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Amino Acid Building Blocks for Efficient Fmoc Solid-Phase Synthesis of Peptides Adenylylated at Serine or Threonine

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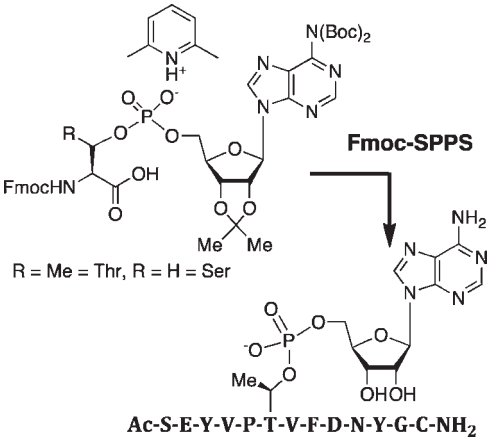
ABSTRACT



The first straightforward building block based (non-interassembly) synthesis of peptides containing adenylylated serine and threonine residues is described. Key features include final global acidolytic protective group removal as well as full compatibility with standard Fmoc solid-phase peptide synthesis (SPPS). The described Thr-AMP SPPS-building block has been employed in the synthesis of the Thr-adenylylated sequence of human GTPase CDC42 (Ac-SEYVP-T(AMP)-VFDNYGC-NH_2). Further, we demonstrate proof-of-concept for the synthesis of an Ser-adenylylated peptide (Ac-GSGA-S(AMP)-AGSGC-NH_2) from the corresponding adenylylated serine building block.

Adenylylation, also known as AMPylation, or adenylation, is a recently rediscovered posttranslational modification, of which much is still unknown.[1] Enzymes that mediate the posttranslational addition of adenosine monophosphate (AMP) on to amino acid side chains of proteins have gained interest as an important regulatory mechanism, especially in a prokaryotic context.[2] Adenosine phosphodiester transferases catalyze the addition of AMP onto amino acid residues, like tyrosine or threonine, thus forming a phosphodiester bond (Figure 1).[1,2] So far, protein adenylylation on a serine residue has not been reported but is expected to occur, in analogy to intracellular phosphorylation and glycosylation.

Recently, it has been discovered that certain pathogenic bacteria are able to influence eukaryotic cell signaling by adenylylating small GTPases on either tyrosine or threonine residues.[3,4] GTPases act as molecular switches when hydrolyzing GTP to GDP, thus regulating signaling at a cellular level.[5] The *Vibrio parahemolyticus* effector protein VopS adenylylates a specific threonine in the Rho (Thr37

(1) In recent literature, "adenylylation" and "adenylation" are both used to describe the addition of adenosine phosphodiesters to proteins. Previously, the terminology "adenylation" was primarily used in the field of RNA chemistry. For a general introduction to protein adenylylation, see: Itzen, A.; Blankenfeldt, W.; Goody, R. S. *Trends Biochem. Sci.* **2011**, *36* (4), 221–228 and references therein.

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(5) For a recent review covering the topic of small GTPases as regulators of vesicular transport, see: Itzen, A.; Goody, R. S. *Sem. Cell Dev. Biol.* **2011**, *22*, 48–56.

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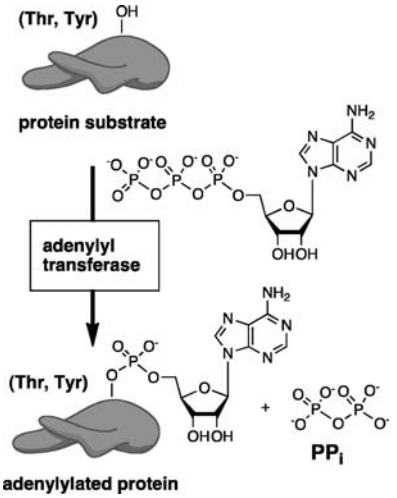

Figure 1. Adenylylation of protein substrate at tyrosine and threonine residues by adenylyl transferase.

in RhoA, Thr35 in Cdc42 and Rac1) subfamily of small GTPases,[3] whereas the protein IbpA of *Histophilus somni* adenylylates a tyrosine residue of the same proteins (Tyr34 in RhoA, Tyr32 in Cdc42 and Rac1).[6] The substrates of the effector protein DrrA/SidM from the human pathogen *Legionella pneumophila* are GTPases from the Rab-subfamily, with the modified tyrosine being in the switch II-region (Tyr77 in Rab1b).[4] VopS and IbpA belong to the FIC family, a protein family with more than 2700 members of sequentially homologous proteins.[7] Although the substrates of VopS and IbpA have been identified, the physiological protein substrates of DrrA and the remaining members of the FIC family proteins are less clear. We hypothesized that identification of physiological substrates of adenylylating proteins (e.g., FIC domains) could be aided by antibodies that specifically recognize adenylylated proteins in eukaryotic cells or cell lysates. Recently, we reported a convenient synthesis of an AMP-tyrosine building block for standard Fmoc solid-phase peptide synthesis, as well as generation of *anti*-adenylyl-Tyr antibodies.[8] Herein, we present the extension from adenylylated tyrosine residues to adenylylated threonine and serine containing peptides by solid-phase peptide synthesis, according to the standard Fmoc-protocol, with preformed stable building blocks. Until now, peptides adenylylated at serine or threonine have only been avaliable through synthesis via the interassembly approach. In the interassembly protocol, the peptide is constructed under SPPS conditions until the amino acid to be adenylylated (Ser, Thr), which is incorporated without side-chain protection, followed by completion of the peptide sequence, phosphitylation on the solid phase, coupling of the adenosine moiety by H-phosphonate activation (Pybop), and subsequent oxidation. The peptide is then removed from the solid support by acidolytic cleavage. The interassembly approach to adenylylated Ser- and Thr-containing peptides carries a number of inherent problems, such as limited applicability to oxidation-sensitive peptide sequences (methionine and tryptophan oxidation), poor overall yield, and low functional group compatibility.[9] Although limited in synthetic scope, the interassembly protocol was recently used to prepare peptides containing adenylylated Thr, which were successfully employed as antigens for raising of antibodies.[10] With our applications in mind, we found this protocol incompatible with our demands on synthesis flexibility, as well as functional group tolerance.

Initially, we investigated serine and threonine building blocks in the form of phosphotriesters masked by β-cyanoethyl protection (CNE) in analogy to caged phosphoamino acid building blocks.[11] In our previous work on tyrosine adenylylation, we successfully employed β-cyanoethyl protection, which was cleaved under the first piperidine treatment after AMP-building block coupling, thus leading to clean conversion into the unprotected phosphodiester.[9] In the case of fully protected adenylylated threonine and serine (**1**, **2**), β-cyanoethyl-protection led to a large amount of β-elimination product under standard coupling conditions (Figure 2, A). In this case, peptide coupling could only be carried out under base-free conditions, which resulted in incomplete couplings, as well as prolonged reaction times, not compatible with efficient parallel peptide synthesis. Therefore, we envisioned a more general strategy, based on unprotected phosphodiester intermediates such as **3** and **4**, as the phosphodiester is a poor leaving group in monoanionic form, it should suppress β-elimination product formation (Figure 2, B).

The general strategy for the synthesis of fully protected adenylylated building blocks of serine and threonine is outlined in Scheme 1. N^{α}-Fmoc-serine (**5**) and N^{α}-Fmoc-threonine (**6**) were converted to corresponding allyl esters (**7**, **8**) by treatment with allyl bromide under standard conditions.[12] Next, N^6,N^6-bis-Boc-2′,3′-isopropylideneadenosine (**9**) was treated with 1-(allyloxy)-1-chloro-*N*,*N*-diisopropylphosphinamine (**10**), under basic conditions (DIPEA) yielding building block **11**.[13,14] We found it essential to purify **11** over a short silica gel column in order to achieve high yields in the subsequent steps. Compound **11** is highly unstable and should be used immediately after

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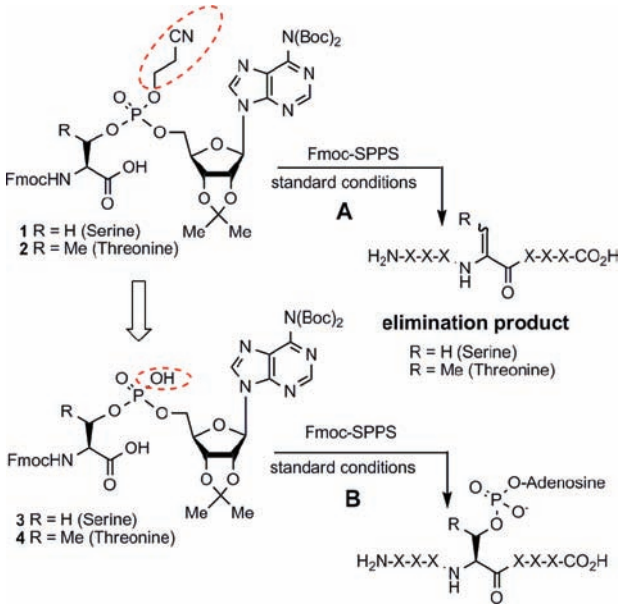

Figure 2. (A) CNE-protected Ser/Thr-adenosine phosphotriesters (**1** and **2**) cause β-elimination upon application to standard Fmoc-SPPS-protocol. (B) Analogous unprotected phosphodiester building blocks (**3**, **4**) are applicable to the standard Fmoc-SPPS protocol.

preparation. Coupling between **11** with amino acid esters **7** and **8** under optimized conditions, employing tetrazole (2 equiv) as activator, followed by in situ oxidation (TBHP, 5 M in decane, 2 equiv) led to the corresponding phosphotriesters **12** and **13** in excellent isolated yields. Attempts to perform the coupling in a reversed manner (phosphoroamidite on amino acid, coupling with **9**) did not result in significant product formation and were not considered further. Column purification resulted in analytically pure allyl phosphotriesters **12** and **13** as 1:1 diastereomeric mixtures at phosphorus. Simultaneous deallylation of the carboxylic acid allyl ester functionality and the allyl phosphotriester was carried out with tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$, 5 mol %) as catalyst, phenylsilane ($PhSiH_3$, 3 equiv) as nucleophile, and 2,6-lutidine (2.5 equiv) as acid scavenger.[15] We found the addition of 2,6-lutidine to be essential for achieving high isolated yields of stable products. Omitting the amine base, or employing a stronger base (DIPEA), resulted in lower yields and inferior purity of isolated products. The resulting crude deallylation reactions were evaporated to dryness, dissolved in minimum amounts of acetonitrile, and applied to reversed-phase (C_{18}) Sep-pak cartridges, subsequently eluted with a water–acetonitrile gradient. Lyophilization of the product-containing fractions resulted in final building blocks **14** and **15** in high yields as mono-2,6-lutidine salts. As a guideline, we typically employed 10 g of Sep-pak C_{18} material per gram of crude deallylation product. The

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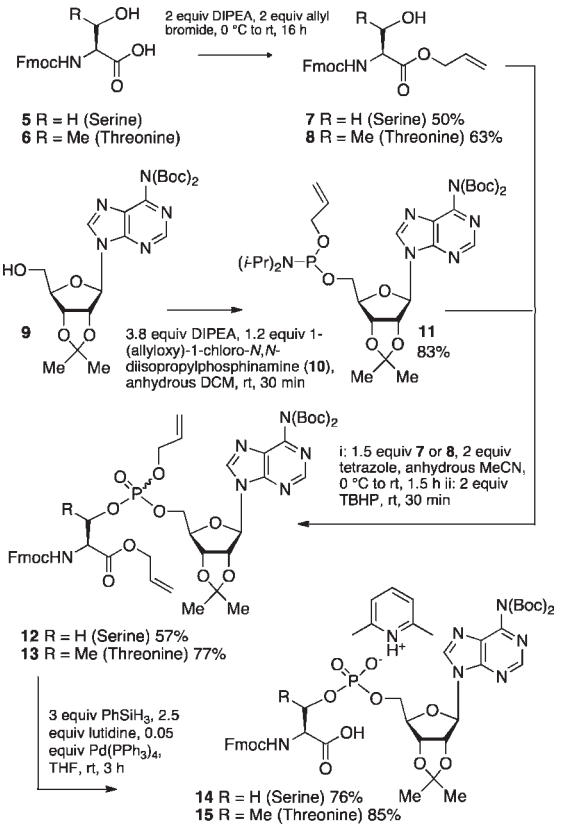
Scheme 1. Synthesis of Protected Adenylylated Amino Acid Building Blocks **14** and **15**



synthesis of **14** and **15** has been carried out on a multigram scale with consistent results.

Next, we evaluated building blocks **14** and **15** in standard solid-phase peptide synthesis according to the Fmoc protocol. We chose to prepare human CDC42 sequence SEYVP-T(AMP)-VFDNYG, which was assembled on Tentagel resin functionalized with a RAM-anchored Fmoc-(Trt)-cysteine amide (**16**, Scheme 2), resulting in Ac-SEYVP-T(AMP)-VFDNYGC-NH_2 (**17**) suitable for immobilization in various applications through the C-terminal cysteine amide.[16] Coupling of the Fmoc-amino acids (10 equiv) was carried out using standard HBTU/HOBt activation on an automated peptide synthesizer, except for the protected adenylylated building blocks **14** and **15** (2.5 equiv), which were coupled manually employing HATU/HOAt as activating reagent, followed by coupling of residual Fmoc-amino acids according to the automated protocol.[17–19] The N-terminal Fmoc-group was removed, and the resulting free N-terminus was capped with an *N*-acetyl group. Subsequent detachment from

(16) Acquired from Rapp Polymere GmbH, www.rapp-polymere.com, accessed October 24, 2011.

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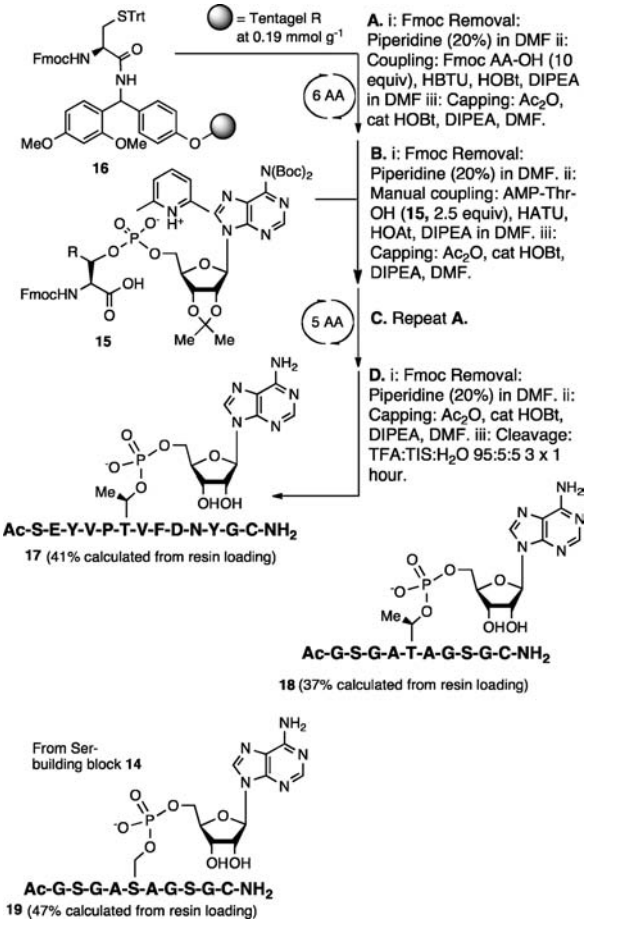
Scheme 2. Solid-Phase Peptide Synthesis of Adenylylated Peptides **17**–**19** According to the Standard Fmoc Protocol



the resin, as well as global deprotection, was carried out by applying a mixture of trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O (90:5:5). After filtering from the resin, an additional 10% H_2O (v/v) was added to the cleavage mixture, which was aged for 30 min to ensure complete hydrolysis of the isopropylidene acetal functionality of the adenosine moiety.[8] Concentration in vacuo at ambient temperature and trituration with diethyl ether yielded the crude peptide. Only traces of depurinated peptide could be detected by LC–ESI–MS. After preparative reversed-phase HPLC purification (C_{18}) and subsequent lyophilization, **17** was isolated in 41% yield (from resin loading). To verify the generality of the methodology, we synthesized one additional peptide sequence carrying adenylylated threonine (Ac-GSGA-T(AMP)-AGSGC-NH_2 (**18**), 37%) as well as a peptide carrying an adenylylated serine in the identical sequence (Ac-GSGA-S(AMP)-AGSGC-NH_2 (**19**), 47%).

In conclusion, we have developed a facile and efficient synthesis of peptides containing adenylylated serine and threonine amino acids, which were introduced by standard Fmoc-based SPPS. The general availability of such building blocks will greatly simplify the synthesis of peptide sequence incorporating adenylylated serine or threonine amino acids, thus extending our previous work on adenylylated tyrosine peptides. The potential biological applications of the complete toolbox of adenylylated peptides are numerous, such as standards for proteomics analysis, antigens for immunization, as well as pull-down probes. A number of serine, threonine, and tyrosine peptides have been synthesized in our laboratory, which are currently under investigation and will be reported in due course.

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Supporting Information Available. Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.