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# Comparison of various coupling reagents in solid-phase aza-peptide synthesis

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ABSTRACT

Aza-peptides are promising drug leads, however extensive study of their properties is hampered by low yielding aza-peptide bond formation during conventional Fmoc SPPS. The kinetics of aza-peptide bond formation in the model peptide H-Ala-AzAla-Phe-$NH_2$ was compared with various conventional amino acid activators. The reaction rates and yields were dependent on the activator structure. The reaction time of aza-peptide formation using oxyma-based agents was approximately 30 times longer than in typical peptide synthesis. Therefore, new activators are required to increase the reactivity of the activated amino acid to achieve effective acylation of the semicarbazide moiety during aza-peptide bond formation.



Biologically active peptides may represent ideal drug candidates due to their biocompatibility and outstanding specificity for various target sites. However, peptide applicability is generally limited by their rapid degradation in living organisms.[1,2] Thus, identifying suitable chemical modifications that increase peptide stability is a major challenge. Several different approaches to peptidomimetic design have emerged.[3] One such approach is chemical modification of the peptide backbone structure *via* nearly isosteric substitution of the α-carbon atoms in conventional amino acids **1** with nitrogen to yield amino acid aza-analogs **2** (Fig. 1).

The first study concerning biologically active aza-petides was reported by Hess and co-workers.[4] As a result, many attempts have been made to synthesize and study peptidomimetics containing aza-amino acids. As expected, these studies revealed that the aza-peptide bond is significantly more stable towards degradation by peptidases, such as α-chymotrypsin[2,5] and subtilisin,[5] than the typical peptide bond. This is in line with the general understanding that the carbonyl C atom within the structural fragment –NH–NR–C(O)–NH– exhibits lower electrophilicity than that of the amide group –NH–CH(R)–C(O)–NH– in the peptide backbone.

The replacement of amino acids with their aza-analogs also induces greater peptide backbone rigidity, since the φ and ψ dihedral angles of the aza-peptide bond are constrained.[6,7]

These constraints favor formation of the β-turn conformation, as observed *via* X-ray and NMR studies and revealed using computational models. This conformational effect may have minimal influence on the binding effectiveness of aza-peptides with different binding sites in proteins.[8] Thus, these peptidomimetics represent promising drug leads.[9,10]

In spite of these challenging features,[5] the properties of aza-peptides have only been less studied.[4,6,11–13] This is due to the difficulties associated with the synthesis of aza-amino acid precursors and building blocks.[11,12] Additionally, common peptide bond synthesis methods, wherein the peptide *N*-terminal amino group is acylated by the subsequent amino acid, are unefficient for aza-peptide bond synthesis. In this case, acylation of the semicarbazide moiety $NH_2$–NR–C(O)– should occur instead of alkylamine acylation, however the nucleophilicities of these two nitrogen atoms are different.[14,15] Unfortunately, most studies do not consider this difference and the similarities between the chemistries of peptide and aza-peptide bond synthesis have been advocated even in textbooks.[16]

The differing reactivities of amino groups in α-amino acids **1** and their aza-derivatives **2** can be theoretically justified by the nucleophilicity parameters N = 13.85 ($s_N$ 0.53) and N = 11.05 ($s_N$ 0.52) listed in Mayr's nucleophilicity database for methylamine and semicarbazide, respectively.[17] The aim of this study was to


**1** **2**

**Fig. 1.** Structure of amino acid (**1**) and aza-amino acid (**2**).

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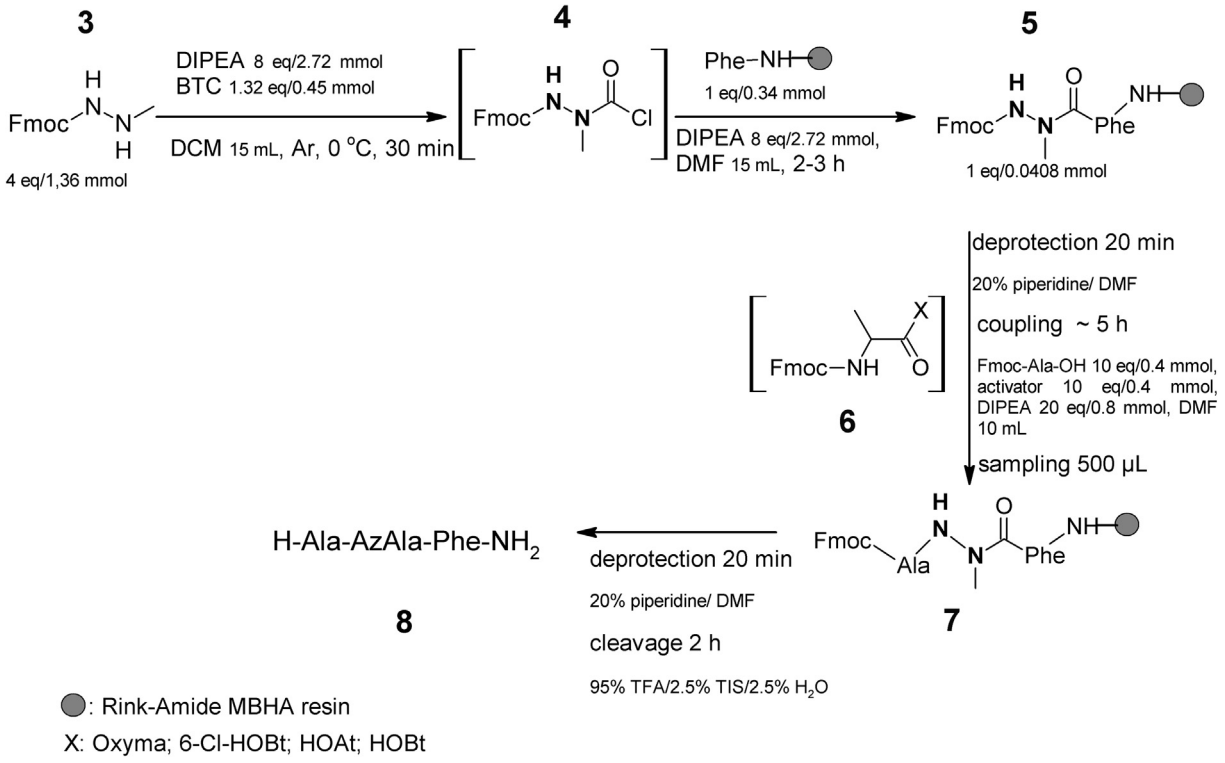



**Scheme 1.** Model aza-peptide H-Ala-AzAla-Phe-$NH_2$ synthesis.

experimentally evaluate these differences, as well as the applicability of the conventional SPPS protocol to aza-peptide bond synthesis in general. For this purpose, a systematic investigation of the kinetics of aza-peptide bond formation in the model aza-peptide H-Ala-AzAla-Phe-$NH_2$ was conducted, and the applicability of various amino acid activators to this reaction was analyzed. To the best of our knowledge, no systematic aza-peptide bond formation kinetic study has been reported thus far.

Scheme 1 outlines the preparation of the model aza-peptide, H-Ala-AzAla-Phe-$NH_2$ **8** from Fmoc-protected methyl hydrazine **3** (see ESI for details). The most critical step is formation of the aza-peptide bond *via* the coupling of activated Fmoc-Ala-OH **6** to the nitrogen atom of the deprotected semicarbazide moiety in **5**. Therefore, a detailed kinetic study of this step was conducted using various activating agents. Each activating agent inserts a different leaving group X into alanine **6** to facilitate coupling of the amino acid to the resin-linked peptide or aza-peptide.

The reaction of activated alanine **6** to the deprotected peptide sequence **5** was studied under conditions defined by the conventional SPPS protocol. At appropriate times, aliquots were taken from the reaction mixture and analyzed *via* HPLC (see ESI for details). As a 10-fold excess of alanine relative to the number of resin-bound reaction sites was used, the process was described using a first-order rate equation

$$Y = e^{-k_{obs}t} + Y_{\infty} \quad (1)$$

where $k_{obs}$ is the observed first-order rate constant, t is the reaction time and $Y_{\infty}$ is the plateau value that is reached at the end of the acylation reaction. Parameter Y characterizes the tripeptide **7** formation process and was calculated using the remaining starting material (dipeptide) and tripeptide peak areas (S) from same chromatographic run:

$$Y = \frac{S_{dipeptide}}{S_{dipeptide} + S_{tripeptide}} \quad (2)$$

**Table 1**
Kinetic study of aza-peptide bond formation *via* the reaction of Fmoc-Ala-OH with the semicarbazide group of the resin-bound H-AzAla-Phe residue at 25 °C using various coupling reagents. Eq. (1) was used to calculate the $k_{obs}$ and yield $(1 - Y_{\infty})$ values using Graphpad 5 software.

| Activator | Leaving group X | $k_{obs}$, $min^{-1}$ | Yield | $pK_a$ of HX[18] |
|---|---|---|---|---|
| COMU[19] | Oxyma | 0.022 ± 0.001 | 0.99 ± 0.01 | 4.24 |
| PyOxim[20] | Oxyma | 0.023 ± 0.001 | 0.95 ± 0.01 | 4.24 |
| HDMC[21] | 6-Cl-HOBt | 0.016 ± 0.001 | 0.55 ± 0.02 | 4.62 |
| HCTU[22] | 6-Cl-HOBt | 0.017 ± 0.002 | 0.68 ± 0.03 | 4.62 |
| HATU[23] | HOAt | 0.017 ± 0.001 | 0.93 ± 0.01 | 4.65 |
| TBTU[24] | HOBt | 0.004 ± 0.001 | 0.69 ± 0.05 | 5.65 |
| PyBOP[25] | HOBt | 0.005 ± 0.002 | 0.65 ± 0.14 | 5.65 |

The observed first-order rate constants ($k_{obs}$) and reaction yields $(1 - Y_{\infty})$ were determined at 25 °C (Table 1), and in some cases also at 40 °C. The reaction time was dependent on the activator used, but product formation was monitored for 300 min in most experiments

Initially, the time evolution of aza-tripeptide **7** formation was compared to synthesis of the typical peptide bond in the resin-bound tripeptide Fmoc-Ala-Ala-Phe. Two oxyma-based coupling reagents, COMU and PyOxim, were used to activate Fmoc-protected alanine in the latter case. The $k_{obs}$ values for resin-bound tripeptide Fmoc-Ala-Ala-Phe formation were 1.02 ± 0.29 and 1.06 ± 0.24, respectively. The results of the study using aza-peptides are listed in Table 1 and the kinetic curves obtained at 25 °C are shown in Fig. 2.

COMU and PyOxim had similar effects on both reactions; nearly complete acylation of the resin-linked aza-peptide and the conventional peptide were achieved. In the case of peptide synthesis, the reaction half-life[26] ($t_{1/2} = \ln 2/k_{obs}$) is about 1 min. This is in line with expectations of the fast conventional Fmoc SPPS protocol, where the amino acid coupling time is 2–5 min at room temperature.[22,27]

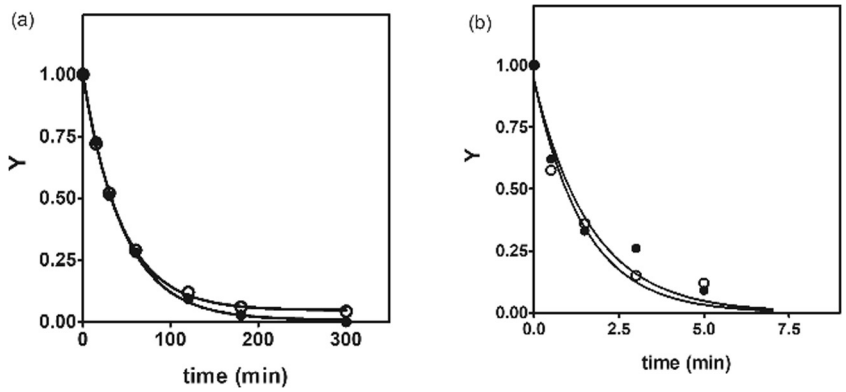



**Fig. 2.** Time evolution of the reactions between activated Fmoc-alanine and resin-bound H-AzAla-Phe during aza-peptide bond synthesis (a), and with resin-bound H-Ala-Phe during peptide bond synthesis (b) at 25 °C in DMF. Fmoc-alanine was activated with either COMU (●) or PyOxim (○).

However, the reaction time of aza-peptide formation is approximately 30 times longer, as can be seen from the $k_{obs}$ values listed in Table 1. This difference is also indicated by the time-scale of the kinetic curve for this reaction (Fig. 2). This data demonstrates that synthesis of an aza-peptide bond is slower than that of a peptide bond. Thus, the conventional SPPS protocol cannot be applied to aza-peptide synthesis without significant changes in activator reactivity.

Aza-peptide formation was faster and characterized by a $k_{obs}$ value of 0.040 ± 0.001 $min^{-1}$ at 40 °C, when COMU was used for Fmoc-alanine activation. Again, practically complete conversion of the dipeptide into the tripeptide was observed. Although this reaction was faster, it was still not comparable to the conventional peptide synthesis protocol, since its half-life was 17 min. Synthesis of the model peptide was then studied with various activating agents. HATU, HCTU, HDMC, TBTU, and PyBOP are all triazole derivatives and produce similar leaving groups in compound **6**. The results using these activating agents are shown in Fig. 3 and the observed rate constants and calculated acylation yields are listed in Table 1.

Fig. 3 shows that product formation reaches an asymptote after 250 min using HATU, HCTU or HDMC. The reaction rates are somewhat lower than those using COMU and PyOxim. The half-lives of the first three reactions were each ~40 min. Although HATU results in nearly complete conversion of the dipeptide into the tripeptide, the reactions using HCTU and HDMC were incomplete. The aza-peptide bond formation yield was only slightly higher than 50% (Fig. 3).

Interestingly, aza-peptide bond formation occurs more slowly with TBTU and PyBOP. The half-lives of these reactions were ~150 min. As above, these reactions were incomplete and are characterized by an extrapolated yield of ~0.6. Tripeptide formation was faster at 40 °C than at 25 °C. The reaction was characterized by $k_{obs}$ values of 0.021 ± 0.005, 0.014 ± 0.003, and 0.007 ± 0.002 for HCTU, PyBOP, and TBTU, respectively. However, tripeptide formation was incomplete at this temperature; he yields were 0.7, 0.5, and 0.8 for HCTU, PyBOP, and TBTU, respectively. Since these yields are similar to those obtained at 25 °C, the reaction rate is not closely related to the yield.

Carbodiimides are another type of amino acid activator used for aza-peptide bond synthesis.[6,28] Thus, the model reaction was studied after Fmoc-alanine activation with DIC.[29] Kinetic curves obtained at 25 °C and 40 °C are shown in Fig. 4.

DIC is a less efficient aza-peptide synthesis coupling reagent than previously considered activators; the half-life of the reaction with DIC-activated Fmoc-alanine was 211 min at 25 °C. However, tripeptide formation is nearly complete after an extended reaction time. Similar results were obtained at 40 °C; the reaction rate increases ($k_{obs}$ = 0.017 ± 0.002 $min^{-1}$), and conversion of the aza-dipeptide was almost complete. However, this increase is not sufficient to make the reaction rate comparable to that of peptide bond synthesis.

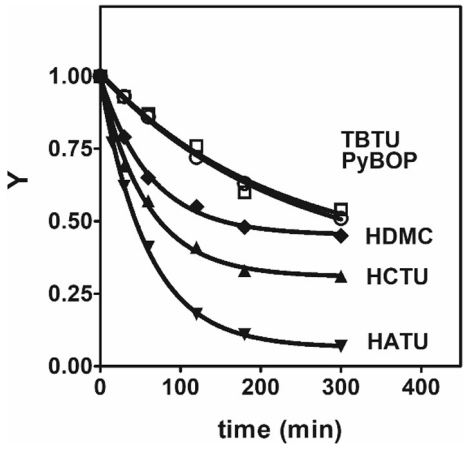



**Fig. 3.** Time evolution of the reaction between activated Fmoc-alanine and resin-bound H-AzAla-Phe during aza-peptide bond synthesis at 25 °C in DMF. Fmoc-alanine was activated with TBTU (□), PyBOP (○), HDMC (◆), HCTU (▲), and HATU (▼).

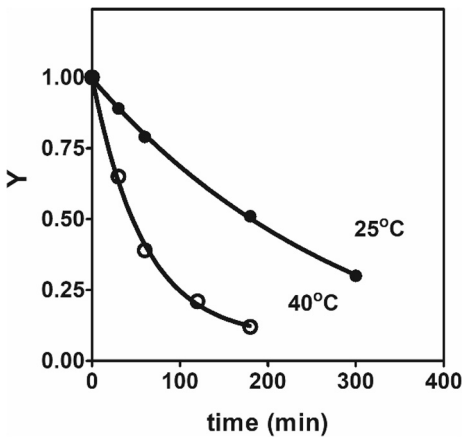
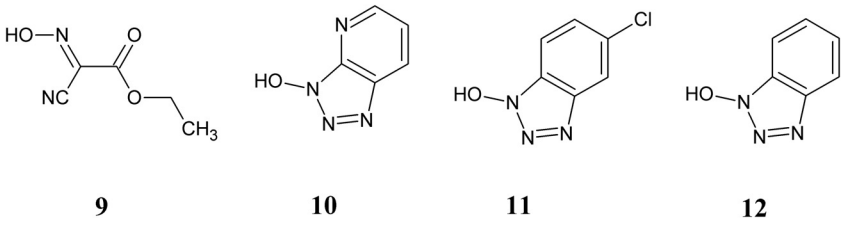



**Fig. 4.** Time evolution of the reaction between DIC-activated Fmoc-alanine and resin-bound H-AzAla-Phe for aza-peptide bond synthesis at 25 °C (●) and 40 °C (○) in DMF.




9 10 11 12

**Fig. 5.** Structures of leaving groups in compound **6**: Oxyma (**9**), HOAt (**10**), 6-Cl-HOBt (**11**), HOBt (**12**).

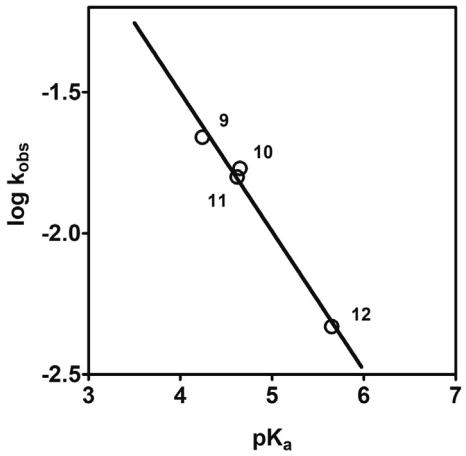



**Fig. 6.** Dependence of $\log k_{obs}$ values of the aza-peptide bond synthesis reaction on the $pK_a$ values of acids that correspond to the leaving group X of activated Fmoc-alanine **6** in Scheme 1 (**9** oxyma, **10** HOAt, **11** 6-Cl-HOBt, **12** HOBt). The $\log k_{obs}$ values were taken from Table 1, and the $pK_a$ values were compiled from the literature.[18]

All model peptide synthesis reactions were conducted under similar conditions, including reagent concentration and sample processing conditions. Therefore, the kinetic data obtained during experiments with different activators should be comparable. Specifically, the rate constant $k_{obs}$ should characterize the reactivities differences of the activated alanines **6** during acylation. This hypothesis was confirmed by the linear $\log k_{obs}$ vs $pK_a$ plot (Fig. 6), where the $pK_a$ values measured in a 95% acetonitrile-water mixture at 25 °C[18] quantify the acidities of compounds that correspond to the leaving group X in the activated Fmoc-alanine (compound **6** in Scheme 1) with following structures (Fig. 5).

However, in parallel to the influence of leaving group acidity that seems to quantify polar effects, it is possible that steric effects may also play some role in this acylation reaction, in analogy with ester hydrolysis.[30] Therefore, before final conclusions about the role of the leaving group in the activated amino acid molecule, the reactivity of more bulky substrates should be analyzed.

To understand why the reaction yield varies by activator, it is important to emphasize that the process was monitored *via* direct measurement of resin-bound H-AzAla-Phe conversion to the aza-tripeptide Fmoc-Ala-AzAla-Phe. This ensures that none of the reagents were consumed in parallel reactions. Therefore, the low reaction yields observed with certain activators should be related to side-reactions of the activated Fmoc-alanine. As the yield is not dependent on the reaction temperature, the aza-peptide synthesis and putative side-reaction should have similar temperature dependencies. Incomplete acylation has also been observed in typical peptide bond formation. In this case, repeating the process was recommended.[27] In our case, these side-reactions may play even more important role, as the aza-peptide bond formation reaction is very slow.

Synthesis of the aza-peptide bond was much slower than that of the typical peptide bond, including when conventional coupling agents were used for amino acid activation. Moreover, both the reaction rates and the yields were dependent on the activator structure. Oxyma-based activators COMU and PyOxim lead to nearly complete aza-peptide bond formation. Triazole based HATU is as efficient in achieving complete coupling, but it requires a longer reaction time. These results demonstrate that the conventional SPPS protocol cannot be directly applied to aza-peptide synthesis, and that new coupling agents are needed to increase the reactivity of the activated amino acid in order to achieve effective acylation of the semicarbazide moiety during aza-peptide bond formation.

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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.2017.07.063.

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