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Solid-Phase S-Alkylation Promoted by Molecular Sieves

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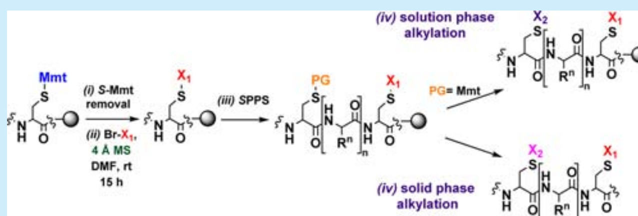
Supporting Information

ABSTRACT: A solid-phase S-alkylation procedure to introduce chemical modification on the cysteine sulfhydryl group of a peptidyl resin is reported. The reaction is promoted by activated molecular sieves and consists of a solid−solid process, since both the catalyst and the substrate are in a solid state. The procedure was revealed to be efficient and versatile, particularly when used in combination with the solution S-alkylation approach, allowing for the introduction of different molecular diversities on the same peptide molecule.



Nature uses covalent modifications of proteins to modulate their functions, so synthetic protocols that enable artificial chemical modification of protein and peptide molecules represent crucial tasks to facilitate biophysical studies for a better understanding of molecular mechanisms, and also to prepare tools for biomedical or biotechnological applications.[1] These chemical modifications generally refer to naturally occurring posttranslational modifications and include the sulfhydryl group farnesylation of a cysteine residue to translocate proteins to the membrane,[2] as well as the introduction of a fluorophore for bioimaging[3] and structural analysis,[4] PEGylation for solubility and bioavailability modulation,[5] and glycosylation for synthetic vaccine and drug development.[6]

One of the most commonly used strategies to perform such modifications involves the labeling of amino acid side chains, and many chemical methods have been developed for such a purpose, by carefully balancing reactivity and selectivity of the chosen functional group.[7]

We recently developed a synthetic strategy for performing postsynthetic peptide modifications via selective alkylation reaction of a nosyl-protected lysine side chain,[8] as well as of the sulfhydryl group of the cysteine residue.[9] The mild conditions employed require only the appropriate alkyl halide and activated molecular sieves to catalyze the reaction. Moreover, a one-pot multialkylation was performed by considering the different reactivities exhibited by the *N*-ε-nosyl-protected lysine with respect to the cysteine side chain.[9b]

Herein we describe a S-alkylation protocol performed on a peptidyl resin. We intended to implement the developed procedure in solid phase, as a routinely peptide synthetic step, considering that the solid phase peptide synthesis is generally a reliable and well consolidated technique. In fact, in addition to the easy and fast purification step, performed by simple filtration, the solid-phase S-alkylation, compared to the solution approach, could allow the modification of peptide sequences that have the tendency to aggregate in solution or to form a secondary structure. Moreover, it could also avoid the cysteine oxidation that can take place in solution where the reaction does not reach completion, offering the great advantage of being able to repeat the procedure.

However, it is worth noting that the reported solid-phase S-alkylation consists of a solid-state procedure, since the molecular sieves promote the reaction on a peptide anchored on the resin. In other words, the big challenge is represented by the investigation of the interaction process that occurs between molecular sites, both immobilized on a solid support.

In order to tune the experimental parameters for a solid-phase S-alkylation reaction, a model peptide, acetylated at the N-terminus and amidated at the carboxylic extremity, was synthesized (peptide **1**). The cysteine to be alkylated was protected with a highly acid labile group, the 4-Methoxytrityl (Mmt).[10] After completion of the peptide sequence, the cysteine residue was freed from its protecting group by treatment of the peptidyl resin with dilute trifluoroacetic acid,[10] and then, the peptidyl resin, suspended in anhydrous DMF and in the presence of activated 4 Å molecular sieves, was alkylated by adding the appropriate alkyl bromide (5 equiv) and keeping the stirring of the obtained mixture at room temperature for 15 h (Scheme 1).

An important concern of the solid-phase S-alkylation process was the investigation of the impact of the resin loading density, as well as of the type of polymer on the developed procedure. In this regard, it must be taken into consideration that an alkylation of the cysteine sulfhydryl group by a fragment of the Wang linker could occur during the cleavage procedure.[11] In particular, we encountered this problem in those cases where the desired S-alkylation partially took place, and a peptide with a cysteine residue alkylated by the p-hydroxyl benzyl, byproduct of Wang linker acidic decomposition, was also collected.

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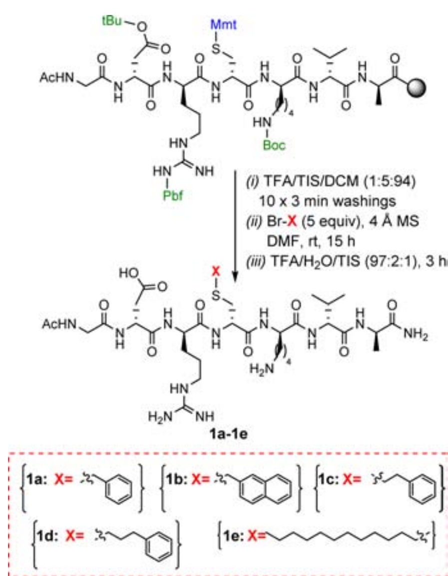
Scheme 1. Solid-Phase S-Alkylation of a Peptide Model with Different Substituents



To finalize the concerns listed above, we used benzyl bromide to alkylate peptide **1** anchored on different solid supports. In particular, we employed two different Rink Amide polystyrene resins, also characterized by a quite different loading value, the Rink Amide MBHA resin (0.9 $mmolg^{-1}$) and NovaSyn TGR (0.2 $mmolg^{-1}$). Since the latter support is characterized by the presence of the hydrophilic polyethylene glycol and a lower loading, both features minimizing the aggregation between peptide molecules on the beads, the reaction reached a high yield. However, the alkylation was also characterized by a quite high yield when performed on the high loaded peptidyl resin (0.9 $mmolg^{-1}$). It proved that the exposure of the sulfhydryl group to the alkylation process is not strongly affected by the characteristics of the solid support employed (see Supporting Information (SI), Figure S1).

Moreover, we also performed the solid-phase benzylation of $AcGlyCysValAlaNH_2$, which has a different sequence compared to compound **1**, by using a highly loaded Rink Amide resin. The high alkylation yield was confirmed not to be related to the specific environment of the sulfhydryl group within the peptide molecule (see SI, Figure S1).

The peptide sequence $AcGlyCys(Bn)ValAlaNH_2$ was also employed to ensure that the chiral center of the cysteine was not affected by the mild conditions used for the solid-phase alkylation (see SI, Figure S2). In fact, the absence of cysteine racemization was already proven for the solution approach; however, this investigation was necessary, since the reaction time of the solid phase protocol was prolonged (15 h).

In order to investigate the efficiency of the solid-phase S-alkylation protocol, upon usage of different alkyl bromides, the peptide series **1** was synthesized, by employing a common Rink amide resin (0.7 $mmol/g^{-1}$) as polymeric support (Table 1).

According to the results obtained for the procedure performed in solution with the same alkylating agents,[9a] high reaction yields were reached with most reactive groups, as well as with the less electrophilic substituents (Scheme 1). The yields were estimated by HPLC integration of the alkylated peptide compared to the starting peptide and other byproducts.

Synthesized peptides were fully characterized by 1D [^{1}H] and 2D [^{1}H, ^{1}H] NMR spectroscopy. Resonance assignments

Table 1. Efficiency of the Solid-Phase S-Alkylation Reaction

entry	peptide	yield (%)
1a	Ac-GDRC(Bn)KVA-NH_2	90
1b	Ac-GDRC(Menaph)KVA-NH_2	90
1c	Ac-GDRC(phenylethyl)KVA-NH_2	80
1d	Ac-GDRC(phenylpropyl)KVA-NH_2	80
1e	Ac-GDRC(dodecyl)KVA-NH_2	70
3a	Ac-GC(Bn)DRC(Bn)TVA-NH_2	85
3b	Ac-GC(Menaph)DRC(Menaph)TVA-NH_2	85
3c	Ac-GC(Menaph)DRC(Bn)TVA-NH_2	85[a]

[a]Second alkylation.

were obtained by a comparison of TOCSY, ROESY, and NOESY experiments and following a standard protocol.[12] Chemical shifts for differently alkylated cysteine residues resulted in agreement with those previously observed for the same compounds prepared with the solution phase alkylation.[9] Some difficulties were encountered in carrying out NMR analysis of the Cys(dodecyl) containing derivative (**1e**) whose 2D NMR spectra contain poor signal intensity likely caused by aggregation phenomena (see SI). As an example, Figure 1 shows the 2D [^{1}H, ^{1}H] TOCSY NMR spectrum of peptide **1b**.

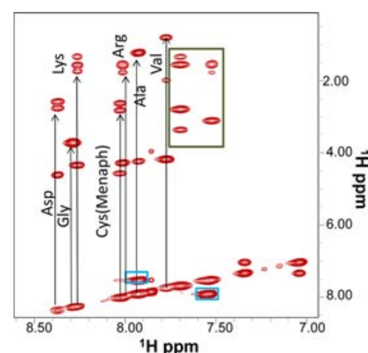


Figure 1. 2D [^{1}H, ^{1}H] TOCSY spectrum of **1b**. Assignment of spin systems belonging to the different amino acid residues are indicated by arrows. Correlations between aromatic protons of the naphthalene-methyl (Menaph) ring are enclosed in the blue boxes. The green rectangle contains contacts involving side chain NH protons of Lys and Arg.

It is important to underline that, under the conditions required to deprotect the cysteine to be alkylated, all the amino acid residues keep their protecting group, except those containing the trityl (Trt), and among them the His(Trt) resulted in being sensitive to the alkylation procedure. Therefore, in the case of a peptide sequence containing a histidine, a *tert*-butyloxycarbonyl (Boc) protection on its side chain could be employed, in order to avoid an undesired alkylated byproduct.

Concerning peptide **2**, it is a lipidated portion of a bioactive molecule (Wnt protein)[13] which was prepared by S-alkylation of Cys2 on solid phase with hexadecyl bromide (Figure 2). It represents an example of a sequence containing multiple

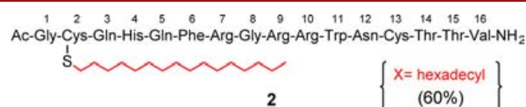


Figure 2. Highly conserved sequence of the Wnt proteins.

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cysteins and a histidine residue; therefore, the solid-phase alkylation approach greatly simplified the synthetic protocol, avoiding the usage of special protecting groups and reducing, at the same time, synthetic and purification steps (see SI, Scheme S1). In fact, we employed a histidine protected with a Boc group on its side chain, while the free cysteine was protected with the diphenylmethyl group (Dpm). It is orthogonal to the Mmt of the cysteine to be alkylated, but is easily removed under the strong acidic cleavage conditions.[17]

Peptides **3a** and **3b** were synthesized to investigate the possibility of poly alkylating in solid-phase a peptide sequence containing more than one cysteine (Table 1; see also SI, Scheme S2). The success of the performed reaction confirmed the efficiency of the S-alkylation process on a solid support.

Moreover, the solid-phase approach can also allow multi-alkylation on a peptidyl resin, by introducing different substituents on cysteines protected with the same Mmt group. After alkylation of the first cysteine introduced into the sequence, elongation of the peptide chain was ended and a different substituent was introduced onto the second cysteine residue (peptide **3c** in Table 1; see also SI, Scheme S3).

It is worth noting that, in the case of an incomplete cysteine alkylation, the sulfhydryl group, during the subsequent N-Fmoc deprotection, can undergo another S-alkylation process that generates the S−Fm protection.[14] However, this group can be easily removed in the presence of 20% piperidine. In other words, this side reaction can be eliminated by prolonging the N-Fmoc time deprotection.[15]

In the case of substituents sensitive to the acidic cleavage conditions, the multialkylation can be performed using both strategies, the solution and the solid-phase S-alkylation. Peptides **3d** and **3e** were obtained by performing the second alkylation in solution, due to the instability of the Dpm group upon treatment of the trifluoroacetic acid required for the cleavage step (Scheme 2).[14]

NMR spectroscopy provided proof of successful insertion of two different substituents into the peptide sequence (Figure 3).

An example of a multialkylated peptide obtained by combining the solution and solid-phase approach is represented by peptide **4**, which consists of a C-terminal N-RAS decapeptide11 (Figure 4).

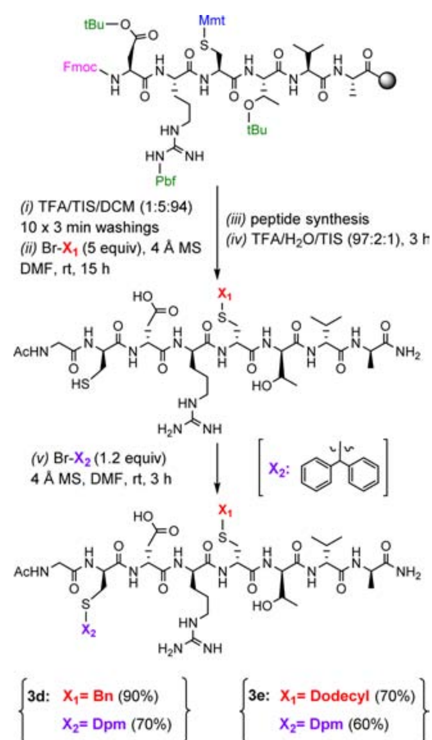
After a selective deprotection of Cys5, the first alkylation was performed on the peptidyl resin by the introduction of the palmityl group. The second alkylation was an S-farnesylation and occurred on the fully deprotected peptide, in order to prevent the acidic treatment, required by the cleavage procedure, from affecting the stereochemistry of the farnesyl group.[17]

In the case of peptide molecules containing cysteine residues involved in the disulfide bridge, the introduction of a functional group via solid-phase S-alkylation could represent the only synthetic strategy that prevents a multistep time-consuming preparation, thus providing the desired compound in high yield.

In this regard, the peptide R, belonging to a novel class of CXCR4 inhibitory compounds,[18] was selected to be modified at its N-terminus with a cysteine residue. The aim was to introduce, via S-alkylation reaction, appropriate functional groups (Scheme 3).

Fluorophores such as coumarin and pyrene were chosen for their peculiar characteristics, both endowed with environmentally and spatially sensitive features. In particular, the pyrene allows monitoring protein conformation, as well as protein−lipid and protein−membrane interactions,[19] while the

Scheme 2. Solution and Solid-Phase Approach To Perform a Peptide Multialkylation



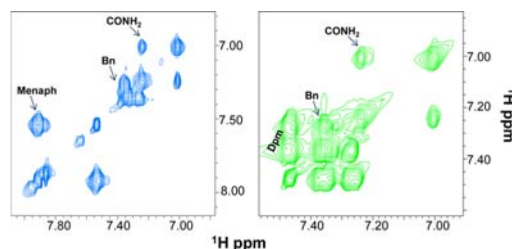


Figure 3. 2D [^{1}H, ^{1}H] TOCSY spectrum of **3c** (left panel) and **3d** (right panel). Spectral regions containing correlations between aromatic protons belonging to different Cysteine-attached groups and amide $CONH_2$ protons from the C-terminal group, are reported in the figure.

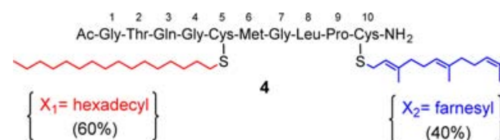


Figure 4. C-terminal portion of N-RAS protein.

coumarin is particularly sensitive to the local polarity and microviscosity.[20]

As shown in Scheme 3, the synthetic protocol employed resulted in being quite simplified by the solid-phase performed alkylation; indeed, it allowed the mild cysteine oxidation to still occur in buffered aqueous solution, after cleavage of the peptide sequence from the resin (peptides **5a** and **5b**).[18,21]

In summary, a mild solid-phase S-alkylation procedure was developed, by deep investigation of the involved synthetic parameters. Different polymeric supports were used in order to study the efficiency of the reaction, and in analogy with the previously reported studies, different alkylating agents were

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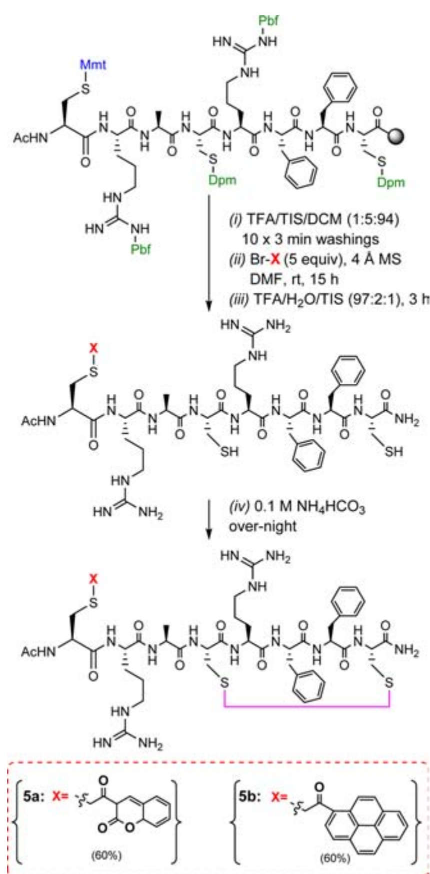
Scheme 3. S-Alkylation Reaction Performed on a Peptide CXCR4 Inhibitor



employed in order to explore their reactivity in solid phase (peptides **1a**–**e**). Multialkylations of the same peptide sequence were also performed (peptides **3a**, **3b**, **3c**), sometimes also combining both strategies, in solution and in solid-phase (peptides **3d**, **3e**, **4**), thus achieving remarkable versatility and efficiency of the developed synthetic procedures.

In conclusion, we believe that the two protocols, solution and solid-phase alkylation, do not represent alternative approaches for introducing molecular diversity on a peptide sequence, but complementary techniques which enhance each other's synthetic, purification, and characterization advantages.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02931.

Experimental section, LC-MS spectra, NMR chemical shift and spectra of S-alkylated peptides (PDF)

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Notes

The authors declare no competing financial interest.

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