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General Method for the Synthesis of Caged Phosphopeptides: Tools for the Exploration of Signal Transduction Pathways

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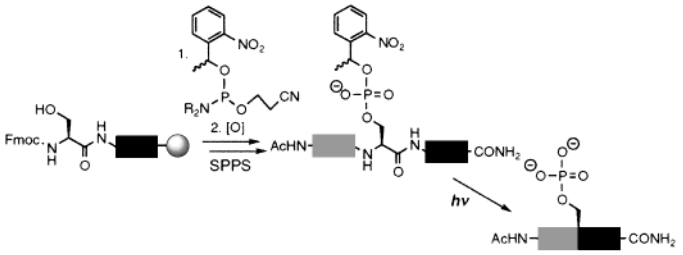
ABSTRACT



An interassembly approach for the synthesis of peptides containing 1-(2-nitrophenyl)ethyl-caged phosphoserine, -threonine, and -tyrosine has been developed. Photochemical uncaging of these peptides releases the 2-nitrophenylethyl protecting group to afford the corresponding phosphopeptide. The peptides described herein are based on phosphorylation sites of kinases involved in cell movement or cell cycle regulation and demonstrate the versatility of the method and compatibility with the synthesis of polypeptides, including a variety of encoded amino acids.

Kinase-mediated phosphorylation of tyrosine, serine, and threonine residues in polypeptides and proteins represents a central mechanism of cell regulation. As such, it is an area of intense study in which there is considerable potential for the development of new chemical tools.[1] The study of protein kinases involved in cell migration is of particular interest in biological and medicinal studies. The present tools available for the study of phosphorylation-dependent signal transduction pathways are limited when information in real time is desired. For example, chemical inhibition, gene knockout, or point mutation studies can often confirm the significance or essentiality of a protein kinase; however, such methods make it difficult to assess the biochemical role of the protein in real time.

A caged compound includes a photocleavable-protecting group that masks an essential functionality; upon removal by photolysis, the functionality is revealed, generating a biologically active molecule.[2] This strategy allows researchers spatial and temporal control over the release of effector molecules in living systems.[3] There are several characteristics required of a caging group to be used in biological systems. The caged compound, as well as the byproducts of photolysis, should be inert in the cell, neither agonizing nor antagonizing the receptor molecule. Only the uncaged effector molecule should alter cell activity. The caged compound must be stable to hydrolysis and enzymatic

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cleavage, and the rate of uncaging must be fast in order to give a concentration burst of the photolysis product on a μs−ms time scale.[4] Derivatives of the *o*-nitrobenzyl group are commonly used to cage biologically active molecules. For example, caged analogues of divalent calcium,[5] ATP,[2] cAMP,[2] peptides,[6,7] and proteins[8] have been prepared. The photochemical release of these species occurs with reasonable quantum efficiencies at wavelengths above 300 nm.[2] Increasing the concentration of a reducing agent such as glutathione in the medium is often necessary to prevent the nitroso photobyproduct from reacting with free amines in the cell.[18]

Therefore, caging biological mediators is an attractive method for introducing specific concentrations of compounds into cells and controlling their release in order to observe a cellular response. Thus, to observe the consequences of phosphorylation in vivo, the controlled release of a physiological concentration of phosphopeptide can be effected by benign photolysis. To date, there are no general strategies for the synthesis of caged phosphopeptides. Bayley and coworkers have recently reported syntheses of nitrobenzyl- and *p*-hydroxyphenacyl-caged thiophosphotyrosine peptides.[9] The preparation of these analogues involves a Hck kinase-mediated thiophosphorylation of tyrosine-containing peptides with ATP(γ)S in the presence of divalent cobalt. The incipient thiophosphate can then be chemically modified with 2-nitrobenzyl bromide or *p*-hydroxyphenacyl bromide due to the intrinsic reactivity of the thiophosphate sulfur. While the resultant caged thiophosphopeptides show good biochemical utility, a disadvantage to this method is that the kinase-mediated thiophosphorylation must be optimized for each substrate and enzyme and the thiophosphate residue unveiled upon photolysis may have altered functionality in downstream events when compared with native phosphorylated substrates.

Herein, we report a general route for the synthesis of peptides containing 2-nitrophenylethyl-caged phosphoserine, -threonine, and -tyrosine residues. The synthesis is based on an interassembly approach,[10,11] integrated into Fmoc solid-phase peptide synthesis (SPPS). The method is general and enables the preparation of various peptides containing 1-(2-nitrophenyl)ethyl-caged phosphoserine, -threonine, and -tyrosine residues. The synthesis of three representative kinase target sequences (Figure 1) is presented to demonstrate

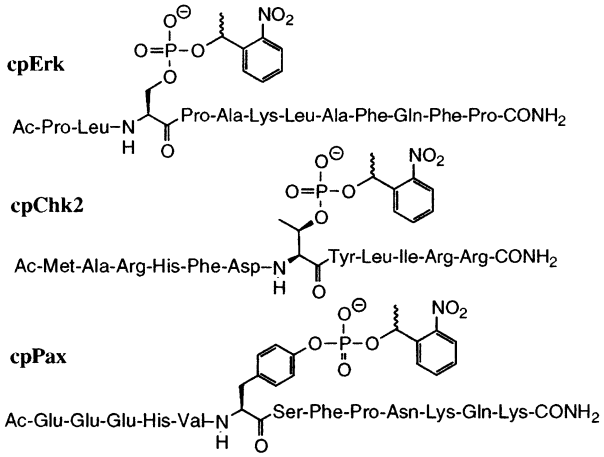

Figure 1. Target caged phosphopeptides, including modified serine, threonine, and tyrosine.

the versatility of the method. The caged phosphoserine peptide, Ac−PL(cpS)PAKLAFQFP−$CONH_2$ (**cpErk**),[12] includes the core recognition motif for the ERK kinase (PLSP)[13] as well as an ERK docking motif (FXFP).[14] The ERK kinases make up a group of mitogen-activated protein kinases, MAPKs. ERK has been implicated in multiple pathways, including cell motility, differentiation, and proliferation. The caged phosphothreonine peptide is Ac−MARHFD(cpT)YLIRR−$CONH_2$ (**cpChk2**). The corresponding uncaged peptide would be an antagonist of Chk2, a homologue of the human Rad53p checkpoint kinase.[15] The caged phosphotyrosine peptide Ac−EEEHV(cpY)SFPNKQK−$CONH_2$ (**cpPax**) mimics a section of human paxillin, which is thought to be involved in focal adhesions during cell movement. Paxillin can be phosphorylated at two sites, Tyr31 and Tyr118: Tyr118 has been identified as the major phosphorylation site of Paxillin by FAK (Focal Adhesion Kinase) in vitro.[16] **cpPax** corresponds to residues 113−125 of paxillin.

The caged oligopeptide sequences derived from the current methodology will also be amenable to integration into full-length proteins via native chemical ligation methods.[17]

Synthesis of Caged Peptides. The general scheme for the assembly of caged phosphopeptides is illustrated with a serine-containing peptide in Scheme 1.

The basic strategy involves the introduction of a 1-(2-nitrophenyl)ethyl-caged phosphate via the intermediacy of a trivalent phosphitylating agent, which is subsequently oxidized. The phosphitylating agent, *O*-1-(2-nitrophenyl)-ethyl-*O*′-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (**1**), was prepared as follows. The precursor 1-(2-nitrophenyl)-ethanol was derived by reduction of 2-nitroacetophenone with sodium borohydride using a method modified from Kaplan

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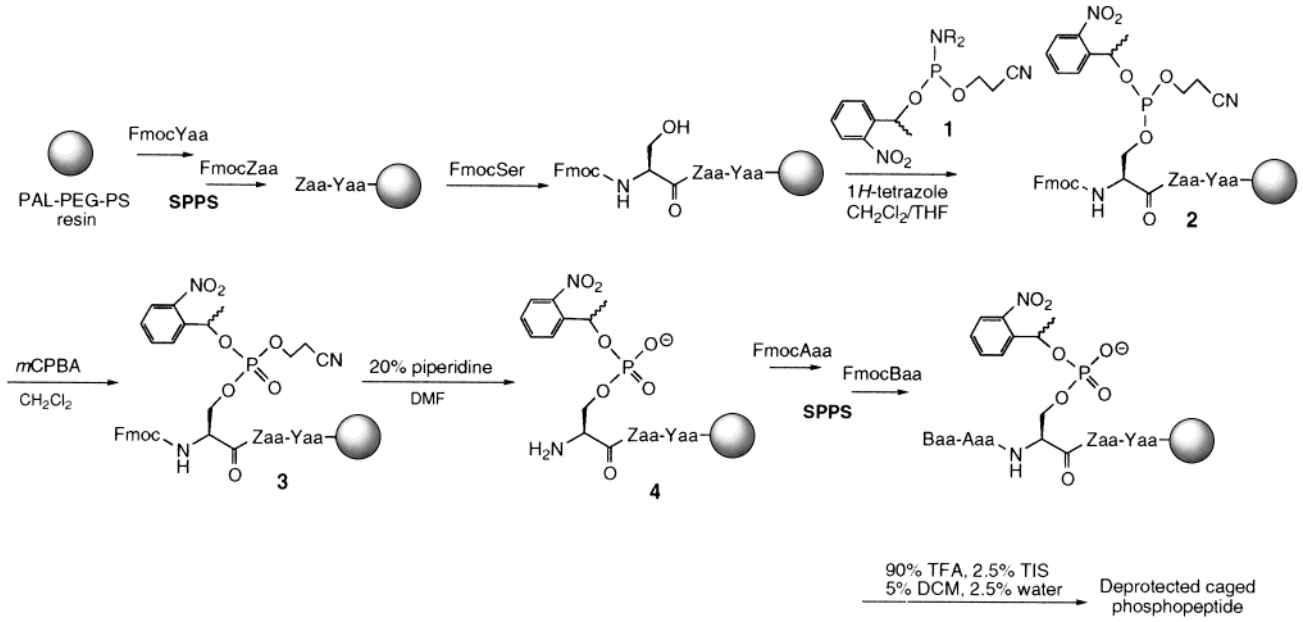
Scheme 1. Interassembly Approach for the Synthesis of Caged Phosphopeptides



and co-workers.[18] Next, **1** was synthesized by substituting 1-(2-nitrophenyl)ethanol for chlorine in 2-cyanoethyl diisopropylchlorophosphoramidite using a procedure similar to that of Kuphiar et al.[19] The preparation of a caged phosphoserine-containing peptide is illustrated in Scheme 1; the method is generally applicable to the synthesis of the corresponding phosphothreonine and phosphotyrosine-containing peptides with noted modifications. In summary, each peptide was synthesized by standard Fmoc SPPS, on a PAL-PEG-PS solid support, up to the key serine, threonine, or tyrosine residue; these three residues were incorporated without side chain protection. For **cpErk**, the free serine residue was phosphitylated with 5 equiv each of **1** and 1*H*-tetrazole in 1:1 dichloromethane (DCM)/tetrahydrofuran (THF) to afford the resin-bound phosphite **2**.[20] Oxidation of **2** with *m*-chloroperoxybenzoic acid (*m*CPBA) afforded the more stable bisprotected phosphate **3**. While phosphotriesters are prone to β-elimination during the basic Fmoc deprotection steps of SPPS,[11] treatment of **3** with 20% piperidine in *N*,*N*-dimethylformamide (DMF) concurrently removed the Fmoc group for chain elongation and the cyanoethyl group[21] on the phosphate to give the caged phosphate **4**. The resulting phosphodiester was stable toward elimination yet suitably protected from side reactions during chain elongation.[11] Upon completion of the synthesis, the peptides were cleaved under various conditions. Notably, it was found that the 2-nitrophenylethyl group was stable to long treatments of 90% TFA that are necessary to remove the relatively stable benzenesulfonyl protecting groups of the arginine precursors. The prevalence of arginine residues in many protein kinase[22] recognition sequences makes this aspect of the synthesis important.

The incorporation of threonine and tyrosine residues into synthetic peptide modules necessitated some minor modifications of the above procedure. Since the secondary hydroxyl group of threonine is less reactive than that of serine, 10 equiv of **1** and 1*H*-tetrazole were required to achieve efficient phosphitylation. The cyanoethyl-protected phosphorylated threonine (threonine version of **3**) appeared as two separate peaks in the HPLC chromatogram. These components may result from the chiral phosphorus center adjacent to the chiral center at the β-carbon of threonine since the two peaks showed identical masses in the mass spectrometry analysis. After deprotection of the cyanoethyl group with piperidine, the HPLC chromatogram for the peptide was converted to a single peak. Due to the potential for methionine oxidation, the **cpChk2** peptide was dissolved in deoxygenated water and stored at −20 °C. It is important to note that residues that are sensitive to oxidation[11] such as methionine and tryptophan can only be incorporated into the sequence of caged phosphopeptides after the phosphite oxidation step.

The tyrosine hydroxyl also showed reduced reactivity in the phosphitylation step; optimal conditions implemented 10 equiv of **1** and 1*H*-tetrazole together with dried 4 Å molecular sieves. It was also found that 4,5-dicyanoimidazole may be used in place of 1*H*-tetrazole with a similar yield. Oxidation of the tyrosine phosphite with *m*CPBA resulted in several side products. Therefore, oxidation was achieved with either *tert*-butyl hydroperoxide solution in decane or hydrogen peroxide as an aqueous solution in THF. No further

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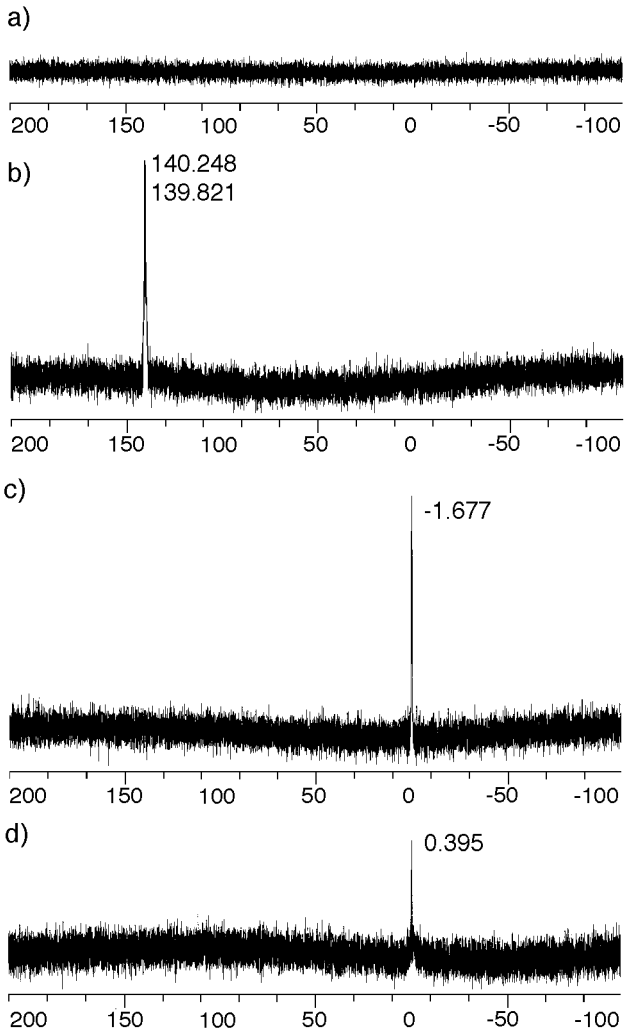

Figure 2. ^{31}P Magic Angle Spinning NMR. Phosphorus NMR taken on AM resin at 202 MHz. (a) Free hydroxyl on serine, (b) phosphite on serine, (c) phosphate on serine, fully protected, and (d) phosphate on serine after cyanoethyl removal.

modifications to the synthesis were necessary after the oxidation step.

The peptides were characterized by reversed-phase high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESMS). The overall phosphorylation efficiency ranged from 75 to 95% as determined by HPLC. The quantum yield for the uncaging of each peptide was determined by a previously reported method.[23] **cpErk** has a quantum yield of 0.26; **cpChk2** has a quantum yield of 0.33, and **cpPax** has a quantum yield of 0.28.[24]

^{31}P Magic Angle Spinning NMR. In addition to **cpErk**, **cpChk2**, and **cpPax**, a resin-bound tripeptide, **cpSer-Pro-Gly**-resin, was prepared on an aminomethylated polystyrene solid support. The higher loading provided by this resin enabled magic angle spinning (MAS) ^{31}P NMR experiments, which could be used as an unambiguous method of following the progression of phosphitylation, oxidation, and subsequent cyanoethyl deprotection on-bead during the course of developing the synthetic strategy. ^{31}P MAS NMR spectra of key intermediates in the synthesis of **cpSer-Pro-Gly**-resin are illustrated in Figure 2.

Herein we have described the generally applicable solid-phase syntheses of peptides containing 1-(2-nitrophenyl)-ethyl-caged phosphoserine, -threonine, and -tyrosine residues. For each representative hydroxyamino acid residue, a biologically active phosphopeptide was synthesized and may be used in real-time studies of kinases in vivo.

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Supporting Information Available: Details of the solid-phase synthesis of **cpErk**, **cpChk2**, and **cpPax**, quantum yield determination, and ^{31}P MAS NMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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