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A general approach towards triazole-linked adenosine diphosphate ribosylated peptides and proteins

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Introduction

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 ribosylation (ADP-ribosylation), 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 glycosidic 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 (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.^{2,3} 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. Methods towards naturally occurring mono-ADP-ribosylated oligopeptides, ADPr oligomers, and NAD⁺-analogues have been reported and employed in studying ADP-ribosyl hydrolase affinity,^{4,5} inhibition of ADP-ribosylating toxins, finding substrate proteins for poly ADPr polymerases,⁶ and determining the structure of poly ADP ribose glycohydrolases.⁷ Such ADPr peptides and related substances are valuable for the interrogation of the complex biology that underlies this PTM.^{8,9} 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 synthesis 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.^{8,10} 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.

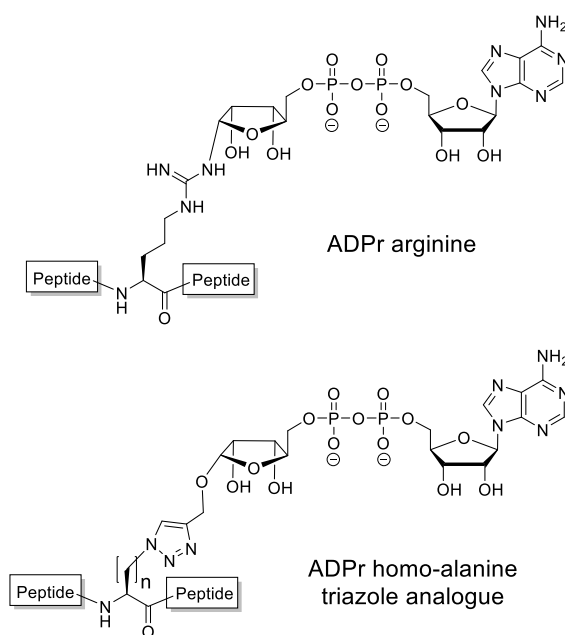
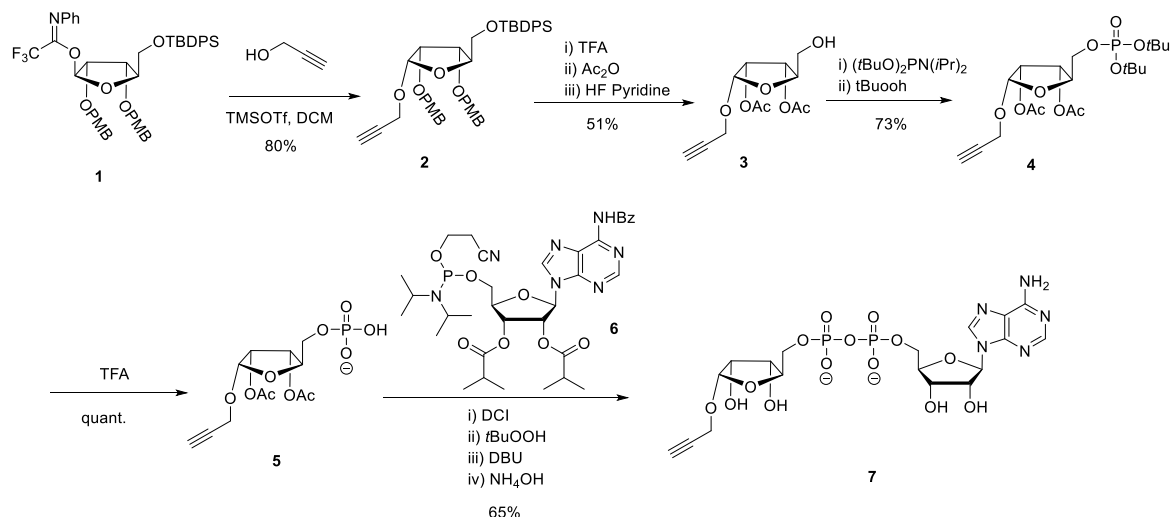


Figure 1. Structure of α -linked ADPr arginine and ADPr triazole analogue linkages.

In this Chapter, an ADPr triazole analogue was selected as a relevant replacement for ADPr amino acids in peptides (Figure 1). Oligopeptides with an azide incorporated can be obtained by standard solid-phase peptide synthesis (SPPS) using an azido-alanine or azido-homoalanine building block at the site of the modification. The azido-oligopeptides, thus obtained, could be “clicked” with α -1-O-propargyl-ADPr (ADPr-pr, **7** in Scheme 1) via a conventional copper catalyzed azide-alkyne cycloaddition (CuAAC), furnishing a triazole linked ADP-ribosylated peptides as bioisosteres of the

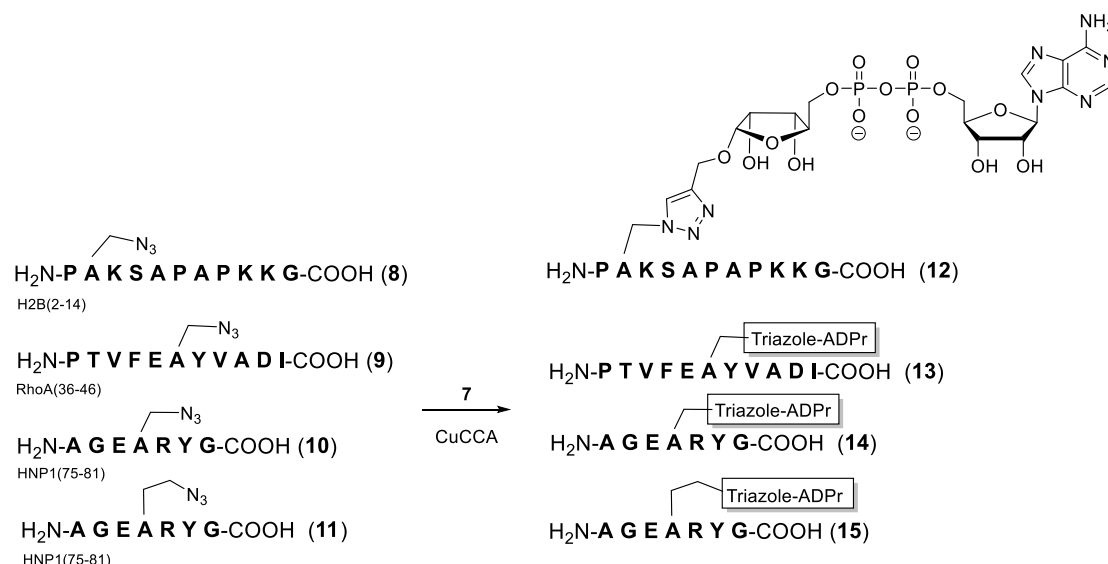
native molecules. Furthermore, this strategy was also applied to the synthesis of ADPr-ribosylated protein mimics in which ADPr-pr was selectively conjugated to a synthetic ubiquitin at a desired site. Importantly, the synthetic ADPr-ubiquitin was found to possess similar bioactivity as the natural counterpart in our auto-ubiquitination assay, highlighting the broad application of this method in the biological interrogation of ADPr biology.



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

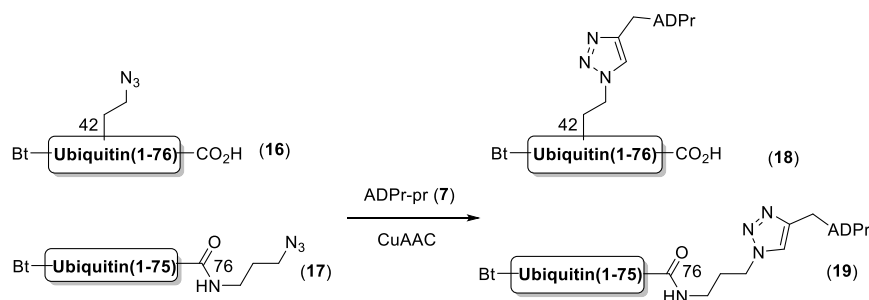
Results and discussion

The synthesis of ADPr-pr **7** started with the condensation of propargyl alcohol with trifluoroacetimidate ribofuranose donor **1**¹⁰ (Scheme 1, see also Chapter 6) to yield an anomeric mixture **2** ($\alpha:\beta$, 71:29). In contrast to two previously reported syntheses that show the preparation of either β -O-alkyne or β -azide containing ADPr analogues, the biologically relevant α -anomer could be isolated^{7, 11} in this work. Protective group manipulations to introduce acetyls on the 2- and 3-hydroxyls allowed alkaline deprotection 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 furnished crucial phosphotriester intermediate **4**. Removal of the *t*Bu groups set the stage for the installation of the pyrophosphate moiety using adaptation of the procedure of Gold *et al.*^{4, 12} Reaction of phosphomonoester **5** with suitably protected adenosine phosphoramidite **6**¹³ using dicyanoimidazole (DCI) as activator and oxidation of the intermediate P(V)-P(III) 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.^{8, 11, 14}



Scheme 2. CuAAC reaction towards ADPr peptides

To assess the viability of ADPr-pr building block **7** in the projected cycloaddition, three peptides derived from mono-ADP-ribosylated proteins were prepared; namely Histone H2B (2–14) (Scheme 2, compound **8**), RhoA (36–46) (compound **9**) and human neutrophil defensin 1; HNP1 (75–81) (compound **10**). In the selected peptides, Gln3, Asn50, and Arg78, respectively, were substituted, for β -azidoalanine to allow conjugation by CuAAC. 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 in the presence of 10 mM CuSO_4 , 60 mM sodium ascorbate, and 10 mM tris triazole ligand.¹⁵ 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.¹¹ To mimic the length of the Arg-ADPr linkage more closely, HNP1-peptide **11** was prepared, in which the Arg was replaced not with β -azidoalanine but with azidohomoalanine. Again CuAAC to ADPr-pr proceeded uneventfully, yielding **15**.



Scheme 3. CuAAC reaction towards Ub-ADPr conjugates

Having established an efficient procedure to prepare ADPr oligopeptides, it was investigated whether this method could be expanded for the 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.¹⁶ 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.¹⁷⁻¹⁹ These SidE effectors are the first reported class of enzymes that are able to ubiquitinate target proteins independently 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 the advantage of pathogene. In analogy to peptide **11**, Ub mutant **16**, in which Arg42 has been replaced by azidohomoalanine, was prepared using the linear SPPS approach of El Oualid *et al.*²⁰ Gly76 modified Ub **17** was prepared by first synthesizing Ub75 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 and finally size exclusion chromatography, gave easy access to ADP-ribosylated ubiquitin analogues **18** and **19**, respectively (Scheme 3).

Of note is that this procedure does not require the use of RP-HPLC purification after the introduction of the ADPr moiety. To assess whether the artificial triazole linkage is tolerated and this method indeed results in useful ADPr protein analogues, Ub-ADPr **18** was compared 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 (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 behavior, an effect that is not fully understood so far, but is reported for all four SidE family members. Comparison of the ability of recombinant SdeA to use **18** in an auto-ubiquitination assay showed 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-ADP-ribosylated wild type Ub shows no auto-ubiquitination of SdeA (Figure 2C). These results further confirmed that Ub-ADPr conjugate **18** functions similar to Ub-ADPr wt.

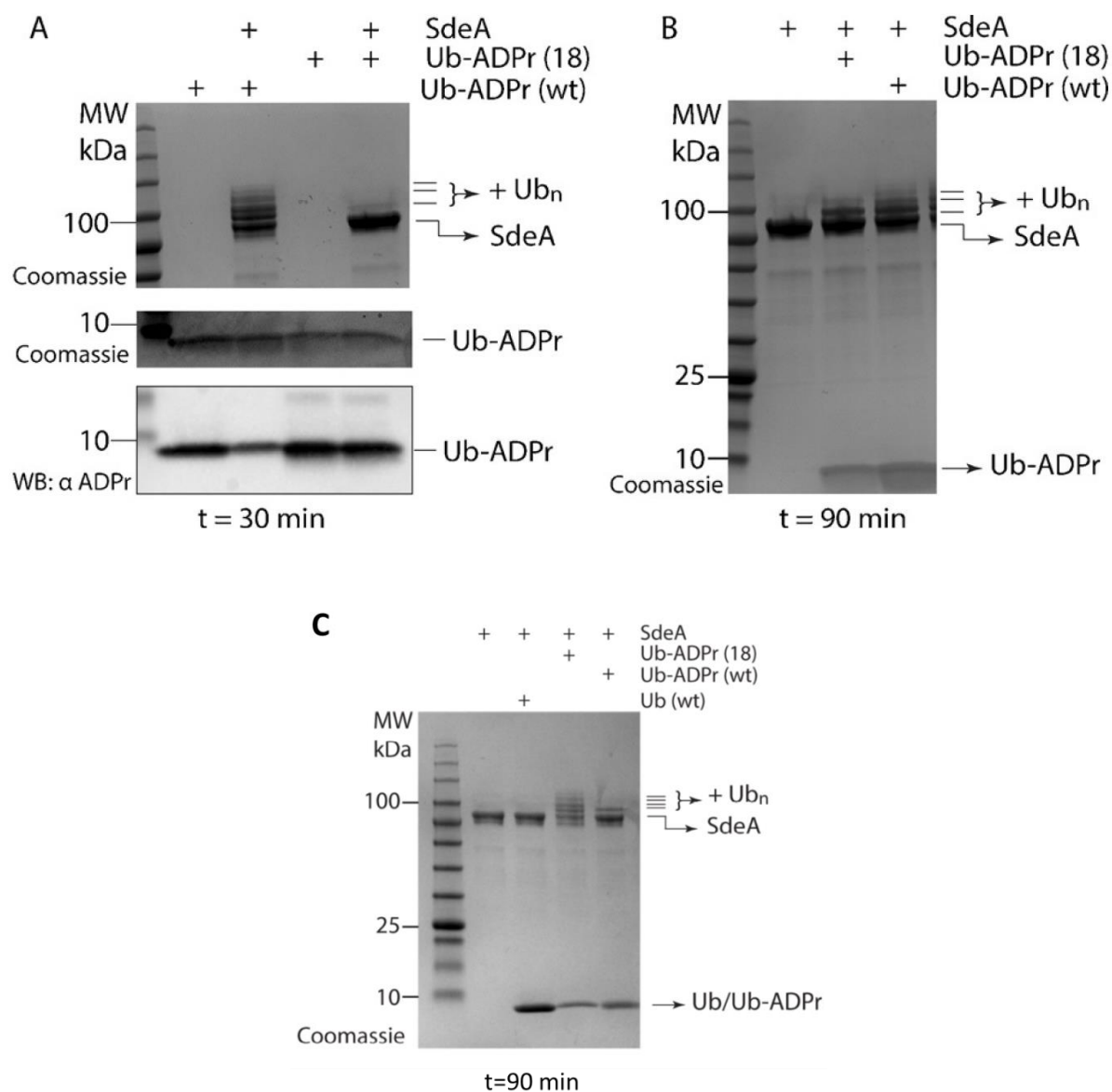


Figure 2. **A/B:** Comparison of Ub-ADPr (wt) and Ub-ADPr analogue (**18**) processing by SdeA at 30 min and 90 min. **C:** Control experiment of Ub-ADPr (wt), Ub-ADPr (**18**) and Ub (wt) processing by SdeA at 90 min.

Conclusion

This chapter presents the design and synthesis of propargylated ADP-ribose **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. Furthermore, two analogues of ADPr ubiquitin, shown to play a role in Legionnaires' disease and DNA repair, were prepared using the same CuAAC chemistry. The effectiveness of these reactions and subsequent purifications provided an easy entry to this interesting class of post-translationally 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 method provides a useful platform for the biological interrogation of ADPr biology.

Acknowledgement

Mengjie Shen is kindly acknowledged for the help in the lyophilization of ADPr-peptide.

Experimental Section

General procedure for synthesis

General reagents were obtained from Sigma Aldrich, Fluka and Acros and used as received. Solvents were purchased from BIOSOLVE or Aldrich. Peptide synthesis reagents were purchased from Novabiochem. TLC, NMR, LCMS, anion exchange, gel filtration, HRMS, IR, optical rotation facilities were used as described in Chapter 2.

Bioassay

Purification of SdeA (193-998) was performed as previously described.¹⁹ For *in vitro* ubiquitination reaction, 2 µg of purified SdeA was incubated with 4 µg of either Ub wt, Ub-ADPr wt or Ub-ADPr **18** in 30 µL reaction buffer containing 50 mM Tris, 50 mM NaCl at pH 7.5. Reaction components were incubated at 37 °C for indicated duration. Reaction was stopped by adding SDS loading buffer and samples analysed by Coomassie staining and western-blot detection of Ub variants using anti-pan ADP-ribose binding reagent (Millipore, catalogue number: MABE1016).

Solid phase peptide synthesis

SPPS was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 25 µmol scale, using fourfold excess of amino acids relative to pre-loaded Fmoc amino acid Wang type resin (0.2 mmol/g, Applied Biosystems®) or pre-loaded Fmoc amino acid trityl resin (0.2 mmol/g, Rapp Polymere GmbH). The Ub (mutant) peptide sequences were synthesized on resin following the procedures as described before.²⁰ Peptides were treated with TFA/TIS/H₂O/Phenol for 2.5 hours followed by precipitation from Et₂O/Pentane and dissolved in lyophilization mixture (MQ: CH₃CN: Formic acid 65/25/ 10, v:v:v) and lyophilized. Bt-PEG2-Ub₇₅ was cleaved of the trityl resin using hexafluoroisopropanol in DCM (1:4 v/v) for 2 times 20 minutes and filtered. The flow through was collected and concentrated *in vacuo*, followed by coevaporation with dichloroethane (3 x) to remove residual HFIP.

Subsequently the protected peptide was dissolved in DCM and reacted with PyBOP, DiPEA and 3-azido-1-propanamine for 16 hours. The reaction was concentrated *in vacuo* and treated with TFA/TIS/H₂O/Phenol for 2.5 hours. The peptide was precipitated from Et₂O/pentane and subsequently purified using RP-HPLC.

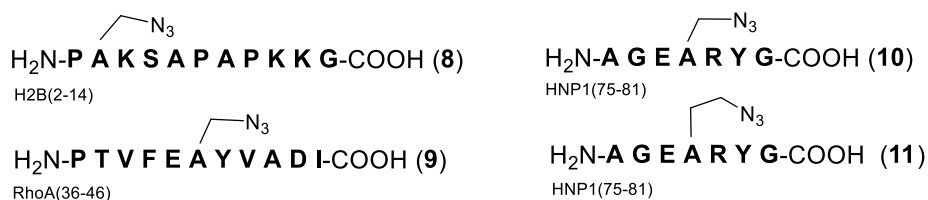
RP-HPLC purifications

A) Waters preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 μ m OBD (30 x 150 mm) column at a flowrate of 37.5 mL/min using 3 mobile phases: A: MQ, B: CH₃CN and C: 1% TFA in MQ. Gradient: 20 $\rightarrow$ 45% B, 5% C.

B) Shimadzu semi-preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 μ m OBD (10 x 150 mm) column at a flowrate of 6.5 mL/min. using 2 mobile phases: A: MQ + 0.05% FA, B: CH₃CN + 0.05% FA. Gradient: 0 $\rightarrow$ 15% B.

C) Gilson preparative RP-HPLC system, equipped with Phenomenex Gemini (10 x 250 mm) column at a flowrate of 5 mL/min, using 2 mobile phases: A: 50 mM NH₄OAc in MQ, B: CAN.

Purification and analytical data of peptides



Azido peptides (**8** – **10**) were dissolved in 0.1% Formic Acid in MQ and purified using RP-HPLC. Pure fractions were pooled and lyophilized. Azidohomoalanine-HNP1 peptide (**11**) was used crude in the CuAAC reaction after cleavage from the resin followed by lyophilization.

Histone H2B(2-14): P[A*]PAKSAPAPKKG ([A*]: Azidoalanine) – Compound (8)

LC-MS: Rt = 0.41 min., ESI MS⁺ (amu) calcd: 1260.47, found 1261.05 [M + H]⁺, 630.86 [M + 2H]²⁺, 420.91 [M + 3H]³⁺, 315.92 [M + 4H]⁴⁺.

RhoA (36-46): PTVFE[A*]YVADI ([A*]: Azidoalanine) – Compound (9)

LC-MS: Rt = 2.14 min., ESI MS⁺ (amu) calcd: 1265.39, found 1266.02 [M + H]⁺, 633.31 [M + 2H]²⁺

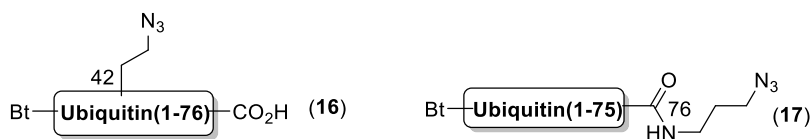
HNP1 (75-81): AGE[A*]RYG ([A*]: Azidoalanine) – Compound (10)

LC-MS: Rt = 0.55 min., ESI MS⁺ (amu) calcd: 763.77, found 764.29 [M + H]⁺, 382.66 [M + 2H]²⁺

HNP1 (75-81): AGE[A*]RYG ([A*]: Azidohomoalanine) – Compound (11)

LC-MS: Rt = 4.84 min., ESI MS⁺ (amu) calcd: 777.78, found 778.3 [M + H]⁺

Purification and analytical data of ubiquitin azide mutants



The crude Ub mutant was taken up in a minimal amount of warm DMSO and diluted in warm MilliQ while the final DMSO concentration was kept as low as possible (<10%). Next, the peptide was purified by preparative RP-HPLC (A). Pure fractions were pooled and lyophilized.

Biotin-PEG2-(R42Azidohomoalanine) Ub₇₆ - Compound (16)

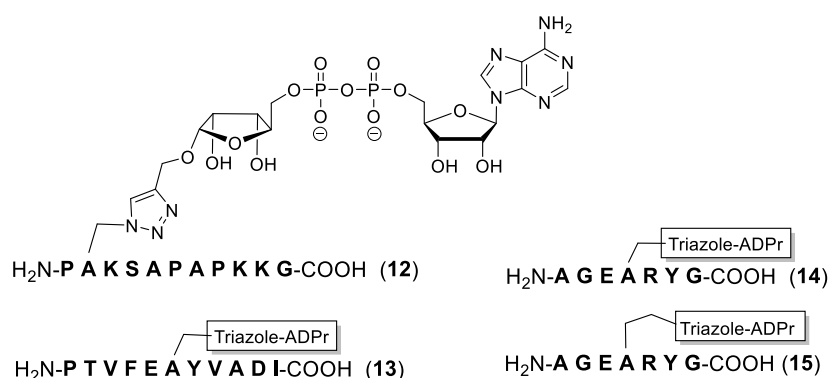
LC-MS: Rt = 2.07 min., ESI MS⁺ (amu) calcd: 8888.26, found 8889.00 (deconv.)

Biotin-PEG2-(G76Azidopropanamine)Ub₇₆ - Compound (17)

LC-MS: Rt = 1.92 min., ESI MS⁺ (amu) calcd: 8943.34, found 8943.00 (deconv.)

General procedure for CuAAC reactions:

150 μ L ADPr-propargyl (1.5 eq, 10 mg/mL in DMSO) was added to azido modified peptide (1.0 eq.) and subsequently added to 500 μ L buffer (20 mM TRIS/150 mM NaCl, pH 7.6). To this was added 60 μ L of freshly prepared click-mixture (1:1:1 v/v/v, CuSO₄ (26 mg/mL in water): Sodium Ascorbate (120 mg/mL in water): TBTA ligand (52 mg/mL in CH₃CN)). The reaction was shaken for 30 min at room temperature or 37°C and followed using LC-MS analysis. Once the azide starting material was fully converted to the ADPr-conjugate the reaction was quenched using 15 μ L EDTA (0.5 M). The reaction was then purified by gel filtration, lyophilized and purified by RP-HPLC purification.



Histone H2B(2-14)-ADPr: P[A*]PAKSAPAPKKG ([A*]: triazolyl ADPr) – Compound (12)

The product was synthesized using the general procedure. HPLC purification using (C) with gradient: 4-10% B, in 12 min. The combined fractions were lyophilized to yield the title compound as a white solid (1.51 mg, 0.81 μ mol, yield: 25%). ¹H NMR (500 MHz, D₂O) δ 8.41 (s, 1H, 2H), 8.13 (s, 1H, 8H), 7.90 (s, 1H, triazole), 6.02 (d, *J* = 6.0 Hz, 1H, H1'-ade), 4.96 (d, *J* = 4.3 Hz, 1H, H1''-rib). ³¹P NMR (202 MHz, D₂O) δ : -10.65. LC-MS: Rt = 4.12 min.

ESI MS⁺ (amu) calcd: 1857.8, found 1858.5 [M + H]⁺, 929.5 [M + 2H]²⁺. HRMS: [C₇₃H₁₁₈N₂₄O₂₉P₂ + 2H]²⁺: found 929.4082, calc. 929.4064, [C₇₃H₁₁₈N₂₄O₂₉P₂ + 3H]³⁺: found 619.9447, calc. 619.402, [C₇₃H₁₁₈N₂₄O₂₉P₂ + 4H]⁴⁺: found 465.2092, calc. 465.2071, [C₇₃H₁₁₈N₂₄O₂₉P₂ + 5H]⁵⁺: found 372.3672, calc. 372.3649.

RhoA (36-46)-ADPr: PTVFE[A*]YVADI ([A*]: triazolyl ADPr) – Compound (13)

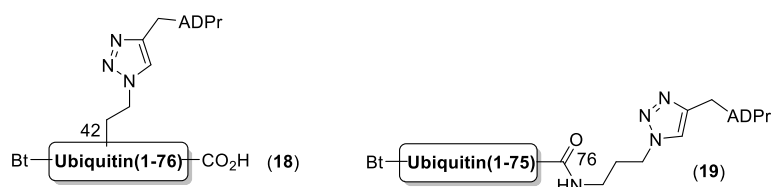
The product was synthesized using the general procedure. HPLC purification using (C) with gradient: 13-19% B, in 12 min. The combined fractions were lyophilized to yield the title compound as a white solid (0.4 mg, 0.21 μmol, yield: 3%). ¹H NMR (500 MHz, D₂O) δ 8.46 (s, 1H, 2H), 8.17 (s, 1H, 8H), 7.89 (s, 1H, triazole), 7.24 – 7.13 (m, 3H, arom. Phe), 7.11 (dd, *J* = 6.8, 1.8 Hz, 2H, arom. Phe), 7.02 – 6.95 (m, 2H, arom. Tyr), 6.76 – 6.67 (m, 2H, arom. Tyr), 6.06 (d, *J* = 5.8 Hz, 1H, H1'-ade), 4.99 (d, *J* = 3.9 Hz, 1H, H1''-rib). ³¹P NMR (202 MHz, D₂O) δ: -10.48, -10.60, -10.63, -10.76. LC-MS: Rt = 7.34 min. ESI MS⁺ (amu) calcd: 1862.8, found 1863.4 [M + H]⁺, 932.3 [M + 2H]²⁺. HRMS: [C₇₆H₁₀₉N₁₉O₃₂P₂ + 2H]²⁺: found 931.8559, calc. 931.8548, [C₇₆H₁₀₉N₁₉O₃₂P₂ + 3H]³⁺: found 621.5708, calc. 621.5732.

HNP1 (75-81)-ADPr: AGE[A*]RYG ([A*]: triazolyl ADPr) – Compound (14)

The product was synthesized using the general procedure. HPLC purification using (C) with gradient: 2-8% B, in 12 min. The combined fractions were lyophilized to yield the title compound as a white solid (2.36 mg, 1.73 μmol, yield: 53%). ¹H NMR (600 MHz, D₂O) δ 8.46 (s, 1H, 2H), 8.18 (s, 1H, 8H), 7.85 (s, 1H, triazole), 7.09 – 7.00 (m, 2H, arom. Tyr), 6.76 – 6.68 (m, 2H, arom. Tyr), 6.07 (d, *J* = 5.8 Hz, 1H, H1'-ade), 5.05 (d, *J* = 4.4 Hz, 1H, H1''-rib). ³¹P NMR (202 MHz, D₂O) δ: -10.47, -10.58, -10.66, -10.76. LC-MS: Rt = 4.30 min. ESI MS⁺ (amu) calcd: 1361.1, found 1361.3 [M + H]⁺, 681.3 [M + 2H]²⁺. HRMS: [C₄₈H₇₀N₁₈O₂₅P₂ + H]⁺: found 1361.4315, calc. 1361.4313, [C₄₈H₇₀N₁₈O₂₅P₂ + 2H]²⁺: found 681.2223, calc. 681.2195, [C₄₈H₇₀N₁₈O₂₅P₂ + 3H]³⁺: found 454.8186, calc. 454.8166.

HNP1 (75-81)-ADPr: AGE[A*]RYG ([A*]: triazolyl ADPr) – Compound (15)

The product was synthesized using the general procedure and purified with gel filtration. The combined fractions were lyophilized to yield the title compound as a white solid (3.13 mg, 2.28 μmol, yield: 76%). ¹H NMR (500 MHz, D₂O) δ: 8.55 (s, 1H, 2H), 8.25 (s, 1H, 8H), 7.89 (s, 1H, triazole), 7.08 (d, *J* = 8.4 Hz, 2H, arom. Tyr), 6.75 – 6.68 (m, 2H, arom. Tyr), 6.12 (d, *J* = 5.7 Hz, 1H, H1'-ade), 5.09 (d, *J* = 3.9 Hz, 1H, H1''-rib). ³¹P NMR (202 MHz, D₂O) δ: -10.43, -10.53, -10.66, -10.76. LC-MS: Rt = 4.36 min. ESI MS⁺ (amu) calcd: 1375.2, found 1375.3 [M + H]⁺, 688.3 [M + 2H]²⁺. HRMS: [C₄₉H₇₂N₁₈O₂₅P₂ + 2H]²⁺: found 688.2247, calc. 688.2274.



Biotin-PEG2-(R42*) Ub₇₆ (R42*: triazolyl ADPr) - Compound (18)

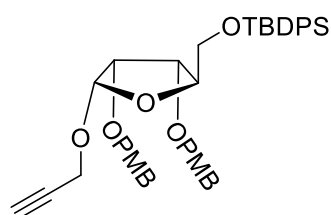
Ubiquitin **16** (5.1 mg), 1.0 eq. was dissolved in 45 μL DMSO and subsequently added to 510 μL buffer (20 mM TRIS/150 mM NaCl, pH 7.6). To this was added 45 μL of freshly prepared click-mixture (1:1:1 v/v/v, CuSO₄ (26 mg/mL in water): Sodium Ascorbate (120 mg/mL in water): TBTA ligand (52 mg/mL in acetonitrile)) and pH was

adjusted to 7.4 before addition of 60 μ L ADPr-propargyl 2.4 eq. (13 mg/mL in DMSO). The reaction was shaken for 90 min at room temperature and followed using LC-MS analysis. Once the azide starting material was fully converted to the ADPr-conjugate the reaction was quenched using 10 μ L EDTA (0.5 M). Dialysis using a 3.5-5 kD MWCO dialysis device removed traces of excess ADPr-propargyl reagent as well as copper/ligand/ascorbate from the reaction, followed by size exclusion chromatography resulted in the title compound (4.6 mg, 0.48 μ mol, 84%. Calculated based on SDS-page /Coomassie stain comparison with standard curve of wt Ub) . LC-MS: Rt = 2.00 min., ESI MS+ (amu) calcd: 9485.36, found 9486.00. HRMS: $[C_{411}H_{675}N_{113}O_{137}P_2S + 7H]^+{}^7$: found 1355.9868, calc. 1355.9886, $[C_{411}H_{675}N_{113}O_{137}P_2S + 8H]^+{}^8$ found 1186.6187, calc. 1186.6161, $[C_{411}H_{675}N_{113}O_{137}P_2S + 9H]^+{}^9$: found 1054.8846, calc. 1054.8818, $[C_{411}H_{675}N_{113}O_{137}P_2S + 10H]^+{}^{10}$: found 949.4930, calc. 949.4944, $[C_{411}H_{675}N_{113}O_{137}P_2S + 11H]^+{}^{11}$: found 863.2679, calc. 863.2684, $[C_{411}H_{675}N_{113}O_{137}P_2S + 12H]^+{}^{12}$: found 791.4152, calc. 791.4133, $[C_{411}H_{675}N_{113}O_{137}P_2S + 13H]^+{}^{13}$: found 730.6146, calc. 730.6129.

Biotin-PEG2-(G76*)Ub₇ (G76*: triazolyl ADPr) - Compound (19)

Ubiquitin **17** (3.8 mg), 1.0 eq. was dissolved in 50 μ L DMSO and subsequently added to 250 μ L buffer (20 mM TRIS/150 mM NaCl, pH 7.6). To this was added 45 μ L of freshly prepared click-mixture (1:1:1 v/v/v, CuSO₄ (26 mg/mL in water): Sodium Ascorbate (120 mg/mL in water): TBTA ligand (52 mg/mL in acetonitrile)) and pH was adjusted to 7.4 before addition of 40 μ L ADPr-propargyl 2.4 eq. (13 mg/mL in DMSO). The reaction was shaken for 30 min at room temperature and followed using LC-MS analysis. Once the azide starting material was fully converted to the ADPr-conjugate the reaction was quenched using 15 μ L EDTA (0.5 M). Dialysis using a 3.5-5 kD MWCO dialysis device removed traces of excess ADPr-propargyl reagent as well as copper/ligand/ascorbate from the reaction, followed by size exclusion chromatography resulted in the title compound (3.2 mg, 0.34 μ mol, 80%). Calculated based on SDS-page /Coomassie stain comparison with standard curve of wt Ub). LC-MS: Rt = 1.96 min., ESI MS+ (amu) calcd: 9540.44 , found 9541.00. HRMS: $[C_{414}H_{684}N_{116}O_{135}P_2S + 7H]^+{}^7$: found 1363.8558, calc. 1363.8586, $[C_{414}H_{684}N_{116}O_{135}P_2S + 8H]^+{}^8$ found 1193.5070, calc. 1193.5023, $[C_{414}H_{684}N_{116}O_{135}P_2S + 9H]^+{}^9$: found 1061.0068, calc. 1061.0029, $[C_{414}H_{684}N_{116}O_{135}P_2S + 10H]^+{}^{10}$: found 955.0046, calc. 955.0034, $[C_{414}H_{684}N_{116}O_{135}P_2S + 11H]^+{}^{11}$: found 868.2764, calc. 868.2766, $[C_{414}H_{684}N_{116}O_{135}P_2S + 12H]^+{}^{12}$: found 796.0090, calc. 796.0042, $[C_{414}H_{684}N_{116}O_{135}P_2S + 13H]^+{}^{13}$: found 734.8512, calc. 734.8506.

Synthesis of α -propargyl-ADPr (7)



1-O-propargyl-2,3-bis-O-(4-methoxybenzyl)-5-O-tert-butyldiphenylsilyl- α,β -D-ibofuranoside (2)

The trifluoroacetimidate donor **1** (2.15 g, 2.75 mmol), described previously,¹⁰ and propargyl alcohol (160 μ L, 2.75 mmol) were co-evaporated with 1,4-dioxane (3 x) and dissolved in dry DCM (30 mL) along with freshly activated 3 Å molecular sieves. The reaction mixture was stirred at room temperature for 1 hour under argon to remove traces of water. The reaction mixture was cooled to -78 °C, TMSOTf (10 μ L, 55 μ mol) was added and the reaction mixture was stirred at -78 °C for 30 minutes. The reaction was quenched by the addition of triethylamine,

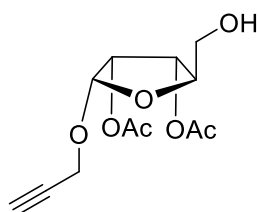
filtered, concentrated *in vacuo* and purified by silica gel chromatography (Pentane/EtOAc, 90/10 – 85/15) to obtain the title compound with the two anomers separated (β -anomer: 419 mg, 0.63 mmol; α -anomer: 1.04 g, 1.57 mmol; $\alpha/\beta=71/29$; 80% in total yield).

β -anomer:

$^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 7.66 (d, $J = 6.9$ Hz, 4H, arom. TBDPS), 7.46 – 7.32 (m, 6H, arom. TBDPS), 7.29 (d, $J = 8.5$ Hz, 2H, arom. PMB), 7.18 (d, $J = 8.5$ Hz, 2H, arom. PMB), 6.87 (d, $J = 8.6$ Hz, 2H, arom. PMB), 6.81 (d, $J = 8.6$ Hz, 2H, arom. PMB), 5.22 (s, 1H, $\text{H1}'$), 4.58 (AB, $J = 36.4, 11.7$ Hz, 2H, CH_2 PMB), 4.39 (AB, $J = 43.3, 11.4$ Hz, 2H, CH_2 PMB), 4.26 – 4.23 (m, 1H, $\text{H4}'$), 4.19 – 4.11 (m, 3H, $\text{H3}'$, CH_2CCH), 3.89 (d, $J = 4.5$ Hz, 1H, $\text{H2}'$), 3.85 (AB, $J = 11.3, 3.3$ Hz, 1H, $\text{H5}'$), 3.80 (s, 3H, CH_3 PMB), 3.78 (s, 3H, CH_3 PMB), 3.69 (AB, $J = 11.3, 4.1$ Hz, 1H, $\text{H5}'$), 2.38 (t, $J = 2.4$ Hz, 1H, CH_2CCH), 1.03 (s, 9H, $t\text{Bu}$ TBDPS). $^{13}\text{C-NMR}$ (126 MHz, CDCl_3) δ : 159.48, 159.38 (Cq. Arom.), 135.73, 135.70 (arom.), 133.55, 133.52, 130.08, 129.98 (Cq. Arom.), 129.84, 129.80, 129.75, 129.53, 127.81, 127.80, 113.96, 113.87 (arom.), 103.17 ($\text{C1}'$), 82.39 ($\text{C4}'$), 79.45 ($\text{C2}'$), 79.32 (CH_2CCH), 77.07 ($\text{C3}'$), 74.60 (CH_2CCH), 72.18, 72.07 (CH_2 PMB), 64.01 ($\text{C5}'$), 55.40, 55.38 (CH_3 PMB), 54.26 (CH_2CCH), 26.95 ($t\text{Bu}$ TBDPS), 19.40 (Cq. $t\text{Bu}$ TBDPS).

α -anomer:

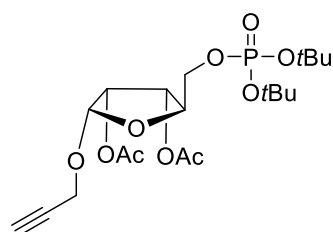
$^1\text{H NMR}$ (500 MHz, CDCl_3) δ : 7.59 (d, $J = 7.8$ Hz, 2H, arom. TBDPS), 7.54 (d, $J = 7.8$ Hz, 2H, arom. TBDPS), 7.43 – 7.21 (m, 10H, arom. TBDPS, PMB), 6.85 (d, $J = 8.5$ Hz, 2H, arom. PMB), 6.81 (d, $J = 8.5$ Hz, 2H, arom. PMB), 5.31 (d, $J = 4.3$ Hz, 1H, $\text{H1}'$), 4.65 (AB, $J = 12.2, 7.5$ Hz, 2H, CH_2 PMB), 4.57 (d, $J = 12.0$ Hz, 1H, CH_2 PMB), 4.51 (d, $J = 12.4$ Hz, 1H, CH_2 PMB), 4.39 (d, $J = 2.3$ Hz, 2H, CH_2CCH), 4.15 – 4.12 (m, 1H, $\text{H4}'$), 3.96 (dd, $J = 6.5, 2.7$ Hz, 1H, $\text{H3}'$), 3.86 (dd, $J = 6.5, 4.4$ Hz, 1H, $\text{H2}'$), 3.78 (s, 3H, CH_3 PMB), 3.77 (s, 3H, CH_3 PMB), 3.60 (AB, $J = 11.2, 3.4$ Hz, 1H, $\text{H5}'$), 3.50 (AB, $J = 11.1, 3.1$ Hz, 1H, $\text{H5}'$), 2.40 (t, $J = 2.2$ Hz, 1H, CH_2CCH), 0.94 (s, 9H, $t\text{Bu}$ TBDPS). $^{13}\text{C NMR}$ (126 MHz, CDCl_3) δ : 159.39, 159.23 (Cq. Arom.), 135.69, 135.62 (arom.), 133.28, 133.14, 130.52, 129.97 (Cq. Arom.), 129.84, 129.78, 129.71, 129.68, 127.79, 127.75, 113.89, 113.76 (arom.), 98.93 ($\text{C1}'$), 84.13 ($\text{C4}'$), 79.84 (CH_2CCH), 77.71 ($\text{C2}'$), 74.85 ($\text{C3}'$), 74.23 (CH_2CCH), 72.19, 71.98 (CH_2 PMB), 64.12 ($\text{C5}'$), 55.32 (CH_3 PMB), 54.22 (CH_2CCH), 26.85 ($t\text{Bu}$ TBDPS), 19.26 (Cq. $t\text{Bu}$ TBDPS). HRMS: $[\text{C}_{40}\text{H}_{46}\text{O}_7\text{Si} + \text{Na}]^+$: 689.2902 found, 689.2902 calculated.



1-O-propargyl-2,3-bis-O-acetyl- α -D-ribofuranoside (3)

Compound **2** (660 mg, 0.99 mmol) was dissolved in DCM (20 mL) and TFA (0.6 mL) was added. The reaction was stirred at room temperature for 20 minutes and co-evaporated with toluene. The crude intermediate was dissolved in pyridine (5 mL) and acetic anhydride (1.9 mL) and DMAP (cat.) were added. The reaction was stirred at room temperature for 1 hour, diluted with DCM and washed with sat. aq. NaHCO_3 . The organic layer was dried (MgSO_4), concentrated and co-evaporated with pyridine (2x). The crude intermediate was dissolved in pyridine (5 mL) and HF-pyridine (1 mL) was added. The reaction was stirred at room temperature for 1 hour and carefully quenched with sat. aq. NaHCO_3 . The mixture was extracted with DCM, dried (MgSO_4) and concentrated under reduced pressure. Column chromatography (Pentane/EtOAc, 60/40 – 50/50) yielded the title compound as a white foam (138 mg, 0.57 mmol, 58% over 3 steps). $^1\text{H NMR}$ (500 MHz, CDCl_3) δ : 5.50 (d, $J = 4.5$ Hz, 1H, $\text{H1}'$), 5.24 (dd, $J = 7.3, 3.4$ Hz, 1H, $\text{H3}'$), 5.03 (dd, $J = 7.3, 4.6$ Hz, 1H, $\text{H2}'$), 4.34 (dd, $J = 33.8, 2.4$ Hz, 2H, CH_2CCH), 4.18 (q, $J = 3.3$ Hz, 1H, $\text{H4}'$), 3.85 (AB, $J = 12.1, 3.1$ Hz, 1H, $\text{H5}'$), 3.81 (AB, $J = 12.1, 3.4$ Hz, 1H, $\text{H5}'$), 2.53 (s, 1H, OH), 2.47 (t, J

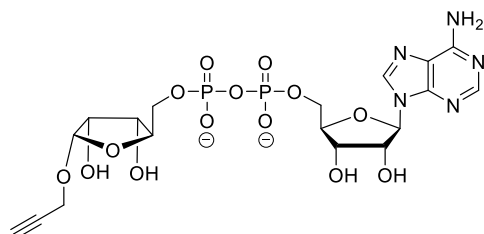
= 2.3 Hz, 1H, CH₂CCH), 2.14 (s, 3H, CH₃ Ac), 2.14 (s, 3H, CH₃ Ac). ¹³C NMR (126 MHz, CDCl₃) δ: 170.90, 170.04 (CO Ac), 98.55 (C1'), 83.07 (C4'), 78.78 (CH₂CCH), 74.98 (CH₂CCH), 71.12 (C3'), 70.02 (C2'), 62.10 (C5'), 54.57 (CH₂CCH), 20.93, 20.62 (CH₃ Ac).



1-O-propargyl-2,3-di-O-acetyl-5-O-(di-tert-butyl)-phosphoryl-α-D-ribofuranoside (4)

Compound **3** (136 mg, 0.50 mmol) and pyridinium chloride (230 mg, 2 mmol) were co-evaporated with pyridine (3x) and dissolved in pyridine (5 mL) under an atmosphere of argon. Di-*tert*-butyl-*N,N*-diisopropylphosphoramidite (0.23 mL, 0.75 mmol) was added and the reaction was stirred at room temperature for 15 minutes. Then *t*BuOOH (5.5 M in nonane) (0.7 mL, 3.75 mmol) was added and the reaction mixture was stirred for 30 minutes. The reaction was quenched upon addition of aq. NaHCO₃ (sat.), extracted with DCM, dried (MgSO₄) and concentrated *in vacuo*. Column chromatography (pentane/EtOAc, 60/40 – 50/50) yielded the title compound as a white foam (172 mg, 0.37 mmol, 74%).

¹H NMR (500 MHz, CDCl₃) δ: 5.49 (d, *J* = 4.5 Hz, 1H, H1'), 5.27 (dd, *J* = 7.2, 3.2 Hz, 1H, H3'), 5.05 (dd, *J* = 7.2, 4.6 Hz, 1H, H2'), 4.33 (d, *J* = 2.2 Hz, 2H, CH₂CCH), 4.27 – 4.25 (m, 1H, H4'), 4.18 – 4.15 (m, 2H, H5'), 2.42 (t, *J* = 2.3 Hz, 1H, CH₂CCH), 2.13 (s, 6H, CH₃ Ac), 1.49 (s, 18H, CH₃ *t*Bu). ¹³C NMR (126 MHz, CDCl₃) δ: 170.47, 169.71 (CO Ac), 98.53 (C1'), 82.85, 82.81, 82.79, 82.75 (Cq. *t*Bu), 81.15, 81.08 (C4'), 78.75 (CH₂CCH), 74.71 (CH₂CCH), 70.82 (C2'), 69.91 (C3'), 65.80, 65.76 (C5'), 54.57 (CH₂CCH), 29.83, 29.82, 29.80, 29.79 (CH₃ *t*Bu), 20.81, 20.54 (CH₃ Ac). ³¹P NMR (202 MHz, CDCl₃) δ: -9.23. HRMS: [C₂₀H₃₃O₁₀P + H]⁺: 465.1885 found, 465.1884 calculated.



α-1-O-propargyl-ADPr (7)

Compound **5** (120 mg, 0.26 mmol) was dissolved in DCM (5.7 mL) and TFA (0.3 mL) was added. The reaction was stirred at room temperature for 15 minutes, co-evaporated with toluene and pyridine (2x). The intermediate phosphate was analysed by ³¹P NMR to confirm the complete removal of both *t*Bu groups (³¹P-NMR (121 MHz, D₆-acetone) δ: -0.14). Intermediate phosphate and dicyanoimidazole (77 mg, 0.65 mmol) were co-evaporated with ACN (3x) and dissolved in dry ACN (2mL). Adenosine amidite **6** (277 mg, 0.39 mmol) was added to the reaction mixture, stirred at room temperature for 15 minutes and *t*BuOOH (5.5 M in nonane) (0.25 mL, 1.38 mmol) was added. The reaction was stirred for 30 minutes, DBU (0.2 mL, 1.4 mmol) was added and the reaction was stirred for an additional 30 minutes. Aq. NH₄OH (35%) (5 mL) was added to the reaction mixture, the reaction was stirred for 16 hours and concentrated under reduced pressure. Size exclusion chromatography followed by lyophilization yielded the title compound as a white powder (100 mg, 0.17 mmol, 65%). ¹H NMR (500 MHz, D₂O) δ: 8.42 (s, 1H, H2), 8.15 (s, 1H, H8), 6.05 (d, *J* = 5.5 Hz, 1H, H1'-ade), 5.04 (d, *J* = 4.0 Hz, 1H, H1'-rib), 4.67 (t, *J* = 5.5 Hz, 1H, H2'-ade), 4.44 (dd, *J* = 5.0, 4.5 Hz, 1H, H3'-ade), 4.32 – 4.29 (m, 1H, H4'-ade), 4.21 – 4.13 (m, 4H, H5'-ade, CH₂CCH), 4.12 – 4.10 (m, 1H, H4'-rib), 4.10 – 4.04 (m, 2H, H2'-rib, H3'-rib), 3.93 – 3.91 (m, 2H, H5'-rib), 2.73 – 2.72 (m, 1H, CH₂CCH). ¹³C NMR (126 MHz, D₂O) δ: 155.49 (C4), 149.03 (C6), 118.59 (C5), 100.62

(C1'-rib), 86.89 (C1'-ade), 83.85, 83.78, 83.66, 83.59 (C4'-rib, C4'-ade), 79.07 (CH₂CCH), 74.29 (C2'-ade), 70.92 (C3'-ade), 70.32 (C2'-rib), 69.48 (C3'-rib), 65.57, 65.54 (C5'-rib), 65.17, 65.13 (C5'-ade), 54.84 (CH₂CCH). ³¹P NMR (202 MHz, D₂O) δ: -10.46, -10.57, -10.65, -10.76. HRMS: [C₁₈H₂₅N₅O₁₄P₂ + H]⁺: 598.0939 found, 598.0946 calculated.

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