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Racemization Studies of Fmoc-Cys(Trt)-OH during Stepwise Fmoc-Solid Phase Peptide Synthesis

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Abstract: *In conventional stepwise Fmoc solid phase peptide synthesis, diisopropylethylamine (DIEA) base-catalyzed acylation methods lead to considerable amounts of racemization of Fmoc-Cys(Trt) residues during the activation/coupling process. The epimerization can be reduced to a negligible degree, via a base-free activation step.*

In connection with the development of a total synthesis of mouse macrophage migration inhibitory factor (mMIF), a cytokine with 115 amino acid residues[1,2,3], *via* the convergent solid phase peptide synthesis (CSPPS)[4] we have been faced with a serious problem: High racemization of the protected cysteine amino acid residue have been observed during the stepwise synthesis of the protected cysteinyl peptide fragment mMIF (70-82), Fmoc-Gly^{70}-Ala-Gln(Trt)-Asn(Trt)-Arg(Pmc)-Asn(Trt)-Tyr(tBu)-Ser(tBu)-Lys(Boc)-Leu-Leu-Cys(Trt)-Gly^{82}-OH. The peptide was synthesized batchwise on an ECOSYN P peptide synthesizer (Eppendorf-Biotronik, Maintal, Germany) *via* the Fmoc/tBu strategy on a highly acid labile 2-chlorotrityl resin[5]. Fmoc deprotections were performed with 25% piperidine/dimethylformamide (DMF) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate(TBTU)-activated couplings (Fmoc-amino acid (3 eq., 0.2 mmol/ml DMF), TBTU (3 eq.), 1-hydroxybenzotriazole (HOBt) (1 eq.), diisopropylethylamine (DIEA) (6 eq.), 35 min, RT) were used for incorporation of the amino acid residues. *Via* Kaiser tests[6] and thin layer chromatography (TLC)[7] each deprotection and coupling step was controlled. After incorporation of Fmoc-Lys(Boc)-OH, an unexpected by-product was detected by TLC (ΔR_f = 0.02, $CHCl_3$/MeOH/HAc = 7:2:0.5).

For further investigations, the peptide fragments mMIF(78-82) and mMIF(77-82) were cleaved from the resin with simultaneous deprotection (trifluoroacetic acid/thioanisole/ethandithiol/phenol/H_20 = 8.4: 0.7:0.5:0.2:0.2, 3h), followed by ether precipitation and lyophilization. Reversed phase-HPLC profiles of the crude peptides are shown in Figure 1 and ion spray mass spectroscopic measurements (IS-MS) resulted

in identical masses for fractions 1 and 2 having different retention times. Racemization as a source of the observed heterogeneity could be verified though by racemization studies[8,9] on the mMIF(78-82) and mMIF(77-82) peptides (Table 1), indicating that a high degree of epimerization occurs during the Fmoc-Cys(Trt)-OH/TBTU/HOBt/DIEA coupling step.

Table 1. D-amino acid analysis of synthetic mMIF(78-82) and mMIF(77-82) peptides[a].

peptide	%D-Cys	%D-Leu	%D-Lys	%D-Ser
H-KLLCG-OH[b]	10.30	1.50	0.14	-
H-KLLCG-OH[c]	0.78	0.79	<0.20	-
H-SKLLCG-OH[b]	11.71	1.60	0.21	1.70
H-SKLLCG-OH[c]	1.20	0.59	<0.20	1.80
H-KLL(D-C)G-OH[b]	80.50	0.82	1.60	-
H-KLL(D-C)G-OH[c]	98.30	0.59	0.16	-
H-SKLL(D-C)G-OH[b]	73.70	1.12	<0.50	1.80
H-SKLL(D-C)G-OH[c]	98.80	0.63	0.34	0.55

[a]Determination by capillary gas chromatography mass spectroscopy (GC-MS); conditions: hydrolyses: 6N D_2O/DCl + 1% thioglycolic acid (TGA), 110° C, 24 h, vacuum; derivatization: esterification: CH_3OD, 10% DCl, 110° C, 15 min; acylation: trifluoroacetic anhydride, 110° C, 10 min; gas chromatographic conditions: GC-column: Chirasil-Val film (20 cm x 0.3 mm silica capillary), T= 60° C/3'/ 3,5'/185° C, detection: selected single ion monitoring mass spectrometry (SIM-MS).
[b]crude deprotected peptides, [c]reversed phase-HPLC-purified peptides.

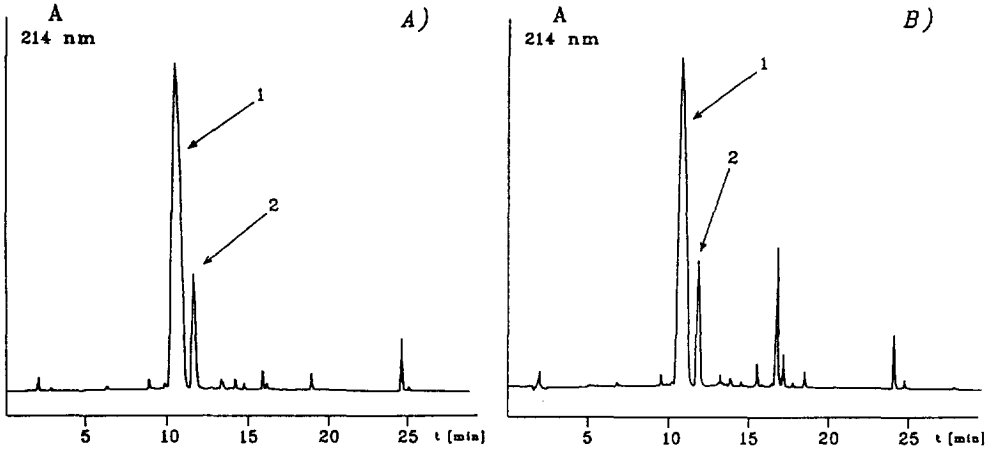

Figure 1. RP-HPLC profiles of crude deprotected synthetic mMIF(78-82) (panel A) and mMIF(77-82) (panel B). Chromatographic conditions: column: 4,6 Nucleosil 120-5 C_{18} (Macherey Nagel, Düren, Germany); eluents: A: 0,05% TFA in H_2O, B: 0,05% TFA in CH_3CN, flow rate: 1 ml/min, gradient elution: 0-1min 95% A, 1-31min 40% A (detection: λ = 214 nm), HPLC system: Gynkotek, Munich, Germany. Peak identification according to IS-MS. Peak 1 = L-Cys-epimer, Peak 2 = D-Cys-epimer.

Similar degrees of racemization were also observed, using Fmoc-D-Cys(Trt)-OH for acylation in the SPPS of the penta- and hexapeptides mMIF (78-82) and mMIF (77-82), enabling the structural proof of the related diastereomers by comparative reversed phase HPLC (Figure 2) and amino acid analysis (Table 1).

Variations of the experimental conditions, using DIEA as tertiary amine causing lowest racemization levels in comparison to other tertiary amines[10] and additional amounts of HOBt for further supression[10], could not reduce the degree of epimerization during the base-catalyzed "in situ" couplings to acceptable amounts (Table 2).

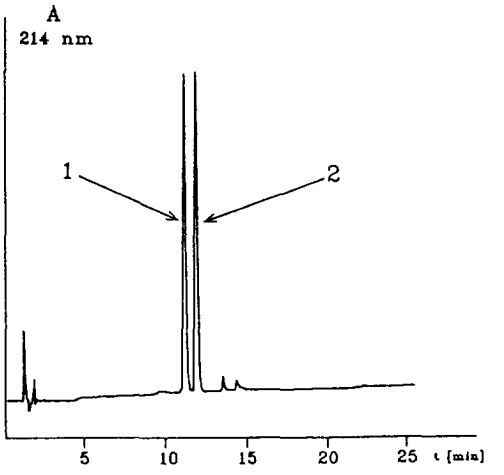

Figure 2. Co-chromatography (RP-HPLC) of the L-Cys and D-Cys mMIF(78-82) peptide diastereomers. Chromatographic conditions see Figure 1. Peak identification according to IS-MS; Peak 1 = L-Cys-epimer, Peak 2 = D-Cys-epimer.

Table 2. Racemization studies of crude deprotected mMIF (78-82) produced under different coupling conditions.

Coupling procedures	Contents of D-Epimer	
	RP-HPLC[a]	D-amino acid analysis[b]
Fmoc-AA/TBTU/HOBt/DIEA = 3 : 3 : 1 : 6	13%	10.30%
Fmoc-AA/TBTU/HOBt/DIEA = 3 : 3 : 1.5 : 6	11%	n.d.
Fmoc-AA/TBTU/HOBt/DIEA = 3 : 3 : 3 : 6	7%	n.d.
Fmoc-PSA = 3	< 1%	1.41%
Fmoc-AA/TBTU/HOBt/DIEA = 3 : 3 : 0 : 6, but PSA for Cys	< 1%	1.14%
Fmoc-AA/TBTU/HOBt/DIEA = 3 : 3 : 1 : 6, but PSA for Cys	< 1%	0.30%

[a]conditions see legend to Figure 1;
[b]conditions see legend to Table 1.

Epimerization as undesired side reaction during DIEA-catalyzed acylation of Fmoc-Cys(Trt)-OH was also observed during the syntheses of other Cys peptides in our laboratory, indicating that this racemization step is not sequence-dependent. Finally, we succeeded to solve this serious and general problem of racemization by using the neutral preformed symmetrical anhydride (PSA) method[11] for the Cys coupling step, which reduced the racemization to negligible amounts at the stage of the penta- and hexapeptides (Table 2, Figure 3).

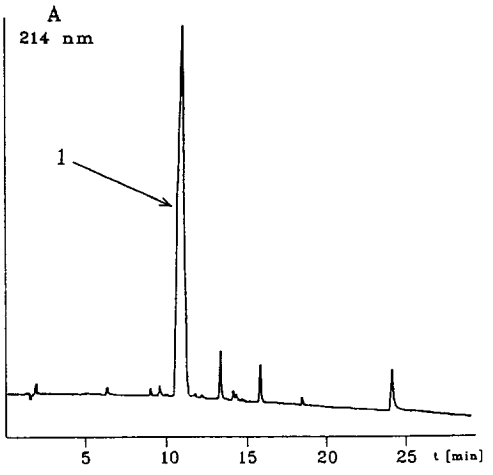

Figure 3. RP-HPLC profile of the crude deprotected synthetic mMIF(78-82), using the PSA coupling for the incorporation of the Fmoc-L-Cys(Trt)-OH residue. Chromatographic conditions see Figure 1.

In conclusion we want to emphasize that only activation/coupling methods in a medium free of base can prevent the racemization during the coupling of Fmoc-Cys(Trt)-residues to peptide fragments, and would like to address this message specially to peptide chemists using automatic solid phase peptide synthesizers to develop appropriate software programs and load their protected amino acid vials with corresponding synthons, if Cys couplings are needed producing their peptide sequence.

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