The development of chemical tools to study the interplay of ubiquitination and ADPribosylation

Kloet, M.S.

Chapter 4

Arginine ADP-ribosylation: Chemical Synthesis of Post-Translationally Modified Ubiquitin Proteins

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Introduction

Post-translational modification (PTM) of cellular proteins can affect their functioning and localization, influencing a wide range of cellular signaling processes. PTMs include relatively small groups such as a phosphate or methyl but can also involve more complex molecular entities such as (poly-)glycosides and ADP-ribose (ADPr)-moieties, or even entire proteins, such as ubiquitin (Ub). In the case of ADPr, mono-ADP-ribosyltransferases (mART) catalyze the displacement of nicotinamide from NAD^+ by a nucleophilic amino acid side chain in the target protein, thereby effectively connecting ADP-ribose to the protein via an alpha configured ribosyl linkage.[1,2] As is the case for most PTMs, ADP-ribosylation is a highly dynamic process and specific writer- (mART) and eraser- (ADPr-hydrolase (ARH)) enzymes can act on specific proteins or amino acids.[3] ARTs can be classified into two families, ART-C and ART-D, named after their first identification in cholera- and diphtheria bacteria, respectively.

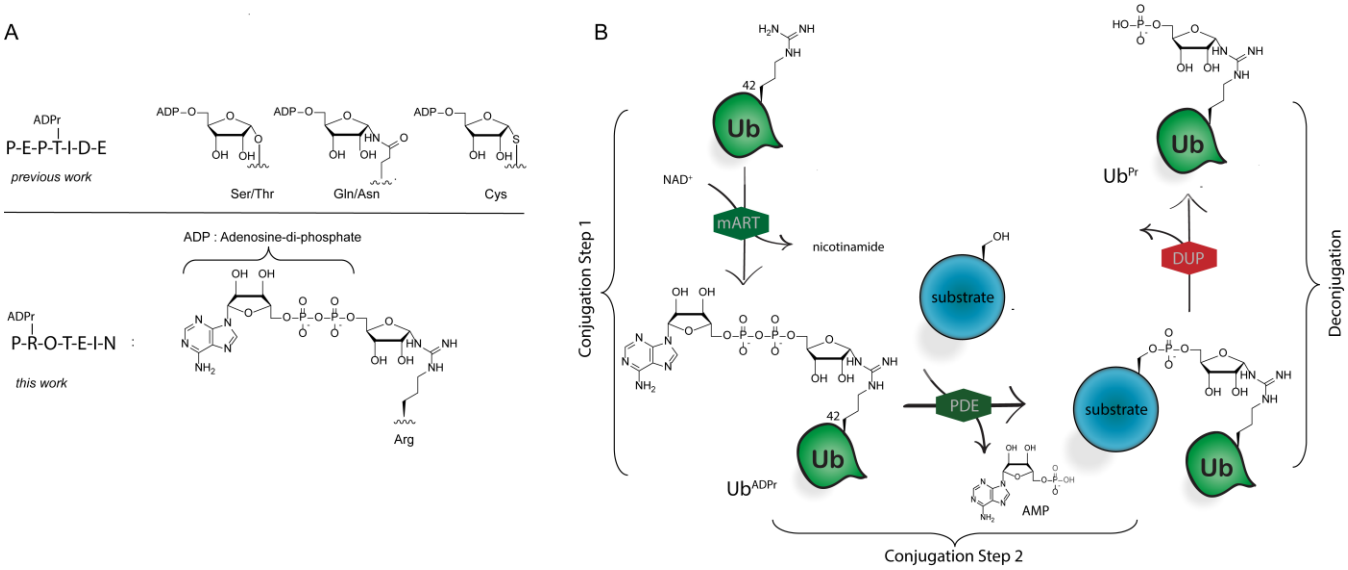


Fig.1 (**A**) Advances presented in this study, (B) Schematic representation of the pathway Legionella pneumophila enzymes use to (de)ubiquitinate host cell substrate proteins.

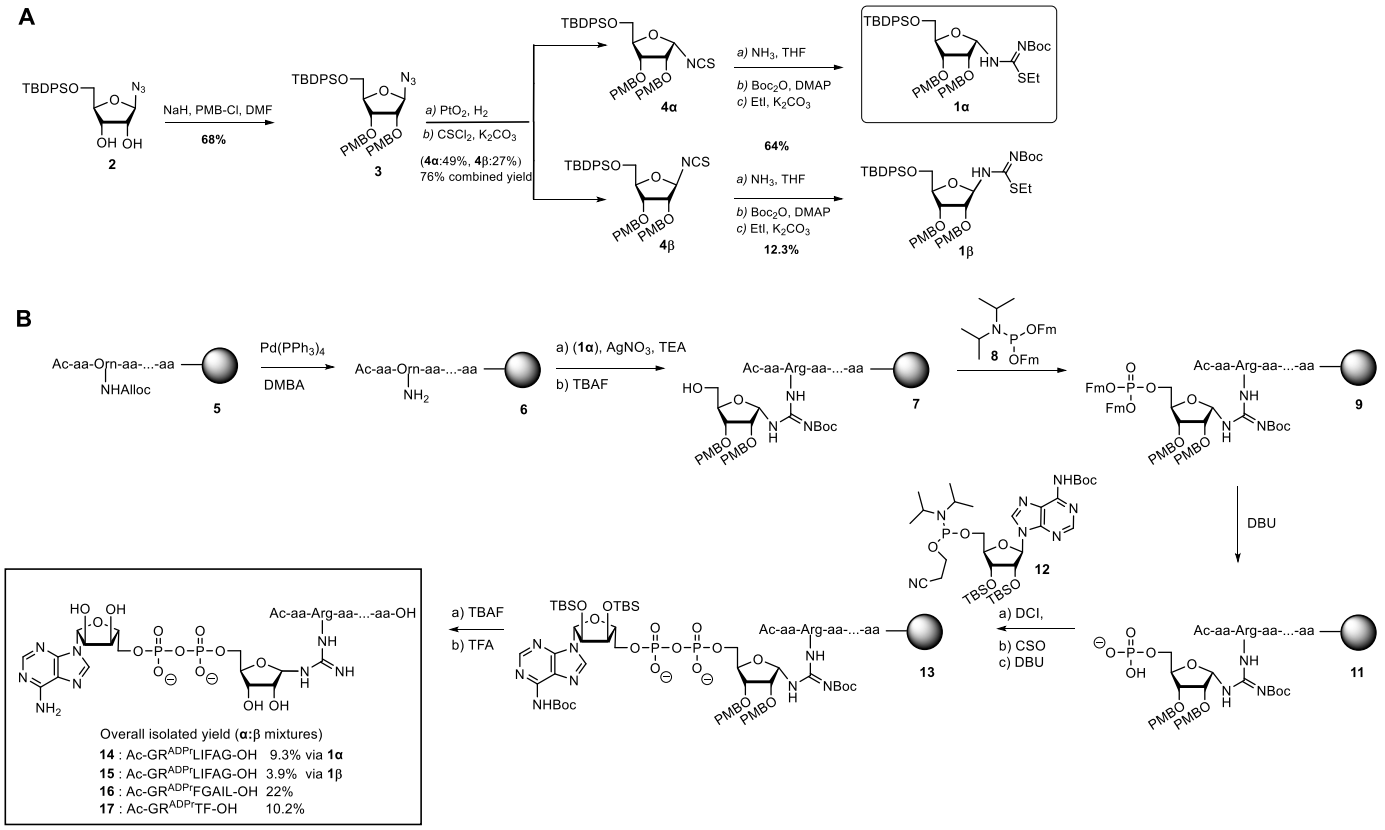
ADP-ribosylation of the δ-guanidinium group of an arginine residue is typically catalyzed by the ART-C subfamily. [3-5] The effector family of *Legionella pneumophila* SidE proteins (SdeA, SdeB, SdeC and SidE) combines multiple domains in a single protein, including an ART-C type domain and a phosphodiesterase (PDE) domain. Legionella uses these SidE proteins to hijack the eukaryotic host cell's ubiquitin pathway and ubiquitinate host cell proteins in an unconventional manner. This multi-step cascade starts with the Legionella SidE mART domain that catalyzes the attachment of ADPr on Arg42 of the host cells ubiquitin proteins. Subsequently, the phosphodiesterase (PDE) domain in SidE catalyzes the formation of a phosphodiester bond between the serine of host cell substrate protein and the arginine linked Ub^{ADPr}, while expelling adenosine monophosphate (**Fig. 1B**).[6-9] In this way, the bacterial effector enzyme effectively links

host Ub to host substrate proteins via an arginine-phosphoribosyl linkage. It contrasts with the canonical ubiquitination process in which an isopeptide bond between the Ub C-terminal Gly76 carboxylic acid and ε-amine of a lysine residue in the substrate protein is formed by host ligases. By using these SidE enzymes to achieve phosphoribosyl ubiquitination of host substrates and so-called Dup hydrolases to release the substrate protein in a deconjugation step, Legionella has dynamic control over part of the host cell's ubiquitinome, predominantly ER- and Golgi-associated proteins, which allow the bacterium to create an environment in which it can effectively replicate.[10-12] These SidE effectors are important for Legionella to proliferate in the host cell and effectively dodge the immune system, as bacterial replication is greatly reduced without these effectors.[13] Synthetic ADP-ribosylated peptides and proteins and reagents based thereon are of great use in studying activities, preferences and molecular mechanisms of (de)ADPribosylating enzymes. Chemical synthesis offers the possibility of preparing well-defined material on a scale that is useful for interrogating the complex biology associated with this PTM. We and others have previously reported on the synthesis of ADP-ribosylated peptides where ADP-ribose is attached to Ser[14,15], Thr[15], Cys[15], Asn [16-18], Gln[16-18] as well as to unnatural amino acids[19-21] (**Fig. 1A**). The closest reported Arg^{ADPr}-mimicking isostere is Cit^{ADPr} [17], resembling the natively linked Arg^{ADPr}, but with the distinction that the guanidinium moiety of the arginine side chain is replaced by the urea side chain of citrulline. Besides the synthesis of mono-ADP-ribosylated peptides, solid support based synthesis protocols for defined poly-ADPr chains have been developed.[22-25] Recent advances in the chemical synthesis of stabilized ADPr-protein conjugates show that copper-catalyzed azide-alkyne cycloaddition (CuAAC)[26,27] can be used to obtain functional mimics of ADP-ribosylated substrates. A semi-synthetic approach based on a native chemical ligation – desulfurization methodology of a synthetic ADPr-peptide and a truncated expressed histone gave rise to ADPribosylated histones, that were used to reveal the impact of serine ADPribosylation on chromatin structure and function.[28] Another powerful approach towards such modified histones is the use of chemo-enzymatic methods to mono- or poly-ADPribosylate synthetic peptides on designated serine sites using PARP1 in isolation or in combination with HPF1 followed by native chemical ligation strategies to obtain modified histones. [29-31] We here set out to develop a methodology that would be generally applicable in the synthesis of peptides ADP-ribosylated at arginine and expand this chemistry to the first entirely chemical synthesis of a natively linked ADP-ribosylated protein, Ub^{ADPr}. We validated the applicability of this approach by synthesizing Ub^{ADPr}, with an ADPr residue on all four different Arg positions in Ub (Arg42, Arg54, Arg72 and Arg74).

Results and Discussion

In recent work, an orthogonally protected ribosylated amino acid was used in solid-phase peptide synthesis to yield a ribosylated peptide that was turned into an ADPr-peptide using on-resin phosphitylation and subsequent pyrophosphate formation.[15] Threonine-, serine- and cysteine-linked ribosyl amino acids were thus prepared via the stereoselective glycosylation of a suitably protected amino acid acceptor with a ribosyl donor. However, such a direct glycosylation reaction is difficult to perform on the guanidinium group of arginine due to its high basicity. An alternative route towards glycosylated arginine building blocks uses a Lewis acid (silver-ion) promoted coupling of the less basic nucleophilic amine in the ornithine side chain to

an alpha oriented isothiourea glycoside,[32-35] that proved to be useful for the solution-phase synthesis of glycosylarginine building blocks. This method is also suitable for Fmoc-based SPPS to synthesize arginine-



Scheme 1. Synthetic scheme towards arginine linked ADPr-peptides. **A)** Solution phase chemistry towards building block **1**, **B)** Solid-phase chemistry towards ADPr-peptides **14**-**17**.

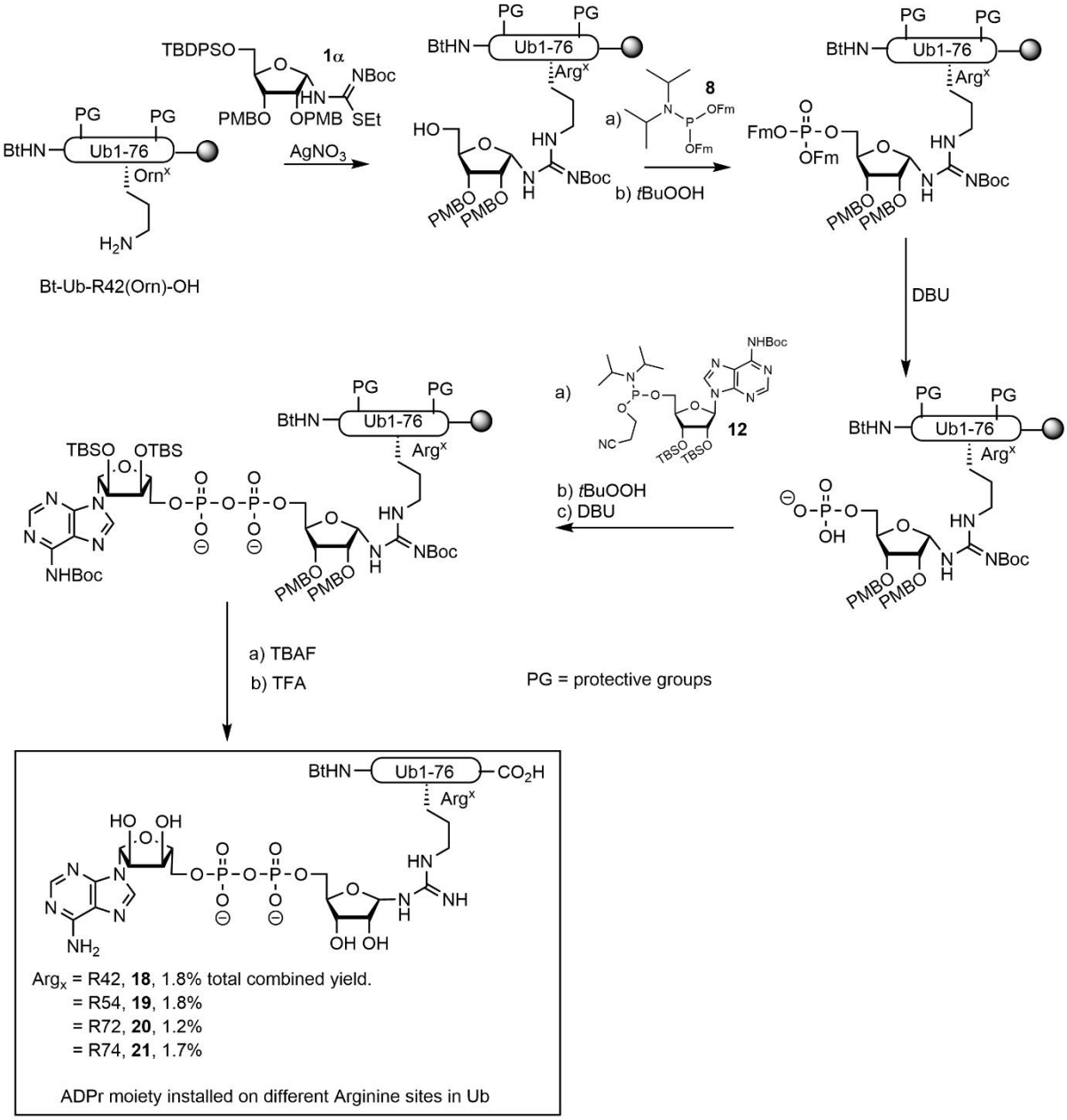
linked glycopeptides,[34,35] and can even be adapted to perform glycosylations on a resin-bound peptide.[32,33] We applied a similar strategy to couple an alpha-configured isothiourea riboside to the δ-amine of ornithine in resin-bound peptides. To our knowledge, this is the first example showing such an isothiourea based guanidinylation for furanoses.

The synthesis of isothiourea ribosyl building block **1α** (**Scheme 1A**) started with the preparation of 5-*O*-((*tert*-butyl)-diphenylsilyl)-β-D-ribofuranosyl azide **2** as described previously.[17,36] PMB protection on the 2'- and 3'-hydroxyls in **2** yielded **3** in 68%. Next, the anomeric azide was reduced using Adam's catalyst and H_2. Attempts to work up the reaction proved unsuccessful as the resulting ribosylamine is highly labile and concentration *in vacuo* led to total degradation of the product. Therefore, after filtration over a pad of Celite to remove the catalyst, the filtrate was directly used without further work-up or purification to install the isothiocyanate. The resulting anomeric mixture of isothiocyanates could easily be separated by column chromatography to obtain the α-anomer **4α** in a yield of 49% over the two steps. In addition, the β-anomer **4β** was obtained in a yield of 27% over the two steps. Next, α-anomer **4α** was subjected to aminolysis using ammonia in THF to give the thiourea that was directly treated with Boc_2O to protect the amine functionality, followed by treatment with iodoethane to furnish ribosyl isothiourea **1α** in 64% yield**.** The same sequence

of steps was performed to synthesize **1β** in 12% yield, respectively. With ribosyl isothiourea **1α** in hand, the on-resin synthesis of model heptapeptide **14** (Ac-GR^{ADPr}LIFAG-OH) was undertaken (**Scheme 1B**). Peptide **14** is derived from the human Ub protein and contains the amino acids 42-47 known to be ADP-ribosylated on the Arg42 residue by *Legionella pneumophila* effector enzymes. On the prospected ADP-ribosylation-site N$^{\delta}$-Alloc protected ornithine was incorporated into the peptide sequence. The Alloc protecting group allows for orthogonal on-resin deprotection with $Pd(PPh_3)_4$ to furnish the primary amine. When, after a test cleavage of an aliquot of resin, full removal of the Alloc-group was observed, peptide **6** was guanidinylated with building block **1α** using $AgNO_3$ as a Lewis acid. After full deprotection and removal from the resin on a test sample, LC-MS analysis showed complete conversion with no notable side-products detected. Next, on-resin desilylation of the 5'-OH on the ribosyl moiety was performed to yield resin **7** and the primary alcohol was subsequently phosphitylated using the appropriate Fm-protected phosphoramidite reagent **8**, followed by on resin P^{III} to P^{V} oxidation. During this phosphorylation reaction, however, along with desired product **9**, a side product **10** originating from the phosphitylation of the guanidine group was observed (see **Supporting Table S1**). We optimized this reaction and suppressed the formation of the side-product by varying the activator (5-ethylthio-1H-tetrazole (ETT), tetrazole or 4,5-dicyanoimidazole (DCI)) and equivalents of the respective phosphitylating reagent (2.5 and 5.0 equivalents) (**Supporting Table S1**). Over-phosphitylation could be largely suppressed when utilizing DCI as an activator with 2.5 eq. of the phosphitylating reagent. Subsequent 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) mediated deprotection of the phosphotriester towards peptide **11** prepared the resin for P^{V} to P^{III} coupling with adenosine amidite **12** that bears TBS and Boc as protecting groups. Subsequent oxidation with (1S)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) and removal of the cyanoethyl protective group on the pyrophosphate moiety with DBU led to protected ADPr-peptide **13**. The silyl ethers on the adenosine moiety were removed by treatment of the resin with a 1M TBAF solution. Finally, the peptide was cleaved from the resin using 10% trifluoroacetic acid (TFA) in DCM with concomitant loss of all remaining protecting groups (Boc and PMB). RP-HPLC purification of the crude mixture led to the isolation of **14** in a 9.3% overall yield (based on initial loading of the resin), as the first example of a synthetic Arg-linked ADPr-peptide. While characterizing the Arg-ADPr peptide **14** by ^{1}H-NMR we observed anomerization in a ratio of 6:4 (α:β), although the isothiourea riboside used in the guanidinylation reaction was of the pure α-configuration. It has been reported by Oppenheimer *et al*. that Arg-ADPr is prone to spontaneous anomerization during purification under both buffered or acidic conditions leading to the 6:4 (α:β) ratio.[37-39] In our methodology, we applied 10% TFA to release the Arg-ADPr peptide conjugates from resin, which might thus potentially induce or even enhance anomerization. To examine this further we coupled the pure β-configured isothiourea ribose **1β** to peptide **6** and conducted the full cycle to obtain ADPr-peptide **15**. Analysis of this ADPr-peptide revealed that a similar 6:4 ratio of anomers was formed, confirming that indeed during the liberation from the resin and deprotection of the peptides or its subsequent purification, anomerization occurs towards the same anomeric equilibrium. We additionally also synthesized a randomized heptamer **16** and a shorter tetramer peptide **17** in 22 and 10 % yield, respectively.

Our next aim was to extrapolate our synthetic methodology from peptides to proteins. Therefore, full-length ubiquitin in which Arg42 was replaced with N$^{\delta}$-Alloc protected ornithine was prepared using SPPS. Chemical synthesis of ^{Arg42}Ub^{ADPr} was performed using procedures similar to those used to obtain **14** (**Scheme 2**) and

monitored via test cleavages on small resin samples. Alloc deprotection using Pd(0) chemistry exposed the amine of the ornithine moiety and on-resin guanidinylation with **1α** proceeded uneventfully (**Figure S1**). Subsequent phosphitylation (**Figure S2**) and P^{V}-P^{III} coupling (**Figure S3**), resulted in fully protected resin-bound $^{Arg42}Ub^{ADPr}$. For the short peptide **14**, 10% TFA in DCM was sufficient to remove all protective groups, and under these conditions, the glycosidic bonds and the pyrophosphate moiety underwent minimal



Scheme 2. Synthetic scheme towards arginine linked Ub^{ADPr}'s **18-21.**

hydrolysis. For synthetic Ub^{ADPr}, however, the Pbf protective-groups on the three remaining arginine residues in Ub needed prolonged reaction times at higher TFA concentrations (routinely, 90.5% TFA is used for 2 hours to deprotect synthetic Ub fully). Strikingly, taking into account the acid-lability of glycosidic bonds and the

intrinsic lability of the pyrophosphate bond, test cleavages in 90.5% TFA for 1.5 hours on Ub^{ADPr} showed no notable traces of cleavage of these bonds and confirmed the formation of Ub^{ADPr}. We confirmed this acid-stability by incubation of Ub^{ADPr} (and heptamer **14**) in TFA (90.5%) for 1.5 hours (**Figure S4, S5**). We observed the full-length protein to be more acid-stable than the heptamer peptide, observing no glycosidic bond nor pyrophosphate bond cleavage, respectively. Using these conditions, full cleavage from the resin and global deprotection followed by HPLC purification yielded synthetic $^{Arg42}Ub^{ADPr}$ **18** in an overall yield of 1.8%. The introduction of the ADPr-group on the other arginine residues in Ub can be achieved straightforwardly by incorporating the N^{δ}-Alloc protected ornithine on another position in the protein during SPPS. Hence, we successfully synthesized Ub^{ADPr} on Arg54, Arg72 or Arg74, obtaining the conjugates **19**, **20** and **21** in 1.8%, 1.2% and 1.7% overall isolated yield, respectively. All four Ub^{ADPr}s were characterized by HRMS and SDS-PAGE (**Figure S6-S10**). Depending on the position of the Arg, we observed between 14 and 30% Ub^{Pr} in our samples and attribute this to inefficient pyrophosphate formation caused by incomplete coupling of the nucleoside phosphoramidite to ribose 5-phosphate on resin, as we established $^{Arg42}Ub^{ADPr}$ to be stable under acidic conditions (**Figure S5**).

To investigate whether Legionella DUPs are able to hydrolyze the pyrophosphate in our synthetic ADPr-peptides or are affected by the anomeric configuration of the arginine-ribosyl linkage, we incubated Ub-derived Arg-ADPr heptapeptide **14**, with DupA and followed the enzyme-mediated hydrolysis of the pyrophosphate bond over time using ^{1}H-NMR (**Fig. 2A**). After 2 hours, we observed hydrolysis of the ADPr-peptide (α-anomer, proton H1': δ = 5.27 ppm, β-anomer, proton H1': δ = 5.08 ppm) to the corresponding phosphoribosyl (Pr)-peptide (α-anomer, proton H1': δ = 5.40 ppm, β-anomer, proton H1': δ = 5.14 ppm) (**Fig. 2B**) and a change in the initial 6:4 (α /β) ratio between the anomeric protons belonging to α- and β-anomers of the remaining ADPr-peptide. This verifies that our synthetic Arg-ADPr peptide is being recognized and processed by the catalytic activity of the enzyme. Additionally, DupA seems to have a preference for α over β, hydrolyzing α-oriented Arg-ADPr peptide **14** (roughly 1.5x) faster than its β-anomer. A similar observation has been reported previously for the recognition of Arg^{ADPr} by ARH1.[39]A measurement of the same sample after overnight incubation in the presence of DupA showed a near completion of the pyrophosphate hydrolysis reaction for both anomers and formation of both α- and β-phosphoribosyl peptide as major products. Although indeed both anomers appear to be processed by the enzyme over this extended time, we cannot conclude that DupA directly hydrolyzes the β-anomer (at a lower rate) or rather this hydrolysis is caused by spontaneous epimerisation of the β-anomer to the α-anomer that only then gets processed by the enzyme to finally re-epimerizes back to the natural equilibrium in the product over time. Estimated $t_{1/2}$ for anomerization of Arg^{ADPr} under physiological conditions are between 3 and 6 hours, although no experimental determination has been conducted and the rate of spontaneous anomerisation of analogues α-NADH to β-NADH was determined to be 3.1×10^{-3} min $^{-1}$ ($t_{1/2}$ = 4 hours).[38]

Encouraged by the fact that DupA processes the synthetic ADPr peptides, we next set out to compare the rate of hydrolysis with that of enzymatically produced $^{Arg42}Ub^{ADPr}$ (**Figure S11-S12**).[6] We also included heptameric peptide **16**, randomized in the amino acid sequence surrounding the Arg42 Ub recognition site, and tetrapeptide **17 (Scheme 1)**, a sequence shorter in length and not derived from Ub. Enzymatic $^{Arg42}Ub^{ADPr}$

was prepared by incubating ubiquitin with SdeA H277A mutant and NAD^+ followed by purification using size-exclusion chromatography under buffered conditions at pH 7.5.[7] The ADPr-peptides were incubated in the presence of DupA under buffered conditions and analyzed using high-resolution mass-spectrometry at indicated times (**Fig. 2C**). In this hydrolysis assay, the enzymatic $^{Arg42}Ub^{ADPr}$ was completely hydrolyzed by DupA to $^{Arg42}Ub^{Pr}$ within 30 minutes. Ubiquitin derived heptamers **14** and **15** were processed at a rate lower than $^{Arg42}Ub^{ADPr}$, showing 48 and 52% hydrolysis after 90 minutes, respectively. The sequence surrounding Arg42 of Ub seems to affect recognition by DupA as scrambled heptamer **16** was processed significantly less (32% after 90 min) and tetramer **17** was not hydrolyzed by DupA at all. It hence seems that DupA can recognize the specific peptide context and/or peptide length of the Ub surrounding position 42. Not surprisingly, the full-length $^{Arg42}Ub^{ADPr}$ protein, being the native substrate for Legionella effector proteins, provides more sequence context and structure, is hydrolyzed more efficiently than **14-15**, although we cannot exclude that anomerization of **14-15** might also contribute to the observed reduced rate of hydrolysis.

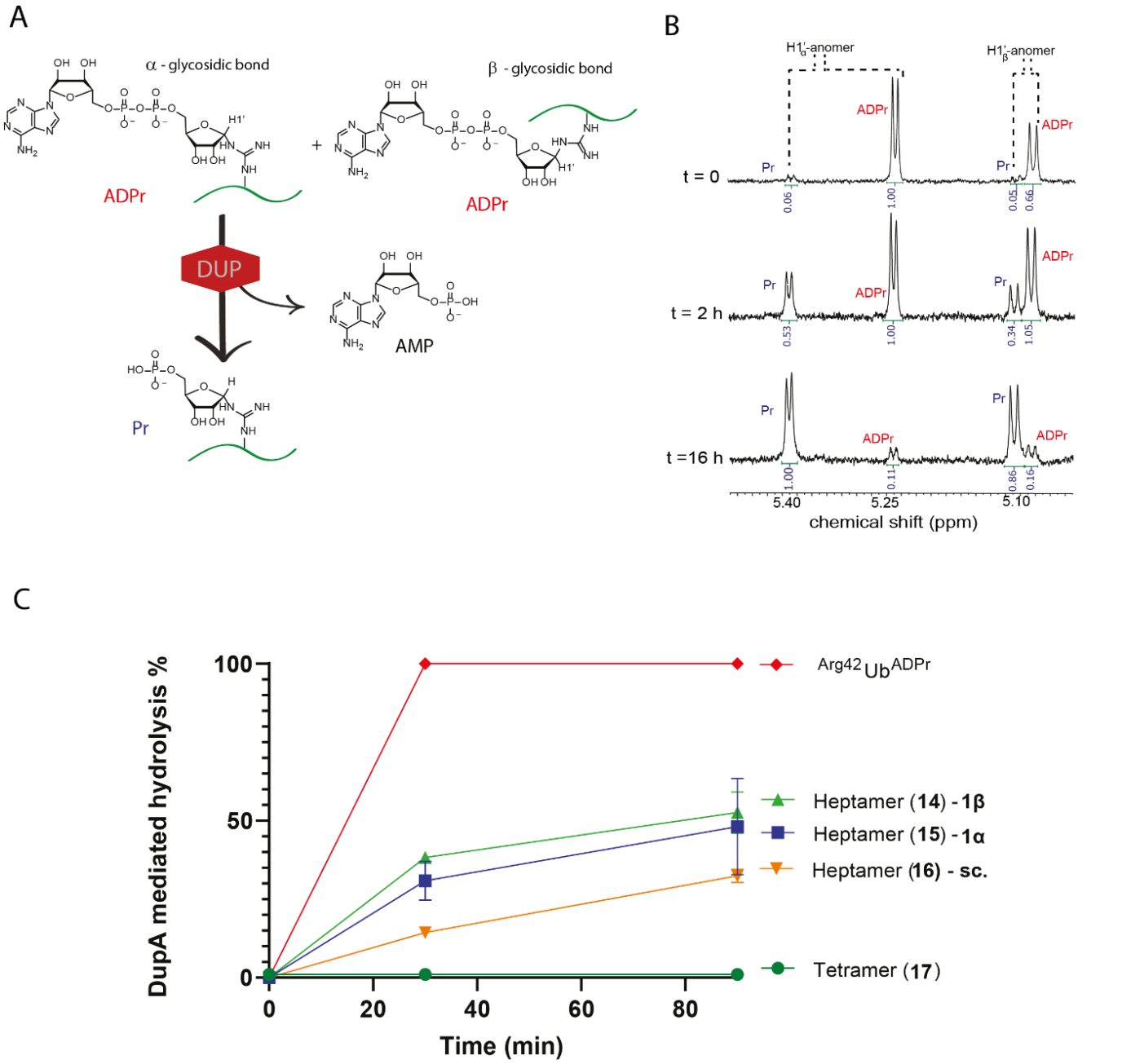


Fig. 2. (**A**) Schematical representation of experimental set-up, where DupA cleaves the pyrophosphate linkage in α- or β-configured Arg-ADPr peptides. (**B**) DupA-mediated hydrolysis of heptamer **14** followed over time using ^{1}H NMR. The anomeric (α- or β-glycosidic linked **14**) is hydrolyzed into the α- or β-linked phosphoribose variant providing different chemical shifts for each product. The associated protons are annotated and integrated. (**C**) DupA-mediated hydrolysis of **14**-**17** as compared to enzymatically produced $^{Arg42}Ub^{ADPr}$. The conversion is measured over time and followed with HRMS. **14** is prepared using **1α**, **15** is prepared using **1β**. **16** is a scrambled sequence and **17** is a tetramer.

We next examined the recognition and hydrolysis of our four synthetic Ub^{ADPr} proteins **18**-**21,** further annotated as (synth.), in comparison to enzymatically prepared $^{Arg42}Ub^{ADPr}$, further annotated as (enz.), by incubating the respective Ub^{ADPr}-analogues with DupA. We first analyzed if the synthetic conjugates were processed at all during overnight incubation with DupA and observed hydrolysis of all four synthetic Ub^{ADPr}'s,

albeit in different amounts (see **Fig. 3A**). This hydrolysis is DupA mediated as incubation of enz. $^{Arg42}Ub^{ADPr}$ in buffer without DupA does not lead to hydrolysis at these prolonged times. Synthetic $^{Arg42}Ub^{ADPr}$ **18** and Arg42-derived Ub^{ADPr} heptamer **14** were almost completely processed, as is enz. $^{Arg42}Ub^{ADPr}$. Although less than $^{Arg42}Ub^{ADPr}$, $^{Arg74}Ub^{ADPr}$ is hydrolyzed significantly in contrast to $^{Arg54}Ub^{ADPr}$ and$^{Arg72}Ub^{ADPr}$. Performing a similar assay and analyzing the conversion at shorter time points (15 – 90 min) showed enz. $^{Arg42}Ub^{ADPr}$ to be completely hydrolyzed after 15 min. The processing of synth. $^{Arg42}Ub^{ADPr}$ was more moderate in this time frame (52% after 30 min) and (65% after 90 min) (**Fig. 3B**), whereas the other three Ub^{ADPr} 's linked via Arg72, Arg74 and Arg54 show significantly less hydrolysis by DupA, complementing the demonstrated preference of DupA for Arg42. The initial swift turnover of roughly half the synth. $^{Arg42}Ub^{ADPr}$, could be processing of the α-anomer in comparable rate to enz. $^{Arg42}Ub^{ADPr}$. The slower continuation of hydrolysis after this 50% mark might be indicating that either the β-anomer is processed by the enzyme at a reduced rate, or that the β-anomer spontaneously anomerizes over time to give the α -anomer that in turn is processed by the enzyme. We set out to examine whether coupling of the β-thioisurea ribose **1β** and additional synthesis of $^{Arg42}Ub^{ADPr}$ would lead to an ADPr-protein that is processed similar or differently by the DupA enzyme, we synthesized $^{Arg42}Ub^{ADPr}$ via β-isothiourea **1β** (**22**). Interestingly, **22** is processed to the same extent as $^{Arg42}Ub^{ADPr}$ synthesized using α-riboside **1α**, indicating a comparable anomeric ratio after synthesis/isolation as was observed for peptides **14** and **15** (**Figure S13)**. The observed difference between enz. $^{Arg42}Ub^{ADPr}$ and synth. $^{Arg42}Ub^{ADPr}$ is striking and we speculate this reduced processing rate to be caused by anomerization during synthesis of the material, as was shown for synthetically prepared heptapeptide **14** (**Figure 2C**). We then wondered whether enzymatically prepared Ub^{ADPr} also anomerizes spontaneously under physiological conditions. It is speculated in literature that such a spontaneous anomerisation of ADPribosylated proteins *in vivo* might not occur due to physical stabilisation of the ADPr group by the protein context, in contrast to the ADPribosylated-Arg amino acid in *in vitro* settings.[39] If indeed the formed α-configurated Ub^{ADPr} is stabilized by Ub's C-terminal tail, this might explain that enzymatically produced $^{Arg42}Ub^{ADPr}$ retains an α-configuration while the synthetic $^{Arg42}Ub^{ADPr}$ anomerizes completely during the unfolded state in the SPPS protocol.

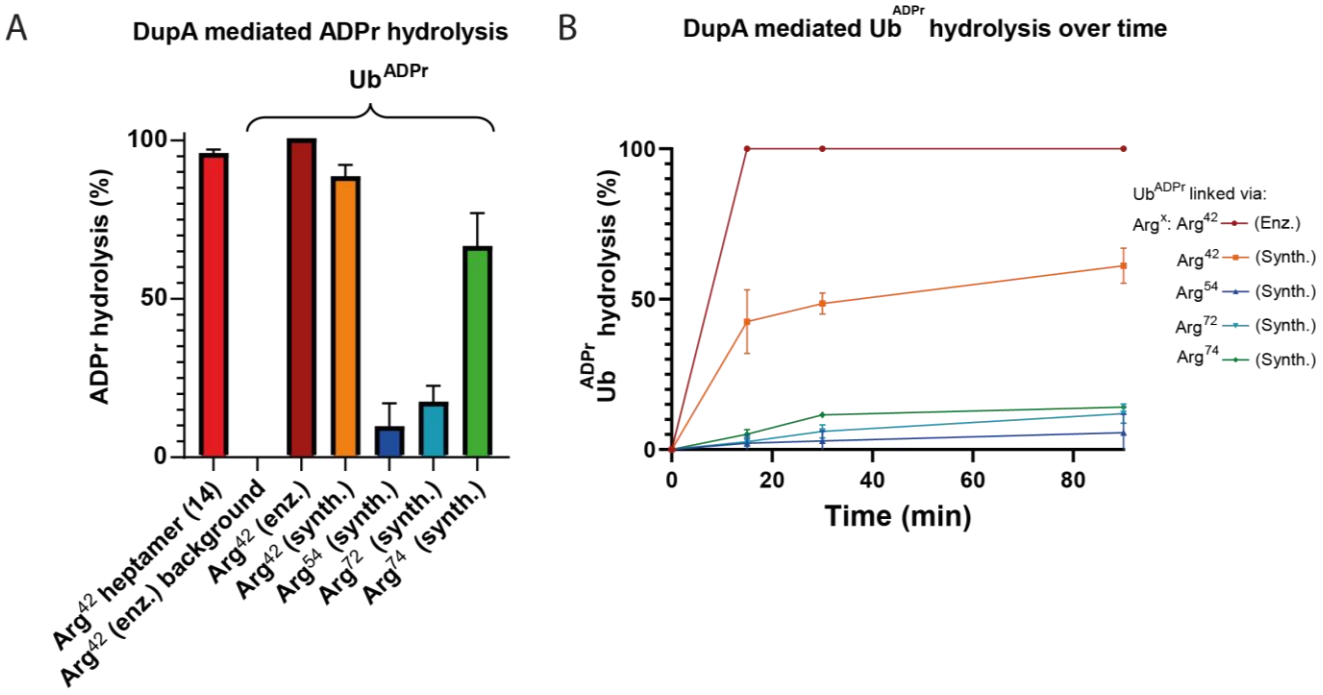


Fig. 3. DupA-mediated hydrolysis of Ub^{ADPr} into Ub^{Pr}. (**A**) DupA-mediated pyrophosphate bond cleavage in Ub^{ADPr} arginine variants after overnight incubation. (**B**) Hydrolysis of Ub^{ADPr} by DupA followed over a time course of 0 - 90 min. Both graphs are analyzed with HRMS. The measurements in both graphs are normalized for background Ub^{Pr} present as impurity associated with the synthesis.

Our next aim was to investigate the SdeA-mediated ligation of substrate ER-proteins to Ub^{ADPr}, the critical biological process in the onset of Legionnaires' disease.[40] We synthesized a 20-mer peptide (sequence on page **S38**) derived from the ER remodeling RTN4b protein (**23**) known to be a substrate of SidE effectors[8], equipped with a rhodamine fluorophore on the N-terminus. We tested whether SdeA, using its PDE domain, would ligate Ub^{ADPr} to this RTN4b peptide to form a fluorescent peptide-Pr-Ub conjugate (**Fig. 4A**). The full RTN4b 20-mer peptide **23** contains six serine residues as potential conjugation sites.[10] Enzymatically produced $^{Arg42}Ub^{ADPr}$ was incubated with SdeA and **23** as control and analyzed by mass spectrometry (**Figure S14).** Under the used conditions, SdeA couples $^{Arg42}Ub^{ADPr}$ to peptide **23** to form the phosphoribosyl linked ^{Arg42}Ub-RTN4b product (**Fig. 4A**) and shows partial hydrolysis of the pyrophosphate bond to $^{Arg42}Ub^{Pr}$, as has been reported.[6,10] This confirms that peptide **23** is a suitable substrate for inducing the PDE mediated ligation of $^{Arg42}Ub^{ADPr}$.

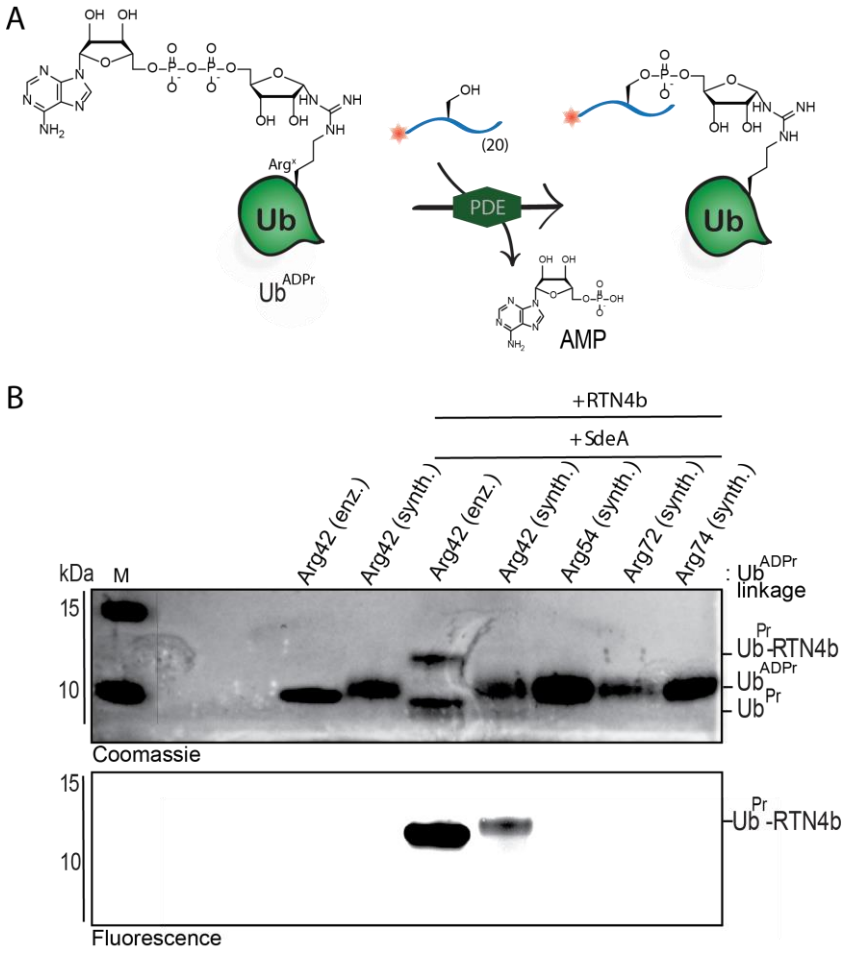


Fig. 4. SdeA-mediated ligation of Ub^{ADPr} and fluorescent RTN4b 20-mer fragment **23**. (**A**) Schematic representation of the conducted assay showing SdeA mediated conjugating of Ub^{ADPr} and peptide **23** to form a fluorescent product. (**B**) $^{Arg42}Ub^{ADPr}$ is recognized and processed by SdeA. SdeA-mediated ligation assay performed for all (synthetic) Ub^{ADPr}'s and analyzed by SDS-PAGE; top panel: gel stained with Coomassie blue protein stain, Bottom panel; Fluorescence scan. M: molecular weight marker.

We next examined if our four synthetic ubiquitin's **18-21** could also officiate in this process. LC-MS analysis confirmed the formation of the product for (synth.) $^{Arg42}Ub^{ADPr}$ **18** although the conversion was more moderate compared to the enzymatic material (**Figure S15**). We then used SDS-PAGE analysis to compared ligation of **23** to the enzymatic- and synthetic Ub^{ADPr}'s. Indeed, fluorescent product formation of the enzymatic- and synthetic $^{Arg42}Ub^{ADPr}$ regio-isomers could clearly be visualized by in-gel fluorescence (**Fig. 4B**). Synth. Ub^{ADPr} modified at Arg54, Arg72 and Arg74 (**19**-**21)** were neither coupled to RTN4b peptide **23** nor hydrolyzed by SdeA, also showing the preference of the SdeA ligase activity for the Arg42 position. Synthetic $^{Arg42}Ub^{ADPr}$ coupling to **23** by SdeA is significantly less then enzymatically produced $^{Arg42}Ub^{ADPr}$, which might be caused by the degree of anomerization in the former. Since β-NAD^+ is coupled to Ub by the mART domain of SdeA the expected product (Ub^{ADPr}) carries the α-orientation and hence the PDE-domain of SdeA would facilitate the coupling of the RTN4b-derived peptide to only the α-Ub^{ADPr}. The presence of the β-Ub^{ADPr} might hinder efficient coupling to peptide **23** by competing for entry towards the active site of the SdeA PDE domain.

Conclusion

We developed a methodology to synthesize arginine-linked ADPr-peptides and Ub^{ADPr} proteins, showcasing the first total chemical synthesis of an ADP-ribosylated protein carrying a native arginine linkage. Our synthetic strategy features a Lewis acid-mediated on-resin guanidinylation of the primary amine in the ornithine side chain of the protein with a thioisourea riboside to furnish the native Arg-ribosyl residue. Subsequent phosphorylation and formation of the adenosine phosphate was also conducted on-resin. After global deprotection and resin release using acidic conditions the ADPribosylated proteins were purified using RP-HPLC. This methodology to install the N-glycosidic linkage and sequentially build up the ADP-moiety was effective and proved resistant to a high percentage of TFA during deprotection. Of note, the final product contains varying amounts of phosphoribosylated protein indicating that the final adenosine-di-phosphate formation reaction was not quantitative. The ADPr-peptides and ADPr-ubiquitin regio-isomers were recognized by Legionella effectors (DupA and SdeA) in hydrolysis and ligation assays, albeit at a lower rate than enzymatic produced Ub^{ADPr}. We speculate this reduced processing to be caused by the anomerization of the N-glycoside linkage in Arg-ADPr that connects ribose to the side chain of arginine. Although anomerisation is known to occur under physiological conditions, the conditions used to prepare synthetic Ub^{ADPr} might contribute to increased degree of anomerisation, leading to a slower processing by the Legionella hydrolase. The ability to site-specifically introduce the ADPr moiety allowed us to synthesize Ub^{ADPr} on every arginine (Arg42, Arg54, Arg72, Arg74), giving access to well-defined material currently not attainable using biochemical methods. In hydrolysis and ligation assays, we demonstrate that Legionella effectors DupA and SdeA, favor the $^{Arg42}Ub^{ADPr}$ linkage. We hence developed a synthetic approach that provides native linked Arg-ADPr peptides and proteins that were used to profile the site-specificity of enzymes involved in installing and removing ADPr-modifications.

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Supporting Information

Arginine ADP-ribosylation; Chemical Synthesis of Post-Translationally Modified Ubiquitin Proteins

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Supporting Table S1 and the synthesis of ribosides and peptides 1α-17 are described in:

MS Kloet & Voorneveld, J. *et al.* Arginine ADP-Ribosylation: Chemical Synthesis of Post-Translationally Modified Ubiquitin Proteins. *J Am Chem Soc* **144**, 20582–20589 (2022).

HRMS spectra of intermediates in the synthesis of $^{R42}Ub^{ADPr}$ (18)

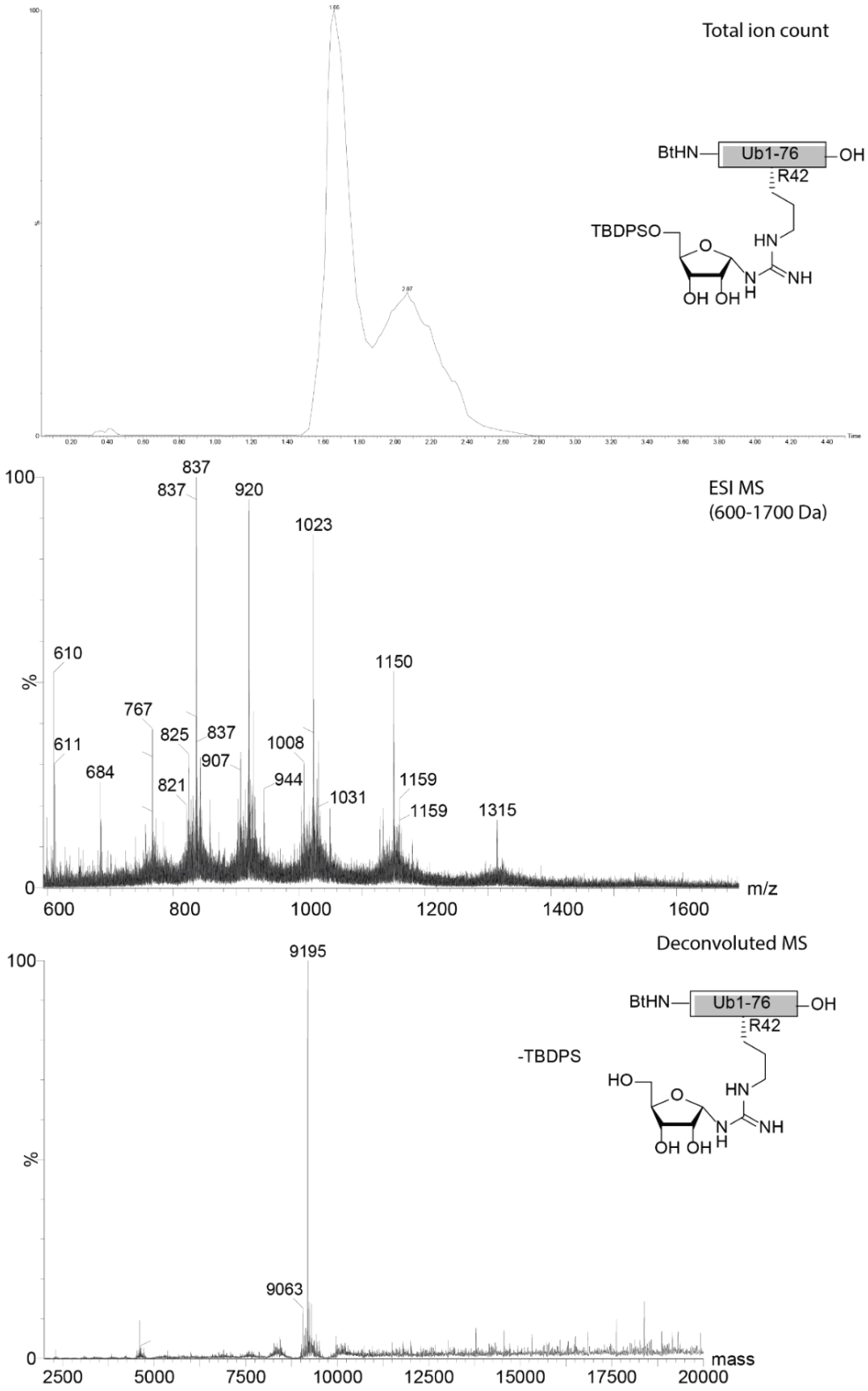


Figure S1. HRMS spectra of Ribosylated Ub_{1-76} (R42 → NH_2 ornithine) using **1α**. During the test cleavage conditions: TFA/TIS/H_2O/Phenol (90.5/2/5/2.5), to release Ubiquitin from the resin the TBDPS group was deprotected (lower panel, deconvoluted mass = 9195).

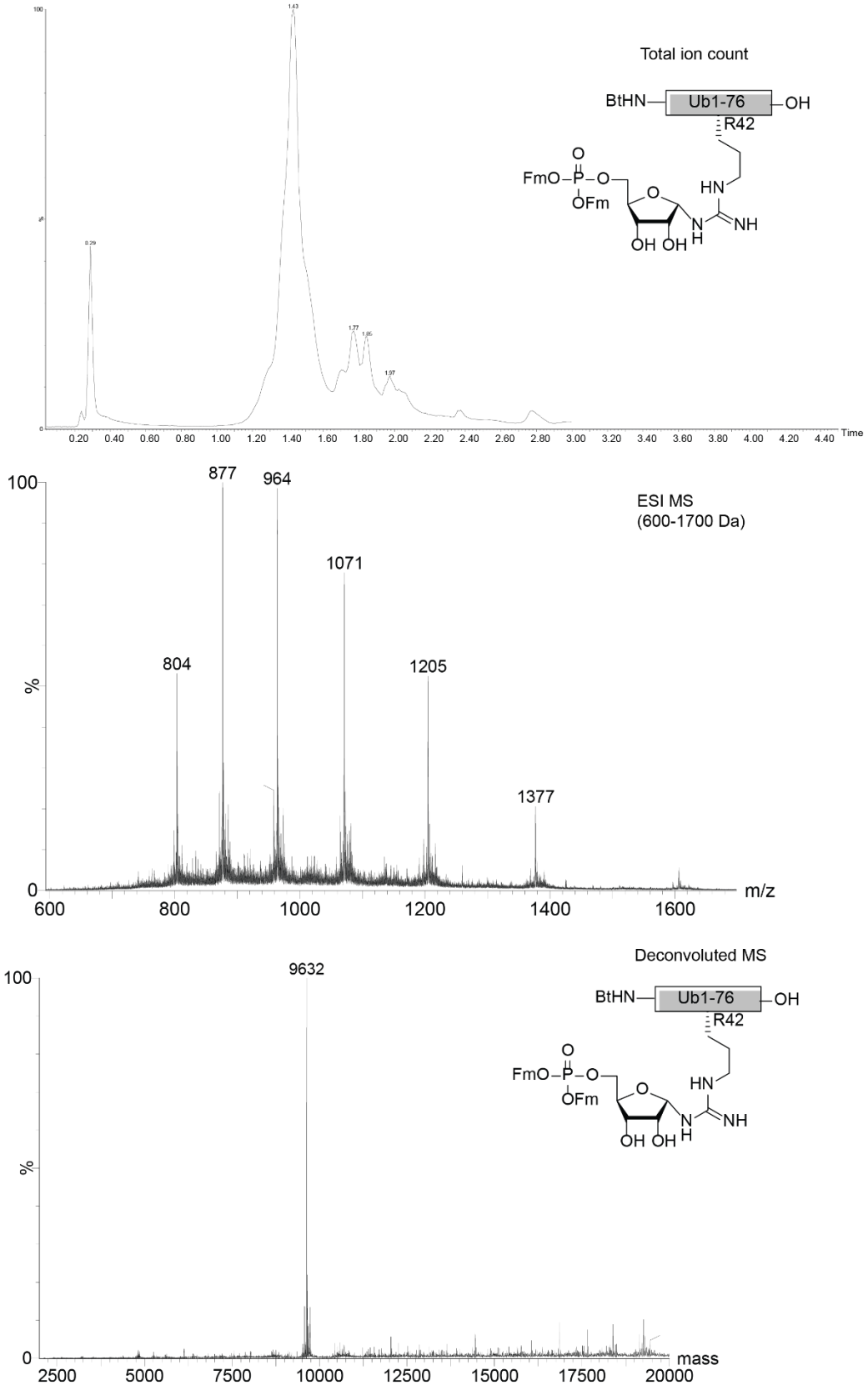


Figure S2. HRMS spectra of the phosphitylation reaction and subsequent oxidation of $Ub_{1\text{-}76}$ using phosphoramidite **8** (lower panel, deconvoluted mass = 9632).

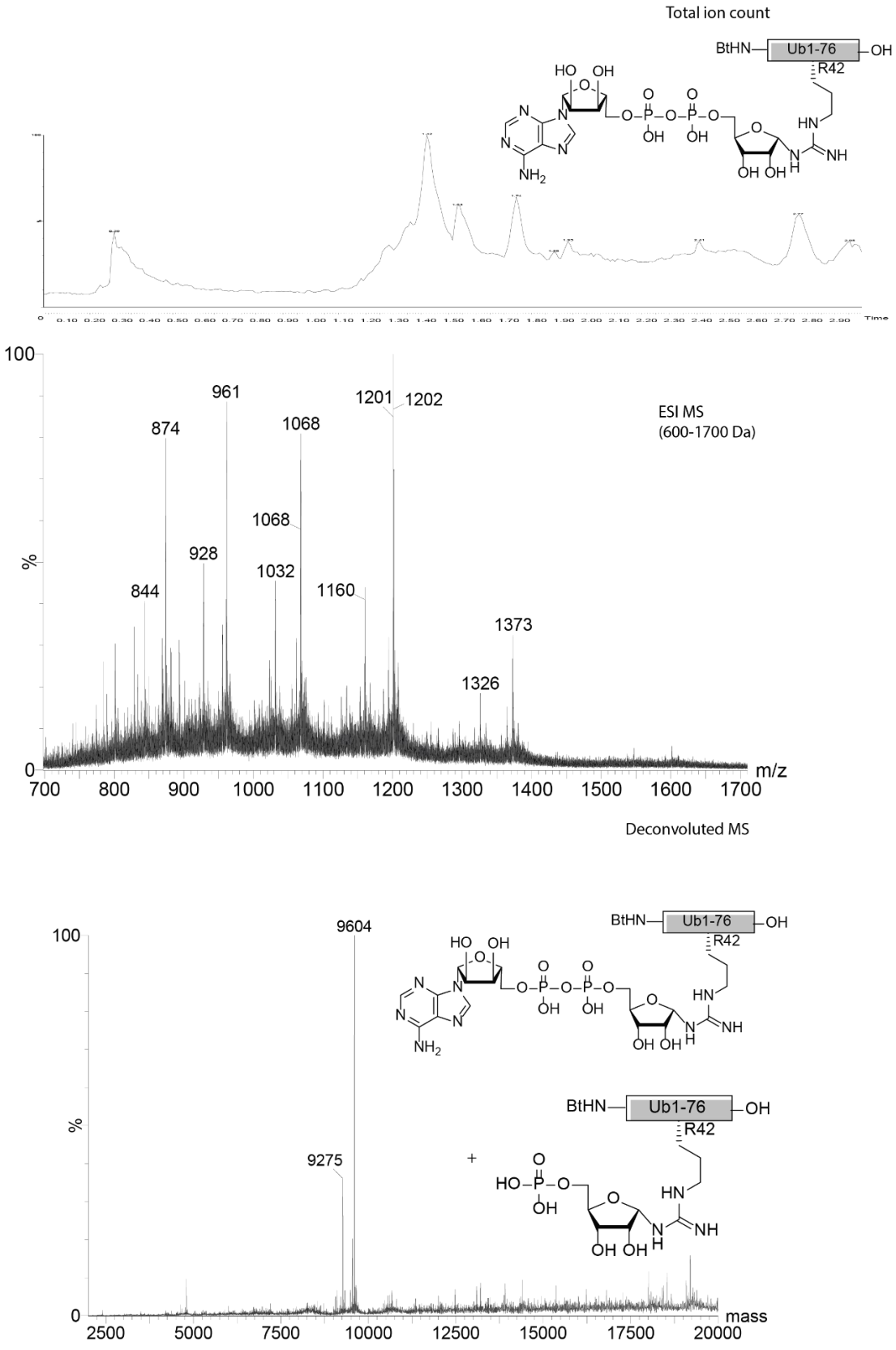


Figure S3. HRMS spectra of the ADPr formation reaction and subsequent oxidation of Ub_{1-76} using nucleoside amidite **12**. The coupling reaction did not go to full conversion leaving uncoupled Ub^{Pr} (deconvoluted mass = 9275) in the mixture. $^{R42}Ub^{ADPr}$ (deconvoluted mass = 9604) was formed.

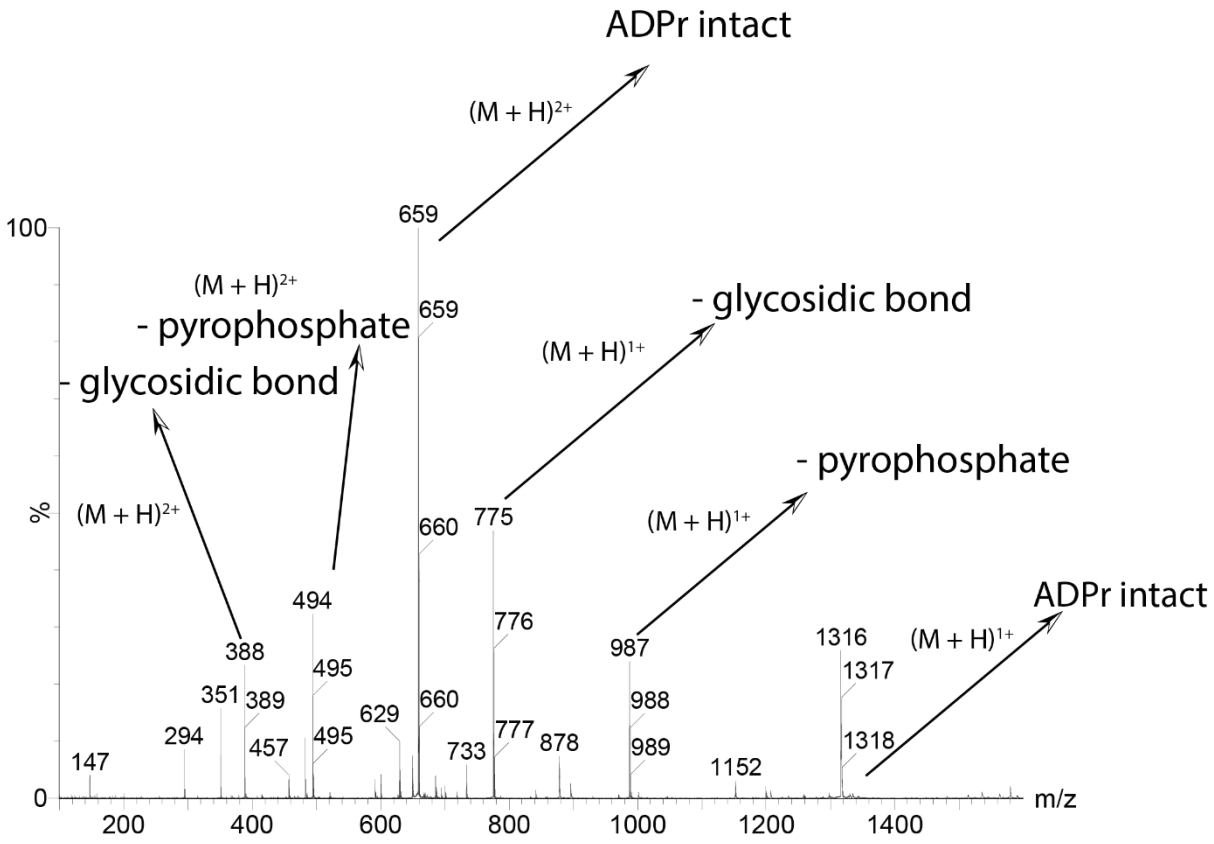


Figure S4. LC-MS of heptamer 14 treated for 90 min with TFA/TIS/H_2O/Phenol (90.5/2/5/2.5).

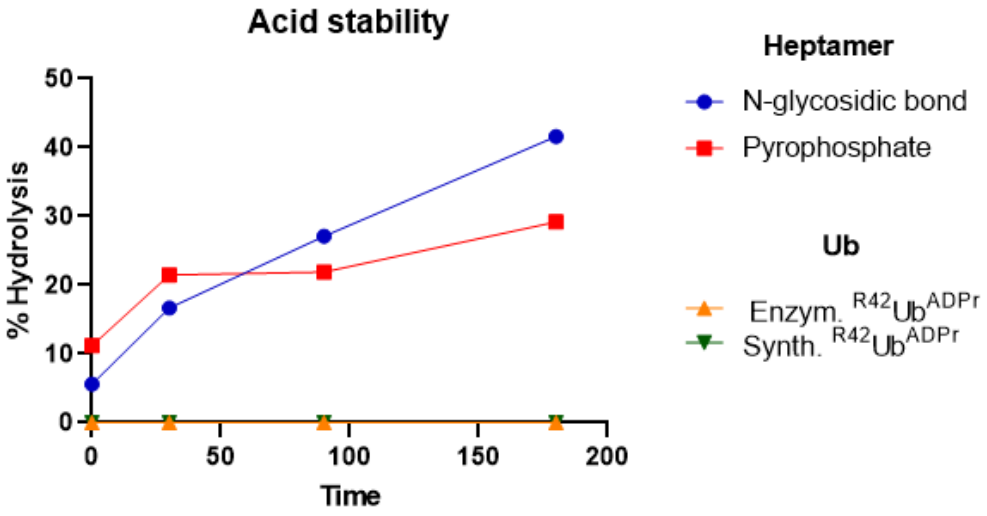


Figure S5. Acid stability of Arg-ADPr compounds. Heptapeptide **14** (1.66 µM), synth. $^{Arg42}Ub^{ADPr}$ **18** (1.66 µM), or enzym. $^{Arg42}Ub^{ADPr}$ (1.66 µM), was stirred in 100 µL TFA/TIS/H_2O/Phenol (90.5/2/5/2.5) and analyzed by LC-MS at the indicated time points. The glycosidic bond cleavage and pyrophosphate hydrolysis were determined as ratio of product versus starting material and plotted.

HRMS spectra of purified $^{R42}Ub^{ADPr}$(18)

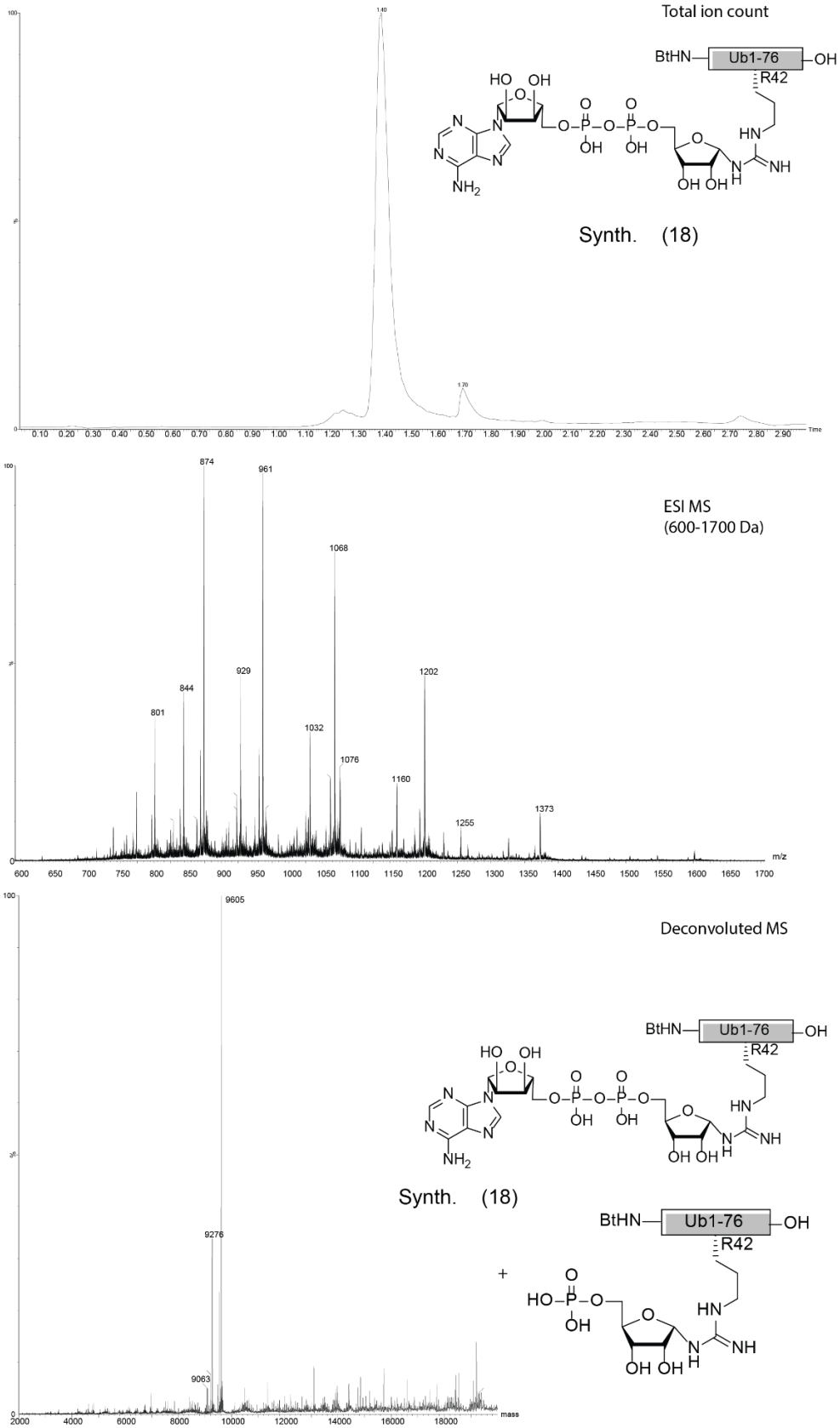


Figure S6. HRMS spectra of $^{R42}Ub^{ADPr}$ after purification.

HRMS spectra of purified $^{R54}Ub^{ADPr}$(19)

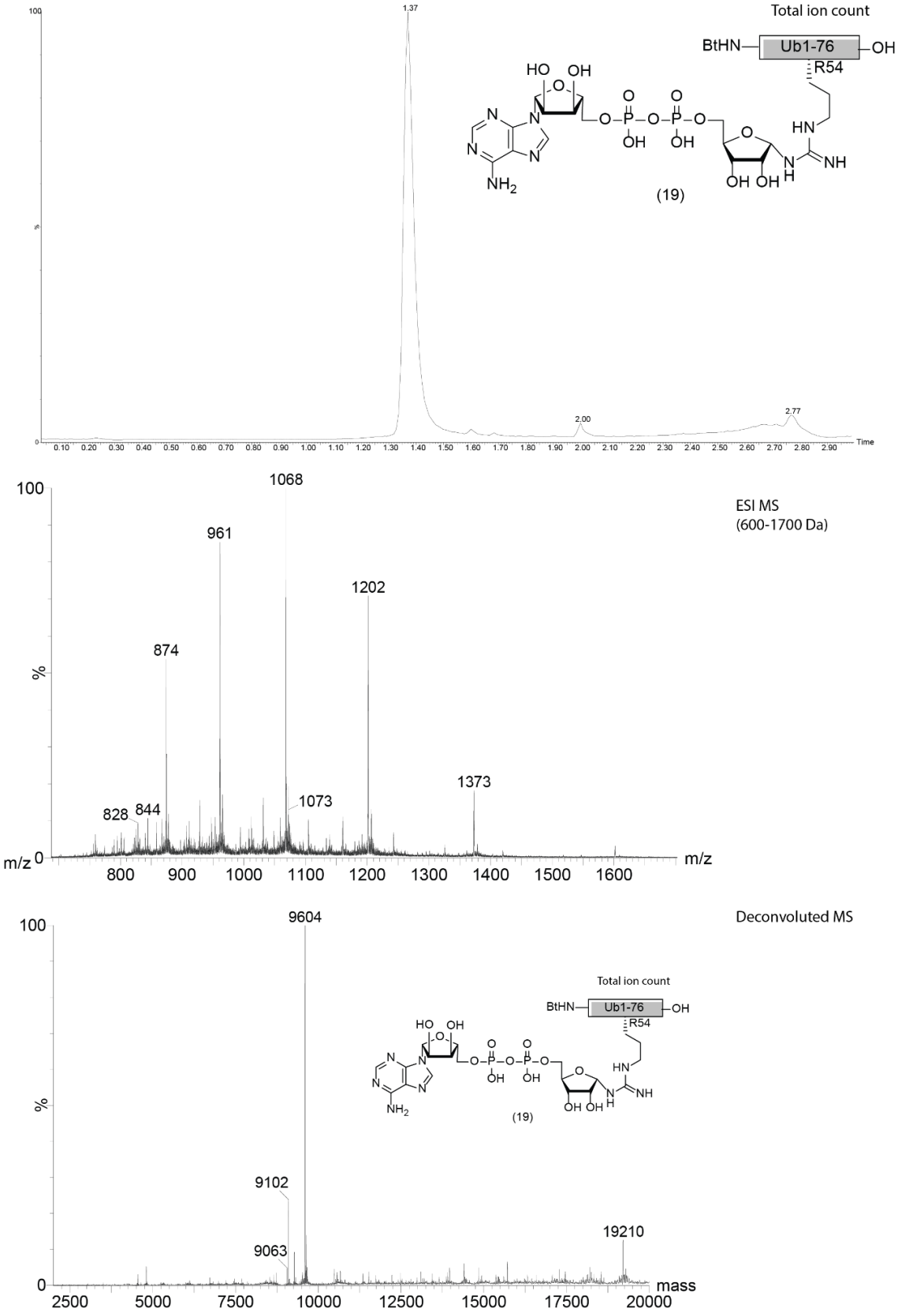


Figure S7. HRMS spectra of $^{R54}Ub^{ADPr}$ after purification.

HRMS spectra of purified $^{R72}Ub^{ADPr}$ (20)

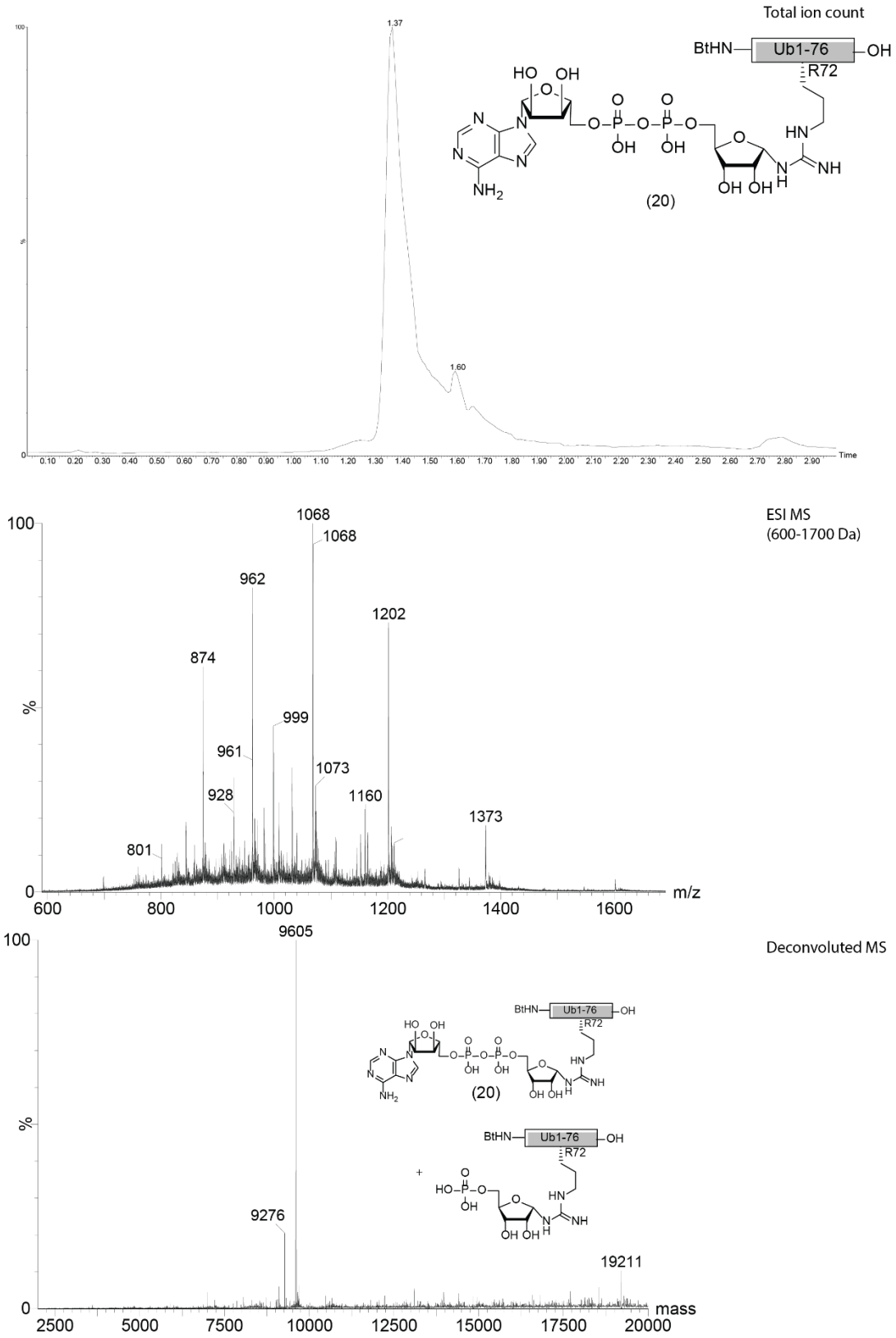


Figure S8. HRMS spectra of $^{R72}Ub^{ADPr}$ after purification.

HRMS spectra of purified $^{R74}Ub^{ADPr}$ (21)

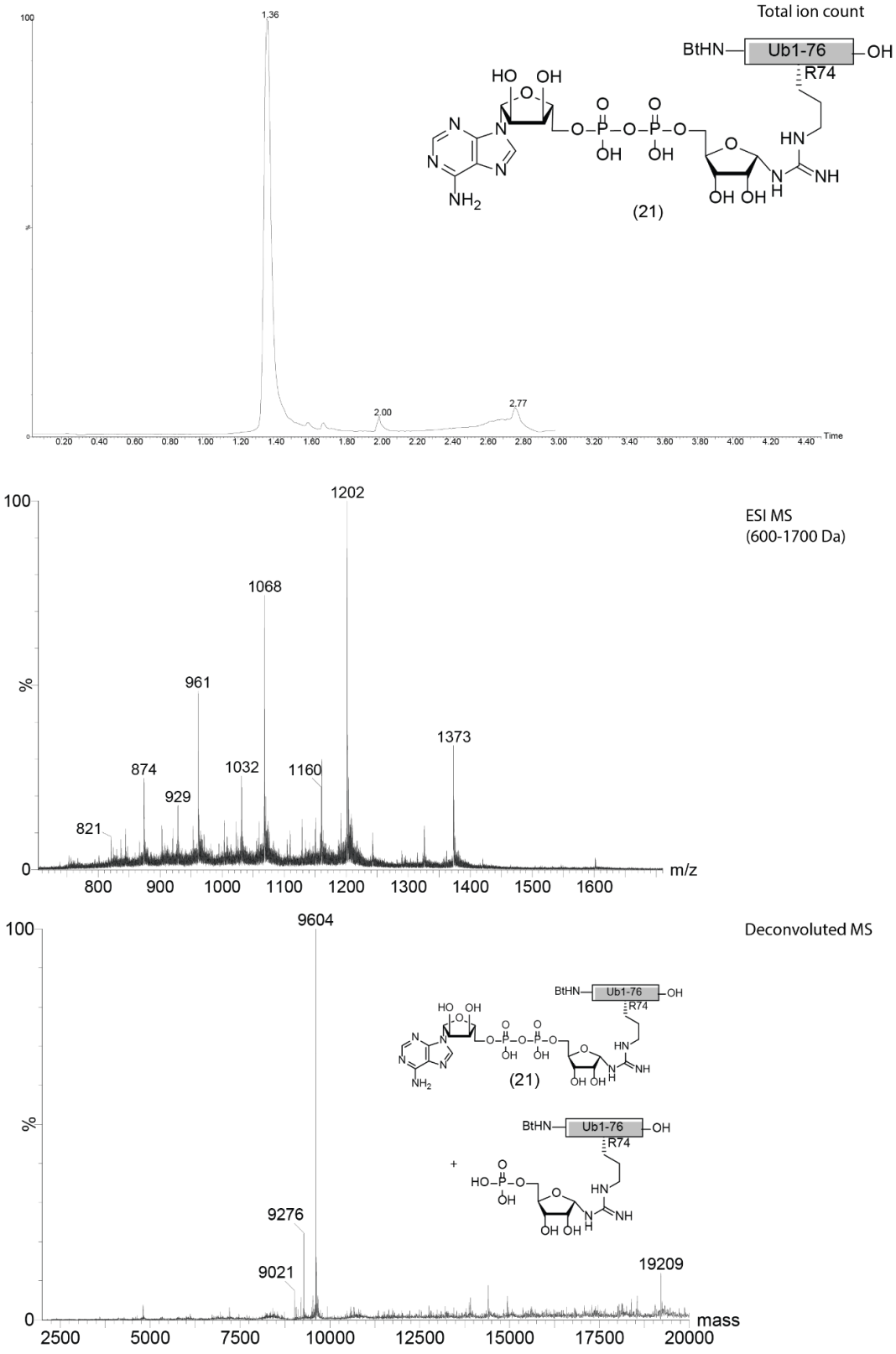


Figure S9. HRMS spectra of $^{R74}Ub^{ADPr}$ after purification.

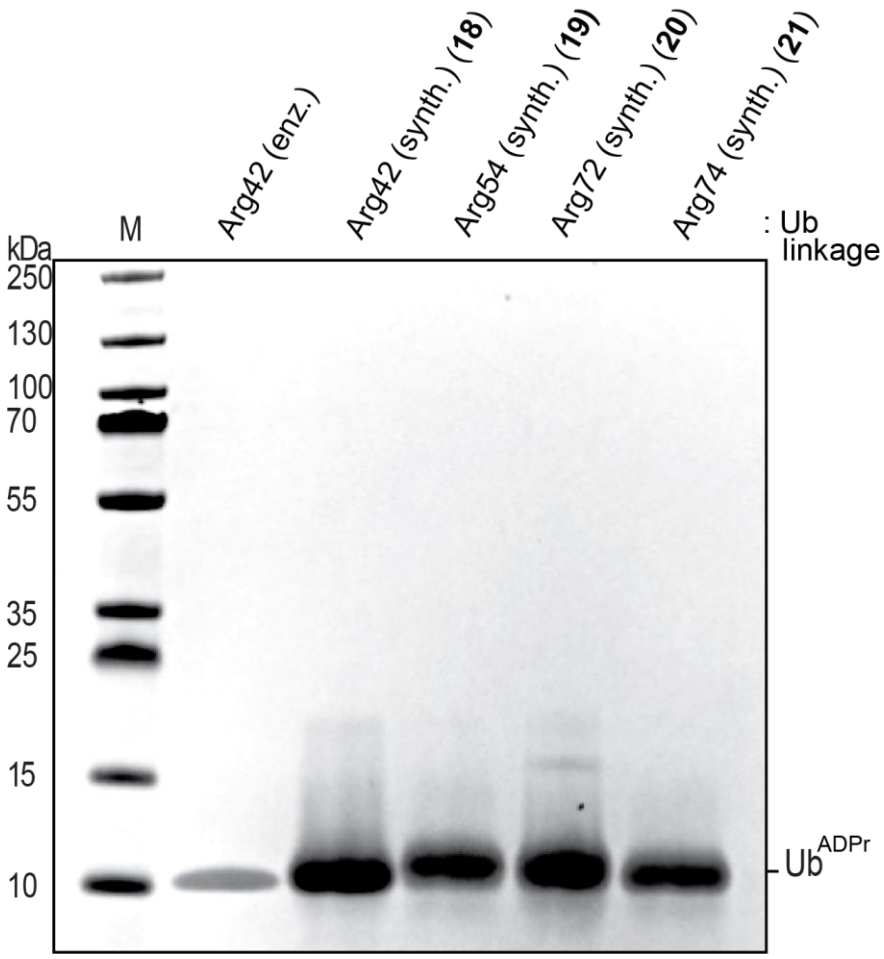


Figure S10. SDS-PAGE analysis of synthetic UbADPr's (**18**-**21**).

HRMS of enzymatically prepared $^{R42}Ub^{ADPr}$

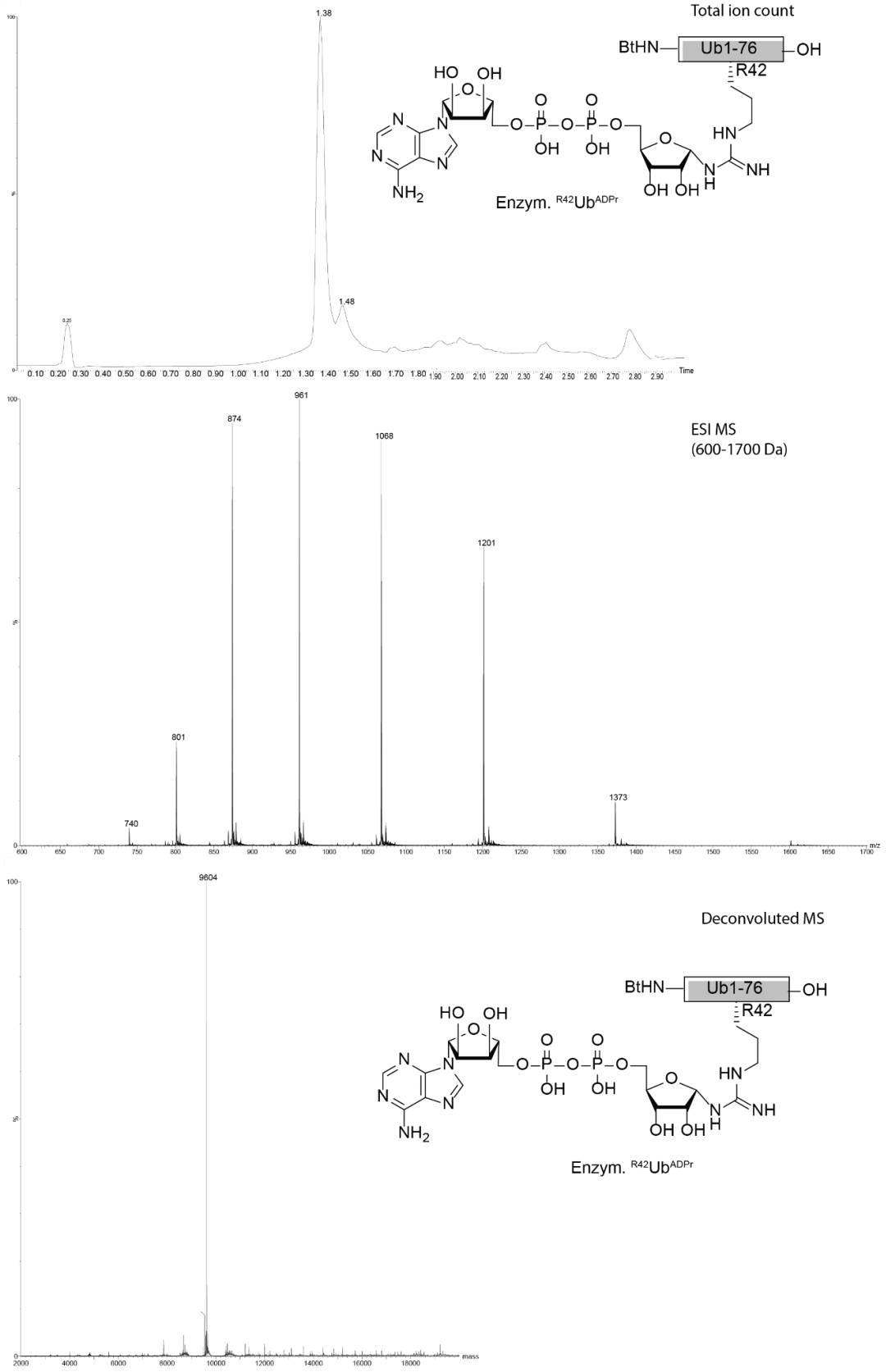


Figure S11. HRMS spectra enzymatically produced $^{R42}Ub^{ADPr}$.

HRMS of the DupA-mediated hydrolysis of enzymatically prepared $^{R42}Ub^{ADPr}$

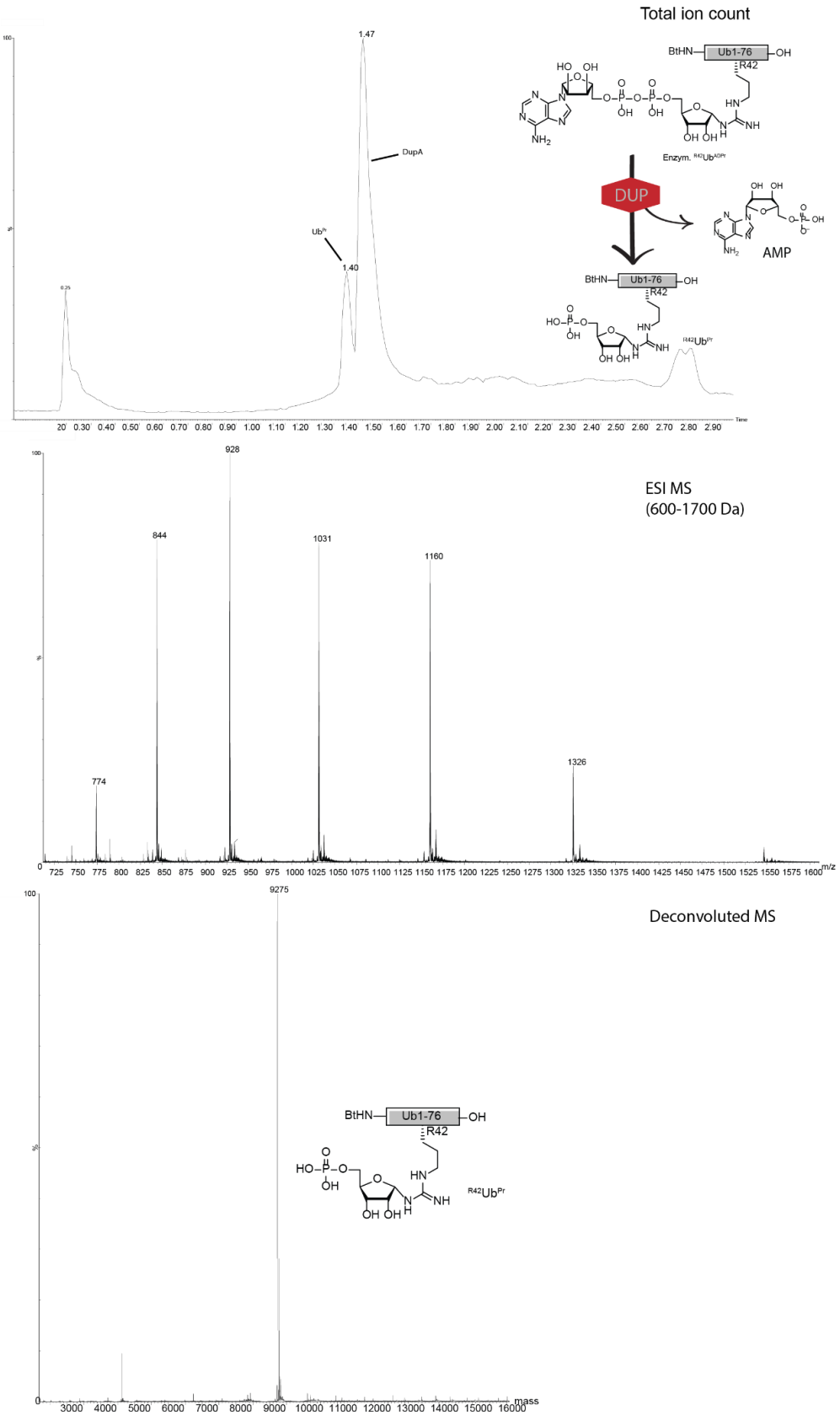


Figure S12. HRMS spectra of the DupA mediated hydrolysis reaction of enzymatically produced $^{R42}Ub^{ADPr}$ to form $^{R42}Ub^{Pr}$.

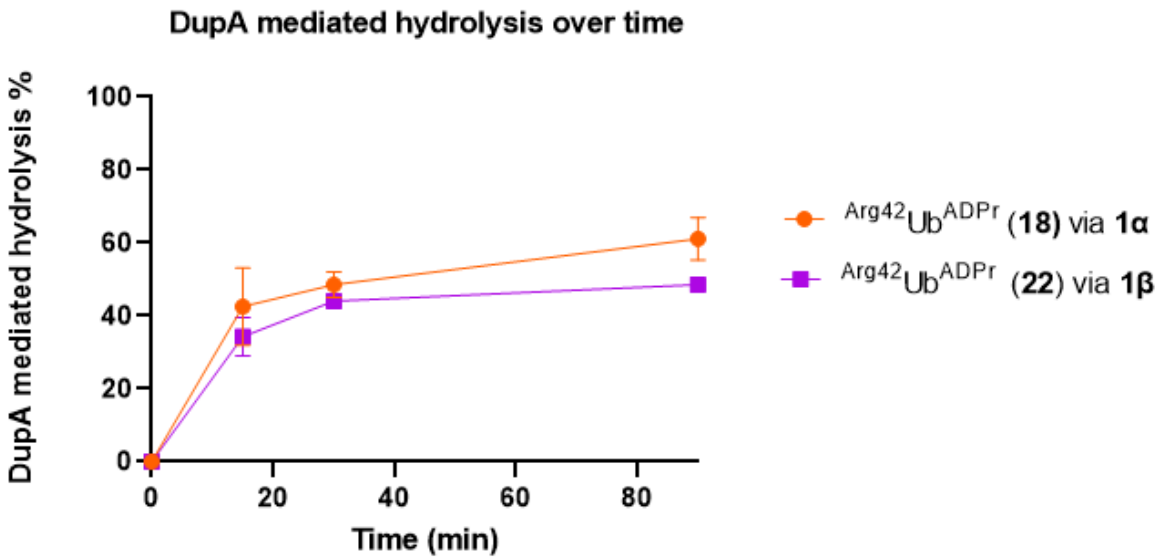


Figure S13. Hydrolysis of $^{Arg42}Ub^{ADPr}$ (**18**) synthesized via **1α** or $^{Arg42}Ub^{ADPr}$ (**22**) via **1β** by DupA followed over a time course of 0 - 90 min. Both graphs are analyzed with HRMS. The measurements in both graphs are normalized for background Ub^{Pr} present as impurity associated with the synthesis.

HRMS of the SdeA-mediated ligation of enzym. $^{R42}Ub^{ADPr}$ and RTN4b (23)

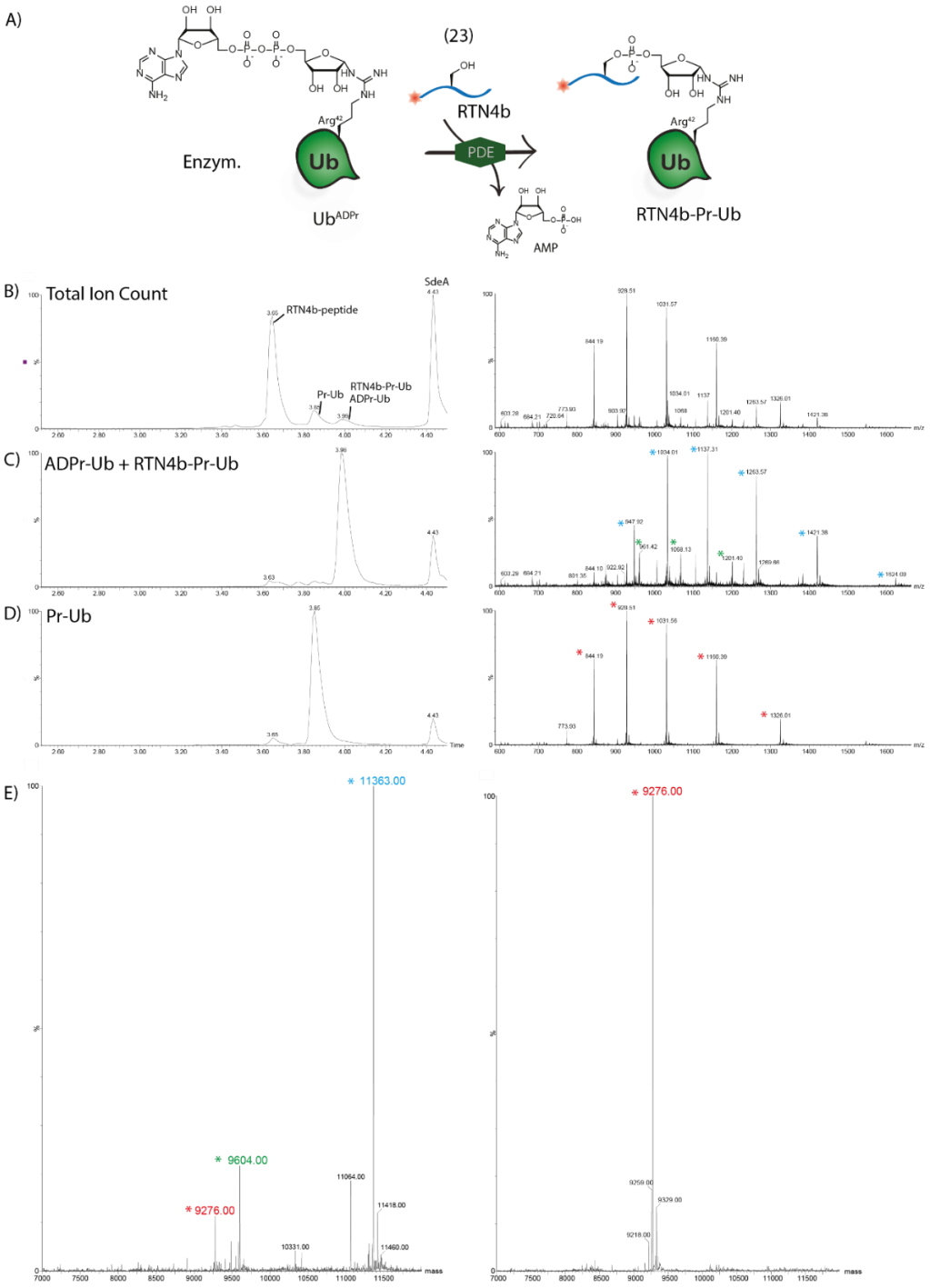


Fig. S14. HRMS spectra of the SdeA mediated ligation reaction of enzymatically produced $^{R42}Ub^{ADPr}$ and RTN4b fragment (**23**). A) The SdeA mediated ligation of enzyme. $^{R42}Ub^{ADPr}$ and fluorogenic RTN4b derived peptide (**23**). Ligation of serine in RTN4b to Ub^{ADPr} forms RTN4b-Pr-Ub as product. B) Total ion count (left) and ESI-MS (right). ESI-MS corresponds to the total region of Pr-Ub, ADPr-Ub and RTN4b-Pr-Ub (retention time: 3.80-4.20). C) Total ion count (left) ADPr-Ub and RTN4b-Pr-Ub and corresponding ESI MS (retention time 3.95-4.20). D) Total ion count (left) Pr-Ub and corresponding ESI MS (retention time 3.80-3.95). E) Deconvoluted mass of C (left) and D (right).

HRMS of the SdeA-mediated ligation of synth. $^{R42}Ub^{ADPr}$ (18) and RTN4b (23)

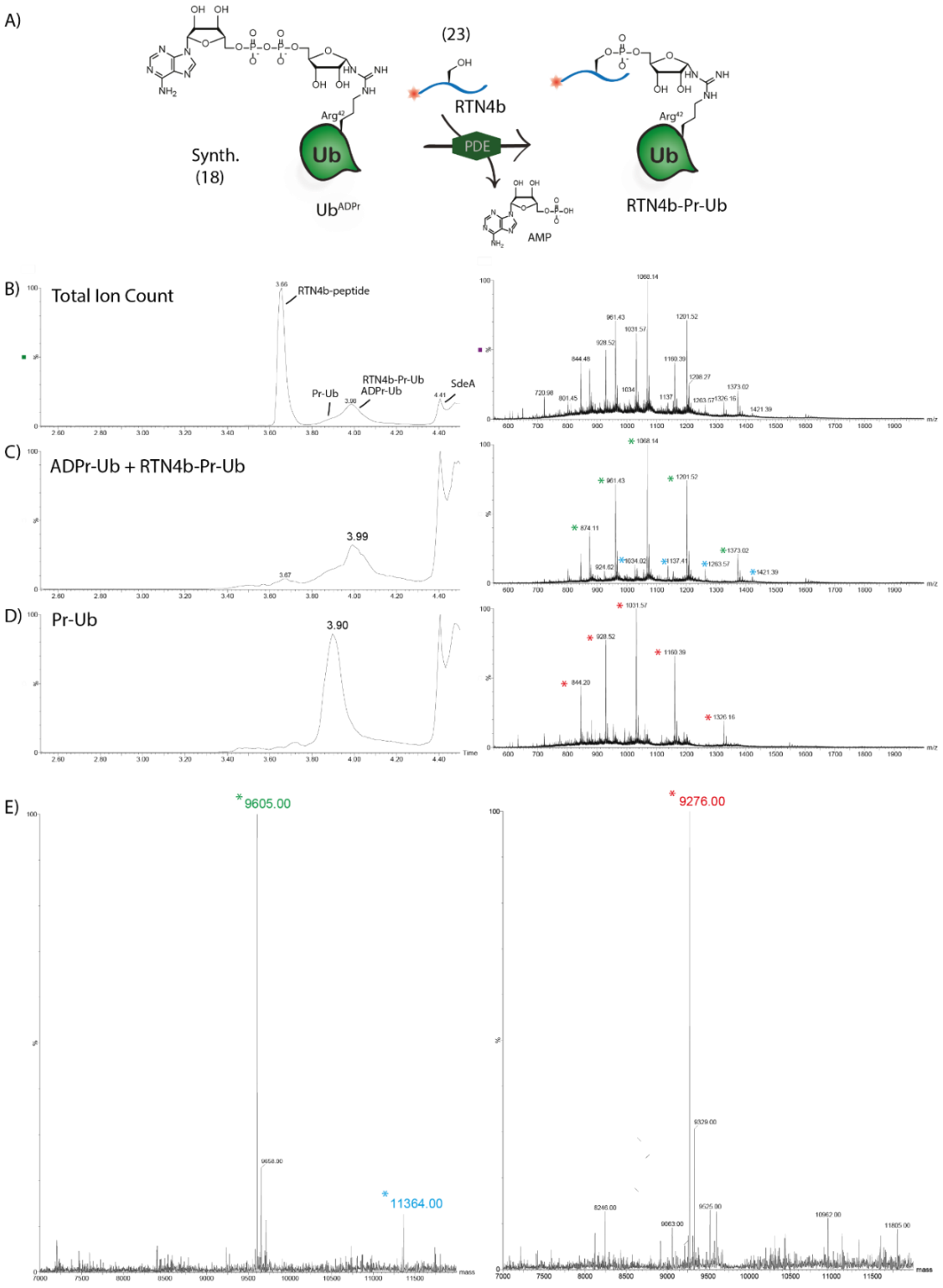


Fig. S15. HRMS spectra of the SdeA mediated ligation reaction of synthesized $^{42}Ub^{ADPr}$ (**18**) and RTN4b fragment (**23**). A) the SdeA mediated ligation of synth. $^{R42}Ub^{ADPr}$ (**18**) and fluorogenic RTN4b derived peptide (**23**). Ligation of serine in RTN4b to Ub^{ADPr} forms RTN4b-Pr-Ub as product. B) Total ion count (left) and ESI-MS (right). ESI-MS corresponds to the total region of Pr-Ub, ADPr-Ub and RTN4b-Pr-Ub (retention time 3.99-4.15). C) Total ion count (left) ADPr-Ub and RTN4b-Pr-Ub and corresponding ESI MS (retention time 3.99-4.15) D) Total ion count (left) Pr-Ub and corresponding ESI MS (retention time 3.80-3.95). E) Deconvoluted mass of C (left) and D (right).

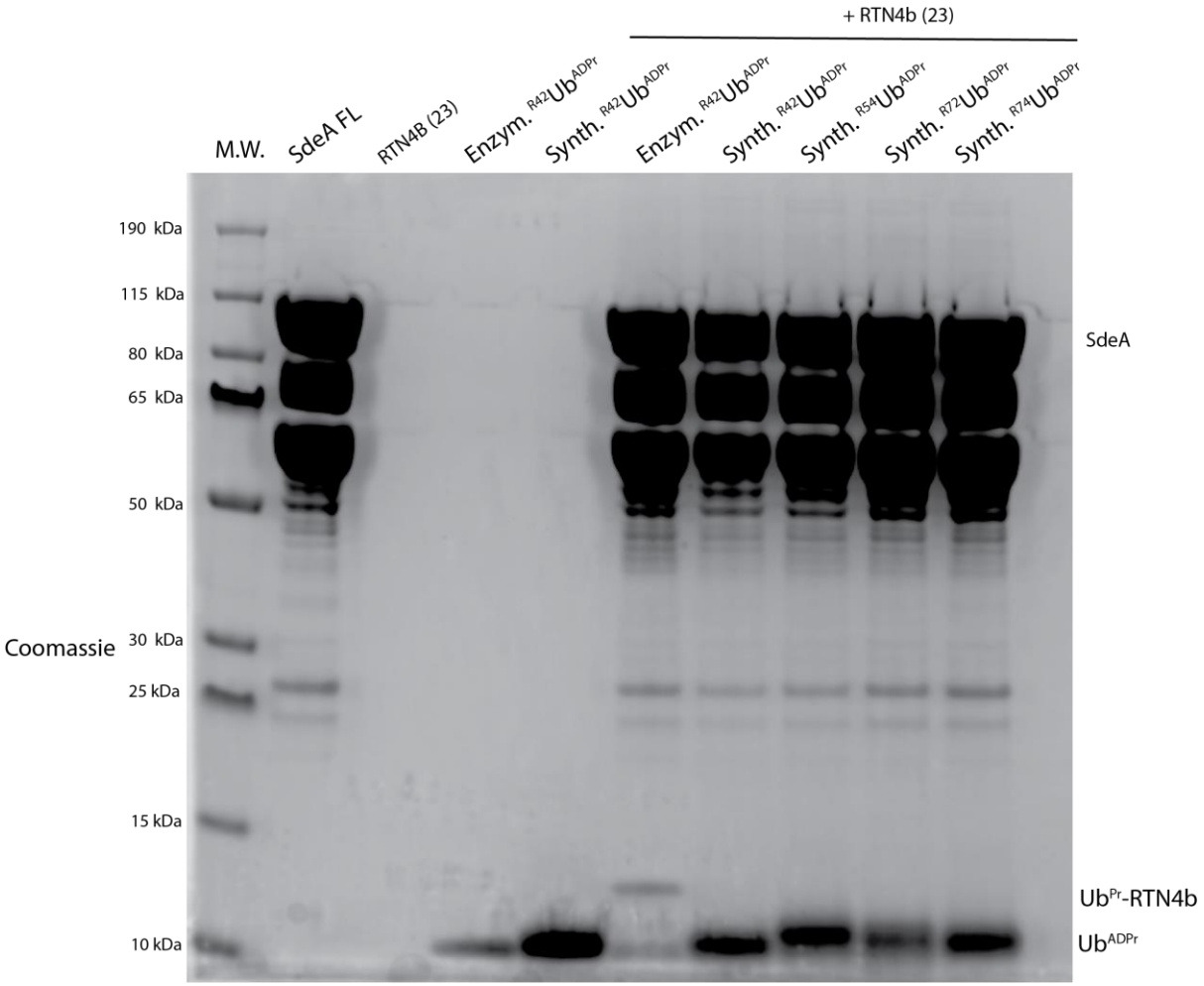


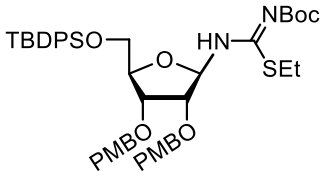
Fig. S16. SdeA-mediated ligation of enzym. $^{R42}Ub^{ADPr}$ or synth. $^{Rx}Ub^{ADPr}$ (**18**-**21**) and RTN4B peptide fragment (**23**).

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 ^oC. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Nucleodur C18 Gravity 3 µm 50 x 4.60 mm column (detection at 200-600 nm) coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI or coupled to a Thermo LCQ Fleet Ion mass spectrometer with ESI. The method used was 10→90% 13.5 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: 10% to 90% MeCN; 8.5→ 11 min: 90% MeCN; 11→13.5 min: 10% MeCN) or 0→50% 13.5 min. NMR spectra were recorded on a Bruker AV-400, AV-500 or AV-600 NMR. Chemical shifts (δ) are given in ppm relative to tetramethyl silane. Coupling constants (*J*) are given in Hz. All given ^{13}C-APT spectra are proton decoupled. In case of synthetic Ub-ADPr, HPLC purification was performed on a Shimadzu semi-preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 µm OBD (10 x 150 mm) column at a flowrate of 6.5 mL/min. using 2 mobile phases: A: MQ + 0.05% FA, B: MeCN + 0.05 % FA. Gradient: 10 -> 70% B. High resolution mass spectra were recorded on a Waters XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.0 kV, desolvation gas flow 900 L/hr, temperature 250 °C) with resolution R = 22000 (mass range m/z = 50-2000) and 200 pg/uL Leu-Enk (m/z = 556.2771) as a "lock mass".

Organic synthesis

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



1-(*tert*-butoxycarbonyl)-3-(5-*O*-((*tert*-butyl) diphenylsilyl)-2,3-di-*O*-(4-methoxybenzyl)-β-D-ribofuranos-1-yl) isothiocyanate **4β** (beta anomer only, 2.36 g, 3.52 mmol) was dissolved in THF (18 mL, 0,2M). The solution was purged with NH_3 for 1 hour after which the reaction was purged with N_2 for 1 minute. The crude thiourea was concentrated *in vacuo* till a yellow foam. The crude product was dissolved in DCM (35 mL, 0.1M). DMAP (46.6 mg, 0.38 mmol, 0.11 eq) and Boc_2O (560 µL, 2.43 mmol, 1.1 eq) were added and the reaction was stirred for 2 hours. The reaction was diluted with DCM (100 mL) and washed with brine (100 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was dissolved in MeCN (61 mL, 0.1M) and K_2CO_3 (8.45, 69.1 mmol, 11.3 eq) and EtI (1.75 mL, 21.88 mmol, 3.6 eq) were added under vigorous stirring. The suspension was stirred overnight and diluted in EtOAc (100 mL). The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (0 -> 45% EtOAC in heptane) obtained

the title compound as a colorless oil (353 mg, 0.43 mmol, 12.3%) Rf**:** 0.45 in 30% Et_2O in pentane. ^1H NMR (300 MHz, $CDCl_3$): δ 7.67-7.59 (m, 4H, TBDPS arom.), 7.46-7.31 (m, 6H, TBDPS arom.), 7.29-7.18 (m, 4H, PMB arom.), 6.88-6.80 (m, 4H, PMB arom.), 5.58 (t, J = 6.4 Hz, 1H, H-1), 4.59 (s, 2H, CH_{2a} PMB), 4.54-4.41 (m, 2H, CH_{2b} PMB), 4.13 (q, J = 3.6 Hz, 1H, H-4), 4.02 (t, J = 6.0 Hz, 1H, H-3), 3.89 (t, J = 6.0 Hz, 1H, H-2), 3.80 (s, 3H, CH_3 PMB), 3.79 (s, 3H, CH_3 PMB), 3.75-3.58 (m, 2H, H-5), 3.04 (d, J = 7.4, 2H, CH_2Et), 1.48 (s, 9H, CH_3 Boc), 1.26 (t, J = 7.5 Hz, 3H, CH_3 Et), 1.01 (s, 9H, CH_3 TBDPS). ^{13}C NMR (75,5 MHz, $CDCl_3$): δ 161.7 (C=O Boc), 159.5, 159.5 (Cq PMB), 135.8, 135.7 (CH arom. TBDPS), 133.4, 133.0 (Cq TBDPS), 129.9, 129.9, 129.7, 129.7 (CH arom. TBDPS/PMB), 127.9, 127.9, 114.0, 114.0 (CH arom. PMB), 86.3 (C-4), 82.8 (C-1), 80.7(C-3), 79.6 (Cq *t*Bu Boc), 76.0 (C-2), 72.1, 71.9 (CH_2 PMB), 63.7 (C-3), 55.4, 55.4 (CH_3 PMB), 28.30 (CH_3 Boc), 27.1 (CH_3 TBDPS), 25.4 (CH_2 Et), 19.4 (Cq *t*Bu TBDPS), 13.9 (CH_3 Et). HRMS: $[C_{45}H_{58}N_2O_8SSi + H]^+$ found: 815.3863, calculated: 815.3756.

Synthesis of peptides 14-17 (general procedure synthesis Arg-ADPr)

Peptide synthesis (Protocol A, peptides **14***,* **15**, **17**)

The intermediate peptides (generalized as **5**, Scheme 1) were synthesized using standard, Fmoc-based solid phase peptide synthesis utilizing (pre-loaded) Tentagel® S AC purchased from Rapp Polymer GmbH on a Syro II MultiSyntech Automated or a CEM Liberty Blue Automated Microwave Peptide Synthesizer Peptide synthesizer. Coupling cycles were as followed: Fmoc deprotection: 2x2 min, 1x5 min treatment with 20% piperidine in DMF. Coupling: treatment of 6 eq. amino acid, 6 eq. HCTU (0.25M in DMF) and 12 eq. DIPEA (1 M in DMF) for 30 minutes. Capping: 2x2 min treatment of the resin with a 10% Ac_2O solution in DMF and catalytic DIPEA. Washing between the steps was done with DMF. For the prospected Arg-ADPr site, commercially available Fmoc-Orn(OAll)-OH was used in the coupling cycle.

Peptide synthesis (Protocol B, peptide **16**)

The intermediate peptides (generalized as **5**, Scheme 1) were synthesized on a CEM Liberty Blue Automated Microwave Peptide Synthesizer. The resin was first swollen for 5 minutes in DMF prior to amino acid coupling. Activation was achieved using DIC/Oxyma. Standard coupling was achieved using 5 eq. amino acid as a 0.2 M amino acid/DMF solution, 5 eq. DIC as a 0.5 M of DIC/DMF solution and 5 eq. Oxyma as a 1M Oxyma/DMF solution which was buffered by DIPEA (0.1M) at 90˚C for 2 minutes. Standard Fmoc deprotection was achieved by 20% $^{v}/_{v}$ piperidine/DMF at 90˚C for 1 minute (2 cycles). Washing between the steps was done with DMF. For the prospected Arg-ADPr site, commercially available Fmoc-Orn(OAll)-OH was used in the coupling cycle. Synthesis quality could be monitored by UV absorption of dibenzofulvene released during Fmoc deprotection.

Deprotection/building block coupling for Arg-ADPr peptides

The Alloc protecting group was removed by treating the resin with a freshly prepared solution of 10 mg $Pd(PPh_3)_4$ and 23 mg 1,3-dimethylbarbituric acid in 1 mL DCM (purged with nitrogen prior to use) for 15 minutes. This procedure was then repeated twice to ensure full deprotection. The resin was washed

extensively with DCM and DMF. Coupling of the ribosyl building block was performed as follows: Ribosyl building block **1α** (or **1β**) (3 eq.) was dissolved in DMF (0.1 M) and added to the resin. TEA (30 eq.) followed by $AgNO_3$ (3 eq.) were added to the reaction and the syringe was wrapped in aluminium foil to protect it from light and shaken overnight. The resin was then extensively washed with DCM and DMF.

Deprotection and phosphorylation

The resin was washed with THF and treated with TBAF (1 M) in THF for 30 minutes. The resin was thoroughly washed with DCM and DMF before the treatment was repeated once, furnishing the desilylated intermediate. The resin was then extensively washed with dry MeCN and flushed with nitrogen to remove traces of water before the resin was subjected to a solution of $(FmO)_2PN(iPr)_2$ **8** (2.5 eq., (0.13 M in MeCN)) and DCI (5.0 eq. (0.25M in MeCN) was added. The resin was shaken for 30 minutes after which the resin was washed with MeCN. The resin was then treated with a 0.5 M CSO solution in MeCN for 30 minutes and treated with a 10% DBU solution in DMF (2x 15 minutes) to furnish the crude, immobilized and partially deprotected phosphoribosyl peptide.

Pyrophosphate synthesis

The resin was extensively washed with MeCN and flushed with nitrogen to remove traces of water. The resin was then treated with a solution of adenosine amidite **12** (3 eq., 0.13 M in MeCN) and DCI (6 eq., 0.25 M in MeCN) for 30 minutes. The resin was thoroughly washed with MeCN before a CSO solution (0.5 M in MeCN) was added to the resin and shaken for 30 minutes.

Final deprotection and cleavage

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

Synthesis of full-length $^{R42}Ub^{ADPr}$ (18) on wang resin

Synthesis was performed using the above mentioned protocol (for the synthesis of peptides **14**-**17**) with exception of the following conditions:

- Alloc deprotection was performed using ($Pd(PPH_3)_4$ (0.2 eq) and PhSiH (20 eq).
- Equivalents were varied in the crucial steps of synthesis (ribosylation (20 eq), phosphitylation (11.2 eq) and ADPr formation (11.2 eq)).
- Instead of CSO a *t*BuOOH solution (0.55 M in MeCN) was used for oxidation. A *t*BuOOH solution of 5.5M in nonane was diluted ten times in MeCN to obtain the solution.
- TFA/TIS/H_2O/Phenol (90.5/2/5/2.5) was used for final resin cleavage/deprotection of ubiquitin.

Solid Phase Peptide Synthesis, biotin-PEG_2 coupling

SPPS was performed according to literature procedure[41] on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 20 µmol scale, using fourfold excess of amino acids relative to pre-loaded preloaded Fmoc-Gly wang resin (0.2 mmol/g, Rapp Polymere GmbH). On position-42 in the peptide sequence arginine was replaced by Fmoc-Orn(Alloc)-OH. After SPPS, 5 µmol Ub_{1-76} (R42 → Alloc ornithine) on resin was treated with PyBOP (3.1 mg, 30 µmol, 5 eq) and Bt-PEG_2-COOH (16.1 mg, 30 µmol, 5 eq) in DMF (2 mL). After 5 min of shaking, DIPEA (16 µL, 90 µmol, 15 eq) was added. The reaction mixture was shaken overnight, after which a test cleavage confirmed full conversion of the conjugation. The resin was then washed with DMF and DCM before resuspension in DCM.

ADPr synthesis (**R42**)

Deprotection conditions (desilylations, Fm and cyanoethyl deprotections) were performed identical to the synthesis of peptides **14**-**17** described above, however the amounts of equivalents used in the crucial steps of the synthesis were varied (ribosylation, phosphitylation and ADPr formation) as well as the oxidations and final resin release/deprotection. The synthesis was performed on 2.5 µmol wang resin containing Ub_{1-76} (R42 → Alloc ornithine. Alloc deprotection was performed by treating the resin with a solution of ($Pd(PPH_3)_4$ (1.4 mg, 1.2 µmol, 0.2 eq) and PhSiH (15 µL, 120 µmol, 20 eq) in anhydrous DCM. This was repeated once more and a test cleavage confirmed complete deprotection of the Alloc-group.

The ribosylation was performed using **1α** (40.7 mg, 50 µmol, 20 eq) and $AgNO_3$ (8.5 mg, 50 µmol, 20 eq). After desilylation, the phosphitylation was performed using $(FmO)_2PN(iPr)_2$ **8** (11.2 eq, 0.13 M in MeCN) and DCI (22.4 eq, 0.25 M in MeCN) and full conversion was confirmed by a test cleavage. Oxidation was performed using a 0.55 M solution of *t*BuOOH in MeCN for 30 minutes. In the final ADPr formation step TBS-protected adenosine amidite **12** (11.2 eq, 0.13 M in MeCN) was used and DCI (22.4 eq, 0.25 M in MeCN). The resin was thoroughly washed with MeCN before a *t*BuOOH solution (0.55 M in MeCN) was added to the resin and shaken for 30 minutes.

Final deprotection, cleavage and purification

After deprotection of the ADPr moiety (cyanoethyl with DBU and TBS with TBAF) identically done as for peptides **14** and **15** the resin was treated with TFA/TIS/H_2O/Phenol (90.5/2/5/2.5) for 1.5 hours before filtrated in an ice-cold solution of Et_2O:pentane (1:1). The precipitate formed was centrifuged (5 min, 3500 rpm) and the supernatant decanted. The pellet was subsequently dried with N_2, taken up in warm DMSO and diluted in warm water before purification by RP-HPLC. Pure fractions were pooled and lyophilized affording $^{R42}Ub^{ADPr}$ **18** (421 µg, 0.044 µmol, 1.75% total yield as a 75.8:24.2 mixture of (Ub^{ADPr}:Ub^{Pr}) as a white powder. LC-MS: Rt = 1.47 min. Deconvoluted mass = 9604. HRMS: $[C_{416}H_{688}N_{114}O_{139}P_2S + 7H]^{7+}$ found: 1373.3523, calculated: 1373.1071. $[C_{416}H_{688}N_{114}O_{139}P_2S + 8H]^{8+}$ found: 1201.8162, calculated: 1201.5938. $[C_{416}H_{688}N_{114}O_{139}P_2S + 9H]^{9+}$ found: 1068.3911, calculated: 1068.1944. $[C_{416}H_{688}N_{114}O_{139}P_2S + 10H]^{10+}$ found:961.6514, calculated: 961.4748. $[C_{416}H_{688}N_{114}O_{139}P_2S + 11H]^{11+}$ found: 874.3194, calculated: 874.1591.

Synthesis of full-length $^{R54}Ub^{ADPr}$ (19) on wang resin

On position 54 in the peptide sequence arginine was replaced by Fmoc-Orn(Alloc)-OH and the procedure described for R42 was followed.

ADPr synthesis (**R54**)

Synthetic procedure was identical to the synthesis described for **$^{R42}Ub^{ADPr}$ 18** with exception of the equivalents used in the following conditions: ribosylation, phosphitylation and ADPr formation. The synthesis was performed on 5 µmol wang resin. The ribosylation was performed using **1α** (61 mg, 75 µmol, 15 eq) and $AgNO_3$(12.7 mg, 75 µmol, 15 eq). Performing the phosphitylation amidite **8** $(FmO)_2PN(iPr)_2$ (15 eq., 0.13 M in MeCN) and DCI (30 eq, 0.25 M in MeCN) were used. In the final ADPr formation step TBS-protected nucleoside amidate **12** (22 eq., 0.13 M in MeCN) and DCI (44 eq., 0.25 M in MeCN) were used. After resin cleavage and precipitation the crude was purified by RP-HPLC. Pure fractions were pooled and lyophilized affording $^{R54}Ub^{ADPr}$ **19** (865 µg, 0.090 µmol, 1.75% total yield as a 85.3:14.7 mixture of (Ub^{ADPr}:Ub^{Pr}) as a white powder. LC-MS: Rt = 1.47 min. Deconvoluted mass = 9604. HRMS: $[C_{416}H_{688}N_{114}O_{139}P_2S + 7H]^{7+}$ found: 1373.0187, calculated: 1373.1071. $[C_{416}H_{688}N_{114}O_{139}P_2S + 8H]^{8+}$ found: 1201.5184, calculated: 1201.5938. $[C_{416}H_{688}N_{114}O_{139}P_2S + 9H]^{9+}$ found: 1068.1235, calculated: 1068.1944 $[C_{416}H_{688}N_{114}O_{139}P_2S + 10H]^{10+}$ found: 961.4104, calculated: 961.4748. $[C_{416}H_{688}N_{114}O_{139}P_2S + 11H]^{11+}$ found: 874.1077, calculated: 874.1591.

Synthesis of full-length $^{R72}Ub^{ADPr}$ (20) on wang resin

On position 72 in the peptide sequence arginine was replaced by Fmoc-Orn(Alloc)-OH.

ADPr synthesis (**R72**)

Synthetic procedure was identical to the synthesis described for ^{R42}**Ub**^{ADPr} **18** with exception of the equivalents used in the following conditions: ribosylation, phosphitylation and ADPr formation. The synthesis was performed on 5 µmol wang resin. The ribosylation was performed using **1α** (81.4 mg, 0.10 mmol, 20 eq) and $AgNO_3$(17.0 mg, 0.10 mmol, 20 eq). During the phosphitylation using amidate **8** $(FmO)_2PN(iPr)_2$ (25 eq, 0.13M in MeCN) and DCI (50 eq, 0.25M in MeCN) a product ratio of (55:45) between mono-phosphorylation $(M + H)^{1+}$ = 9632) and di-phosphorylation $(M + H)^{1+}$ = 10069) was observed. The synthesis was proceeded and in the final ADPr formation step using TBS-protected nucleoside amidate **12** (30 eq, 0.13M in MeCN) and DCI (30 eq, 0.25M in MeCN) we observed the mono- and di-ADPribosylated products. After ADPr-protective group deprotection and additional resin cleavage the mono- and di-ADPribosylated products could be separated by HPLC isolating $^{R72}Ub^{ADPr}$ 20 (650 µg, 0.058 µmol, 1.2% total yield as a 70.3:29.7 mixture of (Ub^{ADPr}:Ub^{Pr}) as a white powder. LC-MS**:** Rt = 1.47 min. Deconvoluted mass = 9605. HRMS: $[C_{416}H_{688}N_{114}O_{139}P_2S + 7H]^{7+}$ found: 1373.0209, calculated: 1373.1071. $[C_{416}H_{688}N_{114}O_{139}P_2S + 8H]^{8+}$ found: 1201.5221, calculated: 1201.5938. $[C_{416}H_{688}N_{114}O_{139}P_2S + 9H]^{9+}$ found: 1068.1351, calculated: 1068.1944. $[C_{416}H_{688}N_{114}O_{139}P_2S + 10H]^{10+}$ found: 961.4160, calculated: 961.4748. $[C_{416}H_{688}N_{114}O_{139}P_2S + 11H]^{11+}$ found: 874.1078, calculated: 874.1591.

Synthesis of full-length ^{R74}Ub^{ADPr} (21) on wang resin

On position 74 in the peptide sequence arginine was replaced by Fmoc-Orn(Alloc)-OH.

*ADPr synthesis (***R74**)

Synthetic procedure was identical to the synthesis described for ^{R42}**Ub**^{ADPr} **18** with exception of the equivalents used in the following conditions: ribosylation, phosphitylation and ADPr formation. The synthesis was performed on 5 µmol wang resin. The ribosylation was performed using **1α** (61 mg, 75 µmol, 15 eq) and $AgNO_3$(12.7 mg, 75 µmol, 15 eq). In the phosphitylation reaction amidate **8** $(FmO)_2PN(iPr)_2$ (15 eq, 0.13M in MeCN) and DCI (30 eq, 0.25M in MeCN) were used. In the final ADPr formation step TBS-protected nucleoside amidate **12** (30 eq, 0.13M in MeCN) and DCI (30 eq, 0.25M in MeCN) were used. After resin cleavage and precipitation, the crude was purified by RP-HPLC. Pure fractions were pooled and lyophilized affording $^{R74}Ub^{ADPr}$ **21** (820 µg, 0.085 µmol, 1.7% total yield as a 75.7:24.3 mixture of (Ub^{ADPr}:Ub^{Pr}) as a white powder. LC-MS: Rt = 1.47 min. Deconvoluted mass = 9604. HRMS: $[C_{416}H_{688}N_{114}O_{139}P_2S + 7H]^{7+}$ found: 1372.9884, calculated: 1373.1071. $[C_{416}H_{688}N_{114}O_{139}P_2S + 8H]^{8+}$ found: 1201.4900, calculated: 1201.5938. $[C_{416}H_{688}N_{114}O_{139}P_2S + 9H]^{9+}$ found: 1068.1035, calculated: 1068.1944. $[C_{416}H_{688}N_{114}O_{139}P_2S + 10H]^{10+}$ found: 961.4286, calculated: 961.4748.$[C_{416}H_{688}N_{114}O_{139}P_2S + 11H]^{11+}$ found: 874.0895, calculated: 874.1591.

Synthesis of full-length $^{R42}Ub^{ADPr}$ (22) on wang resin via β-isothiourea 1β

*ADPr synthesis (***R42**) via β-isothiourea **1β**

Synthetic procedure was identical to the synthesis described for ^{R42}**Ub^{ADPr} 18** with exception of the equivalents used in the following conditions: ribosylation, phosphitylation and ADPr formation. The synthesis was performed on 2 µmol wang resin. The ribosylation was performed using **1β** ribosyl isothiourea β-anomer (21.2 mg, 26 µmol, 13 eq) and $AgNO_3$(4.42 mg, 26 µmol, 13 eq). Performing the phosphitylation amidite **8** $(FmO)_2PN(iPr)_2$ (12 eq., 0.13 M in MeCN) and DCI (24 eq, 0.25 M in MeCN) were used. In the final ADPr formation step TBS-protected nucleoside amidate **12** (22 eq., 0.13 M in MeCN) and DCI (44 eq., 0.25 M in MeCN) were used. After resin cleavage and precipitation the crude was purified by RP-HPLC. Pure fractions were pooled and lyophilized affording $^{R42}Ub^{ADPr}$ **22** (151 µg, 0.0157 µmol, 0.79% total yield as a 55.3:44.7 mixture of Ub^{ADPr}:Ub^{Pr})) as a white powder. LC-MS: Rt = 1.47 min. Deconvoluted mass = 9604. HRMS: $[C_{416}H_{688}N_{114}O_{139}P_2S + 7H]^{7+}$ found: 1373.0187, calculated: 1373.1071. $[C_{416}H_{688}N_{114}O_{139}P_2S + 8H]^{8+}$ found: 1201.5184, calculated: 1201.5938. $[C_{416}H_{688}N_{114}O_{139}P_2S + 9H]^{9+}$ found: 1068.1235, calculated: 1068.1944 $[C_{416}H_{688}N_{114}O_{139}P_2S + 10H]^{10+}$ found: 961.4104, calculated: 961.4748. $[C_{416}H_{688}N_{114}O_{139}P_2S + 11H]^{11+}$ found: 874.1077, calculated: 874.1591.

Synthesis RTN4B fragment: Rho-DPSPVSSTVPAPSPLSAAA (**23**) on rink amide resin

SPPS was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 10 µmol scale, using fourfold excess of amino acids relative to pre-loaded preloaded Fmoc amino Rink amide resin (Rapp Polymere GmbH). After automated peptide synthesis, diBoc-rhodamine (29 mg, 50 µmol, 5 eq.), PyBOP (29 mg, 50 µmol, 5 eq.) and DIPEA (26 µL, 150 µmol, 15 eq.) were added and the mixture was shaken for 1 hour. A test cleavage confirmed conjugation of rhodamine to the N-terminus. The resin was treated with TFA/TIS/H_2O/Phenol (90.5/2/5/2.5) for 1.5 hours before filtrated in an ice-cold solution of Et_2O:pentane (1:1). The precipitate formed was centrifuged (5min, 3500 rpm) and the supernatant decanted. The pellet was subsequently dried with N_2, taken up in warm DMSO and diluted in warm water before purified by RP-HPLC. Pure fractions were pooled and lyophilized affording **23** (7.13 mg, 3.39 µmol, 33.9%) as an orange powder. LC-MS: (26 -> 100% B in A): Rt = 3.66. HRMS: $[C_{97}H_{136}N_{22}O_{31} + 2H]^+$ found: 1053.9845, calculated: 1053.4849.

Procedures DupA-mediated hydrolysis assays and SdeA-mediated ligation

^{1}H-NMR kinetic DupA-mediated hydrolysis of Heptamer 14.

6 µL heptamer **14** (10 mM stock in H_2O) was added to a NMR tube containing 460 µL buffer (Tris 20 mM, NaCl 150 mM, pH 7.6) and 53 µL D_2O . A reference spectrum was measured on a Bruker 600 mHz in which

the H_2O signal was suppressed. The contents of the NMR tube were transferred to an Eppendorf and DupA was added (20 µL of a 889 µM stock solution) to generate final concentrations of DupA (33 µM) and heptamer **14** (111.1 µM). After addition, the mixture was incubated at 37 °C and monitored by HRMS. After 2 hours HRMS indicated conversion and a ^{1}H-NMR was taken suppressing the H_2O signal. The anomeric protons could be visualized and conversion could be monitored as ratio between the integrals of the corresponding intact ADPr moiety protons or dupA-mediated hydrolysis of the pyrophosphate bond phosphoribosyl associated protons. Differences in the hydrolysis-kinetics of the alpha and beta anomer could be visualized in the NMR spectra. An additional ^{1}H NMR spectrum was measured after overnight incubation at 37 °C.

DupA mediated hydrolysis of ADPribosylated peptides 14-17 (0-90min)

The peptides **14**-**17** (5 µM) in buffer (20 mM TRIS, 150 mM NaCl, pH 7.6) were incubated with DupA (3 µM) or without (background hydrolysis) at 37 °C in a total volume of 50 µL. At the indicated time points 15 µL sample was 4 times diluted before measuring HRMS. The ratio of product versus starting material was determined, corrected for t = 0 min and plotted as increase in pyrophosphate cleavage over time. The means of two individual measurements is depicted with standard deviation and compared to enzym. $^{R42}Ub^{ADPr}$

DupA-mediated hydrolysis of synthetically prepared $^{Rx}Ub^{ADPr}$ (18-21) and heptamer 14, analyzed after overnight incubation

One of the ubiquitin's **18**-**21** (5 µM) or heptamer **14** were incubated with DupA (3 µM) or without (background hydrolysis) at 37°C in a total volume of 30 µL. After overnight incubation 15 µL sample was 4 times diluted before measuring HRMS. The ratio of product versus starting material was determined, corrected for t = 0 min and plotted as increase in pyrophosphate cleavage. The means of two individual measurements is depicted with standard deviation and compared to enzym. $^{R42}Ub^{ADPr}$

DupA-mediated hydrolysis of synthetically prepared $^{Rx}Ub^{ADPr}$ (18-21) (0-90min)

The ubiquitin's **18**-**21** (5 µM) in buffer (20 mM TRIS, 150 mM NaCl, pH 7.6) were incubated with DupA (3 µM) or without (background hydrolysis) at 37°C in a total volume of 50 µL. At the indicated time points 15 µL sample was 4 times diluted before measuring HRMS. The ratio of product versus starting material was determined, corrected for t = 0 min and plotted as increase in pyrophosphate cleavage over time and compared to enzym. $^{R42}Ub^{ADPr}$

SdeA-mediated ligation of $^{Rx}Ub^{ADPr}$ (18-21) and RTN4b peptide 23.

The enzymatically prepared $^{R42}Ub^{ADPr}$ or synthetically prepared ubiquitin's **18**-**21** (67 µM) in buffer (20 mM TRIS, 150 mM NaCl, pH 7.5) were incubated with RTN4B fragment **23** (60 µM) and SdeA FL (20 µM), at 37°C in a total volume of 25 µL. The mixture was monitored by HRMS and after 1 hour the enzymatic and synthetic $^{R42}Ub^{ADPr}$'s indicated conversion to the $^{R42}Ub^{Pr}$-RTN4b complex on mass spectrometry (deconvoluted mass = 11363). The ubiquitin's were analyzed by SDS PAGE adding 10 µL of each sample to 5 µL loading buffer (3X). The samples were run on a NuPAGE™ 12% Bis-Tris gel in MES buffer, 190 mV, for 45 minutes. A fluorescence scan on a Typhoon FLA 9500 (rhodamine channel, 473 nm) was performed to visualize the complex formed and additionally, the proteins were stained with Coomassie staining.