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N-terminus FITC labeling of peptides on solid support: the truth behind the spacer

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ABSTRACT

Fluorescein isothiocyanate (FITC) is an amine reactive derivative of fluorescein dye that has wide ranging application in biochemistry. It has been extensively used to label peptides and proteins. However, its use in solid phase peptide synthesis is restricted. Indeed, in acidic conditions required for linker cleavage, N-ter FITC-labeled peptides undergo a cyclization leading to the formation of a fluorescein with subsequent removal of the last amino acid. This can be avoided when a spacer such as amino hexanoic acid is used or if non-acidic cleavage is operated to release targeted peptide from the resin.

Fluorescent dyes are major tools for the labeling of biomolecules. Fluorescein derivatives are probably the most widely used of this kind of reagents displaying a wide range of applications in fluorescence microscopy,[1] flow cytometry,[2] and immunofluorescence-based[3] assays. The fluorescein isothiocyanate (FITC) is more reactive than the carboxy fluorescein which, moreover, has to be activated before use. FITC was used extensively to react with sulfhydryl[4] targeting reduced cysteine side chains and especially amino groups in peptides or proteins.[5] For numerous applications, it is convenient to introduce fluorescent label during the chemical synthesis.[6,7] FITC may react during the course of solid phase peptide synthesis, either with a lysine or an ornithine side chain after selective unmasking of protecting group[8] or with a primary amino group at the N-terminal side of the growing peptide. In the latter case, an alkyl spacer such as aminohexanoic acid (Ahx) is introduced between the last amino acid and the thiourea linkage generated through the reaction of isothiocyanate and amine.[9] This is traditionally justified to take away the bulky fluorescent dye from the bioactive sequence.

As part of a project that aimed to design new cell penetrating peptides, we synthesized a series of fluorescent peptides by Fmoc SPPS. FITC was introduced at the N-terminal side of anchored peptides. In this strategy, we believed that a degradation could happen during TFA by a mechanism similar to Edman degradation induced by phenylthioisocyanate.[10] The proposed mechanism is detailed in Scheme 1 and yields fluorescein thiohydantoïne (FTH) **4**. A recent patent drew profit from this reaction in solution to propose a method for analyzing protein adducts.[11] It is worth noting that some FTH were already obtained in the late seventies from unprotected aminoacids or from albumin.[12]

In order to evaluate the importance of this side reaction on solid support, we performed a study on five model peptides (**1′a–e**), which are synthesized on Fmoc-Rink amide PS resin (0.98 mol/g). After classical Fmoc-SPPS,[13] an aliquot (≈10 mg) of each peptidyl-Rink amide PS resin **1a–e** was submitted to TFA/TIS/H_2O 95:2.5:2.5 for 2 h to check the purity of model peptides. After diethyl ether precipitation of crude peptides and lyophilization, LC/MS analyses showed that expected unprotected peptides **1′a–e** were obtained with good purity (Table 1).

Resins **1a–e** were then treated with FITC and DIEA in the dark for 2 h to yield supported compounds **2a–e**. At this stage, only DIEA and FITC used in excess were detected in coupling solution but no thiohydantoïne **4a–e**. Moreover, Kaiser test[14] performed on resin beads **2a–e** was negative, indicating the efficiency of isocyanate coupling on the primary amine. Then, resins **2a–e** were submitted

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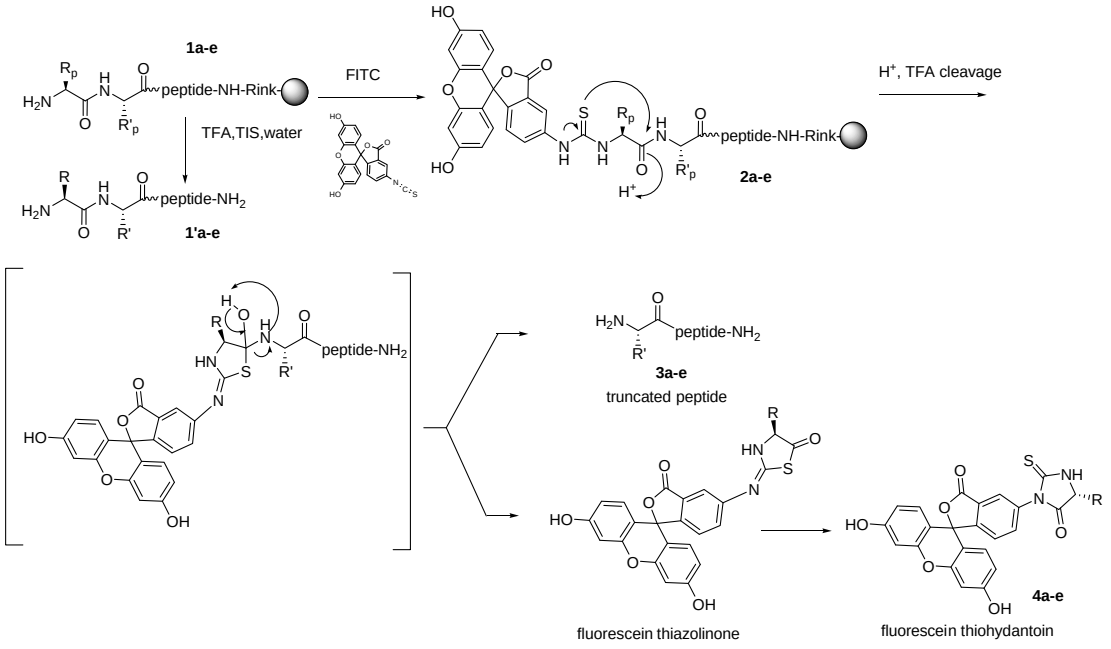


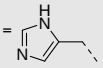
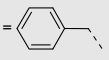
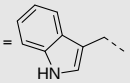
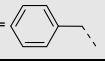
Scheme 1.

Table 1
Model peptides **1′a–e** in Scheme 1

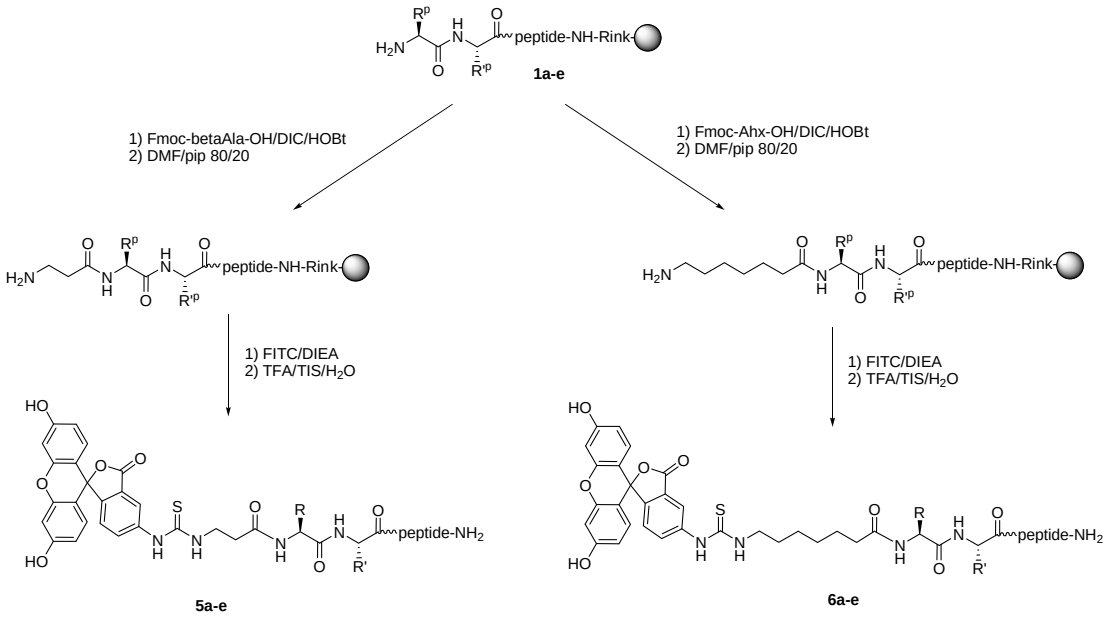
Compound	Sequence and MW calc.	Ions detected (ESI+)	Purity[a] (%)
1′a	Neurokine A H-HKTDSFVGLM-$NH_2$1132.6 Da	$[M+H]^+$ m/z = 1133.3; $[M+2H]^{2+}$ m/z = 516.7	99
1′b	Neurokine B H-DMHDFFVGLM-NH_2 1209.5 Da	$[M+H]^+$ m/z = 1210.5; $[M+2H]^{2+}$ m/z = 605.8	90[b]
1′c	Fulicine H-FNEFV-NH_2 653.3 Da	$[M+H]^+$ m/z = 654.3; $[M+2H]^{2+}$ m/z = 319.3	96
1′d	Nle CCK_4 H-W Nle DF-NH_2 578.4	$[M+H]^+$ m/z = 579.2	96
1′e	FLPAG peptide H-FLPAG-NH_2 502.3	$[M+H]^+$ m/z = 503.2; [2M+H]+ m/z = 1005.6	97

[a] Purity percent was estimated by integration of peak area in LC/MS chromatogram recorded at 214 nm.
[b] 10% of oxidation of methionine was detected.

Table 2
Cleavage products of resin **2a–e**

Truncated peptide **3**	MW calc.	Ions detected (ESI+)	Purity[a] (%)	Hydantoïne **4**	MW calc.	Ions detected (ESI+)
3a, H-KTDSFVGLM-NH_2	995.5	$[M+H]^+$ m/z = 996.7 $[M+2H]^{2+}$ m/z = 498.9	63[b]	**4a**, R = 	526.1	$[M+H]^+$ m/z = 527.2 $[M+2H]^{2+}$ m/z = 264.1
3b, H-MHDFFVGLM-NH_2	1094.5	$[M+H]^+$ m/z = 1095.4 $[M+2H]^{2+}$ m/z = 584.3	71[b]	**4b**, R = 	504.5	$[M+H]^+$ m/z = 505.1
3c, H-NEFV-NH_2	506.3	$[M+H]^+$ m/z = 507.2 $[2M+H]^+$ m/z = 1013.7	96	**4c**, R = 	536.1	$[M+H]^+$ m/z = 537.2
3d, H-Nle DF-NH_2	392.2	$[M+H]^+$ m/z = 393.1 $[2M+H]^+$ m/z = 785.5	62	**4d**, R = 	575.2	$[M+H]^+$ m/z = 576.2
3e, H-LPAG-NH_2	355.2	$[M+H]^+$ m/z = 356.3	75	**4e**, R = 	536.1	[M+H]+ m/z = 537.1

[a] Purity percent was estimated by integration of peak area in LC/MS chromatogram recorded at 214 nm. Yield ranged from 49% to 55% calculated on the basis of theoretical resin loading.
[b] 10–15% of oxidation of methionine was detected.



Scheme 2.

to TFA/TIS/H_2O treatment for 2 h. Thiohydantoins **4a–e** and truncated peptides **3a–e** were detected by LC/MS analysis of an aliquot of TFA cleavage solution (Scheme 1). We noticed that diethyl ether addition led to precipitation of truncated peptides **3a–e** (Table 2). Thiohydantoins **4a–e** were soluble in ether and after evaporation of the discarded diethylether phase, their presence was confirmed by LC/MS analysis.

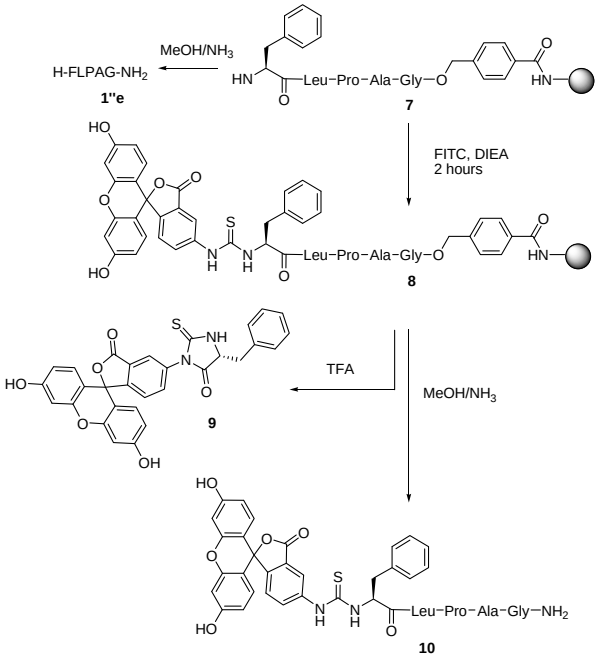
The thiazolinone mechanism is driven by the formation of the five-membered cycle only possible when the last residue in the peptide sequence is an α-amino acid. To complete this study, peptidyl-resins **1a–e** were coupled with a ε-amino or a β-amino acid (Fmoc-Ahx-OH and Fmoc-βAla-OH, respectively) and were reacted with FITC (Scheme 2). TFA-mediated cleavage afforded the expected fluorescent peptides **5a–e** and **6a–e**, respectively. After LC/MS analyses, no truncated peptides **3a–e** were detected, and the labeled peptides were obtained with high purity (Table 3) and average yield of 55%. The alkylene spacer has no incidence on the coupling efficiency of FITC on the free amino group at the N terminus of supported peptides.

According to our observations, the removal of the last amino acid by thiohydantoin formation occurs during final TFA treatment. As an evidence, model peptide **1″e** (identical to **1′e**, previously obtained on Rink amide linker) was synthesized on hydroxymethyl benzamido-PS-resin (HMBA-PS 1.1 mmol/g) using Fmoc SPPS (Scheme 3). The use of the base-labile linker HMBA[15] enabled to cleave the peptide from resin **7** using ammonia-saturated methanol solution for 4 h (purity = 81%, yield = 40%).[16] Resin **7** reacted as usual with FITC and DIEA in the dark for 2 h to yield fluorescent-immobilized peptidyl-resin **8**.

Table 3
FITC-labeled peptides **5** and **6** (Scheme 2)

Compound	MW calc.	Ions detected (ESI+)	Purity[a] (%)
5a	1593.6	$[M+2H]^{2+}$ m/z = 797.4 $[M+3H]^{3+}$ m/z = 532.17	99
5b	1669.6	$[M+2H]^{2+}$ m/z = 836.0 $[M+3H]^{3+}$ m/z = 557.7	63
5c	1113.4	$[M+H]^{+}$ m/z = 1114.5 $[M+2H]^{2+}$ m/z = 557.8	95
5d	1038.4	$[M+H]^{+}$ m/z = 1039.5 $[M+2H]^{2+}$ m/z = 520.4	95
5e	962.4	$[M+H]^{+}$ m/z = 963.5 $[M+2H]^{2+}$ m/z = 482.3	96
6a	1634.6	$[M+2H]^{2+}$ m/z = 818.7 $[M+3H]^{3+}$ m/z = 546.1	99
6b	1711.6	$[M+H]^{+}$ m/z = 857.0 $[M+2H]^{2+}$ m/z = 571.7	99
6c	1155.4	[M+H]+ m/z = 1156.65 $[M+2H]^{2+}$ m/z = 578.9	97
6d	1080.4	$[M+H]^{+}$ m/z = 1081.5 $[M+2H]^{2+}$ m/z = 541.4	95
6e	1004.4	$[M+H]^{+}$ m/z = 1005.6 $[M+2H]^{2+}$ m/z = 503.4	96

[a] Purity percent was estimated by integration of peak area in LC/MS chromatogram recorded at 214 nm.



Scheme 3.

TFA treatment performed on an aliquot (10 mg) of resin **8** led to a yellow TFA solution and white resin beads. After TFA evaporation and LC/MS analysis, fluorescent thiohydantoin **9** was detected as the main product and no peptide was released from the resin as expected. Ammonolysis of the same resin afforded expected fluorescent peptide **10** with 86% purity and 71% yield.

In conclusion, we highlighted a side reaction occurring upon TFA-mediated cleavage of N-terminal FITC-modified peptides on solid support. This reaction follows the Edman degradation pathway affording a fluorescent thiohydantoin (FTH) from the last α-amino acid of the peptide sequence. As expected, this reaction can be avoided by introduction of a non-α amino acid spacer between thiourea bond and the peptide or by using basic conditions for final deprotection of the FITC peptide from the resin. The steric hindrance considerations, sometimes considered as the main reason of using Ahx before fluorescent dye introduction, are not the reason why FITC should not be coupled directly on peptide.

Ahx or βAla were used successfully as spacers to generate FITC-labeled peptides. We also demonstrated that fluorescein-NH-CS-peptides can be obtained using base-labile linker HMBA. It is obvious that this last method suffers consistent limitations as appropriate non-acid labile side chain protecting groups are required to avoid TFA treatment of FITC-labeled peptide.

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16. 9% C-ter free acid peptide was detected by LC/MS as a result of hydrolysis of HMBA linker.