

RACEMIZATION IN AUTOMATED SOLID PHASE PEPTIDE SYNTHESIS

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INTRODUCTION

Automated peptide synthesis allows for preparation of a large number of peptide sequences in a fairly straightforward manner. Currently, the most widely applied methodologies utilize Fmoc-protection in combination with uronium/phosphonium activating agents for synthesis of peptides. The racemization process has been extensively studied with these activating agents on solid support [1]. Most experiments were carried out via manual protocols. Automated peptide synthesis adds another limitation affecting racemization process.

Our recently presented studies covering the two most racemization-prone amino acids, cysteine and histidine pointed out optimal conditions for their incorporation to a peptide chain using an automated peptide synthesis. We have expanded current work to the following commonly used amino acids: Asp, Leu, Ser and Tyr. The previous model tripeptide, Z-Ile-Xxx-Pro-OH, applied to cysteine and histidine residues did not prove to be satisfactory in the current work due to the poor resolution of LLL and LDL diastereoisomers in standard RP-HPLC conditions. In order to obtain a baseline resolution of the studied isomers we have applied in our experiments the previously published model peptide H-Gly-Xxx-Phe-NH₂ [2]. Herein we reevaluate the degree of racemization of Asp, Leu, Ser and Tyr using several popular activating agents applied to a typical automated peptide synthesis using an in-situ activation method.

METHODS

PEPTIDE SYNTHESIS

Model peptides H-Gly-Xxx-Phe-NH₂ where Xxx= Asp, Leu, Ser and Tyr, were used in these studies and prepared on Rink-MBHA resin using the Tetras (Thuramed) multiple peptide synthesizer and in-situ activation. Details of the synthesis are outlined below:

♦ Resin: Fmoc-Phe-Rink-MBHA- resin, 150 mg, 0.6 meq/g, 100-200 mesh, 1% DVB

♦ Amino acids: 0.3 M solutions in NMP

♦ Reagents:

- > 0.4 M solution in NMP (HBTU, PyClock®)
- > 0.6 M solution in NMP (BOP, PyBOP®, HCTU, HATU, COMU)
- > 0.9 M solution in NMP (DIEA)
- > 1.0 M solution in NMP (DIC, HOBt, 6-Cl-HOBt, Oxyma Pure)

♦ Washings after deprotection and coupling: 10 times with 3 mL of DMF for 1 min.
♦ Deprotection: 2 times with 3 mL of 20% piperidine in DMF, for 2 min and 20 min.

♦ Coupling:

> With uronium/phosphonium reagents (order of addition to the resin, no preactivation): Fmoc-amino acid 0.5 mmole, DIEA 0.9 mmole, reagent 0.5 mmole, mixing for 120 min, at RT

> With diisopropylcarbodiimide/additive (order of addition to the resin, no preactivation): additive (HOBt, Cl-HOBt, HOAt, Oxyma Pure) 0.5 mmole, Fmoc-amino acid 0.5 mmole, DIC 0.5 mmole, mixing for 120 min, at RT

> Reference diastereomeric peptides, H-Gly-D-Xxx-Phe-NH₂ where Xxx= Asp, Leu, Ser and Tyr and their L-isomers were prepared as well by automated peptide synthesis using the same starting resin and HCTU/DIEA as the activation protocol.

CLEAVAGE

The cleavage of all studied peptides from the resin was performed with the mixture of TFA : water : phenol : TIPS (87.5:5.5:2.5) for 2 hours at RT. The resin was filtered off and TFA was evaporated under reduced pressure. The product was precipitated by addition of cold diethyl ether, centrifuged and dried.

HPLC ANALYSIS

Chromatographic analysis of the obtained products was performed on Waters Alliance HPLC system using a Vydac C18 column (4.6x250 mm, 21XTP54) with the linear gradients: 1-21 % B in 30 min for H-Gly-Asp-Phe-NH₂, 10-30 % B in 30 min for H-Gly-leu-Phe-NH₂, 1-21 % B in 30 min for H-Gly-Ser-Phe-NH₂ and 5-35 % B in 30 min for H-Gly-Tyr-Phe-NH₂. Buffers used for the analysis where A: 0.1% TFA in H₂O and B: 0.1% TFA in MeCN with flow of 1 ml/min and detection at 220 nm.

The listed above conditions allowed baseline separation of LLL from LDL diastereoisomers (Figures 1-4).

The content of LDL isomer was calculated as relative peak areas (Absorbance) from HPLC as: A (LDL isomer) x 100/[A (LDL isomer) + A (LLL isomer)]. Results of the analysis are presented in Table 1.

RESULTS

We have used model peptides, H-Gly-Xxx-Phe-NH₂ where Xxx= Asp, Leu, Ser and Tyr, as targets to evaluate the degree of racemization during incorporation of Fmoc-Asp(OBut)-OH, Fmoc-Leu-OH, Fmoc-Ser(But)-OH and Fmoc-Tyr(But)-OH to the solid support utilizing a Tetras automated peptide synthesizer with in-situ activation. We have selected the most popular and widely used coupling agents in our experiments. We also included the recently introduced reagents: COMU [3, 4], PyClock [5] and Oxyma Pure [3, 4, 6, 7]. Published racemization results until now have covered mostly either solution or manual solid phase synthesis.

We have applied, in our studies, the in-situ activation method routinely used in batch type automated peptide synthesis. The instrument used in these studies is capable of precise delivery of up to 32 different reagents and amino acids to the reaction vessel. In many cases of the routine peptide synthesis the same activating method is used to prepare an entire peptide sequence. This approach is necessary due to the limitation of instrumentation (lack of sufficient number of reagents precisely delivered on board of synthesizer) or the user's choice. Our previous studies indicated that it is necessary to select proper activation for cysteine and histidine residues in order to minimize racemization. Use of diisopropylcarbodiimide with various additives was found to be a method of choice for introduction of cysteine and histidine residues using an automated peptide synthesis.

In our current studies we only used DIEA (distilled over nihidrin) as base for uronium and phosphonium reagents. Presented results in Table 1 show clearly that in all studied cases (Asp, Leu, Ser, Tyr) the level of racemization was below 1%. The majority of the examples implementing carbodiimide mediated activation with various additives gave lower racemization than uronium/phosphonium reagents. Changing the amount of base to one equivalent in COMU mediated coupling did not reduce significantly racemization as reported earlier [8].

CONCLUSION

Results of our studies limits the application of uronium/phosphonium reagents for cysteine and histidine incorporation into a peptide chain due to extensive racemization. Other amino acids are less prone to racemize in studied conditions. Our data reemphasizes existing problems and reminds practitioners in the field that traditional activating reagents such as carbodiimides in combination with racemization suppressants are good alternatives to minimize epimerization of amino acids including cysteine and histidine residues during activation in solid phase peptide synthesis. This strategy can be a universal activation protocol for other amino acids when the desired peptide sequence to be synthesized is straight forward.

TABLES/DATA

Table 1. Racemization during SPPS of model peptides

Reagent	% LDL isomers					
	H-Gly-Xxx-Phe-NH ₂				Z-Ile-Xxx-Pro-OH	
	Asp	Leu	Ser	Tyr	Cys(Trt)	His
BOP	0.71	0.11	0.32	0.22	5.1	1.1
COMU	0.8	0.12	0.34	0.27	1.5	14.9
COMU 1/2B	0.66	0.13	0.34	0.2	-	-
HATU	0.62	0.09	0.25	0.22	2.9	0.7
HBTU	0.44	0.23	0.35	0.17	4.4	2
HCTU	0.51	0.1	0.37	0.25	6.5	2.8
PyBOP	0.42	0.11	0.53	0.2	5	1.3
PyClock	0.51	0.17	0.25	0.27	7.5	4.1
DIC/Cl-HOBt	0.72	0.15	0.06	0.19	0.6	0.7
DIC/HOAt	0.44	0.14	0.21	0.19	-	-
DIC/HOBt	0.4	0.08	0.07	0.22	0.6	2.3
DIC/OXYMA	0.7	0.09	0.06	0.16	0.1	2.2

Figure 1: HPLC of a mixture of H-Gly-Asp-Phe-NH₂ (1) and H-Gly-D-Asp-Phe-NH₂ (2)

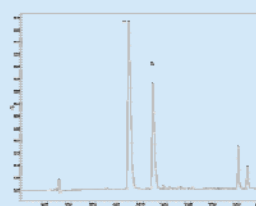


Figure 2: HPLC of a mixture of H-Gly-Leu-Phe-NH₂ (1) and H-Gly-D-Leu-Phe-NH₂ (2)

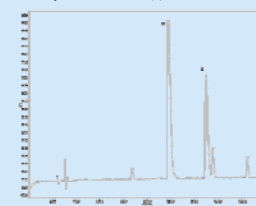


Figure 3: HPLC of a mixture of H-Gly-Ser-Phe-NH₂ (1) and H-Gly-D-Ser-Phe-NH₂ (2)

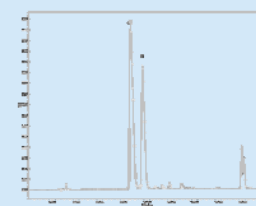
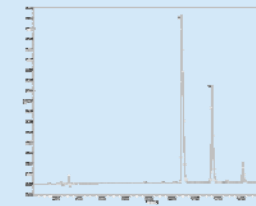


Figure 4: HPLC of a mixture of H-Gly-Tyr-Phe-NH₂ (1) and H-Gly-D-Tyr-Phe-NH₂ (2)



KEY POINTS

- ♦ Most of the studied reagents gave low level of racemization for all studied amino acids except Cys and His.
- ♦ Uronium/phosphonium reagents should be avoided when possible for incorporation of Cys and His residues.
- ♦ Carbodiimide mediated activation gave lower racemization in comparison to uronium/phosphonium reagents.
- ♦ Combination of diisopropylcarbodiimide with proper additive (HOBt, Oxyma Pure) can be used as universal activation method for all studied amino acids including cysteine and histidine.
- ♦ Oxyma Pure is a promising alternative to HOBt and its derivatives.

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