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Phenylglycine racemization in Fmoc-based solid-phase peptide synthesis: Stereochemical stability is achieved by choice of reaction conditions

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ABSTRACT

Phenylglycine-containing peptides have broad applications in medicinal chemistry, but their synthetic accessibility is complicated by the risk of epimerization during solid-phase peptide synthesis (SPPS). Phenylglycine is therefore often considered a troublesome residue. This work studies the extent of Phg racemization under different Fmoc-SPPS reaction conditions. It is shown that the base-catalyzed coupling of Fmoc-Phg is the critical step for racemization. However, racemization can be reduced to a negligible level if DEPBT or COMU combined with TMP or DMP are employed during this step. Resin-bound peptides are remarkably resistant against epimerization during extended incubation under basic conditions and the free peptides were stable in buffer solutions used for biological assays.

Introduction

Phenylglycine-containing peptides received an increasing attention in the past decade, owing to their diverse pharmaceutical applications and importance in chemical biology. For example, phenylglycine (Phg) and its derivatives such as 4-hydroxyphenylglycine (Hpg) and 3,5-dihydroxyphenylglycine (Dpg) are important building blocks within various antimicrobial peptides, such as streptogramins (virginiamycin S, streptogramin B, pristinamycin I or dityromycin) or glycopeptides (vancomycin).[1] Apart from this, peptidic inhibitors incorporating Phg have shown promising biological activity against hepatitis C, dengue and West Nile Virus proteases.[2–9] Hence, Phg plays an important role as a peptidic building block in the development of novel pharmaceutical drugs and biologically active compounds.

Solid-phase peptide synthesis (SPPS) using Fmoc chemistry is a powerful method to obtain pharmaceutically active peptides, primarily because of its high efficiency, universal compatibility, and feasibility.[10] However, acquiring the correct epimer of Phg-containing peptides still poses a non-negligible challenge owing to the increased acidity of the proton at the α-carbon, which facilitates the loss of configuration during SPPS.[11,12] Furthermore, the resulting mixture of epimers can also complicate the purification procedures of Phg-containing peptides. The extent of mesomeric effect and subsequent stability of the anion intermediate at the α-carbon depend on the nature and the position of the phenyl ring substituents, which probably explains the order of susceptibility to racemization for Phg and its derivatives (Hpg < Phg < Dpg).[12] Since the properties of peptides and proteins extensively depend on the chiral centers, loss of configuration should be limited in SPPS.

Some work was conducted to suppress Phg racemization during synthesis in solution,[13,14] but surprisingly, to the best of our knowledge, there have been only two reported studies[11,12] up to now that specifically comment on the racemization of Phg during SPPS, and systematical studies appear to be missing in the current literature. Considering the common notion that Phg is a highly

Abbreviations: COMU, (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylaminomorpholinocarbenium hexafluorophosphate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DEPBT, 3-diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMP, 2,6-dimethylpyridine; DMTMM-BF_4, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate; Dpg, 3,5-dihydroxyphenylglycine; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; Hpg, 4-hydroxyphenylglycine; NMM, 4-methylmorpholine; Phg, phenylglycine; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; RP-HPLC, reverse-phase high performance liquid chromatography; TMP, 2,4,6-trimethylpyridine; TRIS, tris(hydroxymethyl)aminomethane.

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problematic residue with respect to racemization, our own practical experiences during extended synthetic programs that involved this residue indicated that racemization was a manageable issue under the conditions used (20% piperidine for deprotection, HATU/DIPEA/no preactivation for coupling), and the resulting epimerization products, at least for smaller peptides, can be reliably separated on preparative RP-HPLC.[5–8] This led us to investigate this issue, in order to further understand the behavior of this residue in SPSS. A major aim was also to identify conditions that were more robust towards epimerization, less prone to be influenced by modifications of the experimental protocol, and require a less resource-intensive purification step for the final products. Hence, the aim of this contribution is to systematically study the racemization behavior of Phg during SPSS, to identify the critical synthetic step causing Phg racemization, and to establish a straightforward approach to minimize or eliminate this undesired reaction.

Results and discussion

The racemization of Phg during SPPS is proposed to proceed via enolization under basic conditions that are encountered during coupling (pathway A and B, Fig. 1) or Fmoc deprotection (pathway C) steps due to the acidity of the α-carbon.[11,15]

Accordingly, the reagents used for the two main steps, i.e., Fmoc deprotection and amino acid coupling, were investigated. For this purpose and for subsequent stability assessment, the benzoyl-capped dipeptides Bz-(L)-Phe-(L)-Phg-NH_2 (peptide A) and Bz-(L)-Arg-(L)-Phg-NH_2 (peptide B) were chosen as model compounds. Phenylalanine and arginine do not racemize under the conditions tested in the current study, and allow straightforward access to the crude products by precipitation in water or ether, respectively. Rink amide resin was used for the Fmoc-SPPS and the following procedures were regarded as standard reference for further comparison: a) For the coupling step, the amino acid (3.0 equiv) and coupling reagent (3.0 equiv) were dissolved in 1 ml DMF, before the base (4.0 equiv) was added. This reaction mixture was aspired immediately to the resin and the solution was shaken for one hour. b) Fmoc deprotection was performed by adding 1 ml 10% piperidine solution per 100 mg resin twice and shaking for 10 and 5 min, respectively. Peptide stock solution were prepared in DMSO and further diluted in acetonitrile (MeCN) and water (1:1) to analyze the percentage of the correct diastereomer using RP-HPLC.

Earlier investigations indicated a contribution of the Fmoc deprotection procedure to the racemization of Phg during SPPS under mild microwave or conventional conditions.[11] A minor enhancement was reported with 20% piperidine in DMF under microwave SPPS[11] or 1% DBU in DMF for 30 s under conventional SPPS.[12] Following up on these findings, a variety of bases for the removal of the Fmoc group were examined (Table 1). A comparable percentage of the correct diastereomer was observed with all tested deprotection reagents, suggesting no significant effect on the racemization of Phg during SPPS.

Variation of the concentration of piperidine in DMF, the volume of the solution used, and the deprotection time did not influence the extent of Phg racemization. Remarkably, 1 ml 5% piperidine solution in DMF was enough to completely remove the Fmoc protection groups.[16] Attempts to use weaker bases than piperidine and combination with HOAt did not provide a noticeable improvement.[17–19] Among these conditions, it should be noted that Fmoc deprotection was not complete with 2 ml 20% morpholine solution in DMF or 1 ml 3% piperazine solution in DMF. Surprisingly, the use of 1% 1,8-diazabicycloundec-7-ene (DBU) in DMF with short deprotection time (30 s,[12] 1 min, 2 min, and 3 min) did not afford

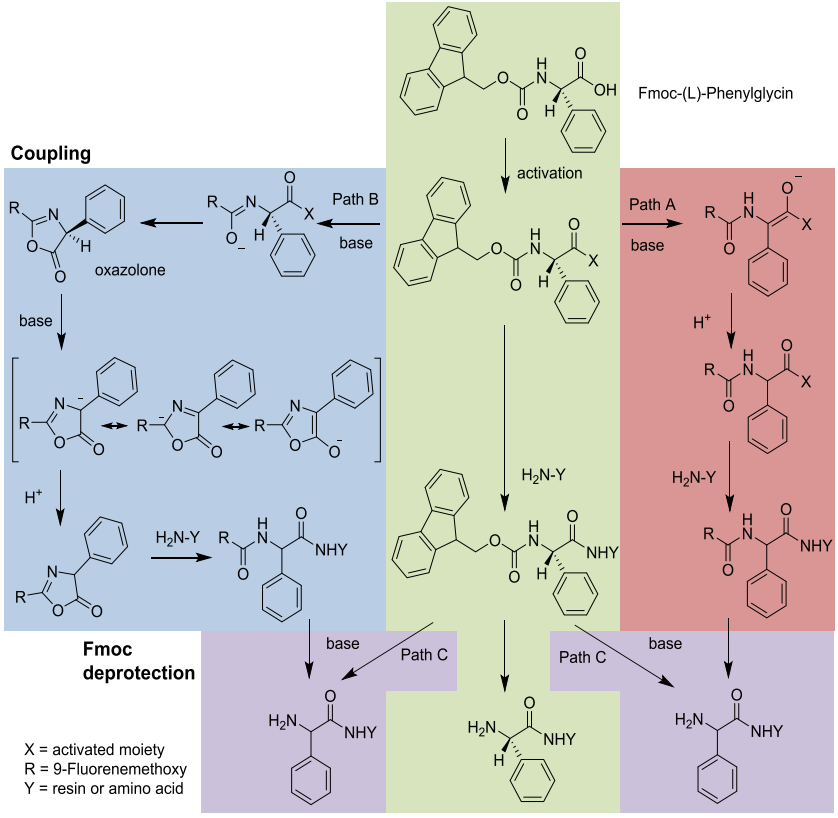


Fig. 1. Potential racemization pathways of Fmoc-(L)-phenylglycine during solid-phase peptide synthesis.

Table 1
Fmoc deprotection. Optimization of reaction conditions.[a]

Entry	Base	Volume (ml)[b]	Deprotection time (min)	Correct diastereomer (%)	
				Peptide A	Peptide B
1	25% piperidine	2	10/5	66	74
2	10% piperidine	2	10/5	70	75
3	5% piperidine	2	10/5	71	75
4	10% piperidine	2	5/5	69	75
5	10% piperidine	1	10/5	71	76
6	10% pyrrolidine	2	10/5	73	76
7	5% piperazine	1	10/5	74	76
8	5% piperazine and 0.1 M HOAt	1	10/5	68	71
9	5% piperazine	1	5/5	69	72
10	10% 4-methylpiperidine	1	10/5	68	77
11	1% DBU	1	5/5	68	73
12	20% morpholine	2	10/5	ntd	ntd
13	3% piperazine	1	10/5	ntd	ntd
14	1% DBU	1	0.5	ntd	ntd

ntd not totally deprotected.
[a] Reaction conditions: Coupling of the Fmoc-protected amino acids (3 equiv) was performed with HATU (3 equiv) as an activator and DIPEA (4 equiv) as a base for 1 h in DMF. The deprotection of the Fmoc group with various bases was performed twice.
[b] Volume of base solution per 100 mg resin.

complete cleavage of the Fmoc group, as observed by HPLC and mass analysis. Total deprotection was achieved by extending the time to 5 min (twice) but did not offer an advantage in reducing the epimerization in comparison to other tested conditions.

A remarkable influence on the racemization of Phg in both model peptides was observed by replacing *N*,*N*-diisopropylethylamine (DIPEA) with alternative bases during the coupling step while maintaining the standard procedure using HATU as amino acid activator and 1 ml 10% piperidine solution in DMF for Fmoc deprotection (Table 2). The results are in agreement with those reported previously,[12,20,21] where bases that are weaker than DIPEA (pK_a 10.1) and have an enhanced steric shielding of the amino function relative to *N*-methylmorpholine (NMM, pK_a 7.38), were proposed to minimize Phg racemization. In our hands, the best outcomes were obtained with 2,4,6-trimethylpyridine (TMP, pK_a 7.43) or 2,6-dimethylpyridine (DMP) as bases catalyzing the coupling reaction. With TMP, the percentage of the correct diastereomer was 93% for peptides A and B.

Using the standard procedure, the exchange of HATU by alternative activators[22] provided a noticeable effect on the degree of racemization of Phg. HATU presented slightly better result than HBTU and PyBOP. A lower degree of racemization was observed with DMTMM-BF_4 and DEPBT, and the best performance was achieved with COMU (92% for peptides A and B), a third generation uronium coupling reagent.

Table 2
Coupling reaction. Optimization of reaction conditions.[a]

Entry	Activator	Base	Correct diastereomer (%)	
			Peptide A	Peptide B
1	HATU	DIPEA	71	76
2	HATU	NMM	56	62
3	HATU	PMP	75	88
4	HATU	TMP	93	93
5	HATU	DMP	93	95
6	HBTU	DIPEA	61	68
7	PyBOP	DIPEA	64	67
8	DMTMM-BF_4	DIPEA	86	80
9	DEPBT	DIPEA	77	79
10	COMU	DIPEA	92	92
11	DMTMM-BF_4	NMM	85	88
12	DMTMM-BF_4	TMP	94	95
13	DEPBT	TMP	98	93
14	DEPBT	DMP	100	98
15	COMU	TMP	96	99
16	COMU	DMP	98	96

[a] Reaction conditions: Coupling of the Fmoc-protected amino acids (3 equiv) was performed with various activators (3 equiv) and bases (4 equiv) for 1 h in DMF. The deprotection of the Fmoc group with 10% piperidine (1 ml per 100 mg resin) was performed twice (10 min and 5 min).

DMTMM-BF_4/NMM coupling conditions were reported by El Sawy et al.[11] to efficiently reduce Phg racemization under microwave SPPS conditions but showed moderate improvement for our model compounds. Replacing DMTMM-BF_4/NMM with DMTMM-BF_4/TMP gave a higher percentage of the correct diastereomer. Unfortunately, the use of DMTMM-BF_4 is limited by its rather poor solubility in DMF under conventional SPPS.

Aiming at racemization-free conditions, COMU or DEPBT were combined with TMP or DMP because of their established potential in suppressing racemization.[12,23,24] Under these conditions a percentage of 93–100% for the correct diastereomer could be attained. Hence, the combination of COMU or DEPBT with TMP or DMP can reduce the racemization to a practically negligible level. Both COMU and DEPBT have better solubility in DMF than HATU, and the coupling time for the model peptides could be reduced to 30 min by using COMU.[23]

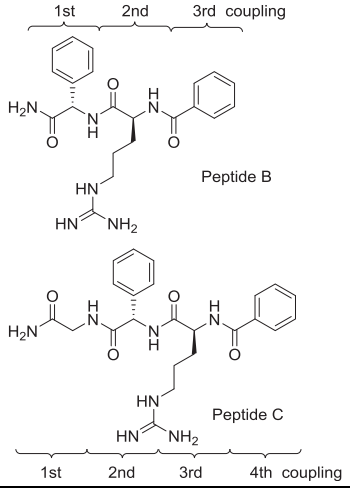
The presented results suggest that the coupling reagents strongly affect the degree of racemization of Phg. To determine whether this effect takes place mainly with the activated Fmoc-Phg amino acid or with the resin-bound Phg-containing peptide, the activator and the base were modified only during the coupling of Fmoc-Phg (Table 3).

When COMU/TMP was used as coupling reagent for Fmoc-Phg and all following coupling steps and other conditions were kept as in the standard procedure, the percentage of the correct diastereomer of the Bz-(L)-Arg-(L)-Phg-NH_2 model dipeptide was more than 98%. The improvement in the percentage of racemization was lost by using HATU/DIPEA as coupling reagent for Fmoc-Phg and COMU/TMP for subsequent steps. In order to exclude an influence of the resin on the coupling process, glycine was inserted in the first coupling step. The outcome remained the same, independent of the position of Phg in the peptide sequence.

These findings further support our reasoning that the crucial step inducing racemization is solely the coupling of Fmoc-Phg and the activated amino acid is most sensitive to the base effect.

After finding the best condition to suppress epimerization during SPPS, the stability of resin-bound peptide B under different basic conditions was assessed. This is particularly relevant for

Table 3
Evaluation of the critical coupling step for epimerization.



1st Coupling	2nd Coupling	3rd Coupling	Correct Diastereomer (%)
HATU/DIPEA	HATU/DIPEA	HATU/DIPEA	76
COMU/TMP	COMU/TMP	COMU/TMP	99
COMU/TMP	HATU/DIPEA	HATU/DIPEA	98
HATU/DIPEA	COMU/TMP	COMU/TMP	69

1st Coupling	2nd Coupling	3rd Coupling	4th Coupling	Correct Diastereomer (%)
HATU/DIPEA	HATU/DIPEA	HATU/DIPEA	HATU/DIPEA	73
COMU/TMP	COMU/TMP	COMU/TMP	COMU/TMP	98
HATU/DIPEA	COMU/TMP	HATU/DIPEA	HATU/DIPEA	99
COMU/TMP	HATU/DIPEA	COMU/TMP	COMU/TMP	71

Table 4
Stability in solution overnight.

Entry	Solution	Correct diastereomer (%)
1	DMF	98
2	5% piperidine in DMF	95
3	10% piperidine in DMF	95
4	20% piperidine in DMF	85
5	1% DBU in DMF	67
6	1% DBU in DMF/H_2O (1:1)	97
7	0.1 M aq. NaOH in DMF	98
8	4.4% DIPEA in DMF[a]	98
9	3.3% TMP in DMF[a]	98
10*	TRIS buffer pH 7.5	99
11*	TRIS buffer pH 9	99

[a] Percentage of base used in the coupling step with 3 equiv of the amino acid per 100 mg resin.
* Not resin-bound peptide.

cases were a prolonged exposure to bases is expected during SPPS, such as during the synthesis of long peptide sequences, or coupling of difficult building blocks, or on-resin chemical derivatization reactions. Therefore, the peptide was treated with the specified solutions (Table 4) and stored overnight. After cleavage from the resin under standard conditions, the degree of epimerization was evaluated by RP-HPLC. Overall peptide B was stable in DIPEA and TMP solutions in DMF overnight. A loss of configuration was noticeable with 20% piperidine solution in DMF, and to a much higher extent with 1% DBU solution in DMF. The outcome of this investigation suggests that exposure to strong bases over extended periods tends to induce the racemization of Phg in peptides.

As mentioned previously, Phg-containing peptides can be used as enzyme inhibitors. The biological activity against certain viral proteases is routinely determined in a pH 9 buffer solution in our laboratory.[25] To study the pH influence on the racemization, peptide B was placed in pH 7.5 and pH 9 TRIS buffer solutions and was incubated in different attempts for 0 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min and overnight. Analysis by RP-HPLC confirmed the stability of the peptide in pH 7.5 and pH 9 buffer solutions. Aqueous conditions were previously shown to increase the stereochemical stability of Phg,[26] an effect that we were able to reproduce by adding water to the racemization-prone 1% DBU conditions (see Table 4, entry 5 vs. entry 6). However, the use of water to suppress racemization is usually not acceptable during the most commonly employed SPSS conditions. The presence of water in the derivatization of Hpg, the details of the experimental protocol,[6] and the increased robustness of Hpg (vs. Phg) against racemization also explains our previous experiences that the final products contained only minor, and separable, amounts of the epimers.

Conclusion

In conclusion, the racemization of Phg-containing peptides during Fmoc-SPPS was systematically assessed using two model peptides. From the obtained results, it can be deduced that not the removal of the Fmoc group, but the base-catalyzed coupling of Fmoc-Phg is the crucial step for racemization. Epimerization free synthesis of both model peptides could be obtained by using DEPBT or COMU combined with TMP or DMP for coupling. Incubation of the resin-bound peptide overnight in different base solutions in DMF generally did not cause significant epimerization, except in presence of 1% DBU in DMF and to a lesser extent in presence of 20% piperidine in DMF. Finally, the Bz-(L)-Arg-(L)-Phg-NH_2 model peptide was stable in buffer solutions at pH 7.5 and pH 9.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2017.04.047.

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