A General Approach Towards Triazole-linked Adenosine Diphosphate Ribosylated Peptides and Proteins

Qiang Liu[a], Hans A. V. Kistemaker[a]#, Sagar Bhogaraju[b], Ivan Dikic[b], Herman S. Overkleeft[a], Gijsbert A. van der Marel[a], Huib Ovaa[c], Gerbrand J. van der Heden van Noort*[c] and Dmitri V. Filippov*[a]

Abstract: Current chemistries to prepare Adenosine Diphosphate ribosylated (ADPr) peptides are not generally applicable due to the labile nature of this post-translational modification and its incompatibility with strong acidic conditions used in standard solid phase peptide synthesis protocols. We present a general strategy to prepare ADPr peptide analogues based on a copper catalysed click reaction between an azide modified peptide and an alkyne modified ADPr counterpart. We expand the scope of this approach to proteins by preparing two ubiquitin ADPr analogues carrying the biological relevant α-glycosidic linkage. Biochemical validation using *Legionella* effector enzyme SdeA shows that clicked ubiquitin ADPr is well tolerated and highlights the potential of this strategy to prepare ADPr proteins.

Regulation of protein activity is controlled by post-translational modifications (PTMs) that are installed on specific side chain functionalities of amino acids in the involved protein. Simple PTMs such as acetylation, methylation and phosphorylation have been subject of a large amount of studies and the focus of PTM research is shifting to more complex PTMs. One of these PTMs is called adenosine diphosphate ribose (ADPr), a modification in which a specific nucleophilic side chain in the target protein displaces β-oriented nicotinamide from NAD$^+$ under the agency of an ADPr-transferase (ART) resulting in an α-oriented linkage to the protein[1]. *Mono*-ADP-ribosylation is not only a PTM effected by bacterial toxins and the starting point for poly-ADP-ribosylation but also a regulatory modification in its own right. *Mono*-ADP-ribosylation is reported to take place on a variety of amino acid side chains including arginine (see **Figure 1)**, glutamic acid, aspartic acid, asparagine and cysteine but recently it was pointed out that serine might be the main point of attachment for ADP-ribosylation.[2] Research in the field of protein ADP-ribosylation benefits greatly from ADP-ribosylated molecular tools. One way to obtain such tools in sufficient quantities is through chemical synthesis. Methodologies towards naturally occurring *mono*-ADP-ribosylated oligopeptides, ADPr oligomers and NAD$^+$-analogues have been reported and employed in studying ADP-ribosyl hydrolase affinity[3], inhibition of ADP-ribosylating toxins, finding substrate proteins for *poly* ADPr polymerases[4] and determining the structure of *poly* ADP-ribose glycohydrolases.[5] Such ADPr peptides and related substances are valuable for the interrogation of the complex biology that underlies this PTM.[6] In the chemical synthesis of peptides and proteins most commonly an acidic step to remove protective groups is employed. Such conditions, however, may cause either epimerization at the anomeric center of ribose or complete loss of the ADPr-moiety. Mild alkaline conditions, carry the risk of degradation of the β-substituted amino acids and are clearly incompatible with the esters of ADP-ribosylated Glu and ADP-ribosylated Asp. The reported syntheses of ADPr amino acids and peptides so far have been carefully tuned to minimize those risks and incorporation of ADPr amino acids asks for a modified protective group strategy in most cases.[6a, 7] In order to prevent the need for highly specialized methods to prepare these amino acid-ribose conjugates we propose a general strategy that would allow a post-synthetic introduction of the ADPr moiety to a peptide or protein of interest.

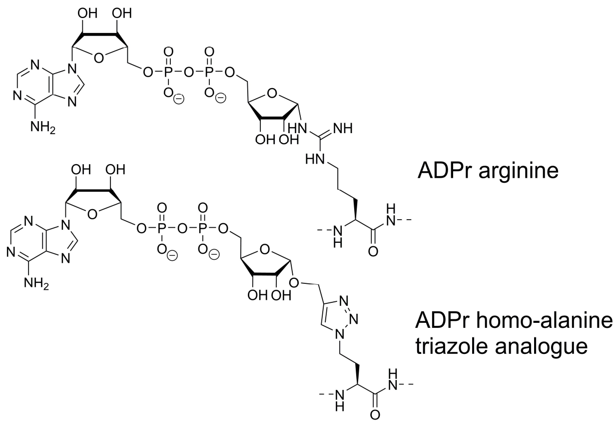

Figure 1. Structure of α -linked ADPr arginine- and ADPr triazole analogue-linkages.

We selected the ADPr triazole analogues as a relevant replacement for ADPr amino acids in peptides (see **Figure 1**).

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[a] Q. Liu, Dr. H. A. V. Kistemaker, Prof. Dr. G. A. van der Marel, Prof. Dr. H. S. Overkleeft, Dr. D. V. Filippov *
Bio-organic Synthesis
Leiden University
Einsteinweg 55, 2333 CC Leiden, The Netherlands
*E-mail: filippov@chem.leidenuniv.nl
Current address: ProQR Therapeutics, Leiden, The Netherlands

[b] Dr. S. Bhogaraju, Prof. Dr. I. Dikic
Institute of Biochemistry II, Goethe University Faculty of Medicine, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany
and
Buchmann Institute for Molecular Life Sciences, Goethe University Frankfurt, Riedberg Campus, Max-von-Laue-Straße 15, 60438 Frankfurt am Main, Germany

[c] Dr. G. J. van der Heden van Noort*, Prof. Dr. H. Ovaa
Department of Chemical Immunology
Leiden University Medical Centre
Einthovenweg 20, 2333 ZC, Leiden, The Netherlands
*E-mail: gvanderheden@lumc.nl

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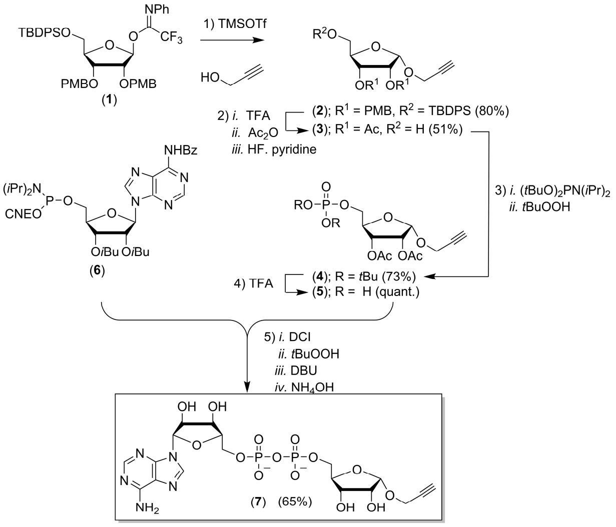
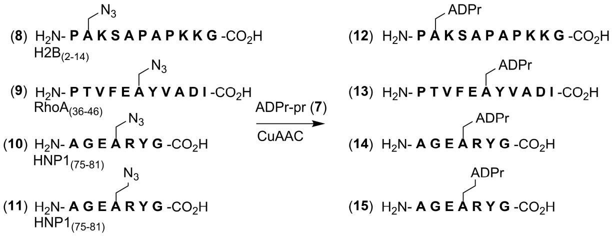
Oligopeptides with an azide incorporated can be obtained by standard solid phase peptide synthesis (SPPS) using an azido-alanine or azido-homo alanine building block at the site of the modification. After conventional synthesis and a copper catalyzed azide alkyne cycloaddition (CuAAC) of the obtained azido functionalized oligopeptide with a suitable propargylated ADPr building block (ADPr-pr, **7** in **Scheme 1**) results in the installation of the ADPr triazole functionality.

The synthesis of ADPr-pr **7** starts with the condensation of propargyl alcohol with imidate ribose donor **1**[7a] (see **Scheme 1**) to yield an anomeric mixture (α:β, 71:29). In contrast to two previously reported syntheses that show the preparation of either β-*O*-alkyne or β-azide containing ADPr analogues we were able to isolate the biologically relevant α-anomer.[5, 8] Protective group exchange allows for an alkaline deprotection scheme in the final stage of the synthesis instead of acid treatment, preventing possible degradation of the ADPr-pr moiety. Phosphitylation of the primary alcohol and subsequent oxidation furnishes crucial phosphotriester intermediate **4**. Removal of the *t*Bu groups set the stage for the installation of the pyrophosphate moiety using our previously reported procedure.[9] Reaction of phosphomonoester **5** with suitably protected adenosine phosphoramidite **6**[10] using dicyanoimidazole (DCI) as activator and oxidation of the intermediate P(III)-P(V) species was followed by a two-step alkaline deprotection procedure. Purification using size exclusion chromatography gave access to ADPr-pr **7** in a quantity of 100 mg. Formation of the pyrophosphate and subsequent deprotection proceeded in an overall isolated yield of 65% which compares favorably to other approaches.[6a, 8, 11]



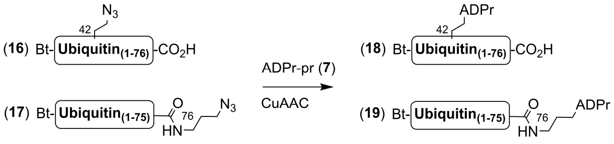
Scheme 1. Synthesis of ADPr-propargyl **7**

To assess the viability of ADPr-pr building block **7** in the projected cycloaddition we prepared three peptides derived from mono-ADP-ribosylated proteins; namely Histone H2B (2-14) (see **Scheme 2**, compound **8**), RhoA (36-46) (see **Scheme 2**, compound **9**) and Human Neutrophil Defensin 1; HNP1 (75-81) (see **Scheme 2**, compound **10**). In the selected peptides, we substituted Gln3, Asn50 and Arg78, respectively, for β-azidoalanine to allow conjugation via copper catalyzed cycloaddition. After completion of the SPPS the immobilized oligopeptides were treated with a cleavage mixture consisting of 90.5% trifluoroacetic acid (TFA), 5% water, 2.5% phenol and 2% triisopropylsilane to globally remove the protective groups and cleave the peptide from the resin. RP-HPLC purification yielded target peptides **8 – 10** that were used in the CuAAC reaction with ADPr-pr **7** in 20 mM tris(hydroxymethyl)aminomethane / 150 mM NaCl buffer at pH 7.6 under the agency of 10 mM $CuSO_4$, 60 mM sodium ascorbate and 10 mM tris triazole ligand.[12] Since these peptides and ADPr-pr **7** dissolved readily in this buffer, the click reaction proceeded efficiently and quickly (within minutes to one hour) in contrast to a previous study.[8] In order to mimic the length of the Arg-ADPr linkage more closely we prepared HNP1-peptide **11** in which the Arg was replaced not with β-azidoalanine but with azido-homoalanine. Again CuAAC to ADPr-pr proceeded uneventfully, yielding **15**.



Scheme 2. CuAAC reaction towards ADPr peptides

Having established an efficient protocol to prepare ADPr oligopeptides we were eager to see whether our methodology could be expanded in preparation of ADPr proteins. Ubiquitin (Ub), a 76 amino acid residue long post-translational modifier itself, has recently been found to be modified with ADPr on different positions. This cross-talk between ADP-ribosylation and ubiquitination is reported to have a regulatory effect on the DNA repair mechanism, where low levels of NAD lead to ubiquitination of histone protein H4, but high levels of NAD lead to ADP-ribosylation of Gly76; the C-terminus of Ub.[13] Other studies show that Arg42 of Ub is ADP-ribosylated by a family of effector proteins originating from *Legionella pneumophila,* the pathogen causing Legionnaires disease.[14] These SidE effectors are the first reported class of enzymes that are able to ubiquitinate target proteins independent of the normally employed enzymatic cascade of E1, E2 and E3 enzymes, utilizing Ub-ADPr as crucial intermediate. Using their unique properties, SidE proteins can hijack the host cells Ub pool and use it to its own advantage. In analogy to peptide **11**, we prepared Ub mutant **16** in which Arg42 is replaced by azido-homoalanine using our previously published linear SPPS approach.[15] Gly76 modified Ub **17** was prepared by first synthesizing Ub_{75} on trityl resin followed by treatment with mild acid (20% hexafluoroisopropanol in dichloromethane). In this step the peptide was liberated from the solid support while leaving all side-chain protecting groups in place. Activation of the free C-terminal carboxylic acid and coupling of 3-azido-1-propanamine followed by strong acid treatment and RP-HPLC purification yielded azide modified Ub **17**. Copper catalyzed click reaction with ADPr-pr building block **7** followed by dialysis to remove traces of excess ADPr-pr and click reagents followed by size exclusion chromatography gave easy access to ADP-ribosylated ubiquitin analogues **18** and **19**, respectively (see **Scheme 3**).



Scheme 3. CuAAC reaction towards Ub-ADPr conjugates.

Of note is that this workflow does not require the use of RP-HPLC purification after introduction of the ADPr moiety. In order to assess whether the artificial triazole linkage is tolerated and this methodology indeed results in useful ADPr-protein analogues, we compared Ub-ADPr **18** to Arg42 Ub-ADPr from natural sources (Ub-ADPr wt). Both Ub-ADPr **18** and Ub-ADPr wt were efficiently recognized by an ADPr-antibody in Western Blot (see **Figure 2A,** lower panel), a first indication that the triazole analogue does not differ too far from its natural counterpart. One of the properties of *Legionella* effector SdeA is its auto-ubiquitination behaviour, an effect that is not fully understood so far, but is reported for all four SidE family members. We compared the ability of recombinant SdeA to use **18** in an auto-ubiquitination assay and found indeed that SdeA is modified with Ub multiple times (see **Figure 2A, B**). Although at a reduced rate compared to Ub-ADPr wt, artificial **18** was processed by SdeA and significant auto-ubiquitination takes place. A control experiment using non-ADPribosylated wild type Ub shows no auto-ubiquitination of SdeA (see **Supporting Information Figure 1**). These results further confirm that Ub-ADPr conjugate **18** functions similar to Ub-ADPr wt.

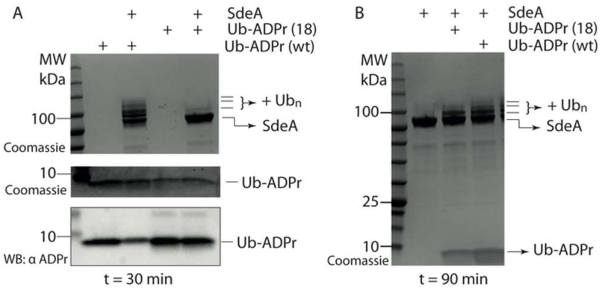


Figure 2. Comparison of Arg42 Ub-ADPr wt and Ub-ADPr analogue **18** processing by SdeA at **A**) 30 min and **B**) 90 min.

In conclusion, we here present the design and synthesis of propargylated ADP-ribose building block (**7**) suitable to take part in an efficient cycloaddition with oligopeptides and proteins having an azide at a predetermined position. In this way oligopeptides and proteins carrying an analogue of the *mono* ADP-ribose post-translational modification are made available. Four ADPr-oligopeptide conjugates derived from known ADP-ribosylated proteins were prepared efficiently. In addition, two analogues of ADPr ubiquitin, shown to play a role in Legionnaires disease and DNA repair, were prepared using the same copper catalyzed chemistry. The effectiveness of these reactions and subsequent purifications provides an easy entry to this interesting class of post-translational modified proteins. Triazole containing Ub-ADPr **18** was shown to be recognized in Western Blot and accepted by SdeA in an auto-ubiquitination assay, indicating that this methodology provides a useful platform for the biological interrogation of ADPr biology.

Acknowledgements

We like to thank Dris el Atmioui and Cami Talavera Ormeňo for SPPS. This work was supported by a NWO VENI-grant to GJvdHvN and by an NWO ECHO grant to HAVK.

Keywords: ADP-ribosylation • Ubiquitination • Post-translational modification • Click chemistry • Protein modification

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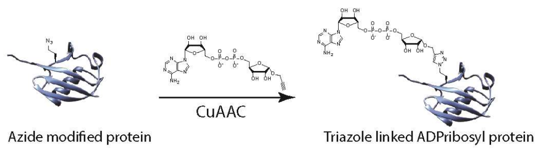

ADPr-Ub-date. A general strategy to prepare ADPr peptide and protein analogues based on a copper catalysed click reaction between an azide modified peptide or protein and an alkyne modified ADPr counterpart is presented. The first fully synthetic ADPribosylated protein analogues carrying the biological relevant α-glycosidic linkage were prepared and biochemical validation shows that clicked ADPr Ubiquitin is tolerated, highlighting the potential of this strategy.

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