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Postsynthetic Modification of Peptides via Chemoselective N-Alkylation of Their Side Chains

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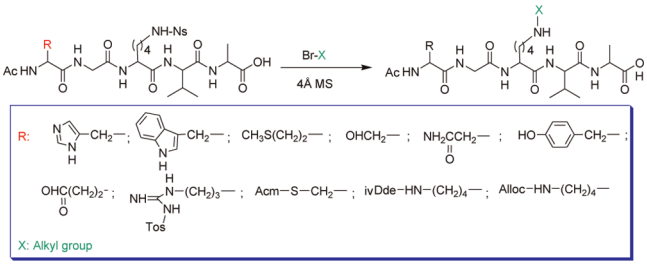
ABSTRACT



A chemoselective, mild, and versatile method for performing postsynthetic modifications of peptide sequences is described. It requires only activated molecular sieves in the presence of an alkyl halide in order to N-alkylate lysine side chains. This reaction is fully compatible with most of the peptide functionalities, discriminates the reactivity of differently protected lysines, and proceeds in good yield. The mild conditions employed were further proved by performing the N-alkylation of a peptide containing a disulfide bridge.

The development of new methodologies for the selective and straightforward chemical modification of peptides is eagerly requested and represents a scientific challenge, due to its important implications in drug discovery, as well as in structure−activity relationship (SAR) studies in peptide chemistry.[1]

Instead of a stepwise synthetic approach where unnatural amino acids are incorporated by a traditional protocol into a peptide chain, direct and selective peptide modification represents a flexible and versatile alternative approach to optimize lead structures.

The main goal of this synthetic strategy is avoiding sterical hindrance problems, which are often encountered during the coupling of modified amino acids with the peptide chain on the resin.[2] However, the key to the success of the postsynthetic peptide modification approach lies in the chemoselective outcome of the employed reaction.

In recent years, many synthetic protocols have been published to introduce modifications on already preformed peptide sequences.[3]

A possible synthetic route to modify peptide side chains is the N-alkylation reaction. In this regard, the most cited Kessler protocol[4] reported a synthetic route to N-alkylate

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nosyl-protected amino acids during the peptide assembly, according to traditional protocols (Mitsunobu conditions or alkyl halides in presence of DBU), which were not fully compatible with Fmoc-chemistry.

We have recently reported the usage of molecular sieves to N-alkylate several basic amino acid derivatives (Fmoc-Lys(Ns)-OH, Fmoc-Orn(Ns)-OH, Fmoc-Dab (Ns)-OH, Fmoc-Dap(Ns)-OH).[5]

Starting from these results, we have developed an innovative protocol to perform peptide postsynthetic modifications. It allows peptides on the amino group of a Lys to be selectively N-alkylated, which were previously protected with the nosyl group,[6] by only employing molecular sieves in the presence of alkyl halides. In particular, it relies on the earlier reported reaction conditions, which does not affect the stereochemistry of the employed amino acid chiral center.[5] To the best of our knowledge, postsynthetic N-alkylation of peptides promoted by molecular sieves is not reported in literature. Herein we report a broad study carried out on several peptide models through a fine-tuning of the reaction parameters.

For each peptide model, amino acids were chosen in order to cover the range of functionalities present on the side chains of natural peptide molecules (Scheme 1).

In detail, the generic peptide sequence **1** was first synthesized, and then several amino acid derivatives were inserted at its N-terminal side (peptides **2**–**12**) (Scheme 1). Each peptide, acetylated at the N-terminus and free at the carboxylic extremity, is alkylated after cleavage from the solid support. The peptide sequences were dissolved in DMF under an Ar atmosphere and in the presence of activated 4 Å molecular sieves, then alkyl bromide was added (1.2 equiv), and stirring of the obtained mixture occurred at rt for 12–24 h.

As we have previously discussed, the molecular sieves are supposed to act as a base, capturing the proton of the amino group.[5,7]

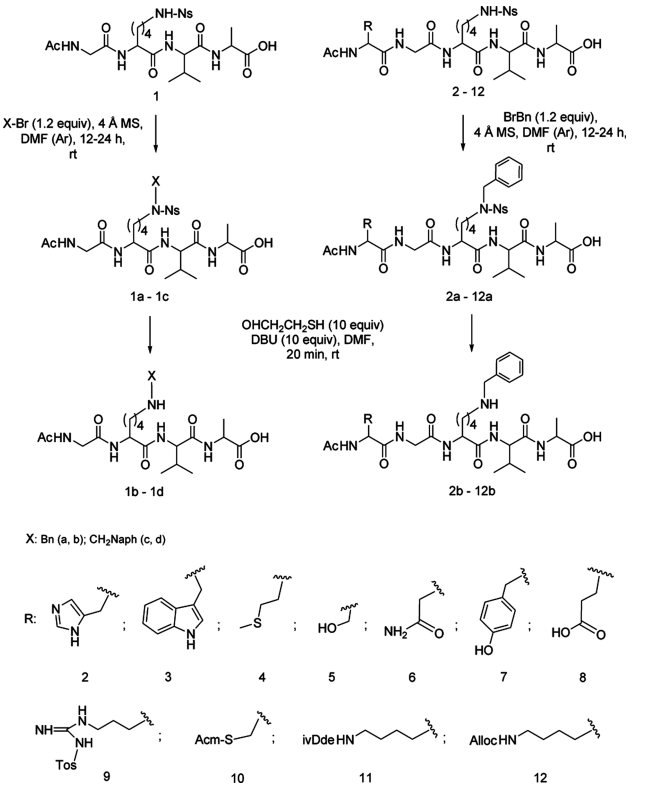
We used benzyl bromide to alkylate the whole set of peptides and methylnaphtyl bromide to obtain peptide **1c**. The aim was to demontrate that the efficiency of the reaction does not rely on the specific employed alkyl group.

The course of the alkylation reaction was followed by HPLC-ES-MS analysis; the yields were estimated by HPLC integration of the alkylated peptide compared to the starting peptide and other byproduct, if any (see Table 1).

The reaction mixture was centrifuged, and the supernatant was collected to remove the Ns group from the Lys side chain. This reaction was easily performed by adding the appropriate amount of DBU (10 equiv) and 2-mercaptoethanol (10 equiv) to the DMF solution and stirring for 20 min at rt. It is worth noting that the whole synthetic protocol does not require intermediate purification steps.

The first alkylation reactions were performed on peptide **1** in good yield (Table 1). Good evidence that benzylation

Scheme 1. Synthetic Strategy of Mono-N-alkylated Peptides



was effectively achieved on the peptide came from comparison of 1D and 2D proton NMR spectra of peptide **1b** (Scheme 1) with those of the parent peptide containing a free Lys. In Figure 1A it can be clearly seen that, upon insertion of the benzyl ring on the Lys side chain, chemical shifts for Ala $_N$H and Lys $_N$Hζ atoms undergo major changes. NMR $^1H/^2H$ exchange experiments noticeably indicated the presence of the benzylic group on **1b**, in fact in an excess of ^2H_2O; signals from labile $_N$H groups disappear whereas peaks from nonexchangeable aromatic protons are still visible in the 1D proton spectrum (Figure 1B). Similar NMR $^1H/^2H$ exchange experiments were carried out to demonstrate the insertion of a naphtylic ring in the AcGlyLys(MeNaph)ValAla peptide (Figure 1C).

The benzylation performed on peptide sequences **2**–**8** (Scheme 1) enabled the assessment that these molecules, containing unprotected functional groups, were chemoselectively alkylated in good yield (Table 1) on the Lys nosyl-protected ε-NH_2, under the mild conditions employed. Most of the features observed in the NMR spectra of **1b** could be also recognized in the spectra of all the mono-alkylated peptides (see Supporting Information (SI)). In particular the shift of the Lys $_N$Hζ group near 7.64 ppm was considered diagnostic of the good accomplishment of the alkylation reaction.

We also analyzed a peptide sequence containing the Lys to be alkylated and an unprotected Arg residue; in this case, the desired monoalkylated peptide was characterized by a lower yield, due to the reactivity of the guanidine

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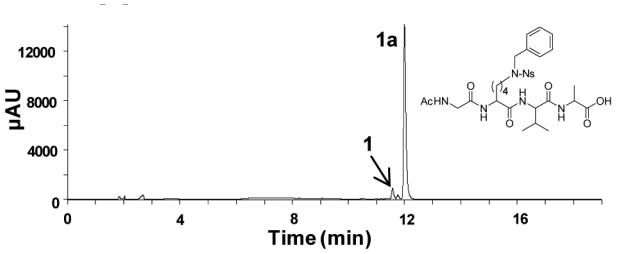
Table 1. Efficiency of the N-Alkylation Reaction Performed on Different Peptide Models



compound	peptide	yield (%)
1a	AcGlyLys(Ns, Bn)ValAla	97
1c	AcGlyLys(Ns, MeNaph)ValAla	95
2a	AcHisGlyLys(Ns, Bn)ValAla	95
3a	AcTrpGlyLys(Ns, Bn)ValAla	97
4a	AcMetGlyLys(Ns, Bn)ValAla	95
5a	AcSerGlyLys(Ns, Bn)ValAla	90
6a	AcAsnGlyLys(Ns, Bn)ValAla	98
7a	AcTyrGlyLys(Ns, Bn)ValAla	95
8a	AcGluGlyLys(Ns, Bn)ValAla	90
9a	AcArg(Tos)GlyLys(Ns, Bn)ValAla	75
10a	AcCys(Acm)GlyLys(Ns, Bn)ValAla	95
11a	AcLys(ivDde)GlyLys(Ns, Bn)ValAla	95
12a	AcLys(Alloc)GlyLys(Ns, Bn)ValAla	80
13a	AcGlyTrpLys(Ns, Bn)ValAla	98
14a	AcGlyTrpLys(Ns, Bn)HisValAla	95
15a	AcLys(Ns, Bn)GlyLys(Ns, Bn)ValAla	80
16a	AcGlyCysTrpMetCysValLys(Dde, Bn)Ala	75

group (pK_a = 12.5) which was alkylated together with the Lys side chain, thus providing a dialkylated byproduct with a yield of 70−80% (data not shown).

This result prompts us to explore the yield of the monoalkylation reaction in a peptide sequence containing a tosyl-protected Arg residue (peptide **9a**). This protecting group, in fact, survives in the employed cleavage conditions (70% TFA in DCM for 30 min)[8] and allows us to obtain benzylation of the Lys side chain with an ~75% yield. The isolated final product contains the tosyl group, clearly identified during NMR analysis, which can be removed in a subsequent step, by using the known reaction protocols.[9]

Afterwards, we explored a peptide containing the Lys to be alkylated and a free Cys residue; in this case, the desired monoalkylated peptide was not obtained in an appreciable yield. In fact, due to the high nucleophilicity of the Cys sulfhydryl group, the dialkylated peptide, with modifications

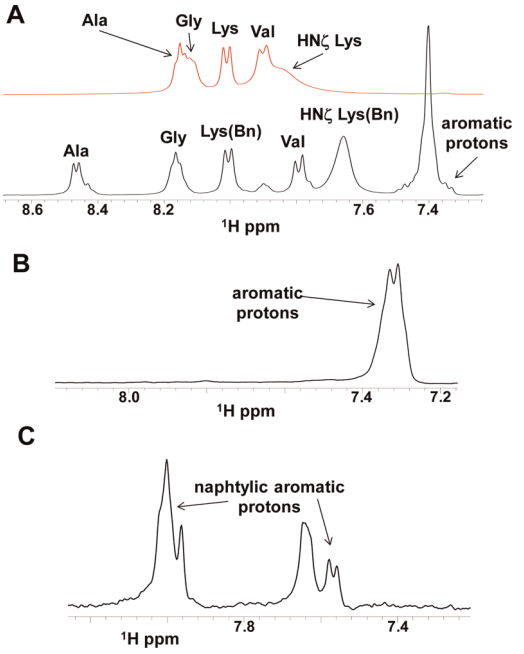


Figure 1. (A) Comparison of 1D NMR spectra, recorded in DMSO-d_6 at 298 K, of AcGlyLys(Bn)ValAla (**1b**, black) and AcGlyLysValAla (red) peptides. Spectral regions containing resonances from backbone and side-chain $_N$H groups as well as aromatic protons are shown. (B, C) $^1H/^2H$ exchange experiments (see SI for details about samples preparation) for AcGlyLys(Bn)ValAla (B) and AcGlyLys(MeNaph)ValAla (C) peptides.

on both the Cys and Lys side chain, resulted in being the main product (data not shown). However, it should not be considered a real synthetic problem, since several Fmoc-Cys derivatives protected on their side chain with no acid-labile substituents are routinely used for the peptide synthesis. For instance, we succeeded in preparing in high yield the peptide **10a** (Table 1) where the Cys protecting group Acm can be removed, by a standard method,[10] after having performed the alkylation.

To explore the reactivity exhibited, upon alkylation, by differently protected lysines, we synthesized peptide sequences **11** and **12**. In our previous work, we investigated the effects of the newly developed N-alkylation procedure on amino groups protected with several substituents which are routinely used in solid-phase peptide synthesis.[5] For instance, Fmoc-Lys(ivDde)-OH and Fmoc-Lys(Alloc)-OH are widely employed. We have already tested the benzylation of the Lys amino group protected with ivDde;[5] thus, we decided to study the same reaction on N-ε-Alloc-Lys (see SI). These N-alkylation reactions were slower with respect to the reactions performed on a nosyl-protected amino group.

Peptides **11** and **12** were alkylated as reported in Scheme 1. After 12 h for **11a** and after 24 h for **12a**, the most abundant products resulted in being monoalkylated (see Table 1).

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We employed 1D ^{1}H and 2D [^{1}H, ^{1}H] NMR spectroscopy (see SI) to further prove that the desidered monoalkylated peptide products were effectively obtained. NMR studies, performed on **11b** and **12b**, confirmed that the chemoselective alkylation occurred on the nosyl-protected Lys, while the ivDde/Alloc-protected lysines resulted free of a benzyl substituent. It is worth noting that the ivDde and Alloc groups were kept on the Lys residues to facilitate NMR analysis. However, the ivDde can be removed together with the Ns group (by preparing an appropriate mixture of hydrazine, 2-mercaptoethanol, and DBU), while the Alloc group can be removed under usually standard conditions.[10]

In 2D [^{1}H, ^{1}H] NMR spectra, such as TOCSY and ROESY, signals from Lys(Bn) and Lys(ivDde)/ Lys-(Alloc) could be easily identified (SI). In 2D [^{1}H, ^{1}H] TOCSY spectra, the characteristic $_N$Hζ spin system of Lys(Bn) at 7.64 ppm is clearly identifiable for both **11b** and **12b** peptides.

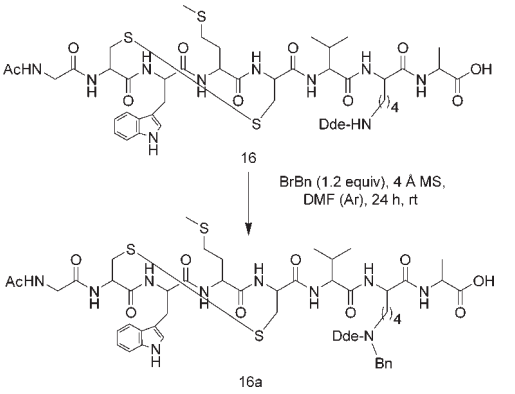
To further examine the chemoselectivity shown by the Lys Ns-NH upon alkylation reaction with respect to the position into the peptide sequence of other competing functional groups, peptides **13a** and **14a** were synthesized in high yield (Table 1). Thus, it was demonstrated that the close proximity of an amino acid, e.g. Trp (**13a**), or even of two nucleophilic amino acids, e.g. Trp and His (**14a**), does not influence the reactivity of the Lys nosyl protected ε-NH_2 during the benzylation reaction.

Peptide **15a** was synthesized to prove that the proposed methodology allowed the one pot synthesis of polyalkylated molecules in good yield (Table 1) and that the reactivity of the substrate does not rely on its specific situation within the peptide sequence. For this peptide, we could not carry out a detailed NMR characterization due to the appearance in the 2D TOCSY and ROESY spectra of many duplicated spin systems indicative of the occurrence of different conformations in solutions and/or aggregation processes.

The very mild conditions of the proposed procedure were further proven by performing the N-alkylation of a peptide containing a disulfide bridge. In fact, in the case of the Cys oxidation reaction being performed in solid phase,[10] the postsynthetic alkylation cannot be accomplished on nosyl-protected Lys residue, whose deprotection conditions (thiol in the presence of a base) are not compatible with a disulfide bridge.

For instance, we synthesized peptide **16a** (Scheme 2) by using the Fmoc-Lys(Dde)-OH derivative. After the oxidized peptide was cleaved from the resin under appropriate reaction conditions, the benzylation reaction was performed on **16** (Table 1). The final peptide was obtained in quite good yield, thus demonstrating that the mild conditions employed to alkylate the peptide do not affect the disulfide bridge. NMR analysis of the peptide confirmed the presence of a benzylated Lys (see SI), whereas Cys residues appear unaffected by peptide postsynthetic chemical modification.

Scheme 2. Synthetic Strategy of N-Alkylation Reaction Performed on Peptide Containing Oxidized Cysteines



In summary, we describe an original method for performing postsynthetic modifications via alkylation of a nosyl-protected Lys side chain, inserted in already preformed peptide sequence. The mild conditions employed (only molecular sieves used to catalyze the reaction) were proven to be compatible with most of the natural peptide functionalities, providing high chemoselectivity and excellent conversion yields. We believe that our versatile and straightforward approach will find wide application in the field of peptide-based drug development. In fact, to the best of our knowledge a protocol that allows generating a large number of analogs by performing the same reaction, with different alkylating agents, on a single presynthesized peptide sequence, has not been reported in literature.

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Supporting Information Available. The content includes the following: (1) experimental section, (2) LC-MS spectra, (3) NMR chemical shift tables, (4) NMR spectra. This information is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.