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

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Chapter 1

General introduction

The human proteome is significantly more complex than would be expected from the analysis of its genome, which is mainly due to two general mechanisms. First of all, alternative splicing, a process that occurs at a transcriptional level, allows for the selection of different combinations of splice sites in pre-messenger RNA, [1] making it possible to derive multiple proteins from a single gene. Further diversification of the proteome takes place after protein biosynthesis through a wide array of modifications that are collectively referred to a post-translational modifications (PTMs). These chemical alterations vary from proteolytic cleavage and protein excision to the covalent attachment of functional groups on amino acid side chains or on one of the termini of the peptide backbone. The latter category not only comprises small modifications such as methylation, acetylation and phosphorylation but also covers the introduction of fatty acid chains, (poly)saccharides and even complete proteins. With a few exceptions, the reversible nature of PTMs allows for rapid and economical tailoring of protein fate by affecting properties such as conformational stability, catalytic functioning, protein-protein interactions, and protein folding, trafficking and degradation. Malfunctions in any of these regulatory processes disturbs the cellular homeostasis and are as a consequence often strongly associated with a variety of human diseases.

Nicotinamide adenine dinucleotide (NAD^+) is well known for its role as redox co-factor in various metabolic biosynthesis routes as well as energy production through processes like the Krebs cycle and oxidative phosphorylation. It also serves as an essential substrate in many biological pathways. For example, the enzymes SARM1, CD38 and CD157 catalyze the conversion of NAD^+ into nicotinamide and adenosine diphosphate ribose (ADPr). Interestingly, the two latter

enzymes are not limited to the hydrolysis of NAD^+, but can also facilitate intramolecular cyclization to produce cyclic ADP-ribose (cADPr).[2] Both ADPr and its cyclic analogue cADPr have been identified as Ca^{2+} mobilizing second messengers[3,4] that act through distinct receptors and can induce local changes when acting alone or synergize to cause global effects on Ca^{2+} flux.[5] Alternatively, the deacetylation of histones by the proteins from the highly conserved sirtuin family is NAD^+-dependent and has been connected to a constantly expanding set of activities that most importantly includes genomic stability and longevity. Last, but certainly not least, NAD^+ is used as a reagent in a wide-spread PTM referred to as ADP-ribosylation, the main subject of this thesis, where one or more ADPr molecules are covalently transferred to a nucleophilic amino acid side chain of the target protein. Here, the developments in the field of ADP-ribosylation, focusing on the transferase and hydrolase enzymes involved in the synthesis and break-down of ADPr chains, are summarized, followed by an in-depth overview of the synthetic advances towards well-defined mono-ADP-ribosylated (MARylated) peptides.

Poly(ADP-ribose)

The initial isolation of an acid-insoluble adenylic polymer from nuclear extracts from hen liver cells by Chambon and coworkers[6] over five decades ago and its subsequent identification as ADP-ribosyl polymer[7] unknowingly opened up the field of ADP-ribosylation. The exact structure of the poly-ADPr (PAR) chain was elucidated by Miwa *et al.*[8-10] in several spectroscopic studies on the fragments that were obtained after digestion with snake venom phosphodiesterase. Comparison of ^{13}C NMR signals from the isolated 5′-phosphoryl-adenosine-5″-phosphoryl-ribofuranoside monomer to 5′-adenosine monophosphate (AMP) and α-/β-configured methyl-ribofuranosides led to the conclusion that the repeating ADPr units are connected via α-(1″→2′)-glycosidic linkages (Figure 1).[8] Shortly after, the hypothesis of a purely linear chain was challenged by the isolation of a branching fragment.[9] Using gas chromatography with a collection of methylated ribitol derivatives as internal standards, Miwa and colleagues identified the 2″-position of the distal ribose in this novel fragment as the branching site. Proximity factors calculated from NOE measurements support an α-configuration of the ribose-ribose linkage.[10] The frequency of branching in the ADPr-chains could be estimated to be 0.7-0.8% with the use of a high-performance liquid-chromatography based fluorescent assay (HP-LC) assay,[11] which also explains why this phenomenon remained unnoticed during previous investigations. Although a lot of information was gathered about the structural basis, a lot of new questions arose surrounding the molecular targets, involved enzymes and most importantly, biological relevance of this novel modification.

ADPr-transferases

Pathogenic bacterial exotoxins were among the first to be identified as enzymes with (ADP-ribosyl)transferase activity, which apply this modification to yield a unique pathology.[12] For example, a multidomain protein secreted by *Corynebacterium diphtheriae*, referred to as diphtheria toxin (DT), was found to effectively block host protein biosynthesis by facilitating the

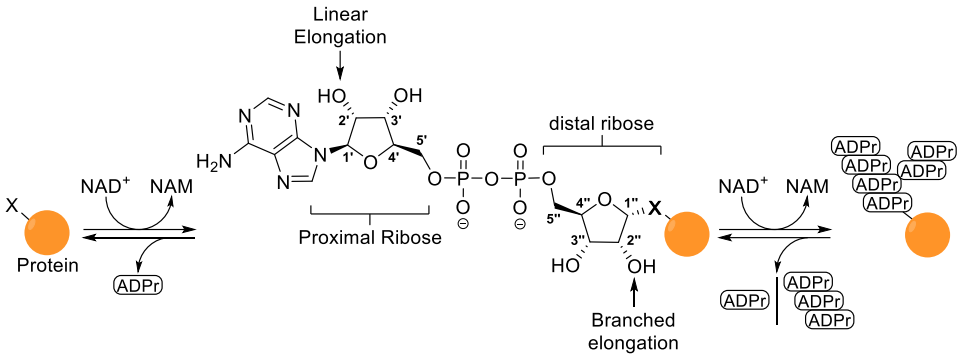


Figure 1| Schematic overview of ADP-ribosylation where NAD^+ is consumed and the ADPr moiety is covalently attached to a defined nucleophilic side chain (X= O, N or S). Numbering denotation and nomenclature used discriminate between the two ribofuranosides is included.

covalent introduction of a single ADPr moiety on the essential elongation factor 2 (EF2).[13] By means of tryptic digestion, the exact modification site on EF2 was found to be a unique residue termed 'diphthamide'[14,15] that is derived from histidine through an multi-step biosynthesis pathway.[16] An NMR analysis of the ADP-ribosylated fragment performed by Oppenheimer and Bodley indicated that, identical to linear and branched elongation of PAR chains, the modification is exclusively α-selective.[17] Similarly, cholera toxin (CT) [18,19] and pertussis toxin (PT)[20], excreted by *Vibrio cholerae* and *Bordetella pertussis* respectively, inhibit G-proteins by mono-ADP-ribosylation (MARylation) of the targets α-subunit and as a consequence interfere with cyclic adenosine monophosphate (cAMP) mediated signaling pathways. Despite the similarities in the target proteins, CT and PT have distinct amino acid substrate specificity as the former was observed to modify arginine residues in an α-selective manner[21] (even though racemization occurs after the introduction),[22] while the latter constructs α-ribosyl linkages to cysteine.[23] With the aid of both crystallographic and mutagenic data, essential amino acids in the catalytic domain of (ADP-ribosylating) bacterial toxins could be identified.[24,25] In the case of DT, a imidazole residue of histidine was observed to interact with the proximal ribose of NAD^+ while a tyrosine moiety engages in π-π stacking with the aromatic system of nicotinamide (Figure 2). Finally, the glutamic acid carboxylate group correctly positions the distal ribose for an incoming nucleophilic attack through hydrogen bonding.[26–29] Although glutamic acid was also found to fulfil a similar role in CT and PT, NAD^+ binding is instead assisted by arginine and serine. The guanidine group of arginine establishes electrostatic interactions with the negatively charged pyrophosphate and serine arranges hydrogen bonds with the distal ribose and nicotinamide.[26,29,30] Sequence alignment and three-dimensional structural studies of bacterial (ADP-ribosyl)transferases not only disclosed that the active sites are superimposable, but also revealed that the above described catalytic triads (H-Y-E and R-S-E) are highly conserved throughout the family.[31,32]

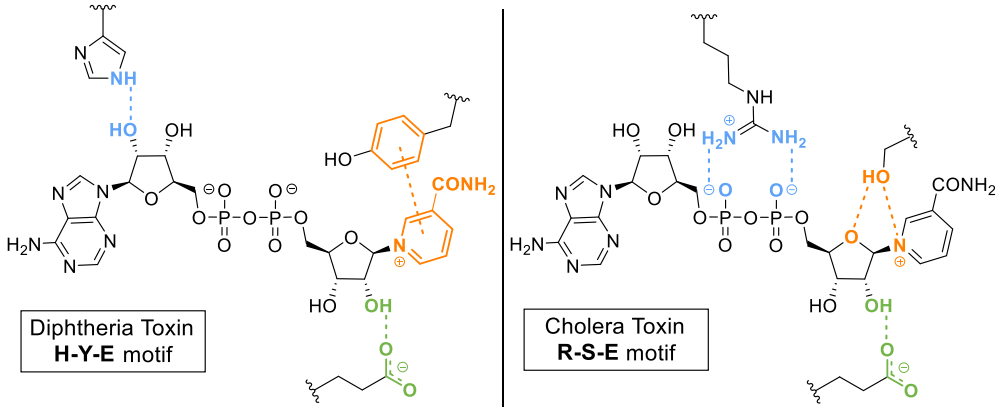


Figure 2| The catalytic triad of DT (left) and CT (right), including their most essential interactions with the NAD^+ substrate, that are both highly conserved among ADP-ribosylating bacterial toxins.

Parallel to the increasing understanding of toxin mediated ADP-ribosylation, advances were made in the search for the eukaryotic orthologs. In separate *in vitro* studies, Gill and colleagues demonstrated that poly-ADP-ribosylation (PARylation) activity in calf thymus extracts appeared to be stimulated by DNA, especially when carrying double-stranded breaks.[33,34] These findings undeniably emphasized that ADP-ribosylation is not only an important virulence trait, but is also an integral part of the normal functioning and regulation of cell physiology in mammals. A breakthrough occurred several years later when the gene encoding the protein responsible for PAR production termed PARP1 (formerly referred to as PARP or poly(ADP-ribosyl)transferase) was finally isolated, sequenced and cloned by different labs.[35–37] For a long time it was believed that PARP1 was the only enzyme mediating ADP-ribosylation in mammalian cells, until comparative analysis of the public nucleotide and protein sequence databases[38] revealed the identity of 20 additional members of the (ADP-ribosyl)transferase family in humans.[39–49] Comparative structure and sequence analyses between bacterial and mammalian ADPr-transferases indicated that both families share a characteristic active site consisting of a central 6-stranded β-sheet with a so-called ADP-ribosylating turn-turn loop.[31,32,49] Moreover, the earlier recognized catalytic motifs of diphtheria (H-Y-E) and cholera (R-S-E) toxins correlate closely with the eukaryotic orthologs, which are distributed accordingly into two subgroups: DT-like and CT-like (ADP-ribosyl)transferases (ARTDs and ARTCs).[50] The ARTD family includes all intracellular and nuclear localized enzymes and is most abundant with a total of seventeen members (PARP1-4, tankyrase 1 and 2 and PARP6-16). In contrast, the members belonging to the ARTC clade are either expressed at the cell surface or secreted in extracellular components (ARTC1 and 3-5).[48,51]

Since the early discovery of diphthamide,[14] arginine,[21] cysteine[23] and asparagine[52] as acceptor sites for ADP-ribosylating bacterial toxins, other endogenous modification sites have been identified *in vitro* using radioactive substrates in combination with purified or recombinantly expressed enzymes.[53–56] However, the true scope of ADP-ribosylation sites and prevalence of the modification was not recognized until advances in mass-spectrometry (MS) enabled the system-wide analysis of the cellular 'ADP-ribosylome'.[57–64] Serine has emerged as the primary target for ADP-ribosylation, especially in DNA damage response,[59] but previously known residues such as glutamate[57], aspartate[57], arginine[62,63], lysine[53,58,60] and cysteine[61], and newcomers like threonine[61], tyrosine[62,65] and histidine[61,64] were found to be frequently modified as well. Covering nearly half of all amino acid residues, it comes as little surprise that ADP-ribosylation is involved in the regulation of numerous vital cellular processes such as chromatin maintainance,[66] DNA-damage repair,[67] protein degradation,[68] cytosolic RNA processing,[69] apoptosis[70] and immune response[71,72] and that abnormal functioning of the involved enzymes has been linked to cancer,[73] metabolic diseases[74] and neurological disorders.[75]

Nevertheless, the underlying mechanisms dictating substrate specificity of ADPr-transferases is still poorly understood due to the complex regulation that seem to adjust their target preferences at various levels. For example, the selectivity of arginine residues observed for the catalytically active ARTC1-2 members is typical for cholera toxin-like (R-S-E triad) transferases,[48] while PARPs have a clear mismatch with their diphtheria toxin-like ancestor and predominantly target acidic residues (glutamate and aspartate)[76] instead of diphthamide and analogues thereof. Furthermore, the canonical activity of PARP1 was found to be redirected through interaction with the chaperone NMNAT1 to glutamate and aspartate residues on histone H2B[77] or in collaboration with the co-factor histone parylation factor (HPF1) to serine residues of various substrates.[78] The steric and charge effects of phosphorylated residues has been shown to affect ADP-ribosylation of surrounding residues, and *vice versa*, demonstrating the additional effect of cross-talk with other PTMs on target specificity.[61,79]

It is noteworthy that PARylation activity has so far only been found in a limited number of human (ADP-ribosyl)transferases. PARP2[51] and tankyrase 1-2[52] were demonstrated, in addition to founding member PARP1,[81] to extend the MARylated modification with additional ADPr molecules. The ability to PARylate target proteins was hypothesized to be dependent on the presence of a glutamate residue in the catalytic triad as the mutation of the respective residue in PARP1 selectively abolished polymer elongation abilities.[82] In order to explain the enzymatic limitations of ARTDs lacking a catalytic glutamate, a substrate-assisted mechanism was proposed in which the acidic target residue of the substrate functions as surrogate for the missing side chain.[76] However, both PARP3 and PARP4 were observed to MARylate rather than PARylate their substrate despite the presence of a catalytic glutamate.[83] This last finding strongly suggests that, similar to substrate specificity, there are additional factors in play that determine the scope of transformations catalyzed by each (ADP-ribosyl)transferase.

ADPr-glycohydrolases

The reversibility of ADP-ribosylation has been known ever since the early demonstrations of PAR degradation by partially purified rat liver and calf thymus extracts containing an enzyme termed PARG.[84,85] Interestingly, PARG was shown to specifically catalyze the hydrolysis of ribose-ribose bonds, while leaving the protein-ribose linkage intact.[85,86] This observation implied the existence of complementary hydrolases that revert the protein-linked ADPr moiety. Although the PARG gene was already cloned in the late 90s,[87] no structural data was available until the first crystal structure was solved by Ahel and colleagues.[86] This revealed that the mechanism likely proceeds via a reactive oxocarbenium intermediate upon activation of the *O*-glycosidic linkage through interactions with a nearby glutamate residue, but also explained that PARG predominantly acts as exo-acting enzyme because the presence of a substituent on the 2'-OH of the proximal ribose would require major reorganizations of the active site. Furthermore, a comparative analysis of the PARG catalytic domain with available structures indicated that its ADPr-binding fold is shared with macrodomain proteins, which suggests that PARG is distant member of this widespread family. Although the macrodomains originally evolved as ADPr-binders,[88,89] some members from the Macrodomain type (MacroD1 and -D2) and ALC1-like (C6orf130, better known as TARG1) subclasses acquired catalytic activity and were found to function as *O*-acetyl-ADPr (OAADPr) deacetylases.[90,91] The chemical similarities between OAADPr and the glycosidic ester linkage of glutamate-ADPr led to the assumption that these three macrodomain proteins potentially function as (ADP-ribosyl)hydrolase for acidic residues. Two separate laboratories confirmed the hypothesis and showed that all three candidates liberated ADPr in a biochemical screening with MARylated peptides that were generated by PARP10.[92,93]

Independent of the developments described above, another ADPr-glycohydrolase with an exclusive specificity towards arginine residues was isolated from turkey erythrocytes and subsequently characterized as founding member of the evolutionary distinct ADPr-hydrolase family (ARH1).[94,95] Several years later, the human homolog was cloned[96] and two additional members of the human ARH family, designated ARH2 and ARH3, were discovered in a sequence alignment study.[48] Apart from its localization in heart tissue, little is known about ARH2.[97] It was demonstrated that ARH3 is capable of processing linear PAR chains, but remains unable to degrade PAR branching points[98] or remove mono-ADPr from cysteine, diphthamide and arginine.[99] The latter substrate was even found to function as inhibitor with nanomolar affinity.[100] Nevertheless, the substrate scope of ARH3 has been expanding ever since and now covers the likes of α-NAD^+,[101] OAADPr,[102] ADPr-5'P DNA[103] and most importantly, serine linked mono-ADPr.[104] At first sight, the active sites of ARH1 and ARH3 are structurally very akin, which is surprising given their distinct substrate preferences, and contain a binuclear magnesium center required for catalytic activity.[105] Closer inspection of the active site residues, made it clear that substrate specificity is tightly regulated by seemingly subtle differences in the Mg(II)-coordination spheres, which are described in more detail by Rack *et al.* [98,105]

Chemical and enzymatic strategies towards mono-ADP-ribosylated peptides

The understanding of the mechanisms of protein ADP-ribosylation has been greatly assisted by the production of well-defined MARylated oligopeptides and analogues thereof. These molecular tools have enabled the production and evaluation of recombinant ADP-ribose specific antibodies,[106] provided valuable structural insights of the modification,[22,107,108] elucidated substrate specificity of ADPr-glycohydrolases,[108–110] were shown to be of use as probes for pull-down experiments to aid in the identification of novel ADPr-binders[111,112] and can be beneficial as internal standards for HP-LC and MS-based analyses. Here, an extensive overview of the reported methodologies towards the synthesis of MARylated peptides and proteins is presented, which have been arranged on the basis of the construction of a native or artificial linkage. The former category is divided further into chemoenzymatic and chemical strategies.

Enzymatic construction of native ADP-ribose linkages

Different laboratories have successfully exploited the (ADP-ribosyl)transferase activity of bacterial exotoxins, and even porcine brain NADase, for the modification of single amino acids and their derivaties,[22,113,114] but the first ADP-ribosylated oligopeptides have been reported by Kharadia and Graves (Figure 3).[79] The cholera toxin mediated modification of arginine residues in synthetically derived hormones angiotensin II and III (**1B** and **1A** respectively) and analogues thereof (**1C-D**), suggested that enzymatic efficiency benefits from hydrophobic residues next to the arginine acceptor site. Nevertheless, the heptapeptide carrying two arginine residues called kemptide (**1E**) proved to be the best substrate and full conversion into two products was observed. Tryptic digestion of the isolated products and subsequent mass-spectrometry analysis of the obtained fragments indicated that the main product was exclusively MARylated on the first arginine reside (Arg^{I}, 70% yield), while the minor product was modified on both arginine side chains (Arg^{I+II}, 30% yield). A similar strategy for the production of mono-ADPr peptides was developed by Scheuring and Schramm[107], after pertussis toxin was found to modify the cysteine residues of oligopeptides originating from guanine nucleotide-binding proteins (G-proteins).[115,116] The 20 amino acid carboxyl-terminus (*C*-terminus) of the $G_{i3}\alpha$-subunit **3** was obtained via solid-phase peptide synthesis (SPPS) and could be converted into ADPr peptide **4** to a maximum extent of approximately 30% using optimized conditions at 0 °C. Extended reaction times and higher concentrations of NAD^+ did not increase the yield. Incubation at room temperature or 37 °C resulted in NADase activity or inactivation of the toxin, respectively.

After more than two decades, Bonfiglio and colleagues developed a chemoenzymatic approach towards peptides ADP-ribosylated on tyrosine or serine side chains (Table 1).[106] Incubation of the target peptides **5**, containing either a serine or tyrosine modification site, under systematically optimized reaction conditions with the PARP1/HPF1-protein complex provided PARylated intermediates **6** that were subsequently truncated to the desired MARylated peptides by PARG and isolated in high purity via boronate-affinity chromatography (Method I).

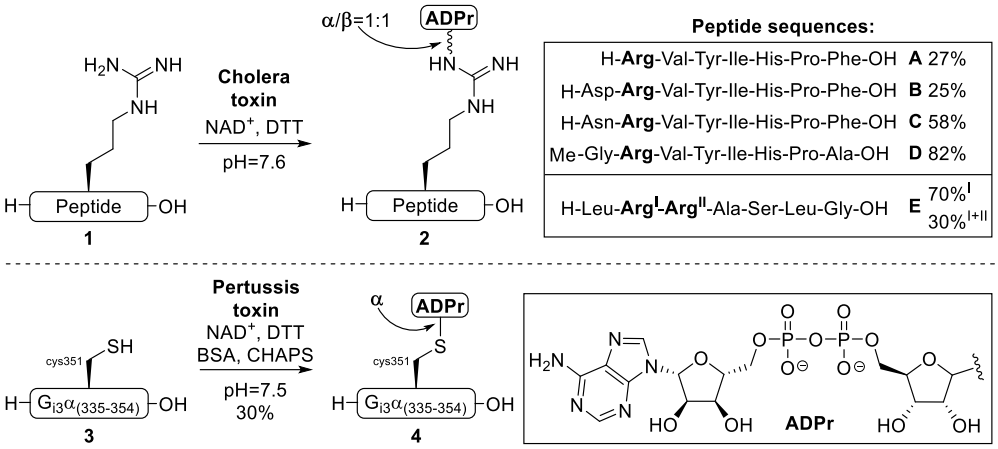


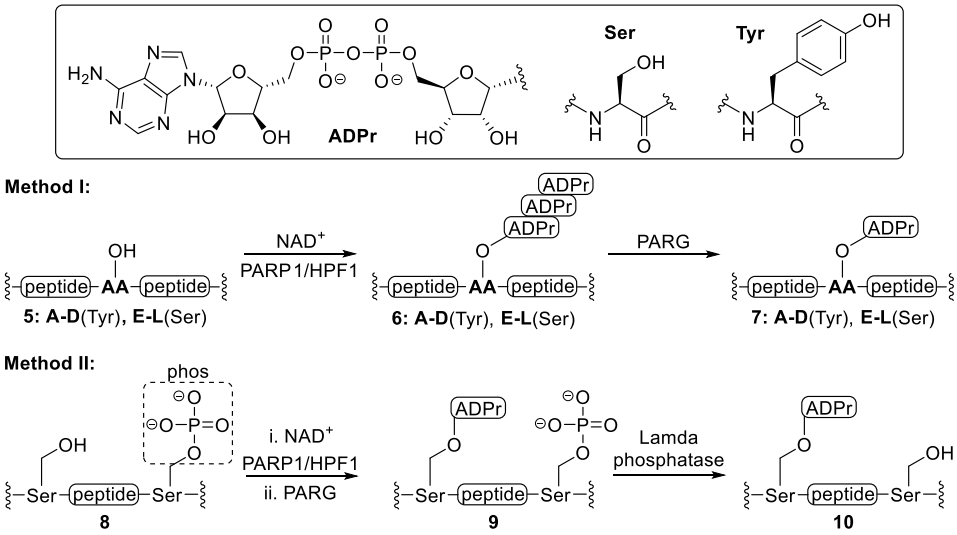
Figure 3| Toxin mediated ADP-ribosylation of synthetic peptides. Modification of arginine in synthetically derived angiotensine peptides (**1A-D**) and kemptide (**1E**) by Kharadia & Graves (Top). ADP-ribosylation of cysteine-351 in $G_{i3}\alpha$(335-354): H-VFDRVTDVIIKNNLKE-**C**-GLY-OH (**3**) by Scheuring & Schramm (bottom). DTT = dithiothreitol and CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

This two-step procedure allows for the installation of a single ADPr moiety on a wide variety of substrates as was demonstrated with the efficient modification of peptides **5A-L** in a scalable manner. However, occasionally sequences were encountered that yielded a complex mixture of peptides ADP-ribosylated at different sites, so a more elaborate strategy was required. In this second approach, the off-target serine residues were orthogonally protected with a phosphate group (**8A-B**) to prevent any non-selective reactions during the two-step MARylation approach described above. Afterwards, all phosphoserines were completely dephosphorylated by Lambda phosphatase while conserving the previously installed ADPr moieties to provide target structures **10**. With the addition of the phosphate protecting group strategy, an even broader pallet of oligopeptides with ADPr on selected serine or tyrosine residues are now within reach.

Whereas the previously described procedures are limited to the production of short peptide constructs, Liszczak and coworkers focused on the development of methodologies that allows for the assembly of full-length proteins with site-specific ADPr modifications. To this extent, hydrazine functionalized *N*-terminal tails of histones H3 **11** and H2B **12** were prepared via Fmoc-based SPPS with DIC/oxyma-mediated peptide couplings on a PEG-resin equipped with an *N*-tritylhydrazine linker (Scheme 1A).[117] Subsequent conversion into thioesters **13** and **14** was initiated by sodium nitrite followed by the addition of sodium 2-mercaptoethanesulfonate (MESNa) and tris(2-carboxyethyl)phosphine (TCEP). In contrast to the procedure described above, the MARylation of peptides **13** and **14**, on Ser-10 and Ser-6 respectively, were realized in a single step by incubation with a cocktail of HPF1, PARP1 and PARG. It is noteworthy that high concentrations of co-factor HPF1 seem to limit the role of PARP1 as ADPr chain elongator

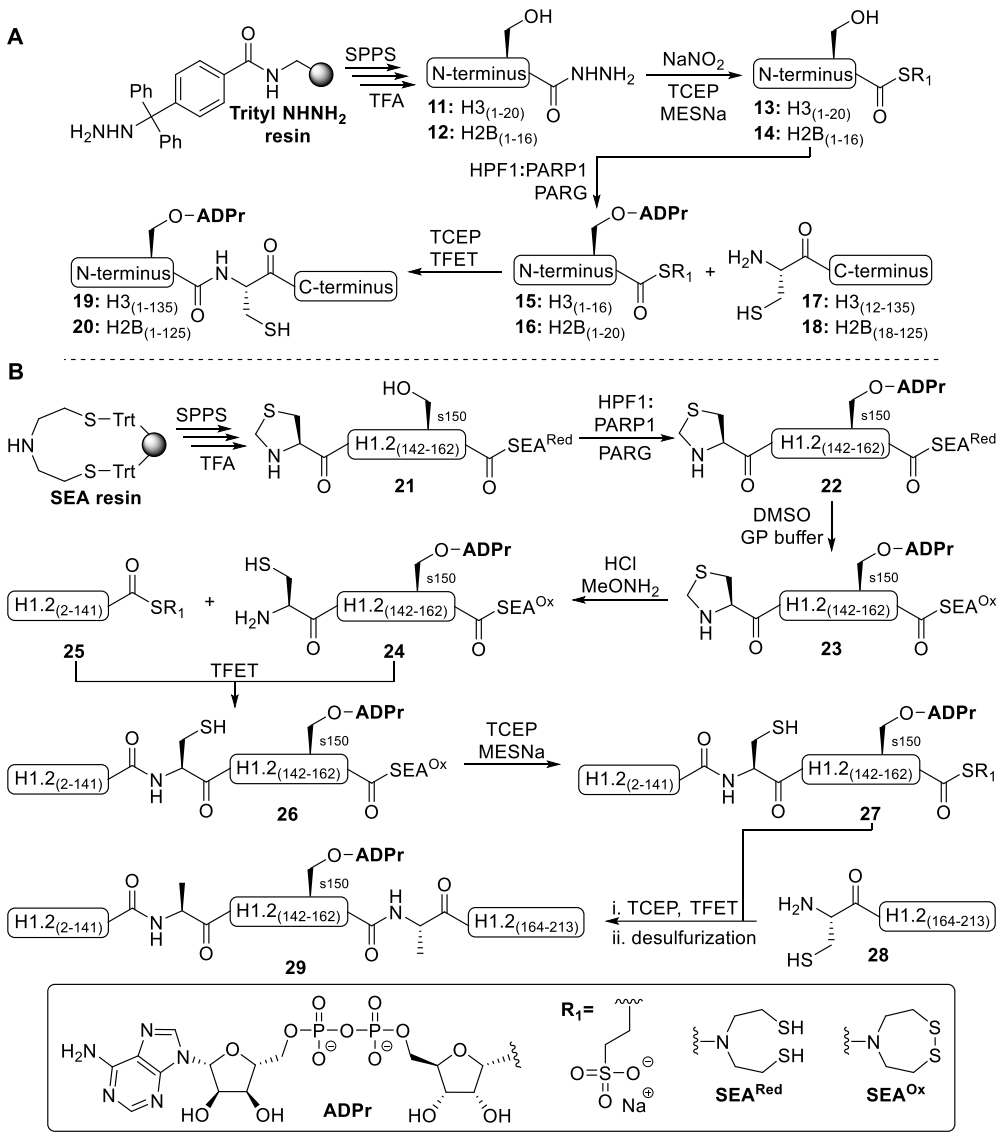
and directs its emphasis to mono-ADPr. This observation is in line with the structure-based hypothesis proposed by Ahel and colleagues.[118] A native chemical ligation (NCL) reaction with the corresponding recombinant *C*-terminal histone fragments **17** or **18** provided the full-length histones **19** and **20** after purification with semi-preparative reversed-phase HP-LC.

Table 1| A chemoenzymatic approach towards peptides ADP-ribosylated on serine (S) or tyrosine (Y) using the PARP1/HPF1 protein complex by Bonfiglio *et al.* A two-step procedure (method I) is compatible with a wide variety of sequences. Substrates with off-target serine residues can be effectively MARylated by implementing phosphate as protecting group (method II). The ADPr acceptor sites in each sequence is depicted bold.



#	Peptide sequence	Method	Residue
5A	Ac-LRKFYKGKK-**Y**-KPLDLRPKKTRAGGK(Biotin)-NH_2	I	Tyr
5B	Ac-KNFTK-**Y**-PKKFYPLGGK(Biotin)-NH_2	I	Tyr
5C	Ac-TGGVKKPHR-**Y**-RPGTVALRGGK(Biotin)-NH_2	I	Tyr
5D	Ac-LVRHRR-**Y**-KHTHGGK(Biotin)-NH_2	I	Tyr
5E	Ac-ARTKQTARK-**S**-TGGKAPRKQLAGGK(Biotin)-NH_2	I	Ser
5F	Ac-ATKAARK-S^{ADPr}-APATGGVKKPHRYRPGGGK(Biotin)-NH_2	I	Ser
5G	Ac-**S**-GRGKGGKGLGKGGAKRHRGGK(Biotin)-NH_2	I	Ser
5H	Ac-KVAKPKKAAK-**S**-AAKAVKPGGK(Biotin)-NH_2	I	Ser
5I	Ac-KATGAATPKK-**S**-AKKTPKKGGK(Biotin)-NH_2	I	Ser
5J	Ac-**S**-GRGKQGGKARAKAKTRSSGGK(Biotin)-NH_2	I	Ser
5K	Ac-RLAKSDEPKK-**S**-VAFKKTKGGK(Biotin)-NH_2	I	Ser
5L	Ac-PKAPKGK-**S**-AGREKKVIHPYSRAGGA-NH_2	I	Ser
8A	Ac-APRGKS(phos)GAALSKK-$\mathbf{S^{ADPr}}$-KGQVGGK(Biotin)-NH_2	II	Ser
8B	Ac-PEPAK-**S**-APAPKKGS(phos)KK AVTKAQKKDGKKRKRGGK(Biotin)-NH_2	II	Ser

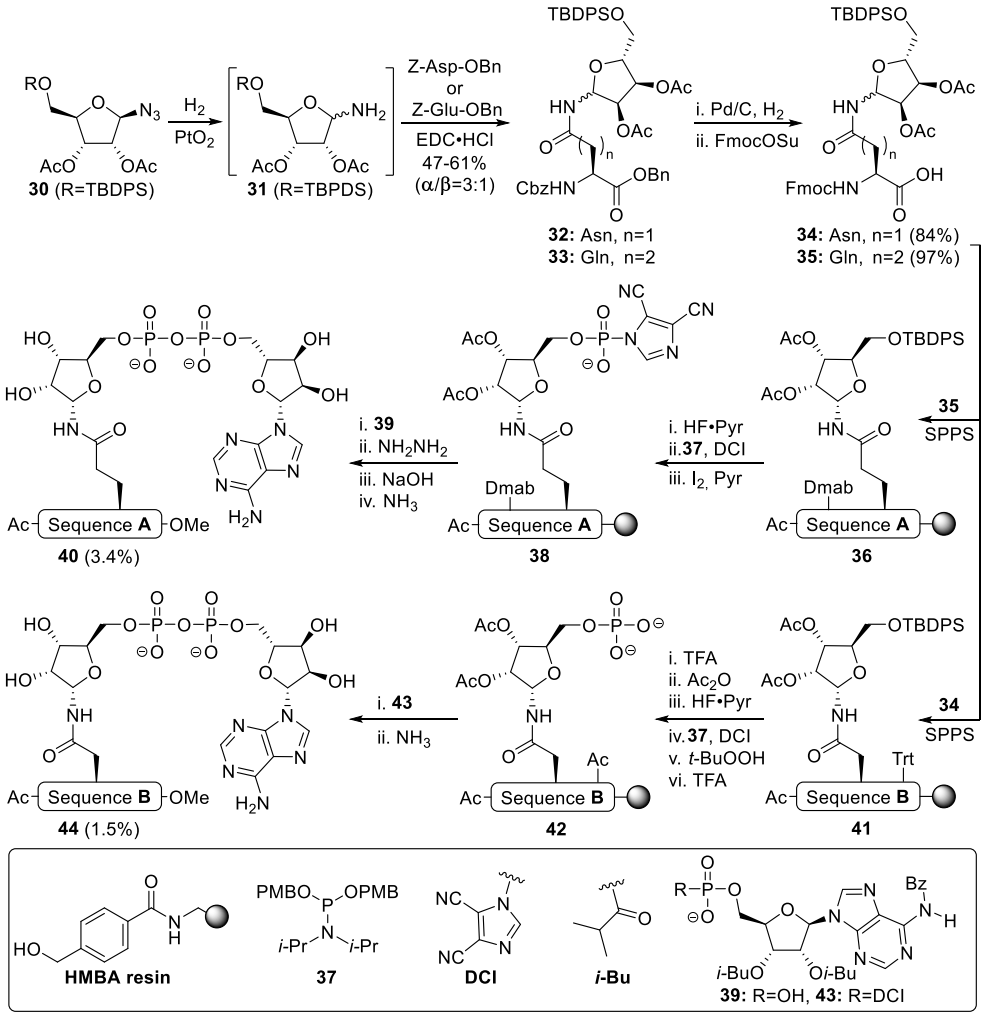
In order to gain access to modification sites throughout the entire protein, Tashiro *et al* pursued a design that supports modular functionalization at both the *N*- and *C*-terminus of serine ADPr peptides. After serine 150 of linker histone 1.2 was validated as target of HPF1:PARP1 mediated ADP-ribosylation, synthetic peptide **21** encompassing this modification site was generated on a polystyrene resin, loaded with a bis(2-sulfanylethyl)amido (SEA)-linker (Scheme 1B).[119] The *N*-terminal cysteine residue was protected with a thiazolidine (Thz) in order to prevent nonenzymatic conjugation to free ADP-ribose. Initially, the use of a *C*-terminal acyl hydrazide was explored, but conditions required for the thioesterfication of this functionality were incompatible with the Thz protecting group.[120] Enzymatic ADP-ribosylation of fragment **21** with near quantitative conversion was followed by the oxidation of SEA to give ADPr peptide **23**. The oxidized SEA group is in contrast to its reduced form inert to NCL reactions. The *N*-terminal cysteine was liberated under acidic conditions and conjugated to recombinant H1.2 (2-141) with trifluoroethanethiol (TFET) as thiol catalyst to yield **26**. The reducing agent TCEP was deliberately omitted to preserve the oxidized SEA-ring and prevent any cross-linking. Incubation of H1.2 (2-162) **26** with TCEP and MESNa facilitated conversion of the SEA-moiety into MES-thioester **27**, which could be coupled to the remaining H1.2 *C*-terminal segment **28** using similar conditions as before. Finally, H1.2-S105-ADPr **29** was obtained through a radical-based desulfurization to restore ligation junction cysteines into native alanine residues and could again be efficiently purified by HP-LC.



Scheme 1| Semisynthesis-based strategies for the preparation of ADP-ribosylated serine containing peptides that are compatible with protein ligation reactions by Liszczak *et al*. **A.** Preparation of histones H2B and H3 with an ADP-modification on the *N*-terminal tail (serine 6 and 10 respectively). **B.** Preparation of histone 1.2 ADP-ribosylated on serine 150. Full sequences of synthetic peptides are H3(1-20): H_2N-ARTKQTARK**S**TGGKAPRKQL-$NHNH_2$, H2B(1-16): H_2N-PEPK**S**APAPKKGSKK-$NHNH_2$ and H1.2(143-162): Thz-GGATPKK**S**AKKTPKKAKKKPA-SEA. GP buffer = guanidine HCl (6 M) + sodium phosphate (0.1 M). MESNa = sodium 2-mercaptoethanesulfonate, Thz = thiazolidine, SEA = bis(2-sulfanylethyl)amide, TCEP = tris(2-carboxyethyl)phosphine and TFET = trifluoroethanethiol.

Chemical synthesis of native ADP-ribose linkages

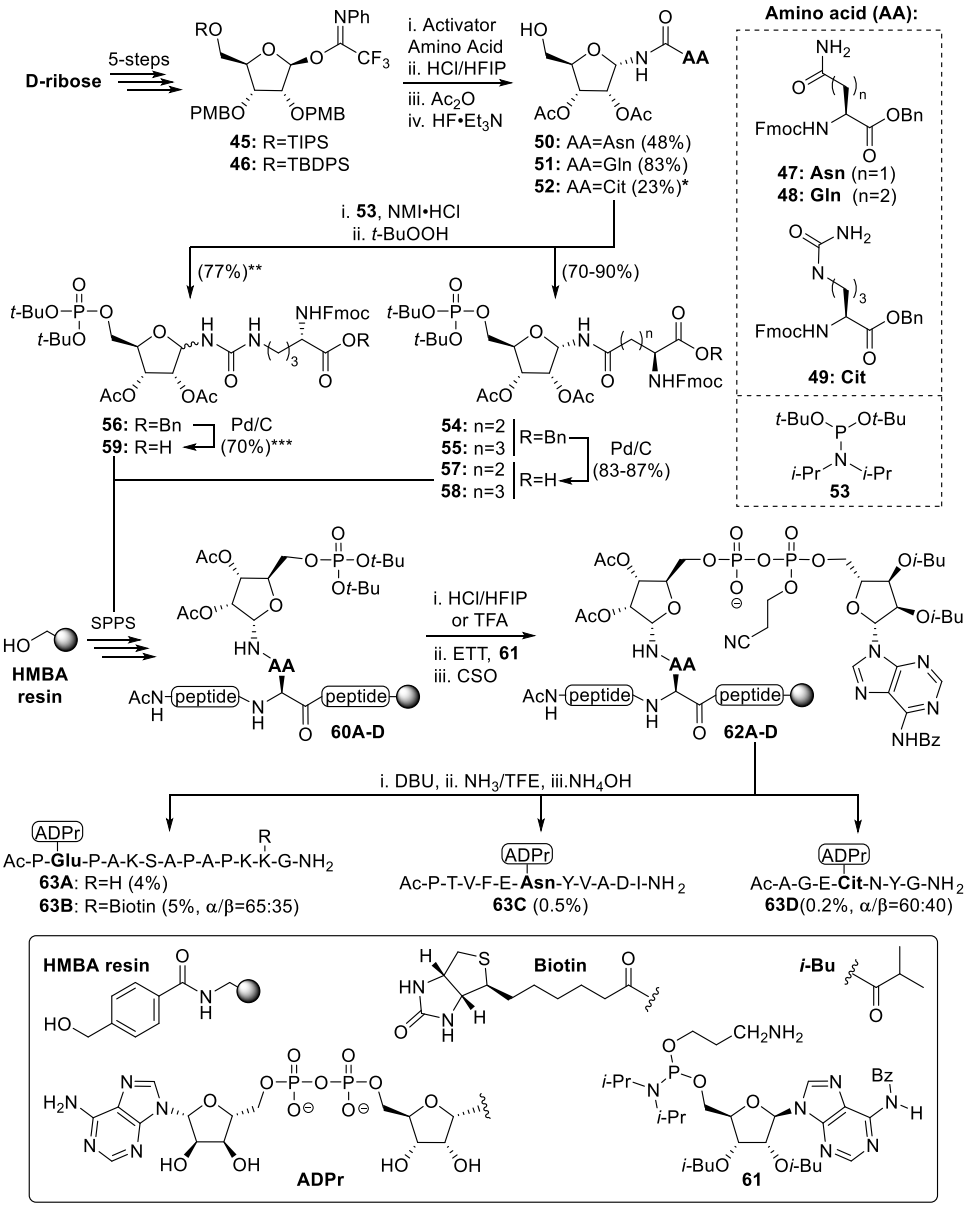
The earliest report of fully synthetic approaches towards ADP-ribosylated peptides was by van der Heden van Noort *et al.*, who prepared ribosylated asparagine **32** and glutamine **33** building blocks via an EDC-mediated peptide coupling between hemiaminal intermediate **31** and carboxybenzyl (Cbz or Z)-protected aspartic or glutamic acid respectively (Scheme 2).[121] Successful separation of the individual anomers by silica gel column chromatography was followed by protective group manipulations to provide α-configured asparagine **34** and glutamine **35** that are compatible with Fmoc-based SPPS conditions. The latter building block was incorporated in a model hexapeptide (sequence A) using a BOP/HOBt-coupling procedure on a Tentagel resin equipped with the base-labile 4-hydroxymethylbenzoic acid (HMBA) linker. Selective deprotection of the 5′-OH of **36** with HF•TEA allowed for the on-resin introduction of the ADPr moiety, which commenced with the DCI-assisted phosphitylation with amidite **37**. The resulting phosphite triester intermediate was both oxidized and liberated from *para*-methoxybenzyl (PMB)-groups using iodine in pyridine.[122] Addition of suitably protected adenosine monophosphate **39** to phosphorimidazolidate **38** led to construction of the pyrophosphate linkage and the desired glutamine ADPr peptide **40** was generated via a sequence of deprotection steps. The Dmab-moiety on the glutamic acid residue was first cleaved via a two-step procedure comprising hydrazine and NaOH,[123] after which the remaining protecting groups were removed while concurrently liberating the peptide from the resin using methanolic ammonia. Liquid chromatography-mass spectrometry (LC-MS) analysis of the crude mixture indicated that formation of ADPr peptide **40** was accompanied by the generation of the *C*-terminal carboxamide, terminal monophosphate and the corresponding H-phosphonate as side products. Although the desired product could be isolated by extensive HP-LC purification, it was hypothesized that formation of the monophosphate and H-phosphonate contaminations could be reduced by reversing the two pyrophosphate precursors. To this extent, asparagine derivative **34** was integrated in a heptapeptide (sequence B) via the same procedure described above to yield peptide **41**. Acid-labile trityl (Trt)-groups on the peptide backbone were exchanged for acetyl moieties and the ribofuranoside was desilylated prior to phosphorylation of the 5-OH with phosphoramidite **37** in the presence of DCI. Oxidation of the phosphite triester intermediate with *t*-BuOOH and PMB removal under acidic conditions now provided phosphoribosylated peptide **42**. The addition of an excess of adenosine phosphorimidazolidate **43** was expected to counterbalance the partial hydrolysis of the reagent and thus minimize residual phosphate **42**. Unfortunately, the obtained by-products after final deprotection and cleavage were very similar to the first procedure, which once more emphasized the complex nature of phosphorus chemistry. Regardless of the challenges remaining in the on-resin construction of ADPr, this approach has enabled the production of well-defined peptides ADP-ribosylated on glutamine (**40**) and asparagine (**44**) in acceptable yields and provided a benchmark for all ensuing synthetic methodologies.



Scheme 2| An SPPS-based approach towards peptides ADP-ribosylated peptides on asparagine and glutamine by Van der Heden van Noort and colleagues. Sequence A = Ac-VA**N**IEV-OMe and sequence B = Ac-P**Q**PSKSA-OMe, where the modification site is highlighted (bold). Dmab =4-(*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino)benzyl, DCI = dicyanoimidazole, EDC = 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, Pyr = pyridine, TEA = triethylamine and TFA = trifluoroacetic acid.

The efforts of Kistemaker and colleagues have led to the discovery a stereoselective glycosylation reaction that enabled the construction of α-configured glycosidic linkages on various amino acid side chains (including glutamate, aspartate and serine)[124] and was first applied in the synthesis of peptides MARylated on asparagine, glutamine and citrulline (Scheme 3).[125] The required trifluoroacetimidate donors **45** and **46** were derived from D-ribose in 5 steps and carried non-participating PMB moieties on the 2- and 3-OH along with an bulky

α-directing triisopropylsilyl (TIPS) or *tert*-butyldiphenylsilyl (TBDPS) ether on the primary 5-OH.[124] Ribosylation of asparagine **47** and glutamine **48**[124] proceeded in an α-selective manner upon activation of donor **46** with TBSOTf or donor **45** with $HClO_4$-SiO_2 respectively. Removal of the PMB ethers with catalytic amounts of HCl in HFIP[126] and subsequent acetylation of the freed 2- and 3-OH positions was followed by desilylation with HF•triethylamine (TEA) as fluorine source to provide ribosylated amino acids **50** and **51** as pure α-anomer. The reaction between donor **46** and the less reactive citrulline **49** mediated by TBSOTf was less selective and provided an anomeric mixture (α/β=78:22). While the α-configured product was isolated and used in the subsequent steps, further isomerization occurred during acidolysis and as a result, furnished functionalized citrulline **52** after acetylation and TBDPS removal as a mixture of anomers (α/β=34:66). All three ribosylated amino acids **50**, **51**, **52** were phosphorylated prior to SPPS in order to circumvent the troublesome on-resin formation of phosphomonoesters as described by Van der Heden van Noort.[121] Activation of *t*-Bu protected phosphoramidite **53** with 1-methylimdazole hydrochloride and subsequent oxidation of the phosphotriester intermediate with *t*-BuOOH[127] yielded asparagine **54** and glutamine **55** without affecting the anomeric configuration. Ensuing removal of the benzyl esters by hydrogenolysis afforded the first pre-phosphorylated building blocks **57** and **58** that are compatible with Fmoc-based SPPS conditions. Since citrulline **52** was configurationally unstable during the two-step phosphorylation procedure as well as under the applied hydrogenation conditions, building block **59** was used as obtained (α/β=62:38) in the following solid-phase synthesis. The peptide sequences of interest were assembled with phosphoribosylated intermediates **57**-**59** on a tentagel resin loaded with an HMBA linker using HCTU/DIPEA. A series of protecting group manipulations, analogous to the work of Van der Heden van Noort (Scheme 2),[121] were required to replace any acid-labile functionalities on the peptide backbone for acetyl moieties. Although deprotection of the phosphate was preferably executed with 1 equivalent of HCl in HFIP to minimize anomerization, trifluoroacetic acid (TFA) was required for efficient removal of the *t*-Bu moieties from biotinylated peptide **60B**. Kistemaker then explored an alternative strategy reported by Gold *et al.*, combining phosphate (P^V) with phosphoramidite (P^{III}) chemistry, for the on-resin construction of pyrophosphate linkages.[128] As such, adenosine phosphoramidite **61** was activated with tetrazole activator ETT and conjugated to the immobilized phosphates, directly followed by oxidation of the P^V-P^{III} intermediate with CSO to yield fully protected ADPr peptides **63A-D**. The cyanoethyl (CNE)-group was cleaved from the pyrophosphate with non-nucleophilic base DBU and Dmab or *N*-Allyloxycarbonyl (Alloc) groups, if present, were removed from the peptide backbone with hydrazine or $Pd(PPh_3)_4$ respectively. A mixture of ammonia in trifluoroethanol (TFE) was found to be superior to methanolic ammonia for liberation of the peptides and exclusively provided the carboxamide products. Addition of NH_4OH in the final step was required to ensure complete removal of the benzoyl group from the exocyclic amine of adenosine. The oligopeptides ADP-ribosylated on glutamine **63A** (R=H, 4%) and **63B** (R=Biotin, 5%, α/β=65:35) were obtained in high purity through a combination of HP-LC and boronate affinity chromatography.

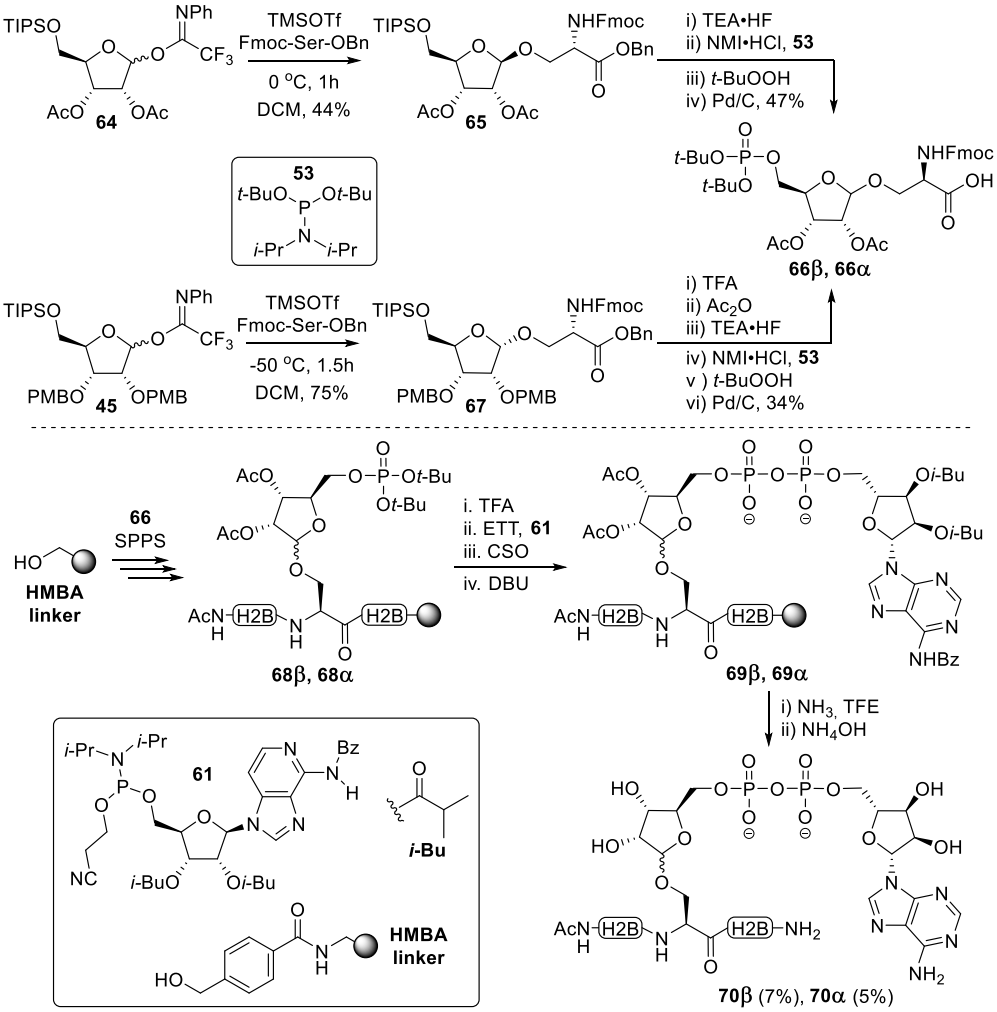


Scheme 3| ADP-ribosylation on asparagine, glutamine and citrulline via an updated methodology by Kistemaker and colleagues. Activator is *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBSOTf) for asparagine and citrulline, and $HClO_4$-SiO_2 for glutamine. Citrulline products were obtained as anomeric mixture: *(α/β=34:66), **(α/β=60:40) and ***(α/β=62:38). CSO = (1S)-(++)-(10-camphorsulfonyl)-oxaziridine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, ETT = 5-ethylthiotetrazole, HFIP = hexafluoro-*iso*-propanol, NMI = 1-methylimdazole and TFE = trifluoroethanol.

The anomeric ratio of biotinylated analogue **63B** clearly demonstrated the impact of the acidic deprotection conditions (5% TFA) on the isomerization of the interglycosidic linkage. ADPr-Asn **63C** (0.5%) and ADPr-Cit **63D** (0.2%, α/β=60:40) were purified with anion-exchange chromatography and the unsatisfactory yield of the latter two peptides was attributed to premature cleavage of the peptide during hydrazine treatment.

Voorneveld and colleagues expanded on the exploratory work of Kistemaker regarding the α-selective ribosylation of serine using a trifluoroacetimidate-based procedure, by preparing a β-directing donor (Scheme 4).[108] Acetimidate donor **64** could be derived from D-ribose in a similar fashion as the known α-selective donor **45**, but was equipped with acetyl groups instead of non-participating PMB ethers on the 2- and 3-OH positions. Upon activation of the donor with TMSOTf, the highly reactive oxocarbenium species that is formed upon activation of the donor with TMSOTf can be stabilized by the formation of a dioxolenium ion with the 2-OH acetyl moiety in a process referred to as neighboring group participation. As a result, a β-oriented attack of the nucleophile is highly favored. After acetyl migration to the serine aglycon[129] was minimized by optimization of the donor/activator ratio and reaction temperature, β-ribosylated serine **65** could be obtained in an acceptable 55% yield. Deprotection of the TIPS group with HF•TEA and the previously reported two-step phosphorylation procedure[127] with *t*-Bu protected amidite **53** was followed by hydrogenolysis to provide SPPS-building block **66β**. The α-selective introduction of Fmoc-Ser-OBn on donor **45** proceeded uneventfully,[124] but initial attempts to remove the PMB-ethers using varying concentrations of HCl in HFIP resulted in degradation of the newly formed *O*-glycosidic linkage. Fortunately, no side reactions were observed upon exposure of **67** to an excess of TFA and the freed 2- and 3-OH could be acetylated with acetic anhydride in pyridine without prior purification. The same sequence of reaction conditions described for the β-anomer could then be applied to provide its α-counterpart **66α**. Incorporation of building blocks **66β** and **66α** in hendecapeptide, originating from the *N*-terminus of histone H2B, and on-resin construction of the ADPr moiety based on the P^{V}-P^{III} coupling were established by adopting the protocol described by Kistemaker et al. (Scheme 3).[125] Cleavage from the resin using ammonia in TFE and a final deprotection with NH_4OH furnished the desired serine ADP-ribosylated peptides **70β** and **70α** after HP-LC purification in satisfying yields of 7% and 5% respectively.

The SPPS-based approach on HMBA-resin described above has facilitated the production of peptide fragments ADP-ribosylated on asparagine, glutamine, citrulline (Scheme 3) and serine residues (Scheme 4), but is limited to *C*-terminal carboxamide products and includes an extensive amount of protecting group shuffling on the peptide backbone in case of residues such as threonine, serine and glutamic acid. These drawbacks have been addressed in the improved methodology developed by Voorneveld *et al*, for which a series of α-configured ribosylated amino acids **74-76** were constructed through coupling of known acetimidate donor **46** with allyl ester derivatives of serine (**71**), threonine (**72**) and cysteine (**73**) (Scheme 5).[109]

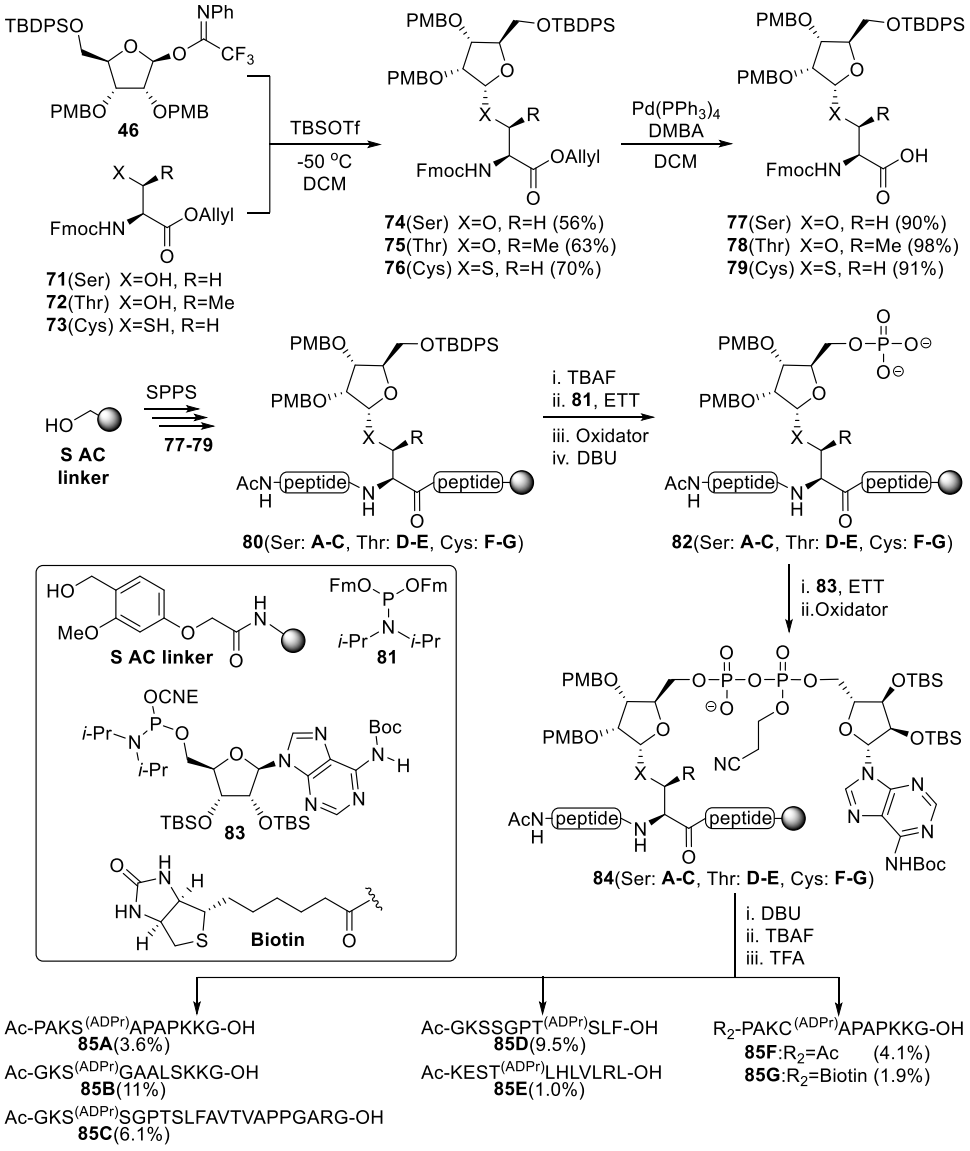


Scheme 4| Synthesis of an $H2B_{4\text{-}14}$ peptide fragment (full sequence = AcNH-PAK**S**APAPKKG-NH_2) with an α- or β-configured ADP-ribosylated serine residue by Voorneveld *et al.* CSO = (1S)-(++)-(10-camphorsulfonyl)-oxaziridine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, ETT = 5-ethylthiotetrazole, NMI = 1-methylimdazole, TEA = triethylamine TFA = trifluoroacetic acid, TFE = trifluoroethanol and TMSOTf = trimethylsilyl trifluoromethanesulfonate.

The convenient palladium(0)-catalyzed deprotection of the allyl ester in the presence of 1,3-dimethylbarbituric acid (DMBA) as allyl cation scavenger furnished building blocks **77-79** from their respective precursor in high yields. In contrast to the previous procedures, phosphomonoester formation was postponed until after SPPS and an highly acid-sensitive S AC linker was selected to provide *C*-terminal carboxylic acids after cleavage to more closely resemble naturally processed peptides. Building blocks **77**-**79** were incorporated in various

peptide sequences that have been identified in proteomics studies[130,131] through HCTU/DIPEA-assisted condensation on Tentagel resin pre-loaded with glycine via the previously mentioned S AC linker. Tetrabutylammonium fluoride (TBAF) was found to facilitate TBDPS removal of ribosylated peptides **80** most efficient with full conversion in less than 30 min, while HF•TEA and HF•pyridine required overnight shaking. Phosphoramidite **81**, decorated with base-labile 9-fluorenylmethyl (Fm) protecting groups, was coupled to the liberated primary 5-OH upon activation with ETT followed by CSO-mediated oxidation of the phosphite triester intermediate. The use of methylsulfonylethyl (Mse) protecting group was investigated as alternative to the Fm functionality, but the latter was preferred due to its more efficient deprotection by DBU. Construction of the pyrophosphate linkage was executed according to a two-step procedure analogous to the 5-OH phosphorylation to yield fully protected ADPr peptides **84**. Removal of the CNE moiety from the pyrophosphate and ensuing desilylation of the proximal ribose was followed by simultaneous deprotection and cleavage from the resin under acidic conditions. The thiol-based scavenger ethane dithiol (EDT) was added to the cleavage cocktail for ADP-ribosylated cysteine peptides to suppress migration of the PMB-cation to the cysteine aglycon. Overall, preparative HP-LC purification provided three distinct ADP-ribosylated serine peptides (**85A-C**), along with two fragments modified on threonine (**85D-E**) as well as ADPr-Cys (**85F**). The *N*-terminal biotin functionality, which facilitates streptavidin enrichment experiments, of ADPr-Cys **85G** was found to be oxidized during CSO treatment. Fortunately, chemoselective oxidation of the P^{III}-intermediates was achieved with the milder oxidizing agent *t*-BuOOH and enabled isolation of the final peptide.

Since a direct use of arginine as an acceptor in a glycosylation reaction is difficult to realize due to the basic nature of the guanidinium moiety, an alternative route towards Arg-ADPr was devised by Voorneveld *et al.*, who opted for the Lewis acid promoted reaction of an isothiourea riboside to ornithine (Scheme 6).[110] To this extent, full protected β-azido-ribofuranoside **87** was derived from commercially available ribofuranose tetraacetate over 4 steps, as described previously.[132] A Pt(IV)-catalyzed reduction of the anomeric azide resulted in a highly labile ribosylamine intermediate that was filtered over a pad of celite and immediately converted into an anomeric mixture of isothiocyanate **88** using thiophosgene. The obtained anomers could be conveniently separated by silica gel column chromatography and the α-anomer was subjected to a three-step procedure. First, thiourea intermediate was produced via ammonolysis, followed by protection of the newly introduced amine with the *tert*-butyloxycarbonyl (Boc) group and finally converted into the desired isothiourea **89** through alkylation of the sulfur atom with ethyl iodide. In order to test and optimize on-resin ADP-ribosylation, various model peptides **90A-C** were assembled on tentagel resin equipped with an acid-labile S AC linker using with an orthogonal alloc-protected ornithine installed at the modification site. After selective deprotection of the alloc-functionality with a palladium(0) catalyst, on-resin guanidinylation of ornithine could be efficiently established in the presence of silver nitrate as Lewis acid.

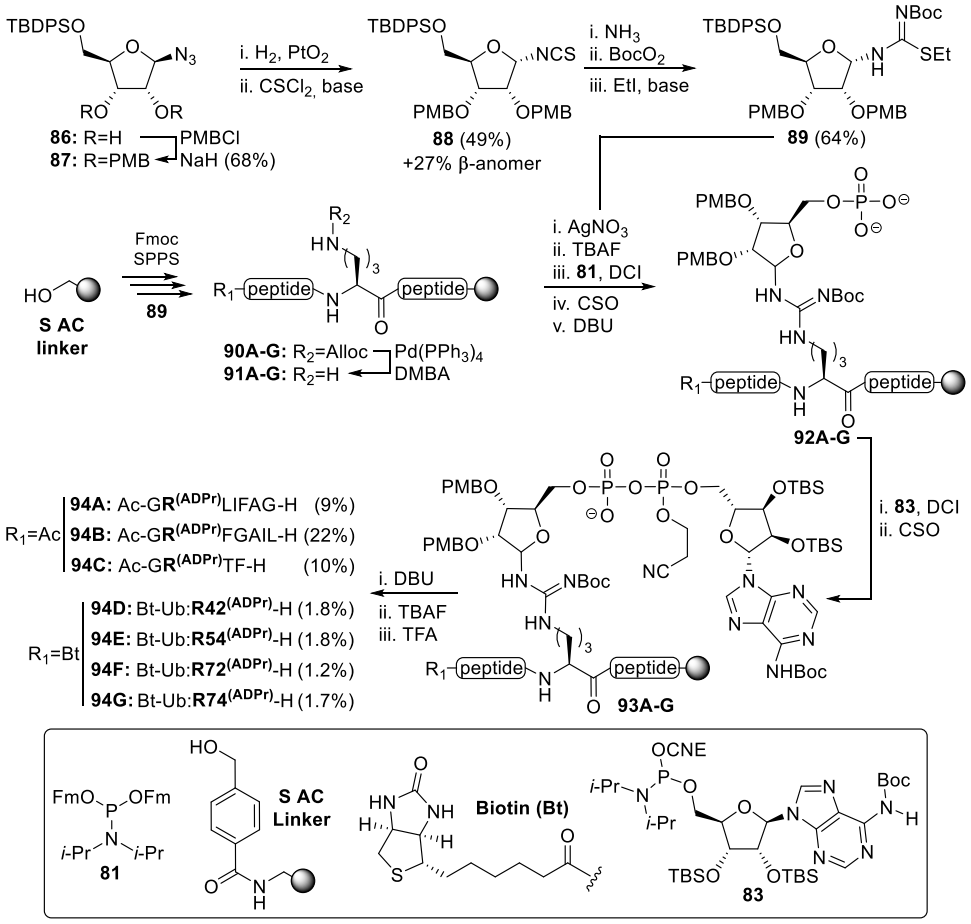


Scheme 5| An improved and comprehensive methodology for synthetic oligopeptides ADP-ribosylated on serine, threonine or cysteine side chains by Voorneveld *et al.* CSO was an suitable oxidation agent for peptides **A-F**, while the milder *t*-BuOOH was used for biotinylated peptide **G**. CSO = (1S)-(++)-(10-camphorsulfonyl)-oxaziridine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DMBA = 1,3-dimethylbarbituric acid, ETT = 5-ethylthiotetrazole, Fm = fluorenylmethyl, TBAF = tetrabutylammonium fluoride and TFA = trifluoroacetic acid.

Desilylation of the 5-OH with TBAF proceeded uneventfully, but the subsequent phosphorylation reaction via the established two-step procedure was hampered by phosphitylation of the guanidine group. Optimization of both the activator and amount of Fm-protected amidite **81**, revealed that DCI was superior over tetrazole and ETT in terms of selectivity and that unwanted phosphitylation could be further suppressed by reducing the original 5 equivalents to a 2.5-fold excess of the phosphoramidite. Ensuing deprotection of the Fm-groups using DBU provided phosphomonoester intermediates **92**, primed for the reaction with amidite **83** through the established P^{V}-P^{III} coupling. Identical deprotection and cleavage conditions as described above (Scheme 5) were applied to obtain ADPr-Arg peptides **94A-C** after preparative HP-LC in satisfying yields of 9-22%. Spectroscopic analysis indicated that the final products were obtained as anomeric mixture (α/β=6:4), which is in accordance with observations of spontaneous anomerization under neutral or acidic conditions made by Oppenheimer *et al.*[22] Then in collaboration with Kloet *et al.*, it was demonstrated that the silver-mediated guanidinylation methodology is not limited to oligopeptides, but is also applicable for the functionalization of whole proteins (Scheme 6). Full-length ubiquitin analogues **90D-G** were synthesized using SPPS with Arg-42, -54, -72 or -74 replaced with alloc-protected ornithine respectively.[133] All four proteins were ADP-ribosylated without major adjustments. However, significantly higher amounts of TFA (90% versus the original 10% used for peptides **94A-C**) were required to ensure complete removal of the Pbf protective groups from the remaining arginine residues. Interestingly, no degradation of the pyrophosphate nor the N-glycosidic bond was observed on LC-MS after prolonged reaction times of up to 1.5 h, suggesting that ubiquitin somehow stabilizes the modification. ADP-ribosylated ubiquitin proteins **94D-G** were successfully isolated by HP-LC in a 1.2-1.8% yield, but were contaminated with varying amounts (14-30 mol%) of their respective phosphoribosylated precursor. The observed stability of ADP-ribosylated ubiquitin under acidic conditions suggests that the formation of this side product is the result of the incomplete coupling of phosphates **92D-G** to adenosine amidite **83** and not due to acid-promoted degradation of ADPr.

Chemical synthesis of artificial ADP-ribose linkages

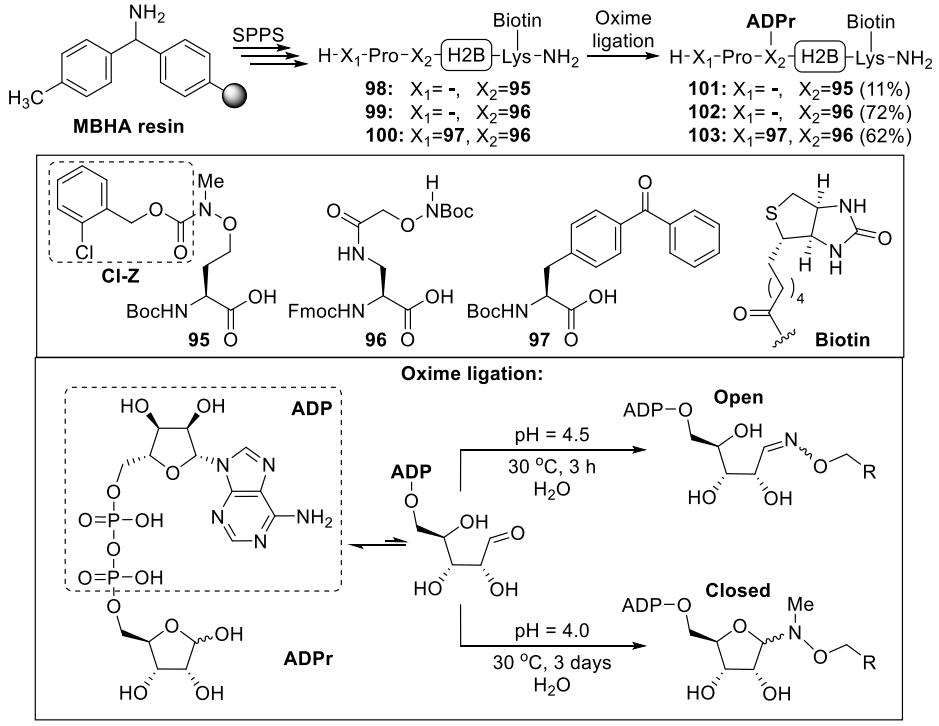
In addition to the modification and construction of native ADP-ribosylated amino acids, several strategies have been developed for the preparation of peptide conjugates that are site-specifically ADP-ribosylated via non-natural linkages. Over a decade ago, Moyle and Muir reported a methodology based on the chemoselective conjugation of free ADPr to aminooxy-containing amino acids **95** and **96** (Scheme 7) as a stable analogue for ester-linked ADPr.[112] Adducts derived from secondary alkoxyamines and reducing carbohydrates exclusively adopt a ring-closed form,[134] opposed to aminooxy groups that mainly generate a ring-opened system.[135] The *N*-methyl aminooxy building block **95** was derived from L-homoserine according to known literature procedures[136] and subsequently appended to the *N*-terminal fragment (3-19) of histone H2B, functionalized with a biotin moiety for streptavidin pull-down purposes, through Fmoc-based SPPS on a 4-methylbenzhydrylamine (MBHA) resin using HBTU/DIPEA. Ligation of



Scheme 6| The use of a lewis acid promoted conjugation between ornithine and isothiourea in the synthesis of arginine-linked ADPr oligopeptides and full-length ubiquitin as reported by Voorneveld *et al*. Alloc = *N*-allyloxycarbonyl, CSO = (1S)-(++)-(10-camphorsulfonyl)-oxaziridine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TBAF = tetrabutylammonium fluoride, TFA = trifluoroacetic acid and Ub = ubiquitin.

the resulting peptide **98** to ADPr was performed in a sodium acetated buffered system (0.5 M, pH=4.0) to render all lysine and arginine residues unreactive. Despite a large excess of ADPr, the reaction never reached completion with a mere 40% conversion after 3 whole days. The use of aniline (0.1 – 100 mM) as nucleophilic catalyst[137] only promoted non-specific reactions and lowering sodium acetate concentrations slowed down reaction rates and reduced product yields. As a result, an inadequate amount of ADPr conjugate **101** was obtained after preparative HP-LC for detailed biochemical evaluation. H2B peptide **99** was assembled with commercially available *N*-Boc-aminooxyacetyl **96** via the same procedure, while Boc-chemistry was used to incorporate **96** alongside benzophenone **97** in bifunctional peptide **100**. Addition

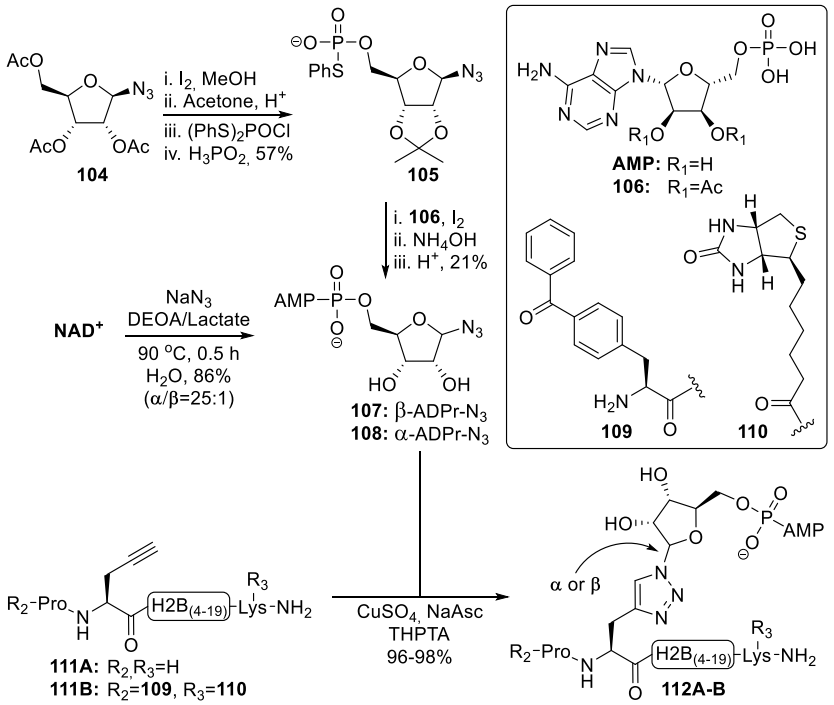
of the photo-induced cross-linker would enable covalent attachment of the probe to low affinity ADPr-binding proteins and possibly aid in the identification of new interaction partners. The conjugation of ADPr to peptides **99** and **100** was completed in less than 1 h and provided, according to spectroscopic analysis, a mixture of E/Z-configured oxime products **102** and **103** in a satisfying 72% and 62% yield respectively. Thus, demonstrating that oxime ligation under acidic conditions can be applied to efficiently produce ADP-ribosylated conjugates.



Scheme 7| Site-specific conjugation of ADP-ribose to aminooxy or N-methyl aminooxy functionalized histone H2B tail fragments by Moyle and Muir. Boc-SPPS was applied for the assembly of **98** and **99**, while Fmoc-based conditions were used for **100**. $H2B_{3\text{-}19}$ sequence = PAK SAPAP KKGSK KAVT.

Li *et al.* were the first to exploit the versatile Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) for the preparation of artificial ADP-ribosylated oligopeptides in a late-stage modification strategy with azido-ADPr analogues **107** and **108** as key components (Scheme 8). Initial efforts were focused on the design of a synthetic route towards β-N_3-ADPr **107** from its azido-ribofuranoside precursor **104**.[132,138] Deacetylation with iodine in methanol and subsequent introduction of the isopropylidene group under acidic conditions was followed by efficient phosphorylation of the 5-OH group with $(PhS)_2POCl$ in the presence of tetrazole as nucleophilic catalyst. Partial deprotection of the dithiophosphate intermediate with hypophosphorous acid in pyridine furnished phenylthiophosphate **105**, which was then subjected to the iodine-

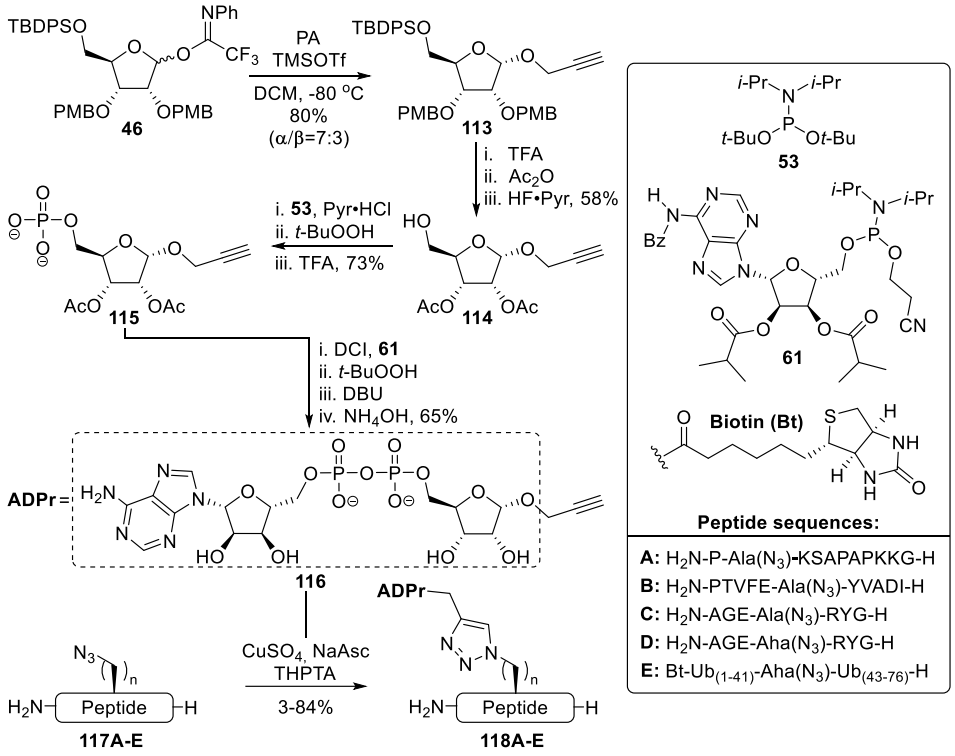
promoted condensation reaction with 2-,3-acetylated AMP **106**.[139] Separate treatments with NH_4OH and formic acid facilitated removal of the remaining acetyl and acetonide protecting groups respectively to furnish β-N_3-ADPr **107** in 12% overall yield over 8 steps after preparative HP-LC. Several years later, Li *et al* have developed a α-selective ribosylation reaction using ionic liquid/water system that enables direct conversion of NAD^+ into the biologically more relevant α-configured N_3-ADPr **108**.[111] The right mixture of cations and anions in ionic liquids have previously been shown to facilitate otherwise impossible chemical transformations through synergistic catalytic effects such as the stabilization of reactive intermediates via noncovalent interactions.[140,141] After optimization of the water content and reaction temperature, an elaborate screening of 54 different ionic liquid systems was performed in which the combination of diethanolamine/lactate was found to be superior in terms of α-selectivity. An increase in scale (0.5 gram) did not seem to reduce stereoselectivity nor reaction yield and provided α-N_3-ADPr **108** in an excellent yield of 86% (α/β=25:1) after a desalination column to remove excess sodium azide. The alkyne-modified amino-terminus of histone H2B **111A** as well as its bifunctional derivative **111B**, decorated with a biotin and photo-cross-linker, were selected as model peptides and were obtained commercially. During optimization of the click reaction conditions with phenylacetylene as test substrate, it was demonstrated that the *in situ* derived Cu(I)-species was most effectively stabilized by the tridentate ligand THPTA[142] in the employed water/ethanol/*tert*-butanol (2:3:5) solvent system, outperforming the TBTA[143] and BBTG[144] ligands. Application of the optimized CuAAC conditions efficiently conjugated H2B peptides **111** to α- or β-configured N_3-ADPr analogues and provided a total of four ADP-ribosylated peptide conjugates (**α-112A**, **α-112B**, and **β-112A** and **β-112B**) in near quantitative yields after preparative HP-LC purification.



Scheme 8| Modular synthesis of ADP-ribosylated histone H2B fragments via a CuAAC between ADPr-N_3 analogues and the target peptide with a propargylglycine at the modification site by Zhang and coworkers. DEOA = diethanolamine, $H2B_{4\text{-}19}$ sequence = PAK SAPAP KKGSK KAVT. DEOA = diethanolamine, NaAsc = sodium ascorbate and THPTA = tris(hydroxypropyltriazolylmethylamine).

Another general strategy for the post-synthesis introduction of a single ADPr moiety on peptides of interest using CuAAC was developed by Liu *et al.*, where in contrast to the work of Li described above, alkyne-ADPr analogue **116** was prepared and conjugated to an azide-modified peptide sequence of interest (Scheme 9).[145] The synthesis of clickable ADPr **116** was initiated with the condensation of propargyl alcohol with acetimidate donor **46**[124] and provided a mixture of anomers (α/β=71:29) that were separable by silica gel column chromatography. The biologically relevant α-anomer **113** was subsequently converted into acetylated ribofuranoside **114** via a series of protecting group manipulations in an overall yield of 58%. A two-step phosphorylation procedure with *t*-Bu protected amidite **53** was followed by immediate removal of the *t*-Bu protecting groups using TFA to furnish phosphomonoester **115**. Pyrophosphate construction was achieved via a DCI-promoted P^{V}-P^{III} coupling with suitably protected adenosine phosphoramidite **61** and subsequent oxidation of the resulting intermediate with *t*-BuOOH. Removal of the cyanoethyl group on the pyrophosphate with DBU and global deprotection of the ester-linked functionalities in NH_4OH finally provided propargyl-ADPr **116**. To assess the viability of the projected cycloaddition, short peptide fragments **117A-D** were synthesized using standard Fmoc-based SPPS conditions, incorporating the

noncanonical β-azidoalanine or β-azidohomoalanine at the site of modification. The reactive Cu(I)-species, which was prepared *in situ* from Cu(II)SO_4 with an excess of the reducing agent sodium ascorbate, was stabilized by tridentate ligand THPTA and added to the alkyne/azide mixture in tris(hydroxymethyl)aminomethane-buffered saline (pH=7.6). All four click reactions proceeded efficiently, with complete conversion in less than 1 hour, and furnished the desired ADP-ribosylated conjugates **118A-D** after HP-LC purification. To investigate the versatility of the established protocol for the MARylation of oligopeptides, full-length ubiquitin modified with a β-azidohomoalanine **117E** was synthetically derived.[133] The CuAAC reaction was successfully performed under identical conditions as described for the peptide fragments. The excess of propargyl ADPr **116** and click reagents were removed by dialysis, after which size exclusion chromatography gave the desired ubiquitin conjugate **118E** in high purity in an 84% yield.



Scheme 9| Preparation of triazole-linked ADPr peptides and full-length ubiquitin via a CuAAC between alkyne modified ADPr **116** and the target sequence **117**, equipped with an azide click handle. β-azidoalanine (n=1) and β-azidohomoalanine (n=2) are abbreviated as A(N_3) and Aha(N_3) respectively. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DCI = dicyanoimidazole, PA = propargyl alcohol, Pyr = pyridine, TFA = trifluoroacetic acid, THPTA = tris(hydroxypropyltriazolylmethylamine) and Ub = ubiquitin.

Overall, the collective of chemoenzymatic and chemical methodologies described here have covered a significant portion of the different MARylation possibilities over these last decades. Despite their limitation to a small subset of residues, enzymatic approaches have proven their worth in the scalable and highly efficient modification of serine and tyrosine residues, but more importantly, have enabled the construction of various full-length histones carrying a site-specific ADPr modification through native chemical ligation. An increased understanding of the mechanisms determining substrate selectivity of (ADP-ribosyl) transferases and, more specifically, the discovery of additional co-factors like HPF1 will potentially broaden the scope of accessible ADP-ribosylation sites in the future. It is noteworthy that enzyme-assisted approaches are, at present, indispensable because the preparation of PARylated oligopeptides[117] has not been attainable so far using fully synthetic means. A clear advantage of the convergent syntheses where ADPr moieties are introduced through CuAAC or oxime ligation is the ease at which a library of ADPr peptides can be generated from relatively few, and sometimes commercially available, precursors. However, results obtained with these artificially linked ADP-conjugates always have to be interpreted with caution. While the on-resin ADPr synthesis approach was initially highly laborious and troubled by the formation of contaminations that were difficult to remove, it has stepwise matured into an effective and versatile means towards MARylated peptides with the incorporation of the two-step P^{V}-P^{III} pyrophosphorylation chemistry and carefully optimized protecting group strategies. Since then, the SPPS-based methodologies have enabled the introduction of ADPr on a substantial fraction of the known ADP-ribosyl acceptor sites and will likely be a platform for new strategies towards the modification of currently missing residues such as tyrosine and histidine.

Aim and outline

The general aim of the research reported in this thesis was to develop new molecular tools to investigate ADP-ribosylation, NAD^+ metabolism and glycohydrolases involved in the turnover of ADP-ribosylated proteins.

Chapter 2 describes a convergent synthesis of 1,4-disubstituted triazole based isosteres of N(τ)-ADP-ribosylated histidine that are obtained through a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) between 1-azido-ADPr analogues and an oligopeptide carrying an alkyne click handle. Screening of the obtained conjugates against a small ADP-ribosyl hydrolase library revealed a slow but consistent cleavage of the N-glycosydic linkage for the Ser-ADPr glycohydrolase ARH3. **Chapter 3** continues on the work described in chapter 2 with an alternative, stepwise solid phase approach towards both 1,4- and 1,5-disubstituted triazole conjugates as isosteres for N(τ)- and N(π)-ADP-ribosylated histidine. Four building blocks were derived from α- and β-configured 1-azido-ribofuranosides and propargylglycine by exploiting Cu(I)- and Ru(II)-catalyzed click chemistry. The desired constructs were finalized using similar procedures as described in chapter 2 and afterwards compared to their respective histidine counterpart in terms of chemical and enzymatic stability. In **Chapter 4** the ribosylation of histidine using a Mukaiyama-type glycosylation under basic conditions is presented, which provided a total of three distinct mono-substituted products. The structure of the synthesized Fmoc-amino acids has been resolved through extensive spectroscopic analysis including NOE-measurements. Subsequent incorporation in a peptide of interest via SPPS followed by on-resin construction of the ADPr moiety provided well-defined ADP-ribosylated histidine peptides for the very first time. Their chemical stability as well as resistance towards (ADP-ribosyl)hydrolases have been evaluated. **Chapter 5** reports on an exploratory study on the synthesis of carba-ribose derivatives carrying a cyclic sulfate warhead as a possible electrophilic trap for common glycosidases. Various reaction conditions have been explored for the essential syn-dihydroxylation reaction and a Barton-McCombie deoxygenation was successfully applied to expand the collection of inhibitors with a 2-deoxy analogue. The inhibitory potential of the ribosyl-configured warheads on a set of glycosidases has been assessed in a fluorescent substrate assay. **Chapter 6** describes the further development of the β-configured cyclosulfate inhibitors from chapter 5 into potential one-step activity-based probes (ABPs) for the membrane-bound glycoprotein CD38. The synthetic approach includes a regioselective phosphorylation with a suitably protected phosphoramidite carrying an alkyne click handle and a final Cu(I)-catalyzed conjugation to an azido-functionalized fluorescent reporter. The desired ABPs were successfully isolated using HP-LC but remain to be tested in an assay with HL-60 lysates. Finally, **Chapter 7** summarizes the research described in this thesis and proposes some potential synthetic targets of interest in the fields of ADP-ribosylation and NAD^+ metabolism.

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