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Simple and Efficient Solid-Phase Preparation of Azido-peptides

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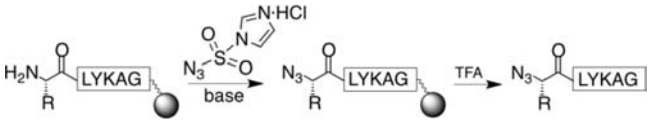
ABSTRACT



A shelf-stable and easily prepared diazotransfer reagent, imidazole-1-sulfonyl azide hydrochloride, was used to transform the N-terminus of a model peptide on solid phase into an azide moiety. It is demonstrated that this conversion was accomplished within 30 min with high efficiency under aqueous conditions on a NovaPEG resin or in DMF on polystyrene beads.

The popularity of the azide functionality has rocketed since the early 2000s with the discovery of the Staudinger ligation[1] and azide−alkyne click chemistry,[2] which both involve azide reactants. In peptide chemistry, azido-peptides are usually prepared by incorporation of azidonorleucine residues,[3] coupling azidocarboxylic acids to the N-terminus of the completed peptide,[4] or azide functionalization of aldehyde resins affording C-terminal azido-peptides.[5] Alternatively, the N-terminus itself can be converted into an azide via a metal-ion-catalyzed diazotransfer reaction using triflyl azide.[6] This reagent, however, is unstable and potentially explosive and requires a metal ion catalyst which may be difficult to remove, even after HPLC purification.[6a] Recently, a number of safer, shelf-stable, and easily prepared diazotransfer reagents have been developed,[7] of which imidazole-1-sulfonyl azide[7c,d] has been used to introduce azide moieties in proteins under copper-free conditions.[8] To the best of our knowledge this diazotransfer reagent has not been applied in solid phase peptide chemistry before, except in a single transformation reported previously by us[9] and Defrancq et al. to introduce azide moieties in a model oligonucleotide in a moderate yield.[10]

Furthermore, diazotransfer reactions on solid support are poorly described in the literature, so to acquire more knowledge of this matter we decided to examine the scope of this reaction. For this exploration we focused on

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a PEG-based resin because of its very good swelling characteristics in a wide range of solvents including water,[11] which is of particular interest as imidazole-1-sulfonyl azide is usually used under aqueous conditions or in polar organic solvents.[8,10,12]

Herein, we describe a protocol for a quick, easy, and copper-free access to N-terminal azido-peptides using imidazole-1-sulfonyl azide as a diazotransfer reagent.

To study the solid phase diazotization of peptides using imidazole-1-sulfonyl azide hydrochloride,[7c] a model peptide ALYKAG was prepared by standard solid phase peptide synthesis on a NovaPEG Rink resin for this purpose (purity > 99%, Supporting Information (SI)).

The same ALYKAG batch and the same resin/solvent ratio were used in all the experiments described below to allow a direct comparison of the diazotization results (a separate ALYKAG batch was prepared on polystyrene resin). To facilitate decarboxylation of any possible N-terminal carbamate moieties (leftover from Fmoc), the resin was washed with an HOBt solution prior to the diazotization (Experimental procedures, SI). After the diazotransfer reaction[13] the peptides were cleaved off the resin with trifluoroacetic acid containing thioanisole and triisopropylsilane as scavengers. It should be noted that thiol-scavengers must be avoided as these have been reported to reduce azides to amines.[14] The peptides were then precipitated in diethyl ether and analyzed by analytical reversed-phase HPLC. The amino-ALYKAG (ALYKAG) and azido-ALYKAG (A^{Az}LYKAG) peaks were assigned by means of LCMS, and the conversion efficiency was reported as the relative amount of azido-ALYKAG to amino-ALYKAG based on integration of their UV-absorption peaks (assuming no significant change in the extinction coefficient of the peptides at 215 nm).

Mostly, the HPLC chromatograms showed only the two peaks from azido- and amino-ALYKAG, though occasionally residual trifluoroacetic acid or thioanisole gave rise to an additional peak (Figure 1).

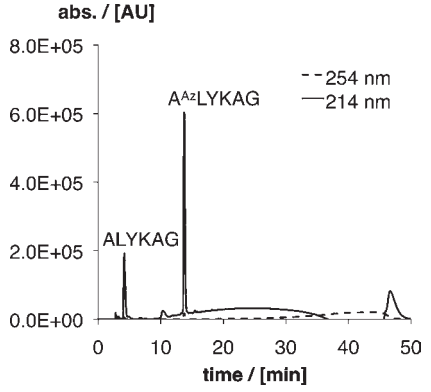

Figure 1. Typical HPLC profile of diazotization of ALYKAG after 5 min of diazotransfer reaction with 3 equiv of imidazole-1-sulfonyl azide hydrochloride.

The diazotransfer reaction is known to be catalyzed by transition metals, *e.g.* Cu(II), so to establish the effect of Cu(II) on the diazotransfer reaction and the time frame of the diazotransfer reaction in general, a kinetic experiment was performed. Two reactions were set up: one including a Cu(II) catalyst and one without, from which samples of the resin-suspension were removed in the course of the reaction. After resin cleavage and HPLC analysis, the conversion of amino-ALYKAG to azido-ALYKAG was plotted as a function of time (Figure 2). As is clear from Figure 2, the diazotransfer reaction is very fast as it reached its conversion maximum of around 95% already after 10 min. Surprisingly, Cu(II) does not seem to have a favorable effect on this conversion, on neither the rate nor the overall conversion. Hence, Cu(II) can be left out of the reaction, and accordingly the following experiments were performed without Cu(II). Copper-free preparation of azido-peptides might be particularly interesting when the azido-peptides are to be used in cellular experiments, as copper is known to be cytotoxic even at low concentrations and complete removal of Cu can be troublesome.[6a,15]

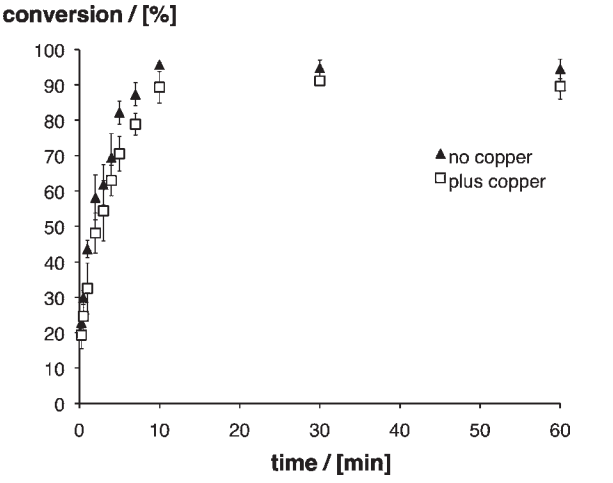

Figure 2. Kinetics of the diazotization of ALYKAG on solid support. The conversion of amino-ALYKAG to azido-ALYKAG using 3 equiv of imidazole-1-sulfonyl azide hydrochloride and 4.5 equiv of K_2CO_3 in water was monitored over time (mean ± SD; $n = 4$).

Even though a conversion of 95% is satisfactory, full conversion might be achieved if more diazotransfer reagent is used in the reaction. Hence, the diazotization efficiency was studied using 1, 3, or 5 equiv of the diazotransfer reagent (Figure 3).

Since the volume was kept the same in all cases this equaled a 3- and 5-fold increase in concentration going

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(13) It should be noted that imidazole-1-sulfonyl azide hydrochloride is shelf-stable but should not be kept as a concentrated aqueous solution for a prolonged time due to the risk of decomposition (see ref 7d).

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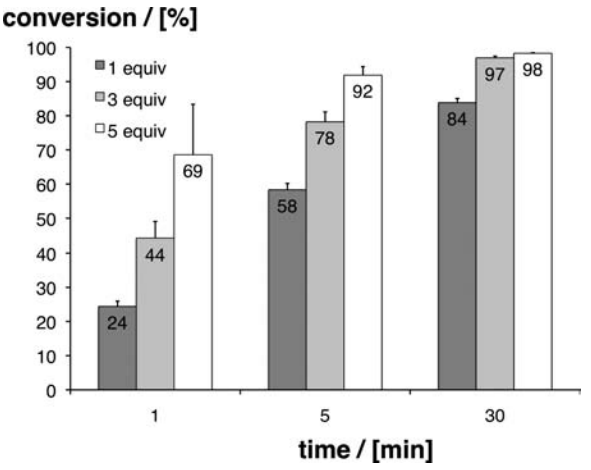

Figure 3. Variation of the amount of diazotransfer reagent. The conversion to azido-ALYKAG using 1, 3, or 5 equiv of imidazole-1-sulfonyl azide hydrochlodride and 1.5, 4.5, or 7.5 equiv of K_2CO_3 in water was studied. Each bar (value shown inside the bars) represents the relative conversion (mean mean ± SD; $n = 4$). Error bars are within the bars when not visible.

from 1 to 3 or 5 equiv, respectively. From this study, it is clear that 'more is better' within a 30 min time frame, as 1 equiv gives less conversion than 3 equiv, which again gives less than 5 equiv. After 30 min there is, however, almost no difference in conversion between 3 and 5 equiv, which gives 97 and 98%, respectively. This also points to the fact that in our hands even with 5 equiv is was not possible to achieve full conversion. It was speculated that the diazotransfer reagent might be rather unstable and might break down before all ALYKAG peptides have had time to react (though no breakdown of the diazotransfer reagent in solution was observed over time according to NMR; result not shown). Therefore, a reaction with 3 equiv of diazotransfer reagent was run for 30 min after which the diazotransfer reagent was replaced with a fresh solution and samples were taken after 1, 5, and 30 min (SI). Unfortunately, this 'double diazotransfer reaction' cycle did not lead to full conversion either, as 'only' 98% conversion was observed.

To ensure that the remaining 2% of amino-ALYKAG was not caused by reduction of the azide moiety during the resin cleavage reaction and thereby regeneration of the starting material, pure azido-ALYKAG peptide was subjected to the same cleavage conditions. However, no amino-ALYKAG or other byproducts were observed confirming that no reduction takes place (result not shown). A less likely explanation could be formation of imidazole-SO_2-NH-peptide or N_3-SO_2-NH-peptide side products, which hydrolyze back to the corresponding amine peptide during the cleavage reaction. However, to our knowledge, these species have never been reported when imidazole-1-sulfonyl azide is used in solution. Unfortunately, this leaves us with no explanation on why 2% of the amino-ALYKAG peptides evade diazotization.

To verify that the diazotransfer reaction is not limited to Ala residues, XLYKAG (where X = 19 different amino acids; Pro was omitted as this contains a secondary amine, which does not undergo diazotization) was prepared and diazotized (Figure 4). Of these 19 peptides only Cys(Trt) and Met did not produce clear amine to azide conversions. Met showed reasonable conversions, but LCMS analysis revealed that diazotization was accompanied by oxidation of the Met moiety. No measures were taken, however, to circumvent or reverse this oxidation by addition of mild reductants, for example. That is, it might be possible to achieve nonoxidized MLYKAG by optimizing the reaction conditions and/or cleavage cocktail.[16]

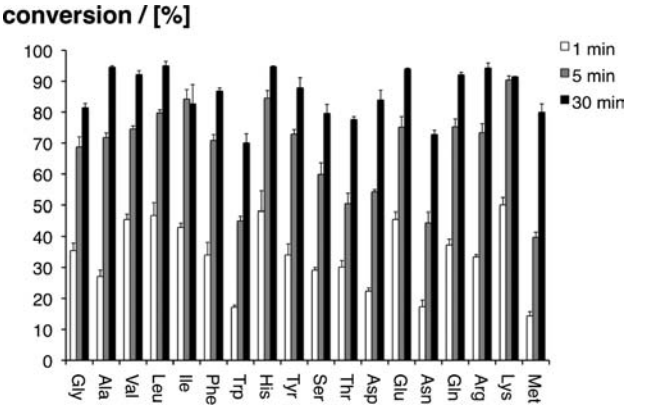

Figure 4. Variation of the N-terminal amino acid. Reactions with 3 equiv of imidazole-1-sulfonyl azide hydrochlodride and 4.5 equiv of K_2CO_3 for each XLYKAG (X = 18 diff. amino acids, excl. Pro) were set up, and samples were taken after 1, 5, and 30 min. Each bar represents the relative conversion of amino-XLYKAG to azido-XLYKAG (mean ± SD; $n = 3$).

HPLC analysis of diazotized CLYKAG showed that the diazotransfer reaction had led to formation of multiple products (SI). Further analysis by LCMS indicated that these products might be disulfide-linked peptides consisting of amino- and azido-CLYKAG peptides (C^{Az}LYKAG). Due to the complexity of the HPLC profiles no quantification of the conversion efficiency was attempted. The remaining 17 XLYKAG peptides, on the other hand, showed clear conversions between 60 and 97% (after 30 min). Remarkably, eight of these peptides exceeded conversions of 90% including some with the more sterically hindered amino acids such as His(Trt), Arg(pbf), and Leu at the N-terminus. However, Gly, the least hindered of all the amino acids, reached a conversion of only 81%. Mediocre conversions of glycine residues relative to other amino acids is not unprecedented in literature though. van Woerkom and van Nispen, for example, reported average acylation efficiencies of Gly in their study of peptide couplings involving various N-terminal amino acids on a growing peptide.[17]

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In this regard, it should be noted that a full kinetic experiment was not carried out for each of these XLYKAG peptides (only 1, 5, and 30 min as shown were included), so the reduced conversion efficiencies might as well reflect more sluggish reaction kinetics.

Nevertheless, most of the peptides showing the lowest conversion efficiencies (Asn, Thr, Ser, Met) share a common feature, it seems, namely the presence of a heteroatom in a short side chain. Unfortunately, the lack of an exact mechanism of the diazotransfer reaction makes speculations on the role of these heteroatoms futile.[18]

Since polystyrene resins are widely used in peptide synthesis, diazotization of ALYKAG was also attempted on a polystyrene resin. However, because of the hydrophobic nature of polystyrene, water could not be used as the main solvent, so DMF was used instead. K_2CO_3 was added to the reaction mixture as an aqueous solution to ease dissolution of K_2CO_3 (final solvent composition DMF/water 6.3:1). After 30 min only 26% conversion was observed on a polystyrene resin and, surprisingly, no conversion at all when NovaPEG resin was used under the same conditions (SI). It is not fully understood why the conversion efficiencies are low, but it might be related to the choice of base, K_2CO_3, which may exist as not fully dissociated ions under these rather nonaqueous conditions. Eventually, this could lead to incomplete neutralization of the diazotransfer reagent hydrochloride salt, which would lead to severely reduced conversion efficiencies (result not shown). Consequently, K_2CO_3 was replaced with Hünig's base (DIPEA or diisopropylethylamine), which in the case of diazotransfer reactions on polystyrene resin gave conversions matching the best results obtained with NovaPEG resin under aqueous conditions (Figures 5 and 3). That is, peptides on polystyrene resin can also be diazotized using imidazole-1-sulfonyl azide under copper-free conditions with very good conversion efficiencies. NovaPEG resin also performed much better in DMF after substitution of K_2CO_3 with DIPEA though reaching neither the high levels achieved under aqueous conditions nor the level of polystyrene under DMF/DIPEA conditions. This clearly indicates that DMF is not a good solvent for this PEG-based resin.

In addition to the two solvents already used (DMF and water) for diazotransfer reactions on NovaPEG resin, dichloromethane and methanol were also evaluated. Dichloromethane was chosen to include an apolar solvent and because this solvent is often used in peptide chemistry. Methanol was included since this solvent is protic and ranks between dichloromethane and DMF in polarity (SI). In dichloromethane only 5% conversion was observed after 30 min. In methanol the diazotization worked better, though still substandard, achieving a conversion of only 40% after 30 min. By comparison of all four solvents (82% in DMF and 97% in water) a clear trend seems to manifest: increasing solvent polarity leads to increased conversion

(18) A mechanism has been proposed for the metal-catalyzed diazotransfer reaction: Nyffeler, P. T.; Liang, C.-H.; Koeller, K. M.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124*, 10773.

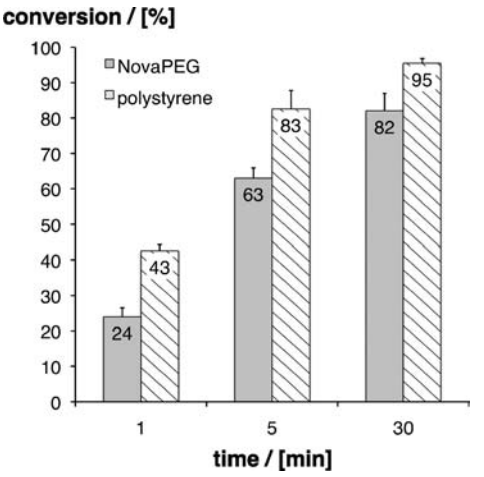

Figure 5. Diazotization of ALYKAG on polystyrene or NovaPEG resin using DMF and DIPEA. A reaction with 3 equiv of imidazole-1-sulfonyl azide hydrochlodride and 4.5 equiv of DIPEA was set up, and samples were taken after 1, 5, and 30 min. Each bar (value shown inside the bars) represents the relative conversion of amino-ALYKAG to azido-ALYKAG (mean ± SD; $n = 3$). Error bars are within the bars when not visible.

efficiency. Solvent proticity, on the other hand, seems to be less important for high conversions as DMF (nonprotic) was better than methanol (protic solvent).

These findings clearly demonstrate that water is the solvent of choice for diazotransfer reactions using the NovaPEG resin.

In summary, we have shown that imidazole-1-sulfonyl azide hydrochlodride can be used to transform the N-terminus of a model peptide, ALYKAG, on a solid support into an azide moiety. This is accomplished in a quick, simple, and efficient way and under metal-free conditions. Several solvent and resin combinations were tested of which NovaPEG resin under aqueous conditions and polystyrene resin in DMF were found to give the highest conversions, up to 98 and 95%, respectively.

Thus, easy access to azido-peptides can be furnished by the easily prepared and shelf-stable imidazole-1-sulfonyl azide hydrochlodride. This might earn this diazotransfer reagent a spot next to the Fmoc/Boc amino acids in the peptide laboratory, as azide introduction can now be accomplished without the need for azido amino acids or preparation of hazardeous triflyl azide prior to each diazotization.

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Supporting Information Available. Experimental procedures and data: HPLC, LCMS, and MS. Additional experiments: Two-cycle diazotization and diazotization in methanol and dichloromethane. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.