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Chemical synthesis of Ser/Thr AMPylated peptides

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ABSTRACT

A method for the solid phase chemical synthesis of AMPylated Ser/Thr containing peptides is described. Peptides were phosphonylated using the H-phosphonate method, then the adenosine moiety was introduced using 2′,3′-isopropylidene adenosine and the condensing reagent PyBOP. Oxidation with iodine yielded the desired AMPylated product in good yield following acidolytic cleavage from the resin support.

1. Introduction

Recent reports have shown that proteins can be modified with an AMP molecule at the hydroxyl group of Thr and Tyr in a process known as AMPylation.[1,2] It has been suggested that AMPylation could be an important post-translational modification (PTM) that is involved in molecular or cellular signaling analogous to phosphorylation.[3] This AMPylation modification, until now, has been found only on Thr and Tyr residues, but it is conceivable that Ser will also be similarly modified in vivo. The ability to chemically synthesize peptides modified with AMP, and to synthesize them in large amounts, would therefore be essential for the identification of AMPylated proteins and peptides, and for the generation of AMP-specific antibodies. Here we describe a solid-phase peptide synthesis (SPPS) method for AMPylating peptides using side chain-unprotected Ser and Thr, with 2′,3′-isopropylidene adenosine as the source of adenosine and benzotriazole-1-yl oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) as the acylating reagent. Two series of peptides were synthesized: Series 1 was EYIPT(amp)VF, corresponding to aa 31–37 region in the Rac protein,[1] whilst Series 2 consisted of a set of 11mers including AAAH AAAT(amp)VHV, and AAAHAAAS(amp)VHV[4] (Table 1).

2. Results and discussion

Peptides were synthesized using Fmoc and side chain-protected amino acids. At the site of AMPylation side chain-unprotected Ser or Thr were used and coupled using standard chemistry.[5] The amino group on the N-terminal amino acid was protected with Boc, due to the instability of the AMP group in the presence of piperidine.

After synthesizing the peptide sequences, the free hydroxyl side chain of Ser or Thr was AMPylated in a three-step process (Scheme 1); the first step involved the phosphonylation of the peptidyl resin **1** using the H-phosphonate approach and the phosphitylating reagent, bis(diethylamino) chlorophosphine,[6] forming **2**. The H-phosphonate method has been used before to synthesize phosphorylated peptides and it is more convenient than the phosphoramidate method, which requires restrictive conditions.[7] The second step involved the coupling of the protected 2′,3′-isopropylidene adenosine to the H-phosphonate intermediate **2** using PyBOP condensing reagent, forming **3**. According to Wada et al. the phosphonium based activators such as PyBOP do not require the protection of the amino group of the nucleotide, thus saving time and expenses by eliminating several unwanted steps and reagents.[8] The final step involved the oxidation of the H-phosphonate-adenosine **3** with iodine forming the AMPylated peptides **4**. 2′,3′-Isopropylidene adenosine is conveniently deprotected under the acidic conditions used to cleave the peptide from the resin, forming the final product **5**.

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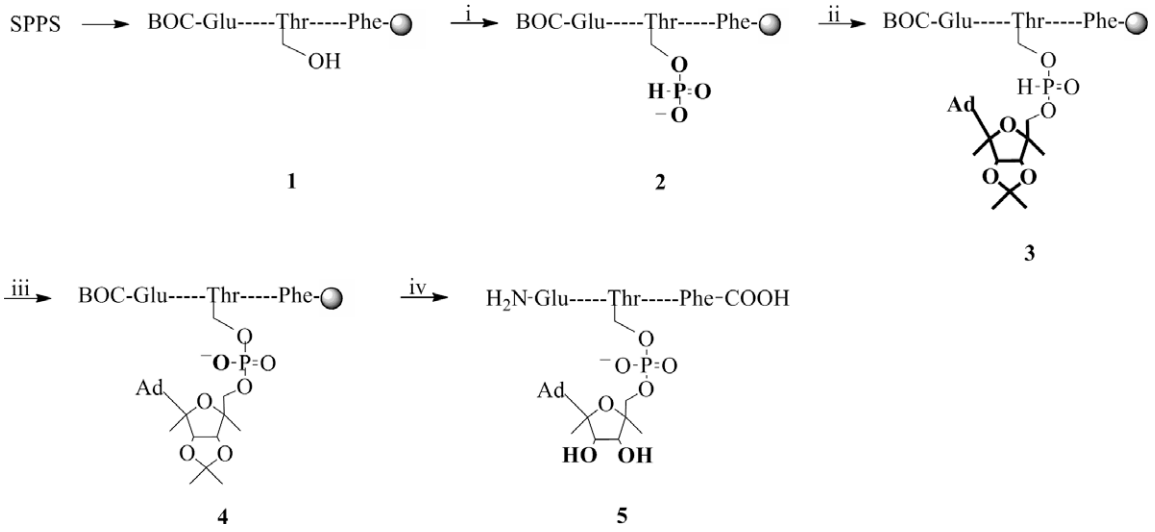
Table 1
Peptide sequences and their molecular weights

Series No.	Peptide sequence	Theoretical MW (Da)	Found MW (Da)
Series 1	NH_2-EYIPT(amp)VF-COOH	1197.20	1197.46
Series 2	NH_2-AAAHAAAS(amp)VHV-COOH	1333.32	1333.48
Series 2	NH_2-AAAHAAAT(amp)VHV-COOH	1347.35	1347.54



Scheme 1. AMPylation of Thr 35 from Rac 31-37 Peptide. Reagents and conditions: (i) pyridine and bis(diethylamino) chlorophosphine in anhydrous 1,4-dioxane; (ii) 2′, 3′-isopropylidene adenosine and PyBOP in pyridine; (iii) I_2 in 98:2 pyridine/water; (iv) acidolytic cleavage.

The time required to couple 2′,3′-isopropylidene adenosine to the peptide was monitored by MS and ranged between 12 and 72 h, and up to 75% yields were obtained.

More work is in progress to find the optimum conditions for synthesizing AMPylated Tyr, as that proved to be difficult using the method described above. It is also possible to couple 2′,3′-isopropylidene adenosine directly to phosphoamino acids [e.g., Tyr(PO_4)] containing peptides. Whilst we were able to obtain some product, sufficient for MS studies,[4] the yields were very low.

In conclusion, we have developed a quick and easy method for the modification of peptides with an AMP molecule making use of the H-phosphonate intermediate, which is more convenient than the phosphoramidate approach. By using the commercially available compound, 2′,3′-isopropylidene adenosine, and the PyBOP activator, we were able to efficiently AMPylate specific Ser/Thr residues in a peptide sequence.

3. Experimental procedure

Peptides were synthesized on an Applied Biosystems 433 automated peptide synthesizer (Foster City, CA), using optimized Fmoc chemistry.[5] All amino acids were purchased from EMD Chemicals (Gibbstown, NJ). All solvents and reagents were purchased from Fisher Scientific (Fair Lawn, NJ), Aldrich (St. Louis, MO), or Alfa Aesar (Ward Hill, MA). Fmoc amino acids were used except for the N-terminal amino acid which was Boc-Glu(tBu) in Series 1 and Boc-Ala in Series 2. Side chain-unprotected Fmoc Ser/Thr was used at the site of AMPylation.

Crude peptides were purified on a Waters 600 HPLC system (Milford, MA) using a Vydac C18 semi-preparative column (250 × 10 mm) at 3 ml/min with a 0–100% *B* gradient in 120 min, where *A* is water/0.045% TFA and *B* is acetonitrile/0.036% TFA. The purified peptides were characterized on a Waters MALDI-MS.

After synthesizing the peptides the Boc-protected peptidyl resin was treated with a fivefold excess of anhydrous pyridine, and a fourfold excess of bis(diethylamino) chlorophosphine in anhydrous 1,4-dioxane, and the mixture was vortexed overnight at room temperature. The resin was then treated with a 10-fold excess of 2′,3′-isopropylidene adenosine, and a 10-fold excess of benzotriazole-1-yl oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) in pyridine. For Series 1 peptides, the second reaction was run for 72 h, and for Series 2 sequences the second reaction was run overnight, both at room temperature. The resin was then washed with pyridine and a 2.5-fold excess of iodine dissolved in 98:2 pyridine/water was added, and the resin was vortexed for 2–3 h.

The progress of each reaction was monitored by cleaving a small amount of the peptide from the resin and analyzing the AMPylated peptide by MALDI-MS. Series 2 peptides were cleaved using the standard cleavage mixture,[5] while Series 1 peptide was cleaved using 5% tri-isopropylsilane in 95% TFA. All AMPylated peptides were then purified by RP-HPLC, and characterized by MS.

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