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The Use of Crown Ethers in Peptide Chemistry IV. Solid Phase Synthesis of Peptides Using Peptide Fragments Nα Protected With 18-Crown-6

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Abstract: The conditions under which peptide synthesis by the fragment condensation approach in the solid phase can be carried out using crown ethers as non-covalent protecting groups for the Nα amino group of peptides were determined. The dipeptide Gly-Gly was complexed with 18-crown-6 to establish the feasibilty of this new protection scheme and to optimise the reaction conditions. Nearly quantitative incorporation of the complex onto resin-bound amino acids possessing either proline or an Nα-alkylated amino acids was achieved using DCM as the activation and coupling reactions solvent. The use of DMF as the solvent and resin-bound primary amino acids were found detrimental to the reaction yields due to the removal of the crown ether protection. Extending the length of the peptide fragment to model pentapeptide complexes bearing no reactive functionalities on the side-chain gave essentially quantitative incorporation yields in the coupling reactions to both resin-bound amino acids and short peptides. The non-covalent nature of the protection afforded by the crown molecule allowed its mild removal from resin-bound complexes by rapid treatments with 1% DIEA solutions. Thus the continuation of chain assembly was possible. The results obtained served as the basis for extending the concept of non-covalent protection to the side chains of Lys and Arg containing peptides.

INTRODUCTION

In previous work the concept of non-covalent protection with crown ethers of the α-amino group of amino acids and small peptide has been introduced[1-3]. The feasibility of this protection scheme for the solution synthesis of peptides by the fragment condensation approach has been demonstrated with the synthesis of an enkephaline analogue[3]. The advantages which may arise from this protection scheme are readily realised when the difficulties encountered in the purification of protected peptides are taken into account. Thus fully protected peptides are poorly soluble and therefore difficult to purify and characterise[4]. Post-purification protection with crown ether of either completely deprotected peptides or partially protected fragments would circumvent these problems. Furthermore, by virtue of the solubility in organic solvents which can be achieved by protecting amino acids or peptides with the crown molecules[1-3,5], it is possible to conduct activation and coupling reactions in either DCM or DMF where minimally protected peptides are often poorly soluble[6].

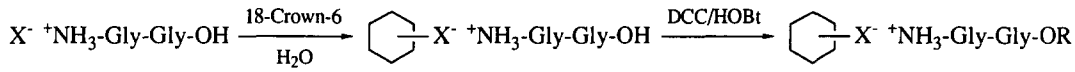
In this paper and in preparation for extending the crown ether protection scheme to peptide fragments containing Lys and Arg residues[8], the solid-phase synthesis of peptides protected at their N^{α}-amino group with crown molecules is described. Initial studies conducted with a di-glycine complex were carried out to comprehend the limits and drawbacks of this approach. The understanding of the mechanisms at the basis of the

side reactions encountered during initial experiments made it possible to design a simple and efficient synthetic protocol which was then used for the nearly quantitative incorporation of larger model peptides onto resin-bound amino acids and peptides.

RESULTS AND DISCUSSION

Initial studies. Preliminary experiments were carried out using the Gly-Gly dipeptide complexed with 18-crown-6 (**1a** and **1b**), which was chosen in order to minimise the effects of steric hindrance on the coupling reactions and to eliminate racemisation, a phenomenon not considered in this study. Activation was performed for 20 mins using 1 eq. each of DCC and HOBt (Scheme 1). A polyamide-based resin was used, prederiviatised with the amino acid of choice. The coupling reactions were conducted for 1 hr in either DCM or DMF. The results of the coupling reactions are summarised in Table 1 and served to show that superior coupling was achieved in apolar solvents like DCM. In particular these initial experiments led to the following conclusions (i) the nature of the complex counterion (i.e. tosylate vs trifluoroacetate) did not affect the solubilty properties of the complex nor its acylating ability, (ii) the yields of the coupling reaction did not appear to depend on the nature of the amino acid bound to resin nor the number of residues attached to the resin, (iii) DCM as the solvent for activation and coupling reactions gave the largest incorporation yields and (iv) coupling reaction yields reached their maximum within the first 30 mins of the addition of activated complex to resin-bound amino acid.

The relatively poor coupling yields, especially with respect to reactions conducted in DMF, were not the result of incomplete activation, as demonstrated when aliquots of the reaction mixture were removed at intervals during the coupling reaction. Each aliquot of reaction mixture was then transferred to freshly deprotected resin-bound Tyr and the coupling efficiency analysed by amino acid analysis (AAA). The results indicated that although maximal incorporation of **1b** in the original reaction had been achieved, the remaining unreacted dipeptide complex was still capable of acylating to fresh resin, even after 24 hrs (data not shown).



1a X = tosylate

1b X = trifluoroacetate

= 18-crown-6

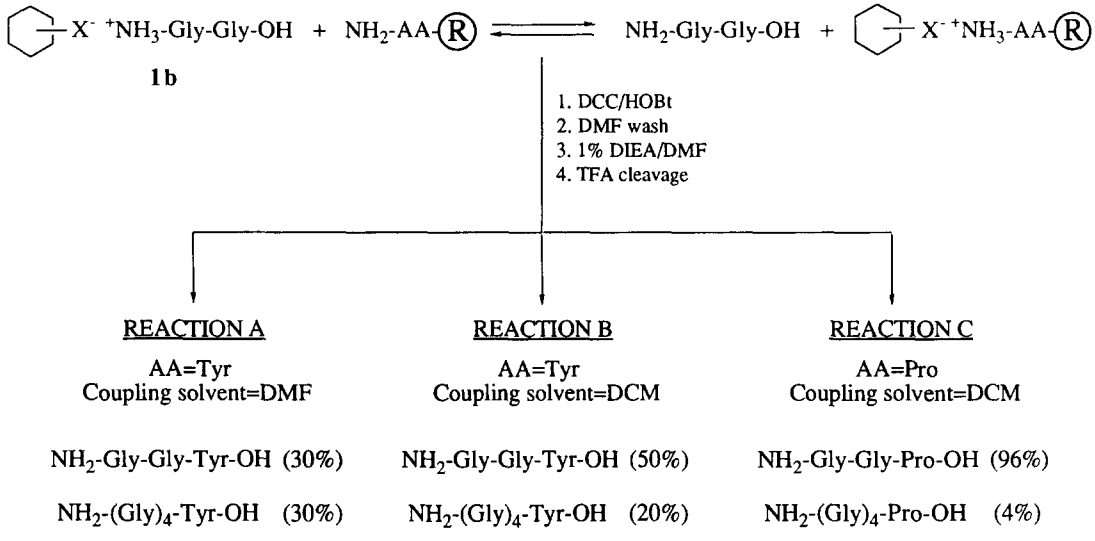
Scheme 1

Table 1. Summary of the preliminary coupling reactions using crown ether/amino acid and peptide complexes.

Peptide Complex	Resin	Resin-bound AA/Peptide	Solvent		% Coupling (AAA)
			Activation	Coupling	
1a	PA	Y	DMF	DMF	30%
1b	PA	Y	DMF	DMF	30%
1b	PA	G-A-Y	DMF	DMF	30%
1b	PA	Y	DCM	DMF	50%
1a	PA	Y	DCM	DCM	70%
1b	PS	Y	DCM	DCM	90%

PA = polyamide resin; PS = polystyrene resin

Since it is known that polyamide-based resins do not swell efficiently in DCM, polystyrene-based (WANG) resin prederivatised with Tyr was used. Applying the same conditions described previously the coupling efficiency was increased from 70% to 90% as measured by AAA. Acid cleavage of the peptidyl-resin, followed by analytical reversed-phase HPLC of the crude material revealed however that the amount of free Tyr was about 30% and larger than that observed by AAA. Furthermore, together with the correct Gly-Gly-Tyr peptide (50%), 20% of the double incorporation product $(Gly)_4$-Tyr was isolated from the reaction mixture (Scheme 2). The existance of the pentapeptide in the crude peptide mixture was ascribed to a deprotection reaction either at the level of activated dipeptide complex or resin-bound Gly-Gly-Tyr complex.



Scheme 2

In previous work it has been shown that when amino acids complexed with 18-crown-6 are activated with DCC, they deprotect and then polymerise[1]. Since these reactions can be prevented by increasing the distance which in the amino acid separates the charged amino group from the carboxylic group[1], a deprotection reaction during activation of the dipeptide **1b** seemed unlikely. Furthermore, when a solution containing freshly activated **1b** was lyophilised and the resulting product analysed by MS and NMR, the $(Gly)_4$ tetrapeptide was not detected, consistent with deprotection taking place during the coupling reaction (data not shown).

Equilibria involving the complexed forms of Gly-Gly and resin-bound Tyr were then considered (Scheme 2). These would be influenced by the nature of both the reaction solvent (i.e. DCM and DMF) as well as the amino acid on the resin support. Thus the stability of crown ether complexes increases in low-polar solvents because of the decreased competition between solvation of the cation and coordination of the latter by the crown ring[8]. Conversely, in solvents of greater polarity such as DMF the charged ammonium ion and its counterion form a "looser" ion pair and the likelihood of a simultaneous exchange of a proton and the crown molecule between **1b** and resin-bound amino acid should increase. The nature of the amino acid should also play a role in these equilibria since secondary amines form complexes with crown ethers which are less stable than those formed by primary amines[9].

To verify the equilibrium hypothesis two new experiments were carried out. The first involved coupling **1b** to tyrosine in DMF. RP-HPLC of the crude peptide mixture indicated that equivalent amounts of Gly-Gly-Tyr and $(Gly)_4$-Tyr were obtained, which contrasted with a much larger ratio of tri- to pentapeptide (ie. 5:2), deriving from the use of DCM (see above and Scheme 2).

In the next experiment resin-bound proline was coupled to **1b** in DCM. AAA and RP-HPLC of the reaction product revealed that the formation of the tripeptide, Gly-Gly-Pro, had gone nearly to completion, with less than 4% of double incorporation product recovered from the crude peptide mixture (Figure 1A and Scheme 2). These results were consistent with the equilibrium hypothesis for the deprotection mechanism and suggested the conditions under which maximal yields of the desired product could be obtained.

In summary, to maximise the coupling reaction yields and reduce to a minimum the formation of by-products, the following were required (i) DCM as the solvent for both activation and coupling reactions (ii) the use of polystyrene-based resins and (iii) the amino acid proline as the N-terminal residue of the resin-bound fragment. Furthermore using four-fold excess of activated peptide complex over available amino groups on the resin, the yield of the coupling reaction reached its maximum value within the first 30-45 minutes of reaction time. It should be noted that subsequent repetition of the reaction between **1b** and proline indicated that the same levels of efficiency as obtained above were best reproduced when an excess of 18-crown-6 was added to the reaction mixture. The reasons for this empirical finding were not further investigated although it was assumed that the excess crown ether would help the peptide stay in the complexed form.

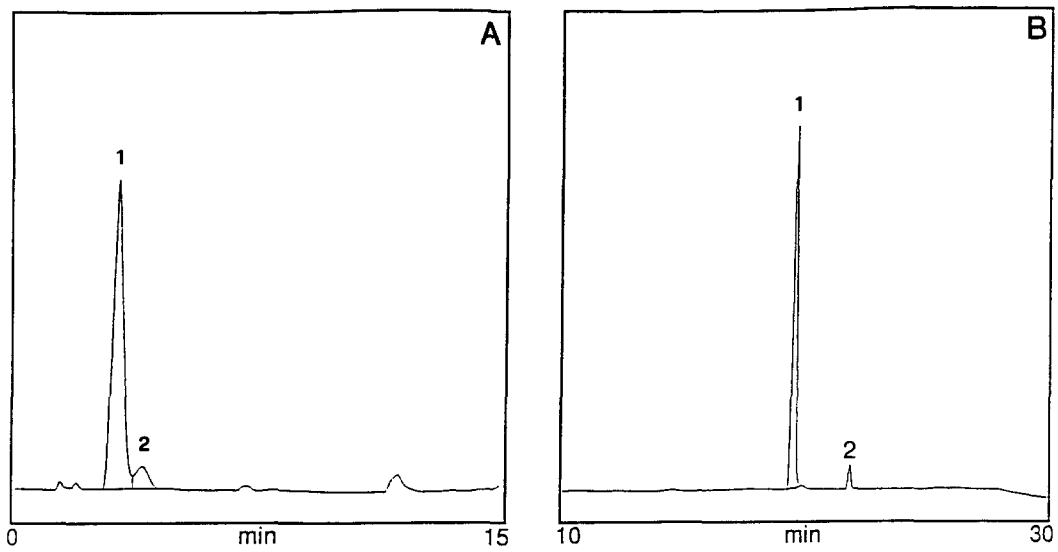


Figure 1. HPLC chromatograms of the crude material deriving from the coupling reactions involving resin-bound amino acid/peptide and oligopeptide complexes with crown ether. (A) Coupling di-Gly complex **1b** to P-resin (Wang) in DCM; Peak 1 = expected peptide, peak 2 = double coupled peptide; HPLC system A, (B) Coupling Val-Ala-Ala-Phe-Gly-Pro *p*-toluenesulphonic acid salt to P-resin (Wang) in DCM; HPLC system A.

The replacement of proline with N-alkylated amino acids.

Extending the crown ether approach to the synthesis of peptides other than those described here requires the presence of a proline as the N^{α}-terminal residue of the resin-bound fragment. A solution to this limitation was suggested by the recently introduced doubly N^{α} protected amino acids, which were developed to facilitate SPPS by decreasing aggregation between growing peptide chains[10]. One such derivative is Fmoc-(FmocHmb)Ala activated as *o*-pentaphenyl ester (Hmb=2-Hydroxy-4-methoxybenzyl), which was used here as an example of secondary amino acids other than Pro.

After addition of the Hmb derivative to Tyr(tBu)-resin and removal of the N^{α} Fmoc protecting group, **1b** activated as symmetrical anhydride was added. The choice of this method of activation was suggested by the reported, negative effects towards acylation due to steric hindrance of N^{α} di-substituted amino acids[10]. After a 24 hr reaction time, AAA indicated that the complex had coupled with an efficiency of about 70%. Since in the above experiments activation of **1b** to symmetrical anhydride had produced inferior results, the resin peptide material was further reacted with **1b** freshly activated with DCC/HOBt. The yields of the reaction were almost quantitative as indicated by AAA and HPLC of the crude peptide mixture (Fig. 2). The peaks were collected and shown by MS to correspond to correct tetrapeptide (peak 1), unreacted Ala-Tyr (peak 2) and material of non-peptidic origin (peaks 3 and 4). Interestingly, unlike the reactions which involved proline, the double incorporation product was not detected. The basic character of the Hmb derivative which is more similar to that of primary amino acids than to proline was probably the reason for this different behaviour.

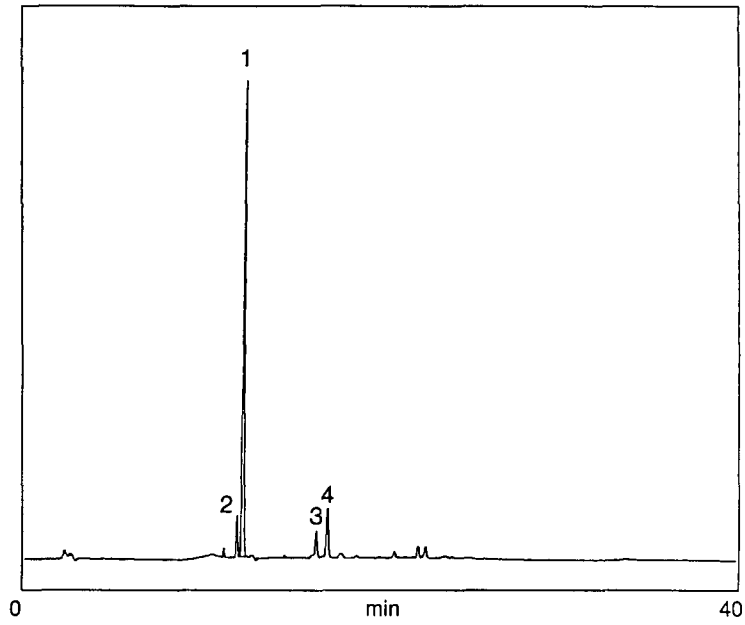


Figure 2. HPLC chromatogram of crude material from the reaction between **1b** and resin-bound Hmb-Ala-Tyr(t-Bu) dipeptide. The main product (labelled 1) was isolated and shown to correspond to the expected tetrapeptide. HPLC system B.

Fragment condensation using larger peptides.

It was the aim of this work to show the feasibility of the protection with crown ethers for the synthesis of peptides by the protected fragment approach. Thus, further attempts were made to demonstrate whether the results obtained with the di-Gly complex **1b** could be equally applied to longer sequences.

The first involved the model pentapeptide Val-Ala-Ala-Phe-Gly and resin-bound proline. The deprotected pentapeptide TFA salt, synthesised using conventional Fmoc chemistry and purified by RP-HPLC, was dissolved in water and treated with one equivalent of 18-crown-6. The complex obtained after lyophilisation was found to have the expected molecular weight but was not completely soluble in the preferred solvent, DCM. To improve its solubility, the TFA counterion was exchanged for *p*-toluensulphonic acid which had been shown to increase the solubility of amino acid complexes through charge delocalisation[1-3]. As expected the new pentapeptide complex thus obtained was completely soluble in DCM. For the subsequent coupling reactions the same conditions which had led to nearly quantitative incorporation of **1b** onto resin-bound proline, were used. The only exceptions were the time allowed for the coupling reaction which was increased to 24 hrs and the mixing of the coupling suspension which was carried out through vortexing. At the end of the reaction between Val-Ala-Ala-Phe-Gly complexed with 18-crown-6 and proline, an aliquot of resin was hydrolysed and the

remainder treated with acid to release the peptidic material. AAA analysis indicated that the reaction had gone nearly to completion (95%). This was confirmed by HPLC of the crude peptide which showed that only one major component (peak labelled 1, Fig. 1B) with the expected MW had been obtained from the reaction. The peak labelled 2 (Fig. 1B) was found to correspond to double coupled product, $(Val\text{-}Ala\text{-}Ala\text{-}Phe\text{-}Gly)_2\text{-}Pro$.

Next, the influence of the length of resin-bound peptide on the coupling yields was investigated using the same pentapeptide complex from above and the Pro-Asp(tBu)-Leu-Tyr(t-Bu) model tetrapeptide **2**. After removal of the N-terminal protection, the resin-bound tetrapeptide was treated with 4-fold excess of activated pentapeptide complex. At the end of the reaction period the coupling yields, as measured by AAA, were larger than 90%. This result was essentially confirmed by the combination of HPLC of crude clevage product (Fig. 3A) and MS of the isolated peaks labelled 1, 2 and 3. The results of the latter analysis revealed that the crude peptide mixture contained, together with the expected nonapeptide (labelled 2; 88%), about 2% of the double incorporation product (labelled 3) and about 10% of unreacted tetrapeptide (labelled 1).

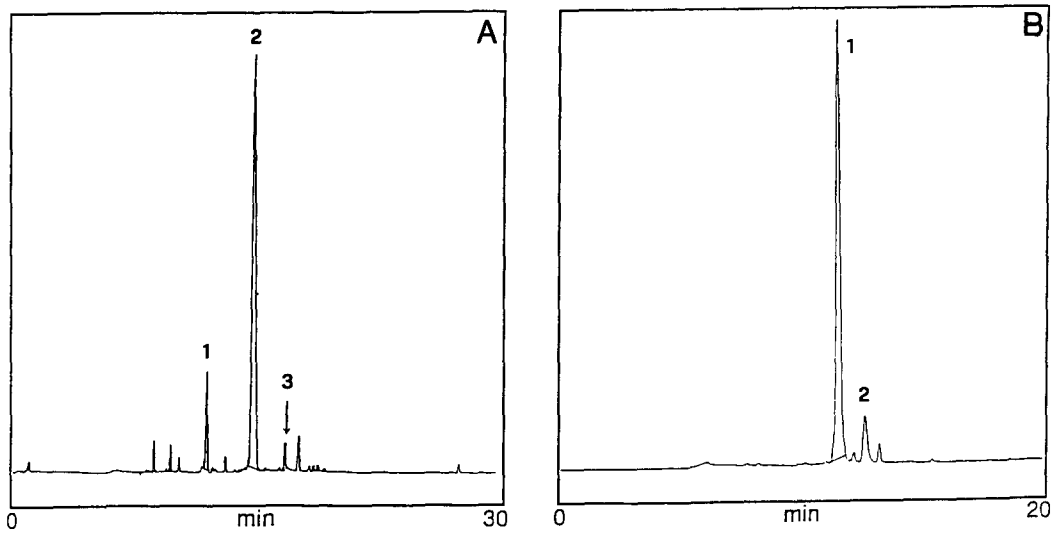


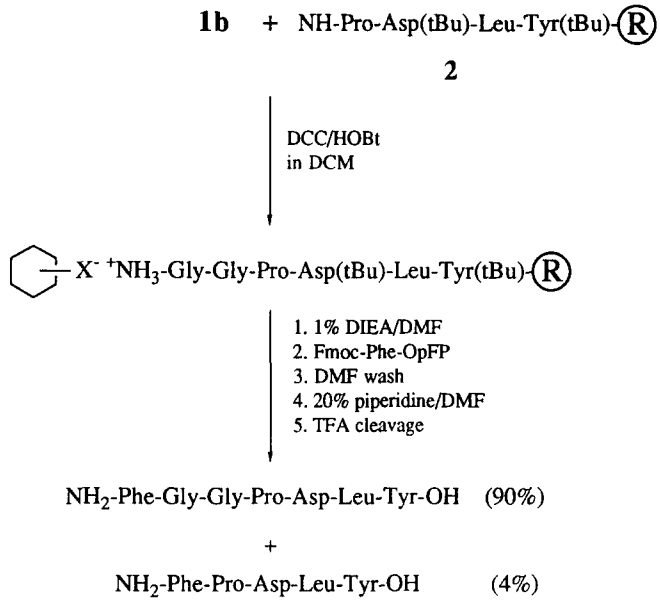
Figure 3. HPLC chromatograms of the crude material deriving from the coupling reactions involving resin-bound amino acid/peptide and oligopeptide complexes with crown ether. (A) Coupling Val-Ala-Ala-Phe-Gly *p*-toluenesulphonic acid salt to Pro-Asp-Leu-Tyr-resin (Wang); Peak 1 = unreacted peptide, peak 2 = expected peptide, peak 3 = double coupled peptide; HPLC system B, (B) Coupling Phe to Gly-Gly-Pro-Asp-Leu-Tyr-resin (Wang) after deprotection of 18-crown-6 with 1% DIEA/DMF; Peak 1 = expected peptide, peak 2 = Phe-Pro-Asp-Leu-Tyr; HPLC system D.

Dipeptide complexes with other crown molecules.

Attempts to solubilise a complex between 18-crown-6 and peptide fragments larger than those described previously or peptides containing unprotected Ser were unsuccessful in most cases (data not shown). Therefore in an effort to improve the solubility of the peptide complexes in DCM other more lipophilic crown compounds were considered. The first crown molecule investigated was a mono-benzo 18-crown-6 derivative which was complexed to Gly-Gly in the usual manner. The product thus obtained although correct by MS was highly hygroscopic and the resulting handling difficulties prevented its further consideration. As an alternative a di-benzo 18-crown-6 complex was formed with Gly-Gly and shown to be stable after lyophilisation and soluble in DCM. Coupling of this new complex to resin-bound proline gave a yield of about 50%. To verify whether the lower than expected yields were to be ascribed to steric hindrance, the same reaction was repeated this time adding to the final solution an excess of 18-crown-6. This, was hoped, would partly exchange with the di-benzo compound thus reducing the postulated steric hindrance. Indeed the results of two separate experiments gave an average incorporation of about 80%, thus confirming this hypothesis.

Deprotection of resin-bound complex.

As with more conventional protection schemes, the use of crown ethers for peptide synthesis requires the removal of the protecting group after coupling. During solution synthesis[3], this was achieved using potassium ions which compete efficiently with ammonium ions for the crown molecule[8]. In the experiments described here, where the use of aqueous solutions of KCl was not practicable and due to the reduced affinity of crown molecules for free amines[8], deprotection with organic base was carried out. The following describes an optimised protocol for the removal of the crown molecules using diisopropylethylamine (DIEA) in DMF as illustrated in Scheme 3. DIEA was ideally suited for this purpose since it is compatible with selective deprotection of peptides bearing both the crown ether and Fmoc group. After coupling **1b** to **2** (Scheme 3), the peptidyl-resin was washed with DMF, treated twice for 3 minutes each with a 1% solution of DIEA in DMF and finally mixed with four eq.s of Fmoc-Phe pentafluorophenyl ester. The reaction was allowed to proceed in DMF for 1 hr after which time AAA analysis performed on resin-bound peptide product indicated that the incorporation of both di-Gly and Fmoc-Phe had gone nearly to completion. This was confirmed by HPLC of the crude peptide mixture (Figure 2D) which showed that a major product (labelled 1) accounting for more than 90% and possessing the molecular mass expected for the heptapeptide had been obtained. The minor component (6%) in Fig. 3B (labelled 2) was also isolated and found to be the Phe-Pro-Asp-Leu-Tyr pentapeptide presumably arising from the incompleteness of the coupling reaction between **1b** and **2**.



Scheme 3

CONCLUSIONS

The synthesis of peptides by the fragment condensation approach is generally hampered by both the poor solubility of fully protected peptides in those solvents used for purification and the poor solubility of minimally protected fragments in DCM or DMF in which coupling reactions are normally carried out. Crown ethers, which are known to solubilise ammonium ions in apolar solvents[5], offer the opportunity to combine this property with the use of minimally protected fragments thus circumventing the solubilty problems mentioned above. Consequently the feasibility of the crown protection scheme for the solution synthesis of peptides has been evaluated[1-3]. In this work model di- and pentapeptides were complexed with 18-crown-6 and their ability to couple as HOBt active esters or symmetrical anhydrides to either resin-bound amino acids or peptides was examined. Initial attempts using a Gly-Gly complex indicated that while this new method of protection was feasible for the solid-phase synthesis of peptides, the coupling reaction yields were not satisfactory. The main obstacle to improved yields was identified in a deprotection mechanism which by exposing the free amino group was leading to the formation of double incorporation products. The optimal protocol devised required the use of DCM for all reactions involving the complexes. Thus the latter were less stable in DMF or DCM/DMF mixtures where more readily generated the corresponding free base. The second key factor identified for optimal coupling efficiency was the nature of the amino acid involved in the coupling reaction with the complex. When this was a primary amino acid competition for the crown molecule led to deprotection of the complex resulting in double incorporation products. However the replacement of primary amino acids with either proline or doubly protected

residues both having a reduced affinity for the crown ether, abolished almost completely the deprotection of the peptide complexes and gave nearly quantitative yields of the desired coupling products.

The application of the optimal reaction conditions to peptides larger than di-glycine required only extending the reaction time to 24 hrs. Thus in all cases attempted which involved the use of a pentapeptide complex and resin-bound tetrapeptide the incorporation yields were essentially quantitative.

The non covalent nature of the interaction between the crown molecules and amino groups permitted then the use of extremely mild basic conditions (ie. 1% DIEA) for the removal of the crown ether protection.

Finally crown ethers other than the 18-crown-6 molecule were used to improve the solubility in organic solvents of peptide complexes. It was thus found that protection with the di-benzo 18-crown-6 derivative was equally efficient although the coupling yields were inferior probably due to greater steric hindrance caused by the more bulky di-benzo crown derivative. However, addition of 18-crown-6 to a suspension of di-benzo complex and resin-bound proline increased the yields from 50% to about 80%, indicating that an equilibrium existed between the two crown molecules.

The results described here served to extend the concept of a non-covalent protection scheme to peptides containing positively charged side chain groups for the condensation of minimally protected fragments[7].

EXPERIMENTAL

The solvents used for the synthesis of the peptides and the coupling reactions were purchased from either Merck or Applied Biosystem (ABI), Warrington UK. DMF was kept over molecular sieves while AcCN and DCM over Na_2SO_4 and filtered prior to use. The resins used for the reactions had the following substitution values: Fmoc-Ala-Pepsyn KA100 (0.09 mmol/g) Milligen; Fmoc-Tyr-Pepsyn KA100 (0.1 mmol/g) Milligen; Fmoc-Tyr-Wang (0.59 mmol/g), Fmoc-Pro-Wang (0.57 mmol/g) NovaBiochem; Fmoc-Gly-Sasrin (0.65 mmol/g) Bachem, Switzerland. Reagents: N-hydroxybenzotriazole (HOBT), dicyclohexylcarbodiimide (DCC) and all Fmoc and t-Boc amino acid derivatives were from Novabiochem; Gly-Gly and propionic acid-hydrochloric acid 50:50 (v/v) from Sigma; the crown ether molecules from Aldrich; *p*-toluenesulphonic acid·H_2O (TosOH) from Merck and TFA, TFMSA and piperidine from ABI, UK.

HPLC analysis. In all solvent systems used eluent A was H_20/0.045% TFA, eluent B = AcCN/0.036% TFA.
System A) HPLC Shimadzu LC10, λ=220 nm; Column Vydac C_{18} RP (150x4.6 mm);
Gradient: t=0' 0%B, t=10' 5%B, t=20' 50%B, t=22' 50%B, t=24' 100%B. Flow rate 1ml/min.
System B) HPLC Kontron Pump System 420, λ=214 nm; Column: Shiseido C_{18} RP (250x4.6 mm);
Gradient: t=0' 0%B, t=40' 80%B, t=41' 100%B. Flow rate 1ml/min.
System C) HPLC Kontron Pump System 420 λ=214 nm; Column Vydac C_{18} RP (250x10 mm);
Gradient: t=0' 0%B, t=10' 10%B, t=40' 20%B, t=50' 50%B, t=60' 100%B. Flow rate 2.5 ml/min.
System D) HPLC System Shimadzu LC10 λ=220 nm; Column Vydac C_{18} RP (150x4.6 mm);
Gradient: 0-100% in 30'; Flow rate 1ml/min.

Mass Spectrometry. Spectra were aquired on a TRIO 2A instrument (VG MASSLAB, Altrincham, UK) in the Fast Atom Bombardment (FAB) mode using 8KeV Xe atoms and *m*-nitrobenzyl alcohol as matrix.

Amino acid analysis (AAA). Samples for AAA were prepared by acid hydrolysis [propionic acid/hydrochloric acid (1:1); 21 hr; 110°C] under vacuum and in the presence of 1% phenol. Analysis of the hydrolysate was carried out using a Beckman System Gold instrument equipped with a Spherogel TM AA-Na^+ (3mmx25cm) column.

Peptide synthesis.

Peptides were synthesised on either an ABI 430A instrument or LKB Biolynx 4175 semi-automatic instrument using standard Fmoc chemistry. When necessary, they were cleaved from the resin support using 95% TFA and purified by conventional semi-preparative reversed phase HPLC (system C). Amino acid analysis and mass spectrometry were used to identify the peptides structure.

Preparation of the peptide complexes. The complexes were prepared as previously described[1-3]. An example of the procedure is here described for the pentapeptide Val-Ala-Ala-Phe-Gly complex with 18-crown-6.

25.5 mg (44 μmoles) of purified pentapeptide as the TFA salt was dissolved in 1 ml of water; while stirring 11.7 mg (44 μmoles) of 18-crown-6 was added. After 1 hr stirring at r.t. 44.1 μmoles of *p*-toluene-sulphonic acid mono hydrate were added, the solution further stirred for additional 30 mins and finally lyophilised. The lyophilised material was redissolved in water and subjected again to lyophilisation. The latter procedure was repeated three times to eliminate any trace of acid. The complex, which was not further purified, was analysed by FAB-MS: Expected 727.9, [M+1] Found 729.1. TFA·Gly-Gly (**1b**): MS: Expected:396.5; Found 397.1; TFA/mono-benzo 18-crown-6·Gly-Gly. MS: Expected 444.1, [M+1] Found 444.9

Coupling reactions. The protocol used for the synthesis of peptides using crown ether complexes was, unless otherwise specified in the text, always the same. The synthesis of Phe-Gly-Gly-Pro-Asp-Leu-Tyr heptapeptide is used as an example. Amino acid composition and FAB-MS results for the other peptides discussed in the text are given after the example.

17.5 mg (34.2 μmoles) **1b** was dissolved in 340 μl of DCM. To the solution were then added sequentially 4.6 mg (34.2 μmoles) of HOBT and 7 mg (34.2 μmoles) of DCC. The mixture was left stirring for eight minutes and then filtered to remove DCU. The clear solution was then transferred to the reactor which contained the tetrapeptide **2** bound to polystyrene resin (Wang resin, substitution value 0.59 mmol/g). The latter had been previously treated with a solution of 20% piperidine in DMF to remove the proline Fmoc protecting group, washed several times with DMF and DCM and then left swelling in DCM for 30 mins. The coupling reaction was carried out for 45 mins while vortexing. At the end of the reaction the resin was washed with DMF and then treated twice (3 mins each) with 1% DIEA in DMF to remove the crown protection. The resin was then washed with DMF resuspended in the same solvent and then treated with 4 eq.s of Fmoc-Phe-O-pFP. The reaction was allowed to proceed for 1hr. The final peptide was first reacted with 20% piperidine to remove the Fmoc group

and then cleaved from the resin by acid treatment (95% TFA, 5% H_2O; 1.5hrs). Distillation of TFA yielded an oily material which solidified upon trituration with anhydrous diethylether. Purification of the peptide was accomplished by semi-preparative HPLC (system C). MS: Expected 767.8; Found [M+1] 769.1.

Gly-Gly-Tyr. MS: Expected 295.3; Found [M+1] 296.1. AAA: Gly(2) 2.0, Tyr(1) 1.0

Gly-Gly-Pro. MS: Expected 229.2; Found [M+1] 229.9. AAA: Pro(1) 1.1, Gly(2) 1.9

Gly-Gly-Ala-Tyr. MS: Expected 336.4; Found [M+1] 367.1. AAA: Gly(2) 2.1, Ala(1) 1.0, Tyr(1) 1.0

Val-Ala-Ala-Phe-Gly-Pro. MS: Expected 560.7; Found [M+1] 561.1. AAA: Pro(1) 1.1, Gly(1), 1.0, Ala(2) 2.0, Phe(1) 1.0.

Val-Ala-Ala-Phe-Gly-Pro-Asp-Leu-Tyr. MS: Expected 952.1; Found [M+1] 952.5. AAA: Asp(1) 1.1, Pro(1) 1.1, Gly(1), 1.0, Ala(2) 1.9, Val(1) 0.9, Leu(1) 1.1, Tyr(1) 1.1, Phe(1) 0.9.

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