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Fmoc-based solid-phase synthesis of adenylylated peptides using diester-type adenylylated amino acid derivatives

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ABSTRACT

Phosphodiester-type adenylylated (AMPylated) Ser, Thr, and Tyr derivatives were developed for Fmoc solid phase peptide synthesis of AMPylated peptides. One-pot/sequential reaction consisting of condensation of an N-nonprotected adenosine derivative and Fmoc-Ser/Thr/Tyr-OAllyl using allyl-*N*,*N*-diisopropylchlorophosphoramidite and subsequent oxidation with *m*-chloroperbenzoic acid gave phosphotriester-type AMPylated Ser/Thr/Tyr derivatives. After Pd(0)-mediated deprotection of allyl groups, the resulting phosphodiester-type AMPylated Ser/Thr/Tyr derivatives were successfully incorporated into peptides by standard Fmoc solid phase peptide synthesis without significant side reactions including dehydroalanine formation.

Introduction

Nucleotidylation of the hydroxyl functionality of proteins has been recognized as an important posttranslational modification. Among such nucleotidylations, uridinylylation or guanylylation of viral proteins found in RNA viruses has been reported to be involved in the initiation of genome replication.[1] Very recently, Fic (filamentation induced by cyclic AMP) domain proteins from two pathogenic bacteria have been reported to use ATP to catalyze the addition of adenosine monophosphate (AMP) to a tyrosine or threonine residue of Rho-family GTPases RhoA, Rac1 and Cdc42, leading to modulation of downstream signaling events (Fig. 1).[2,3]

Such AMP addition or adenylylation is referred to as 'AMPylation' and is predicted to be functionally similar to other posttranslational modifications including phosphorylation and acetylation with respect to transient modification of protein functions. The presence of a Fic protein in higher eukaryotes, including humans, and AMPylation catalyzed by a non-Fic protein (*Legionella* protein DrrA) have also been reported.[4] However, the physiological roles of AMPylation as a newly emerging posttranslational modification have remained elusive. We have therefore studied the synthesis of AMPylated peptides to provide AMPylated materials for uncovering the biological significance of AMPylation events.

Recently, solid-phase synthesis of AMPylated Ser/Thr peptides was achieved using an H-phosphonate approach involving phosphonylation of the hydroxyl group on a Ser/Thr residue followed by coupling of a protected adenosine derivative and subsequent oxidation of the resulting H-phosphonate-adenosine.[5] However, application of this protocol to AMPylated Tyr peptides has yet to be achieved. Synthetic approaches using nucleotidylated Fmoc amino acid building blocks have been applied to the preparation of uridinylylated, guanylylated, and AMPylated Tyr and uridinylylated Ser peptides using nucleotidylated phosphotriester-type amino acid derivatives.[6] Very recently, Hedberg and co-workers reported the synthesis of AMPylated Tyr[7] and Ser/Thr peptides[8] using phosphotriester- and phosphodiester-type protected AMPylated amino acids, respectively. The logical extension of the triester-type compounds used in AMPylated Tyr peptide synthesis to the Fmoc-based synthesis of AMPylated Ser/Thr peptides initially

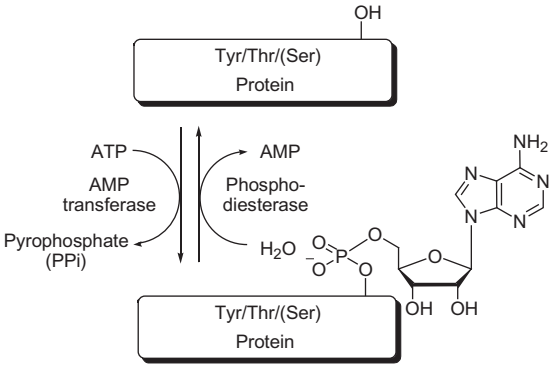

Figure 1. Adenosine monophosphorylation (AMPylation) of proteins.

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failed, and gave β-elimination products; this led to the use of diester-type compounds. These unproductive studies using phosphotriester-type compounds have been well documented in the synthesis of phosphopeptides by Fmoc protocols. Phosphodiester-type amino acid derivatives have therefore frequently been used in Fmoc solid-phase peptide synthesis (Fmoc SPPS), and have been very successful in the synthesis of phosphopeptides (Fig. 2, **1**–**3**).[9] In particular, phosphoserine- and phosphothreonine-containing peptides have been successfully synthesized using monobenzyl-protected phoshoamino acids as diester-type derivatives.

On the basis of this knowledge, we planned to synthesize three types of AMPylated peptides using diester-type AMPylated Ser/ Thr/ Tyr derivatives (**4**, **5**, and **6**) by Fmoc methodology (Fig. 3).

Results and Discussion

Initially, we attempted the synthesis of the desired phosphodiester-type compounds using temporary methyl protection on the phosphotriester unit. In our previous synthetic work on phosphopeptides, we realized that one methyl group on the phosphotriester unit can be easily removed under acidic conditions.[10] However, final deprotection of the methyl group on an AMPylated Ser derivative did not proceed efficiently. We attempted to use temporary allyl protection, which can be removed using Pd(0) reagents. The synthetic routes for Fmoc-diester-type AMPylated amino acids are shown in Scheme 1. In our protocol, 2′,3′-*O*-isopropylideneadenosine **7** was reacted with a slight excess of allyl-*N*,*N*-diisopropylchloro-phosphoramidite[11] **8** (1.1 eqiv) in CH_2Cl_2 in the presence of triethylamine at room temperature for 2 h to yield ally-(2′3′-*O*-isopropylideneadenosin-5′-yl)phosphoramidite **9**. Without purification of the resulting phosphoramidite reagent, an equimolar mixture of Fmoc-Ser-OAllyl **10** and 1*H*-tetrazole was added to the reaction mixture with additional stirring at room temperature for 2 h. Then, oxidation with *m*-chloroperbenzoic acid (*m*CPBA) was performed to give crude fully protected AMPylated Ser derivative **12**. Column chromatographic purification of the crude materials on silica-gel afforded pure triester-type AMPylated Ser derivative **12** as a diastereomeric mixture in 44% isolated yield based on starting adenosine derivative **7**. Without requiring an intermediate purification step, two-step condensation, and subsequent oxidation proceeded efficiently to yield the desired materials.

In previous papers, 6,6-*N*,*N*-diBoc or 6-*N*-benzoyl group was used for N-protection in the nucleobase part.[6c,7,8] However, we doubted the necessity of protecting the 6-amino group on adenine during Fmoc-based SPPS using nucleophilic piperidine,[12] that is, partial loss of the acyl-type protection was anticipated. Furthermore, the accidentally generated 6-amino group is expected to show low nucleophilicity and not to be involved in the acylation reactions commonly used in peptide chain elongation steps. In our synthesis of AMPylated amino acids, the N-non-protected adenosine derivative **7**, which is easily obtainable from major

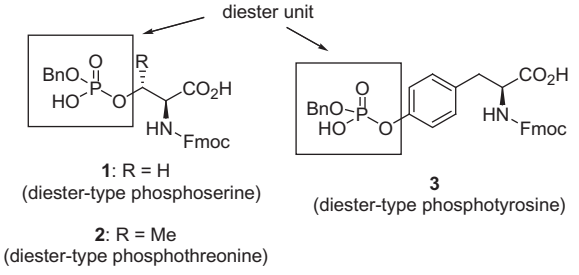


Figure 2. Phosphodiester-type amino acids compatible with Fmoc SPPS.

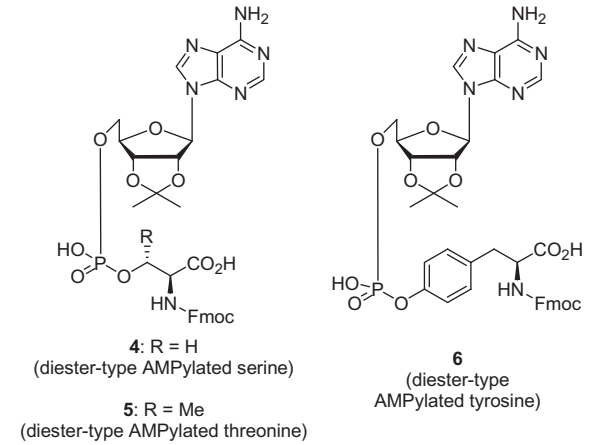
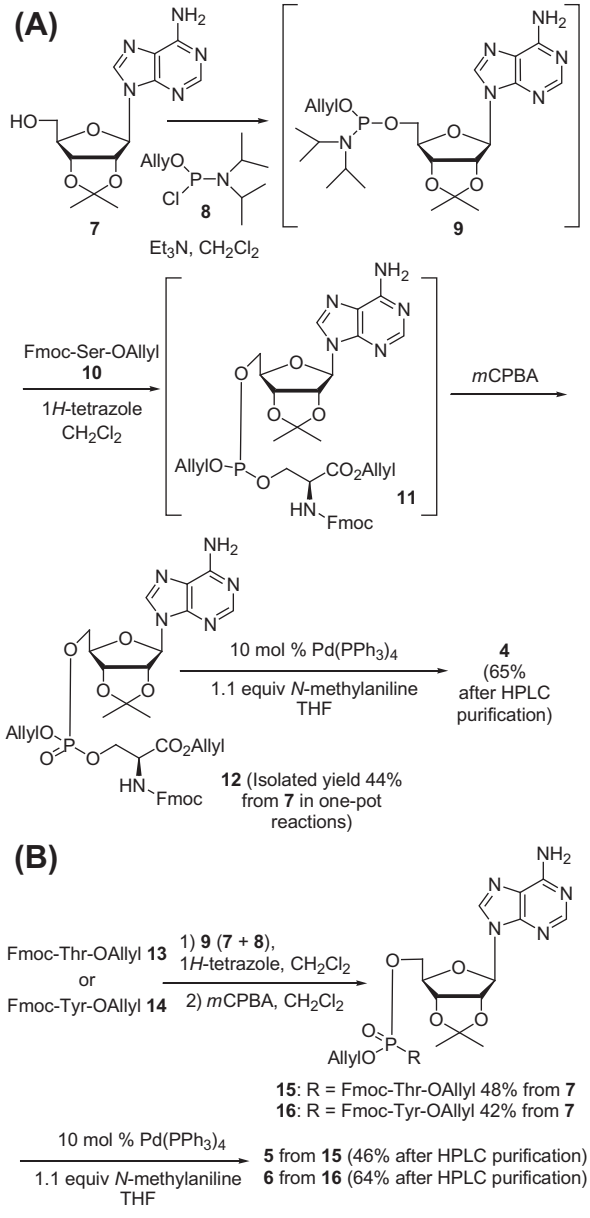
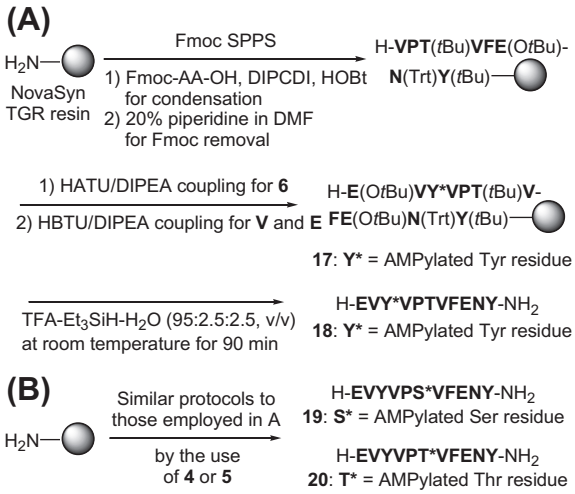


Figure 3. Diester-type AMPylated amino acid derivatives synthesized in this work.



Scheme 1. Synthetic routes to AMPylated amino acid derivatives. (A) AMPylated serine **4**; (B) AMPylated threonine **5** and tyrosine **6**.



Scheme 2. Outline for the Fmoc SPPS for AMPylated peptides using derivatives **4**, **5**, and **6**.

commercial suppliers, was therefore used. The steps in the synthesis of 6-N-non-protected derivatives (**4**, **5**, and **6**) were not accompanied by significant side reactions.

Next, the resulting phosphotriester derivative **12** was subjected to Pd(0)-mediated deprotection of the two allyl groups in the presence of *N*-methylaniline in THF. After reaction for 2 h at room temperature, addition of EtOAc to the reaction mixture gave a yellow soild. After sequential washing of the resulting solid with EtOAc and MeOH, the desired diester-type AMPylated Ser derivative **4**, which is adequate for subsequent synthetic purpose, was obtained in nearly quantitative yield. For analytical purposes, reversed-phase HPLC purification gave purified **4** in 65% isolated yield.

Similar one-pot synthetic protocols were applied to Fmoc-Thr-OAllyl **13** and Fmoc-Tyr-OAllyl **14** to yield the corresponding fully protected triester-type AMPylated Thr and Tyr derivatives (**15** and **16**), respectively. Deprotection of phophotriesters **15** and **16** with Pd(0) in the presence of *N*-methylaniline, followed by precipitation with EtOAc and subsequent washing steps, afforded the Fmoc-SPPS compatible AMPylated amino acid derivatives **5** and **6**, respectively. Reversed-phase HPLC purifications of the crude materials gave pure samples in 46% (**5**) and 64% (**6**) isolated yields (Scheme 1-B).

Having obtained the requisite AMPylated amino acid derivatives **4**, **5**, and **6**, we then tried to incorporate these derivatives into model peptide sequences. The AMPylated Tyr derivative **6** was first installed in a model peptide sequence (H-**EVY***VPTVFENY-NH_2: the asterisk indicates the AMPylated residue) found in RhoA GTPase. Fmoc-SPPS was conducted on NovaSyn TGR resin® (amine content: 0.25 mmol/g) as shown in Scheme 2. Before incorporation of the AMPylated derivatives, peptide chain elongation was performed by coupling of the Fmoc amino acid (5.0 equiv) using diisopropylcarbodiimide (DIPCDI) (5.0 equiv) and 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) (5.0 equiv) in DMF, and removal of the Fmoc group with 20% piperidine in DMF. Incorporation of **6** (2.0 equiv) was achieved using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) (1.9 equiv) in the presence of diisopropylethylamine (DIPEA) (4.0 equiv) with the aim of achieving high coupling efficiency.[13,14] The following couplings were performed using a combination of Fmoc amino acid (5.0 equiv), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (4.9 equiv), and DIPEA (5.0 equiv) in DMF.[14] Final deprotection of the completed resin **17** with TFA–Et_3SiH–H_2O (95:2.5:2.5, v/v) at room temperature for 90 min gave the crude deprotected peptide **18**. Analysis of the crude materials by HPLC indicated that Fmoc SPPS of AMPylated Tyr peptide using **6** proceeded successfully without significant side reactions attributable to non-protection of the adenine moiety (Fig. 4-A). HPLC purification of crude peptides yielded pure peptide in 26% isolated yield based on the starting resin.

Synthetic protocols similar to those used for synthesis of the AMPylated Tyr peptide **18** were applied to the syntheses of model peptides **19** and **20** (H-**EVYVPX***VFENY-NH_2: **19**: X* = AMPlyated Ser; **20**: X* = AMPylated Thr) (Scheme 2-B). Fmoc SPPS followed by acidic deprotection gave the crude desired peptides **19** and **20** with reasonable purities (Fig. 4-B and C: 17 and 21% yields after HPLC purification, respectively). Formation of a dehydroalanine (ΔAla) residue from a diester-type uridinylylated Ser residue was reported by van der Marel and co-workers.[6a]

In this work, the *N*-Fmoc-*O*-*o*-chlorophenyl-phosphotriester-type amino acid **21** was incorporated on the N-terminal position of a model peptide using a standard coupling method under slightly acidic conditions to prevent β-elimination (Fig. 5).

The resulting resin was deprotected with a sequence of reagent systems: (i) tetrabutylammonium fluoride (TBAF)–pyridine–DMF–H_2O (for removal of the *o*-chlorophenyl group on the phosphate); (ii) 20% piperidine in DMF; (iii) TFA–triisopropylsilane–H_2O–

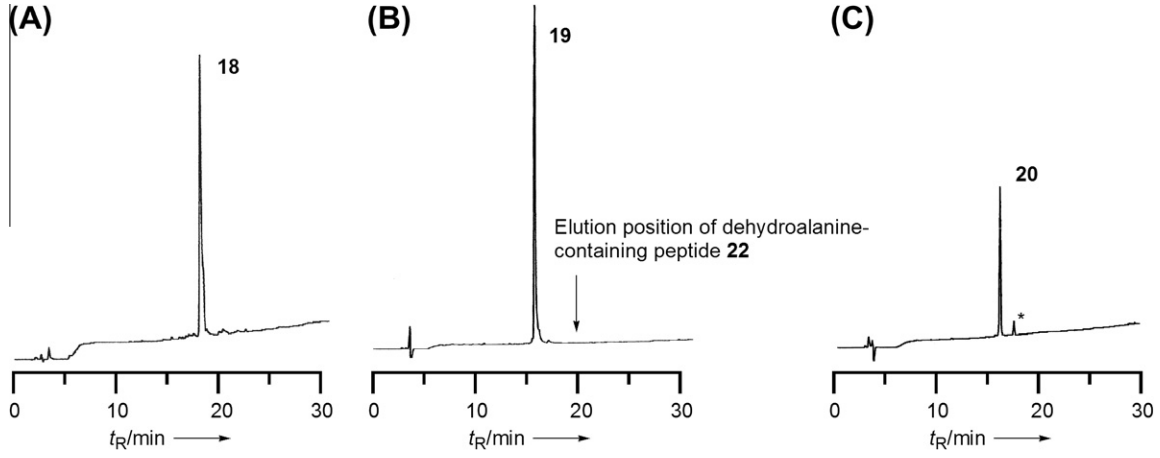


Figure 4. HPLC analyses of crude AMPylated amino acid-containing peptides. (A) AMPylated Tyr peptide **18**; (B) AMPylated Ser peptide **19**; (C) AMPylated Thr peptide **20**. *N-terminal Glu-deleted peptide. HPLC conditions: Cosmosil 5C18 AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA–MeCN 0.1% TFA aq (10:90–60:40 over 30 min) at a flow rate 1.0 mL/min, detection at 220 nm.

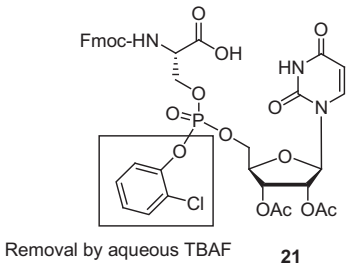
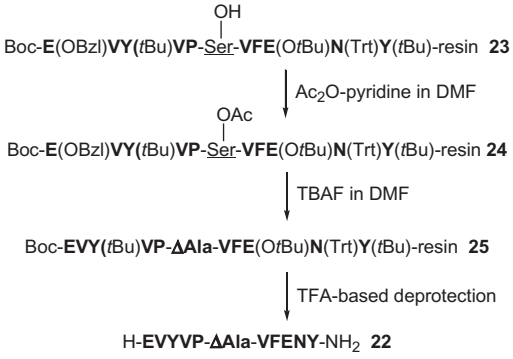


Figure 5. Uridinylylated Ser derivative **21** used in uridinylylated peptide.[6a]



Scheme 3. Synthetic route for dehydroalanine peptide **22**.

PhOH–1,2-ethanedithiol (EDT); and (iv) concentrated ammonia in 1,4-dioxane (for removal of the acetyl group of 2′,3′-*O*-acetyluridine). The major product obtained from the attempted four-step deprotection was a dehydroalanine (ΔAla)-containing peptide. In contrast, subjection of the same model resin to three-step deprotection protocols consisting of (i) the aqueous TBAF system, (ii) the TFA system, and (iii) the concentrated NH_3 system yielded the desired uridinylylated Ser peptide without the formation of ΔAla-peptide. On the basis of these experimental results, the incompatibility of the serine phosphodiester function with the piperidine treatment required for Fmoc removal was addressed. Similar to our approach, Hedberg and co-workers, who used phosphodiester-type AMP amino acids, did not mention the contamination of the dehydroalanine unit in their final synthetic peptide although they paid attention to β-elimination during the coupling step under basic conditions.[8] We therefore tried to prepare a reference peptide **22** (H-**EVYVP-ΔAla-VFENY**-NH_2) and to check its contamination in the AMPylated Ser materials (Scheme 3). On Novasyn TGR resin®, we constructed protected peptide chain **23** using standard Fmoc amino acids, except for Fmoc-Ser-OH and Boc-Glu(OBzl)-OH for the dehydroalanine and N-terminal positions, respectively. The hydroxyl group of the Ser residue in the protected resin (Boc-**E**(OBzl)**VY**(*t*Bu)**VP**-Ser-**VFE**(O*t*Bu)**N**(Trt)**Y**(*t*Bu)-resin **23**) was acetylated using excess acetic anhydride and pyridine in DMF.

The resulting acetylated resin **24** was treated with TBAF (1 M in toluene, 100 equiv) in DMF,[15] giving β-elimination of the acetylated Ser residue and removal of the benzyl group on the Glu residue. Exposure of the treated resin **25** to TFA–Et_3SiH–H_2O (95:2.5:2.5, v/v) at room temperature for 90 min, followed by HPLC purification, afforded the reference ΔAla-containing peptide **22**. HPLC analysis of the crude AMPylated Ser materials showed no contamination by **22** (Fig. 4-B). This observation clearly indicated that diester-type AMPylated Ser and Thr derivatives (**4** and **5**) are useful building blocks for Fmoc-based synthesis of AMPylated Ser and Thr peptides. At this stage, we do not know the possible reason for the different behaviors of uridinylylated and AMPylated peptides with respect to piperidine treatment.

In conclusion, we achieved the Fmoc-based SPPS of AMPylated Ser, Thr, and Tyr peptides using phosphodiester-type amino acid derivatives **4**, **5**, and **6**. In our synthetic schemes for phosphotriester-type intermediates **12**, **15**, and **16**, one-pot/sequential condensations using a phosphoramidite reagent are possible. A robust check of contamination by dehydroalanine-containing byproducts in the crude AMPylated Ser materials using reference peptides clearly showed that the diester-type AMPylated derivatives serve as building blocks of great value in the preparation of AMPylated Ser and Thr peptides. These synthetic protocols will help in future research on AMPylation of proteins.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.04.063.

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