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Multiple Solid Phase Synthesis via Fmoc-Amino Acid Fluorides

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Abstract: In addition to displaying high reactivity, Fmoc-amino acid fluorides are shown to be highly soluble and stable for extended periods in organic solvents such as DMF and therefore to be recommended for use in Multiple Peptide Synthesis. A series of analogs of alamethicin and a partial sequence (22 amino acids) of the CNG-channel forming protein BOVTESTIS have been assembled with excellent results.

The rapidly growing demand for peptides of widely varying sequences requires new techniques for the efficient synthesis of large groups of peptides quickly. One of the most impressive developments during the last decade has been the introduction of the so called Multiple Peptide Synthesis (MPS) approach which allows the simultaneous synthesis of numerous peptides varying in length and amino acid sequence. Many methods have been examined in the search for optimized protocols for highly efficient MPS[1]. However, there is still a need to develop standard protocols which would allow for general and efficient incorporation of any amino acid regardless of sequence. A truly general coupling technique is still not available. Especially important would be a technique which avoids problems with sterically hindered amino acids.

Recently, Fmoc-amino acid fluorides have been shown to be particularly well suited for the solid phase assembly of medium sized peptides incorporating such sterically hindered residues[2]. Fmoc-amino acid fluorides fulfill two of the most important requirements for use in MPS, namely exceptional reactivity and high solubility in organic solvents (>1 M in DMF). The latter effect is especially important since the greater the concentration the more efficient is the acylation. Using conventional coupling reagents the technique of in situ activation always leads to significant dilution of the coupling species, an effect which is especially disadvantageous for difficult couplings.

The construction of most commercial automated multiple peptide synthesizers requires storage of the acid fluorides in solution during the course of synthesis. Therefore, in order to evaluate the use of acid fluorides for MPS, it was first neccessary to check their stability in DMF. The fluorides were stored at room temperature in DMF which had been dried over molecular sieves for 3 days. Aliquots were diluted after an appropriate time with methanol/5%pyridine and analyzed by HPLC at 220 nm. Other than the corresponding methyl ester and the free acid which resulted from slow hydrolysis in DMF the solutions showed no significant amount of any other material over a period of 24h (Fig. 1).

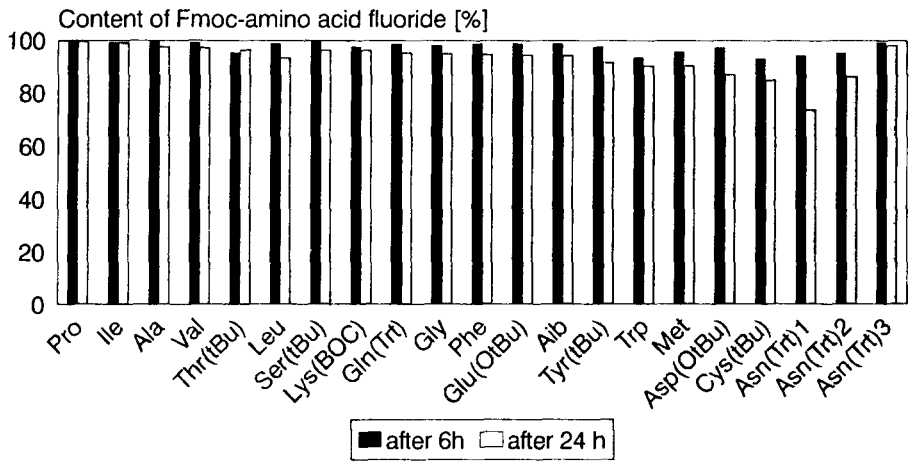

Figure 1. Stabilities of Fmoc-amino acid fluorides in DMF

The relatively fast hydrolysis observed in the case of Fmoc-Asn(Trt)-F [sample Asn(Trt)1], was double checked with a newly prepared sample of the fluoride, which was extensively dried prior to dissolving in DMF. This material showed only 13.7% hydrolysis after 24 h [sample Asn(Trt)2]. If in addition molecular sieves were directly added to the DMF solution hydrolysis was diminished to 1.9% [sample Asn(Trt)3]. It is clear that hydrolysis is caused by water remaining in the sample from the initial preparation of the acid fluorides.
As a model to demonstrate applicability of the Fmoc-amino acid fluorides to MPS alamethicin F30 (sequence 4) and a set of six analogs were chosen for assembly. All syntheses were performed on an ACT 348 Multiple Peptide Synthesizer.

1: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-**Pro**-Gly-Leu-Aib-Val-Aib-Aib-Glu-Gln-Pheol
2: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-**Pro**-Leu-Aib-Val-Aib-Aib-Glu-Gln-Pheol
3: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-**Pro**-Aib-Val-Aib-Aib-Glu-Gln-Pheol
4: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-**Pro**-Val-Aib-Aib-Glu-Gln-Pheol
5: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Val-**Pro**-Aib-Aib-Glu-Gln-Pheol
6: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Val-Aib-**Pro**-Aib-Glu-Gln-Pheol
7: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Val-Aib-Aib-**Pro**-Glu-Gln-Pheol

For each synthesis 100mg (0.032mmol) of a Pheol-loaded[3] o-Cl-Trt-resin (Novabiochem) was used. Peptides were synthesized using single couplings for 30 min, with deblocking by 25% piperidine/DMF for 11 min. The acid fluorides were stored at a concentration of 0.7 M in DMF, but in order to maintain conditions comparable to those used for a recently described successful solid phase assembly of alamethicin[3] were diluted just prior to the coupling step with pure DMF to give a 0.28 M solution. After acetylation of the N-terminus (Ac_2O/DIEA/DMF=1/2/7, 30 min), removal of the protected peptide from the resin was then effected by means of HOAc/TFE/DCM=2/2/6 for 60 min. Complete deblocking was carried out by treatment with 2% triisopropylsilane and 5% water in 50% TFA/DCM. The protected peptides obtained were of remarkably high

quality as demonstrated by the typical HPLC profile for alamethicin F30 (Fig. 2a). After final deprotection the deblocked peptides were of similar quality (Fig.2b). Interestingly, in spite of the potential acidolytic sensitivity of Pro-Aib bonds[4], prolonged treatment with the final deprotection reagent did not cause any deterioration in the quality of the free peptide (Fig. 2c).

Although simultaneous peptide-resin and protecting-group cleavage is useful[3] the two step procedure described here gives more consistent results as far as the purity of the isolated products is concerned.

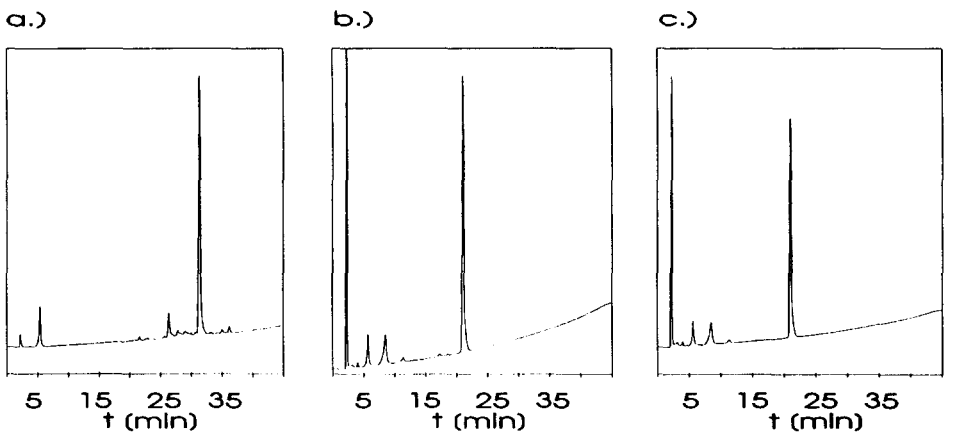

Figure 2. HPLC-profiles of protected alamethicin F30 (ES-MS: calcd. for $[M+H]^+$: 2505.0 found: 2505.5) (a), and of crude alamethicin after final deprotection for 30 min (ES-MS: calcd. for $[M+H]^+$: 1964.3 found: 1965.7) (b) and 240 min (c).

Although difficult to assemble by solid phase techniques because of steric problems, alamethicin lacks many of the trifunctional amino acids which might be expected to give rise to various side reactions on long storage in DMF or during the coupling step. Partial sequence (**1**) of the cyclic nucleotide-gated channel protein chloroac-Gly-BOVTESTIS[5]

chloroac-Gly-Lys-Phe-Ile-Gly-Phe-Gly-Thr-Asp-Ser-Trp-Val-Tyr-Pro-Asn-Val-Ser-Asn-Pro-Glu-Tyr-Gly-NH_2

(1)

was selected as an appropriate second model. Conditions were similar to those used for alamethicin. Fmoc-Gly-F was manually loaded onto the resin (TG S RAM, 0.24 mmol/g) using a single coupling for 30 min. As a check on the efficiency of the synthesis the same sequence was also assembled via TBPIPU[6] activation on a Millipore 9050 continuous flow synthesizer. The final coupling of chloroac-Gly-OH was carried out in both cases by means of TBPIPU (2x30 min). Both peptides were cleaved from the solid support by means of 2% triisopropylsilane, 5% phenol, 5% water in TFA. The HPLC profiles of the crude products clearly demonstrate that the peptide assembled via the acid fluoride-MPS technique is at least of the same purity as the

conventionally assembled peptide (Fig.3) indicating that storage of the activated trifunctional Fmoc-amino acid fluorides does not effect the product quality.

Therefore, the fluoride technique is particularly well suited for efficient Multiple Solid Phase Peptide Synthesis.

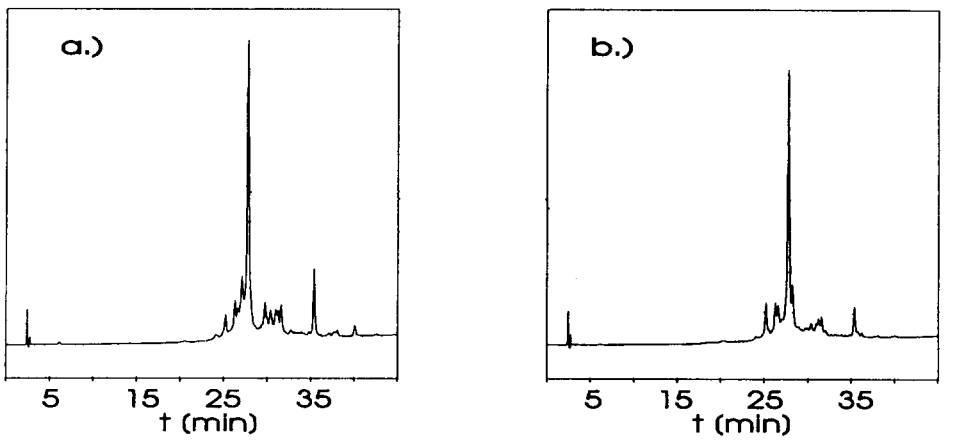

Figure 3. Syntheses of chloroac-Gly-BOVTESTIS (345-365) by conventional synthesis via TBPIPU (ES-MS: calcd. for $[M+H]^+$: 2511,3 found: 2510,2)(a) and by MPS via fluorides (ES-MS: calcd. for $[M+H]^+$:found: 2509.9) (b).

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References:

Abbreviations: DIEA = diisopropylethylamine, Ac_2O = acetic anhydride, HOAc = acetic acid, Pheol = phenylalaninol, TBPIPU = O-(benzotriazole-1-yl)-1,1,3,3-pentamethyleneuronium tetrafluoroborate, chloroac-Gly = chloroacetyl-glycine, TFE = trifluoroethanol, TFA = trifluoroacetic acid.

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