Synthetic peptides, nucleic acids and molecular probes to study ADP-Ribosylation

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Chapter 5

A Methodology for the Preparation of Peptides ADP-Ribosylated on Arginine and Arg-ADPr Modified Human Ubiquitin

Introduction

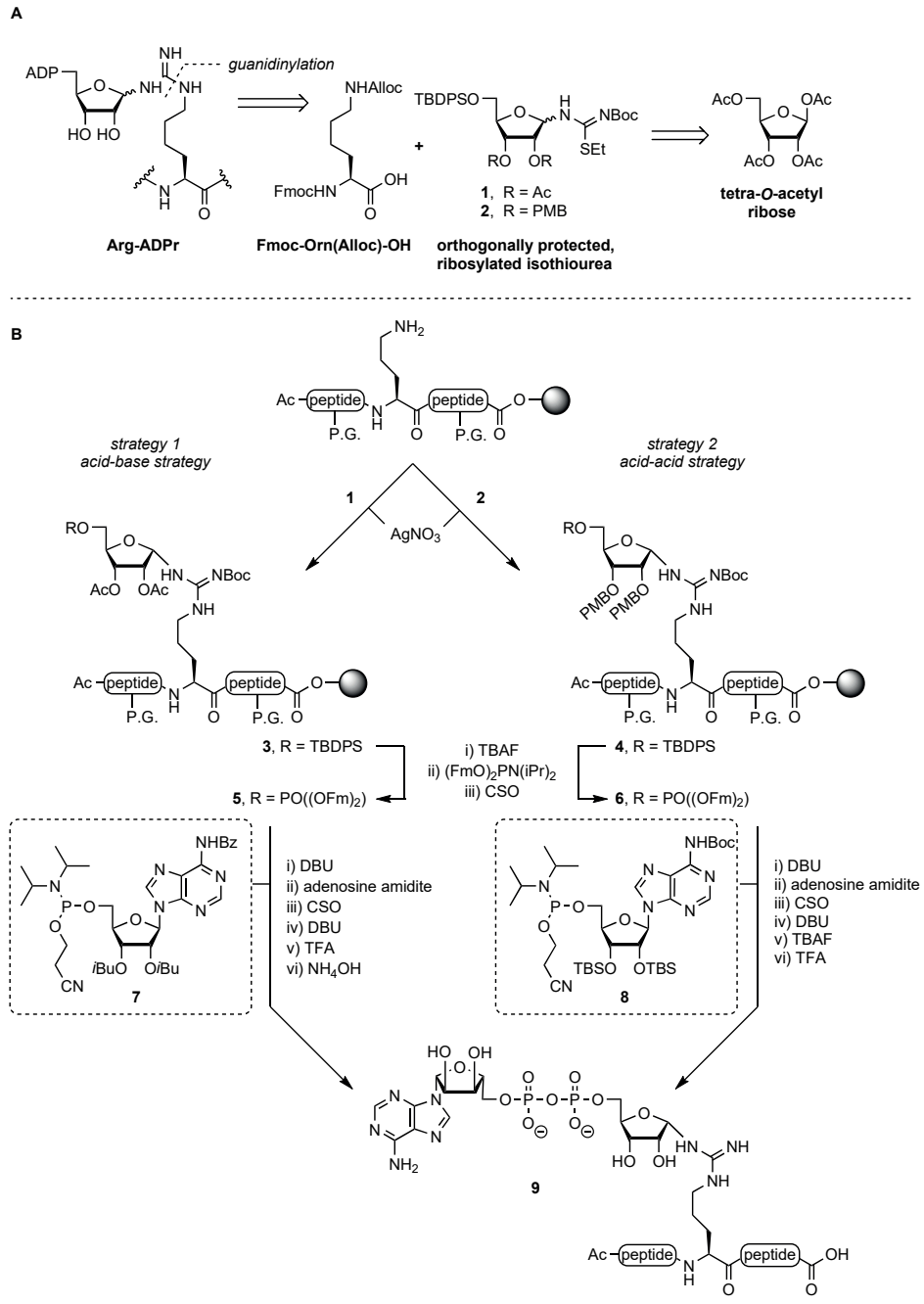
ADP-ribosyl-arginine, the first discovered ADP-ribosylated amino acid, was encountered in *E.Coli* after infection with T4 phages.[1] This finding encouraged a search for identifying more ADP-ribosylated arginine (Arg-ADPr) in proteins originating from both lower and higher organisms. For instance, site-directed mutagenesis of proteins led to the uncovering of several interesting targets to be specifically ADP-ribosylated on their arginine (Arg) residue.[2] However, the mutagenesis studies do not allow for a screening of a wide variety of biological systems for modification sites. Another approach for identifying Arg-ADP-ribosylation sites comprises the generation of antibodies specific for the Arg-ADPr modification.[3–6] Unfortunately, non-specific recognition of the generated antibodies may hinder the proper detection of Arg ADP-ribosylation.[7]

After the initial discovery of Arg-ADPr in 1974, many insights regarding its occurrence and physiological role in various organisms have been acquired.[7] The formation of Arg-ADPr in target proteins is mostly catalyzed by ADP-ribosyl transferases of the cholera toxin-like subfamily (ARTCs) which is characterized by the R-S-E catalytic triad.[8] Specificity of these ARTCs towards guanidinyl groups (Arg and agmatine) is found if the triad is extended with a glutamic acid, leading to an RSE-X-E recognition motif.[9–11] Although ARTCs are extracellular enzymes exclusively,[12] a significant amount of proteins ADP-ribosylated on Arg-residues, are found in the Golgi apparatus or the ER.[13,14] This finding is supported by the fact that the hydrolase ARH1, responsible for the reversal of Arg-ADPr,[15] is localized in the cytoplasm.[12] Despite the information acquired over the past decades, it is still unclear which enzyme is responsible for intracellular Arg ADP-ribosylation. One possibility is that an ARTC ADP-ribosylates Arg whilst being transported to the membrane.[16] Otherwise, an ARTD (ARTs of the subfamily diphtheria toxin-like) that permanently resides inside the cell is likely to be responsible for intracellular Arg ADP-ribosylation. For example ARTD10 which is able to catalyze the formation of Arg-ADPr, albeit that Arg does not appear to be its main target.[17] Up to now, no specific readers that recognize the Arg-ADPr modification are known. Furthermore, the chemical stability of the Arg-ADPr PTM is not properly studied. Knowledge about chemical stability is crucial as some proteomic conditions lead to biased read-outs, intentional[18] or not.[19] This chapter aims for the development of a strategy that allows the preparation of synthetic peptides, ADP-ribosylated on Arg residues as they can provide for useful molecular tools in the research of Arg ADP-ribosylation. To evaluate the ability of the obtained synthetic peptides to be recognized in a biological system a biochemical assay can be performed with DupA (an enzyme encountered in *Legionella pneumophila*) that specifically hydrolyzes the ADP-ribosylated site of Arg42 in human ubiquitin (hUb).

Synthetic strategy

All strategies for the synthesis of peptides, *mono*-ADP-ribosylated (MARylated) at a Ser- (Chapters 2 and 3), Thr- and Cys- (Chapter 3), or Tyr- (Chapter 4) residue relied on the stereoselective coupling of a ribosyl donor and an amino acid acceptor. However, introduction of a guanidinyl group at the anomeric centre of ribose, the linkage by which Arg is bound to ADPr, is usually not achieved via a direct glycosylation procedure. The ubiquity of the guanidine function in bio-molecules and the variety of natural human-made biopolymers has led to the availability of various guanidinylation reagents that also have been applied in the (glyco)peptide chemistry.[20–23] With respect to the synthesis of glycopeptides, two approaches can be discerned. Both strategies utilize the coupling of an *S*-alkyl-isothiourea, situated at the anomeric centre of the desired monosaccharide, with the side chain amine of ornithine (Orn) to synthesise the glycosyl-Arg linkage. In the first approach, the glycosyl-Arg building block is first obtained and then incorporated into a peptide sequence of choice via SPPS.[24,25] In the second approach, the SPPS of a specific peptide with an orthogonally protected Orn at a predetermined position is followed by the selective release of the masked amino group in its side chain. Subsequent on-resin coupling of the free amino group with the S-alkyl-isothiourea functionality yields the prospected Arg-modified glycopeptide.[26,27]

The approach designed to synthesize Arg-ADPr uses the second approach as starting point in which isothiourea function at the anomeric centre of ribose (**1** or **2**, Scheme 1A) is coupled on-resin to the primary amine of an ornithine residue incorporated in an immobilized peptide (Scheme 1A and B). A commercially available ornithine building block was chosen, the Alloc protecting group of which can be selectively removed on-resin relative to acid-labile side chain protecting groups and the TentaGel® S AC linker. Besides, two strategies differing in protecting group patterns on the adenosine and ribosyl moiety have been investigated (Scheme 1B). On the basis of the construction of Tyr-ADPr peptides (See Chapter 4), the first strategy (termed hybrid or more aptly 'acid-base' strategy) uses acetyl groups for the 2' and 3' hydroxyls in the ribosyl moiety and isobutyryl and benzoyl groups for adenosine (Scheme 1B). The side chains of the applied amino acid building blocks are protected with acid labile groups i.e., Trt for Ser- and Thr-residues and Mtt for lysine (Lys), as these are cleavable in low concentrations of TFA. As shown in Scheme 1B, the hybrid strategy entails the silver-promoted coupling of isothiourea **1** with the released amine of the ornithine in the immobilized peptide to give resin **3**. Next, replacement of the silyl-ether by an Fm protected phosphotriester, is followed by DBU mediated deprotection of the phosphate in **5**. With the thus obtained phosphate monoester, the pyrophosphate can be introduced via P^{III} - P^{V} coupling procedure with adenosine phosphoramidite **7** bearing isobutyryl and benzoyl as protecting groups. At the end of the synthesis the immobilized Arg-ADPr peptide is treated with TFA to remove the acid labile protecting groups with



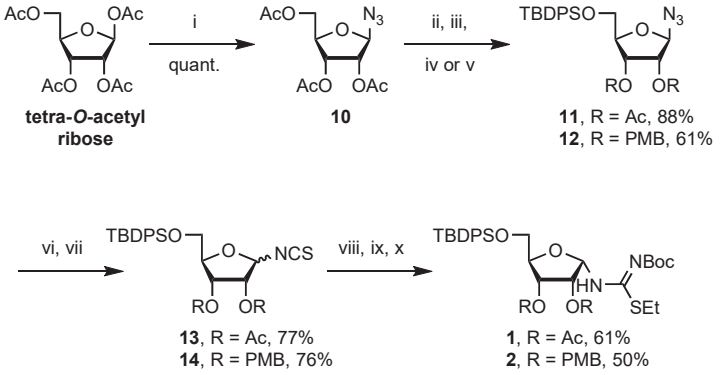
Scheme 1. A) Retrosynthetic analysis for the synthesis of Arg-ADPr via an on-resin guanidinylation approach. **B)** Proposed synthetic strategies to synthesize peptides, MARylated at their Arg-residue. P.G. = protecting group for amino acid side chains i.e., Trt for Ser/Thr/His and Mtt for Lys.

concomitant cleavage from the solid support. Finally, treatment with NH_4OH will result in deprotection of all ester functionalities to obtain MARylated Arg-ADPr peptide **9**. The second, termed here 'acid-acid' strategy, is described in Chapter 3 and circumvents the use of base labile protecting groups by the use of the PMB group for the 2' and 3' hydroxyls in the ribosyl isothiourea **2** (Scheme 1A). The on-resin guanidinylation of **2** with an ornithine residue will result in intermediate peptide **4**. The phosphate moiety is installed in a similar manner as for the hybrid strategy to furnish immobilized phosphotriester **6**. In the next step adenosine phosphoramidite **8**, provided with a Boc-group on the exocyclic amine and silyl ethers on the 2' and 3' hydroxyl, is used to for the introduction of the ADP moiety via the same P^{III} - P^{V} coupling procedure. After removal of the silyl groups, global deprotection and cleavage from the resin is accomplished by treatment with 10% TFA in DCM solution to furnish MARylated Arg-ADPr peptide **9**.

Results and discussion

Building block synthesis

Synthesis of the isothiocyanates **1** and **2** (Scheme 2) commenced with the treatment of the commercially available β-ribofuranose tetra-acetate with $SnCl_4$ as a Lewis acid[28] and TMS-N_3 as an azide donor to give azide **10** in quantitative yield. As described in Chapters 3 and 4, the primary acetate was replaced by the orthogonal TBDPS group by a three-step procedure, comprising deacetylation, silylation and acetylation to provide **11** in 88% overall yield.[29] By replacing the acetylation in this three-step procedure by methoxybenzylation, azide **10** was converted into **12** having the PMB group at the 2'- and 3'-positions with a yield of 61%. Next, the anomeric azides in **11** and **12** were reduced using Adam's catalyst and H_2. Attempts to work up the reaction proved cumbersome as the resulting amine is highly labile and concentration *in vacuo* led to full degradation of the product. Therefore, after filtration over a pad of Celite to rid the reaction mixture of the catalyst, the filtrate was directly treated with thiophosgene to furnish ribosyl isothiocyanates **13** and **14** as anomeric mixtures in 77% and 76% yield respectively. Although Arg-ADPr is known to undergo spontaneous anomerization to its β-anomer after initially being installed α-selectively,[7] the resulting anomers of the isothiocyanates **13** and **14** could easily be separated by column chromatography to obtain the individual anomers in a homogenous fashion. Next, the α-anomer of both **13** and **14** was subjected to ammonolysis by ammonia in THF, followed by protection of the amine in the resulting thiourea with the Boc group and finally treatment with ethyl iodide to furnish building blocks **1** (Ac protected) and **2** (PMB protected), ready for the on-resin, silver-promoted coupling to ornithine.

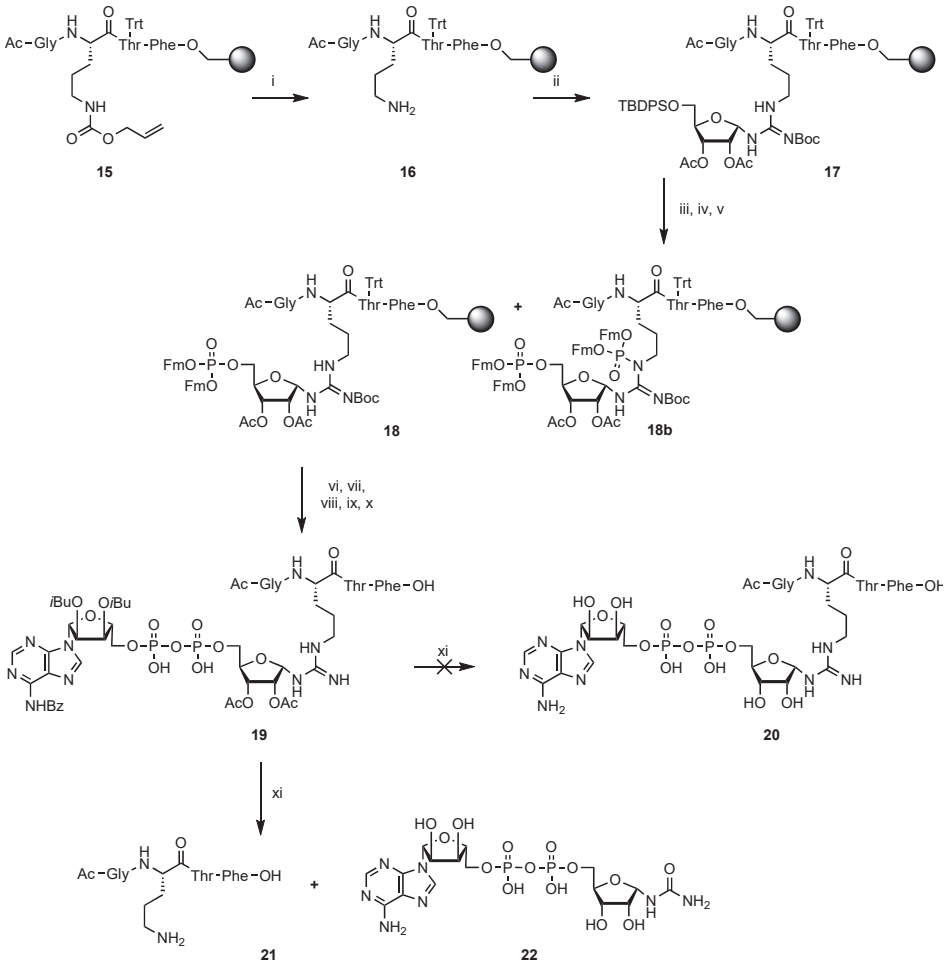


Scheme 2. Synthesis of building blocks **1** and **2**. Reagents and conditions: i) TMS-N_3, $SnCl_4$, DCM. ii) Na, MeOH, 0 °C. iii) TBDPS-Cl, pyr. iv) Ac_2O, DMAP, pyr. v) NaH, PMB-Cl, DMF, 0 °C. vi) PtO_2, H_2, EtOAc. vii) $SCCl_2$, K_2CO_3, water, EtOAc. viii) NH_3, THF. ix) Boc_2O, DMAP, DCM. x) EtI, K_2CO_3, MeCN.

Peptide synthesis

With the appropriate building blocks in hand, the on-resin synthesis of MARylated Arg-ADPr peptide was undertaken. Firstly, the hybrid strategy, using a combination of acid and base labile protecting groups for key building blocks **1** and **7** as outlined in Scheme 3 was investigated. Model tetrapeptide **15** was prepared by standard SPPS with incorporation of Fmoc-Orn(Alloc)-OH on the projected MARylation site. The selective, on-resin removal of the Alloc group with $Pd(PPh_3)_4$ and DMBA as a scavenger in DCM to furnish the primary amine. LC-MS monitoring of this reaction is imperative since, depending on the reagent quality, multiple treatments (up to 5 times) may be required to fully deprotect the ornithine residue. Degassing of the solvent and administering of the reaction solution to the resin in a nitrogen atmosphere did improve the efficiency of this deprotection. After LC-MS analysis showed full deprotection, immobilized peptide **16** was guanidinylated with isothiocyanate **1** using $AgNO_3$ as the Lewis acid. LC-MS analysis of the crude products after treatment with 10% TFA (Figure 1A) showed the lack of starting peptide derived from **16** and the formation of partially protected peptides (with and without the Boc group) derived from **17**. It is noteworthy that during cleavage and deprotection from the resin for a period of 2 hours spontaneous anomerization may occur as analyzed by LC-MS. Next, resin **17** was converted to immobilized phosphotriester **18** by desilylation, phosphitylation of the released alcohol with the bis-Fm-protected phosphoramidite reagent and oxidation of the intermediate phosphite triester. Treatment of resin **18** with 10% TFA and ensuing LC-MS analysis of the released crude product showed partially protected peptides (also with and without the Boc group) derived from **18** and major side products with masses that correspond with one additional Fm-protected phosphotriester (**18b**, Scheme 3, Figure 1B). The formation of **18b** can be explained by the residual nucleophilicity of the urethane protected guanidyl

function. Next, the ADP moiety is appended by deprotection of the phosphotriester in resin **18** (in the presence of **18b**) with a 10% DBU solution and subjecting the formed phosphate monoester to the P^{III} – P^{V} coupling procedure using *i*Bu/Bz protected adenosine amidite **7.** Oxidation was performed with CSO and deprotection of the cyanoethyl group was mediated by DBU. A sample of the resin was taken and treated with 10% TFA. LC-MS analysis of the crude products revealed a complex mixture (Figure 1C) in which peptides derived from **19** (with and without the Boc group) and peptides derived from **18** and **18b** (which was still partially protected with one Fm group) could be assigned. Also, some unreacted but fully deprotected phosphomonoester and the corresponding phosphoramidate were detected, co-eluting with a trityl cation explaining the intense UV-absorption in the LC-MS trace with a retention time of around 4 minutes. Quantification of the obtained products by UV-absorption proved to be cumbersome as products and side products are not base-separated. Therefore, the resin was subjected to a longer TFA treatment to ensure that all Boc protecting groups were removed and the crude product was treated overnight with 28 wt% NH_4OH in water and subsequently analyzed by LC-MS chromatography (Figure 1D). The mass of target Arg-ADPr peptide **20** was not observed, but a major product was detected with the same mass as tetrapeptide **21.** The possible hydrolysis of the guanidine function in **19** leading to **21** is supported by the finding of a similar hydrolysis of Arg-ADP-ribosylated HNP-1 when incubated at pH 9.[30] Moreover hydrolysis of guanidine function in **19** should result in the formation of ADP-ribosyl urea **22**, which indeed is highly abundant in the crude mixture. Due to the lability of the guanidinyl function towards the deprotection conditions of the ester protecting groups, the acid-base strategy was deemed not viable for the synthesis of MARylated Arg-ADPr peptides.



Scheme 3. Attempted synthesis of target tetrapeptide Ac-Gly-Arg^{ADPr}-Thr-Phe-OH **20** using the acid-base strategy. Reagents and conditions: i) $Pd(PPh_3)_4$, DMBA, DCM. ii) **1**, $AgNO_3$, TEA, DMF. iii) TBAF, THF. iv) $(FmO)_2PN(iPr)_2$, ETT, MeCN. v) CSO, MeCN. vi) DBU, DMF. vii) **7**, ETT MeCN. viii) CSO, MeCN. ix) DBU, DMF. x) TFA, DCM. xi) NH_4OH, H_2O.

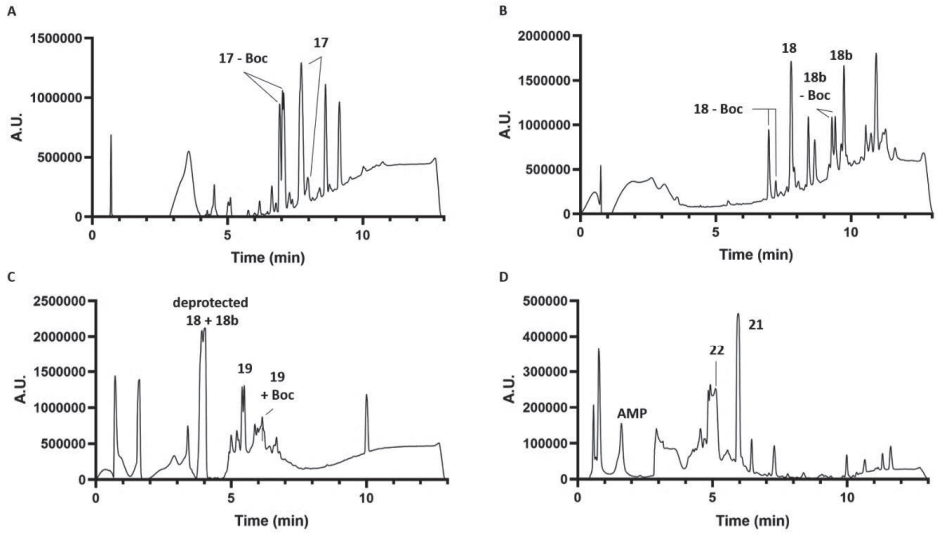
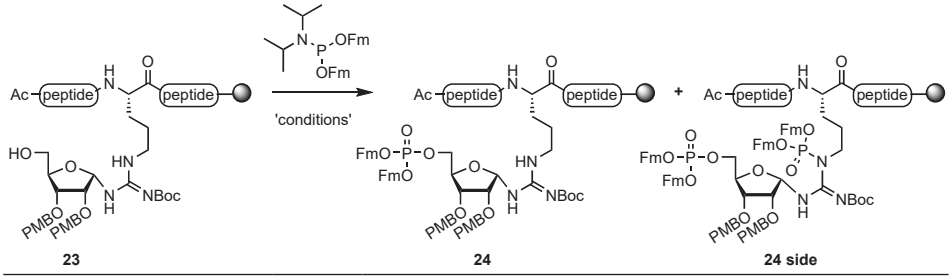


Figure 1. LC-MS traces of the crude intermediates *en route* to peptide **20** via the acid-base strategy as presented in Scheme 3. Resin samples were treated with 10% TFA for 2 hours prior to co-evaporation in a 1:1:1 *t*BuOH:H_2O:MeCN mixture. A) LC-MS analysis of intermediate peptide **17** after guanidinylation of **16** with **1**. B) LC-MS analysis after desilylation of **17** and subsequent phosphitylation with $(FmO)_2PN(iPr)_2$ and oxidation with CSO. C) LC-MS analysis after deprotection of **18** with DBU, P^{III} – P^{V} coupling with adenosine amidite **7** and subsequent treatment with DBU to remove the cyano-ethyl protecting group. D) LC-MS analysis after treatment of the crude product of the TFA cleavage of Figure 1C overnight with NH_4OH.

Therefore, attention was turned towards the acid-acid strategy, characterized by the presence of solely acid labile groups in the protected precursor of the target Arg-ADPr peptide at the final stage of the synthesis. As outlined in Scheme 4, the assembly of Arg-ADPr peptide Ac-Gly-Arg^{ADPr}-Thr-Phe-OH (**20**) started with the SPPS of immobilized peptide **16** as in the hybrid acid-base strategy. Guanidinylation of the amino group in the side chain of ornithine in **16** with isothiourea **2**, of which the cis-diol is protected with PMB ethers, was followed by removal of the primary TBDPS group yielding intermediate peptide **23**. The subsequent introduction of the phosphotriester was investigated more closely to suppress the phosphoramidate formation at the guanidyl function. A set of six reaction conditions was screened varying in activator (ETT, tetrazole and DCI) and equivalents of phosphorylating reagent (2.5 and 5.0 equivalents). The results of this screening are shown in Table 1. Firstly, the same reaction conditions as described above (Scheme 3) led mainly to the formation of phosphoramidate side product (**24 side**, 64%) compared to desired product **24** (36%, entry 1). Reducing the amount of phosphorylating reagent from 5.0 equivalents to 2.5 equivalents did suppress the side product formation in favor of formation of **24** (60% **24** and 40% side product, entry 2). Application of tetrazole

as activator was no improvement as 5.0 equivalents of phosphoramidite led to significant amounts of side product (35% **24**, 65% side product, entry 3) and by reducing the amount of reagent to 2.5 equivalent a ratio of 60% product vs. 40% side product was attained again (entry 4). When DCI was tried with 5.0 equivalents of the phosphitylating reagent, the ratio of product to side product did improve but still showed a slight preference for the side product which was formed in 56% (entry 5). Fortunately, when 2.5 equivalents of the phosphoramidite was used, the amount of product **24** being formed increased to 84% (entry 6).

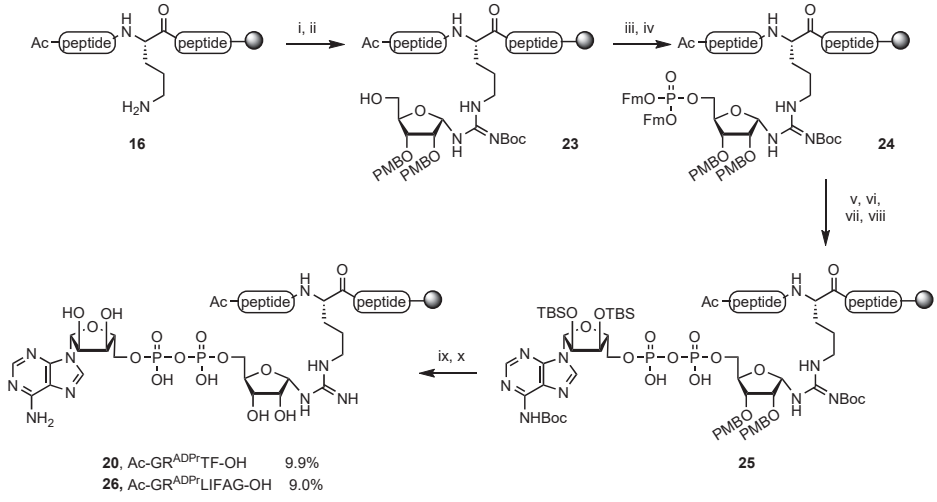
Table 1. Screening of the phosphitylation conditions for immobilized peptide **23** to give **24** and to suppress **24 side**. Product ratios were calculated by integration of peak area of UV absorptions in their LC-MS trace. Every reaction was carried out on a 10 µmol scale with resin **23**. The equivalents in the table are the equivalents of bis-Fm-protected amidite relative to **23**. The concentration is calculated relative to the phosphorylating reagent, 2.0 equivalents of activator was used relative to the phosphoramidite and all reactions were carried out for 30 minutes. After the phosphitylation, all reactions were treated with CSO for 30 minutes to oxidize the phosphite intermediates. The resin was then treated with 10% TFA and 2.5% TIS in DCM for 120 minutes to cleave the peptide from the resin. From the crude reaction mixture, a sample was prepared for LC-MS analysis.



Entry	Activator	Eq	C (mM)	Product 24 (%)	Side-product 24 (%)
1	ETT	5	88	36	64
2	ETT	2.5	44	60	40
3	Tetrazole	5	88	35	65
4	Tetrazole	2.5	44	60	40
5	DCI	5	88	44	56
6	DCI	2.5	44	84	16

Subsequently, the Fm protecting groups in **24**, obtained by applying the latter optimized phosphitylation conditions, were removed with DBU to give the phosphomonoester. The P^{III} - P^{V} coupling procedure to install the pyrophosphate started with the coupling of phosphomonoester was with TBS/Boc protected adenosine amidite **8** (Scheme 1B) using the same, optimized conditions (Table 1 entry 6) to avoid phosphoramidate formation.

Subsequent oxidation with CSO and removal of the cyanoethyl group in the pyrophosphate moiety led to protected and immobilized Arg-ADP-ribosylated target peptide **25**. The silyl ethers on the adenosine moiety were removed by treatment of the resin with a 1 M TBAF solution, as described in Chapter 3. Finally, the peptide was cleaved from the resin with concomitant loss of all remaining protecting groups (e.g., Boc, PMB and Trt) by administration of the resin to a 10% TFA in DCM solution. HPLC purification of the crude mixture led to the isolation of the first synthetic mono-Arg-ADPr linked peptide **20** in 9.9% overall yield. Encouraged by this result the same method was adopted for the synthesis of heptapeptide **26** (Scheme 4), sequence of which is derived from human hUb and known to be ADP-ribosylated on its Arg residue (Arg42 in human Ub). After prep-HPLC purification, peptide **26** was obtained in 9.0% yield.



Scheme 4. Synthesis of Arg-ADP-ribosylated peptides **20** and **26** using the acid-labile strategy. Reagents and conditions: i) **2**, $AgNO_3$, TEA, DMF. ii) TBAF, THF. iii) $(FmO)_2PN(iPr)_2$, DCI, MeCN. iv) CSO, MeCN. v) DBU, DMF. vi) **8**, DCI, MeCN. vii) CSO, MeCN. viii) DBU, DMF. ix) TBAF, THF. x) TFA, DCM.

A next challenge for the newly developed acid-acid strategy to obtain Arg-ADPr peptides comprised the synthesis of the full-length hUb with the ADPr modification on its Arg-42 residue. Therefore, a Ub mutant where Arg42 was replaced by an Alloc-protected ornithine residue was synthesized on a 6 µmol scale using a well-established SPPS approach.[31] Next, the installation of the Arg-ADPr moiety started with the removal of the orthogonal Alloc-protecting group on the resin-bound synthetic Ub. The efficiency of the subsequent coupling of the released amine in ornithine with ribosyl building block **2** was controlled by the following procedure. Cleavage of a test sample from the resin was performed and

LC-MS analysis of the crude product after coupling showed a clean LC-MS trace with the mass corresponding to the Ub-ribosylated intermediate. The protocol to synthesize Ub-ADPr was continued with slight changes as for the purpose of biochemical assays the synthetic Ub was equipped with a biotin tag on the *C*-terminus. Oxidation of the phosphite intermediate species was therefore performed using a 0.5 M *t*BuOOH solution in MeCN to avoid overoxidation of the biotin-tag as described in Chapter 3. Also, human Ub carries three additional Arg residues protected with the Pbf group in the SPPS protocol. These protecting groups require prolonged reaction times in high concentrations of TFA in order to be fully removed, typically 95% TFA for 2 hours. Therefore, a test cleavage, using 95% TFA was performed. Strikingly, the glycosidic guanidine moiety as well as the pyrophosphate bridge remained intact during a 1.5-hour treatment with 95% TFA which was sufficient to fully remove all protecting groups including the Pbf-groups. The remainder of the resin-bound Ub-ADPr was fully deprotected according to the renewed cleavage/deprotection protocol and HPLC purification yielded fully synthetic, human Ub, MARylated on its Arg42 residue in an overall yield of 0.5%.

Biochemical evaluation of synthetic Arg-ADPr peptides

Peptides **20** and **26** bearing the native ADPr-Arg linkage were assessed in their ability to be properly recognized in biologically relevant systems. Therefore, their ability to function as a substrate for DupA, an enzyme originating from *Legionella pneumophila*, was evaluated. In short, *Legionella p.* has multidomain containing SidE ligases which can ADP-ribosylate the Arg42 residue on human Ub with their MARylating domain. Subsequent translocation of the ADP-ribosylated Ub to their phosphodiesterase (PDE) domain allow the ligation of ubiquitin to a serine residue of host substrate proteins. The substrate protein is interconnected with ubiquitin via the phosphoribosyl moiety originating from ADPr and this process is an important factor in the bacterial infection, proliferation and host immune evasion.[32] Legionella carefully controls its host protein ubiquitination activity by the deployment of Dup enzymes, DupA and DupB, which release the ubiquitinated substrate.[33] DupA is also known to accept and hydrolyze the pyrophosphate bridge in Ub-ADPr with concomitant release of AMP, making DupA ideally suited for a hydrolytic assay with synthetic peptides **20** and **26**.

Tetramer **20** with a random sequence and heptamer **26**, a sequence representing the ADP-ribosylated Arg42 residue targeted by DupA, were compared in a DupA mediated hydrolysis assay (Figure 2) with enzymatically obtained Ub, ADP-ribosylated on Arg42[34] as a reference. The peptides were incubated in the presence or absence of DupA under buffered conditions and at indicated times analyzed using mass-spectrometry. Incubation of 5 µM 'nonsense' tetramer **20** and 3 µM DupA did not result in DupA mediated cleavage of the pyrophosphate bond. This indicates the ADP-ribosylated tetramer is not recognized

and processed by the enzyme. Incubation of 5 µM peptide **26** and 3 µM DupA resulted in 40% hydrolysis after 90 minutes. The observed hydrolysis of the pyrophosphate bond can be attributed to the catalytic activity of DupA. This confirms that, although slower than full-length Ub-ADPr, peptide **26** is recognized and processed by DupA. However, since **26** is a smaller fragment of Ub-ADPr as compared to native full-length Ub-ADPr (3 µM Ub-ADPr, 1 µM DupA, 100% hydrolyzed) it is likely that the full-length Ub-ADPr is recognized more efficiently by DupA.

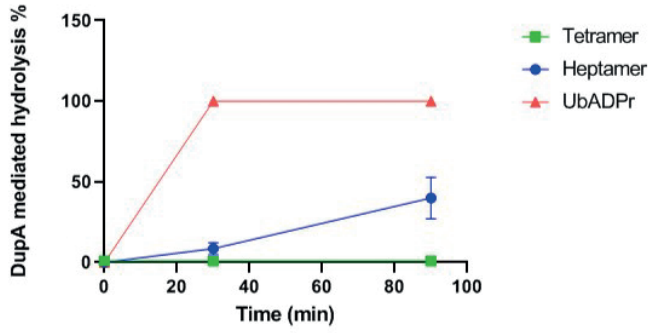


Figure 2. DupA mediated hydrolysis of tetramer **20**, heptamer **26** as compared to enzymatically obtained Ub-ADPr.[34] DupA mediated hydrolysis is measured over time as followed by mass-spectrometry.

Conclusion

This chapter describes the development of the first methodology for the synthesis of Arg-ADP-ribosylated peptides. Key event in the methodology is the coupling of an orthogonally protected, ribosylated isothiocyanate with the amine in the side chain of an ornithine residue furnishing the ribosylated guanidinyl functionality of Arg. The methodology was combined with the phosphoramidite method for the on-resin introduction of a phosphate monoester to give phosphoribosylated Arg. The phosphate monoester was subsequently employed in a P^{III} – P^{V} coupling procedure to install the pyrophosphate bridge to complete the ADPr moiety. With this new methodology, Arg ADP-ribosylated peptides **20** and **26** were obtained in 9.9 and 9.0% yield respectively. In an ultimate test for the applicability of this method for preparation of Arg-ADPr peptides, fully synthetic ubiquitin, ADP-ribosylated on Arg42 was prepared in 0.5% overall yield.

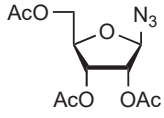
Besides NMR, LC-MS and HRMS analysis of peptides **20** and **26**, their ability to be recognized in biologically relevant settings was evaluated. Therefore, a hydrolytic assay was performed with DupA, an enzyme known to hydrolyze the pyrophosphate bridge in human ubiquitin ADP-ribosylated on Arg42. Tetramer **20** showed no hydrolytic turnover with DupA, most likely due to a mismatch of peptide sequence and recognition of the active site of DupA. Heptamer **26**, having the sequence flanking the Arg42 residue in hUb, in fact was recognized by DupA albeit less efficient than full-length Ub-ADPr. The fact that DupA can recognize heptamer **26**, together with analytical data (NMR, LC-MS, HRMS) concludes that this is the first report on the fully synthetic native Arg-ADP-ribosylated peptides.

Experimental section

General synthetic procedures

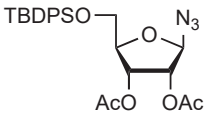
All reagents were used as received unless stated otherwise. Solvents used in synthesis were dried and stored over 4Å molecular sieves, except for MeOH and MeCN which were stored over 3Å molecular sieves. Triethylamine (TEA) and diisopropylethylamine (DIPEA) were stored over KOH pellets. Column chromatography was performed on silica gel 60 Å (40-63 µm, Macherey-Nagel). TLC analysis was performed on Macherey-Nagel aluminium sheets (silica gel 60 F_{254}). TLC was used to visualize compounds by UV at wavelength 254 nm and by spraying with either cerium molybdate spray (25 g/L $(NH_4)_6Mo_7O_{24}$, 10 g/L $(NH_4)_4Ce(SO_4)_4{\cdot}H_2O$ in 10% H_2SO_4 water solution) or $KMnO_4$ spray (20 g/L $KMnO_4$ and 10 g/L K_2CO_3 in water) followed by charring at c.a. 250 °C. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Nucleodur C18 Gravity 3 µm 50 x 4.60 mm column (detection at 200-600 nm) coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI or coupled to a Thermo LCQ Fleet Ion mass spectrometer with ESI. The method used was 10→90% 13.5 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: 10% to 90% MeCN; 8.5→ 11 min: 90% MeCN; 11→13.5 min: 10% MeCN) or 0→50% 13.5 min. NMR spectra were recorded on a Bruker AV-400, AV-500 or AV-600 NMR. Chemical shifts (δ) are given in ppm relative to tetramethyl silane. Coupling constants (*J*) are given in Hz. All given ^{13}C-APT spectra are proton decoupled. In case of synthetic Ub-ADPr, HPLC purification was performed on a Shimadzu semi-preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 µm OBD (10 x 150 mm) column at a flowrate of 6.5 mL/min. using 2 mobile phases: A: MQ + 0.05% FA, B: MeCN + 0.05 % FA. Gradient: 0 -> 100% B. High resolution mass spectra were recorded on a Waters XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.0 kV, desolvation gas flow 900 L/hr, temperature 250 °C) with resolution R = 22000 (mass range m/z = 50-2000) and 200 pg/uL Leu-Enk (m/z = 556.2771) as a "lock mass".

2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl azide (10)



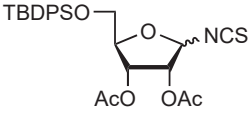
The title compound was prepared according to a modified, literature procedure.[28] Commercially available 1,2,3,5-tetra-*O*-acetyl-β-ribofuranose (7.96 g, 25 mmol) was dissolved in DCM (225 mL, 0.1 M). TMS-N_3 (3.65 mL, 27.5 mmol, 1.1 eq.) and a 1.0 M $SnCl_4$ solution in DCM (25 mL, 25 mmol, 1.0 eq.) were added to the reaction. The reaction was stirred for 2 hours after which TLC indicated full conversion. The reaction was carefully quenched with sat. aq. $NaHCO_3$ and transferred into a separatory funnel. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (30% EtOAc in pentane) furnished the title compound as a colorless oil in quantitative yield. Spectral data was in accordance with literature.[28] **Rf**: 0.43 (30% EtOAc in pentane). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 5.38 (d, *J* = 2.0 Hz, 1H, H-1), 5.34 (dd, *J* = 6.8, 4.8 Hz, 1H, H-3), 5.14 (dd, *J* = 4.8, 2.0 Hz, 1H, H-2), 4.42 (dd, *J* = 12.1, 3.2 Hz, 1H, H-5_a), 4.39 – 4.33 (m, 1H, H-4), 4.15 (dd, *J* = 12.1, 4.2 Hz, 1H, H-5_b), 2.13 (s, 6H, 2x Ac), 2.08 (s, 3H, Ac). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 170.5, 169.5, 169.4 (C=O Ac), 92.6 (C-1), 79.3 (C-4), 74.4 (C-2), 70.4 (C-3), 62.9 (C-5), 20.6, 20.5, 20.4 (CH_3 Ac).

2,3-di-*O*-acetyl-5-*tert*-butyldiphenylsilyl-β-D-ribofuranosyl azide (11)



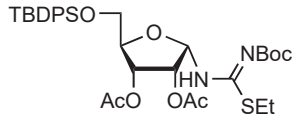
The title compound was prepared according to a modified, literature procedure.[29] Compound **10** (7.53 g, 25 mmol) was dissolved in MeOH (125 mL, 0.2 M) and the solution was cooled to 0 °C. A catalytic amount of Na(s) was added to the solution and the reaction was stirred for 2 hours after which an additional catalytic amount of Na(s) was added. After an additional 30 minutes of stirring, the reaction mixture was acidified with Amberlite H^+ resin till pH ≈ 6.0. The reaction was filtered and concentrated *in vacuo*. The crude triol (Rf = 0.36 in 10% MeOH in DCM) was co-evaporated with pyridine extensively, dissolved in pyridine (250 mL, 0.1 M) after which TBDPS-Cl (7.15 mL, 27.5 mmol, 1.1 eq.) was added and the reaction was stirred overnight. TLC indicated full conversion of the triol into a higher running product (Rf = 0.27 in 10% EtOAc in pentane). To the reaction mixture, DMAP (305 mg, 2.5 mmol, 0.1 eq.) and Ac_2O (11.8 mL, 125 mmol, 5.0 eq.) were added. After 1.5 hours of stirring, TLC indicated full conversion of the starting material. The reaction was quenched by the addition of MeOH and concentrated *in vacuo*. The crude residue was taken up in EtOAc and the resulting solution was washed with 1 M HCl, sat. aq. $NaHCO_3$ and brine successively. The organic layer was dried over $MgSO_4$, filtrated and concentrated *in vacuo*. Flash column chromatography (20 -> 30% Et_2O in pentane) furnished the title compound as a colorless oil (10.9 g, 21.9 mmol, 88% over three steps). **Rf:** 0.75 in 20% EtOAc in pentane. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.78 – 7.63 (m, 4H, TBDPS arom.), 7.48 – 7.35 (m, 6H, TBDPS arom.), 5.50 (t, *J* = 5.3 Hz, 1H, H-3), 5.42 (d, *J* = 3.0 Hz, 1H, H-1), 5.20 (dd, *J* = 4.9, 3.0 Hz, 1H, H-2), 4.21 (dt, *J* = 5.7, 3.6 Hz, 1H, H-4), 3.85 (dd, *J* = 11.4, 3.5 Hz, 1H, H-5_a), 3.72 (dd, *J* = 11.5, 3.6 Hz, 1H, H-5_b), 2.11 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.08 (s, 9H, *t*Bu TBDPS). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 169.7, 169.6 (C=O Ac), 135.8, 135.8 (CH arom. TBDPS), 132.9 (Cq TBDPS), 130.0, 129.9, 127.9, 127.9 (CH arom. TBDPS), 92.9 (C-1), 82.6 (C-4), 74.9 (C-2), 71.0 (C-3), 63.4 (C-5), 26.8 (CH_3 *t*Bu), 20.7 (CH_3 Ac), 19.3 (Cq *t*Bu).

2,3-di-*O*-acetyl-5-*tert*-butyldiphenylsilyl-D-ribofuranosyl isothiocyanate (13)



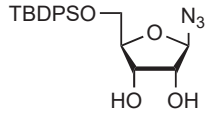
Compound **11** (10.0 g, 20.1 mmol) was dissolved in EtOAc (200 mL, 0.1 M) and the reaction was purged with nitrogen whilst sonicating for 10 minutes. To the stirred solution PtO_2 (913 mg, 4.02 mmol, 0.2 eq.) was added. The solution was purged with H_2 for 2 hours after which the reaction was filtered over a pad of Celite. To the filtrate water was added (200 mL) followed by K_2CO_3 (11.1 g, 80.5 mmol, 4.0 eq.) and thiophosgene (3.1 mL, 40.2 mmol, 2.0 eq.). The suspension was vigorously stirred overnight after which it was transferred into a separatory funnel and sat. aq. $NaHCO_3$ was added. The water layer was extracted thrice with EtOAc and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (20 -> 40% Et_2O in pentane) yielded the title compound as a colorless oil (α-anomer 3.26 g, 6.35 mmol. β-anomer 4.73 g, 9.21 mmol. Combined total yield of 77%). *α-anomer:* **Rf:** 0.71 in 20% Et_2O in pentane. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.66 (m, 4H, TBDPS arom.), 7.39 (m, 6H, TBDPS arom.), 5.66 (d, *J* = 5.2 Hz, 1H, H-1), 5.58 (dd, *J* = 6.0, 1.8 Hz, 1H, H-3), 5.34 (dd, *J* = 6.1, 5.2 Hz, 1H, H-2), 4.34 (q, *J* = 2.4 Hz, 1H, H-4), 3.80 (qd, *J* = 11.5, 2.7 Hz, 2H, H-5), 2.17 (s, 3H, Ac), 2.15 (s, 3H, Ac), 1.06 (s, 9H, *t*Bu TBDPS). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 170.1, 169.4 (C=O Ac), 140.2 (NCS), 135.5, 135.5 (CH arom. TBDPS), 132.5, 132.4 (Cq TBDPS), 130.0, 129.9, 127.9, 127.8 (CH arom. TBDPS), 85.2 (C-1), 85.0 (C-4), 72.4 (C-2), 70.6 (C-3), 63.3 (C-5), 26.7 (CH_3 *t*Bu), 20.7, 20.1 (CH_3 Ac), 19.1 (Cq *t*Bu). *β-anomer:* **Rf:** 0.37 in 20% Et_2O in pentane. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.78 – 7.62 (m, 4H, TBDPS arom.), 7.49 – 7.35 (m, 6H, TBDPS arom.), 5.56 (d, *J* = 3.5 Hz, 1H, H-1), 5.49 (t, *J* = 5.0 Hz, 1H, H-3), 5.42 (dd, *J* = 4.9, 3.6 Hz, 1H, H-2), 4.19 (dt, *J* = 5.0, 3.5 Hz, 1H, H-4), 3.88 – 3.69 (m, 2H, H-5), 2.12 (s, 3H, Ac), 2.05 (s, 3H, Ac), 1.09 (s, 9H, *t*Bu TBDPS). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 169.7, 169.4 (C=O Ac), 143.3 (NCS), 135.8, 135.8 (CH arom. TBDPS), 132.9, 132.7 (Cq TBDPS), 130.0, 129.9, 128.0 (CH arom. TBDPS), 88.0 (C-1), 83.1 (C-4), 75.6 (C-2), 71.3 (C-3), 63.5 (C-5), 26.8 (CH_3 *t*Bu), 20.7, 20.6 (CH_3 Ac), 19.3 (Cq *t*Bu). **HRMS:** $[C_{26}H_{31}NO_6SSi + Na]^+$ found: 536.1544, calculated: 536.1534.

1-(*tert*-butoxycarbonyl)-3-(2,3-di-*O*-acetyl-5-*tert*-butyldiphenylsilyl-α-D-ribofuranos-1-yl)-2-ethylisothiourea (1)



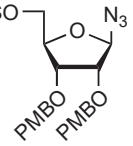
Compound **13** (3.26 g, 6.35 mmol) was dissolved in THF (32 mL, 0.2 M). The solution was saturated with ammonia by bubbling NH_3 (g) through the solution for 1 hour. The reaction was monitored by TLC and after complete conversion of starting material, the reaction was purged with N_2 for 1 minute. The crude thiourea was concentrated *in vacuo* until a white foam was obtained. The crude product was dissolved in DCM (64 mL, 0.1 M) and DMAP (78 mg, 0.64 mmol, 0.1 eq.), TEA (1.77 mL, 12.7 mmol, 2.0 eq.) and Boc_2O (1.60 mL, 6.99 mmol, 1.1 eq.) were added and the reaction was stirred overnight. The reaction was diluted with DCM and the solution was washed with water and brine consecutively. The water layers were extracted with DCM and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was dissolved in MeCN (64 mL, 0.1 M) and K_2CO_3 (8.78 g, 63.5 mmol, 10.0 eq.) and EtI (1.78 mL, 22.2 mmol, 3.5 eq.) were added to the solution. The suspension was stirred overnight and taken up in EtOAc. The organic layer was washed with brine and the water layer was extracted with EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (10 -> 30% EtOAc in pentane) afforded the title compound as a white foam (2.54 g, 3.86 mmol, 61%). **Rf:** 0.26 in 10% EtOAc in pentane. **^{1}H NMR:** (500 MHz, $CDCl_3$) δ 10.66 (s, 1H, NH), 7.79 – 7.60 (m, 4H, TBDPS arom.), 7.47 – 7.31 (m, 6H, TBDPS arom.), 5.86 (dd, *J* = 8.5, 5.7 Hz, 1H, H-1), 5.62 (dd, *J* = 5.4, 1.8 Hz, 1H, H-3), 5.48 (t, *J* = 5.6 Hz, 1H, H-2), 4.22 (q, *J* = 2.6 Hz, 1H, H-4), 3.77 (d, *J* = 2.9 Hz, 2H, H-5), 3.13 (qd, *J* = 7.5, 1.1 Hz, 2H, CH_2 SEt), 2.22 (s, 3H, Ac), 2.10 (s, 3H, Ac), 1.50 (s, 9H, Boc), 1.31 (t, *J* = 7.5 Hz, 3H, CH_3 SEt), 1.09 (s, 9H, *t*Bu TBDPS).**^{13}C NMR:** (126 MHz, $CDCl_3$) δ 172.7 (RHN-*C*-(NBoc)(SEt)), 169.9, 169.1 (C=O Ac), 161.6 (C=O Boc), 135.6, 135.5 (CH arom. TBDPS), 132.6, 132.5 (Cq TBDPS), 129.8, 129.8, 127.8, 127.7 (CH arom. TBDPS), 82.5 (C-4), 81.7 (C-1), 79.2 (Cq *t*Bu Boc), 72.0 (C-3), 70.3 (C-2), 63.6 (C-5), 28.1 (CH_3 Boc), 26.7 (CH_3 *t*Bu TBDPS), 25.1 (CH_2 SEt), 20.4, 20.3 (CH_3 Ac), 19.1 (Cq *t*Bu TBDPS), 13.8 (CH_3 SEt). **HRMS:** $[C_{33}H_{46}N_2O_8SSi + Na]^+$ found: 659.2815, calculated: 659.2817.

5-*O*-*tert*-butyl-diphenylsilyl -β-D-ribofuranosyl azide



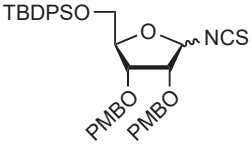
Compound **10** (2.99 g, 9.92 mmol was dissolved in MeOH (50 mL, 0.2 M). A 25 wt% solution of NaOMe in MeOH (0.23 mL, 0.99 mmol, 0.1 eq.) was added and the solution was stirred for 1 hour. The reaction was quenched with Amberlite H^+ resin, filtered and concentrated *in vacuo*. The resulting residue was co-evaporated extensively with pyridine and dissolved in pyridine (100 mL, 0.1 M). TBDPS-Cl (2.8 mL, 11 mmol, 1.1 eq.) was added and the reaction was stirred overnight. The reaction was quenched by the addition of MeOH and the reaction was concentrated *in vacuo*. The residue was taken up in EtOAc and the resulting solution was washed with 1 M HCl, sat. aq. $NaHCO_3$ and brine consecutively. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (20 -> 40% EtOAc in pentane) yielded the title compound as a colorless oil (3.65 g, 8.83 mmol, 89%). **Rf:** 0.54 in 40% EtOAc in pentane. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.77 – 7.62 (m, 4H, TBDPS arom.), 7.51 – 7.32 (m, 6H, TBDPS arom.), 5.31 (d, J = 1.9 Hz, 1H, H-1), 4.36 (t, J = 6.3, 4.8 Hz, 1H, H-3), 4.05 (dt, J = 6.3, 4.2 Hz, 1H, H-4), 3.96 (dd, J = 4.8, 2.0 Hz, 1H, H-2), 3.83 (qd, J = 11.2, 4.2 Hz, 2H, H-5), 3.06 (bs, 1H, OH), 2.60 (bs, 1H, OH), 1.08 (s, 9H, *t*Bu TBDPS). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 135.7 (CH arom. TBDPS), 133.1, 133.0 (Cq TBDPS), 130.0, 130.0, 128.0, 127.9 (CH arom. TBDPS), 94.9 (C-1), 83.9 (C-4), 75.6 (C-2), 71.5 (C-3), 63.9 (C-5), 26.9 (CH_3 *t*Bu TBDPS), 19.3 (Cq *t*Bu TBDPS). **HRMS:** $[C_{21}H_{37}N_3O_4Si + Na]^+$ found: 436.1664, calculated: 436.1663.

5-*O*-((*tert*-butyl)-diphenylsilyl)-2,3-di-*O*-(4-methoxybenzyl)-β-D-ribofuranosyl azide (12)



5-*O*-((*tert*-butyl)-diphenylsilyl) -β-D-ribofuranosyl azide (3.65 g, 8.83 mmol) and TBABr (570 mg, 1.77 mmol, 0.2 eq.) were co-evaporated in toluene before they were dissolved in DMF (44 mL, 0.2 M). PMB-Cl (4.80 mL, 35.3 mmol, 4.0 eq.) was added and the solution was cooled to 0 °C. NaH (60 wt% dispersion in oil, 1.06 g, 26.5 mmol, 3.0 eq.) was added and the reaction was stirred for 15 minutes before the ice bath was removed. The reaction was stirred for an additional hour before TLC showed full conversion of the starting material. The reaction was cooled to 0 °C and carefully quenched by the addition of sat. aq. $NaHCO_3$. After bubbling seized, the emulsion was transferred into a separatory funnel and diluted with sat. aq. $NaHCO_3$. The water layer was extracted thrice with Et_2O and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (20 -> 30% Et_2O in pentane) furnished the title compound (3.91 g, 5.98 mmol, 68%) as a clear oil. **Rf:** 0.56 in 30% Et_2O in pentane. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.67 (ddt, J = 8.1, 6.5, 1.6 Hz, 4H, TBDPS arom.), 7.45 – 7.33 (m, 6H, TBDPS arom.), 7.32 – 7.25 (m, 2H, PMB arom.), 7.22 – 7.14 (m, 2H, PMB arom.), 6.92 – 6.78 (m, 4H, PMB arom.), 5.37 (d, J = 2.2 Hz, 1H, H-1), 4.55 (q, J = 11.8 Hz, 2H, CH_2 PMB), 4.49 – 4.34 (m, 2H, CH_2 PMB), 4.22 (dt, J = 6.6, 3.3 Hz, 1H, H-4), 4.16 (dd, J = 6.7, 4.5 Hz, 1H, H-3), 3.84 (dd, J = 11.5, 3.1 Hz, 1H, H-5_a), 3.78 (s, 3H, CH_3 PMB), 3.77 (s, 3H, CH_3 PMB), 3.73 (dd, J = 4.6, 2.2 Hz, 1H, H-2), 3.69 (dd, J = 11.5, 3.5 Hz, 1H, H-5_b), 1.04 (s, 9H, CH_3, *t*Bu TBDPS). **^{13}C NMR:** (101 MHz, CDCl3) δ 159.5, 159.4 (Cq PMB), 135.7, 135.7, 134.9 (CH arom. TBDPS), 133.2, 133.1 (Cq TBDPS), 129.8, 129.8, 129.8 (CH arom. TBDPS/PMB), 129.7 (Cq PMB), 129.7, 129.6 (CH arom. TBDPS/PMB), 129.5 (Cq PMB), 127.8, 127.8, 114.0, 113.9, 113.8 (CH arom. PMB), 93.2 (C-1), 83.0 (C-4), 79.7 (C-2), 76.0 (C-3), 72.2, 72.1 (CH_2 PMB), 63.1 (C-5), 55.3, 55.3 (CH_3 PMB), 26.8 (CH_3 TBDPS), 19.3 (Cq *t*Bu TBDPS). **HRMS:** $[C_{37}H_{43}N_3O_6Si + Na]^+$ found: 676.2812, calculated: 676.2813.

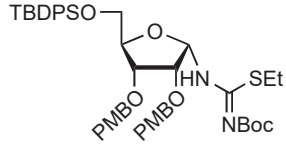
5-*O*-((*tert*-butyl)-diphenylsilyl)-2,3-di-*O*-(4-methoxybenzyl)-α,β-D-ribofuranosyl isothiocyanate (14)



Compound **12** (1.34 g, 2.04 mmol) was dissolved in EtOAc (20 mL. 0.1 M) and the reaction was purged with nitrogen whilst sonicating for 10 minutes. To the stirred solution PtO_2 (93 mg, 0.41 mmol, 0.2 eq.) was added. The solution was purged with H_2 for 2 hours after which the reaction was filtered over a pad of Celite. To the filtrate water was added (20 mL) followed by K_2CO_3 (11.3 g, 8.16 mmol, 4.0 eq.) and thiophosgene (313 µL, 4.08 mmol, 2.0 eq.). The suspension was vigorously stirred overnight after which it was transferred into a separatory funnel and sat. aq. $NaHCO_3$ was added. The water layer was extracted thrice with EtOAc and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (10 -> 15% Et_2O in pentane) yielded the title compound as a colorless oil (α-anomer 670 mg, 1.00 mmol. β-anomer 378 mg, 0.56 mmol. Combined total yield of 76%). *α-anomer:* **Rf:** 0.36 in 20% Et_2O in pentane. **^{1}H NMR:** (400 MHz, CDCl3) δ 7.57 (ddt, J = 8.3, 6.6, 1.5 Hz, 4H, TBDPS arom.), 7.47 – 7.33 (m, 6H, TBDPS arom.), 7.33 – 7.26 (m, 4H, PMB arom.), 6.90 – 6.81 (m, 4H, PMB arom.), 5.31 (d, J = 4.5 Hz, 1H, H-1), 4.74 (d, J = 11.7 Hz, 1H, CH_{2a} PMB), 4.61 (s, 2H, CH_2 PMB), 4.49 (d, J = 11.7 Hz, 1H, CH_{2b} PMB), 4.25 (q, J = 2.7 Hz, 1H, H-4), 4.09 – 4.00 (m, 2H, H-2 + H-3), 3.79 (s, 3H, CH_3 PMB), 3.78 (s, 3H, CH_3 PMB), 3.64 (dd, J = 11.5, 3.3 Hz, 1H, H-5_a), 3.55 (dd, J = 11.4, 2.8 Hz, 1H, H-5_b), 0.97 (s, 9H, *t*Bu TBPDS). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 159.6, 159.3 (Cq PMB), 139.9 (NCS), 135.6, 135.6 (CH arom. TBDPS), 133.0, 132.8 (Cq TBDPS), 130.2 (Cq PMB), 130.0, 130.0, 129.7, 129.6 (CH arom TBDPS/PMB), 129.2 (Cq PMB), 127.9, 127.9, 114.1, 113.8 (CH arom. PMB), 86.1 (C-1), 85.7 (C-4), 79.6, 75.5 (C-2 + C-3), 72.8, 72.6 (CH_2 PMB), 63.7 (C-5), 55.3 (CH_3 PMB), 26.9 (CH_3 TBPDS), 19.3 (Cq *t*Bu TBDPS). *B-anomer:* **Rf:** 0.27 in 20% Et_2O in pentane. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.66 (ddt, J = 8.0, 6.3, 1.7 Hz, 4H, TBDPS arom.), 7.45 – 7.33 (m, 6H, TBDPS arom.), 7.30 – 7.16 (m, 4H, PMB arom.), 6.92 – 6.79 (m, 4H,

PMB arom.), 5.42 (d, J = 2.7 Hz, 1H, H-1), 4.55 (s, 2H, CH_2 PMB), 4.44 (q, J = 11.4 Hz, 2H, CH_2 PMB), 4.20 – 4.11 (m, 2H, H-3 + H-4), 3.96 (dd, J = 4.2, 2.8 Hz, 1H, H-2), 3.80 (dd, J = 11.5, 2.8 Hz, 1H, H-5_a), 3.77 (s, 3H, CH_3 PMB), 3.76 (s, 3H, CH_3 PMB), 3.68 (dd, J = 11.6, 3.2 Hz, 1H, H-5_b), 1.04 (s, 9H, *t*Bu TBDPS). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 159.6, 159.5 (Cq PMB), 140.7 (NCS), 135.7, 135.6 (CH arom. TBDPS), 133.2, 132.9 (Cq TBDPS), 129.8, 129.8, 129.6 (CH arom. TBDPS/PMB), 129.5, 129.1 (Cq PMB), 127.9, 127.8, 127.8 (CH arom. PMB), 114.1, 114.0, 113.9 (CH arom. PMB), 88.4 (C-1), 83.4 (C-4), 81.3 (C-2), 76.0 (C-3), 72.5, 72.3 (CH_2 PMB), 63.2 (C-5), 55.3, 55.3 (CH_3 PMB), 26.9 (CH_3 TBDPS), 19.3 (Cq *t*Bu TBDPS). **HRMS:** $[C_{38}H_{43}NO_6Si + Na]^+$ found: 692.2464, calculated: 692.2473.

1-(*tert*-butoxycarbonyl)-3-(5-*O*-((*tert*-butyl)-diphenylsilyl)-2,3-di-*O*-(4-methoxybenzyl)-α-D-ribofuranos-1-yl)-2-ethylisothiourea (2)



Compound **14** (670 mg, 1.05 mmol) was dissolved in THF (5.25 mL, 0.2 M). The solution was purged with NH_3 for 1 hour after which the reaction was purged with N_2 for 1 minute. The crude thiourea was concentrated *in vacuo* till a white foam. The crude product was dissolved in DCM (10.5 mL, 0.1 M). DMAP (13 mg, 0.11 mmol, 0.1 eq.) and Boc_2O (266 µL, 1.16 mmol, 1.1 eq.) were added and the reaction was stirred for 2 hours. The reaction was diluted with DCM and the resulting solution was washed with brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was dissolved in MeCN (10.5 mL, 0.1 M) and K_2CO_3 (1.45 g, 10.5 mmol, 10.0 eq.) and EtI (296 µL, 3.68 mmol, 3.5 eq.) were added to the solution. The suspension was stirred overnight and taken up in EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (20 -> 40% Et_2O in pentane) furnished the title compound as a white foam (430 mg, 0.53 mmol, 50%). **Rf:** 0.42 in 30% Et_2O in pentane. **^{1}H NMR:** *measured at 59 °C* (500 MHz, $CDCl_3$) δ 7.67 – 7.57 (m, 4H, TBDPS arom.), 7.43 – 7.39 (m, 2H, TBDPS arom.), 7.39 – 7.33 (m, 4H, TBDPS arom.), 7.30 – 7.23 (m, 4H, PMB arom.), 6.86 – 6.79 (m, 4H, PMB arom.), 5.68 (bs, 1H, H-1), 4.65 (d, J = 11.3 Hz, 1H, CH_{2a} PMB), 4.56 – 4.45 (m, 3H, CH_2 PMB + CH_{2b} PMB), 4.18 (ddd, J = 4.4, 3.3, 2.4 Hz, 1H, H-4), 4.12 (dd, J = 5.0, 2.5 Hz, 1H, H-3), 4.09 (t, J = 5.1 Hz, 1H, H-2), 3.77 (s, 3H, CH_3 PMB), 3.76 (s, 3H, CH_3 PMB), 3.70 – 3.62 (m, 2H, H-5), 2.90 (bs, 2H, CH_2 Et), 1.49 (s, 9H, CH_3 Boc), 1.21 (t, J = 7.4 Hz, 3H, CH_3 Et), 1.03 (s, 9H, CH_3 TBDPS). **^{13}C NMR:** (126 MHz, $CDCl_3$) δ 161.1 (C=O Boc), 159.7, 159.5 (Cq PMB), 135.7, 135.6 (CH arom. TBDPS), 133.4, 133.3 (Cq TBDPS), 130.3 (Cq PMB), 129.9, 129.9 (CH arom. TBDPS/PMB), 129.8 (Cq PMB), 129.6 (CH arom. TBDPS/PMB), 127.8, 127.8, 114.1, 114.0 (CH arom. PMB), 83.0 (C-4), 82.1 (C-1), 79.1 (Cq *t*Bu Boc), 78.2 (C-3), 77.4 (C-2), 73.0, 72.6 (CH_2 PMB), 64.1 (C-5), 55.3 (CH_3 PMB), 28.3 (*t*Bu CH_3 Boc), 27.0 (*t*Bu CH_3 TBDPS), 25.2 (CH_2 Et), 19.3 (Cq *t*Bu TBDPS), 13.8 (CH_3 Et). **HRMS:** $[C_{45}H_{58}N_2O_8SSi + H]^+$ found: 815.3745, calculated: 815.3756.

Synthesis of peptides 20 and 26

The following procedures are applicable on 50 µmol resin and scalable for smaller scales.

Peptide synthesis

The intermediate peptides were synthesized using standard, Fmoc-based solid phase peptide synthesis utilizing (pre-loaded) TentaGel® S AC purchased from Rapp Polymer Gmbh. Coupling cycles were as followed: Fmoc deprotection: 2x2 minutes, 1x5 minutes treatment with 20% piperidine in DMF. Coupling: treatment of 6 eq. amino acid, 6 eq. HCTU (0.25 M in DMF) and 12 eq. DIPEA (1 M in DMF) for 30 minutes. Capping: 2x2 minutes treatment of the resin with a 10% Ac_2O solution in DMF and catalytic DIPEA. Washing between the steps was done with DMF. For the prospected Arg-ADPr site, commercially available Fmoc-Orn(Alloc)-OH was used in the coupling cycle.

Deprotection/building block coupling for Arg-ADPr peptides

The Alloc protecting group was removed by treating the resin with a freshly prepared solution of 10 mg $Pd(PPh_3)_4$ and 23 mg DMBA in 1 mL DCM (purged with nitrogen prior to use) for 15 minutes. This procedure was then repeated twice to ensure full deprotection. The resin was washed extensively with DCM and DMF. Coupling of the ribosyl building block was performed as followed: Ribosyl building block **2** (3 eq.) was dissolved in DMF (0.1 M) and added to the resin. TEA (30 eq.) followed by $AgNO_3$ (3 eq.) were added to the reaction and the syringe was wrapped in aluminum foil to protect it from light and shaken overnight. The resin was then extensively washed with DCM, DMF.

Deprotection/phosphorylation

The resin was washed with THF and treated with a 1 M TBAF in THF for 30 minutes. The resin was thoroughly washed with DCM and DMF before the treatment was repeated once, furnishing the desilylated intermediate. The resin was then extensively washed with MeCN and flushed with nitrogen to remove traces of water before the resin was subjected to a solution of $(FmO)_2PN(iPr)_2$ (2.5 eq. as 0.13 M in MeCN) and DCI (5.0 eq. as 0.25 M in MeCN) was added. The resin was shaken for 30 minutes after which the resin was washed with MeCN. The resin was then treated with a 0.5 M CSO solution in MeCN for 30 minutes. The resin was then treated with a 10% DBU solution in DMF (2x 15 minutes) to furnish the crude, immobilized and deprotected phosphoribosylated peptide.

Pyrophosphate synthesis

The resin was extensively washed with MeCN and flushed with nitrogen to remove traces of water. The resin was then treated with a solution of adenosine amidite **8** (3 eq. (0.13 M in MeCN)) and DCI (6 eq. (0.25 M in MeCN)) for 30 minutes. The resin was thoroughly washed with MeCN before a CSO solution (0.5 M in MeCN) was added to the resin and shaken for 30 minutes.

Final deprotection and cleavage

The resin was then treated with a 10% DBU solution in DMF (2x 10 minutes) to remove the cyano ethyl protecting group. The resin was then treated with a 1 M TBAF solution in THF (2x 45 minutes) and washed with DMF followed by DCM. Final cleavage/deprotection occurred by treating the resin with a cleavage cocktail (2.5:10:87.5 TIS:TFA:DCM) for 4 hours. The crude peptide was precipitated by flushing the cleavage cocktail in an ice-cold 1:1 mixture of Et_2O:pentane. The resin was washed twice with cleavage cocktail. The crude products were stored at -20 °C overnight to induce as much precipitation as possible before the products were centrifuged. The supernatant was removed, obtaining the solid crude peptide.

Ac-Gly-Arg(5-*O*-adenosine-diphosphate-α,β-D-ribosyl)-Thr-Phe-OH (20)

The general procedures described above were applied to 25 µmol TentaGel® S AC resin preloaded with Phe. The amino acids used were Fmoc-Gly-OH, Fmoc-Thr(Trt)-OH and Fmoc-Orn(Alloc)-OH. The crude peptide was purified by RP-HPLC in NH_4OAc buffer. The pure fractions were concentrated, co-evaporated extensively with a 1:1 mixture of MeCN:Milli-Q water, redissolved in Milli-Q water and lyophilized to obtain the title compound as a white solid (2.71 mg, 2.47 µmol, 9.9%). **^{1}H NMR:** (500 MHz, D_2O) δ 8.45 – 8.38 (m, 2H, H-2 adenine α/β), 8.20 – 8.10 (m, 2H, H-8 adenine α/β), 7.21 – 7.03 (m, 10H, Phe α/β), 6.02 (d, J = 5.8 Hz, 1H, H-1' adenosine α or β), 5.93 (d, J = 5.4 Hz, 1H, H-1' adenosine α or β), 5.25 (d, J = 4.3 Hz, 1H, H-1' ribosyl α or β), 5.05 (d, J = 5.8 Hz, 1H, H-1' adenosine α or β). **^{31}P NMR:** (202 MHz, D_2O) δ -10.2, -10.3, -10.3, -10.4, -10.7, -10.7, -10.8, -10.8. **LC-MS:** (0 -> 20% B in A): Rt = 7.15. **HRMS:** $[C_{38}H_{56}N_{12}O_{20}P_2 + H]^+$ found: 1063.3277, calculated: 1063.3282.

Ac-Gly-Arg(5-*O*-adenosine-diphosphate-α,β-D-ribosyl)-Leu-Ile-Phe-Ala-Gly-OH (26)

The general procedures described above were applied to 25 µmol TentaGel® S AC resin preloaded with Gly. The amino acids used were Fmoc-Gly-OH and Fmoc-Thr(Trt)-OH, Fmoc-Phe-OH, Fmoc-Ile-OH, Fmoc-Leu-OH and Fmoc-Orn(Alloc)-OH. The crude peptide was purified by RP-HPLC in NH_4OAc buffer. The pure fractions were concentrated, co-evaporated extensively with a 1:1 mixture of MeCN:Milli-Q water, redissolved in Milli-Q water and lyophilized to obtain the title compound as a white solid (6.09 mg, 4.51 µmol, 9.0%). **^{1}H NMR:** (850 MHz, D_2O) δ 8.54 – 8.43 (m, 2H, H-2 adenine α/β), 8.24 (s, 2H, H-8 adenine α/β), 7.28 – 7.25 (m, 4H, Phe arom. α/β), 7.25 – 7.20 (m, 2H, Phe arom. α/β), 7.20 – 7.15 (m, 4H, Phe arom. α/β), 6.09 (d, J = 5.8 Hz, 2H, H-1' adenosine), 5.31 (d, J = 4.3 Hz, 1H, H-1' ribosyl α or β), 5.11 (d, J = 5.8 Hz, 1H, H-1' ribosyl α or β). **^{31}P NMR:** (202 MHz, D_2O) δ -10.2, -10.3, -10.3, -10.4, -10.7, -10.7, -10.8, -10.8. **LC-MS:** (10 -> 90% B in A): Rt = 4.23. **HRMS:** $[C_{51}H_{79}N_{15}O_{22}P_2 + H]^+$ found: 1316.5073, calculated: 1316.5072.

Synthesis of full-length Ub-ADPr

Solid Phase Peptide Synthesis

SPPS was performed according to literature procedure[31] on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 20 µmol scale, using fourfold excess of amino acids relative to pre-loaded preloaded Fmoc amino acid chloro-trityl resin (0.2 mmol/g, Rapp Polymere GmbH). On position 42 in the peptide sequence arginine was replaced by Fmoc-Orn(Alloc)-OH. After SPPS, 6 µmol Ub1-76 (R42 → Alloc ornithine) on resin was treated with PyBOP (3.1 mg, 30 µmol, 5 eq) and Bt-PEG_2-COOH (16.1 mg, 30 µmol, 5 eq) in DMF (2 mL). After 5 minutes of shaking, DIPEA (16 µL, 90 µmol, 15 eq) was added. The reaction mixture was shaken overnight, after which a test cleavage confirmed full conversion of the conjugation.

Deprotection/building block coupling

The resin was then washed with DMF and DCM before resuspended in DCM. Alloc deprotection was performed by treating the resin with a solution of $(Pd(PPh_3)_4$ (1.4 mg, 1.2 µmol, 0.2 eq) and $PhSiH_3$ (15 µL, 120 µmol, 20 eq) in anhydrous DCM. This was done two times and a test cleavage confirmed complete deprotection. Coupling of the ribosyl building block was performed as follows: Ribosyl building block **2** (146 mg, 180 µmol, 30 eq.) was dissolved in DMF (1.8 mL, 0.1 M) and added to the resin. TEA (252 µL, 1800 µmol, 300 eq.) was added followed by the addition of $AgNO_3$ (31 mg, 180 µmol, 30 eq.). The syringe was wrapped in aluminum foil to protect it from light and shaken overnight. The resin was then extensively washed with DCM and DMF and transferred to a new syringe to rid the resin of residual silver salts.

Deprotection/phosphorylation

The resin was washed with THF and treated with a 1 M TBAF in THF for 30 minutes. The resin was thoroughly washed with DCM and DMF before the treatment was repeated once, furnishing the desilylated intermediate. The resin was then extensively washed with dry MeCN and flushed with nitrogen to remove traces of water. The resin was subjected to a solution of $(FmO)_2PN(iPr)_2$ (1.15 mL, 0.13 M in MeCN, 150 µmol, 25 eq.) and a DCI solution (1.20 mL, 0.25 M in MeCN, 50 eq.) was added. The resin was shaken for 30 minutes after which the resin was washed with MeCN. The resin was then treated with a *t*BuOOH solution (a 5.5 M solution in nonane was diluted ten times in MeCN to obtain the solution used for oxidation) for 30 minutes. The resin was thoroughly washed with DCM and DMF and subsequently treated with a 10% DBU solution in DMF (2x 15 minutes) to furnish the crude, immobilized and deprotected phosphoribosylated ubiquitin.

Pyrophosphate synthesis

The resin was extensively washed with MeCN and flushed with nitrogen to remove traces of water. The resin was then treated with a solution of adenosine amidite **8** (1.15 mL, 0.13 M in MeCN, 150 µmol, 25 eq.) and a DCI solution (1.20 mL, 0.25 M in MeCN, 50 eq.) for 30 minutes. The resin was thoroughly washed with MeCN before a *t*BuOOH solution (a 5.5 M solution in nonane was diluted ten times in MeCN to obtain the solution used for oxidation) was added to the resin and shaken for 30 minutes.

Final deprotection, cleavage and purification

The resin was thoroughly washed with DCM and DMF and subsequently treated with a 10% DBU solution in DMF (2x 15 minutes). The resin was washed with THF and treated with a 1 M TBAF in THF for 30 minutes. The resin was thoroughly washed with DCM and DMF before the treatment was repeated once. The resin was treated with TFA:TIS:H_2O:Phenol (90.5:2:5:2.5) for 1.5 hours before filtrated in an ice-cold solution of Et_2O:pentane (1:1). The precipitate formed was centrifuged (5 Minutes, 3500 rpm) and the supernatant decanted. The pellet was subsequently dried with N_2, taken up in warm DMSO and diluted in warm water before purified by RP-HPLC. Pure fractions were pooled and lyophilized affording UbR42-ADPr (301 µg, 0.031 µmol, 0.5%) as a white powder. **LC-MS**: Rt = 1.47 min. **HRMS:** $[C_{416}H_{688}N_{114}O_{139}P_2S + 5H]^{5+}$ found: 1922.0817, calculated: 1922.0005. $[C_{416}H_{688}N_{114}O_{139}P_2S + 6H]^{6+}$ found: 1601.7731, calculated: 1601.6680. $[C_{416}H_{688}N_{114}O_{139}P_2S + 7H]^{7+}$ found: 1373.0911, calculated: 1373.0022.

DupA mediated hydrolysis of peptides ADP-ribosylated at arginine

The peptides **20** and **26** (5 µM) in buffer (20 mM TRIS, 150 mM NaCl, pH 7.6) were incubated with DupA (3 µM) or without (background hydrolysis) at 37°C. At the indicated time points 15 µL sample was 4 times diluted before measuring HRMS. The ratio of product versus starting material was determined, corrected for t = 0 minutes and plotted as increase in pyrophosphate cleavage over time. The means of two individual measurements is depicted with standard deviation.

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