Gly-Gly-OEt. A large amount of the free peptide was still detected. The reaction was left for 10 hours after which time very little Ala-Gly-Gly-OEt was detectable by thin layer chromatography.

The reaction was quenched with acetic acid ($0.3cm^3$) and the solution diluted to $50cm^3$ with water. The reaction products were sorbed onto the first 5cm of an 18cm x 1cm cation exchange column. The first band was eluted with 0.2M pyridinium acetate and then the eluant was changed to 1.0M pyridinium acetate which rapidly eluted the second band. This product was collected and freeze-dried.

Results.

Only two bands were found on the cation exchange column and both were orange in colour. The first band eluted was the $[Co(en)_2Leu]^{2+}$ species identified by its retention volume on the column and the second band eluted was the $[Co(en)_2Leu\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ species identified by analysis of its ligands. Yield of $[Co(en)_2Leu\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt\ ]\ (CH_3COO)_3$ = 33mg, $4.71 \times 10^{-5}$mol. Allowing for reaction mixture used for thin layer chromatography, yield = 70%.

Reduction of $[Co(en)_2Leu\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt](CH_3COO)_3$ and the Isolation of the Free Peptide.

Method.

The freeze-dried $[Co(en)_2Leu\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt](CH_3COO)_3$ (33mg, $4.71 \times 10^{-5}$mol) was dissolved in water ($2cm^3$) and the pH of the solution adjusted to 0.8 with concentrated hydrochloric acid (7 drops). 2% zinc amalgam was added and the mixture stirred rapidly. The reduction was complete in 10 minutes with no increase in pH. The solution of reduced complex,

($4cm^3$) was decanted from the amalgam and loaded onto the gel filtration column and eluted with 2N acetic acid. Fractions of $3.6cm^3$ were collected and every third fraction was tested by thin layer chromatography for ninhydrin reactive compounds. Approximately 10µl of the fraction was applied to a spot on an alumina thin layer and dried. Then the thin layers were developed in Solvent 6, dried, sprayed with ninhydrin solution and heated at 120°C to develop colour. Fractions 30 to 55 inclusive were freeze-dried and weighed.

Results.

The only ninhydrin reactive compound found in the gel filtration fractions was ethylenediamine which gave a purple spot when reacted with ninhydrin with Rf = 0.16. The freeze-dried fractions contained a ninhydrin reactive compound identified as Leu-Ala-Gly-Gly-OEt, Figure 4.12. Yield of $^{+}H_2$-Leu-Ala-Gly-Gly-OEt,$CH_3COO^{-}$ (m.wt. = 404.5) = 6.4mg. An amino acid analysis revealed that only 70% of the freeze-dried material was the tetrapeptide, therefore, actual yield = 4.4mg = $1.1 \times 10^{-5}$mol, i.e. 24% based on the mass of $[Co(en)_2$ Leu-Ala-Gly-Gly-OEt$](CH_3COO)_3$.

Amino acid analysis of an hydrolysate of the peptide confirmed the amino acid composition of the peptide as: leucine 1.05, alanine 0.95, glycine 2.00.

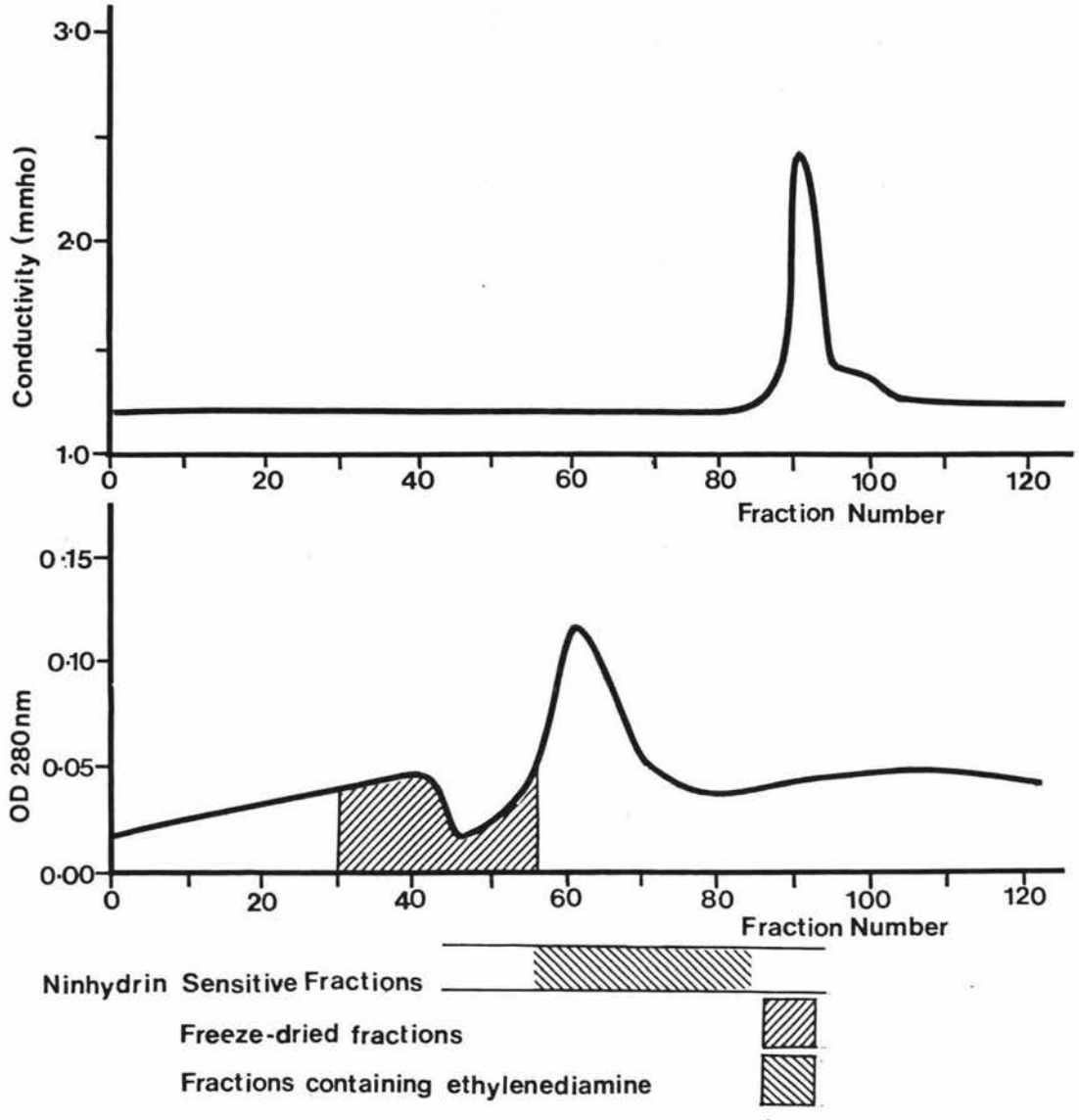



Figure 4.12. The Gel Filtration of Reduced $[Co(en)_2Leu\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt]\,(CH_3COO^-)_3$.

TLC of the product indicates there are some slight impurities in the freeze-dried fraction which are reactive to ninhydrin. One of these is ethylenediamine (a very small amount) and the other reacts with ninhydrin to form a yellow compound. The Rf of this impurity in the silica/solvent 5 system is 0.35. The Rf's of the main product in various solvents on silica thin layers are shown in Table 4.5.

| Solvent | $^{+}H_2$-Leu-Ala-Gly-Gly-OEt, $CH_3COO^-$ | $^{+}H_2$-Ala-Gly-Gly-OEt, $CH_3COO^-$ | Leucine |
|---|---|---|---|
| Solvent 5 | 0.78 | 0.65 | 0.56 |
| Methanol | 0.69 | 0.46 | 0.51 |
| Ethanol | 0.48 | 0.23 | 0.05 |
| Acetone | 0.58 | 0.37 | 0 |
| Ethyl acetate | 0 | 0 | 0 |
| Chloroform | 0 | 0 | 0 |

Table 4.5. Rf's of Leu-Ala-Gly-Gly-OEt on Silica Thin Layers.

Leu-Ala-Gly-Gly-OEt reacts with the sprayed ninhydrin solution at 120°C to form a yellow compound which turns orange after standing at room temperature for 15 minutes. Ala-Gly-Gly-OEt develops as a yellow spot when reacted with ninhydrin but takes longer (15 minutes) to turn orange on standing at room temperature. Leucine develops as a purple spot when reacted with ninhydrin. Analysis of the freeze-dried product by mass spectroscopy reveals the molecular ion at m/e 344. The high resolution data of the fragment ions is given in Table 4.6 and the mass spectrum is shown in Figure 4.13.

| m/e | Molecular Formula | Structure of Fragment Ion |
|---|---|---|
| 344 | $C_{15}H_{28}N_4O_5$ | $[NH_2CH(C_4H_9)CO-NHCH(CH_3)CO-NHCH_2CO-NHCH_2CO-OCH_2CH_3]^{+\cdot}$ |
| 301 | $C_{14}H_{27}N_3O_4$ | $M^{+\cdot}$ minus CO–NH |
| 299 | $C_{13}H_{23}N_4O_4$ | $NH_2CH(C_4H_9)CO-NHCH(CH_3)CO-NHCH_2CO-NHCH_2C{\equiv}O^+$ |
| 273 | $C_{12}H_{23}N_3O_4$ | $M^{+\cdot}$ minus $CH(CH_3)CO-NH$ |
| 242 | $C_{11}H_{20}N_3O_3$ | $NH_2CH(C_4H_9)CO-NHCH(CH_3)CO-NHCH_2C{\equiv}O^+$ |
| 216 | $C_9H_{16}N_2O_4$ | $[CH_2(CH_3)CO-NHCH_2CO-NHCH_2CO-OCH_2CH_3]^{+\cdot}$ |
| 185 | $C_9H_{17}N_2O_2$ | $NH_2CH(C_4H_9)CO-NHCH(CH_3)C{\equiv}O^+$ |
| 157 | $C_8H_{17}N_2O$ | $NH_2CH(C_4H_9)CO-\overset{+}{N}H=CH(CH_3)$ |
| 86 | $C_5H_{12}N$ | $\overset{+}{N}H_2=CH(C_4H_9)$ |

Metastable Transitions: m/e 344→m/e 301→m/e 273

Table 4.6. High Resolution Data from Mass Spectrum of Leu-Ala-Gly-Gly-OEt.

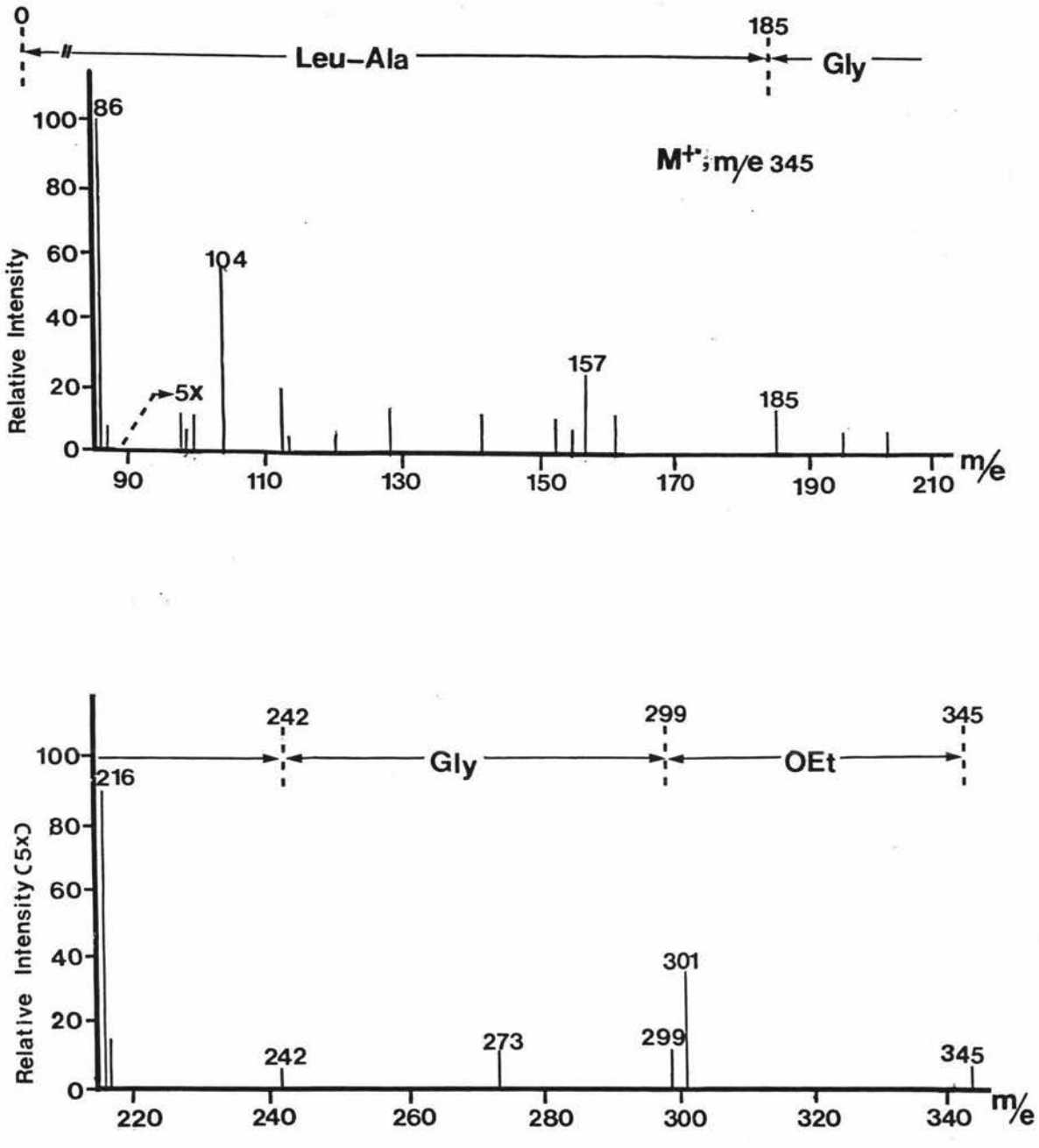



Figure 4.13. Mass Spectrum of Leu-Ala-Gly-Gly-OEt (note that peaks with m/e greater than 90 are shown at five times their actual intensity).

Analysis of the peptide by HPLC shows the peptide is eluted as two peaks at 4.3 and 5.4 minutes. Salts and acetic acid are eluted immediately after the breakthrough volume at 2.1 and 2.4 minutes respectively, Figure 4.14.

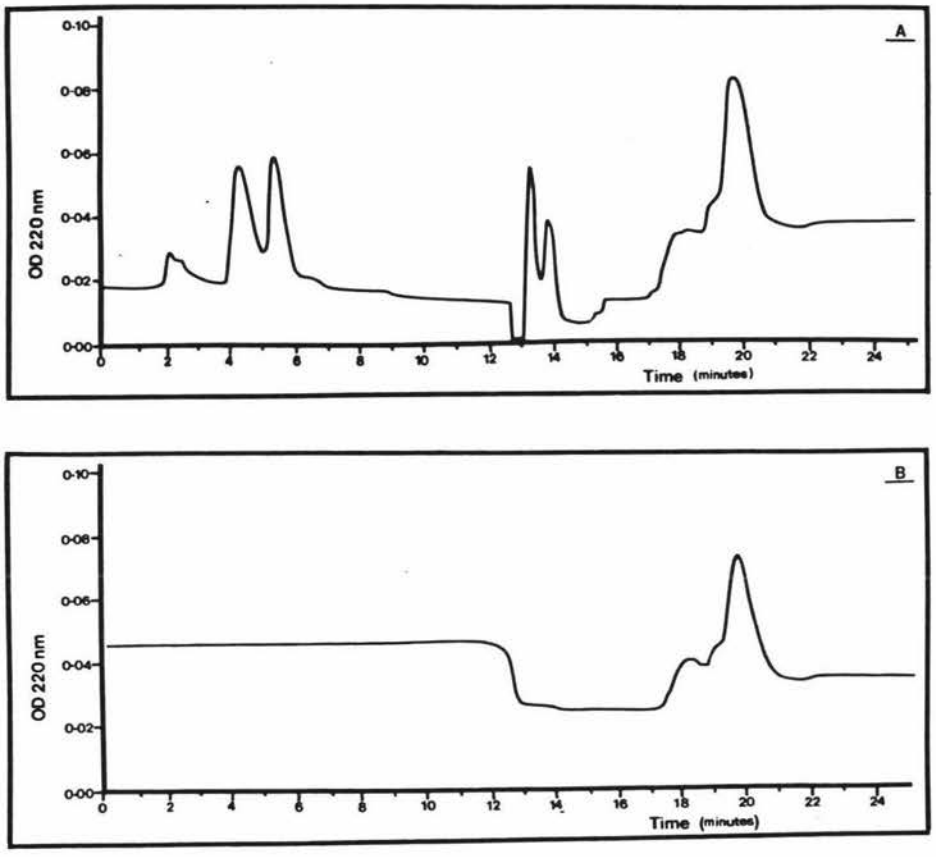



Figure 4.14. HPLC analysis of synthetic Leu-Ala-Gly-Gly-OEt. In A, 50μg of Leu-Ala-Gly-Gly-OEt was analysed at 220nm by elution with 0.1% phosphoric acid for 8 minutes followed by a 10 minute linear 0–75% acetonitrile gradient in 0.1% phosphoric acid. B shows a 10 minute linear 0–75% acetonitrile gradient in 0.1% phosphoric acid starting at 8 minutes.

Discussion.

The reaction of $[Co(en)_2Leu\text{-}OMe]^{3+}$ with Ala-Gly-Gly-OEt to form the tetrapeptide complex $[Co(en)_2Leu\text{-}Ala\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ proceeds very slowly. The pH of the reaction mixture was not checked and, therefore, it is possible that the solution was

acid enough to reform the salt of the N terminal of the tripeptide reactant, thus removing this nucleophile from the reaction. This is unlikely due to the similarity of the reaction conditions to other syntheses which have occurred rapidly.

It has been observed, that for the formation of peptides via Co(III) chelated amino acid esters, the presence of bulky side chains in chelated amino acid ester has a greater effect on the rate of reaction than does the presence of the same side chains in the nucleophilic amino acid or peptide. For example, $[Co(en)_2Ala\text{–}OMe]^{3+}$ is approximately 700 times slower at reacting with Gly–OEt than is $[Co(en)_2$ Gly–OMe$]^{3+}$, whereas Val–OEt is only approximately 10 times slower than Ala–OEt in reaction with $[Co(en)_2Gly\text{–}OMe]^{3+}$, (56). Dekker reports difficulties experienced in the preparation of $[Co(en)_2Leu\text{–}Val]^{2+}$ and $[Co(en)_2Leu\text{–}Ala]^{2+}$ resulting in yields of approximately 50%. It seems likely, therefore, that the slow reaction rate is caused by steric effects of the $[Co(en)_2$ Leu–OMe$]^{3+}$ moiety.

Although the reaction was carried out over 10 hours only the $2^+$ and $3^+$ species were observed on the ion exchange column. Thus no obvious side-reactions had occurred. The yield was good, 70%, of the Ala–Gly–Gly–OEt being coupled.

The reduction of the complex proceeded rapidly under the conditions used and the released ligands and Co(II) species were separated by gel filtration. Note that the increase in peptide length resulted in a more effective separation of

ethylenediamine from the peptide (c.f. Section C).

Isolation of the peptide following the gel filtration column was a problem due to the peptide's dilute form and the low optical density of the product on reaction with ninhydrin. Substantial loss of peptide may have occurred at this stage due to not collecting fractions below 30 in number. No fractions above 55 contained compounds which reacted with ninhydrin to form yellow compounds.

The amino acid analysis of an hydrolysate of the peptide confirms the amino acid content as $Leu_1$, $Ala_1$, $Gly_2$ and establishes that 70% of the freeze-dried material is $^{+}H_2$-Leu-Ala-Gly-Gly-OEt,$CH_3COO^{-}$.

Thin layer chromatography of the prepared peptide shows only two slight impurities. Ethylenediamine is present because the freeze-dried fractions were pooled too close to the ethylenediamine containing fractions. Ethylenediamine gives a very strong purple reaction with ninhydrin and therefore small quantities of the substance are visible. The other impurity reacts with ninhydrin to form a yellow compound. This is not Ala-Gly-Gly-OEt since the compounds have different Rf's. The possibility of the compound being the diastereoisomer D-Leu-Ala-Gly-Gly-OEt should not be overlooked.

The mass spectrum of Leu-Ala-Gly-Gly-OEt firmly establishes the structure of the peptide. The spectrum is more complicated than that of Ala-Gly-Gly-OEt and this may be attributed, in part, to the low intensity of high m/e peaks due to the low vapour pressure of the underivatised sample (54). Similar rearrangements occur in this spectrum as occurred in the spectrum of

Ala-Gly-Gly-OEt, namely, the loss of CHNO from the middle of the molecule. The sequential fragmentation of amino acid units from the molecular ion is pointed out above, Figure 4.13. Another similarity of this spectrum to that of Ala-Gly-Gly-OEt is the appearance of a peak which is $M+C_2H$ or m/e 370 in this case. Either the compound responsible for this peak in the Ala-Gly-Gly-OEt spectrum has reacted to form another compound one leucine residue heavier or this peak is an artifact of decomposition due to heating of the sample in the mass spectrometer.

HPLC analysis of the peptide material is shown in Figure 4.14. Ala-Gly-Gly-OEt is eluted from the column under the same conditions after 3.8 minutes. The addition of a leucine residue to this peptide is expected to increase the retention time slightly due to the interaction of the $C_{18}$ column with the alkyl side chain on leucine. The free amino group will be charged in 0.1% phosphoric acid and this effect is expected to mediate the hydrophobic attraction. Two peaks were found with retention times slightly greater than that of Ala-Gly-Gly-OEt, at 4.3 and 5.4 minutes. Together, these peaks represent 60% of the absorbance of the sample at 220nm. This is consistent with the proportion of $^{+}H_2$-Leu-Ala-Gly-Gly-OEt,$CH_3COO^{-}$ identified in the sample by amino acid analysis, being 70% of the mass of the sample. Both peaks have the characteristic unsymmetrical shape exhibited by peptides analysed by reversed phase HPLC. It is most likely, therefore, that these two peaks are both produced by the Leu-Ala-Gly-Gly-OEt peptide. Under the conditions used for peptide synthesis via Co(III) chelated esters, racemisation of the chelated amino acid ester or chelated amino acid amide is not considered to be prohibitive to the technique (58). A certain degree

of racemisation has been shown to occur (59). It therefore follows that if the chelated amino acid is exposed to the basic coupling conditions for long periods (e.g. when the coupling reaction is slow), a certain proportion of the chelated amino acid will be racemised. For this reason the possibility that the two peaks in question are produced by the diasterioisomers D-Leu-Ala-Gly-Gly-OEt and Leu-Ala-Gly-Gly-OEt, cannot be overlooked. Two contaminants are eluted from the reversed phase column at 11.4 and 11.8 minutes when the acetonitrile concentration is approximately 30%. This indicates that these contaminants are considerably hydrophobic.

The successful preparation of Leu-Ala-Gly-Gly-OEt marks another full cycle of peptide synthesis and reaffirms that this scheme can be applied to the sequential synthesis of peptides larger than two residues in length.

SECTION E: ATTEMPTED PREPARATION OF Pro-Phe-Phe-OBzl

Attempted Preparation of $[Co(en)_2Pro\text{-}Phe\text{-}Phe\text{-}OBzl](CH_3COO)_3$

Pro-Phe-Phe- is the carboxy terminal of a linear decapeptide which can be easily cyclised to form antaminid, a naturally occurring peptide. Antaminid, which contains 4 proline residues, was expected to be a good model peptide for the synthesis method described in this chapter since it tests the repetitive yield of the synthesis scheme, with 9 successive couplings and also requires that problems associated with proline couplings be solved.

Materials.

$^{+}H_2$-Phe-Phe-OBzl,$CF_3COO^-$ was prepared by a standard solution phase reaction as recorded in Appendix I.

Dimethylsulphoxide (A.R. grade) was used without further purification.

Method.

H-Phe-Phe-OBzl,$CF_3COO^-$ (0.51g, $1 \times 10^{-3}$mol) was dissolved in methanol ($3cm^3$) and stirred under anhydrous conditions throughout the course of the reaction. Triethylamine ($0.14cm^3$, $1 \times 10^{-3}$mol) was added whereupon a white compound, unreactive to ninhydrin, precipitated. This precipitate was filtered off and the pH of the filtrate found to be 7.0. $[Co(en)_2Pro\text{-}OMe](CF_3SO_3)_3$ (1.5g, $2 \times 10^{-3}$mol), a twofold excess, was added to the solution and the pH of the reaction mixture checked and found to have pH 8.0. This was adjusted to pH 6.0 with acetic acid (several drops). After 30 minutes acetic acid ($0.5cm^3$) was added to quench the reaction and water ($30cm^3$) was added to dilute the products. An immediate precipitation of the unreacted Phe-Phe-OBzl followed the addition of water and this was filtered off and the filtrate

diluted to 500cm$^3$ with water. This clear red solution was sorbed onto the preparative cation exchange column in the first 2cm of resin. The reaction products were eluted with 0.1M pyridinium acetate. The $[Co(en)_2Pro]^{2+}$ band was eluted rapidly from the column in this eluant. The eluant was changed to 0.3M pyridinium acetate but the $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ band was eluted very slowly in this eluant. This species was, however, eluted rapidly with 1.0M pyridinium acetate. The remaining band on the column, unmoved by 1.0M pyridinium acetate was rapidly removed with 1M hydrochloric acid and was called the polymeric $[(Co(en)_2Pro)_n]^{m+}$ band. The $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ species was reduced in volume to 30cm$^3$ under reduced pressure then freeze-dried.

Results.

The approximate proportions of the reactions products are: $[Co(en)_2Pro]^{2+}$ 50%, $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_2]^{3+}$ 25% and Polymeric $[(Co(en)_2Pro)_n]^{m+}$ 25%. The$[Co(en)_2Pro\text{-}NHCH_2CH_2NH_2]$ was identified by reduction and consequent analysis of its ligands by TLC, mass spectroscopy and amino acid analysis. The $[Co(en)_2Pro]^{2+}$ was identified by its elution volume on the ion exchange column compared to a genuine sample of $[Co(en)_2Pro]I_2$.

The so named Polymeric $[(Co(en)_2Pro)_n]^{m+}$ did contain Co(III) and was easily reduced by the usual method. The colour of this complex was somewhat concentration dependent being red-orange when dilute with a max of approximately 480nm but becoming brown-red in colour when concentrated. The colour of the complex returned to red when the solution was diluted.

Reduction of $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_2]^{3+}$ and Isolation of $Pro\text{-}NHCH_2CH_2NH_2$.

Method.

The freeze-dried red/orange crystals of $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_2]^{3+}$ were dissolved in water ($12cm^3$) and concentrated hydrochloric acid was added to bring the pH to 1.8. 2% zinc amalgam ($5cm^3$) was added and the reaction stirred for two hours after which time the orange complex had been reduced to a light purple Co(II) species. The reduced complex was immediately loaded onto the gel filtration column and eluted with 2N acetic acid. Fractions of eluant ($3.6cm^3$) were collected and every fifth fraction was tested for ninhydrin reactivity by spotting approximately 3µl of the fraction on an alumina TLC plate, then developing the plate in solvent 6 and spraying it with a ninhydrin solution, followed by heating the plate.

Results.

Two ninhydrin reactive species were found in the eluant from the column. The first eluted species, being found in the fractions 50 to 80 inclusive, reacted with ninhydrin on the thin layer to form a brown compound with Rf=0.38. This species was identified as $Pro\text{-}NHCH_2CH_2NH_2$ by its mass spectrum. The second eluted species, fractions 85 to 95, was identified by TLC as ethylenediamine, as shown in Section B.

The $Pro\text{-}NHCH_2CH_2NH_2$ was unstable at room temperature. A methanol solution of this compound contained 5 ninhydrin reactive compounds after two days at room temperature. One of the breakdown products was identified as ethylenediamine by TLC.

Mass spectroscopy of the sample identified it as being Pro-$NHCH_2CH_2NH_2$ as shown in Table 4.7.

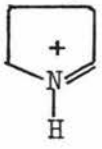
| Molecular Formula | Structural Formula | m/e |
|---|---|---|
| $C_7H_{15}ON_3$ | $[Pro\text{-}NHCH_2CH_2NH_2]^{+\cdot}$ | 157 |
| $C_6H_{11}O_1N_2$ | $[Pro\text{-}NHCH_3]^{+\cdot}$ | 128 |
| $C_4H_8N$ |  | 70 |

Table 4.7. High resolution data from major peaks of mass spectrum of Pro-$NHCH_2CH_2NH_2$.

Mass Spectroscopy also detected a large amount of triethylamine hydrochloride in the sample. Amino acid analysis of this compound gave a proline peak and no trace of any other amino acids. Yield of $\overset{+}{H}_2$-Pro-$NHCH_2CH_2\overset{+}{N}H_3,(CH_3COO^-)_2$ = 0.10g, i.e. 18% based on the total of $[Co(en)_2Pro\text{-}OMe](CF_3SO_3)_3$ added initally. From the amount of proline in the hydrolysate only 90% of the freeze dried product is $\overset{+}{H}_2$-Pro-$NH_2CH_2CH_2\overset{+}{N}H_3,(CH_3COO^-)_2$ and therefore yield = 0.090g, i.e. 16%, which agrees with the proportion of the complex $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ observed on the ion exchange column (approximately 25%), allowing for the usual losses associated with isolation (see previous sections).

Discussion.

An explanation for the formation of polymeric $[(Co(en)_2Pro)_n]^{m+}$ is offered in the discussion of Section F. A similar reaction to the one detailed here was carried out using dimethylsulphoxide as

a solvent instead of methanol and this reaction had as products only $[Co(en)_2Pro]^{2+}$ and $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_2]^{3+}$ species. The failure for a polymeric $[(Co(en)_2Pro)_n]^{m+}$ band to appear in this solvent may be due to the slower rate of aminolysis in dimethylsulphoxide compared with methanol (57), together with the different solvating properties of the two solvents. Another similar reaction to the one detailed here was carried out using methanol as solvent but using the free amine of Phe-Phe-OBzl produced by washing with 10% $Na_2CO_3$. The composition of products in this experiment was exactly the same as that of the original attempt.

There can be no question that the free amine of Phe-Phe-OBzl is being produced since a successful reaction was accomplished under the same conditions using $[Co(en)_2Gly\text{-}OMe]^{3+}$ to produce Gly-Phe-Phe-OBzl. Section F shows that under basic conditions the $[Co(en)_2Pro\text{-}OMe]^{3+}$ can give rise to the three bands seen on ion exchange in this section. Therefore, the failure of this reaction to produce Pro-Phe-Phe-OBzl must lie with a sterically hindered coupling which is very slow. Thus any ligands from reduced Co(III) species will effectively compete for the active cobalt comples $[Co(en)_2Pro\text{-}OMe]^{3+}$. Efforts to synthesise antaminid were thus halted at the tripeptide stage.

Triethylamine was identified in the $Pro\text{-}NHCH_2CH_2NH_2$ fraction despite ion exchange and gel filtration steps to purify the product. This demonstrates the high affinity of triethylamine for $Pro\text{-}NHCH_2CH_2NH_2$. It is unlikely that triethylamine undergoes a ligand exchange reaction with $[Co(en)_2Pro\text{-}OMe]^{3+}$ with one end of an ethylenediamine ligand. If this exchange did occur the amino

group of the ethylenediamine would then be free to attack the activated ester intramolecularly. This explanation is unlikely because no change in the proportions of visible reaction products is observed when $Na_2CO_3$ and not triethylamine is used to produce the free amine of Phe-Phe-OBzl.

## SECTION F: INVESTIGATION OF BASES TO FORM ETHYL GLYCYLGLYCINATE FROM ITS HYDROCHLORIDE SALT IN REACTION WITH $[Co(en)_2Pro-OMe](CF_3SO_3)_3$.

The use of a variety of bases to form the free amine of $^+H_2$-Gly-Gly-OEt,$Cl^-$ *in situ*, for reation with $[Co(en)_2Pro-OCH_3](CF_3SO_3)_3$ to form the complex $[Co(en)_2Pro-Gly-Gly-OEt]^{3+}$ was investigated.

Materials.

Dimethylsulphoxide (A.R. grade) was used without further purification. Pyridine (A.R. grade) was used without further purification. Methanol (A.R. grade) was distilled twice and stored in a sealed bottle. Cation Exchange Resin: SP-C25-12-Sephadex ($Cl^-$ form) was used in 9cm x 1cm columns.

Method.

All trials were performed on a small scale (approximately $1 \times 10^{-5}$ mol of $[Co(en)_2Pro-OCH_3](CF_3SO_3)_3$). The $^+H_2$-Gly-Gly-OEt, $Cl^-$ was suspended in a small amount of solvent (usually $0.5cm^3$) and the base under trial added to the mixture. In the trials with LiOH and NaOH, these solutions were freeze-dried to remove

the water produced then redissolved in the required solvent. In all cases enough base was added to just neutralise the hydrochloride salt which was always in excess with respect to $[Co(en)_2Pro\text{-}OCH_3](CF_3SO_3)_3$. The $[Co(en)_2Pro\text{-}OCH_3](CF_3SO_3)_3$ was then added and the reaction stirred for the stated time. Water ($10cm^3$) was then added and the resulting solution sorbed onto a cation exchange column. Each column was then eluted with 0.2M $NaClO_4$ ($30cm^3$) followed by 0.3M $NaClO_4$ ($30cm^3$) and the ratio of each product produced was estimated from the intensity of colour of each band on the ion exchange resin.

Results.

Figure 4.15 shows a typical elution profile for reaction products. This was, however, often complicated by the appearance of other bands arising from the breakdown of the prolinebis(ethylene diamine)cobalt(III) moiety. Table 4.8 shows the results of each trial to the nearest 5%. In the diisopropylamine trial a yellow band representing 30% of the products was eluted immediately after the $[Co(en)_2Pro\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ band. There were usually three main bands. The first species eluted was identified as $[Co(en)_2Pro]^{2+}$ by its elution volume from the columns, the second species eluted was identified as $[Co(en)_2Pro\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ by reduction and the subsequent analysis of its ligands and the third species eluted was identified as $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ by its elution volume. In addition to these bands an orange band which was not eluted by 0.3M $NaClO_4$ was recognised and is named "polymeric $[(Co(en)_2Pro)_n]^{m+}$" because of its likeness to the slowest moving band found in Section E.

| Solvent | Added Base | Reaction Time | Conc $[Co(en)_2Pro\text{-}OCH_3]^{3+}$ * | Conc. Gly-Gly-OEt * | % $[Co(en)_2Pro]^{2+}$ | % $[Co(en)_2Pro\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ | % $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ | % Polymeric $[(Co(en)_2Pro)_n]^{m+}$ |
|---|---|---|---|---|---|---|---|---|
| DMSO | none | 2 minutes | 0.15M | 0.4M | 100 | 0 | 0 | 0 |
| DMSO | diisopropylanine | 2 minutes | 0.10M | 0.4M | 1 | 60 | 5 | 1 |
| DMSO | LiOH | 2 minutes | 0.11M | 0.4M | 5 | 80 | 5 | 5 |
| Methanol | pyridine | 5 minutes | 0.14M | 0.6M | 40 | 40 | 15 | 0 |
| Methanol | | 25 minutes | 0.14M | 0.6M | 20 | 50 | 30 | 1 |
| Methanol | triethylamine | 4 minutes | 0.15M | 0.6M | 1 | 90 | 2 | 1 |
| Methanol | | 30 minutes | 0.15M | 0.6M | 1 | 80 | 5 | 5 |
| Methanol | NaOH | 2 minutes | 0.14M | 0.6M | 5 | 80 | 10 | 5 |
| Pyridine | none | 2 minutes | 0.10M | 0.4M | 40 | 10 | 30 | 20 |

* Concentrations used in the reaction mixture.

Table 4.8 Reaction Product Proportions With Varied Base and Solvent.

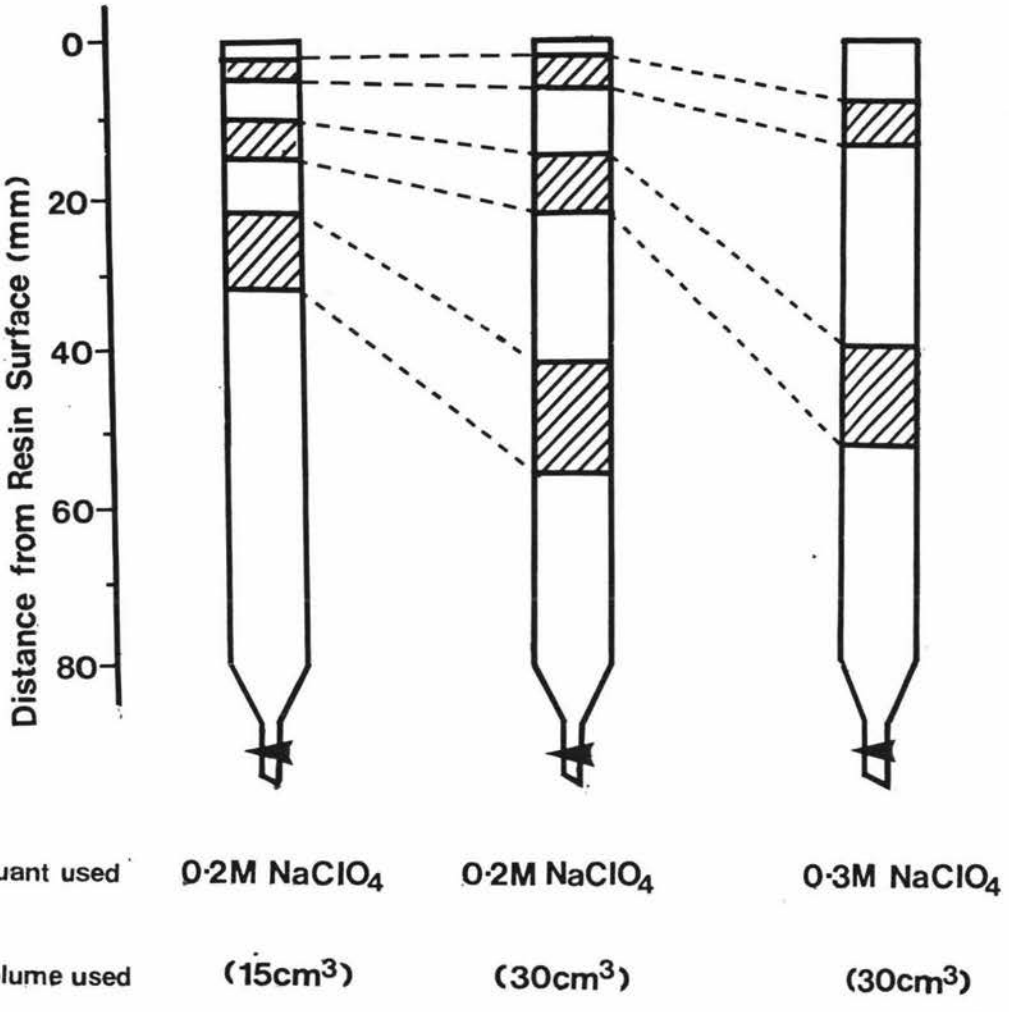



Figure 4.15. Elution Profile of Reaction Products of $[Co(en)_2Pro-Gly-Gly-OEt]^{3+}$ Preparation.

Discussion.

As can be seen from the first line of Table 4.4 no reaction occurs in dimethylsulphoxide between $[Co(en)_2Pro\text{–}OMe]^{3+}$ and $^{+}H_2\text{–}Gly\text{–}Gly\text{–}OEt, Cl^{-}$. It is not until some of the dipeptide free amine is present that the reaction can occur. $[Co(en)_2Pro\text{–}OMe]^{3+}$ is very sensitive to basic solutions and immediately turns a brown colour when it is added to a methanol solution of triethylamine due to decomposition of the complex. When sorbed onto an ion exchange column and eluted with 0.2M $NaClO_4$ this brown species gradually decreases in intensity until it cannot be seen. This is most probably associated with a reduction of the Co(III) moiety and the release of its ligands. Thus, in a mildly basic solution of $[Co(en)_2Pro\text{–}OMe]^{3+}$ a small proportion of this species will be reduced and dissociated from its ligands. These ligands will then be able to react with the remaining $[Co(en)_2Pro\text{–}OMe]^{3+}$ in competition with the Gly–Gly–OEt which is also present, hence the formation of $[Co(en)_2Pro\text{–}NHCH_2CH_2NH_2]^{3+}$. An explanation of the formation of polymeric $[(Co(en)_2Pro)_n]^{m+}$ is that the dangling amino group of the uncoordinated ethylenediamine in the $[Co(en)_2Pro\text{–}NH_2CH_2CH_2NH_2]^{3+}$ species undergoes nucleophillic attack on another $[Co(en)_2Pro\text{–}OMe]^{3+}$ species yielding a third species which is dinuclear, $[Co(en)_2Pro\text{–}NHCH_2CH_2\overline{NH\ Co(en)_2Pro}]^{6+}$. The complete acidification of all the sulphonic acid groups on the ion exchange resin with 1M hydrochloric acid would allow the dinuclear $6^{+}$ species to be eluted rapidly. Thus reaction conditions which give the best yield of $[Co(en)_2Pro\text{–}Gly\text{–}Gly\text{–}OEt]^{3+}$ are those which provide the free amine, H–Gly–Gly–OEt, but are not basic enough to lead to

reduction of the cobalt III moiety. The best conditions for synthesis were found in the methanol/triethylamine trial which gave a 90% yield at 4 minutes but which had decreased to 80% after 30 minutes corresponding to an increase in the proportion of slower moving bands.

Poor yields were obtained using pyridine as the base. This is probably because pyridine ($pK_a$ = 5.18) is a weaker base than Gly-Gly-OEt ($pK_a$ of approximately 9.8) and thus the $^{+}H_2$-Gly-Gly-OEt,$Cl^-$ is left intact. In contrast triethylamine ($pK_a$ = 10.75) is a stronger base and will form the free amine H-Gly-Gly-OEt. When pyridine is used as the base the largest proportions of $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ and polymeric $[(Co(en)_2Pro)_n]^{m+}$ are found. This is probably due to the slow coupling reaction, caused by a very low concentration of the free amine of the peptide. Under these conditions the side reactions described above predominate and $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ and polymeric $[(Co(en)_2Pro)_n]^{m+}$ are formed.

An attempt was made to prepare $[Co(en)_2Val\text{-}Pro\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$. The addition to the reaction mixture of a 1.4 fold excess of triethylamine over the number of moles of $^{+}H_2$-Pro-Gly-Gly-OEt,$CH_3COO^-$ caused reaction conditions to be basic. The analysis of the reaction mixture after 2.0 minutes by elution from the cation exchange resin described in this section, with 0.2M and 0.3M pyridinium acetate showed that five orange species were present. The major species (estimated at 70% of visible products) was $[Co(en)_2Val]^{2+}$. A twofold excess of $[Co(en)_2Val\text{-}OMe](CF_3SO_3)_3$ was used in the coupling. The other dominant species, each estimated to be 15% of the visible products, had mobilities on the resin very similar to $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$ and

polymeric $[(Co(en)_2Pro)_n]^{m+}$ as described in this section. This shows that $[Co(en)_2Val-OMe]^{3+}$ undergoes decomposition in basic solution in the same way that $[Co(en)_2Pro-OMe]^{3+}$ undergoes decomposition in neutral or slightly basic solution. This is one more example showing that the pH of a coupling reaction is critical. If the pH is too low, free amine of the peptide to be coupled is not present and therefore no coupling occurs with the activated ester. If the pH is too high, then a proportion of complexes are reduced to the labile Co(II) species releasing ligands which may react with the activated ester. This side reaction is especially noticeable when $[Co(en)_2Pro-OMe]^{3+}$ is the activated ester.

## SECTION G: PREPARATION OF Pro-Gly-Gly-OEt.

Materials.

$^{+}H_2$-Gly-Gly-OEt,$Cl^{-}$ was obtained from Sigma. The methanol used was A.R. grade, distilled twice. Dansyl arginine is 1-Dimethylaminonaphthalene-5-sulphonyl-$N^{\alpha}$-arginine and fluoresces in uv light.

Preparation of (Ethyl Prolylglycylglycinate)bis(ethylenediamine) cobalt(III) Acetate.

$[Co(en)_2Pro-Gly-Gly-OEt](CH_3COO)_3$.

Method.

$^{+}H_2$-Gly-Gly-OEt,$Cl^{-}$(0.450g, $2.28 \times 10^{-3}$mol) was dissolved in

methanol ($2.0cm^3$) to which had been added enough triethylamine (0.115g, $1.14 \times 10^{-3}$mol) to neutralise one half of the peptide hydrochloride. $[Co(en)_2Pro\text{-}OMe](CF_3SO_3)_3$ (0.840g, $1.14 \times 10^{-3}$mol) was slurried in the solution and the reaction stirred under anhydrous conditions. After 5 minutes methanol ($2cm^3$) was added in an attempt to dissolve the remainder of the complex. After 10 minutes acetic acid ($2cm^3$) was added to quench the reaction and the solution was diluted to $100cm^3$ with water. A small portion of this solution ($5cm^3$) was sorbed onto an analytical cation exchange column and eluted (Figure 4.15). The remainder of the solution ($95cm^3$) was sorbed onto a preparative cation exchange column and eluted with 0.2M pyridinium acetate. The concentration of eluant was increased to 1.0M pyridinium acetate when the major band was being eluted from the column. This complex was collected in a volume of $200cm^3$ of eluant and evaporated to dryness under reduced pressure at 40°C. The complex was dissolved in water ($10cm^3$) and evaporated to dryness under reduced pressure. This procedure was repeated until no pyridine could be smelt on the dark red crystals (one time). The product was not weighed at this stage.

Results.

The analytical cation exchange column (Figure 4.15) indicates a 95% yield of the orange $[Co(en)_2Pro\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ with barely visible traces of the orange coloured species $[Co(en)_2Pro]^{2+}$ and $[Co(en)_2Pro\text{-}NHCH_2CH_2NH_3]^{4+}$, see Figure 4.15 for mobilities.

Reduction of $[Co(en)_2Pro-Gly-Gly-OEt](CH_3COO)_3$ and the Isolation of the Free Peptide.

Method.

The isolated species of $[Co(en)_2Pro-Gly-Gly-OEt](CH_3COO)_3$ was dissolved in a minimum of water ($6cm^3$) and the pH of this solution was adjusted to 0.7 with concentrated hydrochloric acid. Zinc amalgam (2%, $20cm^3$) was added and the solution stirred briskly for 5 minutes after which time the reduction was complete. The solution ($7cm^3$) was decanted from the amalgam and loaded onto a Bio-Rad P2 gel filtration column and elution with 2N acetic acid begun. Every second fraction was tested for reactivity with ninhydrin.

Results.

Two overlapping ninhydrin reactive bands were found (Figure 4.16).

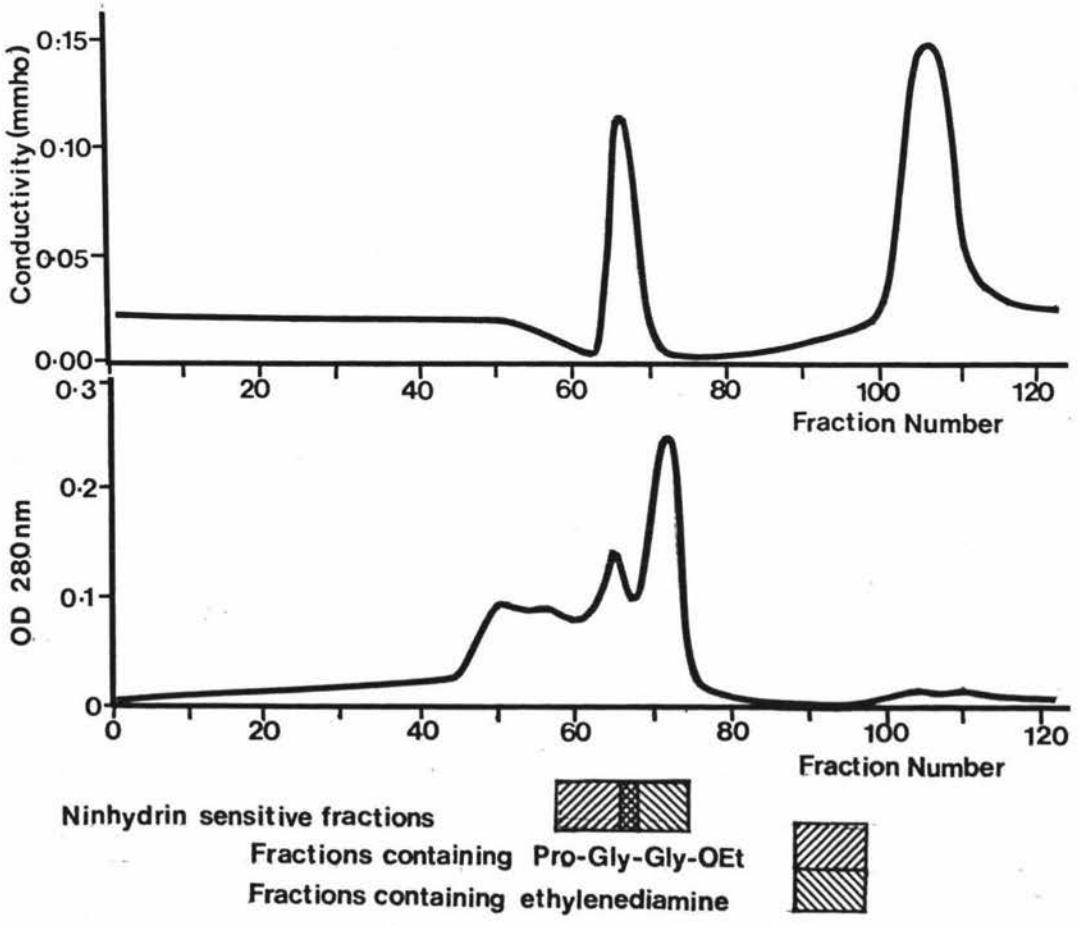



Figure 4.16. Gel Filtration of Reduced $[Co(en)_2$ Pro-Gly-Gly-OEt$]^{3+}$

The first band eluted was Pro-Gly-Gly-OEt and the second, ethylenediamine. Pro-Gly-Gly-OEt was identified by an amino acid analysis of a hydrolysate of the first freeze-dried fraction. The analysis verifies the ratio of amino acids as: proline 0.99, glycine 2.00. Ethylenediamine was identified by TLC and elution volume from the gel filtration column. Pro-Gly-Gly-OEt was found in fractions 58 to 68 inclusive and ethylenediamine was found in the fractions 65 to 74 inclusive. The fractions 58 to 64 and 65 to 70 were freeze-dried separately, thus some Pro-Gly-Gly-OEt was not isolated from ethylenediamine and was, therefore, not calculated in the yield. TLC of Pro-Gly-Gly-OEt is seen in Table 4.9.

| Peptide | Solvent 6 | MeOH | Solvent 8 | Colour of Spots With Ninhydrin |
|---|---|---|---|---|
| Pro-Gly-Gly-OEt | 0.62 | 0.57 | 0.59 | orange |
| $^{+}H_2$-Gly-Gly-OEt,$Cl^{-}$ | 0.56 | 0.62 | 0.52 | yellow turns orange |
| Proline | 0.15 | - | - | yellow |
| Ethylenediamine | 0.18 | - | - | purple |

Table 4.9. Rf's of Peptides*.

* Alumina thin layers were used for Solvents 6 and 8 and silica thin layers for methanol.

The ethylenediamine has an impurity which stains purple with ninhydrin, with Rf 0.33 in Solvent 6 on alumina. It is found only in the more concentrated fractions of ethylenediamine and is

possibly due to reaction of ethylenediamine with the acetic acid (2N) solvent during the evaporation of solvent from thin layers by heating. Pro-Gly-Gly-OEt was further characterised by paper electrophoresis (Table 4.10) and HPLC, Figure 4.17.

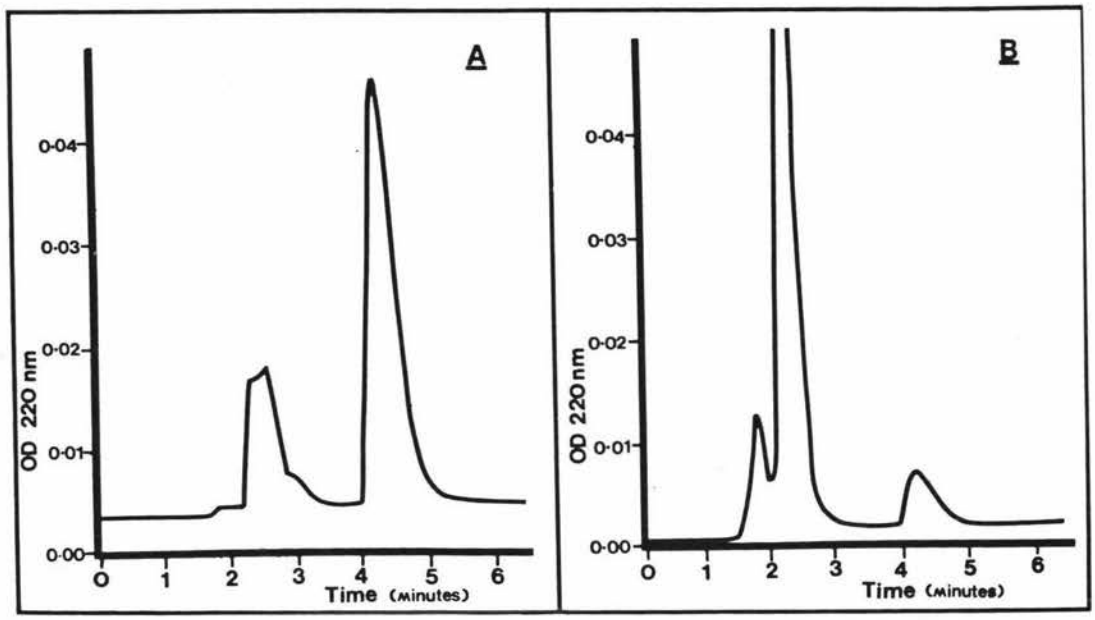



Figure 4.17. HPLC of synthetic Pro-Gly-Gly-OEt. In A, 24µg of Pro-Gly-Gly-OEt and in B, 11µg of the peptide which had been treated with HBr/acetic acid* were analysed at 220nm.

* Pro-Gly-Gly-OEt (1.1mg) was dissolved in HBr/acetic acid (47.49%, 0.1cm$^3$) and stirred for 30 minutes at room temperature. The solution was then blown dry under a jet of compressed air; the residue was dissolved in water and the solution blown dry again.

Analysis by HPLC shows that under the conditions used Pro-Gly-Gly-OEt is eluted at 4.2 minutes. The Pro-Gly-Gly produced by hydrolysing the ester linkage with HBr/acetic acid is eluted at 2.3 minutes under the same conditions.

| Compound | Colour with ninhydrin | Rf w.r.t. Dansyl Arg | Rf w.r.t. serine |
|---|---|---|---|
| serine | purple | 0.70 | - |
| proline | yellow | 0.57 | 0.82 |
| Pro-Gly-Gly-OEt | yellow/grey | 0.84 | 1.21 |
| Gly-Gly-OEt | yellow | 1.09 | 1.56 |
| dansyl arginine | - | - | 1.43 |

Table 4.10. Electrophoresis of Pro-Gly-Gly-OEt.

Overall yield = 46mg, a light orange coloured oil. Weight of $^{+}H_2$-Pro-Gly-Gly-OEt,$CH_3COO^-$ based on glycine in amino acid analysis = 41mg, (90% of the freeze-dried material), 1.36 x $10^{-4}$mol, i.e. yield = 12% based on $[Co(en)_2Pro\text{-}OMe](CF_3SO_3)_3$.

Discussion.

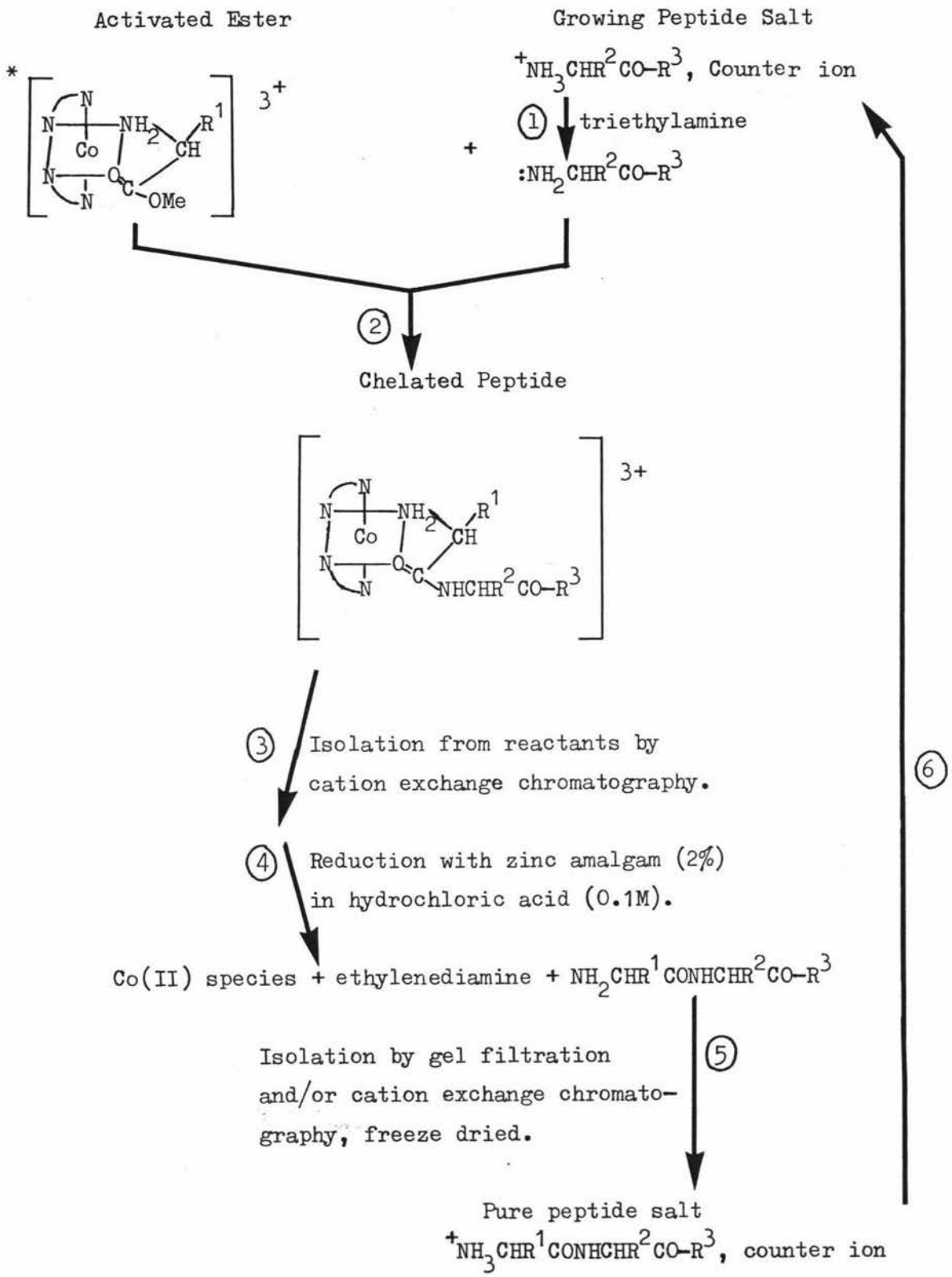
The success of this preparation is due in part to the rapid reaction between $[Co(en)_2Pro\text{-}OMe]^{3+}$ and the sterically unhindered Gly-Gly-OEt. Also, the use of a twofold excess of $^{+}H_2$-Gly-Gly-OEt, only half of which is neutralised with triethylamine, buffers the pH of the solution so preventing reduction of $[Co(en)_2Pro\text{-}OMe]^{3+}$. The use of twofold excesses of the growing peptide chain is extremely wasteful of prepared peptides and is, therefore, not a practical way of buffering reactions entailing longer, more expensive peptides. No identification of the ethyl ester was made during the characterisation of Pro-Gly-Gly-OEt but it has been confirmed in the preparation of Ala-Gly-Gly-OEt that the ethyl ester remains intact under the same conditions of reduction and

purification. The overall yield of 12% is very low. The major source of loss of material between the synthesis of $[Co(en)_2Pro\text{-}Gly\text{-}Gly\text{-}OEt]^{3+}$ and the isolation of Pro-Gly-Gly-OEt is not known. A minor loss occurred (approximately one third of that isolated by gel filtration) because ethylenediamine would not separate completely from the peptide on the gel filtration column.

The purity of the peptide is established by amino acid analysis and HPLC. The 10% of involatile salts are usual in freeze-dried fractions from the ion exchange column. These salts are eluted just after the break-through volume when analysed by HPLC. The decrease in size of the 4.2 minute peak after the peptide was treated with HBr/acetic acid is qualitative evidence that the ethyl ester is intact in the isolated peptide. HBr/acetic acid readily hydrolyses such esters at room temperature.

## SECTION H: DISCUSSION OF SOLUTION PHASE PEPTIDE SYNTHESIS VIA COBALT(III) CHELATES OF AMINO ACID METHYL ESTERS

The synthesis scheme used in sections A - G to prepare peptides via the above method is outlined below, in Figure 4.18.




* N⌒N is ethylenediamine.

Figure 4.18. Reaction scheme for solution phase synthesis.

It is pertinent to discuss each of the steps of the synthesis with reference to the yields obtained in Sections A to G. Step 1, the preparation of the free amine of the growing peptide chain, has been investigated *in situ* in Section F. The addition of an equimolar amount of triethylamine to the peptide ester salt was found to be a convenient and very effective way of preparing the free amine of the peptide. Step 2, the synthesis step, was investigated qualitatively for each synthesis in Sections A to G, by comparison of band intensities on ion exchange columns. Good yields were obtained in all syntheses except for the preparation of $[Co(en)_2Pro\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$, Section E. The quantitative investigation of Step 3 showed that good yields of the peptide cobalt(III) complex were obtainable by this method. Lower yields of $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ and $[Co(en)_2Pro\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ are discussed later. The use of different reagents for effecting Step 4, the reduction of the peptide cobalt complex, has been investigated by another worker (56). The effectiveness of each reagent is shown in Table 4.11.

TABLE 4.11

Effectiveness of Reducing Agents in Releasing Co(III) Ligands. [a] *

| Reagent | Comment |
|---|---|
| Chromous Chloride | Oxygen free conditions required. Rapid reaction. |
| Lithium Aluminium Hydride | Reduction complete in 5 min. |
| Sodium Borohydride | " " " " |
| Lithium tri-(t-butoxy)-Aluminium Hydride | " " " " |
| Sodium Thiosulphate | Very slow reduction ca. 20 hrs. |
| Sodium Cyanide | Rapid reduction. Handling problems. |
| Ammonium Sulphide | Reduction complete after 30min at 40°. |
| Hydrogen Sulphide | " " " " " " |
| Vanadous Sulphate | Reduction complete in 5min. |
| Zn/HCl | Reduction complete after 18 hrs. |

[a] Complex 0.05M, reducing reagent 50% excess.

* Reproduced from Reference (15), p70.

The method of reduction used in the present study was treating an acid solution of the complex with zinc amalgam (2%). Complete reduction occurred in 5 minutes at room temperature using this method. The reaction was followed qualitatively by the disappearance of the orange complex. Step 5, the isolation of the peptide, was investigated quantitatively and a good yield (67%) was obtained for the peptide Gly-Phe-Phe-OBzl. Problems were encountered at this step and these are discussed below. The problems encountered are solvable and this step is not expected to hinder recovery of longer peptides. Step 6, the utilisation of a synthesised peptide in the succeeding synthesis reaction, was accomplished during the preparation of Ala-Gly-Phe-Phe-OBzl and Leu-Ala-Gly-Gly-OEt and shows that the method outlined in Figure 4.18 can be used for preparing peptides of considerable length.

The yields obtained for each synthesis, in Sections A to G, together with the reaction conditions and the number of visible species formed during the coupling reaction, are summarised in Table 4.12.

Generally, the overall yields appear to be moderate. The losses of yield incurred during the preparation of the peptides Ala-Gly-Phe-Phe-OBzl and Leu-Ala-Gly-Gly-OEt were a result of not being able to identify the peptides in fractions eluted from the gel filtration column. These peptides appear to react with ninhydrin slowly or give products with low extinction coefficients making them difficult to detect with this method. The use of a chromogenic ester, attached to the growing peptide chain, would be of great assistance at this stage of purification. The moderate yields of these peptides do not give full justice to the good yields of the cobalt peptide complex obtained, 88% and 70% respectively.

TABLE 4.12. Summary of Solution Phase Peptide Synthesis Yields

| Peptide Syntheses | Coupling Reaction Solvent | Reaction Time | Reagent in excess in Reaction Mixture | Number of Coloured Species after reaction[a] | Elution Rate of $[Co(en)_2peptide\text{-}OR]^{3+}$ [b] | % Yield[c] of $[Co(en)_2peptide\text{-}OR]^{3+}$ | % Yield[d] of Pure Peptide | Overall % Yield[e] |
|---|---|---|---|---|---|---|---|---|
| Gly-Phe-Phe-OBzl | DMSO | 30 minutes | Phe-Phe-OBzl(1.06x) | 2 | slower | 28% | 67% | 19% |
| Ala-Gly-Phe-Phe-OBzl | DMSO | 20 minutes | $[Co(en)_2Ala\text{-}OMe]^{3+}$,(2.7x) | 2 | slower | 88% | 31% | 27% |
| Ala-Gly-Gly-OEt | Methanol | 10 minutes | $[Co(en)_2Ala\text{-}OMe]^{3+}$,(2x) | 2 | slightly faster | - | - | 12% |
| Leu-Ala-Gly-Gly-OEt | DMSO | 10 hours | $[Co(en)_2Leu\text{-}OMe]^{3+}$,(4x) | 2 | slightly faster | 70% | 24% | 17% |
| Pro-Phe-Phe-OBzl | Methanol | 30 minutes | $[Co(en)_2Pro\text{-}OMe]^{3+}$,(2x) | 3 | - | 0% | - | 0% |
| Pro-Gly-Gly-OEt | Methanol | 10 minutes | Gly-Gly-OEt,(2x) | 3 | faster | - | - | 12% |

a. Visible by ion exchange chromatography.

b. Compared to the elution rate of $[Co(en)_2aaNH_2]^{3+}$ where aa is the amino terminal amino acid of the peptide.

c. Based on the reagent not in excess in the coupling reaction.

d. Based on the weight of $[Co(en)_2peptide\text{-}OR](CH_3COO)_3$ used in the reduction step.

e. Based on conditions c and d.

The moderate yield (28%) of Gly-Phe-Phe-OBzl was almost entirely due to a low yield of the cobalt peptide complex since a good yield of free peptide (67%) was isolated from the cobalt peptide complex. The lowering of the yield of the cobalt peptide complex is thought to be due to hydrolysis of the product on the ion exchange column, in the 0.2 to 1.0M pyridium acetate eluant (pH 5). It is interesting to note a trend in the stabilities of these complexes. $[Co(en)_2Phe\text{-}Phe\text{-}OBzl]^{3+}$ undergoes hydrolysis rapidly under the same conditions such that no peptide complex can be isolated (50), $[Co(en)_2Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ appears to undergo hydrolysis slowly such that only about a third of the cobalt peptide complex was isolated (Section A) and $[Co(en)_2Ala\text{-}Gly\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ appears to be very stable to hydrolysis under the same conditions (Section A). Attempts to synthesise $[Co(en)_2Ala\text{-}Phe\text{-}OBzl]^{3+}$ and $[Co(en)_2Pro\text{-}Phe\text{-}OBzl]^{3+}$ resulted in products which did not contain phenylalanine when isolated in the same way as above (50). Thus, it is likely that the particular tertiary structure of the peptide moiety in $[Co(en)_2aa\text{-}Phe\text{-}OBzl]^{3+}$ and, to a smaller extent, in $[Co(en)_2aa\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$ results in a more easily hydrolysed amino terminal peptide bond than is typically observed for these complexes (28, 46). Stability studies of $[Co(en)_2aa\text{-}_D Phe\text{-}OBzl]^{3+}$ would show whether the particular tertiary structure* of the peptide moiety is important in determining the stability of the peptide complex to hydrolysis since the tertiary structure of this cobalt peptide complex would be different.

Under aqueous conditions considerable interaction between the aromatic groups of the ester and the phenylalanine is expected. It may be this interaction which untypically results in the unstable peptide complexes.

* Tertiary structures of peptides and proteins are the three dimensional configuration of the peptide chain determined by disulphide bonds, hydrogen bonds, salt bonds and hydrophobic bonds.

The problem of hydrolysing product could be circumvented by the immediate reduction of all cobalt(III) species following the synthesis then extensive purification of the product. Alternatively, a different ester moiety on the peptide may increase the stability of the cobalt peptide complexes above.

Instability of Proline Complexes.

All cobalt(III) amino acyl complexes, in basic conditions, decompose to form brown polymeric compounds and give Co(II) species (46, 64-66). When the amino acid is proline, however, the decomposition occurs in very mild basic conditions, Sections E and F. The cause of the greater instability of cobalt(III) proline complexes is unknown; however, rate studies suggest that the tetrahedral intermediate formed by the nucleophillic attack of Gly-OEt on $[Co(en)_2Pro\text{-}OMe]^{3+}$ is not as stable as when amino acids other than proline are used (46). Thus, it is likely that whatever destabilises the cobalt(III) complex also destabilises the tetrahedral intermediate. This instability may result from the strained configuration of the proline alkyl chain caused by repulsion between the alkyl chain and the co-ordinated ethylenediamine. If this is true the choice of another $Co^{3+}(amine)_4$ moiety may result in the greater stability of cobalt(III) proline complexes. The basic conditions necessary for reduction of the cobalt(III) species are consistent with a deprotonation reaction, perhaps, as in the base hydrolysis of $[Co(en)_2Br(Gly\text{-}OR)]^{2+}$ where it is thought deprotonation of the ethylenediamine results in a less rigidly co-ordinated ligand trans to the deprotonated ligand and a 5 co-ordinate intermediate results (65). The base sensitivity of $[Co(en)_2Sar\text{-}OMe]^{3+}$ * may give an indication of the cause of instability in $[Co(en)_2Pro\text{-}R]^{3+}$ complexes since both amino acids are secondary amines.

* Sar = N-methylglycine.

Separation Problems

The separation of the smaller peptides, Ala-Gly-Gly-OEt and Pro-Gly-Gly-OEt, from ethylenediamine by gel filtration was difficult. It was this problem which led to lowered yields of isolated peptide in these syntheses. The elongation of the peptide by one residue increases the separation obtained (Section D) and so the separation of peptide from ethylenediamine is not expected to hinder recovery of the peptide in the preparation of longer peptides.

Steric Considerations

The rates of formation of the tetrahedral intermediates in the synthesis of several dipeptides using the aminolysis of cobalt(III) chelated amino acid esters have been measured and are summarised in Table 4.13.*

* The rates cannot be obtained with absolute accuracy because decomposition of the tetrahedral intermediate partially occurs within the same time scale as its formation. The appearence of terms, second order in nucleophile, corresponding to general base catalysed addition of the nucleophile, also complicates the situation. For these reasons the $k_{calc}$ values given in Table 4.13 may have up to 10% error associated with them (46).

TABLE 4.13.

Reaction Rates and Times for Formation of the Tetrahedral Amino-carbinol Intermediate for Various Coupling Reactions[a]

| Cobalt chelated amino acid ester | Nucleophillic amino acid ester | (c) | $\Delta k_{calc}$ (b,d) | $\Lambda k_{calc}$ (b,d) | $\frac{\Lambda k_{calc}}{\Delta k_{calc}}$ | Reference |
|---|---|---|---|---|---|---|
| $[Co(en)_2Gly\text{-}OMe]^{3+}$ | Phe-OEt | A | 4.75 (5.1) | 4.75 (5.1) | 1.00 | (46) |
| $[Co(en)_2Ala\text{-}OMe]^{3+}$ | Phe-OEt | A | 4.16 (5.8) | 11.3 (2.1) | 2.72 | (46) |
| $[Co(en)_2Phe\text{-}OMe]^{3+}$ | Phe-OEt | A | 4.04 (6.0) | 4.20 (5.8) | 1.04 | (46) |
| $[Co(en)_2Val\text{-}OMe]^{3+}$ | Phe-OEt | A | 1.86(13.0) | 0.97(25.0) | 0.52 | (46) |
| $[Co(en)_2Ala\text{-}OMe]^{3+}$ | Val-OEt | A | 2.30(10.5) | 3.47 (7.0) | 1.51 | (46) |
| $[Co(en)_2Gly\text{-}OC_3H_7]^{3+}$ | Gly-OEt | B | 14 (1.7)[e] | | | (72) |
| $[Co(en)_2Gly\text{-}OC_3H_7]^{3+}$ | Ala-OEt | B | 1.3 (18.7) | 2.9 (8.4) | 2.3 | (73) |
| $[Co(en)_2Gly\text{-}OC_3H_7]^{3+}$ | Val-OEt | B | | 2.6 (9.3) | | (73) |
| $[Co(en)_2Ala\text{-}OMe]^{3+}$ | Gly-OEt | B | 2.5-5.1 (9.7-4.8) | 9.8-11.9 (2.5-2.0) | 2.3 | (74) |

(a) Pseudo first order rate conditions are assumed.

(b) Small terms second order in aa-OEt are ignored.

(c) Reaction conditions: A - $CH_3CN$, 25°C, $\mu$=0.1. B - DMSO, 25°C. The nucleophile is in excess.

(d) Units for $k_{calc}$ are $lmol^{-1}$ $sec^{-1}$. The values in parentheses are the time in seconds taken for 99% of the reactants to react given that the reagent in excess is present in a concentration of 0.2M. The times are derived from the equation: 99% reaction occurs at time = $7 \times t_{\frac{1}{2}} = \frac{7 \times 0.693}{k \times 0.2}$

(e) For a mixture of $\Delta$ and $\Lambda$-$[Co(en)_2aa\text{-}OMe]^{3+}$.

These rates are fast and, as shown by the reaction times in parenthesis, all of the reactions are essentially complete within 30 seconds. As can be seen in the first four reactions of Table 4.13 a general trend exists where increased steric hindrance of the chelated amino acid ester results in a decrease of reaction rate (with the exception of $-[Co(en)_2Ala-OMe]^{3+}$ reacting with Phe-OEt). The same trend has been observed by another worker (56) who found a 700fold decrease in the reaction rate of Gly-OEt with $[Co(en)_2Gly-OMe]^{3+}$ compared to the reaction rate of Gly-OEt with $[Co(en)_2Val-OMe]^{3+}$. Decreases in rates of coupling were also observed when the steric hindrance of the nucleophillic amino acid was increased (46,56). This effect was smaller than that of the chelated ester. For example, $[Co(en)_2Ala-OMe]^{3+}$ couples with Phe-OEt twice as fast as with Val-OEt, Table 4.13. These reactions were only carried out at the dipeptide stage, however, and the effect of steric hindrance of the nucleophile may be much greater when longer peptides are used. This effect would depend upon the structure of the peptide and the hindrance of the cobalt(III) chelated amino acid ester.

Dekkers (56) obtained lower than normal yields for the preparation of $[Co(en)_2Leu-Val-OEt]^{3+}$ and $[Co(en)_2Leu-Ala-OEt]^{3+}$. He suggested steric hindrance was the cause.

Reynolds (67) found that $[Co(en)_2Phe-OMe]^{3+}$ and Leu-OAz* would not couple and that the same activated ester reacted only slowly with Leu-OBzl. $[Co(en)_2Gly-OMe]^{3+}$, however, reacted with Leu-OBzl at a faster rate.

Steric hindrance is clearly an important factor in slowing these reactions and is most noticeable when both nucleophillic peptide and chelated amino acid ester are sterically hindered.

* -OAz = the bulky azo-dye ester, see Appendix II.

Two slow coupling reactions were encountered in the present study. The coupling of $[Co(en)_2Pro\text{-}OMe]^{3+}$ with Phe-Phe-OBzl occurred so slowly that only side-products were found in the reaction mixture (Section E) and the coupling of $[Co(en)_2Leu\text{-}OMe]^{3+}$ with Phe-Phe-OBzl also occurred very slowly (Section D), while $[Co(en)_2Gly\text{-}OMe]^{3+}$ couples with the same peptide ester very quickly.

The use of a less bulky ester moiety for the peptide ester may increase the rate of reaction of this nucleophile with $[Co(en)_2Leu\text{-}OMe]^{3+}$ and $[Co(en)_2Pro\text{-}OMe]^{3+}$. It seems likely, however, that all $[Co(en)_2Leu\text{-}OMe]^{3+}$ couplings will be subject to slow reaction rates when the nucleophile is sterically hindered. Other sterically hindered amino acid ester chelates, e.g. $[Co(en)_2Val\text{-}OMe]^{3+}$ and $[Co(en)_2Ile\text{-}OMe]^{3+}$ are also expected to give rise to the same problem.

Slow reaction rates represent a threat to the optical purity of the peptide as outlined in the introduction to this work. As with other active ester coupling methods, catalysts may be necessary to speed up reactions of sterically hindered chelated amino acid esters with sterically hindered amino acid and peptide esters (61 - 63).

Construction of longer peptides.

This work has been further developed by Reynolds (67) to the preparation of Leu-enkephalin. The following reaction scheme was used.

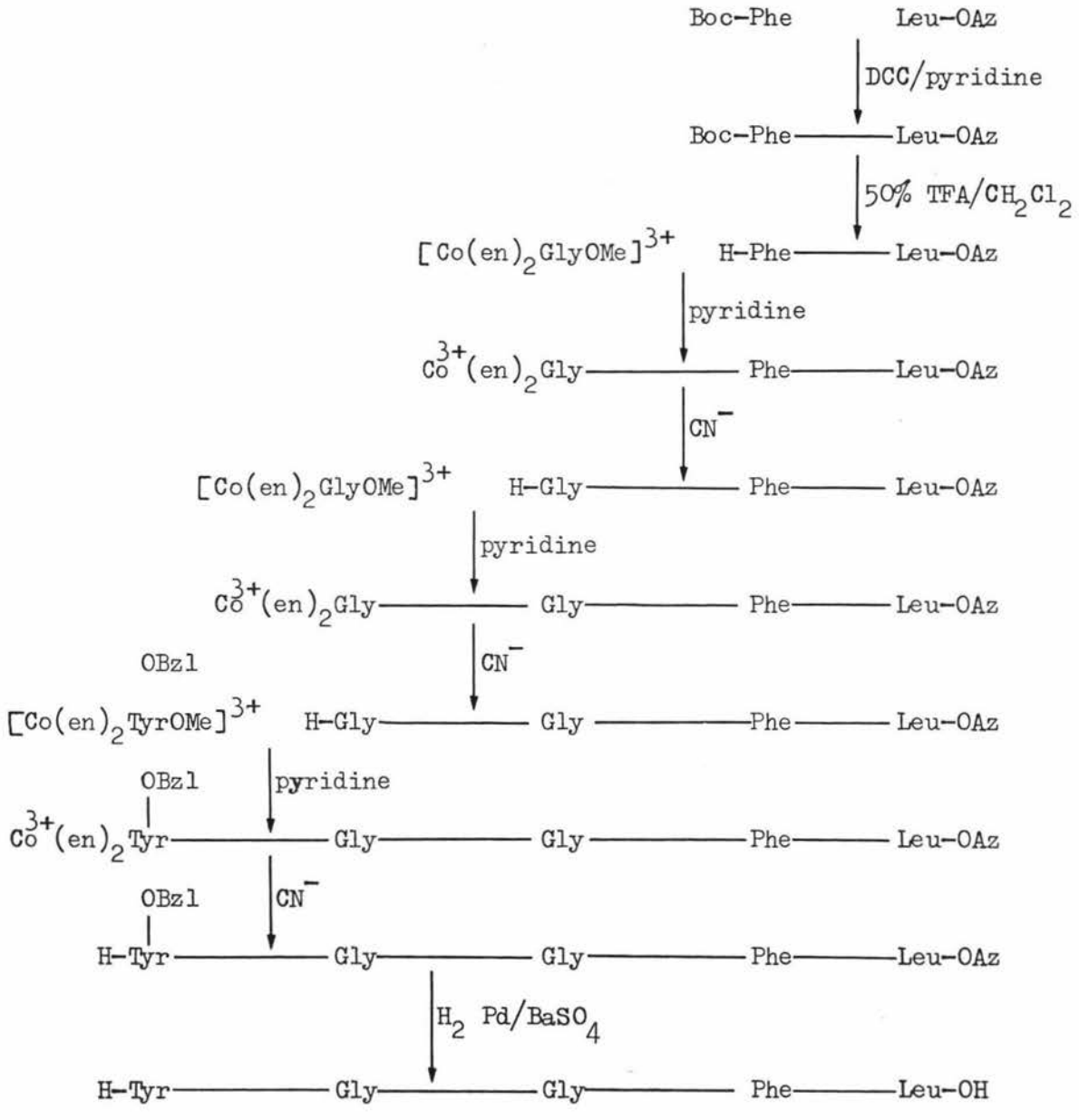



Figure 4.19. Synthesis scheme for Leu-enkephalin.

The product of this reaction scheme co-chromatographed (tlc, HPLC and electrophoresis) with the same peptide prepared by solid phase peptide synthesis using DCC as the acyl activator and Boc protection for amino groups (-OBzl protection on tyrosine side chain).

Reynolds found the use of the azo-dye chromogenic ester (-OAz) an important aid in speeding up purification and obtaining good yields.

Bentley (26) succeeded in coupling $Co(en)_2Gly\text{-}OMe^{3+}$ to several long peptides. This is also evidence that the reaction scheme in Figure 4.18 can be used to prepare peptides of considerable length.

In conclusion, the preparation of target peptides at moderate yields is possible using the aminolysis of cobalt(III) chelated amino acid esters. Further studies should concentrate on the catalysis of slow reactions and on preparing a large range of naturally occurring peptides. A more stable proline complex needs to be found if side reactions occurring during the addition of proline are to be stopped. The optical purity of synthesised peptides are, at present, under study.

# APPENDIX I

## PREPARATION OF BOC-Phe-Phe-OBzl BY CLASSICAL METHODS.

### Materials.

The phenylalanyl benzyl ester tosylate was prepared by a modified method of Jervas, Winitz and Greenstein (68). M.Pt = 168-171°C.

Boc-phenylalanine and dicyclohexylcarbodiimide was obtained from the Protein Research Foundation. Dichloromethane was laboratory reagent purified by distillation followed by passing it through a silica column. Chloroform and hexane eluants were laboratory grade.

### Method and Results.

Phenylalanine benzyl ester tosylate (3.22g, $7.55 \times 10^{-3}$mol) was dissolved in dichloromethane ($15cm^3$) and triethylamine ($1.05cm^3$, $7.54 \times 10^{-3}$mol). BOC-phenylalanine (2.00g, $7.55 \times 10^{-3}$mol) was dissolved in the solution and then dicyclohexylcarbodiimide (10% excess) was added dissolved in dichloromethane ($21cm^3$). Dicyclohexylurea began precipitating after one minute. The reaction mixture was stirred at room temperature for 18 hours, then the dicyclohexylurea was removed by filtration. Impurities were discovered by thin layer chromatography and, as these impurities separated from the product on silica plates developed with hexane or chloroform, the reaction products were chromatographed on a silica column. The reaction mixture was presorbed onto silica (30g) by adding the silica then evaporating the solvent under reduced pressure.

The presorbed silica slurried with hexane ($50cm^3$), was added to the top of a silica column (100–200 mesh, 200g, 32 x 3cm) equilibrated in hexane. The products were eluted with hexane (21), chloroform/hexane (10%, 21) and chloroform/hexane (20%, 41). The second and third litres of 20% chloroform/hexane were evaporated to dryness under reduced pressure at 30°C. Yield = 3.04g, i.e. 80%.

The product had Rf = 0.79 on Silica U.V. plates developed with chloroform and after removal of the Boc group, by spraying the plate with hydrochloric acid (6N) and heating with a drier, the product reacted with ninhydrin to form a red-orange spot. When the plate was not sprayed with hydrochloric acid, the product reacted with ninhydrin weakly after prolonged heating at 120°C confirming the presence of the BOC group preventing the amino group from reacting with ninhydrin. Elemental analysis of product calculated for $C_{30}H_{34}O_5N_2$: C, 71.71; H, 6.77; N, 5.58. Found: C,71.28; H, 7.07; N, 6.01. A $^1H$ NMR spectrum (60MHz) of product dissolved in $CDCl_3$ integrates correctly for BOC–Phe–Phe–OBzl ester. The t-butyl protons are represented by a singlet at 1.38ppm. The aromatic protons are represented by a broad multiplet at 7.00 to 7.22ppm. The benzylic protons on the phenylalanine residues are represented by a multiplet centred at 3.00ppm while those of the benzyl ester are represented by a singlet at 5.08ppm. Mass spectroscopy of the product showed that the molecular ion had m/e = 502.2479 (calculated m/e for $C_{30}H_{34}O_5N_2$ is 502.2466).

The trifluoroacetyl salt of Phe–Phe–OBzl was prepared from Boc–Phe–Phe–OBzl, appendix I, by adding trifluoroacetic acid (20% in chloroform) to the Boc compound and stirring at toom temperature for 30 minutes. The reaction mixture was evaporated to an oil under

reduced pressure, dissolved in chloroform and evaporated to dryness under reduced pressure at 30°C. Analysis of the product by thin layer chromatography showed a homogeneous product reacting to form an orange-yellow spot with ninhydrin. Rf = 0.75 on a silica thin layer using chloroform as solvent, c.f. Boc-Phe-Phe-OBzl Rf = 0.89.

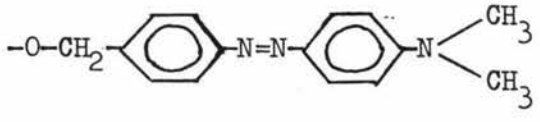
# APPENDIX II

## Abbreviations

a) Amino Acids.

| | | | | | |
|---|---|---|---|---|---|
| Ala | - | l-alanine | Phe | - | l-phenylalanine |
| Arg | - | l-arginine | Pro | - | l-proline |
| Asn | - | l-asparagine | Ser | - | l-serine |
| Gly | - | glycine | Thr | - | l-threonine |
| Gln | - | l-glutamine | Trp | - | l-tryptophan |
| Ile | - | l-isoleucine | Tyr | - | l-tyrosine |
| Leu | - | l-leucine | Val | - | l-valine |
| Lys | - | l-lysine | aa | - | general name for any amino acid. |
| Met | - | l-methionine | | | |

b) Amino acid and peptide blocking groups.

| | |
|---|---|
| z- | benzyloxycarbonyl |
| Boc- | t-butyloxycarbonyl |
| PCP | pentachlorophenyl ester |
| -OBut | t-butyl ester |
| -OBzl | benzyl ester |
| -OEt | ethyl ester |
| -OMe | methyl ester |
| -OAz |  |

c) Ligands.

| | | |
|---|---|---|
| TBP | – | tributylphosphate |
| en | – | ethylenediamine |
| dien | – | diethylenetriamine |
| trien | – | triethylenetetramine |
| tren | – | 2,2',2"–triaminotriethylamine |

d) Solvents.

| | |
|---|---|
| DMF | dimethylformamide |
| DMSO | dimethylsulphoxide |
| $CH_2Cl_2$ | dichloromethane |

e) Reagents.

| | |
|---|---|
| DCC | N,N'–dicyclohexylcarbodiimide |
| TFA | trifluoroacetic acid |

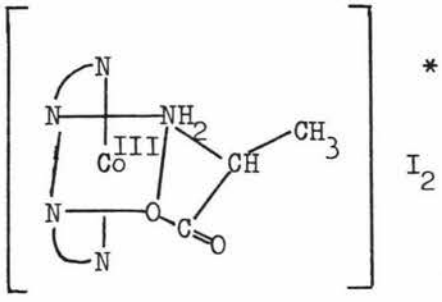
# APPENDIX III

Nomenclature.

Section A: Complexes.

a). Complexes of amino acids such as bis(ethylenediamine) alanatocobalt(III) iodide(I) are represented by the shorthand $[Co(en)_2Ala]I_2$.

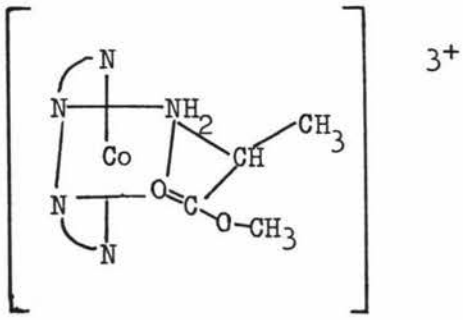
I




Note that peptide chemists would name this ligand Ala-O but this would be confused with an italicized O which defines the ligand as being co-ordinated through an oxygen atom only (69). For this reason the symbol used for the l-alanato ligand is simply Ala. Where *-N,O* is omitted it is implied.

b). Complexes of amino acid esters such as the bis(ethylenediamine) methyl alanate cobalt(III) ion, II, are represented by $[Co(en)_2Ala\text{-}OMe]^{3+}$

II




* $\overset{\frown}{N\ N}$ represents ethylenediamine

c). Complexes containing peptides chelated through their amino terminal amino acid, e.g. bis(ethylenediamine)(benzyl Leucylalanylphenylalanylphenylalanate)cobaltIII ion are represented by $[Co(en)_2Leu\text{-}Ala\text{-}Phe\text{-}Phe\text{-}OBzl]^{3+}$. (Strictly speaking the potential ligand sites should be numbered from the carboxy end of the peptide chain and the particular atoms co-ordinated depicted by numerical superscripts. Thus, the example above would read $[Co(en)_2Leu\text{-}Ala\text{-}Phe\text{-}Phe\text{-}OBzl\text{-}O^{7},N^{8}]^{3+}$. This numbering is confusing to peptide chemists who number amino acids from the amino terminus of the peptide and is, therefore, not used.)

d). The prefix $\Delta$ (delta) or $\Lambda$ (lambda) before a cobalt(III) species refers to the arrangement of chelates around the metal centre. Two enantiomorphous arrangements are possible, as shown in Figure III (70).

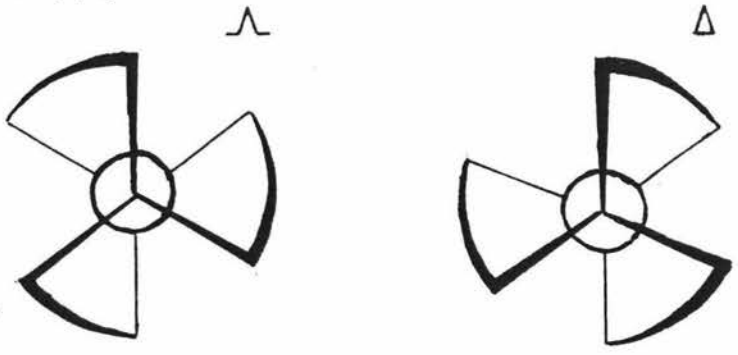



Figure III.

The omission of any prefix implies the two enantiomorphs were not separated.

Section B: Peptides.

The nomenclature used for peptides was adopted from IUPAC rules (71).

The abbreviations of the amino acids are listed in Appendix 2. The capital letter at the front of the amino acid implies the l form, i.e. Ala = l-alanine. The d and racemic forms are depicted by subscripts $_{D}$Ala and $_{DL}$Ala respectively.

The free amine of a peptide ester is named as in the example below. If the H- is omitted the free amino group is presumed.

H-Leu-Ala-Phe-Phe-OBzl

The acetate salt of the above peptide ester is represented by:

$^{+}H_2$-Leu-Ala-Phe-Phe-OBzl, $CH_3COO^{-}$

# BIBLIOGRAPHY


1 Bodanszky, M. and M. A. Ondetti, Peptide Synthesis, New York, Interscience, 1966.

2 Law, H. D. The Organic Chemistry of Peptides, London, Wiley-Interscience, 1970.

3 Merrifield, R. B., J.Amer.Chem.Soc, 85, 2149, (1963).

4 Khosla, M.C., R. R. Smeby and F. M. Bumpus, J.Amer.Chem.Soc, 94, 4721, (1972).

5 Rothe, M. and J. Razanek. Angew.Chem.(Int.Ed.), 1972, Vol.2.4, 293.

6 Gutte, B. and R.B. Merrifield, J.Amer.Chem.Soc., 91. 501. (1969).

7 Erickson, B. W. and R. B. Merrifield, (1976) in The Proteins, Neurath, H. and R. H. Hill, Academic Press, New York, 3rd ed., Vol.2., 255-527.

8 Hancock, W. S., D. J. Prescott, P. R. Vagelos and G. R. Marshall, J.Org.Chem., 38, 774, (1973).

9 Westall, F. C. and A. B. Robinson, J. Org. Chem, 35. 2842, (1970).

10 Gish, D.T., P.G. Katsoyannis, G.P. Hess and R.J. Stedman, J.Amer. Chem.Soc., 78, 5954, (1956).

11 Klausner, Y. S. and M. Bodanszky, Synthesis 1972, 453-463.

12 Bodanszky, M. and Bath, R. J., Chem.Comm. 1969, 1259.

13 Ref.1, p.98-109.

14 Buckingham, D. A., D. M. Foster and A. M. Sargeson, J.Amer.Chem. Soc. 92, 5701, (1970).

15 Dekkers, J. Cobalt(III) Promoted Synthesis of Peptides. Thesis, Ph.D., Australian National University, 1972.

16 Jencks, W.P., Catalysis in Chemistry and Enzymology, New York, McGraw-Hill, 1969.

17 Ref.15., Chapter 4.

18 Isied, S. S. and C. G. Kuehn, J.Amer.Chem.Soc., 100, 6752, (1978).

19 Bentley, K.W., Cobalt(III) Promoted Synthesis and Degradation of Peptides. Thesis, Ph.D., Australian National University, 1972, p.199.

20 Buckingham, D.A., L.G. Marzilli and A. M. Sargeson, J.Amer. Chem.Soc., 89, 2772, (1967).

21 Buckingham, D.A., L.G. Marzilli and A. M. Sargeson, ibid, 89, 4539, (1967).

22 Collman, J. P. and E. Kimura, ibid, 89, 6096, (1967).

23 Wu, Y. and D. H. Busch, ibid, 94, 4115, (1972).

24 Ref. 15, Chapter 4.

25 Ref. 15, Chapter 6.

26 Ref. 19, p.187.

27 Ref. 19, p.196.

28 Ref. 15. Chapter 3.

29 Ref. 15. p.81.

30 Ref. 15, p.68.

31 Buckingham, D.A., D. M. Foster and A. M. Sargeson, J.Amer.Chem. Soc, 92, 5571, (1970).

32 Buckingham, D. A., I.I. Olsen, A.M. Sargeson, J.Amer.Chem.Soc, 88, 5443, (1966); 89, 5129, (1967).

33 Buckingham, D.A., I.I. Olsen and A. M. Sargeson, Aust.J.Chem., 20, 597, (1967).

34 Buckingham, D. A., D.M. Foster and A.M. Sargeson, J.Amer.Chem. Soc., 92, 6151, (1970).

35 Buckingham, D. A., D.M. Foster, L. G. Marzilli and A.M. Sargeson. Inorg.Chem., 9. 11, (1970).

36 Buckingham, D.A., D.M. Foster and A.M. Sargeson, J.Amer.Chem. Soc, 91, 3451, (1969); 91, 4102, (1969).

37 Collman, J. P. and D.A. Buckingham. J.Amer.Chem.Soc., 85, 3039, (1963).

38 Buckingham, D.A., J.P. Collman, D.A.R. Happer and L.G. Marzilli, J.Amer.Chem.Soc., 89, 1082, (1967).

39 Bentley, K.W. and E.H. Creaser, Biochem.J. (1973), 135, 507. Bentley, K.W., J.Biochem, (1976), 153, 137.

40 Meisenheimer, J., Ann. 438, 217, (1924).

41 Ref. 19, p.195.

42 Stewart, J.M. and J.D. Young, Solid Phase Peptide Synthesis, p.32, Freeman, San Francisco.

43 Beyerman, H.C. (1971), Peptides 1969, p.145, North-Holland, New York.

44 Ref. 15, Chapter 6.

45 Williams, and D.H. Busch, J.Amer.Chem.Soc., 87,4644,(1963).

46 Clark, C.R., Private Communication.

47 Alexander, M.D. and D.H. Busch, J.Amer.Chem.Soc., 88, 1130,(1966).

48 Buckingham, D.A., D.M. Foster, L.G. Marzilli and A.M. Sargeson, Inorg.Chem., 9, 11,(1970).

49 Wu, Y and D. H. Busch, J.Amer.Chem.Soc, 92, 3326,(1970).

50 M.Friar, Thesis, M.Sc., Massey University, In print.

51 Buckingham, D.A., C.E. Davis, D.M. Foster and A.M. Sargeson, J.Amer.Chem.Soc., 92, 5571,(1970).

52 Gelotte, B., J.Chromatogr., 3,330,(1960).

53 Schwartz, A.N., A.W.G. Yee and B.A. Zabin, J.Chromatogr., 20, 154,(1965). Fawcett, J.S. and C.J.O.R. Morris, Separation Sci, 1,9,(1966).

54 Biemann, K. Mass Spectrometry Organic Chemical Applications. New York, McGraw-Hill, (1962), p.294.

55 Kent, S.B.H., Peptide Sequencing by Mass Spectroscopy. Thesis, M.Sc., Massey University, 1970.

56 Ref. 15, p.69.

57 Ref. 15, p.68.

58 Ref. 15. p.1.

59 Keyes, W.E., and J.I. Legg, J.Amer.Chem.Soc., 98, 4970,(1976).

60 Walter, M. and L. Ramaley, Anal.Chem., 45, 165,(1973).

61 Beyerman, H.C., C.A.M. Boers-Boonekamp and H. Maassen Van Den Brink-Zimmermannova, Rec.Trav.chim.,1968, 87, 257.

62 Konig, W. and R. Geiger, Chem.Ber., 106, 3626,(1973).

63 Klausner, Y.S. and M. Chorev, J.C.S.Chem.Comm., 1975, 973.

64 Ref. 15., p.79.

65 Buckingham, D.A., D.M.Foster and A.M. Sargeson, J.Amer.Chem. Soc., 91, 4105,(1969).

66 Buckingham, D.A., L.G. Marzilli and A.M. Sargeson, J.Amer.Chem. Soc., 89, 5133,(1967).

67 Reynolds, G., Private communication.

68 Zervas, L., M.Winitz and J.P.Greenstein. J.Org.Chem., 22, 1515,(1957).

69 I.U.P.A.C. Nomenclature of Inorganic Chemistry 1970, 2nd ed. London, Butterworths, 1970, Section 7.34.

70 Cotton, F.A. and G. Wilkinson, Advanced Inorganic Chemistry, Interscience, London, 3rd ed., 1972, p.632.

71 Extracts from the tentative rules of the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature, p.226-243 In Amino-acids, Peptides and Proteins - Volume 2, London, The Chemical Society.

72 Ref. 15., p.80.

73 Ref. 15., p.107.

74 Ref. 15., p.105.