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Chemical Modifications of Peptide Sequences via S-Alkylation Reaction

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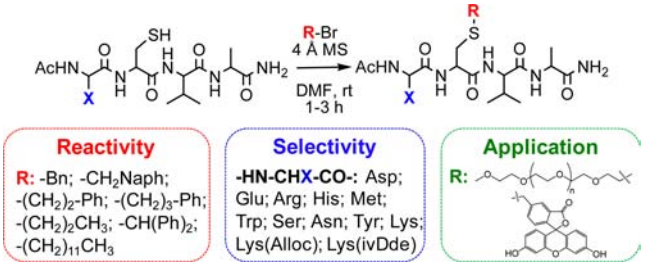
ABSTRACT



A chemoselective, convenient, and mild synthetic strategy to modify peptides on a cysteine sulfhydryl group is described. It simply requires activated molecular sieves to selectively promote S-alkylation in the presence of peptide nucleophilic functionalities. The procedure is easy to perform, fast, and provides high yields even in the case of poor electrophilic groups. Moreover, the method allows an efficient one-pot poly alkylation, proving that the sulfhydryl reactivity does not rely on its specific position within the peptide sequence.

Chemical derivatization of an individual amino acid is a commonly used approach to manipulate and study protein and peptide therapeutics. Indeed, it allows the introduction of naturally occurring post-translational modifications, as well as specific functional moieties that convey to the molecule favorable pharmacokinetic properties, increase in vivo stability and solubility, or reduce immunogenicity.[1] This strategy particularly expands the field of peptide-based bioactive compounds. In fact, peptides have long been recognized as attractive candidates to develop drugs, due to their unique conformational and functional features. In numerous conjugation methods the chemical modification is introduced on nucleophilic residues, such as lysine and cysteine, by using electrophilic reagents.[2]

In this regard, we have recently described a strategy for performing postsynthetic peptide modifications via selective alkylation of a nosyl-protected Lys side chain under mild conditions that requires only the appropriate alkyl halide and molecular sieves to catalyze the reaction.[3]

The current work focuses on cysteine; this amino acid can probably represent a more convenient target for introducing peptide chemical modifications, owing to the stronger nucleophilicity of its sulfhydryl group.

A broad range of peptide models, in order to tune S-alkylation reaction parameters, were synthesized; all prepared peptides contain a protecting acetyl group at the N-terminus and are amidated at the carboxylic extremity.

In analogy with previously reported studies,[3] molecular sieves are employed as base to promote S-alkylation, so it is reasonable to hypothesize that the implementation of very

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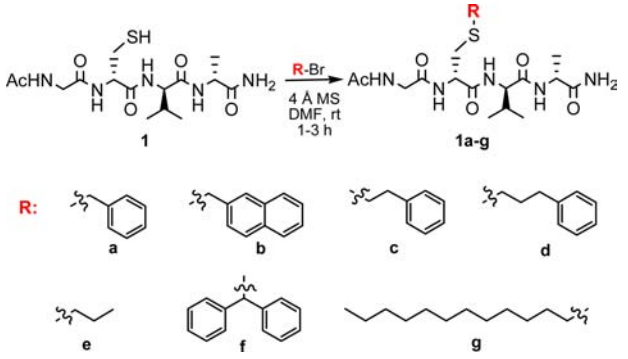
mild reaction conditions should not induce any cysteine racemization, which is one of the main problems encountered when cysteine alkylation is performed under drastically basic conditions.[4]

Peptide series **1** (Table 1) was first designed and synthesized to investigate the efficiency of the S-alkylation protocol upon usage of different alkyl bromides (Scheme 1).

Scheme 1. S-Alkylation of a Peptide Model with Different Substituents



After cleavage from the solid support, the peptide **1** was alkylated by using as solvent DMF, under an Ar atmosphere, and in the presence of activated 4 Å molecular sieves. Then, the appropriate alkyl bromide was added (0.9−1.2 equiv), and the stirring of the obtained mixture was kept at room temperature for 1−3 h. Reaction times and yields are summarized in Table 1.

As expected, high reaction yields were reached after 1 h with the most reactive alkylating reagents (peptides **1a**, **1b**, **1c**). The presence of rather electron-poor (d, e, g in Scheme 1) or sterically hindered (f in Scheme 1) substituents on the alkyl central carbon increases the reaction time from 1 to 3 h (peptides **1d**, **1e**, **1f**, **1g** in Table 1). The reaction yield is still quite high (near 90%), even for the less electrophilic alkyl substituents (d, e in Scheme 1). Surprisingly, even the lowest reactive dodecyl bromide (g in Scheme 1) reacted in 3 h with a good yield of the final product **1g** (Table 1).

Table 1. Efficiency of the S-Alkylation Reaction with Different Alkyl Bromides

entry	peptide	yield (%)	time (h)
1a	AcGlyCys(**Bn**)ValAlaNH$_2$	>98	1
1b	AcGlyCys(**naphthalenemethyl**)ValAlaNH$_2$	>98	1
1c	AcGlyCys(**phenylethyl**)ValAlaNH$_2$	90	1
1d	AcGlyCys(**phenylpropyl**)ValAlaNH$_2$	90	3
1e	AcGlyCys(**propyl**)ValAlaNH$_2$	90	3
1f	AcGlyCys(**diphenylmethyl**)ValAlaNH$_2$	90	3
1g	AcGlyCys(**dodecyl**)ValAlaNH$_2$	85	3

(4) Triola, G.; Brunsveld, L.; Waldmann, H. *J. Org. Chem.* **2008**, *79*, 3646–3649 and references therein.

Peptides were characterized by 1D [^{1}H] and 2D [^{1}H, ^{1}H] NMR spectroscopy. A comparison of 1D and 2D NMR spectra of the peptide model **1** containing a free cysteine and that generated after cysteine benzylation (**1a**) is reported in Figure 1, where it can be seen that insertion of an aromatic ring causes several chemical shift changes.

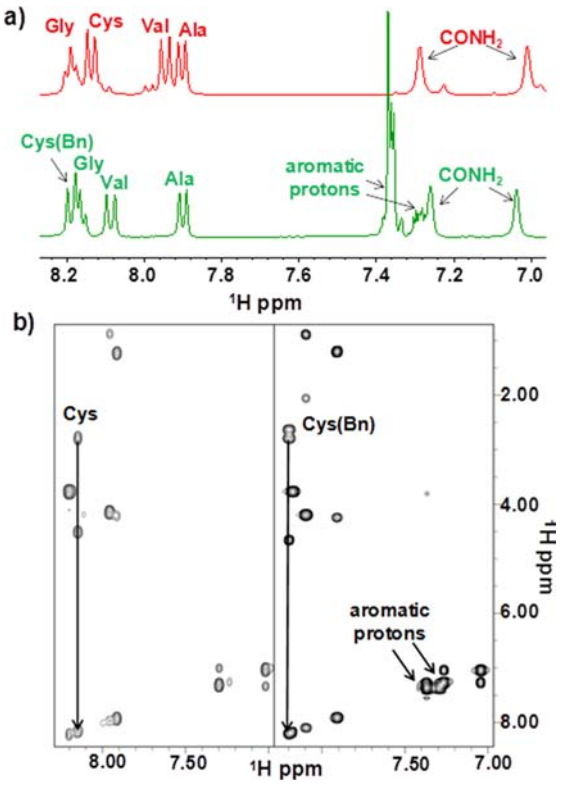


Figure 1. (a) Comparison of 1D [^{1}H] spectra of AcGlyCysValAlaNH$_2$ (**1**, red) and AcGlyCys(Bn)ValAlaNH$_2$ (**1a**, green) peptides. The H$_N$ and aromatic protons region is shown, and resonance assignments are indicated. (b) Region from the 2D [^{1}H, ^{1}H] TOCSY spectra of peptides **1** (left) and **1a** (right). Cys and Cys(Bn) spin systems are indicated by arrows.

The process of resonance assignment was achieved with a standard protocol[5] based on combined analysis of 2D [^{1}H, ^{1}H] TOCSY,[6] and ROESY[7] experiments (see also the Supporting Information). First, spin systems belonging to different amino acids were recognized in the 2D TOCSY spectrum (Figure 1b) and then they were related sequentially by looking for Hαi-H$_N$i+1 ROE contacts.

NMR hydrogen/deuterium exchange experiments were as well conducted to prove the presence of aromatic rings in the peptides after alkylation reactions. Toward this goal, 1D [^{1}H] NMR spectra of the molecules in a large excess of D_2O were recorded. In the presence of D_2O all signals belonging to labile protons (such as backbone amide H$_N$

(5) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.
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and $CONH_2$ of the C-terminal protecting group) disappear while aromatic protons can still be seen (Figure 2).

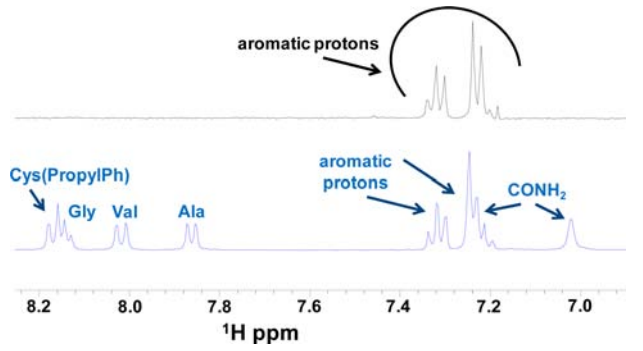

Figure 2. 1D [^{1}H] spectrum of AcGlyCys(PropylPh)ValAlaNH_2 peptide recorded in DMSO (blue trace) and in presence of an excess of D_2O (black trace).

We then investigated the sulfhydryl reactivity in presence of other nucleophilic groups. Indeed, peptides **2**–**10** (see Table 2) were prepared by using the same agent (i.e., benzyl bromide) to alkylate different sequences deriving from addition of a new N-terminal residue to the peptide model **1** (Table 1) (Scheme 2). Benzylation of this set of compounds occurred in high yields (Table 2) and enabled the assessment that these molecules, containing unprotected functional groups, were chemoselectively thioalkylated. Indeed, polyalkylated byproducts were never revealed in yields higher than 5%, as estimated by relative integrations of the HPLC peaks corresponding to the alkylated desired molecules, unreacted starting material, and other byproducts, if any.

For compound **11a**, the presence of a free lysine lowered, to a certain extent (20–30%), the yield of the mono-S-alkylated peptide, since di- and trialkylated products were also detected into the reaction mixture. However, this synthetic problem was easily circumvented by employing differently protected lysines, like Fmoc-Lys(ivDde)-OH and Fmoc-Lys(Alloc)-OH, that are routinely used in solid-phase peptide synthesis and that exhibited a lower reactivity upon alkylation reaction (Scheme 2). In fact, the peptide sequences **12a** and **13a** were successfully synthesized (Table 2).

As concerning with peptide **14a**, the high yield of the performed alkylation reaction demonstrated that the close proximity of other nucleophilic groups, such as Trp and His residues, does not influence the reactivity of the cysteine during the benzylation reaction (Table 2).

The successful outcome of alkylation reactions performed with benzyl bromide and different peptide sequences was also tested by NMR spectroscopy. As an example, in Figure 3, NMR spectra of peptide **10a**, which possesses a tyrosine residue together with a benzylated cysteine, are shown. Different amino acids can be easily identified in the 2D TOCSY experiment (Figure 3b), whereas the 2D ROESY spectrum (Figure 3c) contains strong sequential Hαi-H_Ni+1 connectivities that are generally typical of extended flexible peptides. The benzylic

Scheme 2. S-Alkylation of Different Peptide Sequences

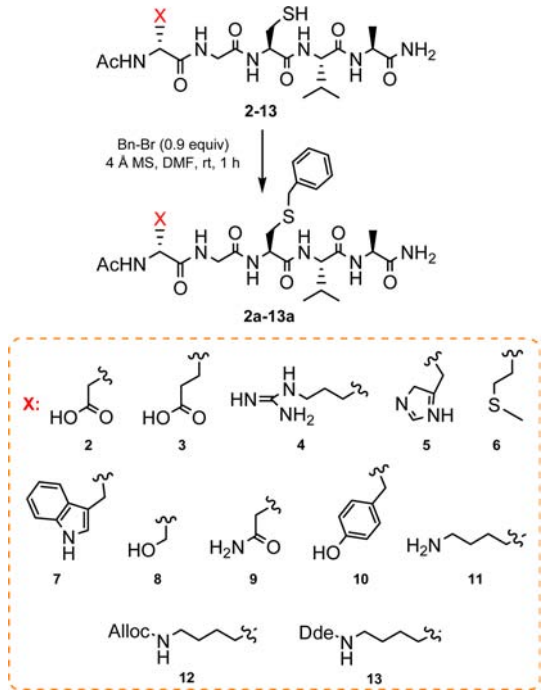

Table 2. Efficiency of the S-Alkylation Reaction Performed on Different Peptide Models

entry	peptide	yield (%)	time (h)
2a	AcAspGlyCys(**Bn**)ValAlaNH_2	90	1
3a	AcGluGlyCys(**Bn**)ValAlaNH_2	90	1
4a	AcArgGlyCys(**Bn**)ValAlaNH_2	90	1
5a	AcHisGlyCys(**Bn**)ValAlaNH_2	90	1
6a	AcMetGlyCys(**Bn**)ValAlaNH_2	90	1
7a	AcTrpGlyCys(**Bn**)ValAlaNH_2	90	1
8a	AcSerGlyCys(**Bn**)ValAlaNH_2	90	1
9a	AcAsnGlyCys(**Bn**)ValAlaNH_2	90	1
10a	AcTyrGlyCys(**Bn**)ValAlaNH_2	90	1
11a	AcLysGlyCys(**Bn**)ValAlaNH_2	70	1
12a	AcLys(Alloc)GlyCys(**Bn**)ValAlaNH_2	90	1
13a	AcLys(ivDde)GlyCys(**Bn**)ValAlaNH_2	90	1
14a	AcGlyTrpCys(**Bn**)HisValAlaNH_2	90	1
15a	AcGlyCys(**Bn**)AlaCys(**Bn**)ValAlaNH_2	95	3
16a	AcCys(**Bn**)GlyCys(**Bn**)AlaCys(**Bn**)ValAlaNH_2	90	3

SCH_2 protons resonate at 3.80 ppm (Figure 3a, d) while aromatic benzylic protons are represented by two multiplets centered at 7.36 and 7.27 ppm (Figure 3a, d). Althought the signal from the benzylic methylene CH_2 group is partially overlapped with that of the Hαα′ protons belonging to the Gly (3.77 ppm) (Figure 3a), ROE cross-peaks between benzylic aromatic, benzylic methylene and Hββ′ Cys protons are neat (Figure 3d).

Compounds **14h**–**i** were synthesized in order to conjugate the peptide **14** to functional substituents useful for various applications in the biochemical field (Figure 4).

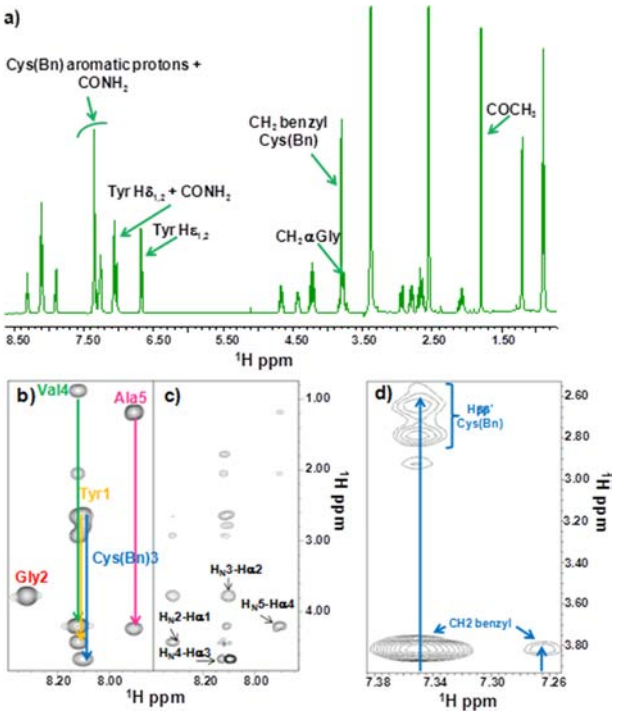

Figure 3. 1D [^{1}H] NMR spectrum of AcTyrGlyCys(Bn)-ValAlaNH$_2$ peptide in DMSO (a). HN-high field correlation regions of the 2D [^{1}H, ^{1}H] TOCSY (b) and ROESY (c) experiments; spin systems assignments and Hαi-HNi+1 ROE contacts are shown in (b) and (c), respectively. (d) Region of the 2D ROESY spectrum containing cross peaks between benzylic aromatic and others Cys(Bn) protons.

For instance, to prove that our method could be also efficient to insert a polymer, such as PEG (poly ethyleneglycol), into a peptide sequence, we prepared compound **14h** (Figure 4). Introduction of PEG is a modification that is commonly carried on bioactive molecules, since it may increase peptide in vivo stability and solubility, nevertheless reduce immunogenicity.[1e,8]

For imaging purposes, we synthesized **14i** by employing 1 equiv of 5-(bromomethyl)fluorescein (5-BMF) that has previously been described as a very effective labeling agent for carboxyl groups.[9] The final alkylated peptide **14i** was obtained in excellent yield (Figure 4). Thus far, fluorescein isothiocyanate has generally been conjugated to peptides with solid-phase synthetic routes. Our novel proposed solution-phase strategy offers the great advantage of requiring a much lower amount of starting fluorescein derivative.

Next, to prove that the fluorescein derivative selectively binds a sulfhydryl cysteine even in the presence of a

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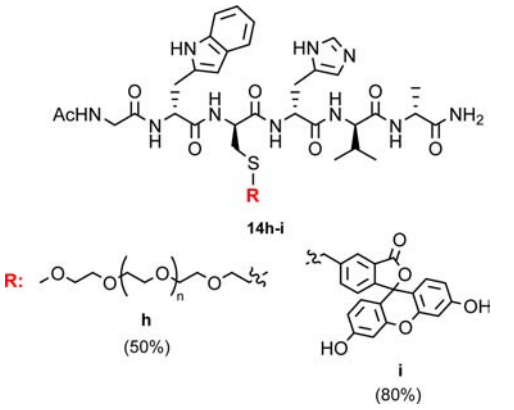

Figure 4. S-Alkylation with useful substituents.

carboxylic function, we tested alkylation with 5-BMF on the AcAspGlyCysValAlaNH$_2$ peptide sequence. NMR analysis of the resulting peptide confirmed that the most abundant product (90%) contained a fluorescein bound via the cysteine S-atom (data not shown).

In order to investigate the possibility to alkylate, under the employed mild conditions, more than one site on the same peptide sequence, peptides **15a** and **16a** were synthesized. Interestingly, they were obtained in high yields, thus demonstrating that the proposed methodology allows an efficient one-pot synthesis of poly alkylated molecules (Table 2) and that the reactivity of the substrate does not rely on its specific situation within the peptide sequence.

In summary, we have described a chemoselective and convenient S-alkylation method which can discriminate the reactivity of the cysteine sulfhydryl group upon that of other sensitive peptide functionalities. In particular, the S-alkylation selectively occurs on peptides containing different residues like arginine, histidine, tryptophan, aspartic acid, and glutamic acid.

This approach can be widely applied to various alkyl groups and allows the introduction of even poor electrophilic substituents, such as long aliphatic chains, in high yield and short time (1−3 h) and functional groups as reporters (i.e., fluorescein) or carriers (i.e., PEG) of peptide molecules. Moreover, the versatility of the method was proven with the efficient one-pot synthesis of poly alkylated molecules.

As an application of this novel strategy, we have planned to synthesize in the close future peptides reproducing natural sequences and containing functional substituents on cysteine residues.

Supporting Information Available. (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.