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Racemization Studies of Fmoc-Ser(tBu)-OH During Stepwise Continuous-Flow Solid-Phase Peptide Synthesis

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Abstract: We observed unexpectedly high levels of racemization of Fmoc-Ser(tBu)-OH during automated stepwise solid-phase peptide synthesis under standard continuous-flow conditions. We set up a model assay, based on the solid-phase assembly of the tripeptide H-Gly-Ser-Phe-NH_2, in order to test the effect of the coupling conditions on serine racemization. Protocols based on collidine as the tertiary base added to the coupling reagent enabled incorporation of serine with less than 1% racemization.

Keywords: Amino acids and derivatives; Racemization; Solid-phase synthesis.

The key step in the preparation of synthetic peptides is the controlled formation of a peptide bond between two amino acids, the so-called coupling reaction. It is well known that this reaction, which requires the activation of the carboxyl group of one amino acid, is particularly prone to racemization. Accordingly, coupling methods which preserve the configurational integrity of the carboxylic component have been widely studied.[1] In stepwise solid-phase peptide synthesis (SPPS) the problem of racemization is generally assumed to be less dramatic than for other strategies. In fact, amino acids N^{α}-protected by a urethane type blocking group, such as 9-fluorenyl-methoxycarbonyl (Fmoc) or tert-butoxycarbonyl (Boc), are resistant to racemization during activation and coupling.[2] Moreover, the large excess of reagents generally used in SPPS makes the coupling reaction faster than in solution, thus minimising the loss of chirality. Consequently, it is widely accepted that in SPPS the risk of racemization is reduced to a negligible level, if not completely absent. However, recent reports indicate that racemization of cysteine residues can be a serious concern in Fmoc SPPS.[3] We now report a similar observation regarding high levels of racemization of Fmoc-Ser(tBu)-OH[4] during stepwise SPPS under standard continuous-flow conditions.

In the course of our structure-activity relationship studies of bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH), which imply the synthesis of several Ser-containing analogues using standard automated continuous-flow SPPS methods,[5] we consistently observed in the crude a side-product with the same mass as the desired peptide, as shown by EI-MS. The racemization analysis of the side-product, isolated by semi-preparative RP-HPLC, indicated that racemization occurred at the level of the Ser residue. We decided to set up a

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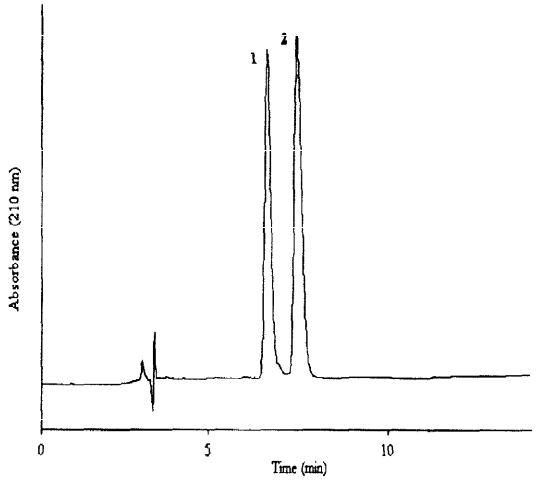

Figure 1. HPLC of a mixture of H-Gly-Ser-Phe-NH_2 (1) and H-Gly-D-Ser-Phe-NH_2 (2). See[7] for chromatographic conditions.

model assay for serine racemization, with the aim of investigating the effect of coupling conditions on this side reaction. By analogy to what recently reported by Han et al.,[3c] we used as a model the solid-phase assembly of the tripeptide H-Gly-Ser-Phe-NH_2 on TentaGel S AM resin.[6] After deprotection and cleavage, it is possible to obtain a base-line separation of the two diastereoisomers, H-Gly-Ser-Phe-NH_2 and H-Gly-D-Ser-Phe-NH_2, by analytical RP-HPLC,[7] as shown in Fig. 1, using an authentic sample of H-Gly-D-Ser-Phe-NH_2.[8]

Most common coupling protocols for SPPS typically involve preactivation of the amino acid with a coupling reagent, such as uronium or phosphonium salts of HOBt and HOAt, in the presence of an additive, i.e. HOBt and HOAt, and a tertiary amine base.[9] For the choice of the coupling conditions to be tested in the model assay, aimed to the development of a "racemization free" protocol, it should be taken into account that several factors have been reported to affect racemization during coupling,[1] including the method of activation, the amount and the chemical character of the organic base added to the reaction mixture, and the solvent. However, when dealing with protocols to be routinely employed in continuous-flow SPPS using an automated synthesizer, some limitations should be considered. In fact, it is not possible to abolish a preactivation period, which corresponds to the time employed by the machine to dissolve the amino acid and the activator and to deliver the solution to the reaction column. Moreover, for most of the routinely users of automated continuous-flow synthesizer, it will be easier to maintain DMF as the sole solvent for coupling and washings. Thus, we decided to limit our study to the effect on serine racemization of coupling reagents of the last generation and the added base. This choice is supported by recent studies indicating that the optimization of these two factors can be very efficient for racemization reduction, at least in the case of cysteine coupling in SPPS[3] and in fragment condensation strategies.[10] Accordingly, as shown in Tab. 1, we tested the two coupling reagents HBTU[11] and HATU[12], either alone or in the presence of HOBt[13] or HOAt[14] as additives, using as base NMM or TMP.[10] Each synthesis was performed essentially as described for the standard peptides,[8] starting from 0.2 g of resin.

The results reported in Tab. 1 are expressed in terms of peak area ratio (D-Ser/L-Ser %) in the analytical HPLC separation of the diastereomeric peptides in the crude mixtures. It appears clearly that all the five coupling protocols based on the use of NMM gave rise to a totally unacceptable level of serine racemization. Thus, more

Table 1. Racemization of Fmoc-Ser(tBu)-OH During the Synthesis of the Model Peptide H-Gly-Ser-Phe-NH_2 by SPPS as a Function of Coupling Conditions.

No	Reagent	Additive	Base	D-Ser/L-Ser %
1	HBTU	--	NMM	6.4
2	HBTU	HOBt	NMM	6.5
3	HATU	--	NMM	22.2
4	HATU	HOBt	NMM	10.4
5	HATU	HOAt	NMM	18.1
6	HBTU	--	TMP	2.0
7	HBTU	HOBt	TMP	1.4
8	HATU	--	TMP	0.6
9	HATU	HOBt	TMP	0.4
10	HATU	HOAt	TMP	0.3

than 6% D-Ser was formed when HBTU was used as the coupling reagent, either alone (entry 1) or in the presence of HOBt as additive (entry 2). The lack of effect of added HOBt was observed also by others.[10a] Surprisingly, the 7-aza-1-hydroxybenzotriazole-based reagent HATU gave the worst result (entry 3; 22% D-isomer). This data is in line with the observation that the performance of this new reagent are optimal when it is used with TMP as the added base.[10] Once again, the use of additives did not improve significantly the results, in terms of racemization suppression (entries 4 and 5).

The situation is much better when we replaced NMM by the more hindered TMP, as suggested by Carpino.[10] In this case the claimed superiority of HATU over HBTU[10,12,14] is more evident: HBTU alone gave 2.0% of D-isomer (entry 6), which was reduced to a still unsatisfactory 1.4% using HOBt as additive (entry 7). It was possible to obtain less than 1% racemization with the HATU-based coupling protocols, both using this reagent alone (entry 8) and in the presence of additives (entries 9 and 10).

Taken together, these data are in total agreement with those previously reported by Carpino and co-workers[10] in the case of fragment condensation and with the findings of Han et al.,[3c] referred to the racemization of cysteine in SPPS. A critical role of the added base was also observed by Kaiser et al.,[3b] within their racemization study of Fmoc-Cys(Trt)-OH in SPPS.

In conclusion, racemization of serine residues during continuous-flow SPPS appears to be an underscored problem; according to the results obtained in our model assay, it can be reduced to acceptable levels simply by the use of TMP as the added base in the coupling mixture. The best results, i.e. less than 0.5% of racemization, have been obtained using HATU as the coupling reagent and HOBt or HOAt as additives.

References and Notes

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4. Abbreviations are in accord with the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All amino acids are in the L configuration unless otherwise specified. Other abbreviations: Boc, t-butyloxycarbonyl; DMF, dimethylformamide; EI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxy-carbonyl; HATU, (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; HBTU, N-[[(dimethyl-amino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; NMM, N-methyl-morpholine; RP-HPLC, reverse-phase high performance liquid chromatography; SPPS, solid-phase peptide synthesis; TMP, 2,4,6-trimethylpyridine or collidine.
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6. TentaGel S AM is a polystyrene-supported polyoxyethylene resin with the 5-(4-amino-methyl-3,5-dimethoxyphenoxy) valeric acid linker for the synthesis of peptide amides.
7. HPLC conditions: Beckman System Gold apparatus, Vydac C_{18} column 0.46 x 15 cm; eluant A: 0.1% TFA/water, eluant B: 0.1% TFA/acetonitrile; gradient from 5% to 65% B over 20 min; flow 1 ml/min; detection UV, 210 nm.
8. Standard peptides were synthesized on a Milligen 9050 automated synthesizer, starting from 0.5 g of TentaGel S AM resin (0.25 mmol/g); Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH or Fmoc-D-Ser(tBu)-OH and Fmoc-Gly-OH were coupled using HBTU/HOBt/NMM activation (4:4:8 equiv.) in DMF (15 min at 8 ml/min). Fmoc removal was carried out with 20% piperidine in DMF (v/v) for 3.5 min at 8 ml/min. Final deprotection and cleavage was obtained using reagent K for 90 min at room temperature.
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