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RESEARCH ESTABLISHMENT

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A NEW N-TERMINAL PROTECTING GROUP FOR PEPTIDE ATTACHMENT TO INSOLUBLE SUPPORTS

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This research was carried out by the author as a Research Fellow at the Research School of Biological Sciences, The Australian National University, Canberra.

ABSTRACT

The cobalt(III) active ester complexes $[Co(en)_2(NH_2CHRCOOR')](ClO_4)_3$, where en is ethylenediamine, R is H or $-CH_2-$ and R' is CH_3, C_2H_5 or $C_3H_7^i$, have been used to provide N-terminal protection before the attachment of peptides to insoluble polymeric supports. The protecting group can be removed readily and quantitatively using normal physiological parameters from peptides attached to non-crosslinked resins.

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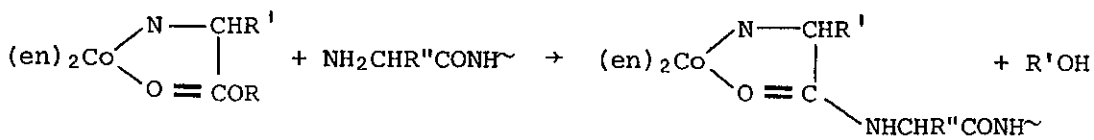
1. INTRODUCTION

Recently, methods have been described which adapt the Edman procedure [Edman 1950] for amino acid sequence determination to a solid phase system. Peptides have been linked by ester [Battaerd & Tregear 1967] or amide bonds [Laursen 1969, Laursen & Bonner 1970] to crosslinked and non-crosslinked resins. Difficulties have arisen using phenylisothiocyanate and methylisothiocyanate N-terminally protected derivatives because of simultaneous cleavage of the C-terminal amino acid-resin linkage under conditions necessary to remove the N-terminal residue [Laursen 1971]. Subsequent attachment and removal of these reagents accentuates the problem during sequence determination. Cobalt(III) complexes of ethylenediamine may be reacted with the α-amino nitrogen atom of amino acids and dipeptides in non-aqueous solution to form stable [cobalt(III) (tripeptide)]$^{2+}$ complexes [Bentley 1972]. In these complexes, the [Co(en)$_2$NH$_2$CHRCO~] moiety is linked *via* a peptide bond, *i.e.*



This work investigates the usefulness of the aminoacido(ethylenediamine)cobalt(III) active ester complexes as acid stable and base labile N-terminal protecting reagents for the attachment of peptides to insoluble supports.

2. EXPERIMENTAL

2.1 Materials and Methods

The resin supports were a crosslinked 2 per cent polystyrene-divinylbenzene copolymer (2% S-DB Pierce Chemicals - Merrifield 1963) and poly[trifluorochloroethylene surface grafted-chloromethylstyrene] (Kel-F-g ICI Aust. Ltd. - Battaerd & Tregear 1967) containing 1.15 meq $CH_2Cl\ g^{-1}$ and 0.075 meq $CH_2Cl\ g^{-1}$ respectively. These resins were aminoacylated with 0.38 and 0.061 meq g^{-1} of valine and 0.36 and 0.057 meq g^{-1} of alanine respectively.

The cobalt(III) monodentate complexes [Co(en)$_2$Br(NH$_2$CH$_2$COOR)]Br$_2$, where R is CH_3, C_2H_5 or $C_3H_7^i$, were synthesised from *trans*-[Co(en)$_2$Br$_2$]Br and the appropriate amino acid ester by a modification of the procedure of Alexander & Busch [1966]. For example, *cis*-[Co(en)$_2$Br(glyOC$_2$H$_5$)Br$_2$: 8.20 g of *trans*-[Co(en)$_2$Br$_2$]Br together with 0.2 g of $CoBr_2.6H_2O$ as

catalyst were ground together with 5.0 mℓ of water. Then 2.32 g of ethylglycinate was added to an equimolar amount of triethylamine in 10 mℓ methanol and, after mixing, this solution was rapidly added to the slurry which was stirred intermittently for 60 min. Ethanol (25 mℓ) was added with stirring and the mixture was filtered. The complex was recrystallised from 0.1 *M* HBr at 80°C. The perchlorate salt was obtained by recrystallising the bromide salt three times from 0.1 *M* hot $HClO_4$ and adding excess $NaClO_4$.

Found: C, 17.34%; H, 4.61%; N, 12.61%; Br, 14.14% (ε_{547} = 79 in water at 25°C).

Anal. Calcd. for *cis*-$[Co(C_2H_8N_2)_2Br(NH_2CH_2COOC_2H_5)](ClO_4)_2$: C, 17.12%; H, 4.49%; N, 12.48%; Br, 14.24%.

The chelated monodentate methyglycinate and isopropylglycinate complexes were similarly prepared.

Found: C, 15.32%; H, 4.08%; N, 12.76%; Br, 13.92%; (ε_{545} = 80 in water at 25°C).

Anal. Calcd. for *cis*-$[Co(C_2H_8N_2)_2Br(NH_2CH_2COOCH_3)](ClO_4)_2$: C, 15.37%; H, 4.24%; N, 12.80%; Br, 14.61%.

Found: C, 18.60%; H, 4.83%; N, 12.45%; Br, 14.19% (ε_{543} = 81 in water at 25°C).

Anal. Calcd. for *cis*-$[Co(C_2H_8N_2)_2Br(NH_2CH_2COOC_3H_7^{i})](ClO_4)_2$: C, 18.79%; H, 4.73%; N, 12.18%; Br, 13.90%.

Monodentate complexes of sarcosine (N-methylglycine) and β-alanine can be prepared in the same manner.

The active ester complexes $[Co(en)_2NH_2CH_2COOR)](ClO_4)_3$ were prepared from the bromoesters by reaction with silver perchlorate, *i.e.* *cis*-$[Co(en)_2Br(NH_2CH_2COOR)](ClO_4)_2$ (5.0 g) in anhydrous methanol (Grignard dried) (30 mℓ) in a blackened Erlenmeyer flask was treated with a 1.1 molar excess of dried $AgClO_4$ (dried with P_2O_5 for 48 h *in vacuo*). After 60 min at 20°C, the solution was filtered through cellulose to remove the precipitated AgBr. An excess of diethylether (sodium dried) was added to the methanolic product to precipitate the complexes.

After standing for 4 h, the products were collected and dried *in vacuo* over anhydrous $Mg(ClO_4)_2$. The quality of the products was tested by dissolving the $[Co(en)_2(NH_2CH_2COOR)](ClO_4)_3$ compound (10 mg) in anhydrous methanol (10 mℓ), then adding a twofold excess of alanine ethyl ester (in methanol, 5 mℓ) and, after about 1 min, bringing the solution to pH 4 with $HClO_4$ (0.1 *M*). The solution was diluted with

water to 50 mℓ and sorbed onto Dowex 50W x 2 (Na^+ form). After elution with 1.0 *M* $NaClO_4$, two orange bands separated, corresponding to the $[Co(en)_2(gly\text{-}L\text{-}alaOC_2H_5)]^{3+}$ and $[Co(en)_2gly]^{2+}$ species. These were collected and identified by proton magnetic resonance and visible spectra. Cobalt analysis indicated that ~ 90-95 per cent of the product was the dipeptide ester complex for each case.

Samples of resin with bound peptide were hydrolysed by the method of Scotchler, Lozier & Robinson [1970] and the hydrolysate was subjected to amino acid analysis using a Beckman 120C analyser.

3. RESULTS

Methods for the preparation of complexes of the form β-$[Co(trien)(aao)_y](ClO_4)_2$, where y is 1-4, by reaction of the appropriate peptide ester with β-$[Co(trien)(OH)(OH_2)]^{2+}$ or $\beta_2[Co(trien)Cl_2]^+$ species have been reported previously [Bentley & Creaser 1974a, 1974b]. The β-$[Co(trien)(peptide)](ClO_4)_2$ complexes may be prepared by acidic ester hydrolysis of the corresponding dipeptide ester complexes. Since this method may be used for the preparation of readily identifiable, analytically pure crystalline materials, it has been used routinely in the present work for coupling peptides up to four residues long.

The preparation of Co(III) complexes from larger peptides by this method was much slower, and the presence of concomitant hydrolysis resulted in a homologous series of peptide complexes of form β-[Co (trien) (n) (n-1) (n-2) peptide]$^{2+}$. For example, in the preparation of β-$[Co(trien)(insulin)]^{2+}$, a minimum of three N-O bonded 2^+ species could be separated on Dowex 50W x 2 (eluent : 0.35 *M* sodium citrate at pH 4.8); this number increased if condensation times were prolonged. Similarly, glucagon gave four products, only one of which could be shown to be a peptide impurity.

The active ester complexes $[Co(en)_2(glyOR)](ClO_4)_3$ react rapidly with exposed amino groups of amino acids and peptides in non-aqueous media to form the corresponding $[Co(en)_2gly\text{-peptide}]^{2+}$ species. In these complexes, the $[Co(en)_2gly]^{2+}$ moiety is linked *via* a peptide bond.

One milligram of insulin, 0.5 mg of hexapeptide (Leu Tyr Met Arg Phe Ala) and 1 mg of glucagon in 1 mℓ of dry DMSO (dimethyl sulphoxide) were each reacted with a tenfold excess of β-$[Co(en)_2(glyOMe)](ClO_4)_3$ for ten minutes at room temperature. To each of these solutions was added 20 mℓ of water and the pH was adjusted to about 3 with HCl (10^{-3} *M*).

The products were separated using Biorad P-2 resin (eluent: 0.001 *M* HCl, column: 60 x 0.9 cm). The Co(III) peptide derivative was eluted with the solvent front. The pH of Co(III) peptide eluates was lowered to 2 with HCl (0.1 *M*) and freeze-dried in 20 mℓ vessels. The freeze-dried preparations were used directly for coupling to the de-protected resins.

3.1 The Coupling of Cobalt(III) Protected Peptides to Resin Supports

(a) Dipeptides

The t-Boc-L-valine and t-Boc-L-alanine resin esters of 2% S-DB and Kel-F-g supports were de-protected with dioxan-4 *M* HCl, neutralised with triethylamine/methylene chloride (10:90 vol.%), washed several times with methylene chloride, acetone and ethanol and dried *in vacuo* for 48 h over anhydrous calcium chloride. The cobalt(III) dipeptide complexes of glycylglycine, glycyl-L-alanine, glycyl-DL-leucine and glycyl-L-phenylalanine were also dried *in vacuo* for 48 h with phosphorus pentoxide.

A quantity of complexes (1 x 10^{-5} mole) was dissolved in 0.2 mℓ of dry dimethylsulphoxide and added to 3 x 10^{-5} mole of available amino groups of the amino acyl resin. Before use, the de-protected aminoacyl supports were swollen by washing with methylene chloride and equilibrated with dicyclohexylcarbodiimide (DCCI) (1.0 mℓ, 50 per cent in methylene chloride) and 1-hydroxybenzotriazole (30 mg $m\ell^{-1}$ ethanol).

After the addition of cobalt(III) dipeptide, the solution was gently agitated for 180 min before filtration and washed three times with DMSO, CH_2Cl_2, ethanol, water and then methanol. The resins were collected, dried *in vacuo* for 48 h over calcium chloride and samples taken for amino acid analysis (Table 1).

(b) Peptides

The lyophilised Co(III) derivatives of insulin A, glucagon and a commercially obtained hexapeptide, prepared by active ester coupling, were taken up in 0.1 mℓ of DMSO and treated in a similar manner to the dipeptide complexes using phenylalanyl aminoacyl resins 25 mg, 2% S-DB or 250 mg of ICI-Kel-F and DCCI as the coupling agent. The results are given in Table 2.

3.2 Removal of the $[Cobalt(III)gly]^{2+}$ Moiety Following Peptide Coupling

Treatment of cobalt-protected resin-bound peptides with 0.2 *M* phosphate buffer at pH 9.6 results in hydrolysis of the N-terminal peptide bond. For example, when the bound hexapeptide referred to above

was treated in this way, over 92 per cent of the terminal glycine was liberated from the Kel-F peptide and 61 per cent from the crosslinked resin. Recovery of all the other amino acids was 100 ± 3 per cent after acidic hydrolysis.

4. DISCUSSION

Using either the $(en)_2$ or the trien cobalt complexes as N-protecting agents, it was possible to couple small amounts of peptides up to the size of glucagon to both Merrifield and Kel-F resins. However, it was only possible to remove the N-terminal glycine cobalt complex completely when the non-crosslinked Kel-F resins were used. It is probable that the hydrophobic nature of the crosslinked styrene-divinylbenzene copolymer allows cleavage of the N-terminal cobalt amino acid complex only with great difficulty.

The coupling of peptides to insoluble supports after initial protection of the N-terminal residue by the β-*cis* hydroxyaquo cobalt(III) procedure results in concomitant hydrolysis. The active ester procedure does not suffer from this drawback and essentially gives a single cobalt peptide derivative.

The specificity of the two cobalt protecting groups for the N-terminal amino acid is not absolute. The trien species will only couple with the N-terminal amino acid and may have problems when N-terminal aspartic acid is present [Bentley 1976]. The reactivity of the active ester species is such that they could be expected to react not only with N-terminal NH_2 groups but with internal NH_2 side chain groups. However, in practice this would not be significant as all the cobalt glycine residues would be cleaved when the pH was raised to 9.6.

The protection procedure seems to hold promise for the attachment of peptides to solid supports, for example, before sequential degradation. In this context, it is possible to degrade attached dipeptides by the cobalt hydroxyaquo hydrolysis in a quantitative manner comparable to that shown for degradation of the same dipeptide in free solution.

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TABLE 1

Coupling of β-[Co(trien)(dipeptide)]$(ClO_4)_2$ Complexes to Crosslinked and Non-Crosslinked Resins

Coupling Agent: DCCI with 1 hydroxybenzotriazole catalyst (five times molar excess). The percentage attachment observed assumes that all sites were de-protected and available for coupling.

Complex*	Resin			
	Aminoacyl-Merrifield % Attachment		Aminoacyl-Kel-F-g-styrene % Attachment	
	Val	Ala	Val	Ala
glycylglycine	74	80	81	78
glycyl-L-alanine	71	-	77	-
glycyl-DL-leucine	66	74	68	71
glycyl-L-phenylalanine	70	77	69	74
L-leucylglycine	76	80	82	82
L-phenylalanyl-L-phenylalanine	78	76	78	82
glycyl-L-leucyl-L-tyrosine	81	84	86	80

**The differences observed in coupling efficiency arise from steric hindrance from the C-terminal amino acid side chain.*

TABLE 2

Coupling of Cobalt(III) Protected Insulin A, Glucagon and a Six Unit Peptide (Sigma) to Solid Supports

The attachment figure refers to the amount of peptide attached/amount of peptide made available (in the presence of a large excess of resin sites).

Peptide	% Attachment (Merrifield-phe)	% Attachment (Kel-F-phe)
$(en)_2$gly-Co-insulin	46	62
$(en)_2$gly-Co-hexapeptide	58	71
$(en)_2$gly-Co-glucagon	61	77