Tetrahedron Letters, Vol. 33, No. 3, pp. 397-400, 1992
Printed in Great Britain

0040-4039/92 $3.00 + .00
Pergamon Press plc

Reversible Protection of Lysine to Facilitate the Purification of Protected Peptide Segments

Josep Rizo§, Fernando Albericio, Ernest Giralt and Enrique Pedroso*

Departament de Química Orgànica, Universitat de Barcelona, Martí i Franquès 1-11, 08028-Barcelona (Spain). §Current address: University of Texas Southwestern Medical Center at Dallas, Department of Pharmacology, 5323 Harry Hines Blvd., Dallas, TX 75235-9041 (U.S.A.).

Key words: peptide synthesis, protected peptide segments, lysine protection, amine trifluoroacetylation, Nbb-resin

Abstract: *The polarity of temporarily free lysine side chains can be exploited to aid in the purification of protected peptides. The amine groups can easily be reprotected after the purification step. Impurities in commercial TFE[1] can cause trifluoroacetylation of free primary amines.*

Convergent solid-phase synthesis is a very promising approach for the chemical synthesis of small proteins[2]. One of the main problems in this methodology, also common in solution peptide synthesis, is the poor solubility of protected peptide segments, which makes their handling and purification difficult[3]. A possible strategy for facilitating the purification step is the temporary deprotection of polar side chains, with reprotection of these side chains in the purified segment before its use in subsequent coupling reactions. Considering the wide variety of amine protecting groups that have been developed for peptide synthesis, we have explored the application of this scheme using the lysine side chain. During our study, we have found that a serious side reaction can occur when primary amines are dissolved in various commercial samples of TFE: impurities in the solvent can cause amine trifluoroacetylation.

The scheme proposed above can only be advantageous if the two additional steps required, selective deprotection and reprotection of the lysine side chains, are easy, clean and quantitative. A very attractive possibility for the temporary protection of Lys side chains is the Fmoc group[4], which is compatible with the synthesis of protected peptide segments using benzyl-type protecting groups for other side chains, Boc protection for the α-amino groups and a photolabile resin linkage[2b]. The Fmoc group can easily be removed from the Lys side chains with a very short (1 min) treatment with piperidine/DMF 1:1, both in solution and in solid phase[4b]. Trifluoroacetylation appears to be the most convenient way to reprotect the Lys side chains as the Tfa group is considered especially useful for Lys protection in peptide synthesis[3b], and very mild, quantitative methods to introduce this group using alkyl trifluoroacetate esters have been described[5]. Thus, addition of 20 equiv. of TfaOEt and 20 equiv. of DIEA to a solution of Boc-Lys-OH[6] in MeOH produced quantitative trifluoroacetylation in 2 h. Isolation of the reaction product yielded Boc-Lys(Tfa)-OH, indistinguishable from a commercial sample[7].

In order to test our deprotection/reprotection scheme and evaluate its possible advantages in facilitating the purification of protected peptides, we chose the segment 10-18 of neuropeptide K as a target, with the following protection:

Boc-Val[10]-Ala-Leu-Leu-Lys(X)-Ala-Leu-Tyr(Dcb)-Gly[18]-OH

K9(Fmoc)	X=Fmoc
K9(Tfa)	X=Tfa
K9	X=H (Lys unprotected)

Our main goals were to evaluate the synthetic route to **K9(Tfa)** *via* the intermediate **K9(Fmoc)** in comparison with the direct synthesis of **K9(Tfa)**, and to compare the solubility properties and chromatographic behavior of **K9** and **K9(Tfa)**. The protected peptides **K9(Tfa)** and **K9(Fmoc)** were synthesized using standard procedures[8] on an α-(4-bromomethyl-3-nitrobenzamido)benzylcopoly(styrene-1%-divinylbenzene) support (Nbb-resin), which provides a photolabile peptide-resin linkage[2b]. Photolyses of the peptidyl-resins were carried out as described in ref. 2b, leading to **K9(Tfa)** and **K9(Fmoc)** crudes[9] with an excellent degree of purity (Figures 1a,b). Reverse-phase MPLC of the first crude gave **K9(Tfa)** in 65% yield and a high degree of purity (~99%, by analytical HPLC)[10]. The crude containing **K9(Fmoc)** was treated with piperidine/DMF 1:1 for 3 min to remove the Fmoc group; after evaporation of the solvents, the residue was washed with diethyl ether and centrifuged three times to remove the dibenzofulvene-piperidine adduct, yielding the segment with the free Lys side chain. Purification of the crude by reverse-phase MPLC gave **K9** with 70% recovery and a high degree of homogeneity (~ 99% by analytical HPLC, Figure 1c)[10]. Finally, trifluoroacetylation of **K9** with 40 equiv. of TfaOEt in DMF, and in the presence of 40 equiv. of EDIA, produced **K9(Tfa)** quantitatively after 2h of reaction (Figure 1d)[11].

The results described above show that the protected nonapeptide segment **K9(Tfa)** can be obtained in good yields by the deprotection/reprotection method involving the purification of the segment with the free Lys side chain, **K9**. Our results show that this scheme is viable and requires only a minimal additional synthetic effort. It should be stressed that **K9(Tfa)** obtained by trifluoroacetylation of purified **K** needs no extra purification step (Figure 1d).

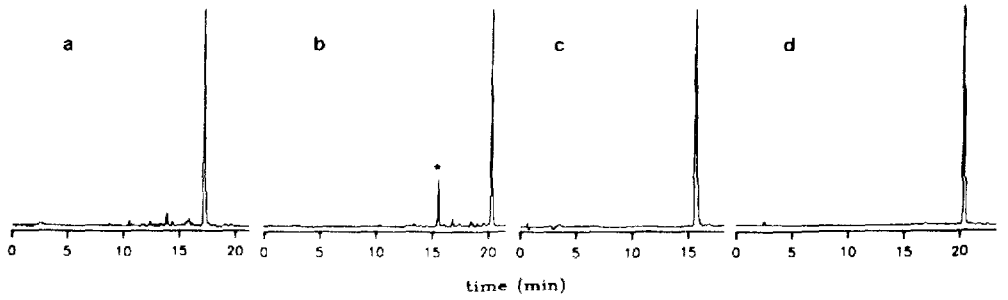

Figure 1: Analytical HPLC on a reverse-phase C_{18} column of: a) **K9(Fmoc)** and b) **K9(Tfa)**, photolysis crudes; c) purified **K9**; d) trifluoroacetylation of **K9**, reaction mixture after 2 h. Elution with A: H_2O (0.045% TFA) and B: acetonitrile (0.036% TFA); linear gradient from 60% B (a) or 40% B (b,c,d) to 90% B in 15 min. UV absorbance at 275 nm. The peak labelled * is non-peptidic.

Furthermore, **K9** presents, as expected, substantially improved chromatographic and solubility properties when compared not only with **K9(Fmoc)** but also with **K9(Tfa)**. For instance, **K9** shows a considerably lower retention time than **K9(Tfa)** in reverse-phase chromatography (Figures 1c,d) and the increase in polarity due to the free Lys side chain allows **K9** to be dissolved in MeOH in concentrations higher than 10 mg/mL, while at least 30% DMF in MeOH is necessary to dissolve **K9(Tfa)** in the same concentrations (minimum to load a sample for semi-preparative reverse-phase chromatography).

In conclusion, the simple and clean reactions involved in this scheme and the improved solubility of the peptide afforded by the Lys side chain should help, in combination with a different approach based on the use of polar picolyl protecting groups[12], to overcome the problems encountered in the purification of protected peptide segments.

Trifluoroacetylation of primary amines in TFE. Removal of the Fmoc on the Lys side chain can also be performed on the peptidyl-resin before the photolysis step. In an attempt to test this sequence of events, we found that the major component in the photolysis crude (in a ratio of 7:3 with respect to **K9**) coeluted with **K9(Tfa)** under various analytical HPLC conditions. When this material was purified, ^{1}H-NMR, ^{19}F-NMR and fast atom bombardment mass spectrometry showed that, indeed, trifluoroacetylation of the Lys side chain had occurred during the photolysis. Repetition of the photolysis gave similar results. A 5 mM solution of 2-phenylethylamine in TFE (same batch used in the photolysis), after standing for 24 h at room temperature gave 90% trifluoroacetylation (by HPLC and ^{1}H-NMR, comparing with a standard sample of trifluoroacetylated amine). An analogous experiment using TFE from a freshly opened bottle from the same commercial source produced 20% trifluoroacetylation in 7 h, while no trifluoroacetylated amine was observed after 24 h when TFE from another brand was used[13]. As esters of TFA are formed spontaneously when this acid is dissolved in alcohols, one may speculate that contamination of the TFE by TFA (or a TFA ester) would produce $TfaOCH_2CF_3$, which would act as the amine trifluoroacetylating agent. Preliminary efforts to characterize this agent in our laboratory have been unsuccesful. Nevertheless, the possibility of undesired trifluoroacetylation needs certainly to be considered when dissolving a primary amine in TFE.

ACKNOWLEDGEMENTS. This work was supported by funds from CICYT (grants PB89-257 and PB88-216)

REFERENCES AND NOTES

1. Abbreviations: Boc, *t*-butyloxycarbonyl; Dcb, 2,6-dichlorobenzyl; DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; MPLC, medium presure liquid chromatography; NMR, nuclear magnetic resonance; Tfa, trifluoroacetyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TLC, thin layer chromatography.
2. (a) Pedroso, E.; Grandas, A.; Saralegui, M.A.; Giralt, E.; C. Granier, C.; Van Rietschoten, J.; *Tetrahedron* **1982**, *138*, 1183-1192 ; (b) Giralt, E.; Albericio, F.; Pedroso, E.; Granier, C.; Van Rietschoten, J.; *ibid.* **1982**, *138*,1193-1208; (c) Kaiser, E.T.; Mihara, H.; Laforet, G.A.; Kelly, J.W.; Walters, L.; Findeis, M.A.; T Sasaki, T.; *Science* **1989**, *243*, 187-192 ; (d) Grandas, A.; Albericio, F.; Josa, J.; Giralt, E.; Pedroso, E.; Sabatier, J.M.; Van Rietschoten, J.; *Tetrahedron* **1989**, *45*, 4637-4648 ; (e) Kneib-Cordonier, N ; Albericio, F.; Barany, G.;*Int. J. Peptide Protein Res.* **1990**, *35*, 527-538.
3. (a) Finn, F.M.; Hofmann, K.; in *The Proteins;* Neurath, H.; Hill, R.L. Eds.; 3rd edn., Vol. 2, Academic Press, New York, 1976, pp. 105-253; (b) G. Barany, G.; Merrifield, R.B.; in *The Peptides;* Gross, E.; Meienhofer, J. Eds.; Vol. 2, Academic Press, New York, 1979, pp.1-284; (c) Bodanzsky, M.; *Int. J. Peptide Protein Res.* **1985**, *25*, 449-474.

4. (a) Albericio, F.; Rizo, J.; Nicolás, E.; Ruiz-Gayo, M.; Cárdenas, F.; Carreño, C.; Andreu, D.; Pedroso, E.; Giralt, E.; in *Peptides: Chemistry, Structure and Biology* ; Rivier, J.E.; Marshall, G.R. Eds.; Escom, Leiden, 1990, pp. 923-924; (b) Albericio, F.; Nicolás, E.; Rizo, J.; Ruiz-Gayo, M.; Pedroso, E.; Giralt, E.; *Synthesis*, **1990**, 119-122 .
5. (a) Weygand, F.; Geiger, R.; *Chem. Ber.* **1959**, *92*, 2099-2106 ; (b) Curphey, T.J.; *J. Org. Chem.* **1979**, *44*, 2805-2807 .
6. Boc-Lys-OH was obtained by treating Boc-Lys(Fmoc)-OH with piperidine/DMF 1:1. The solvents were evaporated after 3 min, the residue was partitioned between CH_2Cl_2 and H_2O/pyridine 19:1, and the aqueous layer was washed twice with CH_2Cl_2. Lyophilization of the aqueous extracts gave Boc-Lys-OH with sufficient purity for the next step, as determined by TLC on silica (*n*-BuOH/H_2O/AcOH 8:1:1, Rf=0.28) and 200 MHz ^{1}H-NMR.
7. The reaction was followed by TLC on silica. Boc-Lys(Tfa)-OH was isolated by evaporation of the solvent, followed by partition between CH_2Cl_2 and 3% aqueous AcOH, and subsequent evaporation of the organic layer. The product obtained presents the same 200 MHz ^{1}H-NMR spectrum as commercial Boc-Lys(Tfa)-OH (Bachem) and has the same chromatographic behavior (TLC on silica: *n*BuOH/H_2O/AcOH 8:1:1, Rf=0.68; acetone/AcOH 98:2, Rf=0.57). The extraction step is not strictly necessary as most reagents and by-products are volatile.
8. The cycles to incorporate each residue consisted of deprotection with TFA/CH_2Cl_2 3:7, neutralization with DIEA/CH_2Cl_2 1:19 and coupling with 2.5 equiv. of Boc-amino acid activated with N,N'-dicyclohexylcarbodiimide (1-2 h, negative ninhydrin test in all cases).
9. Suspensions of 500-800 mg of peptidyl-resin in 100 mL of TFE/CH_2Cl_2 1:4 were sonicated and degassed, and then were exposed to photolysis with four black light mercury lamps for 8-10 hours, at 0-10 °C and under Argon atmosphere.
10. Peptides (20-40 mg batches) were purified by reverse phase MPLC (Vydac C_{18}) eluting with a linear gradient from A: H_2O/MeOH 7:3, to B: H_2O/MeOH 2:8 (total volume 900 mL, flow 3.0 mL/min; both eluents contained 0.2% propionic acid). The amino acid composition of the purified materials is the following: **K9(Tfa)** : G 1.01, A 2.04, V 0.99, L 2.86, Y 1.02, K 1.08; **K9**: G 1.04, A 1.97, V 0.98, L 2.97, Y 1.02, K 1.02.
11. To isolate **K9(Tfa)**, the reaction mixture was evaporated and the residue was treated with water and lyophilized three times. The final product presents the same 200 MHz ^{1}H-NMR spectrum as **K9(Tfa)** obtained by direct synthesis, with a minor impurity corresponding to DIEA (which should not interfere in subsequent segment coupling reactions). **K9(Tfa)** obtained by both synthetic routes was also characterized by fast atom bombardment mass spectrometry [m/e: 1303 (M+1), 1325 (M+Na)] and by ^{19}F-NMR at 188 MHz (signal 3.93 ppm downfield from TFA).
12. Rizo, J.; Albericio, F.; Romero, G.; García-Echeverría, C.; Claret, J.; Muller, C.; Giralt, E.; Pedroso, E.; *J. Org. Chem.* **1988**, *53*, 5386-5389.
13. We prefer to omit the names of the TFE suppliers as no systematic screening of different batches of TFE from the different brands has been carried out. Non-trifluoroacetylated **K9** was obtained when TFE from the second brand was used in the photolysis of **K9**-resin.

(Received in UK 28 October 1991)