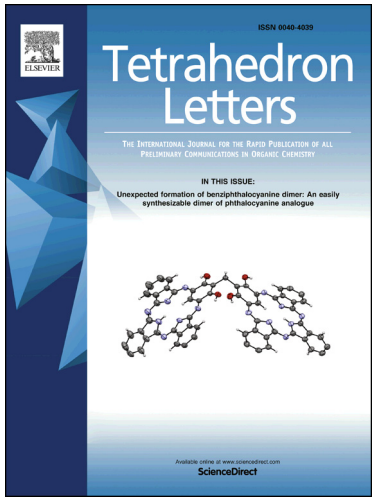

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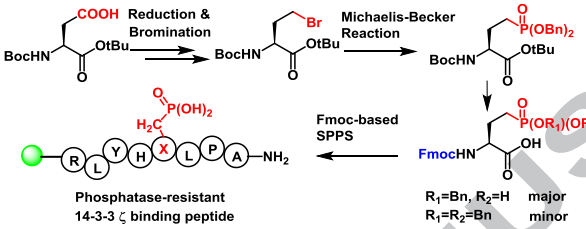
Jie Kang ‡, Hong-Xue Chen‡, Si-Qi Huang, Yun-Lai Zhang, Rong Chang, Fang-Yi Li, Yan-Mei Li, and Yong-Xiang Chen*



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Facile synthesis of Fmoc-protected phosphonate pSer mimetic and its application in assembling a substrate peptide of 14-3-3 ζ

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Key Laboratory of Bioorganic Phosphorus Chemistry and Chemical Biology (Ministry of Education), Department of Chemistry, Tsinghua University, Beijing 100084, China

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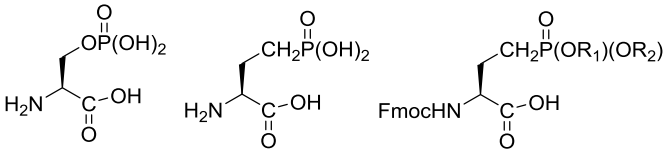
ABSTRACT

Phosphatase-inert peptidomimetics containing phosphonate pSer analogue have been developed as valuable biological tools for probing and regulating pSer-dependent protein-protein interactions (PPIs) in cellular context. Herein, we report a facile and efficient synthesis route of Fmoc-protected phosphonate pSer mimetic and also present the application of this building block in the solid-phase synthesis of a phosphatase-resistant substrate peptide of 14-3-3 ζ, retaining 14-3-3 ζ binding efficacy similar to the parent pSer-containing peptide.

Phosphoserine (pSer **1**)-dependent protein-protein interactions (PPIs) are involved in important cellular signaling pathways, which regulate a variety of vital biological processes including cell growth and proliferation.[1] Many of such interactions are relevant with cancer development and progression.[2] Thus, phosphopeptides derived from phosphoproteins, which can bind tightly to pSer-binding proteins, have been developed as competitive inhibitors of such PPIs.[3] Because serine phosphorylation is a reversible process respectively catalyzed by kinases and phosphatases, the phosphate group is easily removed from serine by phosphatases in cellular context.[4] Thus, nonhydrolyzable CH_2-substituted phosphonate pSer mimetic **2** (Figure 1), in which CH_2 moiety replaces phosphoryl ester oxygen in pSer **1**, has been incorporated into peptides and proteins to avoid cleavage by phosphatases.[5] These phosphopeptide/phosphoprotein mimetics are useful tools for biological studies in cellular context and provide starting points for further design of therapeutics.[5]

14-3-3 ζ, one isoform of highly conserved and ubiquitously expressed 14-3-3 protein family, often binds to specific pSer-containing motifs of interacting proteins in PPIs, which are involved in some vital cellular signal transductions, cell cycle control, and apoptosis.[6a-d] Meanwhile, it was reported that 14-3-3 ζ was upregulated in various types of carcinomas, such as pancreatic adenocarcinoma and lung carcinoma.[6a,6d-f] Thus, 14-3-3 ζ has the implication as prognostic and therapeutic target of some cancers.[6a,6d,6f] The pSer-containing peptide sequences, such as RLYHpSLPA, have been developed as inhibitors of 14-3-3 ζ.[7] Converting these natural phosphopeptides into phosphatases-inert peptidomimetics by incorporating phosphonate pSer mimetic will largely facilitate the regulation of 14-3-3 ζ involved protein-protein interaction networks in cellular environment.



pSer 1 **CH_2-pSer 2** **3:** R_1=Bn,R_2=H **4:** R_1=R_2=Bn

Figure 1. Structures of pSer **1**, CH_2-substituted phosphonate pSer mimetic **2** and its Fmoc-protected forms suitable for solid-phase peptide synthesis.

Dating back to previous pioneering work, a few strategies have been successfully developed to generate the nonhydrolyzable phosphonate pSer mimetics. Walker group prepared racemic phosphonate pSer mimetics through the addition of vinylphosphonate diesters to potassium (*N*-diphenylmethylene) glycine esters.[8a] Weber group employed the Schoellkopf bislactim ether asymmetric amino acid synthesis approach to construct such chirality-pure building blocks,[8b] which had broad applications.[8c] More recently, Mikołajczyk group reported an asymmetric synthetic route toward **2** by diastereoselective addition of α-phosphonate carbanions to sulfinimines.[8d] Haufe group described a straightforward synthesis route starting from

* Corresponding author. Tel.: +86-10-6277-2259; e-mail: chen-yx@mail.tsinghua.edu.cn; ‡These authors contributed equally.

D-methionine to produce Boc-protected phosphonate D-pSer mimetic.[8e]

Herein, we report a facile and efficient synthesis approach toward nonhydrolyzable phosphonate pSer mimetic **3** in a form suitable for Fmoc solid-phase peptide synthesis (SPPS) using commercially available *N*-Boc protected L-aspartic α-*tert*-butyl ester as starting material. Furthermore, we present the application of this easily accessible building block in the solid-phase synthesis of a substrate peptide of 14-3-3 ζ, leading to a phosphatase-resistant inhibitor (Figure 2).

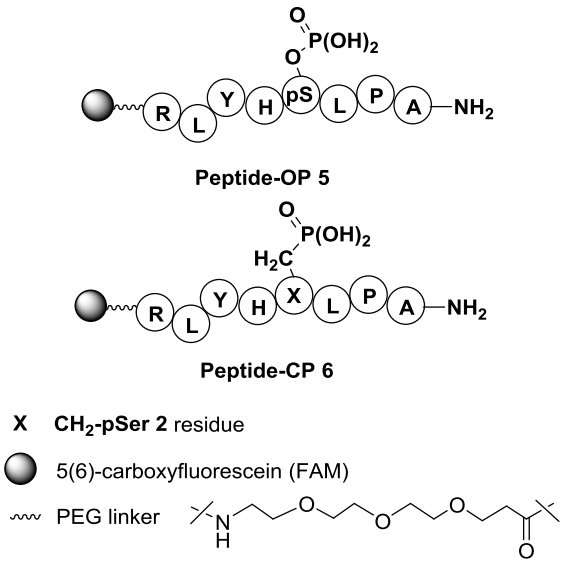
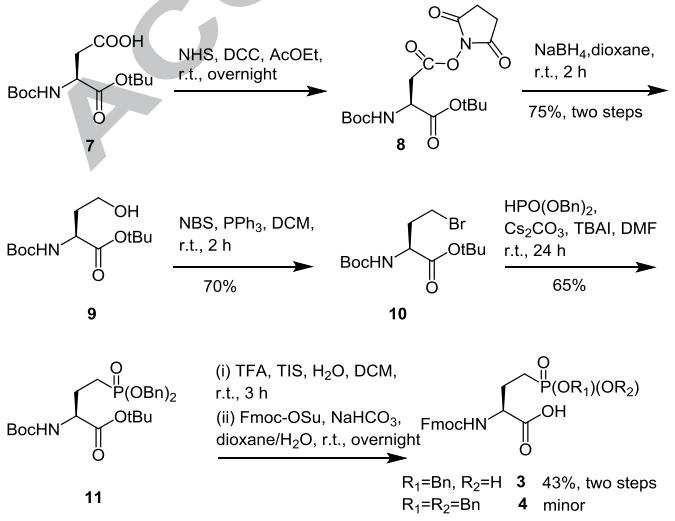


Figure 2. Structures of the parent pSer-containing Peptide-OP **5** and phosphatases-resistant Peptide-CP **6** containing phosphonate pSer mimetic as substrates of 14-3-3 ζ.

Following this concise synthesis route of phosphonate pSer mimetic (Scheme 1), the commercially available *N*-Boc protected L-aspartic α-*tert*-butyl ester **7** was first treated with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (DCC) to give a succinimide ester intermediate **8**, followed by reduction using $NaBH_4$ according to previously reported procedures, [9] leading to an L-homoserine derivative **9**. Next, the hydroxyl group in compound **9** was substituted with bromo group through a derived Appel reaction using triphenylphosphine and *N*-bromosuccinimide to afford the desired bromide **10**.[10]



Scheme 1. Synthesis of Fmoc-protected phosphonate pSer mimetics.

The key step in constructing phosphonate pSer mimetic is the formation of P-C bond,[11] which was achieved by using Michaelis-Becker reaction via nucleophilic attack of bromide **10** by dibenzyl phosphite according to a literature procedure.[8e] The resultant compound **11** underwent a deprotection by treatment with a solution of TFA (50%, v), followed by re-protection using Fmoc-OSu to give the final crude product containing mono-benzyl and bis-benzyl phosphonate derivatives **3** and **4**. After HPLC purification, the major component **3** was obtained in a yield of 43% over two steps. Due to the convenient removal of benzyl groups on phosphonate in the peptide deprotection step, the final crude product actually can be directly used for peptide synthesis.

The obtained Fmoc-protected phosphonate pSer mimetics were applied in the solid-phase synthesis of a phosphopeptide mimetic (Peptide-CP **6**) derived from the 14-3-3 ζ substrate (RLYHpSLPA) (Figure 2). Rink amide resin was chosen for anchoring the peptide leading to an amide *C*-terminus. The peptide was elongated following the standard Fmoc-based SPPS procedures. Considering further evaluation of the binding affinity between peptide target and 14-3-3 ζ, the 5(6)-carboxyfluorescein (FAM) was introduced into the *N*-terminus, while a PEG linker was inserted between FAM and peptide sequence to suppress the effect of fluorophore on the peptide-protein interaction. Finally, the peptide was released from the resin by treatment with a high concentration of TFA (82.5%, v) while all of the protecting groups including benzyl groups on phosphonate were removed simultaneously. After HPLC purification, the desired Peptide-CP **6** was characterized by analytical HPLC and ESI-MS (Figure 3). Meanwhile, the corresponding parent pSer-containing Peptide-OP **5** and nonphosphorylated Peptide-OH **12** were prepared as control following the similar synthetic procedures of Peptide-CP **6**.

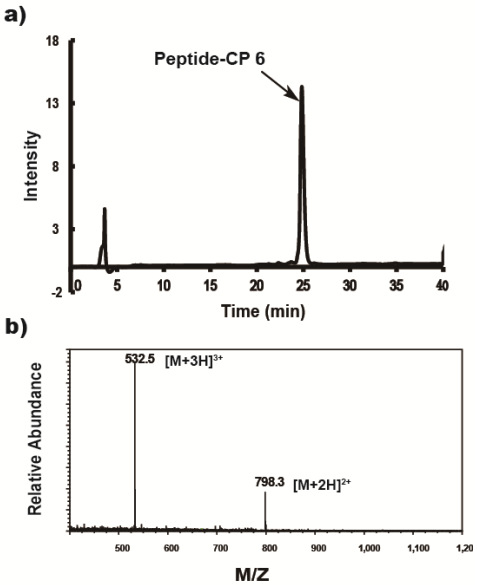


Figure 3. Characterization of Peptide-CP **6**. a) Analytical HPLC profile of Peptide-CP **6**. b) ESI-MS spectrum of Peptide-CP **6**, M_{calcd} =1593.7 Da.

To evaluate the activities of these peptides to inhibit 14-3-3 ζ, we applied fluorescence polarization technique to respectively measure the binding affinities of Peptide-CP **6** and Peptide-OP **5** with 14-3-3 ζ. The nonphosphorylated Peptide-OH **12** was used as a negative control. 100 nM FAM-containing peptides were titrated with increasing amount of 14-3-3 ζ until the polarization signals of Peptide-CP **6** and Peptide-OP **5** reached saturation, while the signal of Peptide-OH **12** displays no obvious increase.

The increasing of fluorescence polarization signal reflects the binding of 14-3-3 ζ to the fluorescent peptides. The polarization signal changes were respectively fitted to the saturation titration Equation 1 (Supporting Information, SI),[12] generating the K_D values. As shown in Figure 4a, Peptide-CP **6** displays a K_D value of 0.86 μM, which is close to the K_D value of Peptide-OP **5** (0.46 μM) binding to 14-3-3 ζ. The results indicate that the substitution of pSer by phosphonate pSer mimetic slightly reduces the binding affinity of peptide substrate (RLYHpSLPA) to 14-3-3 ζ, proving that Peptide-CP **6** can be used as an inhibitor of 14-3-3 ζ. We deduced that the slightly reduced binding affinity might be caused by the less electronegativity of CH_2-substituted phosphonate pSer mimetic compared to that of pSer.

In addition, we detected the association between these peptides and 14-3-3 ζ in the presence or absence of alkaline phosphatase by using fluorescence polarization assay. As mentioned above, the strength of fluorescence polarization signal reflects the degree of 14-3-3 ζ binding to these fluorescent peptides. As shown in Figure 4b, the addition of alkaline phosphatase clearly disrupted the association of Peptide-OP **5** with 14-3-3 ζ due to the removal of phosphate group from serine, while the binding of Peptide-CP **6** to 14-3-3 ζ kept intact in the presence of alkaline phosphatase. These results demonstrate that Peptide-CP **6** containing phosphonate pSer analogue can be developed as useful tools for probing and regulating 14-3-3 ζ involved PPI networks in cellular context.

In conclusion, we have developed a facile and efficient synthesis route for producing Fmoc-protected phosphonate pSer analogue. This synthetic approach has the potential to be used for generating pSer mimetics bearing biorthogonal protecting groups on phosphonate. In addition, the prepared phosphonate pSer analogue has been successfully applied in the solid-phase synthesis of a phosphatase-resistant substrate peptide of 14-3-3 ζ, retaining 14-3-3 ζ binding efficacy similar to the parent pSer-containing peptide.

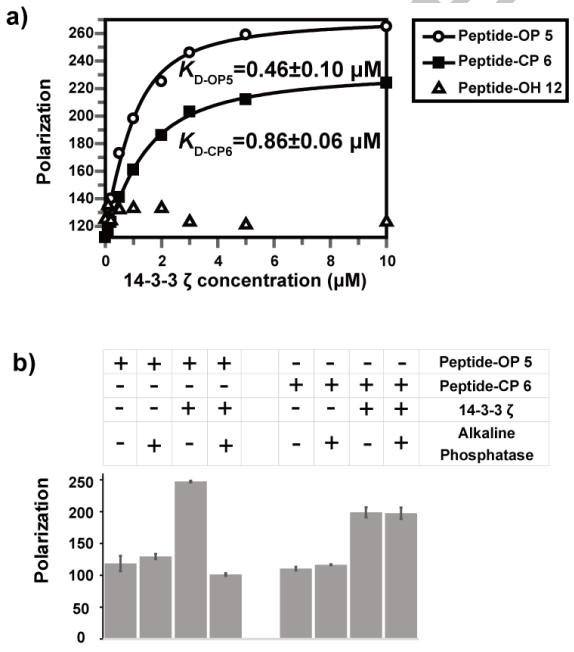


Figure 4. a) Fluorescence polarization measurements of Peptide-OP **5,** Peptide-CP **6** and Peptide-OH **12** respectively upon addition of different concentration of 14-3-3 ζ. The fluorescence polarization changes of Peptide-OP **5** and Peptide-CP **6** were fitted to Equation 1 (SI) to yield K_D values. b) Fluorescence polarization measurements of Peptide-OP **5** and Peptide-CP **6** upon addition of 14-3-3 ζ in the presence or absence of alkaline phosphatases. The concentration of Peptide-OP **5** and Peptide-CP **6** was 100 nM. The concentration of 14-3-3 ζ was 5 μM.

Acknowledgments

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Supplementary Material

The experimental description and characterization data of compounds in this article can be found XXXX, in the online version.

Highlights:

- Developing a facile synthesis method to CH_2-substituted phosphonate pSer mimetic.
- Applying phosphonate pSer mimetic in SPPS synthesis of 14-3-3 ζ substrate.
- The phosphatase-resistant substrate peptide retains 14-3-3 ζ binding efficacy.