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

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Chapter 4

Solid-Phase Synthesis of Peptides with ADP-Ribosylated Tyrosine

Introduction

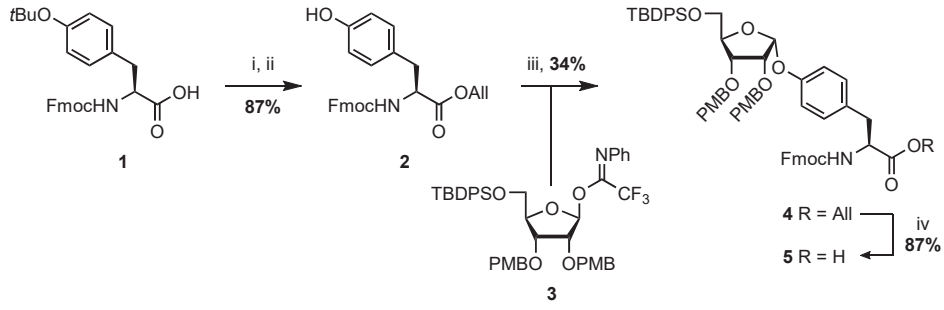
After the emergence of serine (Ser) as an ADPr acceptor,[1] it became apparent that ADPr as a post-translational modification extended to a wider variety of acceptor sources than previously thought. This prompted an unbiased re-evaluation of the LC-MS/MS datasets from past proteomics studies for the possible presence of the ADP-ribosylated amino acids that were not considered before as prevalent sites of modification, for example, Ser, tyrosine (Tyr) and threonine (Thr).[2] Besides redirection of the ADP-ribosylation site from lysine (Lys) to Ser in the apparent Lys-Ser (KS) recognition motif,[3] Tyr proved to be an acceptor of ADPr as well, albeit to a lesser extent than Ser.[4–7] It is noteworthy that this was observed as well in protein phosphorylation patterns, where Ser and Thr are modified more abundantly than Tyr which was estimated to take up less than 1% of the total phosphoproteome.[8–10] Furthermore, both Ser- and Tyr-ADP-ribosylation sites have been found to significantly overlap with phosphorylation sites,[4,11] indicating a possible crosstalk between ADP-ribosylation and phosphorylation of proteins. The importance of phosphoryl Tyr (P-Tyr) as a regulatory modification (excellently reviewed here[12]) is abundantly clear and it is suggested that the functional role of P-Tyr is quite distinct from that of P-Ser and P-Thr.[13] Given the possible interplay between Tyr-ADPr/Ser-ADPr and the phosphorylation of Ser one cannot exclude a regulatory role for Tyr-ADPr.

The identified Tyr-ADPr proteins appear to be mostly localized in the nucleus and are assembled by the PARP1:HPF1 complex.[4,14] ADP-ribosylation of Tyr by PARP1:HPF1 is also dependent on DNA damage thus bearing similarities with its Ser-ADPr counterpart. As described above, Tyr-ADPr occurs to a lesser extent, being responsible for approximately 3% of the ADPr proteome compared to 83% for Ser-ADPr.[5] Interestingly, HPF1 is ADP-ribosylated on Y238, a residue crucial for proper functioning of HPF1[15] which might indicate a regulatory role for the Tyr-ADPr modification on HPF1. Besides HPF1, PARP14 is modified on three Tyr residues.[5] PARP14 is a *mono*-ADP-ribosylating (MARylating) enzyme[16] and plays a regulatory role in RNA stability by modifying PARP13 and several other players in the RNA regulatory proteome.[17] The crosstalk between several members of the PARP family, HPF1 and Tyr-ADPr raises questions as to the exact physiological function of Tyr as ADPr acceptor. Synthetic, well-defined Tyr-ADP ribosylated peptides can greatly assist in elucidating the physiological role of Tyr-ADPr as is demonstrated by recent studies using synthetic peptides ADP-ribosylated at other amino acids.[18–20] This chapter describes a solid phase methodology developed for the synthesis of peptides MARylated at a Tyr residue and the biological evaluation thereof.

Results and Discussion

Building block synthesis

The synthetic strategy to obtain peptides modified with mono ADP-ribose at a predetermined Ser, Thr or cysteine (Cys) side chain as described in Chapter 3, was adopted to acquire peptides MARylated at a Tyr residue. Therefore, the efforts were firstly turned to synthesizing a ribosylated Tyr building block (**5**, Scheme 1) to be used in Fmoc-based solid phase peptide synthesis (SPPS). Commercially available Fmoc-Tyr(*t*Bu)-OH **1** was treated with allyl bromide under basic conditions to orthogonally protect the carboxylic acid as an allyl ester. The crude intermediate was then directly treated with TFA in DCM with TIS as a scavenger to cleave the *tert*-butyl aryl ether furnishing phenolic acceptor **2** in 87% yield over two steps.



Scheme 1. Synthesis of Tyr-ribosylated building block **5**, ready for SPPS. Reagents and conditions: i) All-Br, DIPEA, DMF. ii) 20% TFA in DCM, TIS. iii) TBSOTf, DCM, -50 °C. iv) $Pd(PPh_3)_4$, DMBA, DCM.

Next, the glycosylation reaction of this Tyr acceptor with known ribosyl donor **3** was investigated, starting with the same conditions as described in Chapter 2 for the Ser, Thr and Cys acceptors (Table 1, entry 1).[18] Under these conditions however, solubility issues arose as acceptor **2** did not dissolve properly in 0.1 M DCM at -50 °C. Therefore, a 1:1 mixture of DCM and 1,4-dioxane was used (entry 2) which proved to be effective in addressing similar issues in the past.[21] Unfortunately, the use of this solvent mixture did not improve the solubility of the Tyr acceptor. As the mixture of acceptor **2** and donor **3** is soluble in DCM up to 0.1 M concentration at room temperature, the following glycosylation procedure was attempted. At room temperature, a 0.1 M solution of compounds **2** and **3** in DCM was prepared that was subsequently slowly cooled to -20 °C during which a clear solution persisted. At this temperature, the glycosylation was performed (entry 3) as further lowering of the temperature resulted in the precipitation of acceptor **2**. However, addition of the activator resulted in a complex mixture of products which was inseparable by column chromatography. In line with the results of the similar glycosylation experiments

described in Chapter 3, the formation of multiple products can be explained by the loss of a-stereoselectivity and the unwanted acid catalyzed loss of the PMB protecting groups, at the temperature applied.[21]

Table 1. Optimization of the glycosylation conditions of acceptor **2** with donor **3**. All reactions were carried out at a 0.2 mmol scale. C (M) is the concentration of the donor in the solvent with 0.1 equivalents of activator relative to the donor. n.d. = not determined.

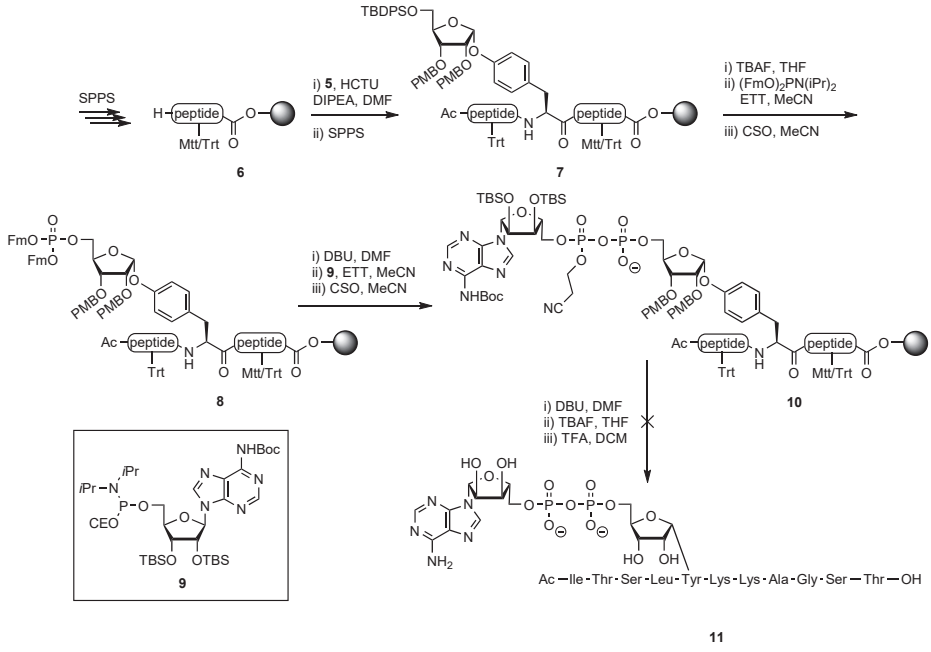
Entry	Solvent	C (M)	Activator	T (°C)	Reaction time	Yield
1	DCM	0.1	TMSOTf	-50	1 h	n.d.
2	DCM:dioxane	0.1	TMSOTf	-50	1 h	n.d.
3	DCM	0.1	TMSOTf	-20	1 h	n.d.
4	DCM	0.03	TMSOTf	-50	1 h	38%
5	DCM	0.03	TBSOTf	-50	2.5 h	47%

Decreasing the concentration of the reaction to 0.03 M allowed the temperature to be maintained at -50 °C without precipitation of acceptor **2** (entry 4). In this way, fully protected, ribosylated Tyr **4** could be obtained in a moderate 38% yield that was attributed to the slower reaction rate at this concentration. After increasing the reaction time to 2.5 hours (entry 5) and changing the activator to TBSOTf, the unwanted side reactions could be suppressed to give ribosylated Tyr **4** in 47% yield. However, scaling up the reaction to a 2.0 mmol scale proved difficult as the yield decreased to 34% (Scheme 1) but no attempts to further optimize this reaction were made as enough material was available to proceed. Pd-catalyzed cleavage of the allyl ester, using 1,3-dimethylbarbituric acid (DMBA) as scavenger, furnished orthogonally protected *O*-ribosylated Tyr **5**, suitable for the intended Fmoc-based SPPS strategy.

Solid phase synthesis of MARylated Tyr-peptide.

The SPPS strategy to acquire peptide **11**, MARylated at its Tyr residue, is depicted in Scheme 2. With the aid of standard Fmoc-chemistry, TentaGel® S AC resin pre-loaded with Thr, was elongated with commercially available Fmoc-amino acids to give immobilized oligopeptide **6.** Subsequent incorporation of Tyr-building block **5** and further elongation led to immobilized peptide **7**. At this stage, the phosphotriester at the 5-OH of the ribose moiety was introduced. Starting with TBAF mediated desilylation, phosphitylation of the released alcohol with Fm protected phosphoramidite and finally oxidation of the phosphite triester produced protected phosphotriester **8**. Next, DBU mediated removal of the Fmoc groups in **8** yielded the corresponding phosphate monoester which was followed by reaction with phosphoramidite **9**. Oxidation of the resulting P^{III} – P^{V} intermediate furnished immobilized, protected MARylated Tyr-peptide **10**. Similar to its Ser, Cys and Thr

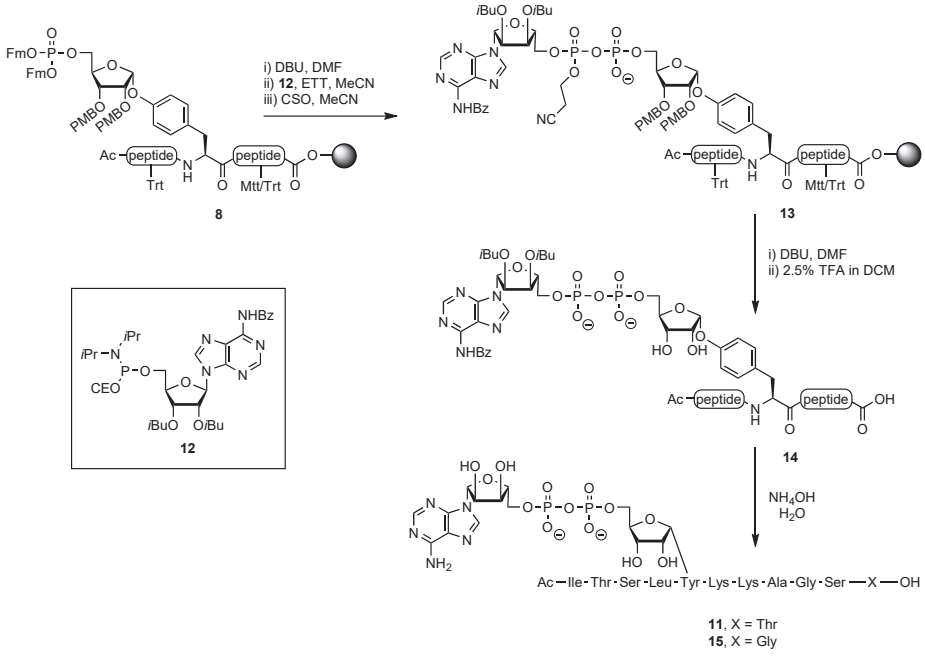
counterparts, the deprotection of **10** comprised of (i) removal of the cyanoethyl protecting group with DBU, (ii) desilylation of the 2'- and 3'-hydroxyls of the adenosine moiety and (iii) cleavage of the target peptide from the resin, together with the accompanying cleavage of the remaining protecting groups with 10% TFA in DCM. LC-MS analysis of the obtained crude mixture revealed a main product (~75% based on the UV-absorption measured in the LC-MS trace) that did not correspond to target **11** but with the free peptide in which the ADPr moiety was lacking. TFA-mediated acidolysis of the glycosidic bond of the tyrosyl ribofuranoside in **11** was suspected. Lowering the concentration of TFA in the cleavage cocktail to 5% did decrease the observed acidolysis to approximately 25% and further reducing the TFA concentration to 2.5% completely prevented this side reaction. Although the Trt, Mtt and PMB groups were completely removed in 2.5% TFA in DCM, the Boc protecting group on the exocyclic amine of the adenine was found to be too stable and required TFA levels of 5% or more for its removal.



Scheme 2. Attempted synthesis of Tyr-ADPr peptide **11** using the strategy developed in Chapter 3.

In order to prevent the unwanted acidolysis of the glycosidic linkage, an alternative for the Boc protecting group on the exocyclic amine of adenine was sought. As outlined in Chapter 2,[18,19] adenosine phosphoramidite **12** (Scheme 3), where the 2'- and 3' hydroxyl groups are protected with *iso*-butyryl and the exocyclic amine is masked with a benzoyl

group, was used for the synthesis of peptides MARylated at Ser. Application of **12** as a replacement for reagent **9** for the introduction of the pyrophosphate moiety using the same sequence of reactions, gave partially protected immobilized peptide **13** (Scheme 3). Removal of the protecting groups started with DBU-mediated cyanoethyl elimination, followed by treatment with 2.5% TFA in DCM, inducing both the removal of the Trt, Mtt, and PMB protecting groups and cleavage of the TentaGel® S AC linker, to give partially protected peptide **14**. Finally, removal of all base labile protecting groups on adenosine in **14** by treatment with a saturated, aqueous ammonium hydroxide solution resulted, after purification, in the isolation of Tyr-ADPr peptide **11** in 4.5% yield. Remarkably, LC-MS analysis showed that approximately 25% of the crude mixture to constitute a side product 18 Da lower in mass. The formation of this side product was attributed to treatment of the intermediate peptide **13** (and probably also **8**) with DBU, that allows for an E1cb reaction thereby eliminating the Trt group of the Thr residue. To prevent this side reaction, Thr was replaced with glycine (Gly) and the peptide synthesis was repeated. Gratifyingly, no dehydrated mass could be detected and *via* this method, peptide Ac-ITSLY^{ADPr}KKAGSG-OH (**15**) was obtained in 14% yield.



Scheme 3. Outline of the strategy in which adenosine amidite **9** is replaced by **12** and the accompanying deprotection sequence to provide **11** without acidolysis of the anomeric Tyr-ADPr bond.

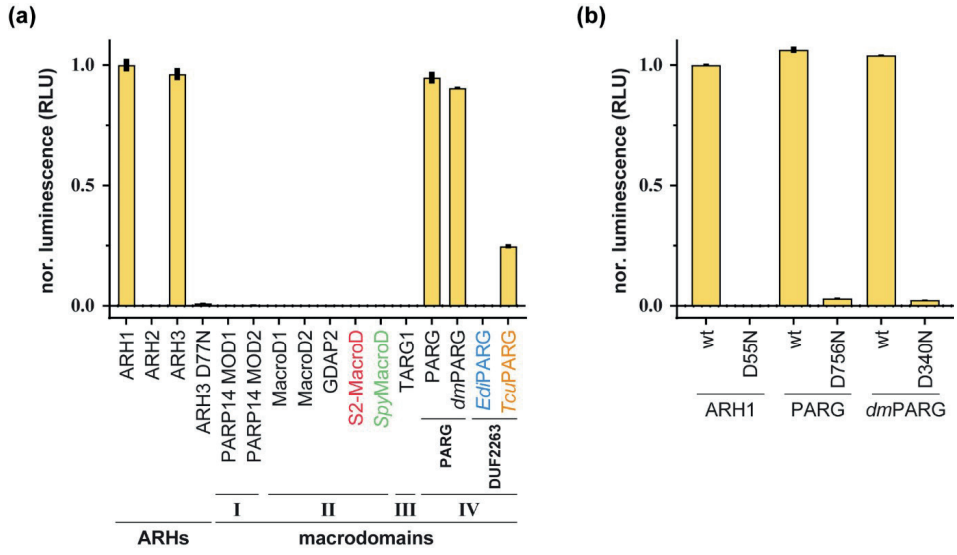


Figure 1. Hydrolysis of Tyr-MARylated peptide **11** by known (ADP-ribosyl)-hydrolases, performed by Johannes Gregor Matthias Rack affiliated with the Sir William Dunn School of Pathology, University of Oxford. Briefly, the ADP-ribose released in the hydrolysis reaction was converted by human NUDT5 to AMP, which in turn was detected via chemiluminescence using the AMP-Glo™ assay (Promega). Samples were background corrected, normalized to ARH1 wt and presented as mean values ± s.d. from triplicate measurements. (a) Hydrolysis of Tyr-MARylated peptide **11** by selected (ADP-ribosyl)-hydrolases of the ARH and macrodomain family. Protein from Animalia are indicated in black, Amoebozoa in blue, viral in red, bacterial in green and Archaea in orange. The macrodomains family is subdivided in several clades: (I) MacroH2A-like, (II) MacroD-type, (III) ALC1-like, and (IV) PARG-like. (b) Control experiment showing that Tyr-ADP-ribosylation reversal by ARH1 and PARG is enzyme-dependent.

Biological evaluation

As Tyr-ADPr is a newly found modification, the enzyme responsible for its reverse reaction is yet unknown. Therefore, a hydrolytic screening assay was performed with peptide **11**, testing a variety of ADP-ribosyl hydrolases in both the ARH- and macrodomain-family, the results of which are summarized in Figure 1a. Starting with the ARH family, ARH2 which is thought to be catalytically inactive,[22] shows no turnover of Tyr-ADPr. ARH3 and ARH1 are both efficient in hydrolyzing Tyr-ADPr which is interesting in the case of ARH1 as this hydrolase is generally thought to be selective towards *N*-linked Arg-ADPr.[22,23] This is the first report of ARH1 being able to hydrolyze an *O*-glycosidic bond despite the distinctly different modes of substrate recognition and ligand binding between ARH1 and ARH3.[24] Also, an unexpected turnover of Tyr-ADPr was encountered in the macrodomain family as PARG was able to hydrolyze peptide **11**. The surprising activity of ARH1 and PARG prompted

further experimentation with ARH1 and PARG mutants rendered catalytically inactive as control (Figure 1b). The catalytic mutants showed no enzymatic turnover of Tyr-ADPr, confirming that the displayed hydrolysis is indeed enzyme-dependent.

Conclusion

This chapter describes the synthesis of a ribosylated Fmoc-Tyr building block (**5**) and the accompanying optimization of the reaction conditions to couple ribosyl donor **3** to Tyr acceptor **2**. Building block **5** was applied in SPPS to produce two peptides MARylated at the Tyr residue. The observed acid lability of the glycosidic bond in Tyr-ADPr led to the development of a new strategy by a combination of the SPPS described in Chapters 2 and 3 by taking the temporary base-labile protecting groups on the adenosine moiety (Chapter 2) and the acid-labile protections on the ribosyl part and amino acid side chains (Chapter 3) to assemble Tyr-ADPr peptides **11** and **15**.

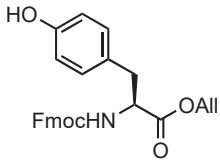
With peptide **11**, the hydrolytic properties of several known ADPr hydrolases towards the Tyr-ADPr modification was tested showing that ARH3 is able to hydrolyze Tyr-ADPr like Ser- and Thr-ADPr. However, an unexpected enzymatic activity of ARH1 and PARG towards **11** was encountered. Although ARH1 is now shown to posess a broader specificity and not limited to Arg-ADPr hydrolysis, the emergence of PARG as a Tyr-ADPr hydrolyzing enzyme is unexpected.

Experimental section

General synthetic procedures

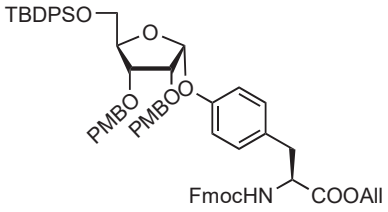
All reagents were used as received unless stated otherwise. Solvents used in synthesis were dried and stored over 4Å molecular sieves, except for MeOH and MeCN which were stored over 3Å molecular sieves. Triethylamine (TEA) and diisopropylethylamine (DIPEA) were stored over KOH pellets. Column chromatography 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 instrument. Chemical shifts (δ) are given in ppm relative to tetramethyl silane as internal standard. Coupling constants (*J*) are given in Hz. For compounds **11** and **15**, 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.

***N*-α-Fmoc-tyrosine allyl ester (2)**



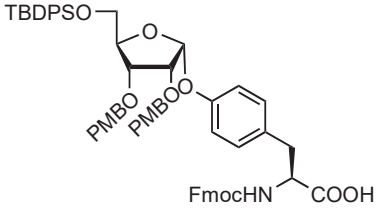
Commercially available Fmoc-Tyr(*t*Bu)-OH **1** (2.22 g, 5.00 mmol, 1 eq.) was co-evaporated thrice with 1,4-dioxane and dissolved in dry DMF (25 mL, 0.2 M). DIPEA (1.04 mL, 6 mmol, 1.2 eq.) was added followed by the addition of allyl-bromide (0.52 mL, 6 mmol. 1.2 eq.) and the reaction was stirred overnight. The reaction was quenched with H_2O (50 mL) and transferred into a separatory funnel. The reaction mixture was extracted thrice with Et_2O and the combined organic layers were washed three times with brine, dried over $MgSO_4$ and concentrated *in vacuo* furnishing the crude *tert*-butyl protected tyrosine allyl ester. The crude product was dissolved in a 4:1 v/v% DCM:TFA mixture (10 mL, 0.5 M), TIS (4.10 mL, 20 mmol, 4 eq.) was added and the reaction was stirred for 3 hours, after which it was diluted with toluene and concentrated *in vacuo*. The residue was co-evaporated thrice with toluene to remove the last traces of TFA. Flash column chromatography (10 -> 40% EtOAc in pentane) afforded the title compound as an off-white solid (1.93 g, 4.36 mmol, 87% over 2 steps) **Rf**: 0.70 in 40% EtOAc in pentane. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.76 (dd, *J* = 7.6, 1.1 Hz, 2H, Fmoc arom.), 7.65 – 7.55 (m, 2H, Fmoc arom.), 7.40 (td, *J* = 7.5, 1.1 Hz, 2H, Fmoc arom.), 7.31 (tt, *J* = 7.4, 1.3 Hz, 2H, Fmoc arom.), 5.91 (ddt, *J* = 16.4, 10.9, 5.7 Hz, 1H, OCH_2CHCH_2), 5.77 (d, *J* = 7.8 Hz, 1H, NH), 5.40 – 5.32 (m, 1H, OCH_2CHCH_{2a}), 5.26 (dd, *J* = 9.4, 1.2 Hz, 1H, OCH_2CHCH_{2b}), 4.69 (d, *J* = 5.7 Hz, 2H, OCH_2CHCH_2), 4.51 – 4.41 (m, 3H, CH_2 Fmoc + CH Tyr), 4.22 (t, *J* = 6.9 Hz, 1H, CH Fmoc), 3.98 (dd, *J* = 25.6, 11.0 Hz, 2H, CH_2 Tyr), 2.28 (s, 1H, OH). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 143.78, 141.47, 131.31 (OCH_2CHCH_2), 127.88, 127.24, 127.21, 125.22, 120.15, 119.18 (OCH_2CHCH_2), 67.34 (CH_2 Fmoc), 66.52 (OCH_2CHCH_2), 63.44 (CH_2 Tyr), 56.22 (CH Tyr), 47.25 (CH Fmoc).

1-*O*-(2,3-bis-*O*-(4-methoxybenzyl)-5-*O*-((*tert*-butyl)-diphenylsilyl)-α-D-ribosyl)-*N*-fluorenylmethoxycarbonyltyrosine allyl ester (4)



Compound[21] **3** (1.60 g, 2.00 mmol, 1.1 eq.) and Fmoc-Tyr(OH)-OAll **2** (734 mg, 1.82 mmol, 1.0 eq. relative to the donor) were co-evaporated thrice with toluene and dissolved in DCM (70 mL, 0.03 M donor concentration). The reaction was cooled to -50 °C and TBSOTf (46 µL, 0.2 mmol, 0.1 eq. relative to the donor) was added. The reaction was stirred at -50 °C overnight after which TLC analysis showed near full conversion of the starting material. The reaction was quenched with TEA and concentrated *in vacuo*. Flash column chromatography (0.5 -> 3% acetone in DCM) yielded the title compound as a clear oil (651 mg, 0.617 mmol, 34%). **Rf:** 0.58 in 3% acetone in DCM. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.78 – 7.70 (m, 2H, Fmoc arom.), 7.67 – 7.51 (m, 6H, Fmoc arom. + TBDPS arom.), 7.48 – 7.24 (m, 14H, Fmoc arom. + TBDPS arom. + PMB arom.), 7.11 – 6.97 (m, 4H, Tyr arom.), 6.86 – 6.75 (m, 4H, PMB arom.), 5.88 (ddd, *J* = 16.5, 10.6, 5.2 Hz, 1H, CH_2CHCH_2), 5.51 (d, *J* = 4.3 Hz, 1H, H-1), 5.38 – 5.19 (m, 3H, NH + CH_2CHCH_2), 4.74 – 4.50 (m, 8H, CH Ser + CH_2CHCH_2), 4.44 (dd, *J* = 10.6, 7.1 Hz, 1H, CH_{2a} Fmoc), 4.40 – 4.29 (m, 1H, CH_{2b} Fmoc), 4.29 – 4.15 (m, 2H, H-4 + CH Fmoc), 4.09 (dd, *J* = 6.5, 2.8 Hz, 1H, H-3), 3.99 (dd, *J* = 6.4, 4.3 Hz, 1H, H-2), 3.78 (s, 3H, CH_3 PMB), 3.76 (s, 3H, CH_3 PMB), 3.58 (ddd, *J* = 53.4, 11.3, 3.1 Hz, 2H, H-5), 3.10 (d, *J* = 5.7 Hz, 2H, CH_2 Tyr), 0.95 (s, 9H, *t*Bu TBDPS).**^{13}C NMR:** (101 MHz, $CDCl_3$) δ 171.3 (C=O COOAll), 159.4, 159.3 (Cq PMB), 156.9 (C=O Fmoc), 155.7 (Cq Tyr), 143.9, 143.8, 141.4 (Cq Fmoc), 135.7, 135.6 (CH arom. TBDPS), 133.2, 133.1 (Cq TBDPS), 131.5 ($CHCHCH_2$), 130.4 (Cq PMB), 130.3 (CH arom. Tyr), 129.9, 129.8, 129.7, 129.7 (CH arom.), 128.9 (Cq PMB), 127.9, 127.9, 127.8, 127.8, 127.8, 127.2, 125.3, 125.2 (CH arom.), 120.1, 120.0 (CH arom. Fmoc), 119.3 (CH_2CHC, 117.5 (CH arom. Tyr), 113.9, 113.9, 113.8 (CH arom. PMB), 100.0 (C-1), 84.2 (C-4), 77.7 (C-2), 75.0 (C-3), 72.4, 72.1 (CH_2 PMB), 67.1 (CH_2 Fmoc), 66.2 (CH_2CHCH_2), 64.0 (C-5), 55.3, 55.3 (CH_3 PMB), 54.9 (CH Tyr), 47.2 (CH Fmoc), 37.5 (CH_2 Tyr), 26.9 (CH_3 *t*Bu), 19.3 (Cq *t*Bu). **HRMS:** $[C_{64}H_{67}NO_{11}Si + Na]^+$ found: 1076.4374, calculated: 1076.4376.

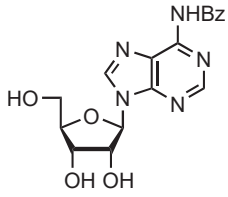
1-*O*-(2,3-bis-*O*-(4-methoxybenzyl)-5-*O*-((*tert*-butyl)-diphenylsilyl)-α-D-ribosyl)-*N*-fluorenylmethoxycarbonyl tyrosine (5)



Compound **4** (626 mg, 0.594 mmol, 1.0 eq.) was dissolved in DCM (6.0 mL, 0.1 M). DMBA (184 mg, 1.18 mmol, 2.0 eq.) and $Pd(PPh_3)_4$ (6.9 mg, 5.9 µmol, 0.01 eq.) were added and the reaction was stirred for 1 hour before TLC showed full conversion of the starting material into a lower running product. The reaction was diluted with DCM, washed with 1 M HCl and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography in (4 -> 6% MeOH in DCM) yielded the title compound as a white foam (518 mg, 0.511 mmol, 87%). **Rf:** 0.50 in 5% MeOH in DCM + 0.1% AcOH. **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 7.5 Hz, 2H, Fmoc arom.), 7.65 – 7.49 (m, 6H, Fmoc arom. + TBDPS arom.), 7.47 – 7.20 (m, 14H, Fmoc arom. + TBDPS arom. + PMB arom.), 7.09 – 6.97 (m, 4H, Tyr arom.), 6.88 – 6.75 (m, 4H, PMB arom.), 5.48 (d, *J* = 4.3 Hz, 1H, H-1), 5.32 (d, *J* = 8.1 Hz, 1H, NH), 4.70 – 4.51 (m, 5H, 2x CH_2 PMB + CH Ser), 4.44 (dd, *J* = 10.5, 7.2 Hz, 1H, CH_{2a} Fmoc), 4.32 (dd, *J* = 10.8, 7.1 Hz, 1H, CH_{2b} Fmoc), 4.28 – 4.15 (m, 2H, H-4 + CH Fmoc), 4.08 (dd, *J* = 6.5, 2.6 Hz, 1H, H-3), 3.97 (dd, *J* = 6.5, 4.4 Hz, 1H, H-2), 3.76 (s, 3H, CH_3 PMB), 3.74 (s, 3H, CH_3 PMB), 3.63 (dd, *J* = 11.1, 3.3 Hz, 1H, H-5_a), 3.49 (dd, *J* = 11.2, 2.8 Hz, 1H, H-5_b), 3.18 – 3.01 (m, 2H, CH_2 Tyr), 0.94 (s, 9H, *t*Bu TBDPS). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 174.8 (C=O COOH), 159.4, 159.3 (Cq PMB), 156.8 (C=O Fmoc), 155.9 (Cq Tyr), 143.9 9, 143.8, 141.4 (Cq Fmoc), 135.7, 135.6 (CH arom. TBDPS), 133.2,

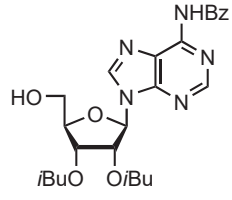
133.1 (Cq TBDPS), 130.4 (CH arom. Tyr), 130.2 (Cq PMB), 129.9, 129.9, 129.8, 129.8, 129.6 (CH arom.), 129.0 (Cq PMB), 127.9, 127.9, 127.8, 127.2, 125.3, 125.2, 120.0 (CH arom.), 117.5 (CH arom. Tyr), 114.0, 114.0, 113.8 (CH arom. PMB), 99.8 (C-1), 84.3 (C-4), 77.8 (C-2), 75.0 (C-3), 72.4, 72.1 (CH_2 PMB), 67.2 (CH_2 Fmoc), 64.0 (C-5), 55.3 (CH_3 PMB), 54.7 (CH Tyr), 47.2 (CH Fmoc), 37.0, (CH_2 Tyr), 26.9 (CH_3 *t*Bu), 19.3 (Cq *t*Bu). **HRMS:** $[C_{61}H_{63}NO_{11}Si + Na]^+$ found: 1036.4060, calculated: 1036.4063.

***N*⁶-benzoyl adenosine**



Commercially available adenosine (5.34 g, 20.0 mmol) was co-evaporated thrice with anhydrous pyridine (3 x 20 mL) and suspended in anhydrous pyridine (100 mL, 0.2 M) after which trimethylsilyl chloride (22.8 mL, 180 mmol, 9 eq.) was carefully added. After stirring for 30 minutes the solution had turned clear and benzoyl chloride (11.6 mL, 100 mmol, 5 eq.) was added dropwise over 5 minutes and stirring continued for another 3 hours. The solution was cooled in an ice bath and quenched by the addition of H_2O (20 mL). The mixture was allowed to warm to room temperature, followed by the addition of an aqueous ammonium hydroxide solution (30 wt. % NH_4OH in H_2O, 40 mL), resulting in the precipitation of the title compound as a white solid. The suspension was stirred for 1 hour, concentrated *in vacuo* and the white residue was partitioned between EtOAc (80 mL) and H_2O (280 mL). The aqueous layer was cooled to 0 °C to induce crystallization of the product which was collected by filtration, rinsed with ice cold Et_2O (30 mL) and dried under high vacuum at 60 °C to afford the title compound as a white crystalline solid (7.43 g, 20 mmol, quant.) Spectral data was in accordance with literary precedence. **Rf:** 0.25 (5% MeOH in DCM). **^{1}H NMR:** (400 MHz, DMSO-d_6): δ 10.50 (br. s, 1H, 6-NH), 8.76 (s, 1H, H-2), 8.73 (s, 1H, H-8), 8.09 – 8.02 (m, 2H, *o*-Bz), 7.70 – 7.61 (m, 1H, *p*-Bz), 7.60 – 7.51 (m, 2H, *m*-Bz), 6.04 (d, 1H, *J* = 5.8 Hz, H-1'), 5.58 (d, 1H, *J* = 6.1 Hz, 2'-OH), 5.27 (d, 1H, *J* = 4.9 Hz, 3'-OH), 5.15 (t, 1H, *J* = 5.6 Hz, 5'- OH), 4.66 (app. q, 1H, *J* = 5.6 Hz, H-2'), 4.19 (app. q, 1H, *J* = 4.9 Hz, H-3'), 3.99 (app. q, 1H, *J* = 3.9 Hz, H- 4'), 3.70 (app. dt, 1H, *J* = 11.9, 4.7 Hz, H-5'), 3.58 (ddd, 1H, *J* = 12.0, 6.1, 4.0 Hz, H-5'). **^{13}C NMR:** (101 MHz, DMSO-d_6): δ 166.2 (C=O), 152.7 (C-6), 152.1 (C-2), 150.9 (C-4), 143.7 (C-8), 133.8 (*ipso*-Bz), 133.0 (*p*-Bz), 129.0 (*o*-Bz, *m*-Bz), 126.3 (C-5), 88.1 (C-1'), 86.2 (C-4'), 74.2 (C-2'), 70.9 (C-3'), 61.8 (C-5').

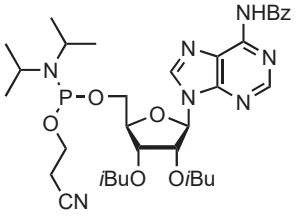
***N*⁶-benzoyl-2',3'-di-*O*-iso-butyryladenosine**



*N*⁶-benzoyl adenosine (1.86 g, 5.0 mmol) was co-evaporated thrice with anhydrous pyridine (3 x 10 mL) and dissolved in anhydrous pyridine (25 mL, 0.2 M) after which *tert*-butyldimethylsilyl chloride (50 wt. % in toluene, 1.91 mL, 5.5 mmol, 1.1 eq.) was added. After stirring overnight, isobutyric anhydride (3.0 mL, 18.1 mmol, 3.6 eq.) was added and stirring continued for a further 7 hours. The mixture was quenched by the addition of H_2O (0.36 mL, 20 mmol, 4 eq.), stirred for 30 minutes and concentrated *in vacuo*. The residue was taken up in EtOAc (100 mL) and washed successively with sat. aq. $NaHCO_3$ (50 mL), 1 M citric acid (50 mL) and H_2O (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography (0 -> 50% EtOAc in pentane) furnished the intermediate product as a white foam (2.67 g, 4.27 mmol, 85%). The silylate compound was then dissolved in MeCN:H_2O (4:1; v/v, 43 mL, 0.1 M), after which *para*-toluene sulfonic acid monohydrate (1.14 g, 5.97 mmol, 1.4 eq.) was added. After stirring for 7 hours, the solution was neutralized by the addition of solid $NaHCO_3$ until pH ~ 7 and concentrated to dryness. The residue was partitioned between EtOAc (200 mL) and sat. aq. $NaHCO_3$ (50 mL) and the organic layer washed thrice with H_2O (3 x 50 mL). The combined water layers were extracted twice more with EtOAc (2 x 50 mL) and the resulting organic

layers combined, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (0 -> 1% MeOH in DCM) afforded the title compound as a white crystalline solid (2.22 g, 4.27 mmol, quant.). Spectral data was in accordance with literary precedence. **Rf:** 0.52 (3% MeOH in DCM). **^{1}H NMR:** (400 MHz, $CDCl_3$) δ 9.48 (br. s, 1H, 6-NH), 8.72 (s, 1H, H-2), 8.18 (s, 1H, H-8), 8.05 – 7.97 (m, 2H, *o*-Bz), 7.61 – 7.52 (m, 1H, *p*-Bz), 7.52 – 7.43 (m, 2H, m-Bz), 6.14 (d, 1H, *J* = 7.4 Hz, H-1'), 5.98 (dd, 1H, *J* = 7.5, 5.4 Hz, H-2'), 5.69 (dd, 1H, *J* = 5.4, 1.6 Hz, H-3'), 4.33 (app. q, 1H, *J* = 1.8 Hz, H-4'), 3.98 (dd, 1H, *J* = 12.9, 1.9 Hz, H-5'), 3.86 (dd, 1H, *J* = 12.8, 1.9 Hz, H-5'), 2.64 (hept, 1H, *J* = 7.0 Hz, $CH(Me)_2$*i*Bu), 2.49 (hept, 1H, J = 7.0 Hz, $CH(Me)_2$ *i*Bu), 1.24 – 1.20 (m, 6H, CH_3 *i*Bu), 1.09 (d, 3H, *J* = 7.0 Hz, CH_3 *i*Bu), 1.05 (d, 3H, *J* = 7.0 Hz, *i*Bu). **^{13}C NMR:** (101 MHz, $CDCl_3$) δ 175.8, 175.2 (C=O *i*Bu), 165.0 (C=O Bz), 152.3 (C-2), 151.0 (C-6), 150.3 (C-4), 142.4 (C-8), 133.4 (*ipso*-Bz), 132.9 (*p*-Bz), 128.8 (*m*-Bz), 128.0 (*o*-Bz), 124.3 (C-5), 88.3 (C-1'), 86.2 (C-4'), 73.0 (C-2'), 72.2 (C-3'), 62.4 (C-5'), 33.9, 33.6 (Ct *i*Bu), 19.0, 18.8, 18.8, 18.7 (Cp *i*Bu).

5'-*O*-(N^6-benzoyl-2',3'-di-*O*-iso-butyryladenosine)-2-cyanoethyl-*N*-*N*-diisopropylphosphoramidite (12)



N^6-benzoyl-2',3'-di-*O*-isobutyryladenosine (2.56 g, 5.0 mmol) was co-evaporated thrice in toluene and dissolved in dry DCM (20 mL, 0.3 M). DIPEA (2.28 mL, 12.5 mmol, 2.5 eq.) and 2-cyanoethyl-*N*,*N*-diis opropylchlorophosphoramidite (1.2 mL, 5.5 mmol, 1.1 eq.) were added and the reaction was stirred for 1 hour. The reaction was diluted with DCM washed with sat. aq. $NaHCO_3$ followed by brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (50% EtOAc in pentane + 1% TEA) yielded the title compound as a white foam (3.45 g, 4.84 mmol, 97%). Spectral data was in accordance with literary precedence[25]. **^{1}H NMR:** (500 MHz, $CDCl_3$) δ 9.26 (bs, 1H, NH), 8.84 – 8.74 (m, 1H, H-2), 8.57 – 8.50 (m, 1H, H-8), 8.07 – 7.97 (m, 2H, *o*-Bz), 7.60 (dt, 1H, *p*-Bz), 7.52 (dt, J = 9.0, 6.9, 1.7 Hz, 2H, *m*-Bz), 6.44 (dd, J = 22.9, 7.0 Hz, 1H, H-1'), 5.83 (ddd, J = 18.4, 7.0, 5.4 Hz, 1H, H-2'), 5.63 (ddd, J = 13.1, 5.4, 2.3 Hz, 1H, H-3'), 4.41 (p, J = 2.6 Hz, 1H, H-4'), 4.10 – 3.77 (m, 4H, H-5' + OCH_2CH_2CN), 3.74 – 3.55 (m, 2H, 2x CH N-*i*Pr), 2.76 – 2.63 (m, 3H, OCH_2CH_2CN + CH *i*Bu), 2.52 (pd, J = 7.0, 5.3 Hz, 1H, CH *i*Bu), 1.28 – 1.16 (m, 18H, 4x CH_3 N-*i*Pr + 2x CH_3 *i*Bu), 1.14 – 1.03 (m, 6H 2x CH_3 *i*Bu). **^{13}C NMR:** (126 MHz, $CDCl_3$) δ 176.0, 175.9, 175.5, 175.3 (C=O *i*Bu), 164.8 (C=O Bz), 153.0, 152.9 (C-6), 152.1, 152.0 (Cq Bz), 149.7, 141.2 (Cq-4), 141.3 (C-2, signal taken from HSQC) 133.8, 132.8 (CH *p*-Bz), 128.9, 128.9 (CH *m*-Bz), 127.9 (CH *o*-Bz), 123.1, 123.0 (C-5), 117.7, 117.6 (Cq OCE), 85.5, 85.1 (C-1'), 84.0, 83.9, 83.6, 83.5 (C-4'), 74.1, 74.0 (C-2'), 72.0, 71.7 (C-3'), 63.3, 63.1, 63.0, 62.9 (C-5'), 58.9, 58.7, 58.6 (OCH_2CH_2CN), 43.3, 43.2 (CH N-*i*Pr), 33.9, 33.9, 33.7, 33.6 (CH *i*Bu), 24.8, 24.8, 24.7, 24.7 (CH_3 N-*i*Pr), 20.5, 20.4, 20.4, 20.4 (OCH_2CH_2CN), 19.0, 19.0, 18.9, 18.8, 18.7 (CH_3 *i*Bu). **^{31}P NMR:** (202 MHz, $CDCl_3$): δ 149.6, 149.1.

General procedures for solid phase synthesis

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. Ribosylated amino acid **5** was incorporated in the sequence by adding a solution of 2 eq. building block in a 0.25 M HCTU solution (2 eq.) in DMF and a 1 M DIPEA solution (4 eq.) in DMF to the resin in a fritted syringe. The resin was shaken overnight and thoroughly washed.

On-resin phosphorylation
The resin was treated with a sufficient amount of 1 M TBAF in THF (enough so that the entirety of the resin is submerged) 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 5 eq. of $(FmO)_2PN(iPr)_2$ (0.25 M in MeCN) with 10 eq. ETT solution (0.25 M in MeCN). The resin was shaken for 30 minutes after which the resin was washed with MeCN. The resin was then treated with a sufficient amount of CSO solution (0.5 M 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.

Construction of the pyrophosphate
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 compound **12** (3 eq., 0.3 M in MeCN) and ETT (6 eq., 0.25 M in MeCN) for 30 minutes. The resin was thoroughly washed with MeCN before a sufficient amount of CSO (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 (2.5:2.5:95 TIS:TFA:DCM) for 1 hour. The crude products were collected by filtration and the resin was washed with a solution of 1:1:1 water:*t*BuOH:MeCN. The solvents were evaporated *in vacuo* and co-evaporated with a 1:1:1 water:*t*BuOH:MeCN solution.

Ac-Ile-Thr-Ser-Leu-Tyr(5-*O*-adenosine-diphosphate-α-D-ribosyl)-Lys-Lys-Ala-Gly-Ser-Thr-OH (11)
50 µmol TentaGel® S AC resin was loaded by treating the resin with 2.5 mL of a 0.2 M Fmoc-Thr(Trt)-OH solution (10 eq.) and DIC (77 µL, 0.5 mmol, 10 eq.) in DMF together with a catalytic amount of DMAP for 2 hours after which the general procedures described above were applied to the resin. The amino acids used were: Fmoc-Ile-OH, Fmoc-Thr(Trt)-OH), Fmoc-Ser(Trt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Ala-OH, Fmoc-Gly-OH and **5**. 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 (3.98 mg, 2.28 µmol, 4.5%). **^{1}H NMR:** (500 MHz, D_2O) δ 8.35 (s, 1H, H-2 adenine), 8.10 (s, 1H, H-8 adenine), 6.88 (d, J = 8.5 Hz, 2H, Tyr arom.), 6.74 (d, J = 8.5 Hz, 2H, Tyr arom.), 5.97 (d, J = 5.5 Hz, 1H, H-1' adenosine), 5.38 (d, J = 4.5 Hz, 1H, H-1' ribosyl). **^{31}P NMR:** (202 MHz, D_2O) δ -11.1, -11.2, -11.3, -11.4. **LC-MS:** (0 -> 50% B in A) Rt = 5.87. **HRMS:** $[C_{69}H_{112}N_{18}O_{31}P_2 + 2H]^{2+}$ found: 876.3677, calculated: 876.3681.

Ac-Ile-Thr-Ser-Leu-Tyr(5-*O*-adenosine-diphosphate-α-D-ribosyl)-Lys-Lys-Ala-Gly-Ser-Gly-OH (15)
The general procedures described above were applied to 50 µmol TentaGel® S AC resin preloaded with Gly. The amino acids used were: Fmoc-Ile-OH, Fmoc-Thr(Trt)-OH), Fmoc-Ser(Trt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Ala-OH, and **5** 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 (12.4 mg, 7.13 µmol, 14%). **^{1}H NMR:** (500 MHz, D_2O) δ 8.43 (s, 1H, H-2 adenine), 8.18

(s, 1H, H-8 adenine), 6.96 (d, *J* = 8.6 Hz, 2H, H arom. Tyr), 6.83 (d, *J* = 8.7 Hz, 2H, H arom. Tyr.), 6.06 (d, *J* = 5.4 Hz, 1H, H-1' adenosine), 5.47 (d, *J* = 4.2 Hz, 1H, H-1' ribosyl). **^{31}P NMR:** (202 MHz, D_2O) δ -10.4, -10.5, -10.7, -10.8. **LC-MS:** (0 -> 50% B in A) Rt = 5.01. **HRMS:** $[C_{67}H_{108}N_{18}O_{30}P_2 + 2H]^{2+}$ found: 854.3545, calculated: 854.3550.

Biochemical evaluation

Expression plasmids and protein purification

The construction of the expression plasmids and the purification procedures were described earlier.[26–28] Briefly, expression plasmids were transferred into Rossetta (DE3) cells and grown to an OD_{600} of 0.6 in LB medium supplemented with appropriate antibiotics. For metal-coordinating proteins the medium was further enriched either by addition of 2 mM $MgSO_4$ (ARHs) or 100 µM $ZnCl_2$ (*Spy*MacroD). Expression was induced with 0.4 mM isopropyl β-D-1-thioglactopyranoside (IPTG) and cultured were allowed to grow further overnight at 17 °C. Cultures were harvested by centrifugation, pellets resuspended in lysis buffer (50 mM TrisHCl [pH 8], 500 mM NaCl and 25 mM imidazole) and stored at -20 °C. Proteins were purified by Ni^{2+}-NTA chromatography (Jena Bioscience) according to the manufacturer's protocol using the following buffers: all buffers contained 50 mM TrisHCl (pH 8) and 500 mM NaCl; additionally, the lysis buffer contained 25 mM, the washing buffer 40 mM, and the elution buffer 500 mM imidazole. Proteins were dialyzed overnight against 50 mM TrisHCl (pH 8), 200 mM NaCl, 1 mM dithiothreitol and 5% (v/v) glycerol and stored at -80 °C. For the purification of ARH and ARH-like proteins all purification buffers were additionally supplemented with 10 mM $MgCl_2$.

(ADP-ribosyl)-hydrolase activity assay

The peptide demodification assay was described earlier[18]. Briefly, peptide concentration for the assay was estimated using absorbance at I_{260nm} using the molar extinction coefficient of ADP-ribose (15,400 M^{-1} cm^{-1}). 20 µM indicated peptide were demodified by incubation with 1 µM hydrolase for 45 minutes at 30 °C in assay buffer (50 mM TrisHCl [pH 8], 200 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 0.2 µM human NUDT5[29]). Reactions were stopped and analyzed by performing the AMP-Glo™ assay (Promega) according to the manufacturer's protocol. Luminescence was recorded on a SpectraMax M5 plate reader (Molecular Devices) and data analyzed with GraphPad Prism 7. Control reactions were carried out in absence of peptide.

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