Post-Translational Modification

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Efficient Synthesis and Applications of Peptides containing Adenylylated Tyrosine Residues

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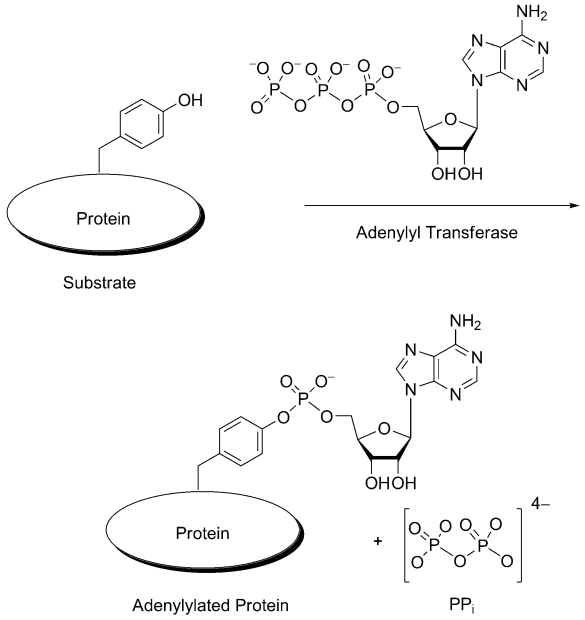
Post-translational modification (PTM) is a versatile strategy to influence the biological activity of proteins and enzymes. One fascinating example of the control of enzymatic activity is the regulation of bacterial glutamine synthetase. Adenylylation, which consists of the covalent transfer of an adenosine monophosphate (AMP) group from an adenosine triphosphate (ATP) precursor to the hydroxy function of a specific tyrosine residue of glutamine synthetase, renders the enzyme less active (Scheme 1).[1a] Recently, it was discovered that certain pathogenic bacteria are able to influence the signaling of eukaryotic cells by adenylylating small GTPases at either tyrosine or threonine residues.[1b,2,3] Small GTPases act as molecular switches and are active or inactive when bound to GTP or GDP, respectively.[4] The *Vibrio parahaemolyticus* effector protein VopS adenylylates a specific threonine residue in the Rho subfamily (Thr37 in RhoA, Thr35 in Cdc42 and Rac1) of small GTPases.[2] The substrates of the effector protein DrrA/SidM from the human pathogen *Legionella pneumophila* are GTPases from the Rab subfamily, with the modified tyrosine residue being in the switch 2 region (Tyr77 in Rab1b).[3] VopS belongs to the Fic family of adenylyltransferases, which contains more than 2700 members of sequence-homologous proteins.[5] Although the substrate of VopS has 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. The generation of a specific anti-tyrosine-AMP antibody has been hampered by the lack of efficient synthesis methods, which has not allowed the preparation of pure multimilligram necessary quantities of a peptide antigen bearing an adenylylated tyrosine for immunization.[6,7,8a]



Scheme 1. Adenylylation of proteins at tyrosine residues.

We report herein a readily applicable and general procedure for the synthesis of peptides carrying adenylylated tyrosine residues by using a standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis. We envisioned a Tyr-AMP building-block approach that relies fully on the global removal of protective groups under acid conditions, thereby allowing its direct and uncomplicated application in the Fmoc solid-phase synthesis of peptides.

As shown by a recent example in the literature, 2′,3′-bis(ester)-protected adenosine moieties are prone to depurination under acidic conditions (Scheme 2 A).[8b] We hypothesized that the depurination reaction of the commonly used 2′,3′-bis(ester)-protected adenosine is induced by nucleophilic participation of the 2′-ester group. To circumvent the decomposition under acidic conditions, the 2′,3′-bis(ester) protecting groups were replaced with a 2′,3′-isopropylidene acetal, and the deactivating N^6,N^6-bis(Boc) protecting groups were introduced at the adenosine moiety to further stabilize the system (Scheme 2 B).[9] We employed *O*-cyanoethyl (CNE) as the protecting group for the phosphodiester, thus allowing for instant deprotection during the removal of the first Fmoc groups. This resulted in stabilization of the phosphodiester linkage in a monoanionic form and generation of the amino acid building block **1** (Scheme 2 C). The phosphodiester moiety of **1** was cleaved by either direction D or E, thereby creating two different sets of phosphoramidite precursors (**3** and **5**), together with their coupling partners (**2** and **4**).

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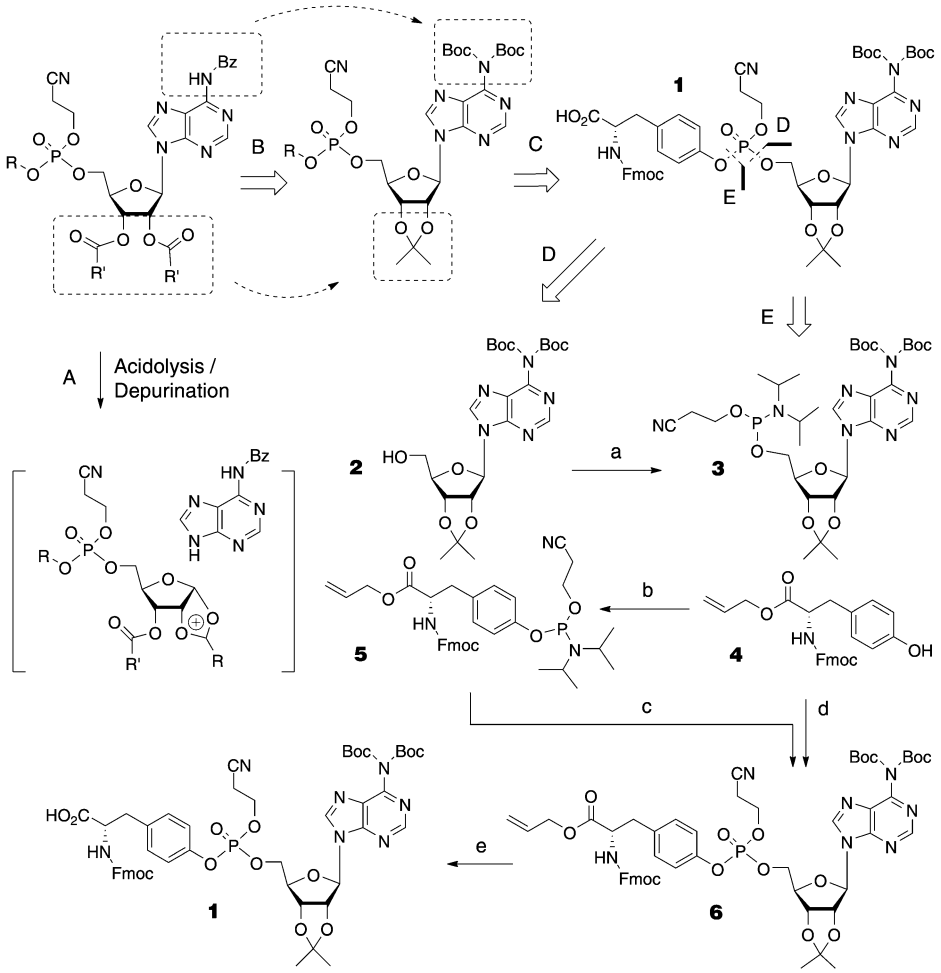
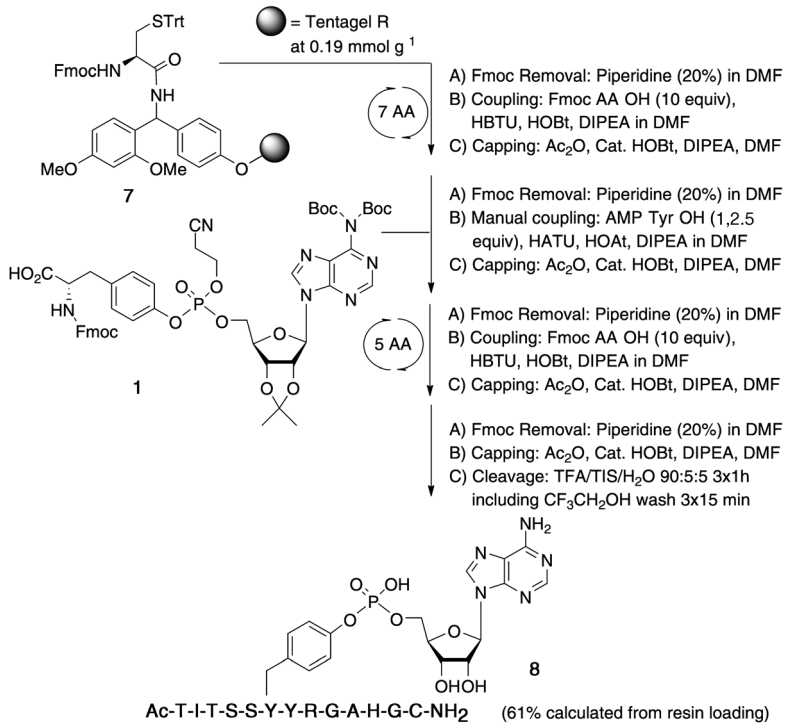


Scheme 2. A) Participation of the 2′-ester leads to depurination upon peptide cleavage under acidic conditions. B) A switch in the protecting-group strategy allows for cleavage. C) Transformation into the final building block. D), E) Alternative disconnection sites for the preparation of the CNE-protected phosphadiester lead to two different routes. Synthesis of the protected Tyr-AMP building block. Reagents and conditions: a) $(i\text{Pr})_2\text{NP(Cl)O(CH}_2)_2\text{CN}$ (1.2 equiv), CH_2Cl_2, DIPEA (3 equiv) 30 min, 0 °C→RT, quant. b) $(i\text{Pr})_2\text{NP(Cl)O(CH}_2)_2\text{CN}$ (1.2 equiv), CH_2Cl_2, DIPEA (3 equiv) 30 min, 0 °C→RT, quant. c) 1. Tetrazole (3 equiv), acetonitrile, 0 °C→RT 3 h; 2. TBHP (2.8 M in $(CH_2Cl)_2$, 1.3 equiv), 45 %. d) 1. tetrazole (3 equiv), acetonitrile, 0 °C→RT, 3 h; 2. TBHP (2.8 M in $(CH_2Cl)_2$, 1.3 equiv), 56 %. Optimized protocol: d) 1. tetrazole (1.2 equiv), diisopropylammonium tetrazolide (2.2 equiv), acetonitrile, 0 °C→RT, 3 h; 2. TBHP (5 M in decane, 1.3 equiv) 76 %. e) $[Pd(PPh_3)_4]$ (5 mol %), THF, $PhSiH_3$ (1.5 equiv), RT, 5 h, 95 %. Boc = *tert*-butoxycarbonyl, Bz = benzoyl, DIPEA = *N*,*N*-diisopropylethylamine.

The synthesis commenced with an investigation of the coupling efficiency of the two phosphoramidites, where **5** was prepared from Fmoc-L-tyrosine allyl ester (**4**),[10] as well as the conversion of N^6N^6-bis(Boc)-2′,3′-isopropylideneadenosine **2**[9] into the corresponding phosphoramidite building block **3**.[11] Diisopropylaminocyanoethyl phosphochloridate (1.2 equiv) together with excess DIPEA (3 equiv) in anhydrous dichloromethane were used for preparation of building blocks **3** and **5** (Scheme 2, steps a and b). Coupling of stoichiometric amounts of tyrosine phosphoramidite **3** with adenosine derivative **2** in acetonitrile by employing an excess of anhydrous tetrazole (2 equiv) as the promoter, followed by oxidation with anhydrous *tert*-butylhydroperoxide (TBHP) in 1,2-dichloroethane (2.8 M, 1.3 equiv) at 0 °C to ambient temperature over two hours, resulted in phosphodiester **6** in 45 % yield (Scheme 2, step c; 1 mmol scale) after chromatographic purification. Next, we investigated the coupling between Fmoc-tyrosine allyl ester **4** and adenosine-derived phosphoramidite **3** under identical conditions and we retrieved 56 % of phosphodiester **6** after chromatographic purification (Scheme 2, step d). Further optimization of the protocol according to route d, by employing a mixture of tetrazole/diisopropylammonium tetrazolide as the activator, resulted in a yield of 76 % of **6** on a 10 mmol scale (protocol 3, see the Supporting Information). Deallylation of **6** with $PhSiH_3$ and $[Pd(PPh_3)_4]$[12] gave the desired amino acid building block **1** in 95 % yield as a white amorphous solid after purification and lyophilization on a C_{18} Sep-Pak cartridge.

The Rab1 (AMP) antigen was synthesized on a tentagel resin[13] functionalized with a RAM-anchored Fmoc-(Trt)-cysteine amide (**7**, Scheme 3; RAM = Rink amide resin, Trt = triphenylmethyl). Coupling of the Fmoc-protected amino acids (10 equivalents) was carried out using standard HBTU/HOBt activation[14] on an automated peptide synthesizer,[15] except for the protected adenylylated Fmoc-protected tyrosine building block **1** (2.5 equiv). In this case, **1** was coupled manually by employing HATU/HOAt as the activating reagent,[16] followed by coupling of residual Fmoc-protected amino acids according to the automated protocol. The N-terminal Fmoc group was replaced with an *N*-acetyl group and subsequently detached from the resin, as well as globally deprotected by applying a mixture of trifluoroacetic acid/triisopropylsilane/water (TFA/TIPS/H_2O 90:5:5).[17] Concentration of the solution in vacuo at ambient temperature and subsequent trituration with diethyl ether yielded the crude peptide. At this stage, only traces (< 3 %) of depurinated peptide could be detected by ESIMS, but was not visible by ^{31}P NMR spectroscopy. After purification by reverse-phase HPLC (C_{18}) and lyophilization, **8** was isolated in 61 % yield. To verify the generality of the method, we synthesized two additional peptide sequences with adenylylated tyrosine



Scheme 3. Solid-phase synthesis of Rab1 Y-77 (adenylyl)-peptide **8**. AA = amino acid, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = 7-aza-1-hydroxybenzotriazole, TIS = triisopropylsilane.

residues (Ac-GSGA-Y*(AMP)-AGSGC-NH_2 (**S4**, 63%) and EVYRGAE-Y*(AMP)-AVDG (**S5**, 59%), both on a 0.1 mmol scale; see the Supporting Information).

The Rab1-antigen peptide **8** was conjugated to Keyhole Limpet Hemocyanin (KLH) carrier protein through MBS coupling (MBS = *N*-hydroxysuccinimidyl-3-maleimidobenzoate) and injected in two rabbits by employing Freund's complete adjuvant.[18] Five booster immunizations were carried out over 8 weeks, and final bleeding, serum isolation, selection over antigen peptide **8** immobilized on sepharose, and depletion over the non-adenylylated Rab1 sequence Ac-TITSSYYRGAHGC-NH_2 (**S3**, see the Supporting Information) resulted in approximately 6 mg of monoselective polyclonal IgG from each animal. For immunosorbent assay analysis, Rab1-antigen peptide **8** was conjugated to bovine serum albumin (BSA) through an MBS linkage, and purified by ultrafiltration (30 kDa cut-off; see the Supporting Information), which resulted in BSA-**8**. Comparison of antisera from each animal by immunosorbent assay over BSA-**8** showed no major differences in the antibody titer (see the Supporting Information). In a Western blot analysis, Rab1b-AMP could be clearly discriminated from wild-type (wt) Rab1b by the derived antibody (Figure 1 A). The level of binding of the α-Tyr-AMP antibody to Rab1b-AMP is approximately 20-fold higher than for unmodified Rab1b. This finding suggests a major contribution of the AMP moiety to the antibody binding and only a weak recognition of the peptide backbone that was part of the immunization. Under these experimental conditions, as little as 10 ng Rab1b-AMP could be detected with the α-Tyr-AMP antibody. The experiments were repeated with different adenylylated proteins to investigate the specificity of the derived α-Tyr-AMP antibody. In addition to Rab1b-AMP and BSA-**8**, the adenylylated

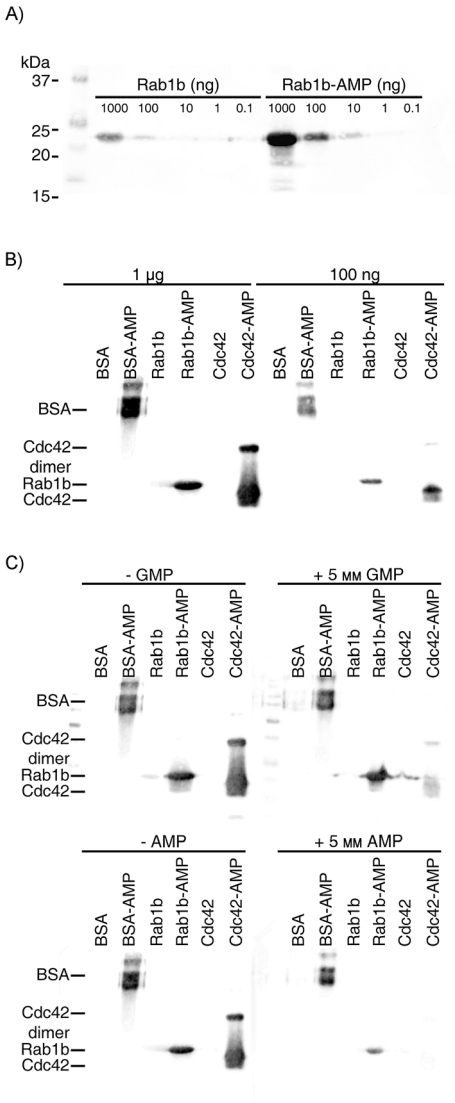


Figure 1. Affinity and specificity of the derived α-Tyr-AMP antibody. A) Western blot analysis with the indicated amounts of highly purified Rab1b and Rab1b-AMP by using the α-Tyr-AMP-antibody (1:100 dilutions), thus demonstrating the specific recognition of the Tyr-AMP modification. B) Western-blot analysis of highly purified adenylylated and unmodified forms of BSA, Rab1b, and Cdc42 (1 or 0.1 µg, protein sample per lane). The α-Tyr-AMP antibody (1:100 dilution) strongly binds to all the tested adenylylated proteins, thus indicating additional binding activity for Thr-AMP (Cdc42). C) Competition of the α-Tyr-AMP antibody in the presence of GMP or AMP. Samples (0.1 µg each) were carried out as indicated in (B). The Western blots were incubated with the α-Tyr-AMP antibody in the presence and absence of 5 mM GMP or AMP. AMP, but not GMP, competes moderately with antibody binding to Rab1b-AMP and BSA-AMP. Both AMP and GMP compete with the antibody for Cdc42-AMP detection. In all Western blots, IRDye800-conjugated donkey anti-rabbit IgG was used as a secondary antibody.

Cdc42 (Cdc42-AMP) that had been preparatively adenylylated using VopS was investigated. VopS has been reported to adenylylate Cdc42 specifically on Thr35,[2] and can thus serve as a control as to whether the α-Tyr-AMP antibody can discriminate between adenylylated threonine and tyrosine residues (Figure 1B). Apparently, the α-Tyr-AMP antibody recognizes adenylylated BSA-**8** and Rab1b-AMP specifically over the unmodified proteins. However, the antibody also recognizes Cdc42-AMP, thus indicating that it can also detect adenylylated threonine residues. This again suggests a major contribution of the AMP group to antibody binding, with only a small influence by the modified amino acid side chain and the peptide backbone.

To evaluate the binding specificity of the generated α-Tyr-AMP antibody further, we have performed nucleotide competition experiments. The binding of the α-Tyr-AMP to **8**-BSA (wt-BSA as control), AMP-Rab1b (wt-Rab1b as control), and AMP-Cdc42 (wt-Cdc42 as control) was investigated in the presence and absence of either GMP or AMP (Figure 1C). In the presence of GMP, no impairment of the binding of the α-Tyr-AMP antibody to BSA-**8** or Rab1b-AMP could be detected. However, the signal corresponding to the binding of the α-Tyr-AMP antibody decreased significantly on incubating the samples with AMP, thus demonstrating the relevance of the adenine base of the AMP moiety for interaction with the antibody. Intriguingly, both GMP and AMP competed with the binding of the α-Tyr-AMP antibody to Cdc42-AMP, although the competition with AMP appeared to be slightly more effective than with GMP. This observation could possibly hint at a strong recognition of the furanoside residue and the phosphate group of GMP/AMP, thereby leading to a substantial degree of competition between AMP and GMP in the absence of the Rab1b peptide sequence.

To further investigate the applicability of the Rab1 antibody we performed pull-down experiments of adenylylated proteins from cell lysates. For this purpose we exogenously added adenylylated Rab1 and Cdc42 to mammalian (simian) Cos7 cell lysates. We were able to preferably pull down adenylylated Rab1, thus indicating a specificity of the α-Tyr-AMP antibody for Rab1-AMP binding even in the presence of the competitive environment of the cell lysate (Figure 2).

The generated tyrosine-AMP-specific antibody will help identify the physiological substrates of proteins containing an FIC domain, for example, BepA[19] well as DrrA.[3] In addition, the peptide-derived tools could be employed to establish whether adenylylation of proteins is a more general, currently underappreciated, process in eukaryotic cells in the absence of pathogenic adenylylating proteins.

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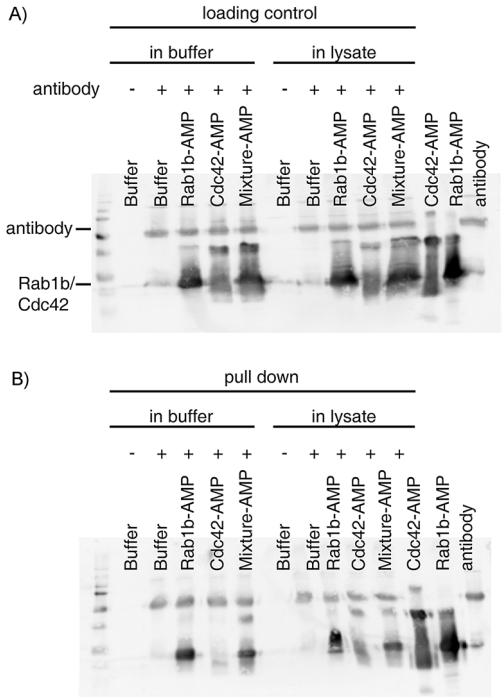

Figure 2. Pull down of adenylylated Rab1/Cdc42 from mammalian cell lysates. Preparative adenylylated Rab1 and Cdc42 (0.1 μg each) were exogenously added to buffer or 100 μg Cos7 cell lysate. A biotinylated α-Tyr-Rab1-AMP antibody was immobilized on magnetic streptavidin beads and used to pull down the Rab1-AMP and Cdc42-AMP samples. A) Loading control containing sample mixtures before pull down. B) Pull-down experiment of (A). In all Western blots, IRDye800-conjugated donkey anti-rabbit IgG was used as a secondary antibody.

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