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The Use of Crown Ethers in Peptide Chemistry-V. Solid-phase Synthesis of Peptides by the Fragment Condensation Approach using Crown Ethers as Non-covalent Protecting Groups

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Abstract: We have previously described the conditions by which peptide synthesis by the solid-phase fragment condensation approach can be carried out using crown ethers as non-covalent protection for the N^{α}-amino group. Here we demonstrate that the procedure can be extended to large, partially protected peptide fragments possessing free Lys and/or Arg residues. The first step was to ensure that complex formation on the side chain of amino acids was not detrimental to the methodology and exhibited the same solubility and coupling properties as N^{α}-complexed peptides. Thus, a model hexapeptide was synthesized using Fmoc chemistry containing Lys and Arg residues, which, when complexed with 18-Crown-6, was readily soluble in DCM and coupled quantitatively to a resin-bound tetrapeptide. Two tripeptides were then prepared, one containing a free Ser residue, the other free Tyr, to examine the possible occurrence of side reactions. After coupling using standard conditions only the former tripeptide exhibited the formation of the *O*-acylation by-product (5%). Another model hexapeptide containing Lys, Tyr, Ser and Asp protected with a TFA-stable adamantyl group was complexed with 18-Crown-6 and coupled to the resin-bound tetrapeptide with near quantative yield. Extending the length of the peptide to 21 and 40 residues, which represent sequences Gly52 to Leu72 (21-mer) and Pro33 to Leu72 (40-mer) from *Rattus norvegicus* chaperonin 10 protein, respectively, resulted in partially protected fragments that were readily soluble in water, thus enabling purification by RP-HPLC. Complexation with 18-Crown-6 gave two highly soluble products that coupled to resin-board tetramer with 68% and 50% coupling efficiencies for the 21-mer and 40-mer, respectively. Treatment with 1% DIEA solutions followed by acidolytic cleavage and purification of the major product confirmed that the correct product had been formed, when analysed by amino acid analysis and ESI-MS. These results served to extend the methodology of non-covalent protection of large partially protected peptide fragments for the stepwise fragment condensation of polypeptides.

Keywords: crown ether; fragment condensation; peptide synthesis

Abbreviations: AAA, amino acid analysis; Ada, 2-adamantyl; 18-Crown-6, 1,4,7,10,13,16-hexaoxa-cyclooctadecane; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; ESI-MS, electrospray ionisation mass spectrometry; FAB-MS, fast atomic bombardment mass spectrometry; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high performance liquid chromatography; NMP, *N*-methyl-pyrrolidone; TFE, 2,2,2-trifluoroethanol; TFMSA, trifluoromethane sulphonic acid; XCE, counterion/18-Crown-6 complex.

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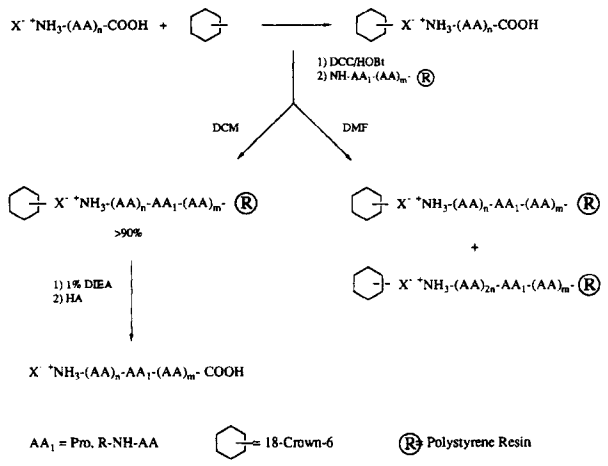
INTRODUCTION

Convergent peptide synthesis which requires the assembly of fully protected peptide segments, their purification and coupling on either a solid support or in solution is often limited by the poor solubility of the peptide intermediates.

With the aim of overcoming this drawback we have introduced the concept of non-covalent protection with crown ethers of amino acids and peptides [1–4]. Thus, peptide intermediates can be obtained either in their free form or with minimal protection (e.g. the side chains of Asp and Glu residues) and after purification using conventional chromatographic techniques reprotected with the crown molecules. The latter form stable complexes with charged amino groups [5]. Furthermore, complexes thus obtained become soluble in most organic solvents [1–5]. This novel protection scheme has been applied to the α-amino group of amino acids (Scheme 1) as



Scheme 1

well as peptides bearing no sensitive functions on their side chains and it has been shown to be suitable for both solution and solid-phase peptide synthesis by the fragment condensation approach [3, 4]. In particular, the conditions which minimize the extent of side reactions (e.g. 'deprotection' of the amino group followed by double incorporation reactions) have been identified. In the case of solid-phase synthesis maximal and nearly quantitative coupling yields were obtained using DCM as the reaction solvent and resin-bound peptides containing the N^{α}-terminal residue as either Pro or residues having their N^{α} doubly substituted (Scheme 1) [4]. The latter were found necessary to avoid the shift of crown complexation from the amino terminal of the peptide segment in solution to that of the resin-bound fragment [4]. Thus, the stability of crown ether–amine complexes decreases with substitution at the nitrogen atom [6]. As to the nature of the reaction solvent, DCM gave results superior to those obtained with DMF owing to the increased stability of crown complexes in less polar solvents [6].

Activation of 18-Crown-6 protected segments was carried out with DCC/HOBt. This combination, which emerged from a limited study on the influence of the activation parameter on the reaction yields, gave virtually quantitative yields after 24 h or less, dependent on the fragment's length [4].

In this study the application of the crown-ether protection scheme has been extended to the side chains of Lys- and Arg-containing peptides. The results obtained with model peptide intermediates or sequences from mammalian chaperonin 10 (cpn10) protein which contain Ser, Tyr, Thr, Lys and Gln in their free form and protected Asp and Glu residues indicate that (i) complexation of Lys and Arg side chains with 18-Crown-6 leads to compounds which are extremely soluble in DCM and DMF and (ii) DCC/HOBt coupling of these complexes to resin-bound Pro or tetrapeptides proceeds efficiently and in most cases with virtually quantitative incorporation yields.

MATERIALS AND METHODS

General Procedures

Solvents and coupling reagents were obtained from Applied Biosystems (Warrington, UK), protected amino acid derivatives and Wang resin from Nova-Biochem (Laufelfingen, Switzerland), and Fmoc-Gly-Sasrin resin from Bachem (Bubendorf, Switzerland).

Amino acid analyses were carried out on a Beckman System Gold (San Ramon, CA, USA) instrument using acid-hydrolysed [propionic acid/hydrochloric acid (1 : 1); 21 h; 110°C). Analysis for Ser was not quantitative and therefore not reported. Mass analyses were performed on either a TRIO 2A instrument (VG MASSLAB, Altringham, UK) in the fast atom bombardment (FAB) mode or on a Finnigan MAT 700 (San Jose, CA, USA) using the electrospray ionisation (ESI) technique.

HPLC Analysis

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Shimadzu

(Kyoto, Japan) LC10 system or Kontron (Tegimenta, Germany) 420 LC system. Analytical HPLC was conducted using a Vydac (Hesperia, CA, USA) C_{18} column (0.46 × 150 mm) or a Shiseido (Tokyo, Japan) C_{18} column (0.46 × 250 mm), with a flow rate of 1 ml/min of eluent A (water, 0.045% TFA) and eluent B (acetonitrile, 0.036% TFA).

System A: Shiseido C_{18} RP. Gradient 0 to 80% B in 40 min, detection at 214 nm.

System B: Vydac C_{18} RP. Gradient: 0 to 100% B in 30 min, detection 220 nm.

Peptide Synthesis

The synthesis of the resin-bound tetrapeptide Boc-Pro-Lys(Boc)-Leu-PAM has been described earlier [4]. All other peptides were synthesized on an ABI 430A synthesizer (Division of Perkin Elmer, Foster City, CA) using optimized Fmoc chemistry described previously [7, 8]. In brief, single coupling cycles were performed using amino acids activated with HBTU/HOBt [7, 8]. After the addition of each residue, capping with *N*-(2-chlorobenzyloxycarbonyloxy) succinimide [9] was used to terminate unreacted amino groups. Cleavage of the peptide from the resin support, together with TFA-labile side-chain protecting groups, was achieved using 95% TFA. Cleaved peptide was precipitated with diethyl ether, centrifuged down, and the solid dissolved in 20% acetic acid solution before lyophilization to obtain the crude dry product. The peptides were purified by semi-preparative RP-HPLC: Vydac C_{18} column (9 × 250 mm) with a flow rate of 2.5 ml/min of eluent A (water, 0.045% TFA) and eluent B (acetonitrile, 0.036% TFA).

Gradient 0 to 100% B in 360 min, detection at 214 nm.

Preparation of Peptide Complexes

A detailed description of how the complexes are formed has been reported elsewhere [1–4]. An example of the procedure is described below for the hexapeptide Fmoc-Lys-Thr-Tyr-Asp(Ada)-Ser-Gly-OH complex with 18-Crown-6 (**3**). The experimental details are the same for all peptides unless stated as otherwise in the text.

Purified hexapeptide, TFA salt, weighing 32 mg (32 μmole) was dissolved in 1 ml of water, containing 30% ethanol. To the solution were added 8.4 mg (32 μmole) of 18-Crown-6, whilst stirring vigorously. After 1 h stirring at r.t., the solution was lyophilized, giving a white powder.

Fragment Condensation

The procedure for coupling peptide complexes to resin-bound amino acids and peptides was always the same unless otherwise indicated in the text. As an example, the coupling of hexapeptide /18-Crown-6 (**3**) complex to a peptidyl-resin containing four residues (H-Pro-Asp(tBu)-Leu-Tyr(tBu)-Resin) (**1**), is described.

A quantity of **3**, 30 mg (24 μmole), was dissolved in 0.5 ml DCM. To the solution were added 3.5 mg (26 μmole) of HOBt and 52 μl (26 μmole) of 0.5 M stock solution of DCC in DCM. The mixture was left to activate for 30 min at r.t.. Any precipitated *N,N'*-dicyclohexylurea was filtered off and the clear solution transferred to the micro reaction vessel which contained the tetrapeptide bound to polystyrene-based resin (**1**). The tetrapeptide was synthesized on Wang resin (substitution 0.59 mmol/g) using Fmoc-based chemistry. 16.3 mg (9.6 μmoles) of **1** were deprotected with 20% piperidine in NMP to remove the N^{α}-terminal protecting group, washed with NMP and DCM and finally left to swell in DCM for 30 min immediately prior to the fragment condensation procedure. The coupling reaction was carried out for 18 h with vigorous vortexing. At the end of the reaction, the resin was filtered and washed with DMF, followed by 20% piperidine in DMF to remove the crown protection and N^{α}-terminal Fmoc group. The peptide was cleaved from the resin, together with protecting groups from the C-terminal fragment, using 95% TFA for 90 min. The resin was filtered and the cleaved peptide precipitated with dry diethyl ether. The solid was collected by centrifugation (4600 r.p.m. for 5 min), the supernatant discarded and the crude peptide redissolved in 20% acetic acid solution. After lyophilization 10.2 mg (82%) crude material were obtained. Purification of the peptide was achieved by semi-preparative RP-HPLC using the same method described above. This gave about 6 mg (about 47% total yields) of pure product. FAB-MS: expected 1292.5; found [*M*+1] 1293.0. AAA: Asp(2) 2.0, Thr(1) 1.0, Pro(1) 1.0, Gly(1) 1.1, Leu(1) 1.0, Tyr(2) 2.0, Lys(1) 1.0.

2[Fmoc-Phe-Arg-Ala-Lys-Ala-Gly] + **1**[H-Pro-Asp(tBu)-Leu-Tyr(tBu)-Resin]: coupling efficiency 100% (AAA); total yields of purified peptide 40%.

FAB-MS: expected 1137.3; found [*M*+1] 1138.0. AAA: Asp(1) 1.1, Pro(1) 1.1, Gly(1) 1.0, Ala(2) 1.9, Leu(1) 1.0, Tyr(1) 1.1, Phe(1) 0.9, Lys(1) 1.0, Arg(1) 1.1.

H-Val-Tyr-Gly-OH + Pro-Resin: coupling efficiency 94% (AAA); total yields after purification, 42%. FAB-MS: expected 434.5; found [*M*+1] 435.0. AAA: Pro(1) 1.0, Gly(1) 1.0, Val(1) 1.0, Tyr(1) 1.0.

H-Val-Ser-Gly-OH + Pro-Resin: coupling efficiency 72% (AAA); total yields after purification, 29%. FAB-MS: expected 358.4; found [*M*+1] 359.0. AAA: Pro(1) 0.9, Gly(1) 1.1, Val(1) 1.0.

4[Fmoc-Gly-Lys-Gly-Lys-Gly-Gly-Glu(OBzl)-Ile-Gln-Pro-Val-Ser-Val-Lys-Val-Gly-Asp(Ada)-Lys-Val-Leu-Leu-OH] + **1**: coupling efficiency 68% (AAA); total yields after purification, 20% ESI-MS: expected 3043.7; found 3044.0.

5[Fmoc-Pro-Glu(OBzl)-Lys-Ser-Gln-Gly-Lys-Val-Leu-Gln-Ala-Thr-Val-Lys-Ala-Val-Gly-Ser-Gly-Gly-Lys-Gly-Lys-Gly-Gly-Glu(OBzl)-Ile-Gln-Pro-Val-Ser-Val-Lys-Val-Gly-Asp(Ada)-Lys-Val-Leu-Leu-OH] + **1**: coupling efficiency 50% (AAA); total yields after purification, 5%. ESI-MS: expected 5000.0; found 4999.6.

RESULTS AND DISCUSSION

The first experiment which indicated that amino acid side-chain protection with crown ether was feasible was as follows. Protected Boc-Pro-Lys(Boc)-Leu tripeptide was assembled on PAM resin and then treated with TFA to remove the Boc protection from both Pro and the side chain of Lys. After neutralization with base, resin-bound tripeptide was suspended in DCM/THF 95:5 (v/v), treated with 1 equivalent of *p*-toluensulphonic acid, to form ammonium salts, and two equivalents of 18-Crown-6. Previous studies have shown that *p*-toluensulphonic acid as the counterion forms complexes which are more stable than those with other acids, e.g. TFA [1, 2]. Excess of the crown molecule was added to favour complex formation. In the assumption of a low concentration of complex between crown ether and the uncharged amino groups due to the scarce affinity of crown molecules for the latter, three different scenarios were at this point expected: (i) complexation at both amino groups (i.e., N^{α} and Lys side chain) and either (ii) preferential complexation at Pro or (iii) preferential complexation at the side

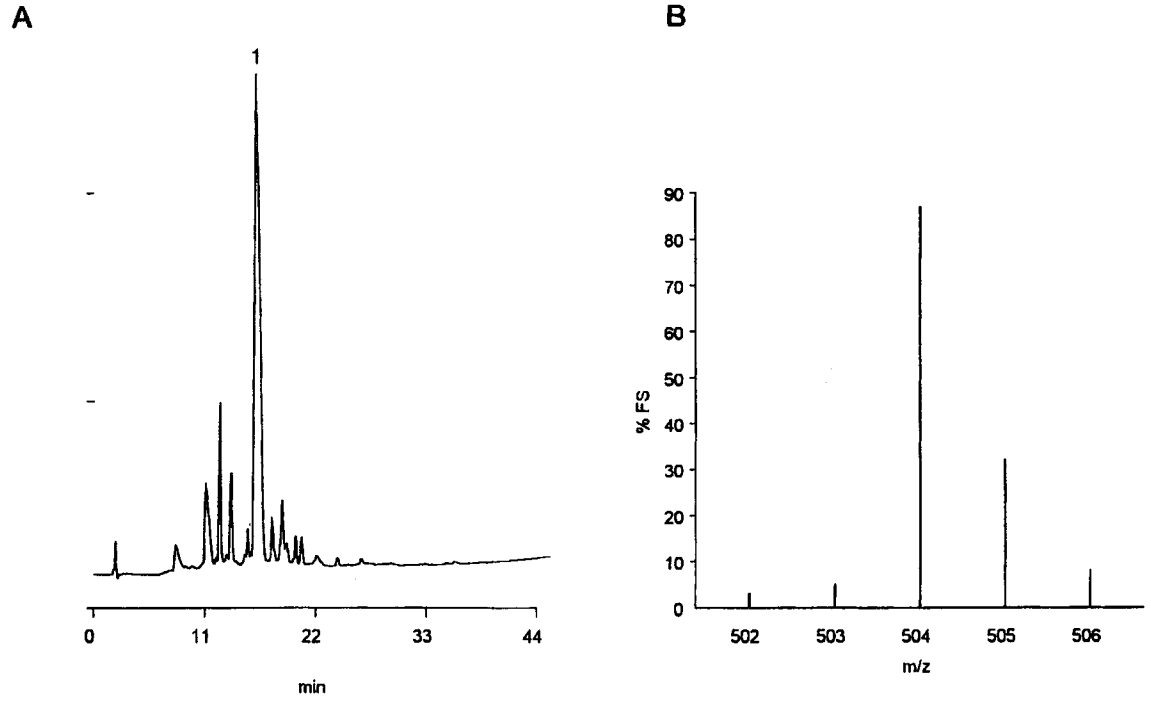
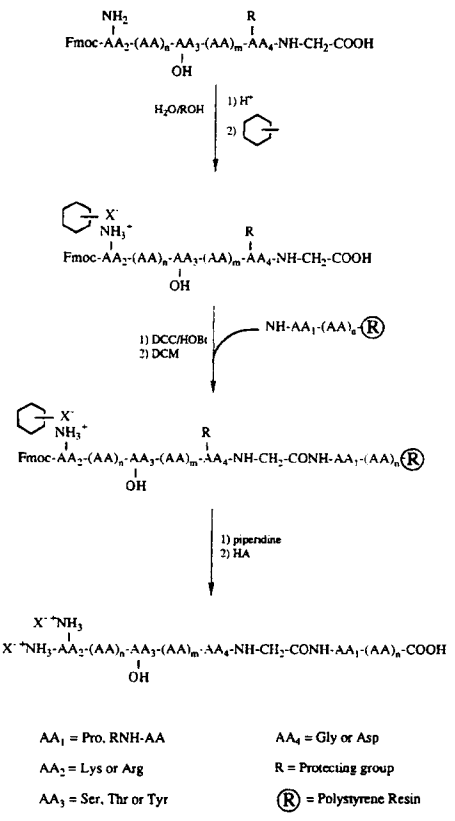


Figure 1 (A) RP-HPLC (System A) chromatogram of crude material from the reaction of Fmoc-Phe pentafluorophenyl ester and resin-bound H-Pro-Lys(Boc)-Leu tripeptide. FAB-MS analysis showed that peak 1 corresponded to the expected tetrapeptide (calculated mass 503.7).

chain of Lys. In fact, preferential complexation at Pro was less likely to occur owing to the fact that the stability of crown ether amine complexes correlates inversely with substitution at the nitrogen atom [6]. To distinguish between the various hypotheses, the thus protected resin-bound tripeptide was treated with 1 equivalent of Fmoc-Phe pentafluorophenyl ester. The reaction was allowed to proceed for 24 h and was followed by removal of the Fmoc group and acidolytic TFMSA release of peptidic material from the resin support. The HPLC profile of crude peptide indicated that a major product (peak labelled 1, Figure 1(A)), which accounted for 70% of the total crude peptide material, had been obtained. The peak labelled 1 was isolated and identified by mass spectrometry as the linear Phe-Pro-Lys-Leu tetrapeptide (Figure 1(B)), thus, demonstrating that protection with crown ether had occurred principally at the side chain of Lys.

Next, complexes between 18-Crown-6 and Fmoc-Arg-OH or Fmoc-Lys-OH were prepared using the same approach described previously [1–4]. As with crown ether protection at the Nα group of amino acid and peptides, the two complexes had the expected molecular mass and were readily soluble in DCM and DMF whilst their free forms were insoluble (data not shown).

Having established that complexation at the side chain of Arg/Lys residues and consequent solubilization of the complexes in organic solvents was possible, the model hexapeptide Fmoc-Phe-Arg-Ala-Lys-Ala-Gly (**2**) was synthesized on an acid-labile resin using Fmoc chemistry. Following acid cleavage which maintained the Fmoc protection, the peptide was purified by RP-HPLC and then treated with two equivalents of 18-Crown-6. Removal of the solvent yielded a product with a molecular weight of 1135.0, consistent with the presence of only one crown molecule. Whether the second molecule was present, but not tightly bound to either Lys or Arg and removed during the mass spectrometry process of atom bombardment and volatilization, or completely absent, could not be concluded based on these results only. The complex was, however, soluble in DCM which supported the former argument. As with other peptide complexes, this new complex was converted to its active form with DCC and HOBt (Scheme 2) in preparation for coupling experiments to H-Pro-Asp(tBu)-Leu-Tyr(tBu) (**1**). The latter was attached to either Wang or PAM polystyrene-based resins. Various reactions were carried out to identify the optimal conditions for coupling. It was found that using a Wang resin and 2.5-fold excess of



Scheme 2

complex, after 18 h the coupling yields were quantitative as judged by AAA. RP-HPLC confirmed this conclusion by showing the existence of only one major product (labelled 1, Figure 2(A)) in the crude peptide mixture which after isolation was identified by FAB-MS (expected 1137.3; found $[M+1]$ 1138.0) as the expected decapeptide (Figure 2(B)).

The application of crown ethers to the synthesis of peptides by the minimal protection scheme required testing the hypothesis on peptide containing Ser and or Tyr residues in their free form. Two new model tripeptides, Val-Tyr-Gly and Val-Ser-Gly, were therefore synthesized and then complexed at their N^{α}-terminus with 18-Crown-6. As with the other examples, the Val-Tyr-Gly complex was soluble in DCM and DMF whilst the second complex was completely soluble in DMF but less so in DCM.

Reaction between the Val-Tyr-Gly complex and resin-bound Pro, using the same conditions as described above, gave a coupling efficiency of 94% as determined by AAA. The combination of HPLC and FAB-MS data confirmed this conclusion and indicated the absence in the crude acid cleavage mixture of detectable amounts of by-products corresponding to *O*-acylation of tyrosine (Figure 3(A)).

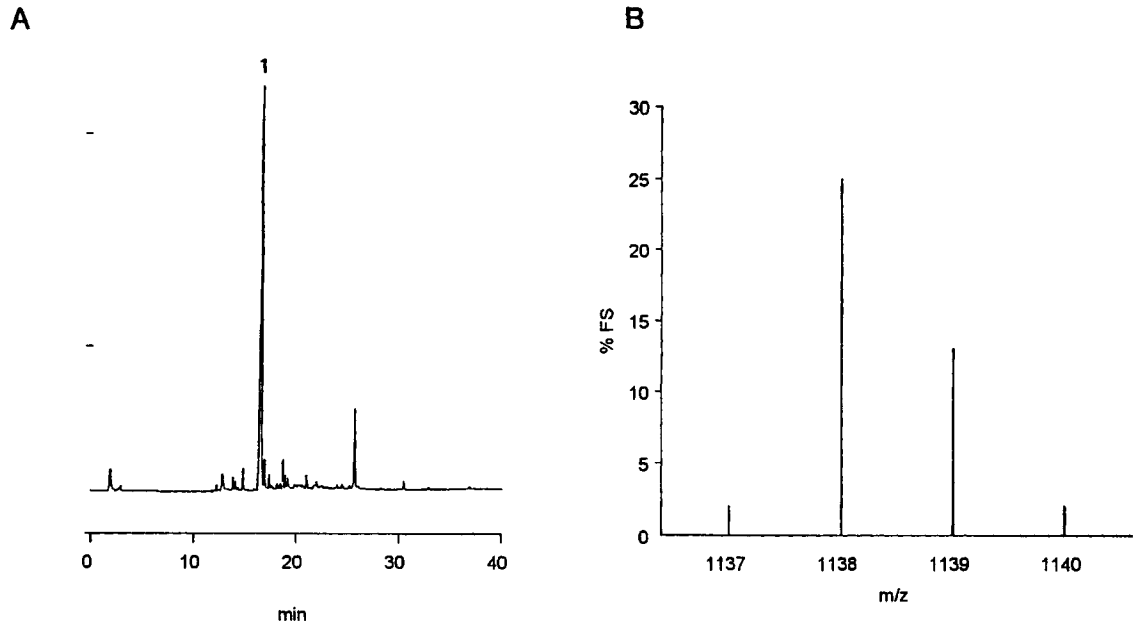


Figure 2 Reaction between hexapeptide (**2**) and resin-bound tetrapeptide (**1**). (A) RP-HPLC (System A) chromatogram of crude product after cleavage from the resin. (B) FAB-MS analysis of peak 1 after purification showing the expected molecular ion for the decapeptide (calculated mass 1137.3).

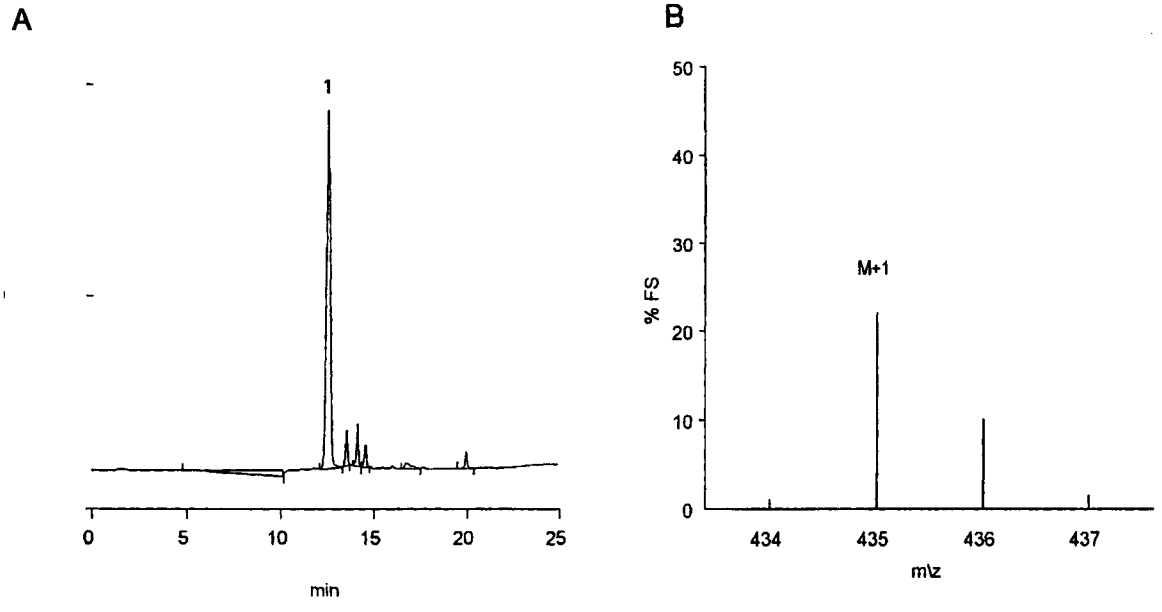


Figure 3 Effect of coupling conditions when phenolic hydroxyl side chain of Tyr is unprotected. (A) RP-HPLC (System A) chromatogram of crude cleavage material after coupling of XCE-Val-Tyr-Gly-OH to resin-bound Pro. (B) FAB-MS analysis of peak 1 after purification showing the expected molecular ion for the tetrapeptide (calculated mass 434.5).

Branching of the peptide at the hydroxyl of Ser (5%) was instead found when the second tripeptide complex was coupled to Pro in a mixture of DMF and DCM (1 : 4) (labelled 2, Figure 4(A)). DMF was used here to increase the solubility of the complex. Furthermore 18% of the linear double-incorporation product [(Val-Ser-Gly)$_2$-Pro)] was also isolated from the reaction mixture (labelled 3, Figure 4(A)). The presence of the double incorporation by-product was not totally unexpected since in previous work we

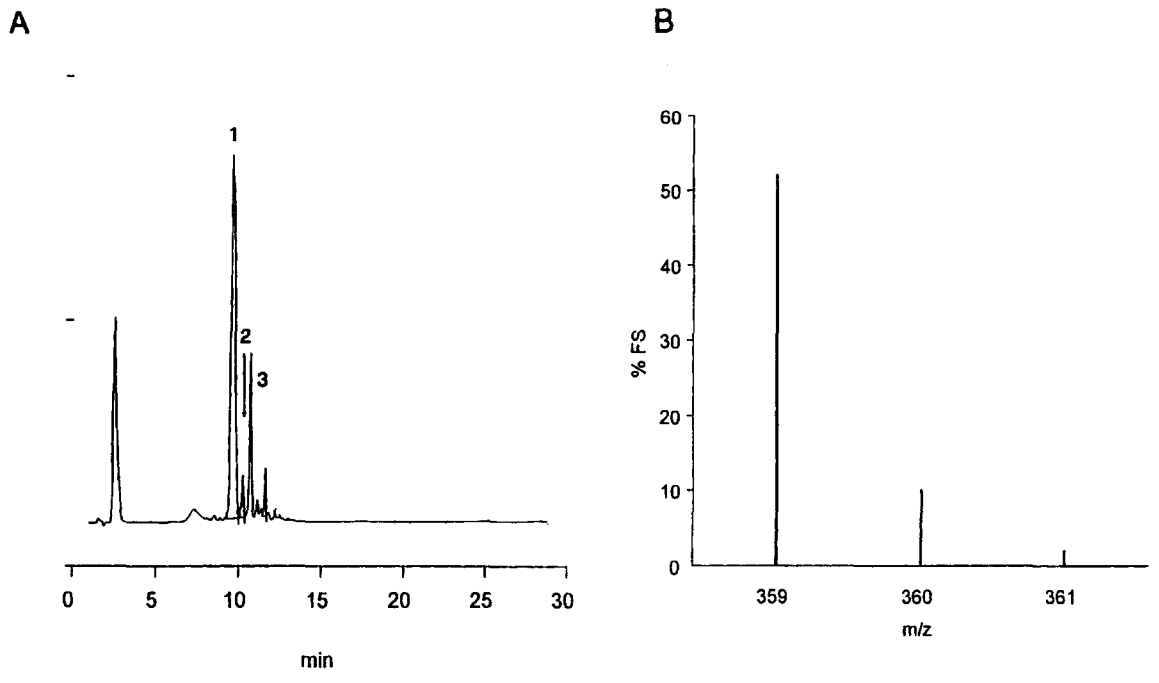


Figure 4 Effect of coupling conditions when hydroxyl side chain of Ser is unprotected. (A) RP-HPLC (System A) chromatogram of crude cleavage material after coupling of XCE-Val-Ser-Gly-OH to resin-bound Pro. Peak 1 corresponds to the correct tetrapeptide product, while peaks 2 and 3 are the *O*-acylation and double incorporation by-products, respectively. (B) FAB-MAS analysis of peak 1 after purification showing the expected molecular ion for the tetrapeptide (calculated mass 358.4).

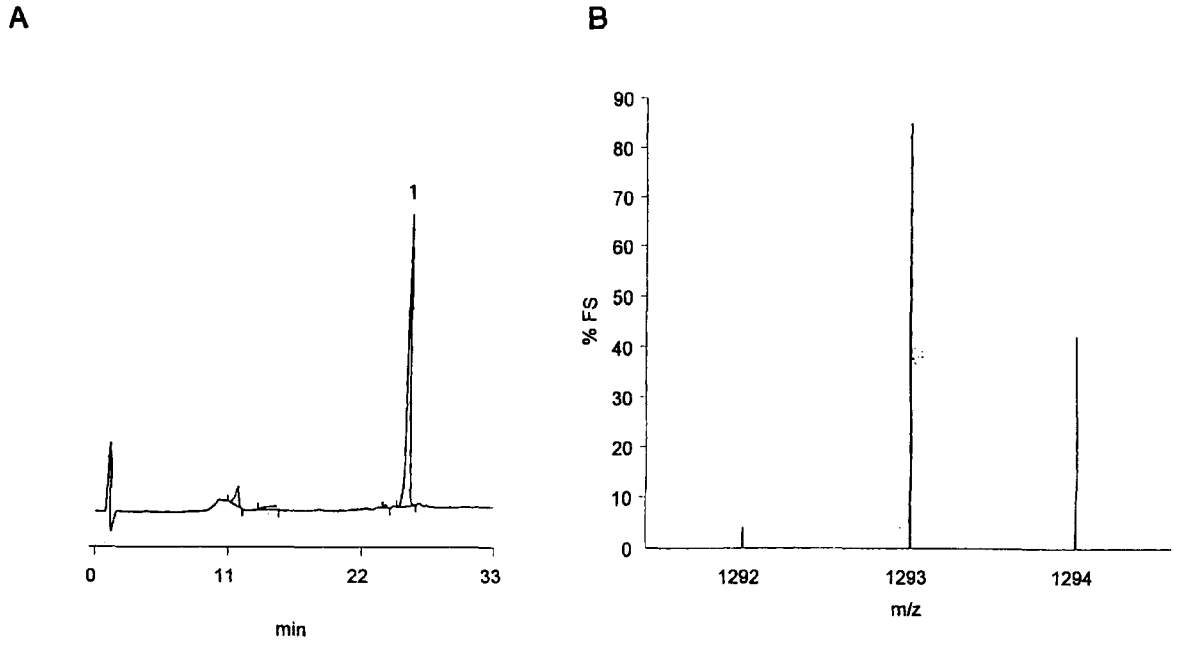


Figure 5 Reaction between hexapeptide (**3**) and resin-bound tetrapeptide (**1**). (A) RP-HPLC (System A) chromatogram of crude product after cleavage from the resin. (B) FAB-MS analysis of peak 1 after purification showing the expected molecular ion for the decapeptide (calculated mass 1292.5)

have shown that peptide–crown ether complexes partially deprotect in DMF (Scheme 1 and [4]).

Branching of the tripeptide was attributed to the free OH group of Ser known to be readily accessible to acylating reagents. This was confirmed by the results of a reaction between Fmoc-Val-Ser-Gly and resin-bound Pro in DCM/DMF mixtures. It should be noticed, however, that the percentage of branched

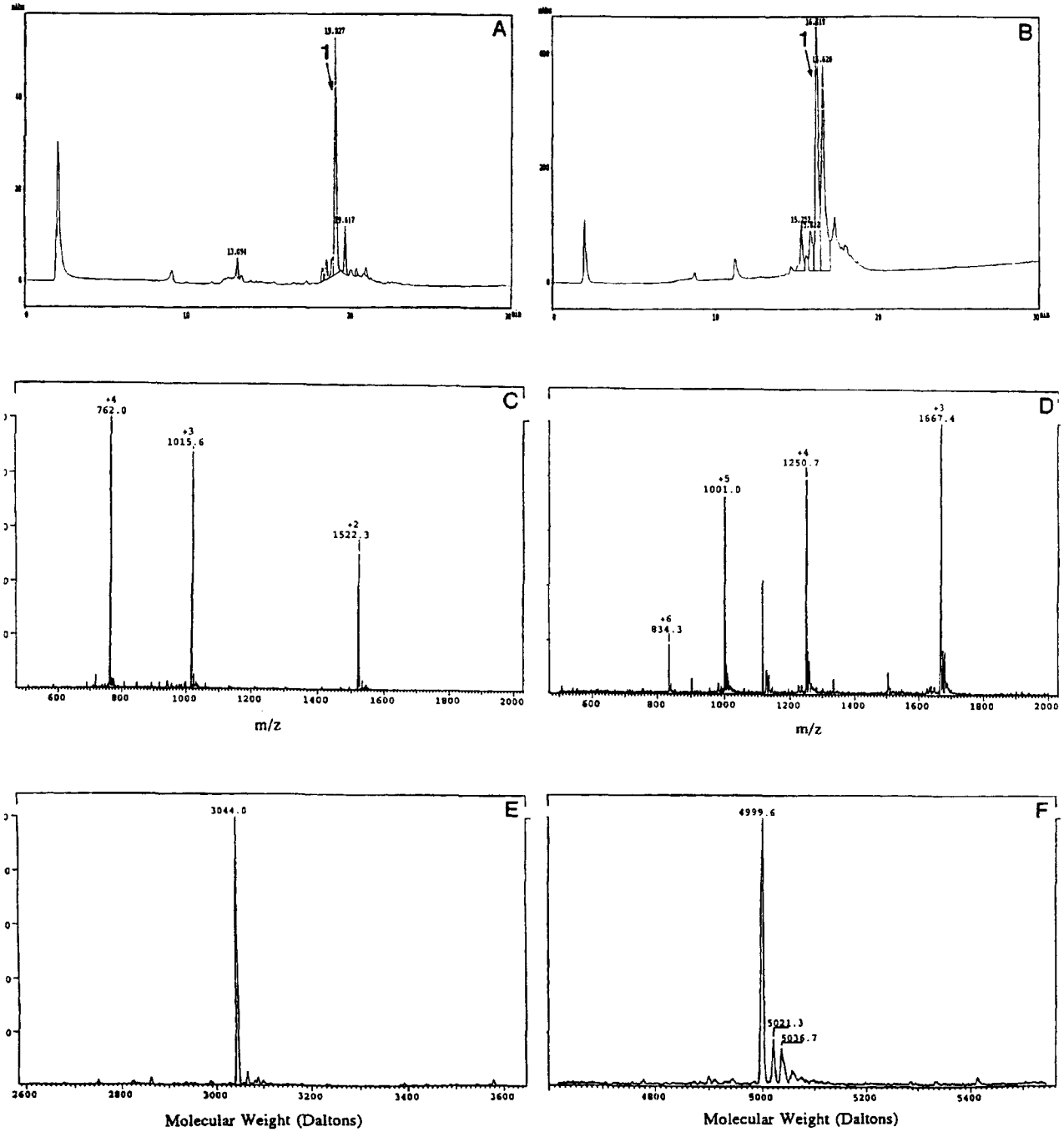


Figure 6 Fragment condensation of 21-mer (**4**) and 40-mer (**5**) onto resin-bound tetrapeptide (**1**). (A) and B) correspond to the RP-HPLC (System B) chromatograms of the crude cleavage products for **4** + **1** and **5** + **1**, respectively. (C), (D), (E) and (F) are the ESI-MS of peaks 1 from panels A and B after purification. All peaks annotated with numbers are different charged states of the target peptide. The calculated molecular mass of **4** + **1** and **5** + **1** are 3043.7 and 5000.0, respectively.

heptapeptide thus obtained was approximately 8%, indicating that not only was *O*-acylation of Ser independent of the crown molecule, but that the latter afforded a certain degree of protection towards this side reaction, probably due to a steric effect (data not shown).

Next, the model hexapeptide Fmoc-Lys-Thr-Tyr-Asp(Ada)-Ser-Gly was synthesized which contained an Asp residue protected with the 2-adamantyl group. After purification by semi-preparative RP-HPLC the partially protected peptide was complexed with 18-Crown-6 in the usual manner. The complex (**3**) had the expected molecular weight and its solubility properties were similar to those of the other complexes, i.e. it was completely soluble in DCM and DMF while its free form was insoluble. The complex was activated with DCC and HOBt and then separately coupled to resin-bound Pro and resin-bound H-Pro-Asp(t-Bu)-Leu-Tyr(t-Bu) tetrapeptide (**1**). Amino acid analysis of resin-bound peptide products indicated that after 18 h the yields of both coupling reactions were virtually quantitative. HPLC of crude peptide material, from the coupling of **3** to **1**, confirmed this conclusion. Thus, a major product accounted for 95% (labelled 1, Figure 5(A)) was isolated and shown by mass spectrometry to possess the expected molecular mass (Figure 5(B)).

To study the effect of chain length on both solubility and performance towards coupling reactions two peptides of 21 and 40 residues were prepared which were taken from the chaperonin 10 sequence of *Rattus norvegicus*. The 21-mer contained four Lys residues, one serine, two Glu protected with the Bzl group and one aspartic acid whose side chain was protected with the adamantyl group. The 40-mer, in addition to the above residues, contained three further Lys, two Ser, one Glu and one Thr. Both sequences were made on acid-labile Sasrin support using Fmoc chemistry. Thus, during the final TFA cleavage all protecting groups were removed with the exception of those of Glu and Asp residues and the Fmoc group from the N^{α}-terminal amino acid. Crude peptides were soluble in aqueous buffer containing 10% acetonitrile and consequently their purification was carried out by conventional semi-preparative RP-HPLC. The purified peptides were analysed by RP-HPLC, AAA and ESI-MS, confirming that the correct material had been obtained. Complex formation was achieved by dissolving the purified peptides in water containing 60% TFE to aid dissolution and one equivalent of 18-Crown-6 for each of the Lys residues contained in the sequence. Mixing for 30 min afforded a clear solution which was frozen and lyophilized.

The dry peptide–crown ether complexes (**4** and **5**) were then activated in DCM with DCC/HOBt and coupling reactions performed using the same resin-bound tetrapeptide (**1**) described above. Reactions were conducted in DCM for 24 h using a 2.5-fold excess of complex. At the end of reaction the peptidyl-resins were treated first with DIEA base to remove the crown protection [4], then with acid to cleave the peptide from the resin support and remove all TFA-labile protecting groups. The N^{α}-terminal Fmoc protecting group, Bzl and 2-adamantyl groups were not affected by the DIEA and TFA treatments and remained attached throughout the experiment. The HPLC chromatograms of crude peptide materials are shown in Figure 6. The main product (labelled 1, Figure 6(A)) from the reaction using the 21-mer accounted for about 68%. It was isolated and shown by mass spectrometry to represent the expected product (Figure 6(C) and (E)). Also in the case of the 40-residue long sequence the main reaction product corresponded to the desired product (Figure 6(B)). ESI-MS (Figure 6(D) and (F)) confirmed that the correct molecular mass had been purified. However, in this case the coupling yield was only 50% (by AAA), although there were no traces of unreacted resin-bound tetrapeptide. Thus, a number of side reactions involving the latter and the complex, and probably accounting for the number of less intense peaks before and after the main product, had occurred during the coupling reaction.

CONCLUSIONS

The ability of a crown ether molecule to complex charged amines and the subsequent solubility in organic solvents thus achieved has been used to explore the feasibility of this non-covalent protection scheme in peptide synthesis. Although the instability of amino acid complexes with 18-Crown-6 in the presence of coupling agents [1, 2] makes this method unsuitable for stepwise assembly of polypeptide chains, the conditions whereby the same protection scheme can be applied to the N^{α}-group of peptide fragments for the assembly of larger sequences either in solution or in solid phase have been found [3, 4].

The results presented here show that the same approach of non-covalent protection can be used for the side chains of Lys and Arg. Thus, both these

residues as well as fragments containing them were readily complexed with 18-Crown-6 and then solubilized in organic solvents such as DMF and DCM. These complexes were then reacted quantitatively with resin-bound model tetrapeptides. Activation was achieved by the DCC/HOBt combination and coupling reactions were performed for about 18 h in DCM.

The protection of peptide amino groups with the crown molecule is compatible with the presence of free hydroxyl groups as shown by the quantative incorporation of a Tyr-containing model tripeptide. In the case of a similar peptide containing Ser in place of Tyr, *O*-acylation occurred during the coupling reaction. This observation was, however, independent of the crown protection since the same peptide protected covalently with the Fmoc group gave similar amounts of *O*-acylated by-product. In addition to the *O*-acylated derivative the reaction using the Ser-containing tripeptide yielded 18% of the doubly incorporated by-product. This was ascribed to the presence in solution of 20% DMF required to improve the solubility of the complex and further served to reiterate the negative effects of this solvent on the stability of these complexes [4]. The different solvent composition of the reactions involving the two tripeptides (DCM for Val-Tyr-Gly, DCM/DMF mixture for Val-Ser-Gly) probably also accounted for the absence of *O*-acylation of the otherwise more reactive Tyr residue.

The feasibility of the method for the assembly of larger peptides was then assessed using two sequences of 21 and 40 residues, respectively. The two peptides containing free Lys and Ser residues and carboxyl functions of Asp and Glu protected with 2-Ada and Bzl groups, respectively, were soluble in aqueous solution. After purification by conventional RP-HPLC the two peptides were complexed at the side chain of Lys with 18-Crown-6, thus generating molecules which were completely soluble in organic solvents. Using 2.5-fold excess of the complex, after 24 h reaction, the yields of incorporation on a resin-bound tetrapeptide were 68% and 50% for the 21-mer and 40-mer, respectively.

Finally, while more examples are needed to demonstrate its general applicability, the results shown here indicate that non-covalent protection with crown ethers for peptide fragment condensation combines the advantages derived from a full protection scheme (i.e. solubility in organic solvents) with those from a minimal side-chain protection scheme (i.e. ease of purification and characterization). The necessary requirements for success using this method are (i) complete solubility of the complexes in either DCM or solvents with similar characteristics to the latter and (ii) coupling of the complexes to fragments having at their N-terminus residues with a secondary nitrogen like in the case of Pro or doubly N^{α} substituted amino acids (e.g. Fmoc-(FmocHmb) AA, see [4] and [10]).

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