Optimized Coupling Protocols for the Incorporation of Cys Derivatives during Fmoc-SPPS

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Introduction

Incorporation of cysteine during Fmoc-SPPS can result in significant racemization depending on the coupling conditions [1-4]. During the preparation of cysteine containing peptides as APIs, we identified the necessity to investigate coupling conditions for Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH based on previous results of other groups [2-4] in more detail.

Using the model peptide Z-Ala-Cys(PG)-Pro-OH, we studied the influence of coupling reagent, solvent, activation time, and temperature during preactivation and coupling on the racemization of Cys. The LDL-isomer of the model peptide can be separated from the LLL-form with standard RP-HPLC.

Experimental Procedures

The model peptide Z-Ala-Cys(PG)-Pro-OH was synthesized on H-Pro-2-chlorotrityl resin. Couplings were performed with 3 equiv. of amino acid derivative / coupling reagent / base or additive. Fmoc-cleavage was performed using 20 % piperidine in DMF (5′ + 10′). Peptides were cleaved from the resin with 1 % TFA in DCM. After evaporation, the residue was analyzed on HPLC (Bakerbond C18, 300 Å, 5 µ, 4.6 x 250 mm; gradient of ACN in 0.1 % TFA; flow 1 ml / min, λ = 220 nm). The content of the LDL-isomer was determined using the relative peak areas from HPLC as: A(LDL-isomer) / [A(LDL-isomer) + A(LLL-isomer)].

Coupling Reagent

To investigate the influence of the coupling reagent, DMF was used as solvent. As described previously for other peptides [2-4], TBTU as coupling reagent with DIPEA or collidine as base lead to significant formation of the LDL-isomer with Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH (see Table 1). With Fmoc-Cys(Acm)-OH, replacing DIPEA with the weaker base collidine resulted in a clear reduction of racemization.
Couplings with the carbodiimides DCCI and DIC in the absence of base gave < 1.0 % of the LDL-isomer.

Table 1

Reagent	Base	Additive	LDL-Isomer (%) Cys(Trt)	Cys(Acm)
TBTU	DIPEA	---	2.6	4.6
TBTU	collidine	---	2.5	0.6
DCCI	---	HOBt	0.6	n.d.
DIC	---	HOBt	0.9	0.7

Solvent

As the carbodiimides yielded lower levels of the LDL-isomer compared to TBTU (see Table 1), the influence of the solvent on the degree of racemization during the coupling of Fmoc-Cys(Trt)-OH was examined using DIC.
The widely used standard solvents for SPPS, DMF and NMP, were compared to their less polar 1 / 1 mixtures with DCM, toluene, and THF (see Table 2).
In accordance with the results of Han et al. [3], the 1 / 1 mixture of DMF and DCM gave less racemization than neat DMF. Compared to DMF, also NMP yielded less LDL-isomer. Replacing DCM with toluene or THF retained the positive effect of DCM in mixtures with DMF and NMP.

Table 2

LDL-Isomer (%)			
DMF	DMF/DCM	DMF/toluene	DMF/THF
0.9	0.2	0.3	0.1
NMP	NMP/DCM	NMP/toluene	NMP/THF
0.4	0.3	0.3	0.3

Preactivation Time and Temperature

For coupling of Fmoc-Cys(Trt)-OH with DIC / HOBt, the influence of preactivation time and temperature during preactivation and coupling was investigated (see Table 3). With DMF as well as with DMF / toluene = 1 / 1, best results were obtained with preactivation times of 5′ and 15′ at 25 °C. These findings differ from previously published data [3].
Therefore, we chose a preactivation time of 5′ for further experiments and varied the temperature during preactivation and coupling. The coupling rate was clearly reduced at 0 °C, but complete coupling was obtained after 20 h. At 0 °C and at 32 °C, we found higher levels of racemization than at 15 °C or at 25 °C.

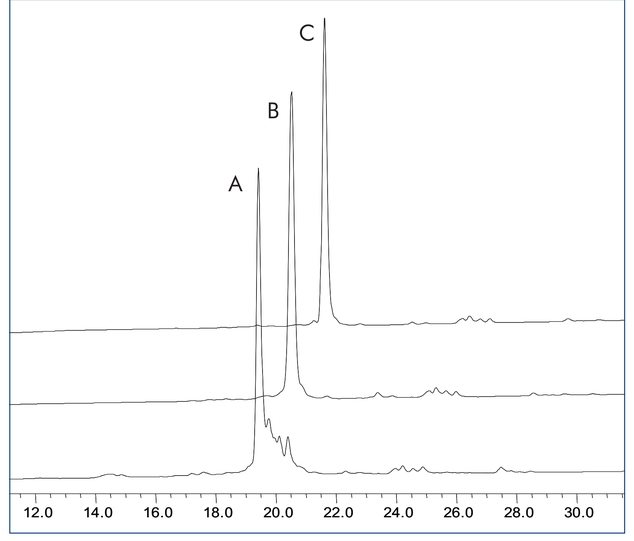
Table 3

Preactivation Time (min)	Temp. (°C)	LDL-Isomer (%) DMF	DMF/toluene
0	25	1.2	0.8
5	25	0.9	0.5
15	25	0.9	0.6
30	25	1.0	0.8
60	25	1.3	0.9
5	0	1.8	1.0
5	15	0.9	0.6
5	25	0.9	0.5
5	32	1.6	1.4

A Case-Study: SPPS of an Enterotoxin-like Peptide

Recently, we developed the SPPS for an Enterotoxin-like peptide containing 6 Cys and the following cysteine pattern: ---CCXXCCXXXCXXCX---.
As a control experiment, we performed the SPPS using Fmoc-Cys(Trt)-OH and TBTU / DIPEA in DMF. HPLC analysis of the resulting crude, linear peptide showed large amounts of related, mostly later eluting impurities (see Figure 1, line A).
By changing the coupling reagent to DIC / HOBt, a clear increase in quality of the crude peptide was achieved (see Figure 1, line B).
A minor additional improvement was observed with DMF / DCM = 1 / 1 as solvent and DIC / HOBt as coupling reagent (see Figure 1, line C).

Figure 1



Conclusion

Coupling of Fmoc-Cys(Trt)-OH or Fmoc-Cys(Acm)-OH with TBTU / DIPEA can result in significant racemization. Carbodiimides like DCCI and DIC with an additive (e.g. HOBt) in the absence of base are the reagents of choice for coupling of Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH. With a preactivation time of 5′ and a reaction temperature of 15 - 25 °C, the resulting amount of LDL-isomer in the model peptide Z-Ala-Cys(PG)-Pro-OH was always below 1 % with DMF or NMP as solvent. The level of racemization can be further reduced by using less polar solvents, for example 1 / 1 mixtures of DMF or NMP with DCM, toluene, or THF, respectively.

References

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