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Puzzling pyrophosphates: synthetic methodologies for ADP-ribosylated biopolymers

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Citation
Wijngaarden, S. (2026, February 20). *Puzzling pyrophosphates: synthetic methodologies for ADP-ribosylated biopolymers*. Retrieved from https://hdl.handle.net/1887/4292395

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Chapter 6

Convergent approach towards ADP-ribosylated-peptides via a chemoselective phosphate condensation

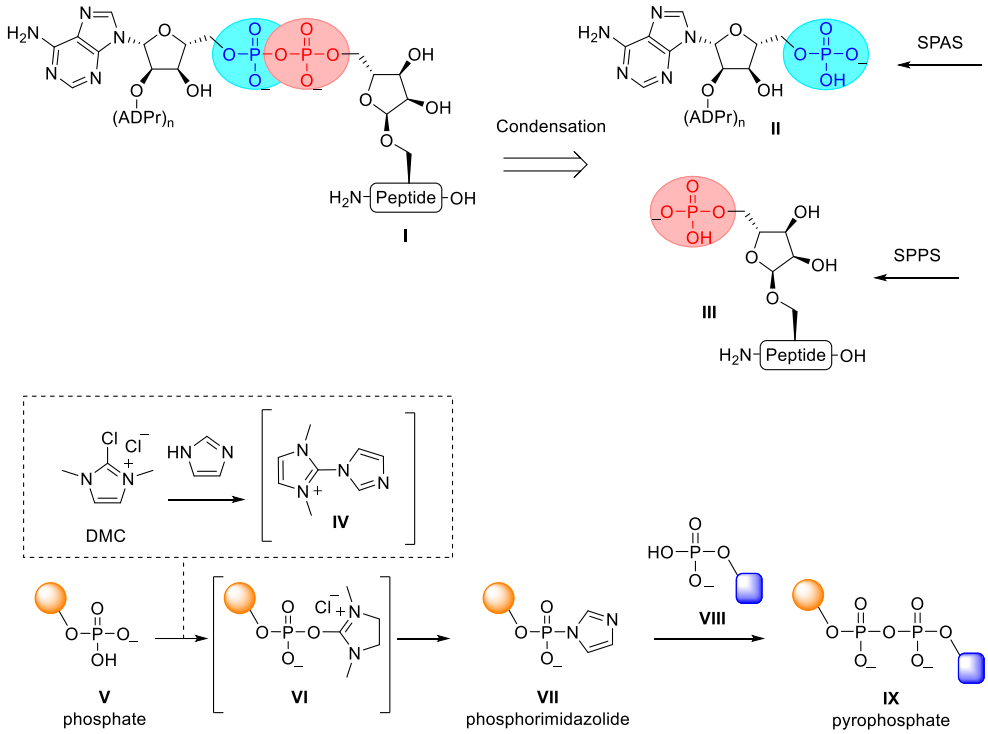
This work was published:

S. Wijngaarden, F. L. A. M. van der Heijden, C. J. Bogaart, M. Siskou, I. Tsoumani, D. J. Robinson, G. A. van der Marel, J. D. C. Codée, H. S. Overkleeft, D. V. Filippov, Convergent approach toward ADP-ribosylated peptides via a chemoselective phosphate condensation. *Chem. Eur. J.* **2025**, *31*, 1-12.

Introduction

Adenosine diphosphate ribosylation (ADP-ribosylation) is a widespread post-translational modification of which the functional role in molecular biology remains poorly understood.[1] Transferase enzymes (ARTs, PARPs) glycosylate various amino acid side chains, using NAD^+ as the glycosyl donor, generating mono-ADP-ribosylated proteins. Subsequent iterative PARP-mediated glycosylation of the 2'-OH of adenosine in ADP-ribosyl proteins leads to the formation of sugar-pyrophosphate chains known as poly-ADPr or PAR. Many amino acid side chains can be modified with ADP-ribose, and the reported acceptor sites include serine, glutamic acid, aspartic acid, arginine, tyrosine, cysteine and histidine.[2,3] Although the majority of these sites are thought to be modified with mono-ADPr exclusively, poly-ADP-ribosylation has been established for serine[4,5] and glutamic acid[6,7] side chains. The ADP-ribosylation of proteins is regulated by specific hydrolases such as PARG that degrades the ADP-ribose chain and ARH1 and ARH3 that cleave the glycosidic bond between the "distal" ribose in ADPr and the amino acid side chains.[8-12] Access to synthetic peptides modified with ADP-ribose chains of defined lengths would be invaluable to better understand the biosynthetic routes and signaling pathways involving ADP-ribose as post-translational modification. ADP-ribosylated peptides and proteins, such as core histones and other chromatin architecture proteins, prepared by chemo-enzymatic methods and containing short stretches of oligo-ADP-ribose, have already provided insights into the impact of the ADPr-chain length attached to a serine side chain on the chromatin structure at DNA damage sites.[13-16]

Over the past two decades, significant advances have been made in the chemical synthesis of well-defined ADP-ribosylated peptides. The method of choice for the synthesis of mono-ADPr-peptides comprises of the incorporation of pre-ribosylated amino acid building blocks in a solid-phase peptide synthesis, followed by on-resin phosphorylation and pyrophosphorylation via P(III)-P(V) phosphate-phosphoramidite chemistry.[17] This method enabled the production of ADP-ribosylated peptides of almost all biologically relevant amino acid acceptor sites.[18-20] However, the challenges in combining the protection schemes for the peptide and ADPr-moiety remain, for example, in the case of the very acid-sensitive phenolic glycosyl bond of Tyr-ADPr.[21] As well, "free" oligo-ADP-ribose fragments up to the pentamer on a solid-support were prepared using P(III)-P(V) chemistry for the installation of the inter-ribose pyrophosphate functionality.[22-24] Alternatively, Lambrecht *et al.* employed a solution-phase approach for the preparation of an ADPr-dimer via a synthetic route that relied on a P(V)-based phosphorimidazolide-phosphate coupling.[25]



Scheme 1. *Above*: Retrosynthetic analysis for the convergent synthesis of ADP-ribosylated peptides (**I**). Disconnection of ADPr-peptide **I** at the peptide proximal pyrophosphate linkage, produces two phosphates which are chemo- and regioselectively condensed. Phosphates **II** and **III** are accessed via solid-phase ADPr synthesis and solid-phase peptide synthesis. *Below:* General reaction scheme of the phosphorimidazolide-based methodology for producing pyrophosphates. Phosphate activation via dehydrating reagent **IV** and subsequent pyrophosphorylation. Imidazole and DMC give dehydrating reagent **IV** that chemoselectively dehydrates phosphate **V** to produce electrophile **VI**. Nucleophilic catalysis by imidazole gives phosphorimidazolide **VII**, which can chemoselectively pyrophosphorylate phosphate **VII** to produce pyrophosphate **IX**.[26]

Existing synthetic methods for ADPr-peptides and poly-ADPr fragments rely on two distinct solid-phase approaches, which are intrinsically hard to combine. The difficulties arise from the necessity to develop protective group strategies for all the amino acid side chains, which would be incompatible with the presence of acid-sensitive oligo-ADP-ribose in the final construct. In this chapter, a convergent strategy was applied that avoids this potential incompatibility by chemoselective ligation of phosphomonoesters to install the pyrophosphate at the final stage of the synthesis of ADPr-peptides (**Scheme 1**). This approach involves retrosynthetic disconnection of ADP-ribosyl peptide **I** at the peptide proximal pyrophosphate bond, generating two key phosphate components: an ADP-ribose fragment **II** lacking the "distal" 5''-O-phosphoribosyl moiety and a phosphoribosyl-functionalized peptide (Pr-peptide) **III**.

Fully deprotected and purified ADPr fragment **II** is accessed via solid-phase ADPr synthesis (SPAS), while the phosphoribosyl-peptide **III** can be obtained through standard solid-phase peptide synthesis (SPPS). Fragments **II** and **III** are then coupled through a suitable chemoselective phosphate condensation. Here a methodology was chosen that relies on the coupling of phosphate nucleophiles with phosphorimidazolide electrophiles, which offers notable chemo- and regioselectivity[27,28] and has been shown to be compatible with aqueous conditions[26] required to dissolve unprotected peptides and ADP-ribose fragments (**Scheme 1**). The method of choice generates phosphorimidazolides via selective dehydration of phosphates using 2-chloro-1,3-dimethylimidazolinium chloride (DMC) and imidazole (**Scheme 1**). This reaction proceeds via formation of imidazolinium species **IV**, which can selectively dehydrate phosphates such as **V** via intermediate **VI** to generate phosphorimidazolide **VII**.[26,29] Nucleophilic attack by phosphate **VIII** on phosphorimidazolide **VII** then yields pyrophosphate diester **IX**. There is ample literature precedent for the application of this phosphorimidazolide-based methodology in both organic solvents and aqueous media for the synthesis of NDP-sugars,[26,29,30] 5',5'-oligophosphorylated dinucleosides,[29,31-33] pyrophosphorylated peptides,[34] proteins[35] and simple ADPr-peptides.[36] Catalysis with metal salts, such as zinc- or magnesium chloride has proven essential to enhance reactivity and selectivity of the phosphate-phosphate condensation.[26,37]

In this chapter, first the compatibility of the phosphate condensation reaction with a range of ADP-peptides modified on serine, cysteine, tyrosine, and arginine was investigated. Hereafter, a phosphoadenosyl-ADPr fragment, containing the 5'-O-phosphate, was condensed to a phosphoribosyl peptide, to produce for the first time a di-ADPr-peptide via a fully synthetic approach.

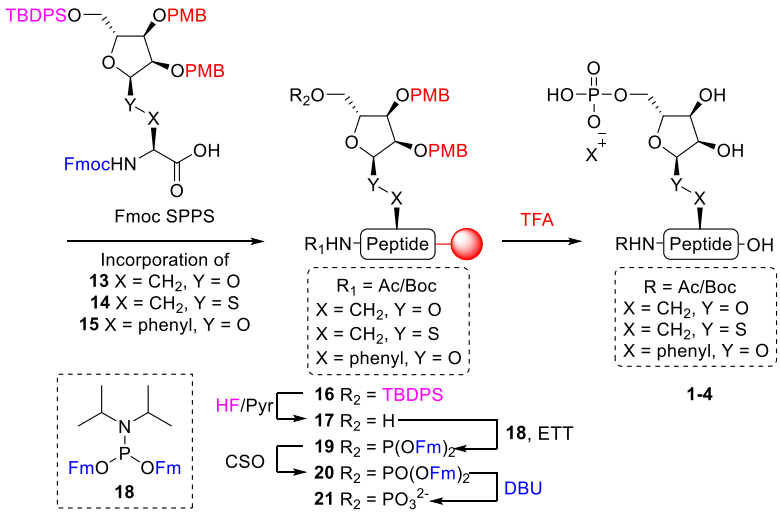
Results and discussion

Phosphoribosyl-peptides bearing an O-, S- or N-glycosidic linkage (serine, cysteine, tyrosine and arginine) were accessed by SPPS (**Scheme 1-3**). These peptides were prepared by adaptation of established protocols, via incorporation of their corresponding ribofuranosylated building blocks, followed by on-resin phosphorylation.[21,38-41] The peptide sequences were derived from proteins known to be ADP-ribosylated on the indicated amino acids (**Table 1**).

Table 1. Overview of Pr- and ADPr-peptides synthesized in this chapter.

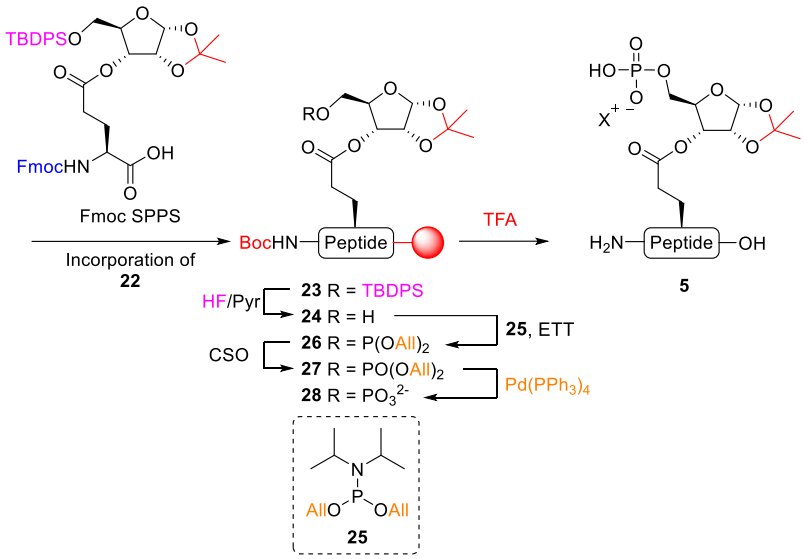
Code	Parent protein	Modification site	Sequence
		Pr-peptides	
1	H2B1C	Ser 6	Ac-PAK**S**^{Pr}APAPKG-OH
2	HPF1	Ser 97	H-TKKK**S**^{Pr}TGLN-OH
3	PARP7	Cys 721	Ac-QEDF**C**^{Pr}FLSS-OH
4	CBX4	Tyr 207	Ac-AAKG**Y**^{Pr}LGAV-OH
5	HPF1	Glu 240	H-VGYR**E**^{Pr}LPET-OH
6	rS1	Arg 139	Ac-V**R**^{Pr}RV-NH_2
		Mono-ADPr-peptides	
7	HPF1	Ser 97	H-TKKK**S**^{ADPr}TGLN-OH
8	PARP7	Cys 721	Ac-QEDF**C**^{ADPr}FLSS-OH
9	CBX4	Tyr 207	Ac-AAKG**Y**^{ADPr}LGAV-OH
10	HPF1	Glu 240	H-VGYR**E**^{ADPr}LPET-OH
11	rS1	Arg 139	Ac-V**R**^{ADPr}RV-NH_2
		Poly-ADPr-peptide	
12	H2B1C	Ser 6	Ac-PAK**S**$^{di\text{-}ADPr}$APAPKG-OH

Serine, cysteine and tyrosine phosphoribosylated peptides were prepared by incorporating the corresponding ribofuranosylated building blocks (**13**-**15**) into the desired peptides sequence by SPPS (**Scheme 2**). Incorporation of appropriately protected ribosylated serine **13** yielded immobilized peptide **16**, of which the silyl-ether was cleaved using the HF-pyridine complex as fluoride source, generating the primary hydroxyl in **17**, ready for phosphitylation by 9-fluorenylmethyl (Fm)-protected phosphoramidite **18**[42] under the aegis of ETT, to produce phosphite **19**. Oxidation of the P(III) phosphite by camphorsulfonyloxaziridine (CSO) triester produced the P(V) phosphotriester **20**, which was deprotected by 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) mediated elimination of the Fm esters, to furnish phosphomonoester **21**.[23,40,43] Global acidolysis and cleavage from the solid support furnished phosphoribosylated-serine peptides **1** and **2**. Using a similar strategy, cysteine-peptide **3**, bearing a thioglycosidic phosphoribosyl, and tyrosine-peptide **4**, containing a phenolic phosphoribosyl, were prepared. Acidolysis of cysteine-Pr-peptide **3** required thioether scavengers to prevent side reactions that could cleave the thioglycosidic linkage.[39,40] For tyrosine-Pr-peptide **4**, a reduced concentration of acid (2.5% TFA in DCM) was necessary to preserve the phenolic aglycon, which is prone to degradation under strong acidic conditions.[21]



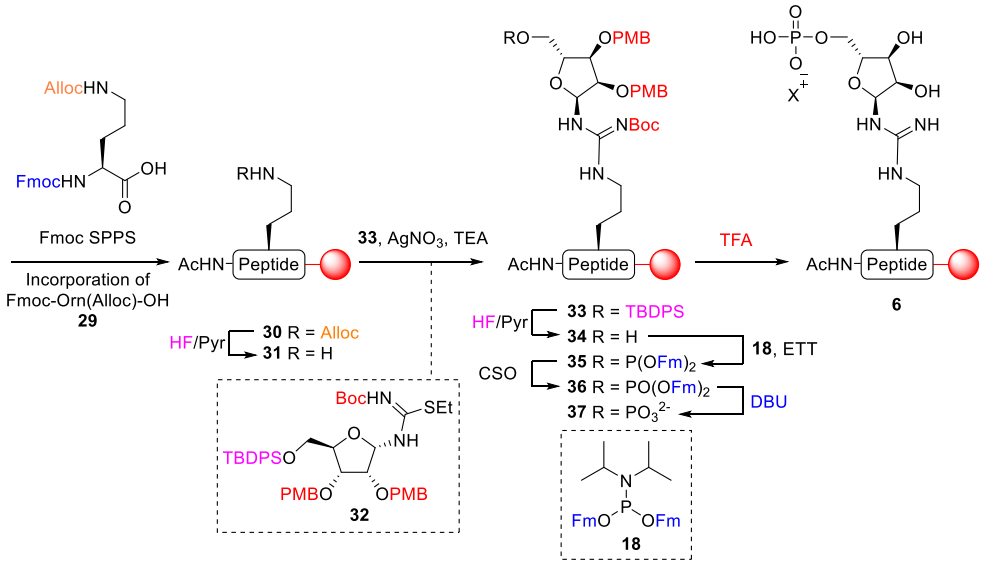
Scheme 2. Synthetic scheme for the preparation of serine, cysteine and tyrosine phosphoribosyl-peptides **1**-**4**. Pr-peptides were prepared using SPPS starting from Fmoc-building blocks **13** for Pr-Ser,[38,40] **14** for Pr-Cys[40] and **15** for Pr-Tyr.[21] Protecting groups depicted in red were cleaved by acid, in blue by base and in purple by a fluoride source.

Glutamyl phosphoribose peptide **5** was synthesized following to the synthetic strategy outlined in **Scheme 3**.[16] Incorporation of glutamylribofuranose **22** in a Fmoc-based SPPS gave immobilized ribosylated peptide **23**, which was deprotected to yield the primary hydroxyl **24**. Phosphitylation with allyl (All)-protected phosphoramidite **25** gave phosphite intermediate **26**, which was oxidized using CSO to form phosphotriester **27**. Allyl protection was chosen for the phosphate, as DBU-mediated deprotection of the Fm group as in **Scheme 2** would result in undesired cyclization to the glutarimide.[16,44] Cleavage of the allyl-ester in phosphate **27** to furnish the phosphomonoester **28** was facilitated by Pd(0) catalysis using DMBA as allyl scavenger.[45] Global acidolysis and cleavage of the solid-phase provided glutamyl-phosphoribose peptide **5**.



Scheme 3. Synthetic scheme for the preparation of Glutamyl phosphoribose peptide **5**.[16] Protecting groups depicted in red were cleaved by acid, in blue by base, in purple by a fluoride source and in orange by palladium mediated deallylation.

Arginyl phosphoribose peptide **6** was synthesized following the strategy outlined in **Scheme 4**.[41] Alloc-protected ornithine **29** was incorporated into the peptide sequence via SPPS, to give immobilized peptide **30**. On-resin cleavage of the Alloc group using Pd(0) mediated deallylation, produced amine **31**, which was guanidinylated with isothiourea **32** under silver nitrate catalysis, forming ribosylated peptide **33**. Desilylation of the TBDPS-ether generated primary hydroxyl **34**, which was phosphitylated with Fm-protected amidite **18** under ETT catalysis to give the phosphite triester **35**. Oxidation of the phosphite produced phosphotriester **36**, after which the Fm-phosphate ester was eliminated using DBU, to give the phosphomonoester **37**. Final, global acidolysis and cleavage from the solid support gave arginyl-phosphoribosyl-peptide **6**.



Scheme 4. Synthetic scheme for the preparation of arginyl phosphoribose peptide **6**.[41] Protecting groups depicted in red were cleaved by acid, in blue by base, in purple by a fluoride source and in orange by palladium mediated deallylation.

Following the synthesis of phosphoribosyl-peptides **2**-**6**, the phosphate activation of adenosine mono phosphate was examined. Adenosine monophosphate (AMP) was converted to phosphorimidazolide (AMP-Im) by reaction with 10 equivalents of DMC and 20 equivalents of imidazole at a concentration of 200 mM in D_2O which was confirmed by ^{31}P-NMR (**Figure 1**). After 30 minutes, a small amount of symmetrical pyrophosphate (AppA) was detected, originating from the hydrolysis of AMP-Im into AMP, followed by nucleophilic attack of this phosphate on AMP-Im. After 6 hours of reaction time, almost all AMP-Im was either hydrolysed back to the parent phosphate or converted to the symmetrical pyrophosphate AppA. The optimal reaction time for conversion of AMP into phosphorimidazolide was determined at 40 °C for 45 minutes.

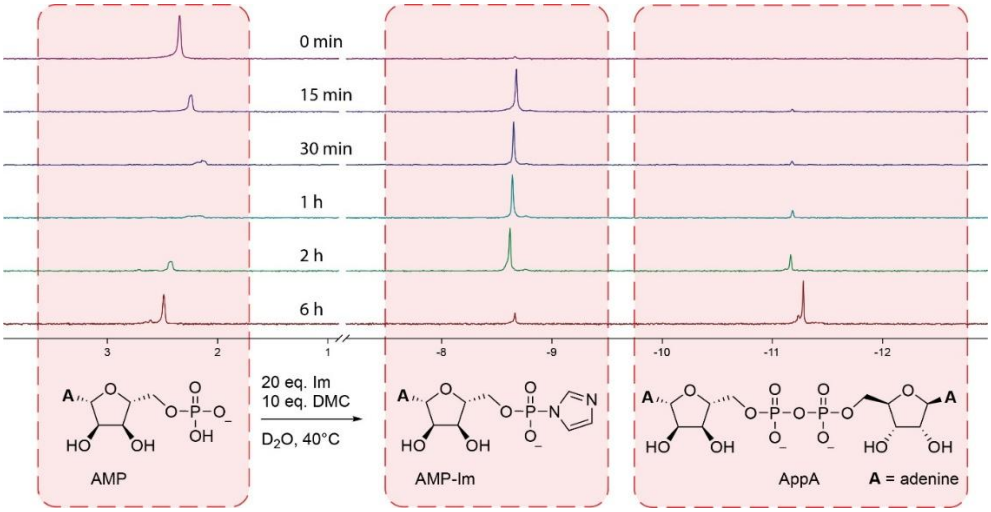
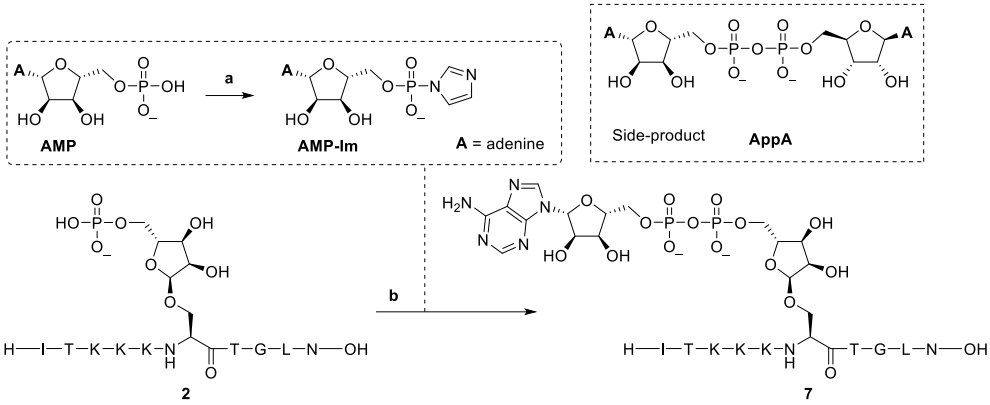


Figure 1. Activation of AMP to AMP-Im monitored over time by ^{31}P-NMR. The dehydration reaction was performed at 40 °C. Samples were taken from the activation cocktail and diluted with D_2O at each indicated time point. The peak at 1 ppm corresponds to AMP, at -6 ppm to AMP-Im and at -11 ppm to symmetrical pyrophosphate AppA.

Hereafter, the coupling of AMP-imidazolide (AMP-Im) to seryl-phosphoribose peptide **2** to produce mono-ADPr-peptide **7** was examined (**Table 2**). Key parameters assessed included stoichiometry, concentration and the effect of additives on the phosphate condensation reaction. AMP was reacted with DMC and imidazole in a molar ratio of 1:2:4 to form the activated AMP-Im in 30 minutes (**Figure 1**). Condensation using 5 eq. of AMP-Im, 20 eq. of $ZnCl_2$ as a Lewis acid, and a final phosphoribosyl-peptide concentration of 8 mM, gave 37% conversion after 5 hours at 40 °C (**Table 2**, entry 1), based on UV integration after LCMS analysis. The most prominent side product, as identified by MS, was the symmetrical pyrophosphate **AppA**. Increasing the amount of zinc chloride from 20 to 100 equivalents improved conversion to 52% (entry 2).[37] Increasing the concentration of phosphoribosyl-peptide **2** from 8 mM to 25 mM further improved conversion to 67% (entry 3). Notably, no evidence of homodimerization of phosphoribosyl-peptide **2** was found in this last experiment. Combining both optimizations (higher peptide concentration and increased amount of zinc chloride) further improved conversion to 79% (entry 4). The reaction progress was followed over time by LCMS analysis for the conditions stated in entry 4, which showed full consumption of **AMP-Im** after 5 hours (**Figure S2**). The reaction progress likely halts due to competing hydrolysis of the phosphorimidazolide. Therefore, to further improve the reaction conversion, an additional 5 equivalents of phosphorimidazolide AMP-Im were added after 2.5 hours, leading to 77% conversion (entry 5). Finally, increasing the concentration of zinc chloride to 100 equivalents resulted in near quantitative conversion of 95% to mono-ADPr-peptide **7** (entry 6).

Table 2. Optimization of reaction condition for the phosphate condensation reaction to produce mono-ADPr-peptide **7**.



Entry	Eq. AMP-Im	Concentration [2] (mM)	Eq. $ZnCl_2$	Conversion*
1	5	8	20	37%
2	5	8	100	52%
3	5	25	20	67%
4	5	25	100	79%
5	2x5	25	20	77%
6	2x5	25	100	95%

Reagents and conditions: **a**) 20 eq. imidazole, 10 eq. DMC, D_2O, 40 °C, 45 min. **b**) AMP-imidazolide, $ZnCl_2$, 40 °C, 5 h. *Activation of AMP to AMP-Im was performed at 200 mM in D_2O at 40 °C for 45 minutes. Test reactions were performed on a scale of 1 µmol at 40 °C and analyzed after 5 hours by LCMS. Conversion was based on UV integration at 200 nm of the peaks corresponding to the Pr-peptide **2** and the ADPr-peptide **7** (**Figure S1**).

Using the conditions described in **Table 2** (entry 6), a diverse set of phosphoribosylated peptides was condensed with commercially available AMP to produce the corresponding ADP-ribosylated peptides (**7**-**11**, **Table 3**). Reactions were conducted on a scale of 1-5 µmol, after which the ADPr-peptides were isolated via size exclusion chromatography, followed by reversed-phase HPLC. The reaction of serine phosphoribosyl-peptide **2** produced ADPr-peptide **7** with a conversion of 90%, consistent with the previously described optimization (**Table 2**, entry 1). Cysteinyl-ADPr-peptide **8** was obtained by condensing phosphoribosyl-peptide **3** with AMP, achieving a 59% conversion and a 20% isolated yield (entry 2). Despite the affinity of thioglycosides for Lewis acids such as zinc chloride,[46] no significant degradation or adverse effects on the coupling reaction were observed. Pyrophosphorylation of tyrosyl-phosphoribosyl peptide **4** with AMP to produce ADPr-peptide **9** proceeded in 75% and peptide **9** was isolated in a yield of 13% (entry 3). The coupling of glutamyl-phosphoribose peptide **5** showed near-complete consumption of the starting material

(entry 4, **Figure S4**) but hydrolysis of the ester linkage occurred under the neutral conditions of the imidazole-like buffer (pH ~7) used in the reaction, which prevented the isolation of the ADPr-peptide.[16] Lastly, arginyl-phosphoribosyl-peptide **6** was transformed into ADPr-peptide **11** with a conversion of 63% based on LCMS analysis and was isolated in a yield of 28%. It is important to note that the difference observed between reaction conversions and isolated yields for peptides **7**-**11** can be attributed to using a two-step purification procedure combined with a small reaction scale of 1-5 µmol. Overall, successful synthesis of mono-ADP-ribosyl-peptides **7**-**11** demonstrates the broad applicability of phosphorimidazolide-assisted phosphate condensation reaction for the synthesis of ADP-ribose-containing biopolymers, with nearly all relevant ADPr-amino acid acceptors being tolerated in the reaction.

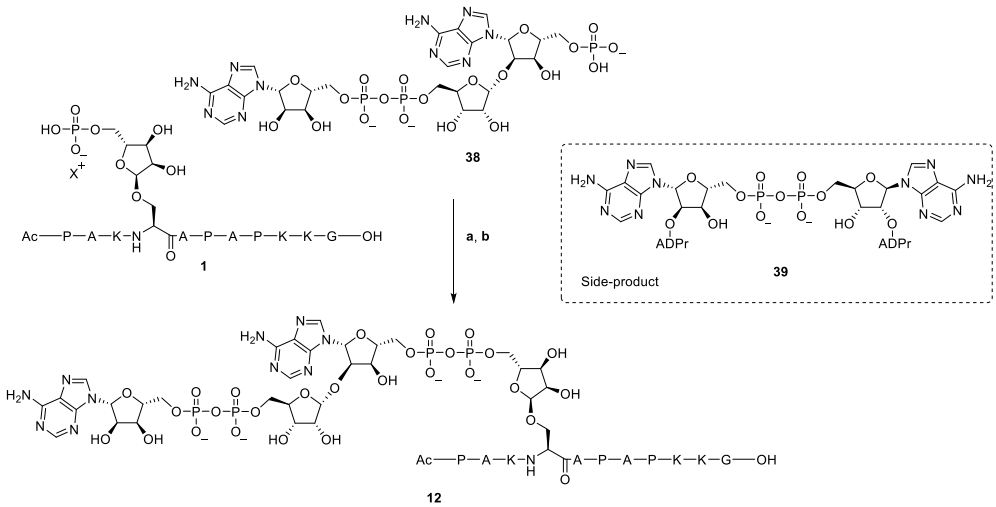
Table 3. Chemoselective condensation of phosphoribosyl peptides **2**-**6** to AMP to produce mono-ADPr-peptides **7**-**11**.

Entry	ADPr-peptide	Linkage type	Scale (µmol)	Conversion*	Isolated yield
1	**7**	Ser	5	90%	10%
2	**8**	Cys	1	59%	20%
3	**9**	Tyr	5	75%	13%
4	**10**	Glu	5	~70%**	degraded
5	**11**	Arg	1	63%	28%

Activation of AMP to AMP-Im was performed at a final concentration of 200 mM in D_2O at 40 °C for 45 minutes. Coupling reactions were performed with 2x5 eq. of AMP-Im, 100 eq. $ZnCl_2$ at 40 °C for 5 hours. *Conversion of the phosphoribose-peptide to the ADPr-peptide was based on UV integration at 200 nm obtained from the LCMS spectrum after 5 hours of reaction time (**Figure S3**).

Next, the condensation of phosphoadenosyl-ADPr fragment **38** to seryl-phosphoribose peptide **1**, yielding di-ADPr-peptide **12**, was investigated (**Scheme 5**). Given the complex synthesis of poly-ADPr fragment **38**, initial efforts focused on activating the more synthetically accessible phosphoribosyl peptide **1** as its phosphorimidazolide. However, activation of the phosphoribosyl-peptide yielded a suspension with no detectable product formation. Therefore, it was decided to activate phosphoribosyl-ADPr fragment **38** instead. The resulting phosphorimidazolide was then reacted in a small stoichiometric excess with the phosphate of peptide **1**. The amount of zinc chloride used in the synthesis of mono-ADPr-peptides was reduced from 100 to 20 equivalents to maintain stoichiometric consistency. The reaction proceeded overnight to ensure complete consumption of the phosphorimidazolide, yielding di-ADPr-peptide **12** in a reasonable conversion, as determined by UV integration after LCMS analysis (**Figure 2**). The most abundant side product which could be identified, originated from homodimerization of phosphoribosyl-ADPr **38**, forming symmetrical pyrophosphate **39**. After the reaction, the crude mixture was desalted by size exclusion

chromatography and purified by ion exchange chromatography affording di-ADPr-peptide **12** in 12% yield. The successful isolation of di-ADPr-peptide **12** suggests a compatibility of the phosphate coupling reaction with larger, more complex ADPr-substrates containing multiple pyrophosphate moieties.



Scheme 5. Convergent synthesis of di-ADPr-peptide **12** via the chemoselective phosphate condensation reaction. Phosphoribosyl-ADPr **38** was activated to its corresponding phosphorimidazolide and coupled with phosphoribosyl peptide **1** to produce di-ADPr-peptide **12**. Reagents and conditions: **a**) phosphoribosyl-ADPr **38**, 20 eq. imidazole, 10 eq. DMC, D_2O, 40 °C, 45 min. **b**) 1.2 eq. peptide **1**. 20 eq. $ZnCl_2$, D_2O, 40 °C, overnight, **12**: 12%.

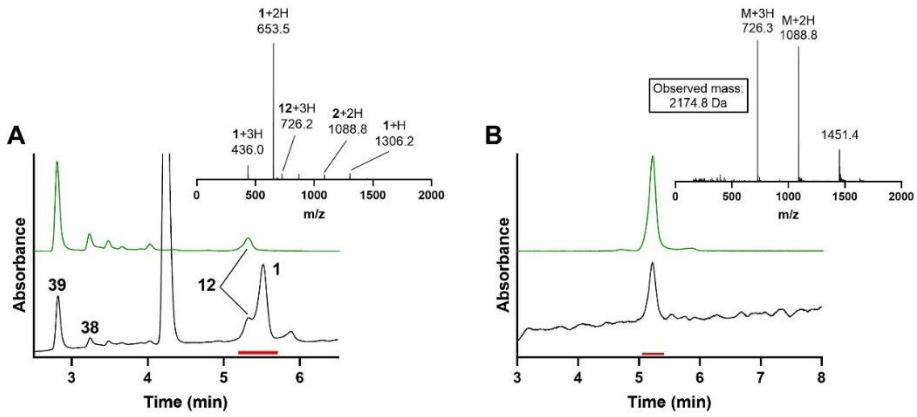


Figure 2A. Analysis of the condensation reaction towards di-ADPr-peptide **12**. Analytical HPLC (linear gradient 00-20% ACN over 10 min) of di-ADPr-peptide **12**. ESI mass spectrum of the peaks under the red line (5.2-5.7 min). Ionization patterns of **1** and **12** are depicted. **B**. Analytical HPLC (linear gradient 00-20% ACN over 10 min) of purified peptide **12**. ESI mass spectrum of the peaks

under the red line (5.1-5.5 min). Expected mass for di-ADPr-peptide **12** is 2174.8 Da. Graphs in black depict the total absorbance measured from 220-680 nm, graphs in green the absorbance of adenine (260 nm).

Conclusion

This work describes a convergent synthesis strategy for preparing ADP-ribosylated peptides via a chemoselective phosphate condensation reaction. The regio- and chemoselectivity of the reaction eliminates the need for protecting functional groups, allowing for the late-stage formation of the peptide proximal pyrophosphate bond. The condensation reaction demonstrates broad applicability for preparing mono-ADP-ribosylated peptides modified on various amino acid acceptors. Additionally, the methodology was extended for the condensation of phosphoadenosyl-ADPr fragments, enabling the first chemical synthesis of a di-ADPr-peptide, which serves as a model for oligo-ADP-ribosylated substrates. Overall, this strategy provides a valuable addition to the chemical toolbox for studying ADP-ribosylation.

Acknowledgements

The following persons are kindly acknowledged for their contribution to this chapter. Femke van der Heijden for supplying the poly-ADP-ribosyl phosphoribosyl building block. Cindy Bogaart and Maria Siskou for their work in optimizing the mono ADP-ribose condensation reactions.

Appendix

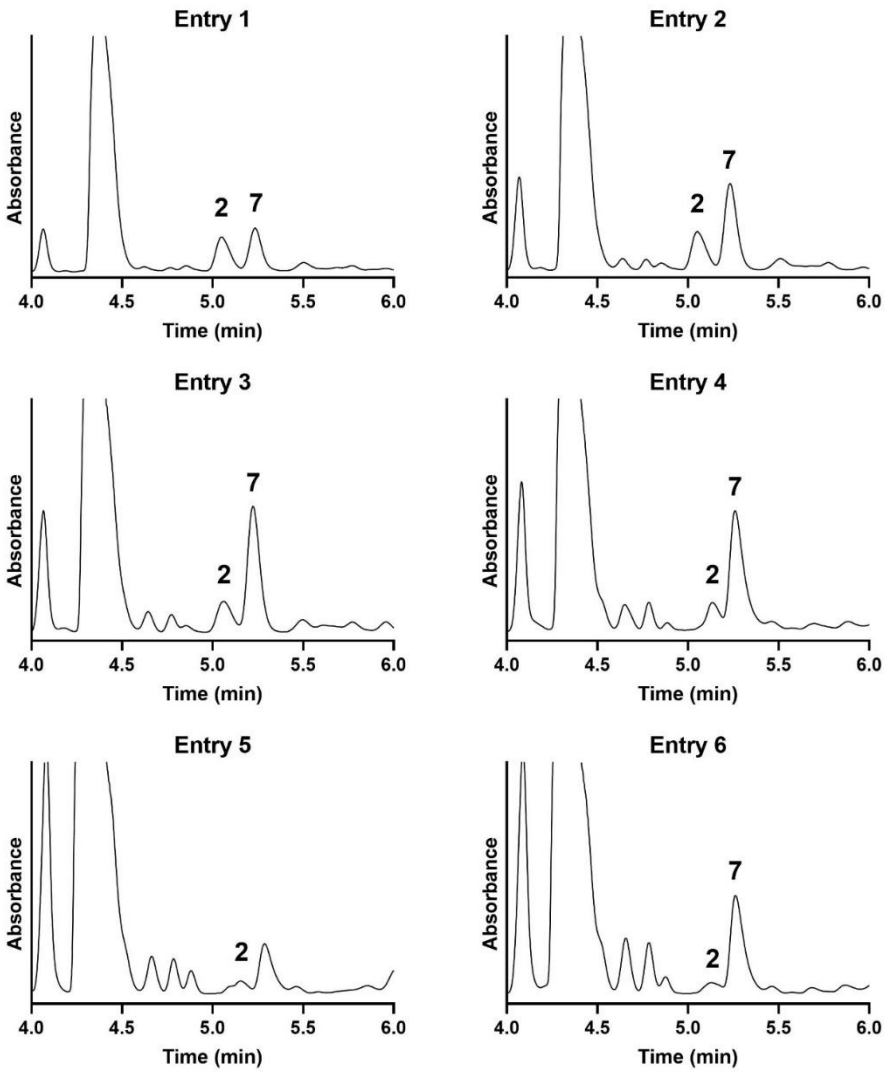


Figure S1. Analytical LCMS (linear gradient 0-20% ACN over 10 minutes) of crude reaction mixtures obtained from optimization of the phosphate condensation reaction in **Table 2**. Total scan chromatogram of UV absorption from 200-600 nm.

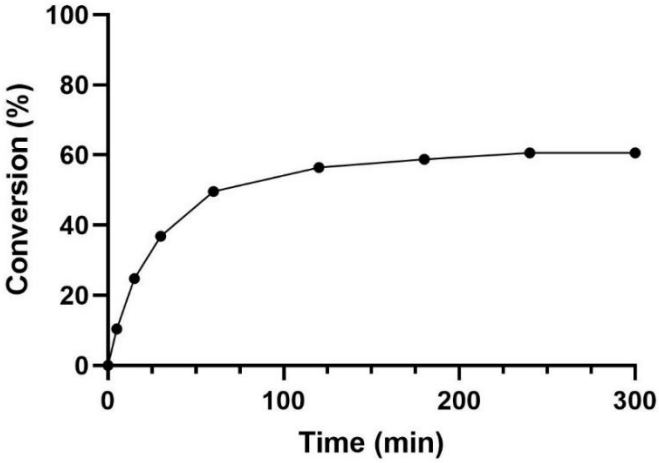


Figure S2. Reaction progress of Entry 4, **Table 2** followed over time. Conversion was based on UV integration at 214 nm of the peaks corresponding to the Pr-peptide **2** and the ADPr-peptide **7**.

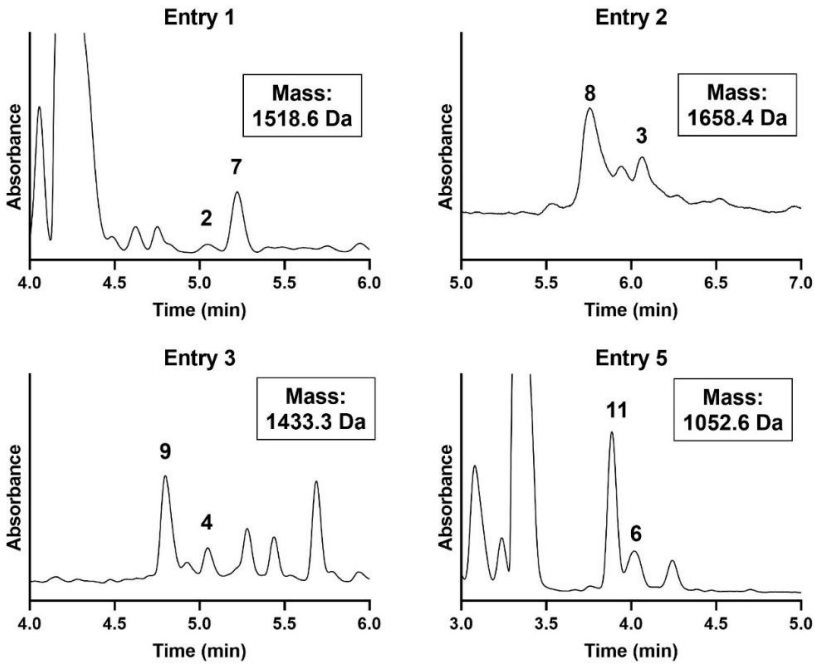


Figure S3. Analytical LCMS of crude reaction mixtures obtained from reactions towards ADP-ribosylated peptides from **Table 3**. The observed mass corresponding to the ADPr-peptide is indicated.

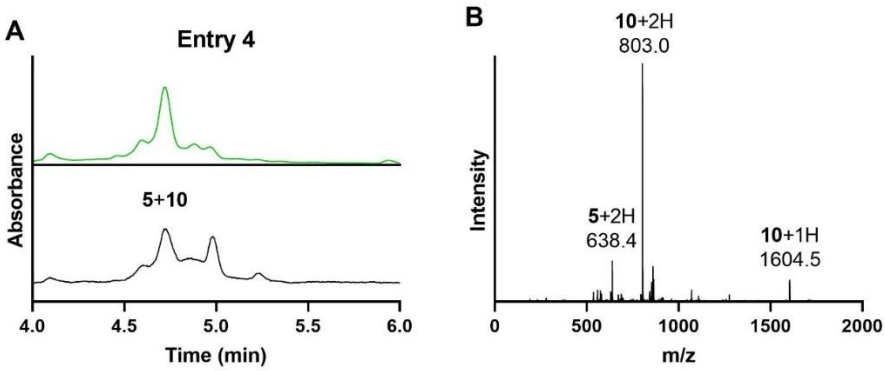


Figure S4. Analytical LCMS of crude reaction mixtures obtained from reactions towards Glu-ADP-ribosylated peptide **10** from **Table 3**, entry 4. ESI mass spectrum of the peak corresponding to peptides **5** and **10** (RT=4.7 min). Graphs in black depict the total absorbance measured from 220-680 nm, graphs in green the absorbance of adenine (260 nm).

Experimental section

General synthetic procedures

All reagents were of commercial grade and used as received unless stated otherwise. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400, AV-500 or AV-600 NMR. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as internal standard. Coupling constants (J) are given in Hz. For pyrophosphate-containing compounds, a small amount of EDTA was added to the NMR sample to sharpen the peaks for ^{31}P-NMR. All given ^{13}C-APT spectra are proton decoupled and are presented with even signals (Cq. and CH_2) pointing upwards and odd signals (CH and CH3) pointing downwards. LCMS 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 a Thermo Scientific Vanquish UHPLC coupled to a Thermo Scientific LCQ Fleet ion mass spectrometer with ESI. Buffers used were A= H_2O, B= MeCN and C= 1% TFA/H_2O. The methods used were 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. HPLC purification was performed on a Gilson GX-281 preparative HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column or on a Waters autopurifier HPLC/MS system coupled to a Phenomenex Gemini 5µm 150x21.2 mm column. Buffers used were A= 1% AcOH/H_2O,

B= 10% H_2O/MeCN. HRMS was recorded on a Thermo Scientific Q Exactive HF Orbitrap mass spectrometer equipped with an electrospray ion source. For HW-40 gel filtration purification, an ÄKTA explorer system equipped with a Superdex-30-HR column (16 mm x 100 cm, flow 1 mL/min) was used.

General procedure A: peptide synthesis

The amino acids (obtained from Novabiochem and Sigma Aldrich) applied in the synthesis were: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Glu(O-2-PhiPr)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Mmt)-OH, Fmoc-Pro-OH, Fmoc-Ser(O*t*Bu)-OH, Fmoc-Ser(Trt)-OH, Fmoc-Thr(O*t*Bu)-OH, Fmoc-Thr(Trt)-OH, Fmoc-Cys(Trt)-OH. TentaGel® S AC and S RAM were bought from Rapp-Polymere GmbH and loaded by hand with the appropriate Fmoc-amino acid. Peptides were synthesized with automated solid phase peptide synthesis on an CEM Liberty Blue Automated Microwave Peptide Synthesizer. Automated synthesis of ADPr-peptides was performed on 50 µmol scale. The first amino acid was manually loaded on the resin using 2 eq. Fmoc-AA-OH, 2 eq. DIC and cat. DMAP while shaking overnight. Resin was first swollen for 5 minutes in DMF prior to amino acid coupling. Activation was achieved using DIC/Oxyma. Standard coupling was achieved using 5 eq. Fmoc-amino acid as a 0.2 M DMF solution, 5 eq. DIC as a 0.5 M of DIC/DMF solution and 5 eq. Oxyma as a 1M solution in DMF which was buffered by DIPEA (0.1M) at room temperature for 60 minutes. Non-commercially available ribosylated Fmoc-amino acids were coupled using 3 eq. amino acid as a 0.1M solution in DMF, 3 eq. DIC as a 0.5M of DIC/DMF solution and 3 eq. Oxyma as a 1 M Oxyma/DMF solution which was buffered by DIPEA (0.1M) at room temperature for 180 minutes. When a free N-terminal amine was required in the peptide sequence, a Boc protected amino acid was used in the last coupling. When an acetylated N-terminus was required in the peptide sequence, the resin was treated with a solution of 10% Ac_2O, 20% DIPEA in DMF (8 ml/gr resin) for 2x10 minutes. Standard Fmoc deprotection was achieved by 20% v/v piperidine/DMF at RT for 10 minutes (2 cycles). Synthesis quality could be monitored by UV absorption of dibenzofulvene released during Fmoc deprotection. The quality of the peptide was checked by a test deprotection. A small amount of resin (tip of spatula) was washed 3 times with DCM. 100 µl of cleavage condition 1 or 2 was added, and the resulting mixture was shaken for 1 h at RT. The mixture was filtered into 1 ml of cold Et_2O and centrifuged at maximum speed for 3 min. The supernatant was discarded, and the pellet dissolved in Magic solution (~1 mg/ml) and analysed by LCMS. After the last coupling, the resin was washed 3 times with DCM, dried by N_2 and stored at -20 °C.

General procedure B: on-resin deprotection and phosphorylation

After peptide elongation, the resin was treated with an 8.8M HF/pyridine solution (10 ml/gr resin) for 30 minutes while shaking. The resin was washed with pyridine and the treatment was repeated once, after which it was extensively washed with pyridine and DCM yielding the desilylated ribosyl peptide intermediate. Hereafter, the resin was washed extensively with MeCN and flushed with N_2 to remove all traces of water. A solution of $(AllO)_2PN(iPr)_2$ **25** or $(FmO)_2PN(iPr)_2$ **18** (5 eq., 0.25M in MeCN) was added, followed by ETT activator (10 eq., 0.25M in MeCN). The resin was agitated for 30 minutes, followed by washing with MeCN. A solution of CSO (8 ml/gr resin, 0.5M in MeCN) was added and the mixture was shaken for 30 minutes, after which the resin was washed with MeCN and DCM.

General procedure C: deprotection of Fm groups

The Fm groups were cleaved by treatment of the resin with DBU (10% $^{v}/_{v}$ in DMF, 8 ml/gr resin) for 15 minutes followed by washing with DMF. The treatment was repeated after which the resin was washed with DMF and DCM, yielding the phosphoribosylated intermediate.

General procedure D: deprotection of Allyl groups

The All groups were cleaved by treatment of the resin with a solution consisting of freshly prepared $Pd(PPh_3)_4$ (8.7 mM) and DMBA (147 mM) in DCM (8 ml/gr resin, DCM was purged with N_2 prior to use) for 15 minutes. The resin was rinsed with DCM after which the treatment was repeated thrice yielding the phosphoribosylated peptide intermediate.

General procedure E: Global deprotection

The resin was globally deprotected and cleaved from the resin with cleavage cocktail **A** (16 ml/g resin, 50% $^{v}/_{v}$ TFA, 2.5% $^{v}/_{v}$ TIS in DCM) or cleavage cocktail **B** (20 ml/g resin, 5% $^{v}/_{v}$ TFA, 2.5% $^{v}/_{v}$ TIS in DCM) for 1 hour, after which the solution was filtered into cold Et_2O (5 times the volume of cleavage cocktail). The resin was washed with the respective cleavage cocktail (2 ml/g resin), which was again filtered into the Et_2O. The precipitated peptide was centrifuged for 5 minutes after which the supernatant was discarded. The resulting pellet was resuspended in Et_2O and again centrifuged, followed by removal of the supernatant. The crude peptide was dissolved in a NH_4OAc solution of 1:1 MilliQ:MeCN (100 mM) and lyophilized.

General procedure F: mono-ADPr-peptide condensation reaction

DMC·HCl (169 mg, 1.0 mmol, 10 eq. relative to AMP) and imidazole (136 mg, 2.0 mmol, 20 eq. relative to AMP) were dissolved in D_2O (250 µl) in an Eppendorf tube. D_2O was added until a total volume of 500 µl, after which the mixture was shaken for 5 minutes. The mixture was added to an Eppendorf containing AMP (39 mg, 0.1 mmol), after which it was shaken for 45 minutes. An Eppendorf tube was loaded with Pr-peptide (1 eq.) and $ZnCl_2$ (100 eq.) after which it was dissolved in D_2O (66 mM relative to Pr-peptide). A solution of the activated AMP-im (5 eq., 200 mM) was added to the Pr-peptide (final concentration of 25mM) and the mixture was shaken for 2.5 hours at 40°C. After 2.5 hours, another freshly prepared portion of activated AMP-Im (5 eq., 200 mM) was added to the condensation reaction. After 5 hours of total reaction time, the mixture was diluted with 5 times the reaction volume of MilliQ and desalted using SEC. The fractions containing the mono-ADPr-peptide were collected, lyophilized repeatedly and purified using HPLC.

Synthesis of phosphoribosyl-peptides

H2B1C Ser6Pr: Ac-Pro-Ala-Lys-Ser(O^{γ}-[5'-O-phosphate-α-D-ribosyl])-Ala-Pro-Ala-Pro-Lys-Lys-Gly-OH (**1**)

The general procedures **A**-**C** and **E** were followed as described to 50 µmol Tentagel® S AC resin. The amino acids used were Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH and Fmoc-Ser(ribosyl)-OH building block **13**[40]. On-resin phosphorylation was achieved with $(FmO)_2PN(iPr)_2$ **18**. Global deprotection was achieved using cleavage cocktail **A** for 1 hour. The Pr-peptide was purified by automated column chromatography (C-18 silica, 0% -> 50% H_2O/ACN +10 mM NH_4OAc) and compound **1** was obtained as a white solid (9.85 mg, 7.5 µmol, 8%). **^{1}H-NMR** (600 MHz, D_2O) δ 5.11 (d, J = 4.4 Hz, 1H, H-1) **^{31}P-NMR** (202 MHz, D_2O) 3.99. **LCMS** (00% -> 50% MeCN) Rt = 3.56 min. **LRMS** calculated $[M+H]^+$ = 1305.6, $[M+2H]^{2+}$ = 653.3; observed m/z 1305.8, 653.5.

HPF1 Ser97Pr: H-Thr-Lys-Lys-Lys-Ser(O^{γ}-[5'-O-phosphate-α-D-ribosyl])-Thr-Gly-Leu-Asn-OH (**2**)

The general procedures **A**-**C** and **E** were followed as described to 50 µmol Tentagel® S AC resin. The amino acids used were Fmoc-Thr(*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH and ribosyl building block **13**[40]. On-resin phosphorylation was achieved with $(FmO)_2PN(iPr)_2$ **18**. Global deprotection was achieved using cleavage cocktail **A** for 1 hour. The Pr-peptide was deemed of sufficient quality to continue crude with the condensation reactions. **LCMS** (00% -> 50% MeCN) Rt = 0.89 min. **LRMS** calculated $[M+H]^+$ = 1188.6, $[M+2H]^{2+}$ = 594.8; observed m/z 1188.7, 595.0.

PARP7 Cys721Pr: Ac-Gln-Glu-Asp-Phe-Cys(S^{γ}-[5'-O-phosphate-α-D-ribosyl])-Phe-Leu-Ser-Ser-OH (**3**)

The general procedures **A**-**C** and **E** were followed as described to 50 µmol Tentagel® S AC resin. The amino acids used were Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Phe-OH, Fmoc-Leu-OH, Fmoc-Ser(*t*Bu)-OH and ribosyl building block **14**[40]. On-resin phosphorylation was achieved with $(FmO)_2PN(iPr)_2$ **18**. Global deprotection was achieved using cleavage cocktail **A** for 1 hour. The Pr-peptide was deemed of sufficient quality to continue crude with the condensation reactions. **LCMS** (10% -> 50% MeCN) Rt = 0.89 min. **LRMS** calculated $[M+H]^+$ = 1329.5, $[M+2H]^{2+}$ = 665.7; observed m/z 1329.3, 665.3.

CBX4 Tyr207Pr: Ac-Ala-Ala-Lys-Gly-Tyr(O^{η}-[5'-O-phosphate-α-D-ribosyl])-Leu-Gly-Ala-Val-OH (**4**)

The general procedures **A**-**C** and **E** were followed as described to 50 µmol Tentagel® S AC resin. The amino acids used were Fmoc-Ala-OH, Fmoc-Lys(MMt)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Val-OH and ribosyl building block **15**[21]. On-resin phosphorylation was achieved with $(FmO)_2PN(iPr)_2$ **18**. Global deprotection was achieved using cleavage cocktail **B** for 1 hour. The Pr-peptide was deemed of sufficient quality to continue crude with the condensation reactions. **LCMS** (10% -> 50% MeCN) Rt = 3.74 min. **LRMS** calculated $[M+H]^+$ = 1103.5, $[M+2H]^{2+}$ = 552.8; observed m/z 1103.6, 552.2.

HPF1 Glu240Pr: H-Val-Gly-Tyr-Arg-Glu(O^{ε}-[5'-O-phosphate-α,β-D-ribosyl])-Leu-Pro-Glu-Thr-OH (**5**)

The general procedures **A**-**B** and **D**-**E** were followed as described to 50 µmol Tentagel® S AC resin. The amino acids used were Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH and ribosyl building block **22**[16]. On-resin phosphorylation was achieved with $(AllO)_2PN(iPr)_2$ **25**. Global deprotection was achieved using cleavage cocktail **A** for 1 hour. The Pr-peptide was deemed of sufficient quality to continue crude with the condensation reactions. **LCMS** (10% -> 50% MeCN) Rt = 3.42 min. **LRMS** calculated $[M+H]^+$ = 1275.5, $[M+2H]^{2+}$ = 638.3; observed m/z 1275.8, 638.5.

rS1 Arg139Pr: Ac-Val-Arg(N^{η}-[5'-O-phosphate-α-D-ribosyl])-Pro-Val-NH_2 (**6**)

The general procedure **A** was followed as described to 50 µmol Tentagel® S AC resin. The amino acids used were Fmoc-Val-OH, Fmoc-Pro-OH and Fmoc-Orn(Alloc)-OH. The Alloc on ornithine was deprotected using a solution consisting of freshly prepared $Pd(PPh_3)_4$ (8.7 mM) and DMBA (147 mM) in DCM (8 ml/gr resin, DCM was purged with N_2 prior to use) for 15 minutes. The resin was rinsed with DCM after which the treatment was repeated thrice. The resin was washed with DMF, after which the ribosyl building block **32**[41] was coupled. **32** (3 eq., 0.1M in DMF) was added to the resin after which TEA (30 eq.) and $AgNO_3$ (3 eq.) were added. The syringe was wrapped in aluminium foil to

protect it from light and shaken overnight. The resin was washed with DMF and DCM after which General procedures **B**-**C** and **E** were followed. On-resin phosphorylation was achieved with $(FmO)_2PN(iPr)_2$ **18**. Global deprotection was achieved using cleavage cocktail **A** for 1 hour. The Pr-peptide was deemed of sufficient quality to continue crude with the condensation reactions. **LCMS** (10% -> 50% MeCN) Rt = 3.42 min. **LRMS** calculated $[M+H]^+$ = 723.3; observed m/z 723.4.

Synthesis of ADPr-peptides

HPF1 Ser97ADPr: H-Thr-Lys-Lys-Lys-Ser(O$^{\gamma}$-[5'-O-adenosine-diphosphate-α-D-ribosyl])-Thr-Gly-Leu-Asn-OH (**7**)

General procedure **F** was applied to 5 µmol of crude Pr-peptide **2**. The activated AMP-Im cocktail (125 µl, 25 µmol, 5 eq.) was added to a solution of Pr-peptide (5.9 mg, 5 µmol, 1 eq.) and $ZnCl_2$ (68 mg, 500 µmol, 100 eq.) in D_2O (75 µl, 66mM). HPLC purification using an ammonium acetate buffer, followed by repeated lyophilization yielded the titled ADPr-peptide as a white powder (0.77 mg, 0.51 µmol, 10%). **^{1}H-NMR** (400 MHz, D_2O) δ 8.47 (s, 1H, H-2), 8.23 (s, 1H, H-8), 6.10 (d, *J* = 5.9 Hz, 1H, H-1'), 5.01 - 4.97 (m, 1H), H-1"). **^{31}P-NMR** (162 MHz, D_2O) δ -10.44, -10.57, -10.58, -10.71. **LCMS** (00% -> 20% MeCN) Rt = 4.82 min. **HRMS** $[C_{56}H_{98}N_{18}O_{27}P_2 + 2H^+]$ found: 759.3234, calculated: 759.3235.

PARP7-Cys721ADPr: Ac-Gln-Glu-Asp-Phe-Cys(Sγ-[5'-O-adenosine-diphosphate-α-D-ribosyl])-Phe-Leu-Ser-Ser-OH (**8**)

General procedure **F** was applied to 1 µmol of crude Pr-peptide **3**. The activated AMP-Im cocktail (25 µl, 5 µmol, 5 eq.) was added to a solution of Pr-peptide (1.3 mg, 1 µmol, 1 eq.) and $ZnCl_2$ (14 mg, 100 µmol, 100 eq.) in D_2O (15 µl, 66mM). HPLC purification using an ammonium acetate buffer, followed by repeated lyophilization yielded the titled ADPr-peptide as a white powder (0.31 mg, 0.19 µmol, 20%). **^{1}H-NMR** (400 MHz, D_2O) δ 8.45 (s, 1H, H-2), 8.14 (s, 1H, H-8), 7.30 - 7.07 (m, 10H, Phe arom.), 6.04 (d, *J* = 5.7 Hz, 1H, H-1'), 5.38 (d, *J* = 4.6 Hz, 1H, H-1"). **^{31}P NMR** (162 MHz, D_2O) δ -10.29, -10.42, -10.54, -10.66. **LCMS** (00% -> 50% MeCN) Rt = 5.80 min. **HRMS** $[C_{64}H_{89}N_{15}O_{31}P_2S + 2H^+]$ found: 829.7600, calculated: 829.7595.

CBX4 Tyr207ADPr: Ac-Ala-Ala-Lys-Gly-Tyr(O$^{\eta}$-[5'-O-adenosine-diphosphate-α-D-ribosyl])-Leu-Gly-Ala-Val-OH (**9**)

General procedure **F** was applied to 5 µmol of crude Pr-peptide **4**. The activated AMP-Im cocktail (125 µl, 25 µmol, 5 eq.) was added to a solution of Pr-peptide (5.5 mg, 5 µmol, 1 eq.) and $ZnCl_2$ (68 mg, 500 µmol, 100 eq.) in D_2O (75 µl, 66mM). HPLC purification using an ammonium acetate buffer, followed by repeated lyophilization yielded the titled ADPr-peptide as a white powder (0.94 mg, 0.66 µmol, 13%). **^{1}H NMR** (400 MHz, D_2O) δ 8.49 (s, 1H, H-2), 8.22 (s, 1H, H-8), 6.99 (d, *J* = 8.4 Hz, 2H, Tyr arom.),

6.84 (d, *J* = 8.2 Hz, 2H Tyr, arom.), 6.06 (d, *J* = 5.6 Hz, 1H, H-1'), 5.51 (d, *J* = 4.5 Hz, 1H, H-1"). **^{31}P NMR** (162 MHz, D_2O) δ -10.36, -10.49, -10.61, -10.75. **LCMS** (00% -> 50% MeCN) Rt = 4.78 min. **HRMS** [$C_{56}H_{87}N_{15}O_{25}P_2$+ 2H$^+$] found: 716.7808, calculated: 716.7809.

HPF1 Glu240ADPr: H-Val-Gly-Tyr-Arg-Glu(O$^{\varepsilon}$-[5'-O-adenosine-diphosphate-α,β-D-ribosyl])-Leu-Pro-Glu-Thr-OH (**10**)

General procedure **F** was applied to 5 µmol of crude Pr-peptide **5**. The activated AMP-Im cocktail (125 µl, 25 µmol, 5 eq.) was added to a solution of Pr-peptide (6.4 mg, 5 µmol, 1 eq.) and $ZnCl_2$ (68 mg, 500 µmol, 100 eq.) in D_2O (75 µl, 66 mM). The reaction was analyzed by LCMS. **LCMS** (00% -> 50% MeCN) Rt = 4.78 min.

rS1 Arg139ADPr: Ac-Val-Arg(N$^{\eta}$-[5'-O-adenosine-diphosphate-α-D-ribosyl])-Pro-Val-NH$_2$ (**11**)

General procedure **F** was applied to 1 µmol of crude Pr-peptide **6**. The activated AMP-Im cocktail (25 µl, 5 µmol, 5 eq.) was added to a solution of Pr-peptide (0.72 mg, 1 µmol, 1 eq.) and $ZnCl_2$ (14 mg, 100 µmol, 100 eq.) in D_2O (15 µl, 66mM). HPLC purification using an ammonium acetate buffer, followed by repeated lyophilization yielded the titled ADPr-peptide as a white powder (0.28 mg, 0.28 µmol, 28%). **^{1}H NMR** (400 MHz, D_2O) δ 8.58 - 8.45 (m, 1H, H-2), 8.23 - 8.20 (m, 1H, H-8), 6.09 (d, *J* = 6.0 Hz, 1H, H-1'), 5.29 (d, *J* = 4.4 Hz, 0.4H, H-1" α/β), 5.09 (d, *J* = 5.5 Hz, 0.3H, H-1" α/β), 4.85 (s, 1H). **^{31}P NMR** (162 MHz, D_2O) δ -10.19, -10.22, -10.36, -10.49, -10.59, -10.64, -10.72, -10.77. **LCMS** (00% -> 50% MeCN) Rt = 3.86 min. **HRMS** [$C_{38}H_{63}N_{13}O_{18}P_2$+ H$^+$] found: 1052.3989, calculated: 1052.3962.

H2B1C Ser6(ADPr)2: Ac-Pro-Ala-Lys-Ser(Oγ-[ADPr2])-Ala-Pro-Ala-Pro-Lys-Lys-Gly-OH (**12**)

DMC-HCl (169 mg, 1.0 mmol) and imidazole (136 mg, 2.0 mmol) were dissolved in D_2O (250 µl) in an Eppendorf tube. D_2O was added until a total volume of 500 µl, after which the mixture was shaken for 5 minutes. The mixture was added to an Eppendorf containing ADPr-phosphoribosyl **38** (1.36 µmol, 1.2 eq) to a concentration of 0.2M of phosphate, after which it was shaken for 45 minutes. An Eppendorf tube was loaded with Pr-peptide **1** (1.5 mg, 1.13 µmol, 1 eq.) and $ZnCl_2$ (3.1 mg, 23 µmol, 20 eq.) after which it was dissolved in D_2O (38 µl, 30 mM relative to Pr-peptide). A solution of the activated phosphoribosyl (7.55 µl, 1.36 µmol, 1.2 eq., 200 mM) was added to the Pr-peptide and the mixture was shaken at 40°C overnight. The mixture was diluted with 5 times the reaction volume of MilliQ and desalted using SEC. The fractions containing the titled ADPr-peptide were collected, lyophilized repeatedly. IEX purification using an ammonium acetate buffer gradient of 20 mM to 1.0 M, followed by repeated lyophilization yielded the titled ADPr-peptide as a white powder (0.28 mg, 0.13 µmol, 12%). **^{1}H-NMR** (500 MHz, D_2O) δ 8.36 (s, 1H, H-2), 8.34 (s, 1H ,H-2), 8.12 (s, 1H, H-8), 8.10 (s, 1H, H-8), 6.14 (d, *J* = 3.6 Hz, 1H, H-1'), 5.96 (d, *J* = 6.0 Hz, 1H, H-1'), 5.24 (d, *J* = 4.3 Hz, 1H, H-1"), 4.95 (bs, 1H, H-1"). **^{31}P-NMR** (162 MHz, D_2O) δ -11.14 - -11.61 (m).

LCMS (00% -> 20% MeCN) Rt = 5.36 min. **HRMS** [$C_{79}H_{126}N_{24}O_{40}P_4$+ $2H^+$] found: 1088.3856, calculated: 1088.3830.

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