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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

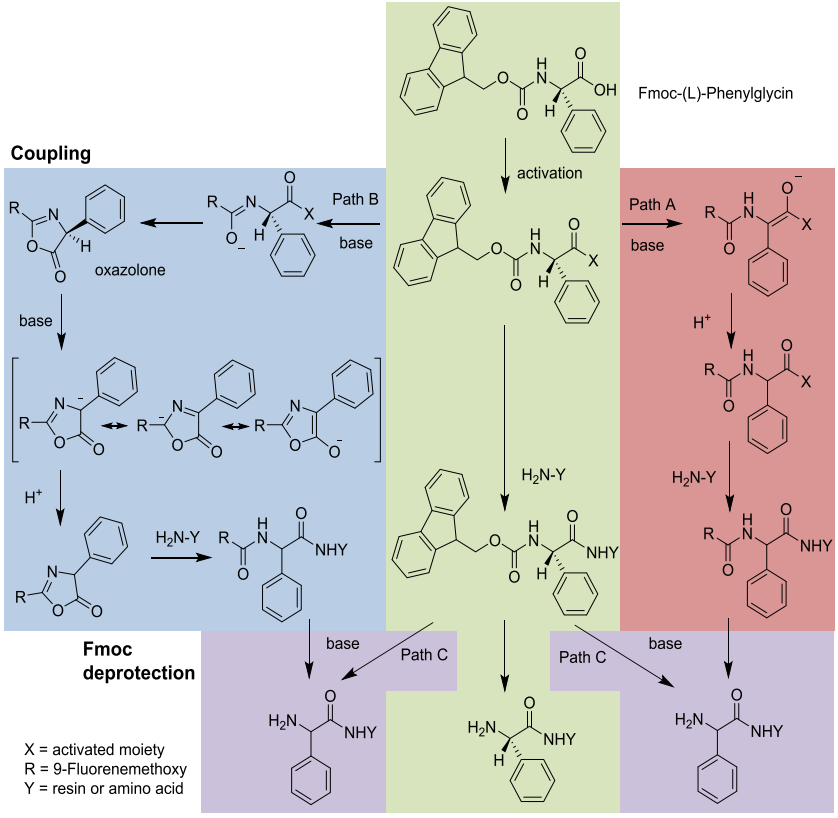

Fig. 1. Potential racemization pathways of Fmoc-(L)-phenylglycine during solid-phase peptide synthesis.