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Development of caged non-hydrolyzable phosphoamino acids and application to photo-control of binding affinity of phosphopeptide mimetic to phosphopeptide-recognizing protein

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ABSTRACT

The design and synthesis of caged non-hydrolyzable phospho-serine, -threonine, and -tyrosine derivatives that generate parent non-hydrolyzable phosphoamino acids, containing a difluoromethylene unit instead of the oxygen of a phosphoester, after UV-irradiation are described. The caged non-hydrolyzable amino acids were incorporated into peptides by standard Fmoc solid-phase peptide synthesis, and the obtained peptides were successfully converted to the parent non-hydrolyzable phosphopeptides by UV-irradiation. Application of the caged non-hydrolyzable phosphoserine-containing peptide to photo-control the binding affinity of the peptide to 14-3-3β protein is also reported.

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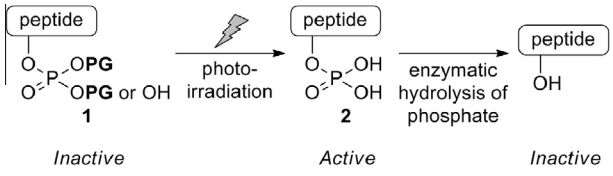
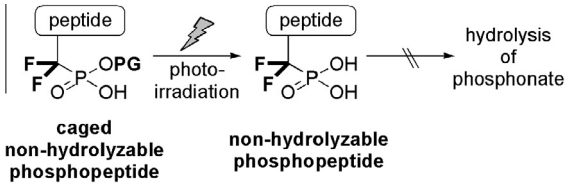
1. Introduction

Protein phosphorylation/dephosphorylation is an indispensable post-translational regulatory mechanism in a wide range of cellular processes.[1] Numerous chemical tools have been developed for controlling or monitoring phosphorylation and/or dephosphorylation events, enabling clarification of their biological significance.[2] A caged phosphopeptide is a useful tool for spatiotemporal regulation of phosphopeptide biological activity (Scheme 1).[3] Caged peptide **1** has a photo-removable protective group on a phosphate moiety to mask its key functionality. Its biological activity derived from interaction between the phosphate and a target biomolecule is therefore turned off. On photo-irradiation followed by removal of the protective group, the biologically active parent phosphopeptide **2** is generated. Although this technique is useful in the study of phosphorylation/dephosphorylation events, there is a risk of conversion of the active phosphopeptide **2** to an inactive form by hydrolysis of the phosphate by endogenous phosphatases. It is therefore desirable to avoid removal of the phosphate of phosphopeptides.

We and other groups have already developed non-hydrolyzable phosphopeptides in which an oxygen of a phosphoester moiety has been replaced by a difluoromethylene (CF_2) unit to prevent phosphate hydrolysis (Scheme 2).[4–6] Because the physical properties of difluoromethyl phosphonates are similar to those of the corresponding phosphates (e.g., pK_{a2}, charge under physiological conditions, and bond angle of C–X–P in $H_2NCH_2XPO_3H_2$: 5.6,



Scheme 1. Caged phosphopeptide (**PG**: protecive group removable by photo-irradiation).



Scheme 2. Design of caged non-hydrolyzable phosphopeptide (PG: protective group removable by photo-irradiation).

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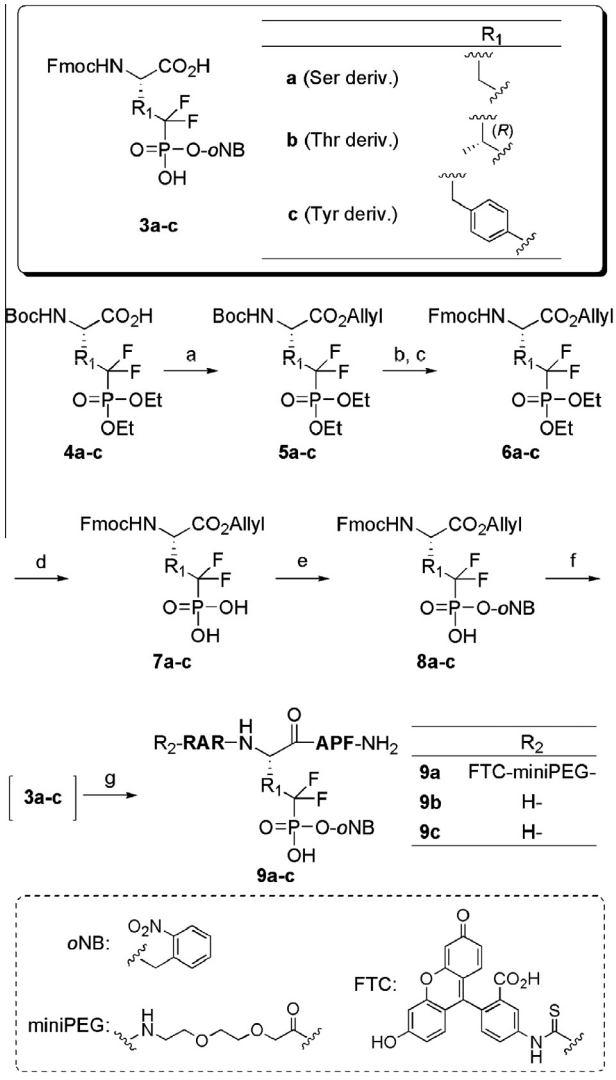
−2, and 117°, respectively, for CF_2 derivative, X is CF_2; 6.5, −2, and 119°, respectively, for the monoalkyl phosphate, X is oxygen),[7] they can be successfully used to elucidate the biological functions of phosphorylated peptides and proteins.[8,9] We therefore designed a caged non-hydrolyzable phosphopeptide that should work as a caged phosphopeptide mimetic without enzymatic hydrolysis of the phosphate.[10] Although introduction of two photo-responsive protective groups into the phosphonate is possible, monoprotection was used in this study because the bulkiness of the group was expected to be enough to prevent binding of the caged peptide to the phosphate-recognizing pockets of proteins. The mono-protected phosphonate was presumed to be compatible with solid-phase peptide synthesis (SPPS) because amino acid building blocks containing the mono-protected phosphate or non-protected phosphonate have been employed for preparation of phosphopeptides and their analogs.[11] In this paper, the synthesis and photoreactions of phosphopeptide mimetics with caged non-hydrolyzable phospho-serine, -threonine, and -tyrosine are described. Application of the serine derivative to photo-control of binding affinity to 14-3-3β protein is also reported.



Scheme 3. Synthesis of caged non-hydrolyzable phosphoamino acid derivatives (*o*NB: *o*-nitrobenzyl). Reagents and conditions: (a) allyl bromide, K_2CO_3 for **5a** and **5c** or Na_2CO_3 for **5b**, DMF, 97% (**5a**), 98% (**5b**), 85% (**5c**); (b) TFA; (c) *N*-(9*H*-fluoren-2-ylmethoxycarbonyloxy)succinimide (FmocOSu), MeCN, 10% (w/v) Na_2CO_3 aq, 60% (**6a**), 97% (**6b**), 77% (**6c**) (two steps); (d) TMSBr, CH_2Cl_2, then MeCN aq, 97% (**7a**), 81% (**7b**), 51% (**7c**); (e) *o*NB alcohol, TsCl, pyridine, DMF for **8a** and **8c**, or *o*NB bromide, DIEA, DMF, 50 °C for **8b**, 68% (**8a**), 84% (**8b**), 54% (**8c**); (f) $(Ph_3P)_4Pd$, *N*-methylaniline, THF; (g) Fmoc SPPS using HBTU/DIEA or HATU/HOAt/DIEA system (A: alanine; F: phenylalanine; P: proline; R: arginine).

2. Results and discussion

2.1. Synthesis of caged non-hydrolyzable phosphoamino acid derivatives and their incorporation into peptides

Fmoc-protected non-hydrolyzable phosphoserine **3a** containing a photo-removable protection was first synthesized as shown in Scheme 3. In this study, an *o*-nitrobenzyl (*o*NB) group was chosen as the photo-responsive protective group because of its widespread use in a chemical biology field, simple structure without a chiral center and commercial availability.[3a,12] Carboxylic acid **4a**[4a] was treated with allyl bromide in the presence of K_2CO_3 to afford ester **5a**. The Boc group of **5a** was replaced by an Fmoc group to give **6a**. The ethyl groups of **6a** were removed by treatment with TMSBr in CH_2Cl_2 followed by aqueous MeCN. Monoesterification of phosphonate **7a** was then achieved by the use of a *p*-toluenesulfonyl chloride (TsCl)-pyridine system. Compound **8a** was used for a subsequent reaction without hesitation because of its instability. Finally, the allyl group of **8a** was removed in the presence of palladium(0) to generate Fmoc-protected non-hydrolyzable phosphoserine **3a**. Purification of the product was highly complicated because of its high polarity, so crude **3a** without column chromatography purification was used in subsequent reactions.[13]

To examine the photo-reactivity and photo-control of the binding affinity of the phosphoserine mimetic-containing peptide, serine derivative **3a** was then incorporated into a model peptide. In this study, a ligand analog of a 14-3-3β protein[14] was synthesized because a phosphate moiety on the ligand is critical for binding[15,16] and the ligand sequence has been well studied.[17] The caged phosphopeptide mimetic **9a**, in which a phosphoserine of the original sequence was replaced by the caged non-hydrolyzable derivative, was prepared using standard Fmoc SPPS. For the coupling reaction, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIEA) were used before introduction of serine derivative **3a**, whereas 2-(1*H*-7-azabenzotriaol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxyl-7-azabenzotriazole (HOAt), and DIEA were used for introduction of **3a**. An HBTU/DIEA system was used for further chain elongation.[18] After introduction of an 8-amino-3,6-dioxaoctanoyl (miniPEG) linker, a 5-fluoresceinylaminothiocarbonyl (FTC) group was incorporated by treatment with 5-fluorescein isothiocyanate (FITC) in the presence of DIEA for the subsequent fluorescence-based binding assay.

Fmoc-protected threonine derivative **3b** and tyrosine derivative **3c** were also prepared, starting from **4b**[5a,19] or **4c**,[6a] similarly to the serine derivative **3a** except for the introduction of the *o*NB group on **7b**. When **7b** was treated with TsCl and pyridine, the yield of monoester **8b** was not sufficient but a mass spectrometric analysis of the crude reaction mixture gave a main peak identical to $[\mathbf{7b}-H_2O-H]^-$ (data not shown). We thought that dehydrative cyclization took place via electrophilic activation of the phosphonate by TsCl, therefore, *o*-NB bromide was employed to avoid the cyclization induced by TsCl. Crude **3b** and **3c**, without column chromatography purification, were successfully used in Fmoc SPPS to prepare model peptide **9b** and **9c**, respectively.

2.2. UV-induced generation of non-hydrolyzable phosphopeptides

Next, UV-irradiation experiments were performed on caged peptide **9a** possessing the serine derivative (Fig. 1A and B). Caged peptide **9a** in sodium phosphate buffer (pH 7.6) was subjected to

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