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Premature Removal of N^{α}-9-Fluorenylmethyloxycarbonyl in Thermodynamically Controlled Enzymatic Coupling Trials

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Abstract: Incubation of N′-Fmoc-ISDR-OH and amino components in mixtures of N,N-dimethylformamide/1,1,1,3,3,3-hexafluoroisopropanol (DMF/HFIP), DMF/2,2,2-trifluoroethanol (TFE) or DMF/1,4-butanediol (BD), 0.2M Tris/HCl buffer, triethylamine (TEA) and trypsin, apparent pH range 8.0-8.9, led to the undesired formation of variable amounts of dibenzofulvene in the reaction media. Such side reaction, which resulted from a premature Fmoc removal, is minimised by the use 1M buffer or by the increment of the trifluoroethanol proportion in the mixtures.

In some aspects, the use of proteases for peptide fragment condensation is more attractive than the chemical methods. For instance, because enzymatic condensations employ minimally protected substrates, solubility problems are greatly reduced in this methodology.[1-3]

Fmoc (9-fluorenylmethyloxycarbonyl) is a well-established urethane-type α-amino protecting group.[4] This base labile blocker has been widely used in chemical peptide synthesis [5,6] and, as a consequence, its stability in several anhydrous solvent systems generally used is quite known. In terms of enzymatic peptide synthesis, however, only a few reports using N^{α}-Fmoc protected carboxyl components can be found in the literature.[7-9] None of them discusses the stability of such protecting group during the enzymatic peptide bond formation under the experimental conditions employed. This knowledge is crucial for those working on enzymatic peptide segments condensation.

Herein we report for the first time the occurrence of premature Fmoc loss in thermodynamically controlled peptide fragment condensations. This secondary reaction leads to the partial consumption of N^{α}-protected compounds present in the reaction media (carboxyl component and coupling product), lowering the condensation yields. Besides, it may also cause secondary couplings due to the generation of a new nucleophile.[10] The data shown in this paper were obtained in thermodynamically controlled coupling trials between Fmoc-ISDR-OH, a N^{α}-protected fragment of cholecystokinin (CCK), and Phe-NH_2 or unsulfated CCK-8 using trypsin as catalyst and the experimental conditions reported by Nishino et al. [11] and Davey et al..[12]

The RP-HPLC monitoring of entries 1 and 2 (Table 1) revealed that after 24h incubation two main

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products were formed for entry 1: the desired N^{α}-protected pentapeptide (fast atom bombardment (FAB)-mass analysis: $M+H^{+}$found = 858; calcd mass = 857) and a very hydrophobic byproduct. In the same period of time, conditions for entry 2 gave rise only to the desired pentapeptide. The secondary product was then isolated by RP-HPLC and characterised, furnishing the following results: no amino acid (amino acid analysis); strong absorption at 310nm, $M+H^{+}$=178 (FAB-mass analysis). Such data indicated that this compound corresponded to the dibenzofulvene (DBF; $M+H^{+}$calcd.= 178) resulting from premature removal of the Fmoc group from the carboxyl component Fmoc-ISDR-OH and/or from the pentapeptide formed Fmoc-ISDRF-NH_2.

The premature deprotection observed was not expected to be that extensive because the apparent pH of the reactions were carefully kept in the range 8.0-8.9 (optimum pH range for trypsin activity), which is relatively low when compared to the high pH normally employed for Fmoc cleavage.[4] As the removal was faster in DMF/HFIP (1:1,v:v; entry 1) than in DMF/TFE (1:1,v:v; entry 2), in spite of the higher acidity of HFIP compared to that of TFE, we associated this difference to the fact that the molar proportion in DMF/HFIP was 1.00:0.73 and in DMF/TFE 1.00:1.06. Thus, the higher content of the acidic solvent in DMF/TFE seemed to prevent the premature loss of the Fmoc group.

It is important to note that the difference between the enzyme concentrations in reactions 1 and 2 is not relevant because Fmoc is not removable by trypsin.

Table 1. Coupling trials between Fmoc-ISDR-OH and H-Phe-NH_2 in different experimental conditions[11]

Entry	Organic Solvents mixtures (1:1,v:v)	Enzyme (mg/mL)
1	DMF/HFIP	0.12
2	DMF/TFE	4.0

Experimental Conditions: Fmoc-ISDR-OH (4.4μmol), H-Phe-NH_2 (1.0μmol), organic solvents mixtures (480μL) containing 4% (20μL) 0.2M Tris-HCl buffer (100mM NaCl and 10mM $CaCl_2$) and trypsin, 24h incubation. The reactions had their apparent pH adjusted to ~8.3 with TEA according to Nishino et al.[11] RP-HPLC monitoring: solvent A: 0.1%TFA; solvent B: 99% acetonitrile/water 0.09%TFA; linear gradient: 5 to 95%B in 30min; λ= 210nm; 1mL/min; column: Vydac C_{18}, 5μm, 300Å, 0.46 X 25cm.

Based on the data described above, we investigated the stability of Fmoc-ISDR-OH for some experimental conditions listed in Table 2. Initially, the conditions for entry 3 were studied in detail. For that, Fmoc-ISDR-OH was incubated in DMF/BD for 48h (**A**), in a mixture of DMF/BD and 0.2M buffer (pH 8.6; containing trypsin) for 48h (**B**) and, finally, in a mixture of DMF/BD, 0.2M buffer (pH 8.6; containing trypsin) and TEA, in amounts needed for final apparent of 8.0 - 8.9, for 48h (**C**). The analytical RP-HPLC profiles obtained are shown in Figure 1. As it can be seen, Fmoc-ISDR-OH was fully stable in the mixture of DMF/BD (**A**), almost as stable in DMF/BD containing 4% 0.2M buffer and enzyme (**B**) and fully unstable in the presence of TEA (**C**). These results indicated that it was the presence of this base that promoted the substantial Fmoc cleavage observed.

The effect of TEA was confirmed when Fmoc-ISDR-OH and an amino component (unsulfated CCK-8) were incubated in a mixture of DMF/BD (1:1,v:v) and 1.0M buffer (entry 4). By using such a concentrated buffer solution, no base was needed for the adjustment of the apparent pH to the range 8.0-8.9 and the Fmoc

group was relatively stable after 48h (**D**). The small amount of DBF detected in this reaction medium probably resulted from aminolysis by the amino component.[10] Despite this, such experimental conditions showed to be a good alternative for thermodynamically controlled enzymatic condensations without significant premature Fmoc removal.

Next, increments of TFE proportion in the DMF/TFE mixtures were tested (entries 5-7). The comparative results obtained after 96h incubation showed that when the proportion of TFE increased and the amount of TEA added was kept constant, the percentage of DBF formed decreased (Figure 2). Similar results were also observed during the coupling trials between the fragments Fmoc-SQQLLGLWGCSGK-OH and H-LICTTTVPWN-NH_2, in different proportions of TFE to DMF (data not shown). Since all these incubations were also performed at apparent pH 8.0-8.9, such data led us to speculate that TFE causes a diminution of TEA activity, probably due to a higher solvation through hydrogen bond formation. This would result in a higher stability of Fmoc in its presence. A similar mechanism has been suggested to explain the formation suppression of arginossuccinyl peptides by using acidic alcohols as additives.[13]

Table 2. Experimental conditions used to verify the stability of the Fmoc group in Fmoc-ISDR-OH

Entry	Fmoc-ISDR-OH (μmol)	Organic solvents (v:v)	Buffer (M)	TEA (μmol)
3 [12]	12.0	DMF/BD (1:1)	0.2	-[i]
4[ii]	12.0	DMF/BD (1:1)	1.0	-
5	0.3	DMF/TFE (4:1)	0.2	0.8
6 [11]	0.3	DMF/TFE (1:1)	0.2	0.8
7	0.3	DMF/TFE (1:4)	0.2	0.8

[i]TEA to adjust the apparent pH to 8.0-8.9, [ii] 120μmol of unsulfated CCK-8 was present in the reaction medium. Experimental Conditions: 3 and 4; organic solvents (35μL), Tris-HCl buffer containing 100mM NaCl and 10mM $CaCl_2$ pH 8.6 (15μL). 5-7; organic solvents (120μL), Tris-HCl buffer containing 100mM NaCl and 10mM $CaCl_2$ pH 8.3 (5μL). All the reaction conditions tested contained 6.6mg/mL of trypsin. RPLC Monitoring: solvent A: 0.1%TFA; solvent B: 80% acetonitrile/water 0.09%TFA (3 and 4) or 99% acetonitrile/water 0.09%TFA (5-7); linear gradient: 5 to 95%B in 30min; λ = 301nm; flow = 1mL/min; column: Vydac C_{18}, 5μm, 300Å, 0.46 X 25cm.

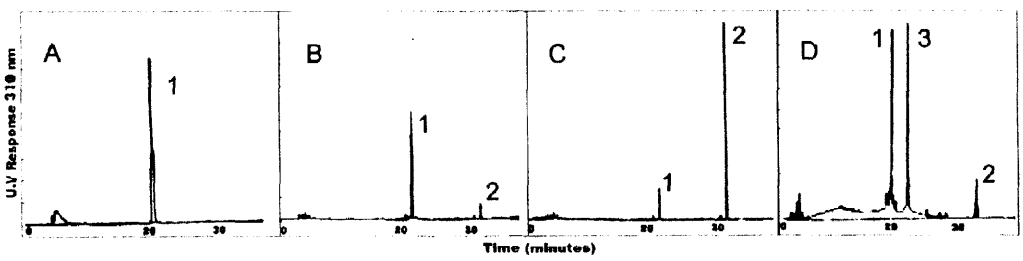

Figure 1. RP-HPLC monitoring of the DBF formation for entries 3 (**A-C**) and 4 (**D**). 1= Fmoc-ISDR-OH, 2= DBF, 3= unsulfated CCK-8. (**A**): 48h incubation of Fmoc-ISDR-OH + DMF/BD, (**B**): 48h incubation of Fmoc-ISDR-OH + DMF/BD + 0.2M buffer, (**C**): 48h incubation of Fmoc-ISDR-OH + DMF/BD + 0.2M buffer + TEA to apparent pH 8.0-8.9, (**D**): 48h incubation of Fmoc-ISDR-OH + unsulfated CCK-8 + DMF/BD + 1.0M buffer, apparent pH 8.6. For buffers description see Table 2.

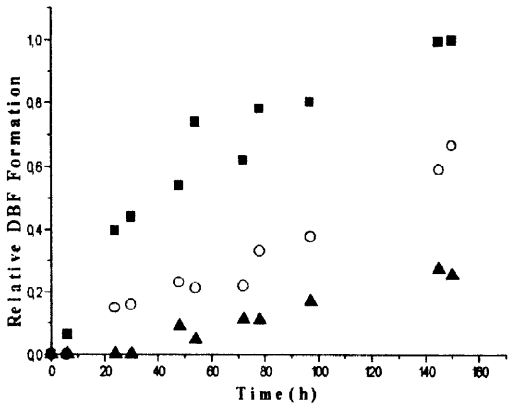

Figure 2. Relative DBF formation from Fmoc-ISDR-OH in different DMF/TFE proportions: ■ 4:1 (v:v), O 1:1 (v:v), ▲ 1:4 (v:v). The values are relative to the maximum formation of DBF in entries 5-7.

In conclusion, a study has been performed to demonstrate that a substantial undesired Fmoc removal occurs simultaneously to thermodynamically controlled peptide bond formation. Therefore, Fmoc stability under some experimental conditions used for such reactions has been determined. Additionally, conditions have been found to minimise the premature cleavage observed.

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