The development of molecular tools for investigating NAD+ metabolism and signalling

Minnee, H.

Chapter 2

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N(τ)-ADP-ribosylated histidine isosteres through copper(I)-catalyzed click chemistry

Introduction

A wide array of posttranslational modifications (PTMs), varying from small alterations, e.g. methylation, to the introduction of complete proteins, e.g. ubiquitination, are essential for regulatory control of protein activity. Although nicotinamide adenine dinucleotide (NAD^+) is commonly known for its role as a redox cofactor, it also participates in a PTM called adenosine diphosphate-ribosylation (ADP-ribosylation). Here, the adenosine diphosphate ribose (ADPr) moiety is covalently attached to specific amino acid side chains of the targeted protein by substitution of the nicotinamide residue. In humans, this process is primarily facilitated by ADP-ribosyl transferases termed PARPs,[1] and the resulting modification can be either emono-ADP-ribosylation (MARylation) when a single ADPr molecule is introduced or poly-ADP-ribosylation (PARylation) when longer linear or even branched ADPr polymers are formed. The latter process can be mediated by only a small subset PARPs: PARP1, PARP2, PARP5a, and PARP5b. In contrast to APD-ribosyl transferases, members of two different protein families, (ADP-ribosyl)hydrolases (ARHs) and macrodomains, are able to reverse the modification.[2,3] Substrate specificity varies greatly among the ADP-ribosyl hydrolyzing enzymes as some are limited to a particular type of glycosidic bond, e.g. ARH1, while others, e.g. ARH3 and macrodomain (MacroD) 1, tolerate a broader target spectrum.[2] On top of that, members of the macrodomain family that lack hydrolytic activity still show strong interaction with the ADPr moiety and are of great importance in the interpretation of ADP-ribosylation as well as NAD^+ derived second messengers.[3] Thus a complex regulatory network of "writer", "reader" and "eraser" proteins is responsible for the huge structural diversity of the "ADP-ribosylome".[4] Due

to such structural diversity, ADP-ribosylation allows for the spatiotemporal and context specific regulation of a wide variety of cellular processes including DNA damage response, replication, transcription, and cellular signaling.[5–8]

Ever since the first isolation of poly-ADPr chains[9] and the identification of the transferase enzyme,[10] this PTM has received ever increasing attention because of the many roles it plays in various biological events. Unfortunately, progress has been slowed down due to the lack of appropriate tools to study this modification. However, recent advances in mass spectrometry did reveal the true range of possible amino acid acceptors, which now includes glutamate,[11,12] aspartate,[11] arginine,[13] lysine,[14] cysteine,[15] histidine,[16–18] tyrosine,[18–20] and serine.[21,22] Among the different acceptor residues, serine has emerged as the primary target in DNA damage-induced ADP-ribosylation.[22–24] Nevertheless, current proteomic studies have also drawn attention towards the occurrence of lower-frequency modifications at tyrosine and histidine sites.[16,17,25] The identification of these new flavors of stress-induced ADP-ribosylation is suggestive of a specialized control mechanism for sub-processes within the DNA-damage response (DDR). For an identification and characterization of the responsible ADPr-interacting proteins as well as an examination of their cellular function, well-defined molecular tools are indispensable.

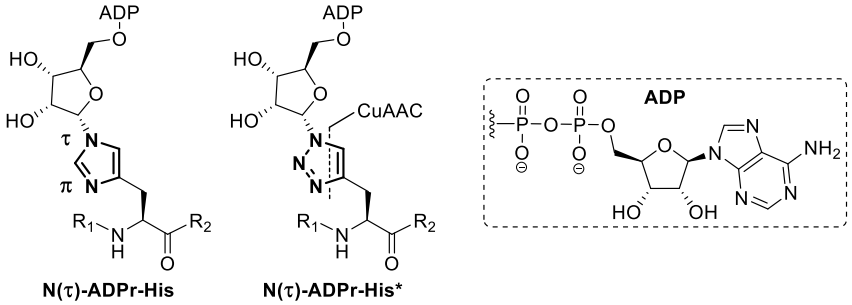


Figure 1| Proposed structure of ADP-ribosylated histidine (N(τ)-ADPr-His), including the τ and π nomenclature for the imidazole nitrogen atoms, versus the ADP-ribosylated histidine isostere (N(τ)-ADPr-His*) that has the imidazole functionality replaced by a triazole moiety.

Although the exact structure of ADP-ribosylated histidine (His-ADPr) has not yet been determined, it is hypothesized here that ADPr is introduced at the N(τ)-position of the imidazole functionality, most likely via an α-configured linkage (Figure 1, N(τ)-ADPr-His). This hypothesis is based on the isolation of ribosylated and ADP-ribosylated histidine metabolites[26,27] combined with the known stereospecificity of PARP enzymes. To generate tools to study histidine ADP-ribosylation, it was reasoned that click chemistry could be exploited to create a nearly perfect isostere of ADP-ribosylated histidine (Figure 1, N(τ)-ADPr-His*). The use of Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), known for its high stereoselectivity, has

been successfully implemented before in the synthesis of ADP-ribosylated oligopeptides and proteins.[28–30]

In this chapter, a convergent synthesis of nearly perfect N(τ)-ADP-ribosylated histidine isosteres is described. A total of four N(τ)-ADPr-His* conjugates (**1-2**) were obtained via a late-stage CuAAC between α- or β-configured azido-ADPr analogues (**5** and **6** respectively) and an alkyne-modified oligopeptide of choice (Figure 2). The desired oligopeptides, originating from histone PARylation factor 1 (HPF1, **3**) and PARP1 (**4**), were made accessible via standard 9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase peptide synthesis (SPPS) by incorporating propargylglycine at the ADP-ribosylation site. The Azido-ADPr analogues could be obtained through construction of a pyrophosphate linkage between the corresponding phosphorylribofuranoside **7** or **8** and the known adenosine phosphoramidite **9** using the well-established $P^{III}-P^{V}$ coupling procedure.[31] Synthesis of the required building blocks was initiated from commercially available D-ribofuranose, ribose tetraacetate and adenosine. Finally, the value of the resulting conjugates as tools for investigating ADPr-His binders or hydrolases was demonstrated in a screening against a small library of human (ADP-ribosyl)hydrolases.

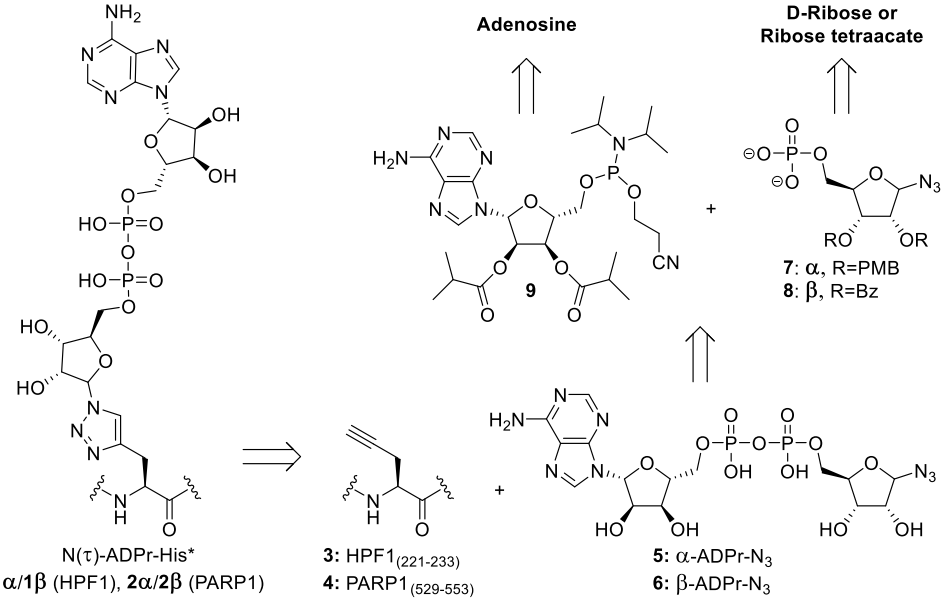
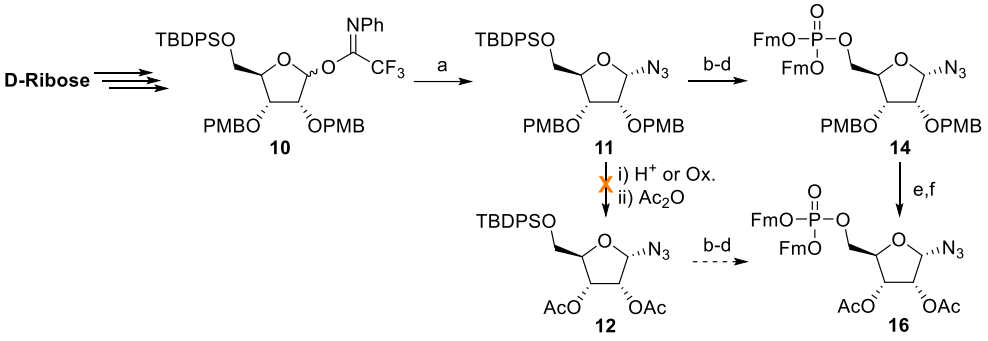


Figure 2| Retrosynthesis of 1,4-triazolyl-linked ADP-ribosylated peptide conjugates **1-2**, referred to in this thesis as N(τ)-ADPr-His*. Azido-ADPr-analogues **7** and **8** were used as stable triethylammonium and pyridinium salts, respectively. Full peptide sequences are $HPF1_{(221-233)}$ = H-Thr-Phe-Pra-Gly-Ala-Gly-Leu-Val-Val-Pro-Val-Asp-Lys-OH and $PARP1_{(529-553)}$ = H-Gly-Gly-Ala-Ala-Val-Asp-Pro-Asp-Ser-Gly-Leu-Glu-Pra-Ser-Ala-OH, where Pra = propargylglycine.

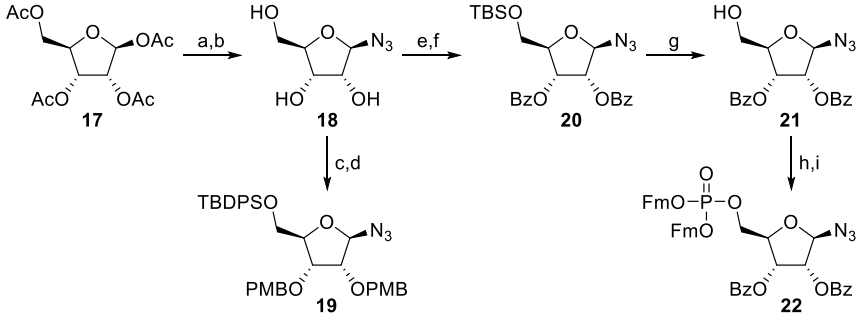
Results and discussion

The assembly of the α-configured azido-5-phosphorylribofuranosides **14** and **16** requires a nonparticipating protection group at the 2-OH moiety for stereoselective introduction of the azido functionality. To this end, suitably protected imidate **10** (Scheme 2) was derived from D-ribose in a total of 5 steps according to a previously reported procedure.[32] Activation of the ribosyl donor **10** with trimethylsilyl triflate (TMSOTf) at −60 °C in the presence of an excess of TMS-azide provided ribofuranosyl azide **11** with excellent stereoselectivity (α/β = 14:1). Removal of the *para*-methoxybenzyl (PMB) protecting groups proved difficult at this point. Acidic conditions such as trifluoroacetic acid (TFA) in DCM or HCl in hexafluoroisopropanol (HFIP)[33] only led to degradation of the starting material. Alternatively, the use of oxidizing agents like 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) or ceric ammonium nitrate (CAN) mainly yielded 2,3-*O*-*para*-methoxybenzylidene side products as was observed by mass ($M+Na^+$=554.2) and NMR analysis. Therefore, the removal of the PMB ethers was postponed to a later stage and instead the *tert*-butyldiphenylsilyl (TBDPS) functionality in fully protected azide **11** was removed with HF-pyridine as a fluorine source. Then, phosphitylation with bis-(fluorenylmethyl)-N,N-diisopropyl phosphoramidite under influence of 4,5-dicyanoimidazole (DCI) as an activator provided the corresponding phosphite which was subsequently oxidized with *t*-BuOOH to yield 1-α-azido-5-phosphorylribofuranoside **14**. With the phosphate group in place, additional attempts to remove the PMB functionalities were made. Addition of a single equivalent of HCl to a cooled solution of phosphorylribofuranoside **14** in HFIP immediately provided a characteristic deep red solution and the desired product was obtained in high purity after standard silica gel column chromatography, albeit in a moderate yield. Initially, the acetyl groups were installed using an excess of pyridine as base and nucleophilic catalyst, which led to removal of the 9-fluorenylmethyl (Fm) functionalities on the phosphate. Limiting the amount of base proved to be sufficient to prevent this side reaction and phosphorylribofuranoside **16** was obtained in a satisfying yield of 95%.



Scheme 2| Synthesis of α-configured phosphorylribofuranosides **14** and **16**. Reagents and conditions: a) $TMSN_3$, TMSOTf, DCM, -60 °C to rt, 16 h (78%, α:β = 14:1). b) HF-pyridine, pyridine, rt, 1 h (87%). c) $(FmO)_2PN(i\text{-}Pr)_2$, DCI, MeCN, rt, 2 h. d) *t*-BuOOH, MeCN, rt, 1.5 h (73% over 2 steps). e) HCl, HFIP, 0 °C, 20 min (63%). f) Ac_2O, DMAP, DCM, rt, 40 min (95%).

Synthesis of the β-configured 5-phosphorylribofuranosides **19** and **22** started with commercially available ribofuranose tetraacetate **17** (Scheme 3). The desired β-azide was acquired with excellent stereoselectivity, owing to neighboring group participation of the 2-*O*-acetyl group,.[34] The remaining acetyl groups were all removed under basic conditions to yield precursor **18**, which allowed for the introduction of orthogonal protecting groups. As a means to unmistakably validate the assigned α-configuration of azido ribofuranoside **11**, the identical protecting group strategy was applied on triol **18** to yield the β-configured counterpart **19**. NMR data of the latter corresponded to the minor contaminant found in the glycosylation reaction with imidate **10**. Thus, confirming that the predominant product of this reaction, ribofuranosyl azide **11**, is the α-anomer.

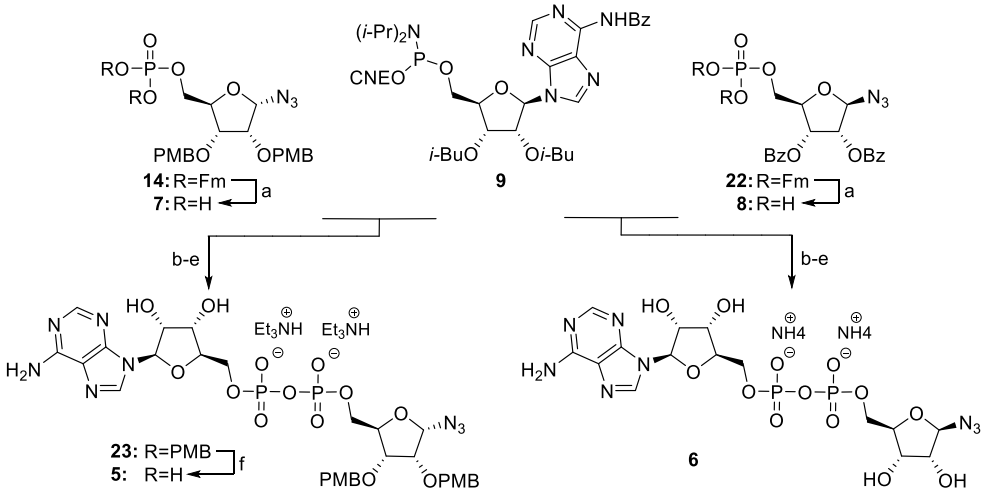


Scheme 3| Synthesis of β-configured ribose 5-phosphate **19** and **22**. Reagents and conditions: a) $TMSN_3$, $SnCl_4$, DCM, rt, 1.5 h (97%). b) NaOMe, MeOH, rt, 1.5 h (92%). c) TBDPSCl, pyridine, rt, 3 days. d) PMBCl, NaH, DMF, rt, 16 h (25% over 2 steps). e) TBSCl, pyridine, rt, 5 h. f) BzCl, pyridine, rt, 1.5 h (92% over 2 steps). g) *p*-TsOH•H_2O, H_2O/MeCN (1:1), rt, 1.5 h (92%). h) $(FmO)_2PN(i\text{-}Pr)_2$, pyridine-1-ium chloride, pyridine, rt, 1 h. i) *t*-BuOOH, pyridine, rt, 2.5 h (76% over 2 steps).

In further efforts towards the desired β-azide **22** building block, a *tert*-butyldimethylsilyl (TBS) group was introduced at the primary alcohol of triol **19**, directly followed by the installation of benzoyl groups at the 2- and 3-OH moieties. The silyl ether of ribofuranoside **20** was selectively removed under acidic conditions. Then, phosphitylation and subsequent oxidation, using similar conditions as described above for α-azide **14**, provided 1-β-azido-5-phosphorylribofuranoside **22**.

The pyrophosphate linkage in the target α- and β-azido ADP-ribose building blocks was installed using a well-established P(III)– P(V) coupling method (Scheme 4).[31] The required adenosine phosphoramidite **9** was synthesized from adenosine in 6 steps according to a previously reported method.[35] Initially, the use of 1-α-azido ribosylfuranoside **16** in the synthesis of α-ADPr-N_3 was explored. Unfortunately, these endeavors were hampered by purification difficulties of the final product. Instead, this strategy was investigated for its PMB protected precursor **14**. First, the phosphate was liberated by removal of the Fm-groups with triethylamine. Next, the fully deprotected phosphate **7** was coupled with adenosine amidite **9**

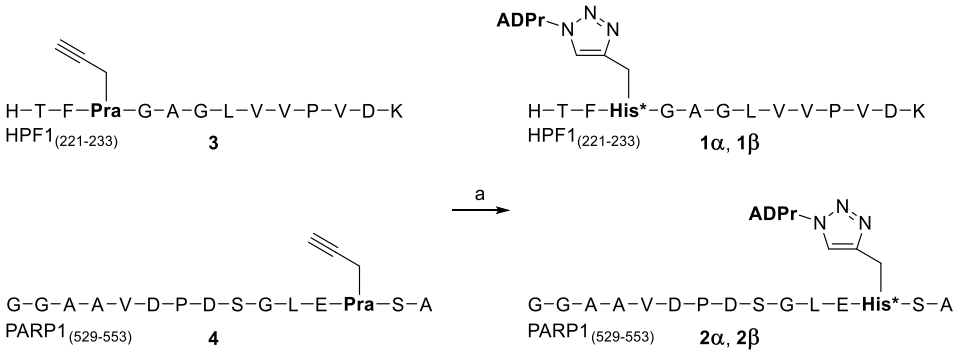
upon activation with DCI. Subsequent *t*-BuOOH mediated oxidation of the $P^{III}-P^{V}$ phosphotriester intermediate provided the partially protected pyrophosphate. Deprotection of this intermediate started with the removal of the cyanoethyl (CNE) group with DBU, after which treatment with aqueous ammonia provided partially protected α-azido-ADPr **23**. Due to the hydrophobic interactions of its two PMB ethers, size-exclusion chromatography (SEC) was not efficient, and preparative reversed-phase high-performance liquid chromatography (RP-HPLC) was required to obtain the pure compound. Final removal of the PMB groups was executed using a catalytic amount of HCl in hexafluoro-2-propanol to yield α-N_3-ADPr **5** as triethylammonium salt after workup and lyophilization. The same methodology, with exception of the final acid treatment, could be used to derive β-N_3-ADPr **6** from its respective precursor 1-β-azido ribosylfuranoside **22**. In this case, only minor contaminants were observed after purification by SEC. Nevertheless, a small sample was further purified by RP-HPLC for analytical purposes and 1mol% of disodium ethylenediaminetetraacetic acid (EDTA) was added for sharpening of ^{1}H NMR signals.



Scheme 4| Synthesis of α- and β-configured N_3-ADPr analogues **17** and **19**. Reagents and conditions: a) Et_3N, MeCN, rt, 2-3 days. b) DCI, MeCN, rt, 0.5-1 h. c) *t*-BuOOH, MeCN, rt, 2-4 h. d) DBU, MeCN, rt, 0.5 h. e) NH_4OH, rt, 16-19 h (28% & 47% yield over 5 steps for **16** and **19** respectively). f) HCl, HFIP, 0 °C, 20 min (quant).

The target oligopeptides **3** and **4** (scheme 5) are based on two potential histidine ADP-ribosylation sites, located on HPF1 and PARP1 respectively, that have been identified in recent proteomic studies.[18] The required peptides were synthesized using standard Fmoc-based solid phase peptide synthesis (SPPS) conditions, incorporating propargylglycine (Pra) at the positions that are to carry the histidine-like ADPr modification. Both peptides were obtained in satisfactory yields and purities after RP-HPLC an NH_4OAc buffered eluent system.

For the final Cu(I)-catalyzed conjugation, a 1.5-fold molar excess of α- or β-N_3-ADPr was added to an aqueous solution of the oligopeptide, after which the solution was degassed with argon. In parallel, a fresh "click mixture" was prepared for every reaction by adding an aqueous solution of sodium ascorbate to $CuSO_4$ directly followed by tris(3-hydroxypropyltriazolylmethyl)amine (THPTA). After addition of this mixture to the solution of the azide and the alkyne, the conversion of the oligopeptide was monitored with liquid chromatography–mass spectrometry (LC-MS). Upon complete conversion, the crude products were desalted by SEC and subsequently purified by preparative RP-HPLC. Unfortunately, this tandem purification method provided the desired products in moderate yields. Direct preparative RP-HPLC proved to be more efficient and furnished the desired N(τ)-ADPr-His* conjugates **1α**, **1β**, **2α** and **2β** in high purity.



Scheme 5| Finalization of the histidine mimetics through a Cu(I)-mediated cycloaddition between the azide-modified ADPr analogues and an oligopeptide, originating from PARP1 or HPF1, that carries a propargylglycine (Pra) at the modification site. Reagents and conditions: a) **5** or **6**, $CuSO_4$, sodium ascorbate, THPTA, H_2O, rt, 0.5-8 h (33% for **1α**, 15% for **1β**, 8% for **2α** and 26% for **2β**).

Having obtained the triazole mimetics of ADP-ribosylated histidine peptides, the enzymatic turnover of this modification was investigated (figure 3A). ADPr conjugates **1-2** were incubated in the presence of different purified human ADPr hydrolases and nudix hydrolase 5 (NudT5) for 1 h at 30 °C. The former may catalyze the breakage of the N-glycosidic bond of the ribosyltriazole, while the latter converts the released ADPr into adenosine monophosphate (AMP), which was quantified using the AMP-Glo assay.[36] As a positive control, the samples were incubated in the presence of NudT16, which in contrast to NudT5 can hydrolyze ADPr that is conjugated to a peptide.[37] Although most human hydrolases were unable to remove ADPr from the peptides, we observed a consistent minor turnover (~8.2%) for the HPF1 peptide **1α**, indicating that our developed isostere is indeed a functional mimic of ADP-ribosylated histidine. Interestingly, ARH3 appeared unable to convert PARP1 peptide **2α**, which could suggest that the removal of His-ADPr modifications is sequence-dependent.[38] These findings were substantiated in a time-course experiment (figure 3B), which showed the steady enzymatic conversion of **1α** and the resistance of **2α** towards enzymatic turnover.

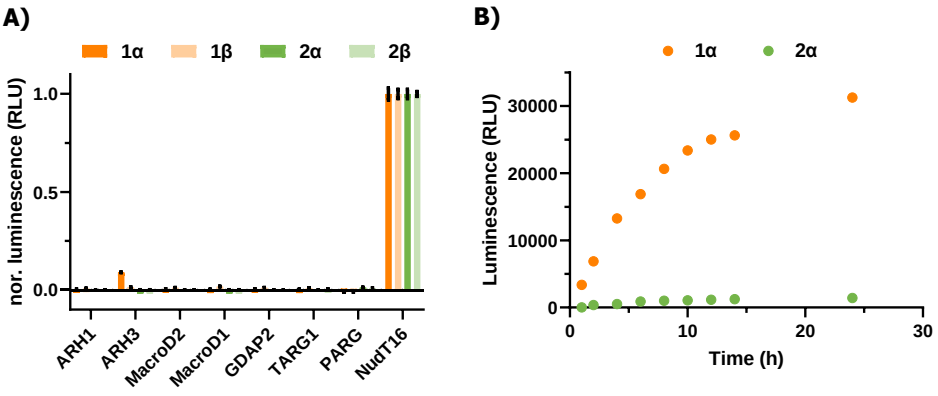


Figure 3| Enzymatic hydrolysis of interglycosidic linkages in N(τ)-ADPr-His* conjugates **1α**, **1β**, **2α** and **2β**. A. Hydrolase activity against the various peptides was assessed by monitoring AMP release directly (NudT16) or converting released ADPr via NudT5 to AMP. AMP was measured using the AMP-Glo assay (Promega). Samples are background corrected and normalized to NudT16 activity. B. Time-course experiment of N(τ)-ADPr-His* **1α** and **2α** with ARH3. Time points taken up to 24 h. Data of both experiments represent mean values ± standard deviation measured in triplicates.

Conclusion

This chapter describes the synthesis of both α-and β-configured 1-azido-phosphoryl-ribofuranosides **7** and **8** from D-ribose and ribose tetraacetate respectively. These precursors were successfully coupled to adenosine phosphoramidite **9** using P(III)-P(V)[31] chemistry to provide key components α-N_3-ADPr and β-N_3-ADPr. Two oligopeptides of interest, originating from PARP1 and HPF1, were obtained via Fmoc-based SPPS where propargylglycine was incorporated at the modification site. Finally, N(τ)-ADPr-His* conjugates (**1α**, **1β**, **2α** and **2β**) were prepared from these four building blocks using CuAAC and investigated *in vitro*. Initial screening of the mimetics against a collection of human ADPr hydrolases revealed that ARH3 is able to hydrolyze the *N*-glycosidic triazole-ribose linkage of HPF1 peptide **1α**, while PARP1 conjugate **2α** remained unscathed. Not only do these results suggest that ARH3 is likely able to remove the ADPr modification from histidine residues in the right sequential context,[38] but it also demonstrates that the peptides presented here provide useful tools for the further study of the interactions of the His-ADPr modification with either binders or hydrolases.

Experimental section

Expression plasmids and protein purification

The construction of the expression plasmids and the purification procedures were described earlier.[37,39–41] Briefly, expression plasmids were transferred into Rossetta (DE3) cells and grown at 37 °C to an OD600 of 0.6 in LB medium supplemented with 1% (w/v) D-glucose and appropriate antibiotics. For (ADP-ribosyl)hydrolases (ARH1 and ARH3) the medium was further enriched by addition of 2 mM $MgSO_4$. Expression was induced with 0.4 mM isopropyl β-D-1-thioglactopyranoside (IPTG) and cultures were allowed to grow 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 until use. 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 ARH1 and ARH3 all purification buffers were additionally supplemented with 10 mM $MgCl_2$.

(ADP-ribosyl)hydrolase activity assay

The peptide demodification assay was described earlier.[24,36] Briefly, peptide concentrations for the assay were estimated using absorbance at λ_{260nm} using the molar extinction coefficient of ADP-ribose (15,400 $M^{-1}cm^{-1}$). 10 µM indicated peptide were demodified by incubation with 0.5 µM hydrolase for 60 min 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).[24] Reactions were quenched and analysed 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 analysed with GraphPad Prism 7. Control reactions were carried out in absence of peptide.

General synthetic procedures

All chemicals were used as received unless stated otherwise. $SnCl_4$ (1 M in DCM) and *t*-BuOOH (5.5 M in nonane) were purchased at Sigma Aldrich. Dowex 50WX8 hydrogen form (100-200 mesh) was purchased at Sigma Aldrich and washed with H_2SO_4 (5 M, 3x) and MeOH (3x) prior to use. Molecular sieves were flamedried (3x) in vacuo before use. Solvents were dried over activated 4Å molsieves for 24 h except for MeCN and MeOH which were dried over 3Å molsieves. A solution of HCl (0.2 M in HFIP) was freshly prepared prior to the reaction by dissolving HCl (37%, 0.1 ml) to HFIP (5.9 ml). Reactions were performed under N_2 atmosphere unless stated otherwise. A Julabo FT902 cryostat was used for low temperature glycosylation reactions. Reaction mixtures were concentrated under reduced pressure using rotary evaporators at 40-45 °C unless state otherwise. Reactions were monitored by thin layer chromatography (TLC) analysis using silica gel 60 F254 coated aluminum sheets from Merck. TLC plates were visualized with ultraviolet light (254 nm) or sprayed with H_2SO_4 (20% v/v in MeOH), potassium permanganate (1 g $KMnO_4$, 5 g K_2CO_3, in 200 ml H_2O) or ceric ammonium molybdate (1 g $Ce(NH_4)_4(SO_4)_4 \bullet 2H_2O$, 2.5 g $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$, 10 ml H_2SO_4 in 90 ml H_2O). Infrared (IR) values are reported in cm^{-1}. ^1H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded on Bruker AV-300 (300 MHz), AV-400 (400 MHz) or AV-500 (500 MHz) spectrometer. ^{13}C NMR spectra are acquired via the attached proton test (APT) experiment and are presented with even signals (C_q and CH_2) pointing upwards and odd signals (CH and CH_3) pointing downwards. The chemical shifts are noted as δ-values in parts per million (ppm) relative to the tetramethylsilane signal (δ = 0 ppm) or solvent signal of D_2O (δ = 4.79 ppm) for ^1H NMR and relative to the solvent signal of $CDCl_3$ (δ = 77.16 ppm) for

^{13}C NMR. Phosphorylation reactions were monitored with ^{31}P NMR using an acetone-D_6 insert for a locking signal and the resulting spectra were indirectly calibrated with H_3PO_4. HRMS samples were prepared in either MeOH, MeCN or milliQ grade H_2O with an approximate concentration of 1 mM and measured on a Thermo Scientific LTQ Orbitrap XL.

Solid phase peptide synthesis

Fmoc-L-propargyl glycine (cat# 05138) was purchased at Chem-Impex International. Fmoc-Asp(OEpe)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Ala-OH and Fmoc-Phe-OH were all obtained from Merck Novabiochem. Lysine(Boc) was purchased pre-loaded on tentagel S AC resin from RAPP Polymere GmbH. Alanine was loaded manually on tentagel S AC resin from the same manufacturer.

Oligopeptides were prepared using a Liberty Blue peptide synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at a 100 µmol scale. A 5 fold excess of the amino acids, with exception of 4 equivalents for Fmoc-L-propargyl glycine, relative to the resin loaded amino acid was added in each prolongation step. A total of 5 equivalents of the additives Diisopropylcarbodiimide (DIC) and OxymaPure were added simultaneously. The coupling was established in the microwave reaction chamber at 90 °C for 2.5 minutes. After each coupling the peptide was subjected three consecutive times to a 20 v/v% piperidine solution in DMF at 90 °C for 1 minute to remove the Fmoc protection group. The oligopeptide was deprotected and simultaneously cleaved by treating it with a TFA/TIS/H2O (95:2.5:2.5, 7 ml) mixture in a reaction syringe for 2 h at rt. Precipitation in 8 volumes of ice-cold Et_2O followed by centrifugation (5 min) yielded the crude residue that was purified by preparative RP-HPLC.

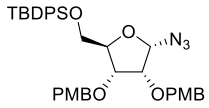
General Cu(I)-catalyzed click reaction procedure:

The alkyne carrying oligopeptide (1.0 eq) and N_3-ADPr analogue (1.5-2.0 eq) were dissolved in milliQ grade H_2O with a final concentration of 3 mM and bubbled with argon gas for 10 min. In the meantime, a click mixture was freshly prepared by adding a solution of NaAsc (0.61 M in H_2O, 8 eq.) to a solution of $CuSO_4$ (0.16 M in H_2O, 1.0-1.3 eq.) directly followed by THPTA (0.1 M in H_2O, 1.0-2.0 eq.). After addition of the click mixture, the reaction was stirred at rt and monitored using LC-MS (C18-column, 10-50% MeCN/H_2O + 1% TFA, 12.5 min) until the oligopeptide was fully depleted. Upon completion, the reaction mixture was quenched with EDTA (0.5 M in H_2O, 2 eq.) and concentrated under reduced pressure. The crude residue was purified by a combination of SEC and preparative HPLC and subsequently lyophilized.

Purification methods

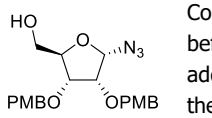
Silica 60 M (0.04-0.063 mm) from Macherey-Nagel GmbH was used in combination with solvents of technical grade from Sigma Aldrich for silica gel column chromatography. Size exclusion chromatography (SEC) was performed by constant elution (1 ml/min) with an aqueous NH4OAc (0.15 M) + 10% MeCN buffer system over an HW-40-S resin (16x 600 mm) from TOYOPEARL. Purification by preparative high pressure liquid chromatography (HPLC) was carried out on a Gilson-preparative-system equipped with a Phenomenex-Gemini-NX C18 column (5µm, 10x250 mm) using Buffer A (25 mM NH4OAc in H_2O) and Buffer B (MeCN). Yields for the ADPr analogues after size exclusion chromatography were calculated assuming its obtained as NH_4 salt and corrected for residual salts like NH_4OBz and NH_4OAc using NMR analysis.

1-α-Azido-2,3-bis-*O*-(4-methoxybenzyl)-5-*O*-tert-butyldiphenylsilyl-D-ribo-furanoside (11).



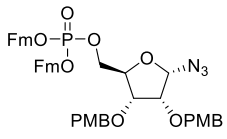
Compound **10**[32] (1.02 g, 1.28 mmol) was co-evaporated with anhydrous toluene (3x) before transferring to a flame dried flask carrying activated 3Å molecular sieves using anhydrous DCM (40 ml). $TMSN_3$ (0.65 ml, 4.9 mmol) was added and the reaction mixture was stirred for 1.5 h at rt before cooling to -60 °C. TMSOTf (54 µl, 0.30 mmol) was added in one go and after 20 h the resulting yellow solution was quenched with Et_3N (0.3 ml). The reaction mixture was concentrated under reduced pressure and the crude residue was subjected to silica gel column chromatography (pentane/Et_2O = 95:5 → 80:20) to provide an anomeric mixture of title compound **11** (654 mg, 1.00 mmol, 78%, α:β =14:1) as a clear oil. $\mathbf{R_f}$ = 0.5 (pentane/EtOAc = 85:15). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.63 – 7.56 (m, 4H), 7.49 – 7.36 (m, 6H), 7.32 – 7.23 (m, 4H), 6.91 – 6.83 (m, 4H), 5.23 (d, *J* = 4.4 Hz, 1H), 4.66 – 4.58 (m, 3H), 4.51 (d, *J* = 12.1 Hz, 1H), 4.28 (q, *J* = 2.9 Hz, 1H), 4.05 – 3.98 (m, 2H), 3.81 (s, 6H), 3.68 (dd, 11.4, 3.2 Hz, 1H), 3.55 (dd, 11.3, 2.8 Hz, 1H), 0.98 (s, 9H). **^{13}C NMR** (101 MHz, CDCl3): δ 159.3, 135.6, 135.5, 133.1, 132.8, 129.9, 129.9, 129.7, 129.7, 129.5, 127.8, 127.8, 127.8, 127.7, 114.0, 113.9, 113.8, 90.6, 84.9, 78.3, 75.1, 72.6, 72.3, 63.7, 55.3, 26.8, 26.8, 19.2. **IR**: 2930.57, 2857.60, 2836.13, 2109.21, 1611.24, 1511.08, 1243.49. **HRMS** $[C_{37}H_{43}N_3O_6Si + Na]^+$ = 676.28112 found, 676.28133 calculated; $[C_{37}H_{43}N_3O_6Si + NH_4]^+$ = 671.32556 found, 671.32594 calculated.

1-α-Azido-2,3-bis-*O*-(4-methoxybenzyl)-D-ribofuranoside (13).



Compound **11** (1.45 g, 2.22 mmol) was co-evaporated with anhydrous toluene (2x) before dissolving in anhydrous pyridine (35 ml). HF-pyridine (3.20 ml, 24.9 mmol) was added and the solution was stirred at rt for 3.5 h. $NaHCO_3$ (sat., 70 ml) was added and the H_2O fraction was extracted with DCM (2x 75 ml). The combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the crude residue by silica gel column chromatography (pentane/EtOAc = 90:10 → 60:40) yielded title compound **13** (805 mg, 1.94 mmol, 87%) as a clear oil. $\mathbf{R_f}$ = 0.3 (pentane/EtOAc = 60:40). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.34 – 7.21 (m, 4H), 6.88 (dd, *J* = 8.5, 6.3 Hz, 4H), 5.14 (dd, *J* = 3.2, 1.6 Hz, 1H), 4.66 – 4.56 (m, 3H), 4.46 (d, *J* = 11.9 Hz, 1H), 4.26 (m, 1H), 3.89 (dd, *J* = 3.1, 1.4 Hz, 2H), 3.80 (d, *J* = 1.8 Hz, 6H), 3.71 (dt, *J* = 12.3, 3.3 Hz, 1H), 3.50 – 3.40 (bs, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 55.4, 62.2, 72.4, 72.8, 75.0, 78.1, 84.0, 90.7, 113.9, 114.0, 129.4, 129.7, 129.8, 159.5, 159.6. **IR**: 2109.21, 1611.24, 1512.51, 1243.49, 1027.42, 815.64. **HRMS** $[C_{21}H_{25}N_3O_6 + Na]^+$ = 438.16359 found, 438.16356 calculated; $[C_{21}H_{25}N_3O_6 + NH_4]^+$ = 433.20829 found, 433.20816 calculated.

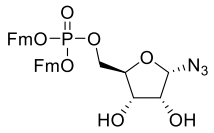
1-α-Azido-2,3-bis-*O*-(4-methoxybenzyl)-5-(di(9H-fluoren-9-yl))-phosphoryl-D-ribofuranoside (14).



Compound **13** (270 mg, 0.647 mmol) and DCI (229 mg, 1.94 mmol) were co-evaporated with anhydrous toluene (3x) and dissolved in anhydrous MeCN (4.5 ml). A solution of $(FmO)_2PN(iPr)_2$ (507 mg, 0.971 mmol) in anhydrous MeCN (3 ml) was added dropwise and the suspension was stirred at rt under argon atmosphere. After 1.5 h, *t*-BuOOH (5.5 M, 0.59 ml, 3.2 mmol) was added and the reaction was monitored by ^{31}P NMR until no residual phosphotriester signal (~125 ppm) was observed. Upon completion the reaction was diluted H_2O (75ml) and extracted with DCM (2x 75 ml). The combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the crude residue by silica gel column chromatography (pentane/EtOAc = 80:20 → 50:50, 10% steps) resulted in title compound **14** (402 mg, 0.647 mmol, 73%) as a clear foam. $\mathbf{R_f}$ = 0.5 (DCM/acetone = 95:5). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.77 – 7.67 (m, 4H), 7.55 – 7.14 (m, 14H), 7.11 (d, *J* = 8.7 Hz, 2H), 6.79 (dd, *J* = 20.2, 8.6 Hz, 3H), 4.97 (d, *J* = 4.0 Hz, 1H), 4.52 – 4.17 (m, 9H), 4.11 (m, 2H), 3.92 – 3.79

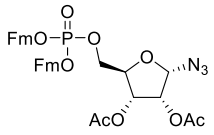
(m, 1H), 3.79 – 3.61 (m, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 47.8, 47.9, 55.3, 55.4, 66.8, 66.9, 69.5, 69.5, 72.6, 73.1, 75.3, 77.7, 81.6, 81.7, 90.4, 113.9, 114.0, 120.2, 120.2, 125.0, 125.0, 127.3, 127.3, 128.1, 128.2, 128.9, 129.0, 129.8, 129.9, 141.5, 142.9, 142.9, 159.8. **^{31}P NMR** (122 MHz, CDCl3): δ -2.33. **IR:** 2110.64, 1512.51, 1246.35, 1011.68, 756.97. **HRMS** $[C_{49}H_{46}N_3O_9P + Na]^+$ = 874.28627 found, 874.28639 calculated; $[C_{49}H_{46}N_3O_9P + NH_4]^+$ = 869.33045 found, 869.33099 calculated.

1-α-Azido-5-(di(9H-fluoren-9-yl))-phosphoryl-D-ribofuranoside (15).



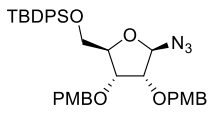
A solution of **14** (220 mg, 0.258 mmol) in HFIP (5.2 ml) was cooled to 0 °C before adding HCl (0.2 M in HFIP, 0.13 ml). The deep red solution was stirred for 20 min, diluted with toluene (20 ml) and quenched with pyridine (40 µl). The reaction mixture was concentrated under reduced pressure and the crude residue was subjected to silica gel column chromatography (DCM/acetone = 90:10 → 70:30) to yield the title compound **15** (101 mg, 0.165 mmol, 64%) as a clear oil. **R_f** = 0.6 (DCM/Acetone = 70:30). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 7.73 – 7.61 (m, 4H), 7.56 – 7.15 (m, 12H), 5.12 (d, J = 4.8 Hz, 1H), 4.22 (dt, J = 9.6, 6.1 Hz, 4H), 4.13 – 4.02 (m, 3H), 3.97 – 3.76 (m, 4H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 142.9, 142.8, 142.8, 142.8, 141.4, 141.4, 141.3, 128.0, 128.0, 127.2, 127.2, 127.2, 125.0, 125.0, 124.9, 120.1, 120.1, 91.8, 83.4, 83.3, 71.7, 69.9, 69.5, 69.4, 69.4, 69.4, 66.9, 66.8, 47.8, 47.8, 47.7, 47.7. **^{31}P NMR** (202 MHz, $CDCl_3$): δ -2.13.

1-α-Azido-2,3-bis-*O*-(acetyl)-5-(di(9H-fluoren-9-yl))-phosphoryl-D-ribo-furanoside (16).



Compound **15** (50 mg, 0.082 mmol) was co-evaporated with toluene (3x) before dissolving in anhydrous DCM (1.4 ml). Ac_2O (0.31 ml, 3.3 mmol), pyridine (15 µl, 0.18 mmol) and DMAP (cat.) were added. The solution was stirred for 1 h, diluted with toluene (15 ml) and concentrated under reduced pressure. The crude residue was co-evaporated with toluene (2x) before subjecting to silica gel column chromatography (pentane/EtOAc = 80:20 → 60:40 → 40:60) to yield title compound **16** (54 mg, 0.078 mmol, 95%, α:β =10:1) as a clear oil. **R_f** = 0.3 (pentane/EtOAc = 60:40). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.74 – 7.63 (m, 4H), 7.50 (m, 4H), 7.41 – 7.19 (m, 8H), 5.30 (d, J = 5.1 Hz, 1H), 5.16 (dd, J = 6.5, 3.6 Hz, 0H), 4.95 (dd, J = 6.5, 5.1 Hz, 1H), 4.34 – 4.19 (m, 4H), 4.10 (dt, J = 12.3, 6.5 Hz, 2H), 3.98 (m, 1H), 2.10 (s, 3H), 2.07 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 169.9, 169.2, 142.9, 142.9, 142.8, 141.3, 141.3, 141.3, 127.9, 127.9, 127.8, 127.1, 127.1, 125.0, 125.0, 120.0, 119.9, 89.5, 81.5, 81.4, 71.0, 69.6, 69.3, 69.3, 69.2, 66.2, 66.1, 47.8, 47.8, 47.7, 47.7, 20.5, 20.3. **^{31}P NMR** (162 MHz, $CDCl_3$): δ -1.05. **HRMS** $[C_{37}H_{34}N_3O_9P + Na]^+$ = 718.19220 found, 718.19249 calculated; $[C_{37}H_{34}N_3O_9P + NH_4]^+$ = 713.23664 found, 713.23709 calculated.

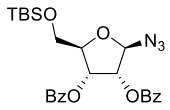
1-β-Azido-2,3-bis-*O*-(4-methoxybenzyl)-5-*O*-*tert*-butyldiphenylsilyl-D-ribo-furanoside (19).



Compound **18**[34] (510 mg, 2.91 mmol) was co-evaporated with anhydrous toluene (3x) before dissolving in anhydrous pyridine (15 ml). TBDPSCl (1.21 ml, 4.66 mmol) was added and the clear solution was stirred at rt for 3.5 h before adding additional TBDPSCl (0.60 ml, 4.33 mmol). After 3 days, the reaction was diluted with H_2O (60 ml) and extracted with DCM (3x 30 ml). The combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was co-evaporated with anhydrous toluene (3x) before dissolving in anhydrous DMF (10 ml). PMBCl (1.18 ml, 8.74 mmol) was added followed by portionwise addition of NaH (60 wt%, 350 mg, 8.74 mmol) at 0 °C. The reaction mixture was stirred overnight while slowly warming to rt. The resulting yellow suspension was carefully quenched at 0 °C with H_2O (2 ml). The solution was diluted with H_2O (60 ml) and extracted with Et_2O (4x 40 ml). The combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure.

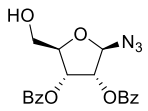
Purification of the crude residue by silica gel column chromatography (pentane/Et_2O = 90:10 → 60:40) provided an anomeric mixture of title compound **19** (470 mg, 2.91 mmol, 25% over 2 steps) as a clear oil. $\mathbf{R_f}$ = 0.7 (Pentane/Et_2O = 60:40). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.69 (ddd, *J* = 9.4, 7.7, 1.7 Hz, 4H), 7.37 (dtd, *J* = 8.3, 6.3, 5.8, 2.0 Hz, 6H), 7.26 (d, *J* = 8.7 Hz, 2H), 7.19 (d, *J* = 8.6 Hz, 2H), 6.84 (dd, *J* = 14.3, 8.6 Hz, 3H), 5.38 (d, *J* = 2.1 Hz, 1H), 4.62 – 4.34 (m, 4H), 4.28 – 4.21 (m, 1H), 4.18 (dd, *J* = 6.7, 4.5 Hz, 1H), 3.86 (dd, *J* = 11.5, 3.0 Hz, 1H), 3.78 – 3.61 (m, 9H), 1.05 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 190.6, 159.4, 159.3, 135.5, 135.5, 133.1, 132.9, 131.9, 129.7, 129.7, 129.6, 129.6, 129.4, 129.3, 127.7, 127.7, 127.6, 114.2, 113.8, 113.7, 113.7, 93.0, 82.8, 79.5, 75.9, 72.0, 71.9, 63.0, 55.4, 55.1, 55.1, 26.7, 19.1. **HRMS** $[C_{37}H_{43}N_3O_6Si + Na]^+$ = 676.28086 found, 676.28133 calculated; $[C_{37}H_{43}N_3O_6Si + NH_4]^+$ = 671.32543 found, 671.32594 calculated.

1-β-Azido-2,3-bis-*O*-benzoyl-5-*O*-*tert*-butyldimethylsilyl-D-ribofuranoside (20).



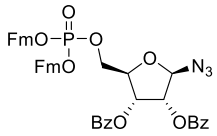
Compound **18** (748 mg, 4.27 mmol) was co-evaporated with anhydrous pyridine (3x) before dissolving in anhydrous pyridine (21 ml). TBSCl (2.4 ml, 6.8 mmol) was added and the reaction was stirred for 1.5 h at rt. BzCl (1.7 ml, 15 mmol) was added and the resulting suspension was quenched with H_2O (0.5 ml) after 40 min. The solution was concentrated under reduced pressure and subsequent purification of the crude residue by silica gel column chromatography (pentane/Et_2O = 97.5:2.5 → 95:5) resulted in title compound **20** (1.99 g, 4.27 mmol, 94%) as a clear oil. $\mathbf{R_f}$ = 0.5 (pentane/Et_2O = 90:10). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.02 – 7.90 (m, 4H), 7.60 – 7.49 (m, 2H), 7.44 – 7.32 (m, 4H), 5.76 (t, *J* = 5.1 Hz, 1H), 5.60 (d, *J* = 3.2 Hz, 1H), 5.53 (dd, *J* = 5.1, 3.2 Hz, 1H), 4.49 (dt, *J* = 5.2, 3.4 Hz, 1H), 4.00 – 3.88 (m, 2H), 0.95 (s, 9H), 0.15 (d, *J* = 1.0 Hz, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ -5.4, -5.3, 18.4, 25.9, 63.2, 72.0, 75.5, 83.5, 92.9, 128.5, 128.5, 129.0, 129.2, 129.7, 129.8, 133.5, 133.6, 165.1, 165.3. **IR**: 2126.38, 1737.17, 1269.25. **HRMS** $[C_{25}H_{31}N_3O_6Si + Na]^+$ = 520.18774 found, 520.18743 calculated; $[C_{25}H_{31}N_3O_6Si + NH_4]^+$ = 515.23243 found, 515.23204 calculated.

1-β-Azido-2,3-bis-*O*-benzoyl-D-ribofuranoside (21).



To a solution of compound **20** (1.8 g, 3.6 mmol) in MeCN/H_2O (7:1, 21 ml) was added *p*-TsOH•H_2O (1.0 g, 5.4 mmol). After stirring for 1 h at rt, the reaction was quenched with $NaHCO_3$ (sat., 6 ml) and diluted with EtOAc (150 ml). The organic fraction was washed with H_2O (3x 30 ml) and subsequently dried, filtered and concentrated under reduced pressure. Purification of the crude residue by silica gel column chromatography (pentane/EtOAc = 90:10 → 80:20) yielded title compound **21** (1.27 g, 3.61 mmol, 92%) as a clear oil. $\mathbf{R_f}$ = 0.5 (pentane/EtOAc = 70:30). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.05 – 7.97 (m, 2H), 7.95 – 7.87 (m, 2H), 7.62 – 7.49 (m, 2H), 7.46 – 7.30 (m, 4H), 5.71 (dd, *J* = 6.3, 5.1 Hz, 1H), 5.64 (d, *J* = 2.2 Hz, 1H), 5.54 (dd, *J* = 5.0, 2.2 Hz, 1H), 4.50 (ddd, *J* = 6.2, 4.0, 3.1 Hz, 1H), 4.02 (dd, *J* = 12.4, 3.2 Hz, 1H), 3.87 (dd, *J* = 12.4, 4.1 Hz, 1H), 2.45 (bs, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 62.4, 71.3, 75.8, 83.3, 93.4, 128.5, 128.6, 128.8, 128.9, 129.8, 129.9, 133.6, 133.7, 165.2, 165.6. **IR**: 2113.51, 1717.13, 1269.25, 1093.24, 685.42. **HRMS** $[C_{19}H_{17}N_3O_6 + Na]^+$ = 406.10123 found, 406.10096 calculated, $[C_{19}H_{17}N_3O_6 + NH_4]^+$ = 401.14594 found, 406.14556 calculated.

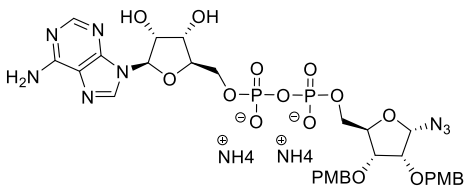
1-β-Azido-2,3-bis-*O*-benzoyl-5-*O*-(di(9H-fluoren-9-yl))-phosphoryl-D-ribo-furanoside (22).



Compound **21** (200 mg, 0.52 mmol) and pyridine-1-ium chloride (241 mg, 2.09 mmol) were co-evaporated with anhydrous toluene (2x) and anhydrous pyridine (1x) before dissolving in anhydrous pyridine (4 ml). A solution of bis((9H-fluoren-9-yl)methyl)diisopropylphosphoramidite (408 mg, 0.783 mmol) in anhydrous pyridine (1.5 ml) was added dropwise to the reaction mixture. After stirring for 1

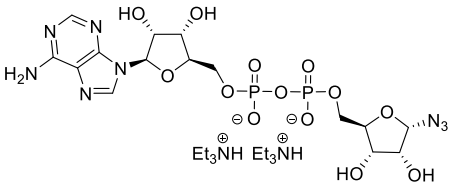
h at rt, *t*-BuOOH (5.5 M, 0.7 ml) was added and the reaction was monitored by ^{31}P NMR until no residual phosphotriester signal (~125 ppm) was observed. Upon completion the reaction was diluted with DCM (15 ml) and washed with $NaHCO_3$ (sat., 30 ml). The layers were separated and the H_2O fraction was extracted with DCM (2x 15 ml). The combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the crude residue by silica gel column chromatography (pentane/EtOAc = 70:30 → 60:40) provided title compound **22** (325 mg, 0.522 mmol, 76%) as a white foam. $\mathbf{R_f}$ = 0.6 (pentane/EtOAc = 50:50). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.96 (dd, *J* = 8.4, 1.3 Hz, 2H), 7.86 (dd, *J* = 8.3, 1.4 Hz, 2H), 7.76 – 7.65 (m, 4H), 7.62 – 7.48 (m, 6H), 7.46 – 7.29 (m, 8H), 7.30 – 7.19 (m, 6H), 5.63 (dd, *J* = 6.7, 4.9 Hz, 1H), 5.60 (d, *J* = 1.9 Hz, 1H), 5.45 (dd, *J* = 5.0, 1.9 Hz, 1H), 4.38 – 4.28 (m, 4H), 4.25 – 4.08 (m, 4H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 47.97, 47.99, 48.05, 48.07, 66.72, 69.58, 69.62, 69.64, 69.69, 71.21, 75.31, 80.57, 80.65, 93.38, 120.09, 120.11, 125.27, 125.31, 127.26, 127.28, 127.97, 127.99, 128.59, 128.69, 128.75, 128.89, 129.88, 129.95, 133.75, 133.85, 141.48, 141.50, 141.52, 143.15, 143.18, 143.22, 165.13, 165.23. **^{31}P NMR** (162 MHz, CDCl3): δ -2.52. **IR**: 2114.94, 1728.58, 1263.52, 1243.49, 988.78, 734.07. **HRMS** $[C_{47}H_{38}N_3O_9P + Na]^+$ = 842.22375 found, 837.22379 calculated; $[C_{47}H_{38}N_3O_9P + NH_4]^+$ = 837.26815 found, 837.26839 calculated.

1''-α-Azido-2'',3''-bis-*O*-(4-methoxybenzyl)-ADPr (23).



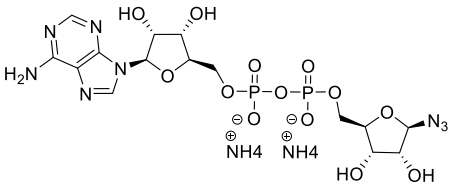
A solution of compound **14** (400 mg, 0.470 mmol) and Et_3N (3.30 ml, 23.5 mmol) in anhydrous MeCN (10 ml) was stirred for 3 days at rt. The solution was concentrated under reduced pressure and the crude residue was co-evaporated with a mixture of anhydrous pyridine (3x). DCI (139 mg, 1.17 mmol) was added and the mixture was co-evaporated with anhydrous MeCN (3x) before adding anhydrous MeCN (12 ml). Compound **9**[35] (401 mg, 0.563 mmol) was co-evaporated with anhydrous toluene (3x) before adding dropwise to the white suspension using anhydrous MeCN (5 ml). The reaction mixture was vigorously stirred under argon atmosphere for 1 h before adding *t*-BuOOH (5.5 M, 0.43 ml, 2.4 mmol). The reaction was monitored by ^{31}P NMR until no residual phosphotriester signals at 125-127 ppm were observed. Upon completion, DBU (0.35 ml, 2.4 mmol) was added and the mixture was stirred for 30 min before adding ammonium hydroxide (28%, 17 ml). After 16 h, the reaction mixture was concentrated under reduced pressure. Purification by preparative HPLC and subsequent lyophilization provided the NH_4 salt of title compound **23** (109 mg, 0.47 mmol, 27 % over 5 steps) as a white solid. **^{1}H NMR** (500 MHz, D_2O): δ 8.38 (s, 1H), 8.04 (s, 1H), 7.12 – 7.06 (dd, *J* = 10.7, 8.7 Hz 4H), 6.77 (dd, *J* = 8.7, 3.7 Hz, 4H), 5.95 (d, *J* = 5.1 Hz, 1H), 5.36 (d, *J* = 4.9 Hz, 1H), 4.56 (t, *J* = 5.1 Hz, 1H), 4.46 (t, *J* = 4.7 Hz, 1H), 4.34 (m, 4H), 4.28 – 4.13 (m, 4H), 4.10 (s, *J* = 5.4 Hz, 1H), 3.99 (dd, *J* = 5.9, 3.0 Hz, 1H), 3.95 (t, *J* = 3.2 Hz, 2H), 3.74 (s, 3H), 3.73 (s, 3H). **^{13}C NMR** (126 MHz, D_2O): δ 179.3, 158.6, 158.5, 154.3, 151.2, 148.4, 139.7, 130.2, 130.1, 129.3, 129.2, 118.3, 113.7, 113.7, 90.3, 87.2, 83.5, 82.9, 77.9, 75.4, 74.6, 72.5, 71.8, 70.0, 65.4, 65.1, 55.2. **^{31}P NMR** (202 MHz, D_2O): δ -10.54. **HRMS** $[C_{15}H_{22}N_8O_{13}P_2 + Na]^+$ = 825.20032 found, 825.20046 calculated.

α-N_3-ADPr (5).



To a solution of **23** (30 mg, 0.035 mmol) in HFIP (3.5 ml) at 0 °C was added HCl (0.2 M in HFIP, 0.35 ml) in one go. The resulting deep red solution was stirred for 20 minutes before quenching with Et_3N (0.05 ml). The crude residue was participated between milli Q grade H_2O (5 ml) and Et_2O (10 ml). The water fraction was lyophilized to yield title compound **5** (28 mg, 0.035mmol, quantified as Et_3NH^+ salt) as a white solid that was used in the click reaction without additional purification.

β-N_3-ADPr (6).



A solution of compound **22** (540 mg, 0.659 mmol) and Et_3N (1.58 ml, 11.3 mmol) in anhydrous MeCN (15 ml) was stirred for 2 days at rt. The solution was concentrated under reduced pressure and the crude residue was co-evaporated with a mixture of anhydrous MeCN/pyridine (1:1, 3x). DCI (191 mg, 1.62 mmol) was added and the mixture was co-evaporated with anhydrous MeCN (3x) before adding anhydrous MeCN (12 ml). Compound **9**[35] (460 mg, 0.646 mmol) was co-evaporated with anhydrous toluene (2x) before adding dropwise to the white suspension using anhydrous MeCN (3 ml). The reaction mixture was vigorously stirred under argon atmosphere for 1 h before adding *t*-BuOOH (5.5 M, 0.25 ml, 1.4 mmol). After 1 h, a second portion of *t*-BuOOH (5.5 M, 0.25 ml, 1.4 mmol) was added. The reaction was monitored by ^{31}P NMR until no residual phosphotriester signals at 125-127 ppm were observed. Upon completion, DBU (0.49 ml, 3.2 mmol) was added and the mixture was stirred for 30 min before adding ammonium hydroxide (28%, 12 ml). After 19 h, the solution was diluted with H_2O (15 ml) and washed with Et_2O (15 ml). The H_2O fraction was concentrated under reduced pressure. Purification by size exclusion chromatography and subsequent lyophilization provided the NH_4 salt of title compound **6** (188 mg, 0.304 mmol, 47% over 5 steps, corrected for the residual salt) as a white solid. A sample was subsequently purified by HPLC for analytical purposes. EDTA-Na_2 (1 mol%) was added to sharpen the signals. **^{1}H NMR** (500 MHz, D_2O): δ 8.52 (s, 1H), 8.25 (s, 1H), 6.12 (d, J = 5.6 Hz, 1H), 5.29 (d, J = 2.3 Hz, 1H), 4.75 (t, J = 5.4 Hz, 1H), 4.53 (dd, J = 5.1, 3.8 Hz, 1H), 4.42 – 4.37 (m, 1H), 4.30 – 4.19 (m, 3H), 4.17 – 4.09 (m, 2H), 4.05 – 3.99 (m, 1H), 3.95 (dd, J = 4.7, 2.3 Hz, 1H). **^{13}C NMR** (126 MHz, D_2O): δ 154.1, 150.9, 148.8, 140.4, 118.5, 94.4, 87.2, 83.9, 83.9, 83.8, 82.1, 82.1, 74.5, 74.4, 70.3, 70.2, 65.7, 65.6, 65.2, 65.1. **HRMS** $[C_{15}H_{22}N_8O_{13}P_2 + H]^+$ = 585.08557 found, 585.08543 calculated.

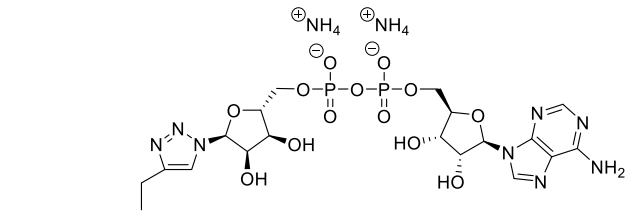
HPF1 (221 – 233): H_2N-Thr-Phe-Pra-Ala-Gly-Leu-Val-Val-Pro-Val-Asp-Lys-COOH (Pra = propargylglycine) (3).

Peptide sequence **3** was synthesized according to the general SPPS method as described above. The crude residue was subjected to size exclusion chromatography followed by HPLC purification. The product fractions were collected and lyophilized to provide title compound **3** (35 mg, 26 µmol, 26%) as a white solid. **LC-MS** R_t = 5.33 min (10-50% MeCN/H_2O, NH_4OAc). **HRMS** $[C_{61}H_{96}N_{14}O_{17} + H]^+$ = 1298.71880 found, 1298.71802 calculated; $[C_{61}H_{96}N_{14}O_{17} + 2H]^{2+}$ = 649.36177 found, 649.36117 calculated.

PARP1 (529 - 553): H_2N-Gly-Gly-Ala-Ala-Val-Asp-Pro-Asp-Ser-Gly-Leu-Glu-Pra-Ser-Ala - COOH (Pra = propargylglycine) (4).

Peptide sequence **4** was synthesized according to the general SPPS method as described above. The crude residue was subjected to size exclusion chromatography followed by HPLC purification. The product fractions were collected and lyophilized to provide title compound **4** (24 mg, 19 µmol, 19%) as a white solid. **LC-MS** R_t = 4.11 min (10-50% MeCN/H_2O, NH_4OAc). **HRMS** $[C_{61}H_{96}N_{14}O_{17} + H]^+$ = 1341.60029 found, 1341.59935 calculated; $[C_{61}H_{96}N_{14}O_{17} + 2H]^+$ = 670.80180 found, 670.80187 calculated.

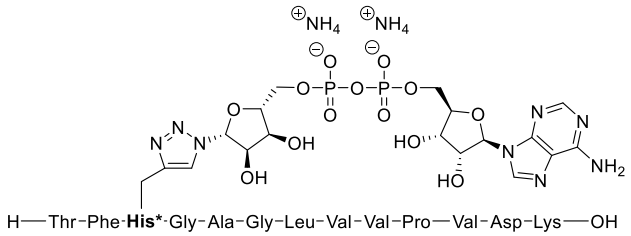
α-HPF1-ADPr: TF[H*]GAGLVVPVDK (H* = Triazolyl ADPr) (1α).



H—Thr-Phe-**His***-Gly-Ala-Gly-Leu-Val-Val-Pro—Val-Asp-Lys—OH

Title compound **1α** was synthesized using the general click chemistry procedure as described above. Compound **5** (13.1 mg as Et_3NH^+ salt, 17.0 µmol, 1.4 eq.) was reacted with **3** (15.0 mg, 12.0 µmol, 1.0 eq.) for 3 h. The molar ratios of the click mixture components were $CuSO_4$:THPTA: NaAsc = 1.3:8.0:2.0. Purification by preparative HPLC and subsequent lyophilization yielded title compound **1α** (7.22 mg, 3.84 µmol, 33%) as a white solid. **^{1}H NMR** (500 MHz, D_2O): δ 8.50 (s, 1H), 8.21 (s, 1H), 7.98 (s, 1H), 7.31 – 7.21 (m, 2H), 7.14 (m, 2H), 6.33 (d, *J* = 5.3 Hz, 1H), 6.11 (d, *J* = 5.9 Hz, 1H). **^{31}P NMR** (202 MHz, D_2O): δ -10.39, -10.49, -10.74, -10.85. **LC-MS** R_t = 5.25 min (10-50% MeCN/H_2O, NH_4OAc). **HRMS** $[C_{76}H_{118}N_{22}O_{30}P_2 + 2H]^{2+}$: 941.40044 found, 941.40025 calculated; $[C_{76}H_{118}N_{22}O_{30}P_2 + 3H]^{3+}$: 627.93637 found, 627.93592 calculated.

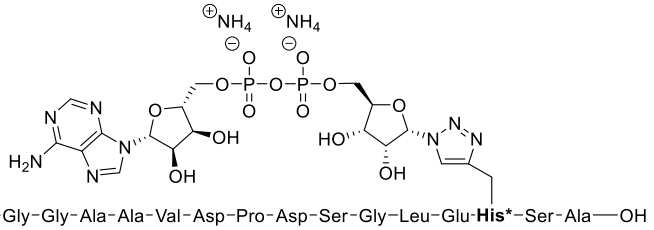
β -HPF1-ADPr: TF[H*]GAGLVVPVDK (H* = Triazolyl ADPr) (1β).



H—Thr-Phe-**His***-Gly-Ala-Gly-Leu-Val-Val-Pro—Val-Asp-Lys—OH

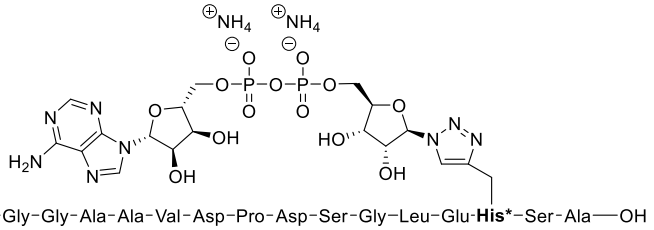
Title compound **1β** was synthesized using the general click chemistry procedure. **6** (10.2 mg, as NH_4^+ salt 12.0 µmol, 1.5 eq.) was reacted with **3** (10.0 mg, 7.71 µmol, 1.0 eq.) for 1.5 h. The molar ratio of the click mixture components were $CuSO_4$:THPTA:NaAsc = 1.3:7.8:1.3. Purification by SEC and preparative HPLC and subsequent lyophilization yielded title compound **1β** (2.1 mg, 1.11 µmol, 15%) as a white solid. **^{1}H NMR** (500 MHz, D_2O): δ 8.50 (s, 1H), 8.24 (s, 1H), 8.08 (s, 1H), 7.22 (dd, *J* = 13.7, 7.1 Hz, 3H), 7.08 (d, *J* = 7.1 Hz, 2H), 6.07 (d, *J* = 5.7 Hz, 1H), 6.04 (d, *J* = 4.6 Hz, 1H). **^{31}P NMR** (202 MHz, D_2O): δ -10.67, -10.77. **LC-MS** R_t =5.23 min (10-50% MeCN/H_2O, NH_4OAc). **HRMS** $[C_{76}H_{118}N_{22}O_{30}P_2 + 2H]^{2+}$: 941.40062 found, 941.40025 calculated; $[C_{76}H_{118}N_{22}O_{30}P_2 + 3H]^{3+}$: 627.93630 found, 627.93592 calculated.

α-PARP1-ADPr: GGAAVDPDSGLE[H*]SA (H* = Triazolyl ADPr) (2α).



Title compound **2α** was synthesized using the general click chemistry procedure as described above. Compound **5** (4.69 mg as Et_3NH^+ salt, 5.96 µmol, 1.4 eq.) was reacted with **4** (5.7 mg, 4.26 µmol, 1.0 eq.) for 7 h. The click mixture ratio was $CuSO_4$:THPTA:NaAsc = 1.3:7.2:1.2 Purification by SEC and preparative HPLC and subsequent lyophilization yielded title compound **2α** (0.67 mg, 0.35 µmol, 8%) as a white solid. **^{1}H NMR** (500 MHz, D_2O): δ 8.55 (s, 1H), 8.29 (d, *J* = 4.1 Hz, 1H), 7.95 (s, 1H), 6.26 (d, *J* = 5.3 Hz, 1H), 6.12 (d, *J* = 5.6 Hz, 1H). **^{31}P NMR** (202 MHz, D_2O): δ -10.70. **LC-MS** R_t = 3.78 min (10-50% MeCN/H_2O, NH_4OAc). **HRMS** $[C_{76}H_{118}N_{22}O_{30}P_2 + 2H]^{2+}$: 962.84102 found, 962.84095 calculated.

β-PARP1-ADPr: GGAAVDPDSGLE[H*]SA (H* = Triazolyl ADPr) (2β).



Title compound **2β** was synthesized using the general click chemistry procedure as described above. **6** (1.5 mg as NH_4^+ salt, 2.42 µmol, 1.5 eq.) was reacted with **4** (2.17 mg, 1.62 µmol, 1.0 eq.) for 1.5 h. The molar ratios of the click mixture components were $CuSO_4$:THPTA:NaAsc = 2.0:8.0:1.2. Purification by SEC and preparative HPLC and subsequent lyophilization yielded title compound **2β** (0.8 mg, 0.42 µmol, 26%) as a white solid. **^{1}H NMR** (500 MHz, D_2O): δ 8.44 (s, 1H), 8.20 (s, 1H), 7.94 (s, 1H), 6.07 (d, *J* = 5.8 Hz, 1H), 5.98 (d, *J* = 4.5 Hz, 1H). **^{31}P NMR** (202 MHz, D_2O) δ -10.69. **LC-MS** R_t = 2.99 min (10-50% MeCN/H_2O, NH_4OAc). **HRMS** $[C_{70}H_{107}N_{23}O_{37}P_2 + 2H]^{2+}$: 962.84054 found, 962.84095 calculated.

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