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

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Chapter 1

General Introduction

Biological background

ADP-ribosylation

Post-translational modifications (PTMs) enable proteins to exist in multiple isoforms, adding another layer of complexity and functionality to the protein-encoding genes. With over 300 PTMs known e.g. phosphorylation, glycosylation and acetylation, the activity or functionality of a given protein that is modified after ribosomal synthesis can be widely varied.[1] One such PTM is adenosine diphosphate ribosylation (ADP-ribosylation) which occurs by the transfer of β-nicotinamide dinucleotide adenine (β-NAD^+) onto a nucleophilic amino acid side chain of the protein target. Nicotinamide (Nam) is then expulsed, resulting in a covalent glycosidic linkage with the ribosyl moiety by inversion of stereochemistry leading to an α-configured ADP-ribose (ADPr) unit to the protein (Figure 1). ADP-ribosylation is catalyzed by the ADP-ribosyl transferase family (ARTs, otherwise known as PARPs[2]). After the initial installation of one ADPr unit (*mono*-ADP-ribosylation, MARylation), some ART family members are able to continue their transferase activity (*poly*-ADP-ribosylation, PARylation[3]) adding multiple ADPr units to the 2'-OH[4] of adenosine. In doing so, long polymers of up to 200 ADPr units can be achieved.[5–7] If PARylation occurs, the chain can be branched at the 2'-OH position of the ribosyl moiety,[4,8] adding to a complex occurrence in heterogeneity of PARylated and MARylated protein populations in the cell.

ADP-ribosylation is a dynamic PTM which can be reversed by several hydrolases. If a protein is PARylated, the protein is first trimmed down to its MARylated isoform by *poly*-ADP-ribosyl glycohydrolase (PARG),[9] capable of cleaving the glycosidic linkages of the ADPr chain but showing no activity towards MARylated proteins.[10] Depending on the amino acid that is modified with ADPr, ADPr-hydrolases (ARHs, e.g. ARH1 for arginine[11] (Arg), ARH3 for serine[9,12,13] (Ser)) then remove the final ADPr unit, returning the protein to its unmodified form.

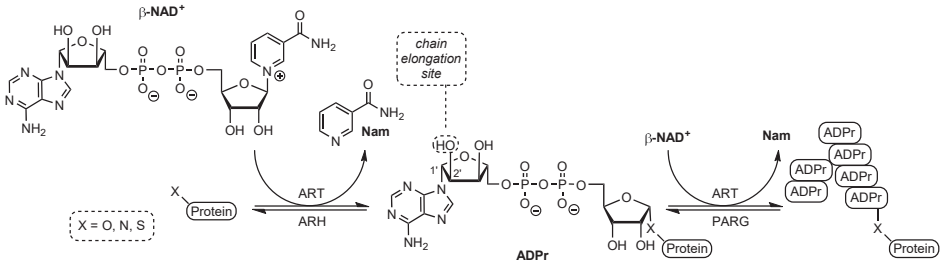


Figure 1. ADP-ribosylation of proteins on the side chains of nucleophilic amino acids by ARTs and their reverse reaction by PARG (in case of PAR) and ARHs (in case of MAR).

ADP-ribosyl transferases

ARTs are divided in subclasses based on two catalytic triad motifs[2,14]: H-Y-E and R-S-E containing transferases, both of which are structurally well conserved throughout viral, bacterial and mammalian ARTs. The H-Y-E motif was first encountered in *Corynebacterium diphtheriae* that secretes diphtheria toxin (DT).[15,16] DT is an ART that targets the diphthamide residue in elongation factor 2 with lethal effects[17] and carries this catalytic triad.[18] ARTs carrying the same H-Y-E motif are therefore named as diphtheria toxin-like ARTs (ARTDs). ARTs with the R-S-E motif are similarly named after cholera toxin (CT), an ART secreted by *Vibrio cholerae*, [19,20] and termed cholera toxin-like ARTs (ARTCs). In human cells, the ARTD subfamily is most abundant with seventeen family members, of which ARTD1 (commonly referred to as PARP1) is the most well studied.[5,21] Upon the detection of single stranded DNA breaks, PARP1 is activated and starts PARylating both itself and the core histones within seconds.[22] The resulting PAR chains are then involved in the recruitment of enzymes associated with DNA repair mechanisms.[23–26] Contrarily to ARTDs, which reside mostly in the nucleus but are found in several other compartments of the cell as well,[2,27] ARTCs are either membrane-bound (ARTC1, 3 and 4) or secreted to the exoplasm (ARTC5).[28–30] Despite these differences, mechanistically they work in a similar fashion (Figure 2). For the H-Y-E triad, encountered for example in human PARP1, the His862 residue provides hydrogen-bonding with the 2'-hydroxyl and exocyclic NH_2 on the adenosine part.[20,31,32] The Tyr896 engages in π-π stacking with the aromatic nicotinamide ring[32] and the Glu988 is involved in several processes i.e. polarization of the *N*-glycosidic bond of the distal ribose with Nam by hydrogen-bonding with its 2'-hydroxyl, stabilizing the hypothesized oxocarbenium ion[33–36] and proper positioning of the incoming nucleophile. This glutamic acid (Glu) residue is found in ARTCs carrying the R-S-E catalytic triad as well where it carries out the same function.[31,37–39] An Arg residue however provides electrostatic interactions with the pyrophosphate and the Ser helps the proper positioning of the nicotinamide ribosyl moiety by hydrogen bonds.[20,28–30] In both cases, the nucleophile is installed, forming an α-glycosidic bond with ADPr.

ADPr reversal

The importance of ADPr as a PTM is highlighted by *in vitro* studies of cells treated with ARTD inhibitors with lethal consequences backed up by *in vivo* studies with PARP1 knockout mice. [40] However, the reverse reaction can be considered equally important. The glycohydrolase PARG is solely active in the highly efficient turnover of PAR chains[9] and undegraded PAR chains, due to dysfunctional PARG, can drive neurodegeneration in Parkinson's disease[41] and embryonic lethality.[42] Another enzyme, called ARH3, was first identified in 2006 and also showed PAR degrading properties[43] albeit less efficient than PARG.[9,44] Its primary target later turned out to be Ser-linked mono-ADPr[12,13] but *O*-acetyl-ADP-ribose is efficiently hydrolyzed as well.[45] Not much is known about the substrate specificity

of ARH3 but crystal structures of this protein co-crystallized with different substrates were recently published[46] to provide insights into this process and the mechanism of hydrolysis. Unfortunately, progress in this area is hampered due to a lack of well-defined ADP-ribosylated material such as MARylated peptides. Synthetic, homogenous material can make for a great molecular tool to aid in this progress.

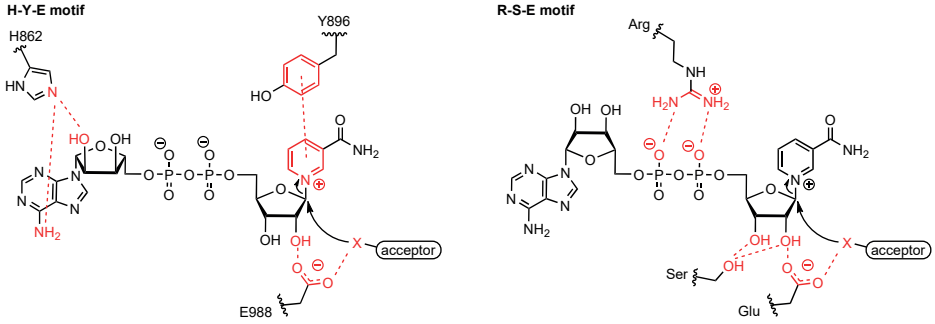


Figure 2. Mechanism of ADP-ribosylation by the ARTD class with the H-Y-E motif (left) of human PARP1 and the general ARTC class with the R-S-E motif (right). The interacting residues are colored red and the X signifies a generic nucleophile of the ADPr acceptor.

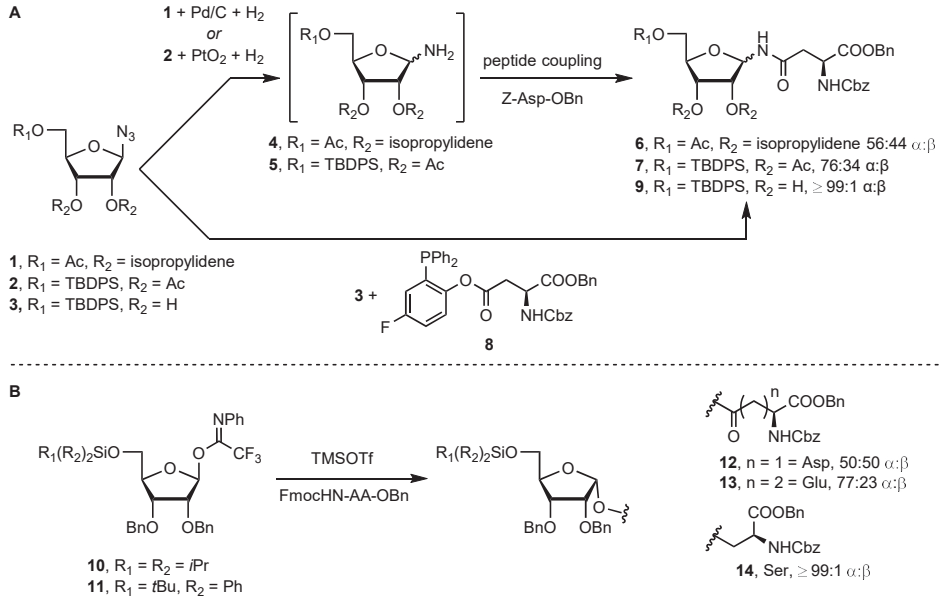
Synthetic studies on ADP-ribosylation

After its initial discovery, the PTM of ADP-ribosylation has gained increasing interest as area of biological research which led to a plethora of literature concerning its physiological role in the cell and mechanism of action.[5,20,40] In contrast, research on the acquirement of ADP-ribosylated biomolecules is still immature. Nevertheless, over the past decade, important steps have been taken to synthesize ADP-ribosylated molecules and their usefulness have been demonstrated in docking studies with macrodomain containing proteins, crystallization studies and MS-based proteomic studies as will be discussed in the next part.

α-Selective construction of ribosylated amino acids

ARTs are known to glycosylate their substrates with α-selectivity (see Figure 2). The first synthetic challenge towards ADP-ribosylated peptides is thus to construct a glycosidic bond that connects the (former) nicotinamidic ribosyl moiety with the amino acid side chain in an α-fashion. One of the earlier examples of ribosylated amino acids is found in the form of Asn-ribosyl **6** (Scheme 1A).[47] Therefore, the anomeric azide in **1** is reduced with Pd/C followed by a peptide coupling of the intermediate amine with a protected asparagine (Asn) giving a 56:44 α:β anomeric mixture. Although this procedure does yield

a decent amount of α-ribofuranosylated Asn **6**, van der Heden van Noort *et al*. successfully increased the α-selectivity of this reaction sequence by both careful kinetic control of the reduction step (10 °C and PtO_2 instead of Pd/C) and a change in the protecting group pattern on the 5' OH to a more bulky silylidene group.[48] These changes increased the α-selectivity, allowing the formation of **7** which was ultimately used for the construction of the first, synthetic MARylated peptides (discussed in more detail later). Although **7** can be obtained in sufficient amounts via this peptide coupling approach, Nisic *et al*. employed a different strategy to equip glycosyl azides with anomeric amides. In this method the glycosyl azide **3** is reduced with a phosphine, appropriately functionalized with an acyl group, via a Staudinger ligation.[49] By removing the β-steering acetyl groups and treating **3** with functionalized phosphine **8**, ribosylated Asn **9** was furnished in a fully α-selective fashion.[50]



Scheme 1. Stereoselective ribosylation of amino acids via **A:** reducing an anomeric azide followed by peptide coupling or Staudinger ligation or **B**: glycosylation of a ribofuranosyl donor with an amino acid acceptor.

Although the above-described method works well for the synthesis of *N*-ribosylated Asn and glutamine, another strategy has to be developed for the synthesis of *O*-ribosylated amino acids. This prompted Kistemaker *et al*. to develop a different approach relying on the glycosylation reaction, providing a more universal approach and an overall enhancement of stereoselectivity (Scheme 1B).[51] The steric bulk of a 5'-OH silyl protecting group in

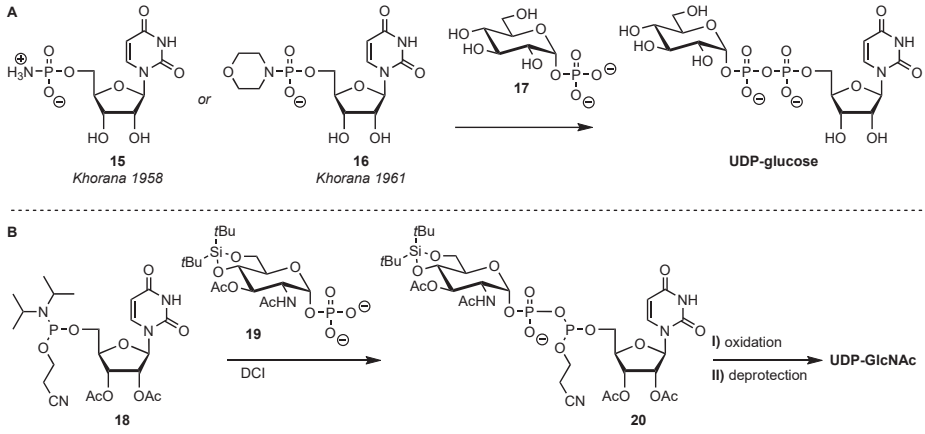
combination with benzyl ether protection of the 2'- and 3'-OH groups, promote α-selective glycosylations,[52,53] which has led to the choice of donors **10** and **11**. The glycosylation procedure was optimized by varying the protecting group patterns on the acceptors and donors as well as the use of different activators (TMSOTf, $HClO_4$-SiO_2), temperatures (-50 °C to room temperature) and solvent systems. Ultimately, these experiments led to an efficient glycosylation procedure for the α-selective formation not only of *N*-glycosylated ribofuranosides (Asn/Gln) but also of *O*-ribosylated **12** and **13** (Asp/Glu) and in particular **14** (Ser). Although α-selectivity is lost in the case of *O*-ribosylated **12** (Asp), column chromatography allowed for the isolation of the α-anomer and this is the first reported synthesis of ribosyl building blocks functionalized with Asp. Ser, although not officially confirmed as ADPr acceptor at that time, was also glycosylated with donors **10** and **11**, giving solely the α-anomer in both cases. Thus, with these results Kistemaker *et al.*[51] paved the way for the generation of a wide variety of both *N*- and *O*-ribofuranosylated amino acid building blocks, which can be used for synthesizing ADP-ribosylated peptides.

Synthesis of the pyrophosphate moiety

The second challenge in the synthesis of ADP-ribosylated molecules is the introduction of the non-symmetric pyrophosphate bridge, connecting the 5'-OH of adenosine with the 5-OH of ribosyl residue. Although a variety of methods are known to construct pyrophosphates, they can roughly be divided into two categories: i) coupling of a phosphate monoester to a different, activated phosphate ester which will henceforth be regarded as P^V – P^V coupling and ii) coupling of a phosphoramidite to a phosphomonoester (P^{III} – P^V coupling).

The P^V – P^V coupling method was pioneered by the group of Khorana who first used phosphoramidates, such as **15** to construct non-symmetric pyrophosphates in the form of UDP-glucose and FAD (Scheme 2A).[54] Not long after, they improved their method by the use of phosphormorpholidate (such as **16**) as activatable intermediate.[55] The employment of phosphormorpholidates has become the foundation of pyrophosphate chemistry ever since as is evidenced by the improvements and optimizations still being reported today (partly reviewed elsewhere[56]). The P^{III} – P^V coupling, is inspired by the extensive use of phosphoramidites in the synthesis of oligonucleotides. Early-stage development of oligonucleotide synthesis involved the condensation of deoxynucleosides with dichlorophosphines,[57,58] a procedure that was hampered by the need of temperatures of -78 °C and the formation of unwanted symmetrical and oxidized side products. The group of Caruthers came with the solution in the form of nucleoside phosphoramidites[59,60] as shelf stable synthons that provide fast reaction rates at room temperature. Shortly after their introduction, protocols with nucleoside phosphoramidites became the method of choice for automated oligonucleotide synthesis[61] and have been employed for solution-phase synthesis of nucleotide analogues as well.[62] Gold *et al.* were the first

who implemented phosphoramidites in the synthesis of pyrophosphates.[63] To efficiently obtain nucleotide sugars as substrates of glycosyltransferases, they synthesized protected 5'-phosphoramidite uridine nucleoside **18** and coupled it with the *tetra*-butylammonium salt of protected GlcNAc phosphate **19** (Scheme 2B). The resulting phosphate-phosphite intermediate **20** is then oxidized and deprotected resulting in the isolation of UDP-GlcNAc in 76% in a one-pot reaction sequence. This method of pyrophosphate construction results in a cleaner reaction profile as no unwanted symmetric pyrophosphates is formed.

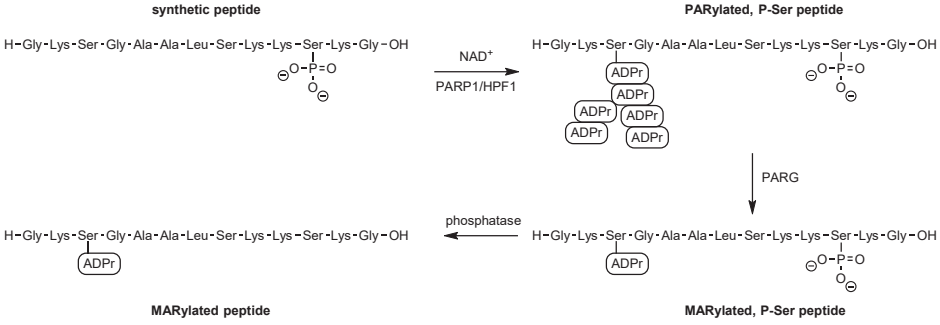


Scheme 2. Pyrophosphate construction in UDP derivatives via **A)** P^{V} – P^{V} coupling methods as pioneered by Khorana and co-workers[54,55] or **B)** P^{III} – P^{V} method as was developed by Gold *et al.*.[63]

Synthetic ADP-ribosylated peptides

As mentioned above, the availability of homogenous ADP-ribosylated biomolecules can greatly assist in studying ADP-ribosylation, either for crystallization studies, proteomics or modified ADPr analogues as molecular tools. Therefore, in the past decade a lot of research has been done towards the synthesis of fragments of biomolecules involved in ADP-ribosylation e.g., MARylated peptides, PAR chains with a well-defined chain length, trimeric branching point of ADPr chains or PARG substrates, all bearing their own synthetic challenges. In this framework, two main strategies can be discerned: i) the (chemo)enzymatic approach in which enzymes are utilized to generate ADP-ribosylated material *in vitro* and ii) a fully synthetic approach whereby protected building blocks are first synthesized and subsequently used to generate well-defined and homogenous ADP-ribosylated material. The next part will discuss the efforts made thus far to obtain synthetic ADP-ribosylated biomolecules with a focus on native MARylated peptides and analogues thereof.

When utilizing ARTs to generate ADP-ribosylated material *in vitro*, some inherent problems arise. PARP1 is a ubiquitous nuclear protein with a broad substrate selection that synthesizes PAR chains with varying polymer lengths and introducing seemingly random branching points. Therefore, a common practice in the field of studying ADP-ribosylation is the use of PARP1 followed by a second step where the proteins are treated with PARG to effectively trim down the chain length to furnish the MARylated proteins. However, PARP1 also modifies multiple amino acid side chains complicating the resulting proteomic mixture. The group of Matic elegantly addressed this problem by exploiting the recently found substrate selectivity of the PARP1:HPF1 complex towards Ser.[64,65] They used synthetic peptides found to be ADP-ribosylated with the aid of proteomics and incubated them with the PARP1:HPF1 complex and NAD^+ (Scheme 3).[66] The KS motif is specifically recognized by the PARP1:HPF1 complex[67,68] and ADP-ribosylates the Ser residue.[69] Incubation by PARG to trim the PAR chain furnished the chemoenzymatically generated MARylated peptide. Unwanted ADP-ribosylation of other Ser-residues are countered by protecting these Ser-residues with a phosphate group. In this case, a phosphatase treatment is incorporated to hydrolyze the phosphate monoesters in order to liberate Ser side chain residues.

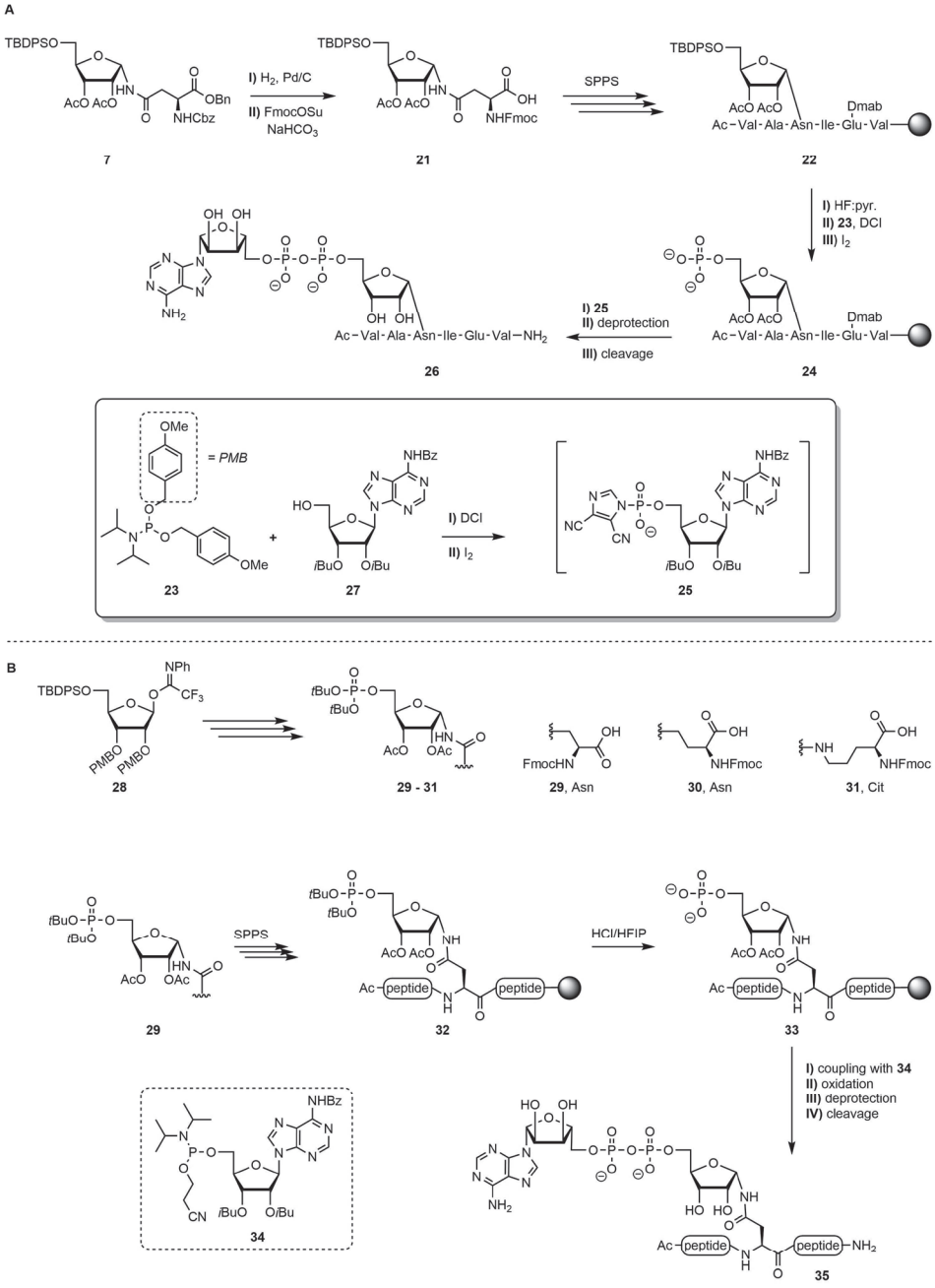


Scheme 3. Chemoenzymatic approach towards MARylated Ser-ADPr peptides developed in the group of Matic.[66]

Chemical synthesis can provide well-defined material and several studies have successfully employed fully synthetic MARylated peptides, PAR oligomers or branch points as molecular tools to study ADP-ribosylation.[70] The first report of fully synthetic MARylated peptide material originated from 2010 when van der Heden van Noort *et al.* developed ribosylated Asn building block **7** (described above, see Scheme 1A and a similar Gln analogue) to synthesize MARylated peptide **26**, Scheme 4A. To make **7** suitable for solid phase peptide synthesis (SPPS) the benzyl ether and Cbz-protecting group were removed by dehydrogenation and the released amino function was selectively masked with an Fmoc-group to furnish building block **21**. Implementation of ribosylated Asn **21** in

standard Fmoc-based SPPS led to immobilized hexamer **22**. Next, a phosphomonoester was introduced at the 5-OH of the ribose residue by desilylation, phosphitylation with phosphoramidite **23** and treatment with iodine which oxidized not only the intermediate phosphite but deprotected the resulting phosphotriester as well, to give immobilized phosphoribosylated **24**.[71] To install the ADP moiety, the phosphomonoester **24** was treated with *in situ* prepared phosphoramidate **25** and finally the resulting peptide was subjected to a set of deprotection conditions and finally cleavage from the resin, yielding the first fully synthetic ADP-ribosylated peptide **26**.

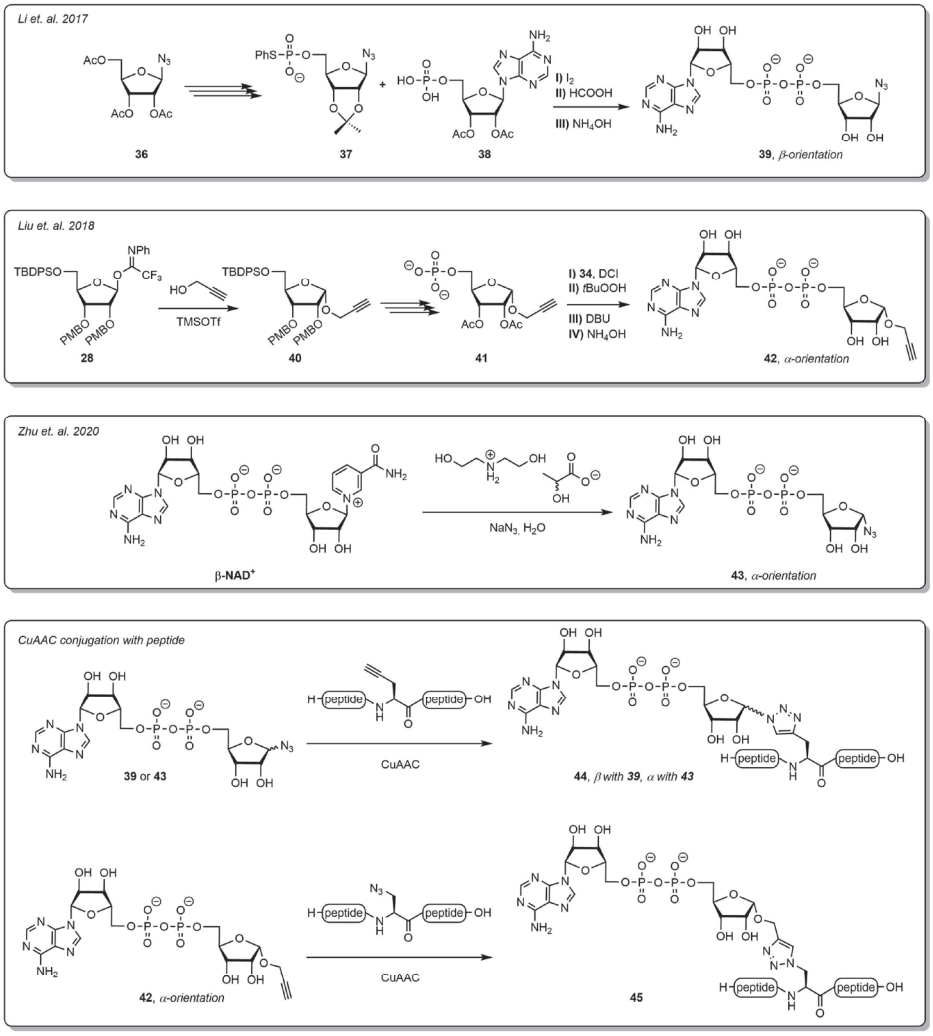
Kistemaker *et al.* furthered the field of synthetic ADPr peptides by providing the ribosylated amino acid building blocks at the 5'-OH with a tert-butyl protected phosphotriester, circumventing on-resin phosphorylation (**22** to **24**) and thereby avoiding the unwanted formation of an H-phosphonate as a result of hydrolysis during the iodine mediated oxidation.[72] To obtain these newly designed amino acid building blocks, ribose donor **28** (Scheme 4B) was coupled with the side chains of appropriately protected Asn, Gln and Cit, the latter of which can serve as an isostere for Arg. As described above, this glycosylation was also optimized to get sufficient α-selectivity and yield.[51] A multi-step synthesis route then furnished Asn, Gln and Cit building blocks **29** – **31** respectively, all of which prove to be compatible with standard Fmoc-based SPPS. Application of building blocks **29** – **31** in SPPS resulted in the formation of immobilized peptide **32**. The phosphotriester in **32** is converted to phosphomonoester **33** using HCl/HFIP, a process that conveniently was monitored by ^{31}P-NMR where phosphotriester **32** (δ≈10 ppm) shifts to phosphomonoester **33** (δ≈0 ppm). Next, the pyrophosphate moiety was introduced on-resin by adaptation of the P^{III} – P^{V} procedure of Gold *et al.* (see Scheme 2B). Accordingly, adenosine phosphoramidite **34** was coupled with immobilized phosphomonoester **33**, followed by oxidation of the phosphate-phosphite intermediate with CSO and finally removal of the cyanoethyl group with a DBU solution. To isolate the MARylated peptide the resin was then treated with a saturated NH_3 solution in 2,2,2-trifluoroethanol, resulting not only in removal of both the acetyl and iso-butyryl (*i*Bu) esters but also in cleavage from the solid support by the selective formation of the carboxamide at the *C*-terminus of the peptide. Addition of aqueous NH_4OH to the cleavage cocktail was needed to ensure complete removal of all protecting groups (i.e., Bz on the adenine ring and also possible side-chain protecting groups like trifluoroacetyl on lysine (Lys) residues) yielding generic peptide **35**. In this way, a variety of peptides was synthesized, MARylated on a Asn, Gln or Cit residue.



Scheme 4. Synthetic MARylated peptides on Asn, Gln or Cit residues via the **A)** van der Heden van Noort procedure[48] or **B)** the Kistemaker approach.[72]

Triazoles have been reported as isostere for the side chain functional group of Arg and as functionality by which generic ADP-ribosylated peptides can be obtained.[73–75] Triazoles are the products of copper catalyzed azide alkyne cyclo-additions (CuAAC), a bio-orthogonal reaction often employed in the field of chemical biology. In 2017, Li *et al.* used b-azide **36** (Scheme 5), a known ribose derivative (see also the synthesis of **1** – **3** in Scheme 1) to prepare phosphorothioate **37**.[74] Upon activation of phosphodiester **37** with I_2 in the presence of adenosine phosphate **38**, the pyrophosphate was formed and ensuing deprotection of the intermediate furnished β-azido-ADPr **39**. Guided by the fact that all known ADPr modifications are α-selective, Zhu *et al.* used an approach that deviates from conventional synthetic strategies (e.g. masking reactive groups with protecting groups) and they developed a reaction whereby a nucleophilic azide directly displaces the nicotinamide ring on β-NAD^+, mimicking the ART enzymatic reaction.[75] They found that the attack of a nucleophile could be steered towards the α-face of β-NAD^+ by the use of ionic liquids. After careful screening of 54 ionic liquids, they found that a mixture of 5:1 H_2O:[diethanolamine-lactate] resulted in the formation of a 24:1 α:β mixture which could be separated by prep-HPLC purification, yielding azide **43**. Next, oligopeptides provided with propargylglycine at the prospected modification site were synthesized and CuAAC reaction of these peptides with azides **39** or **43** in the presence of $CuSO_4$ yielded generic *N*-ADP-ribosylated peptide **44**.

Liu *et al.* reported an alternative approach in which an oligopeptide is provided with an azide at a predetermined position and the ADPr building block is functionalized with an alkyne.[73] In a high-yielding reaction, donor **28** was coupled with propargyl alcohol to give the α-configured alkyne **40**. A set of protecting group manipulations then gave phosphomonoester **41** which was used in a one-pot, three-step (coupling, oxidation and deprotection) P^{III} – P^{V} procedure with adenosine amidite **34** (see Scheme 4) to yield α-ADPr alkyne **42**. SPPS using azido-alanine furnished oligopeptides with an azide at the selected site of modification. CuAAC reaction of these peptides with ADPr building block **42** in the presence of $CuSO_4$ gave *O*-ADP-ribosylated peptide **45**. Liu *et al.* also synthesized a derivative of ubiquitin (Ub), [73] a 76 amino acid protein with azido-homoalanine on the position of Arg-42 (a known ADPr modification site) and upon CuAAC with **43**, homogenous triazole linked Ub-ADPr was isolated. This modified Ub-ADPr was recognized by ADPr-specific antibodies showing that although the triazole link is not native, it can be recognized as such in biological systems.

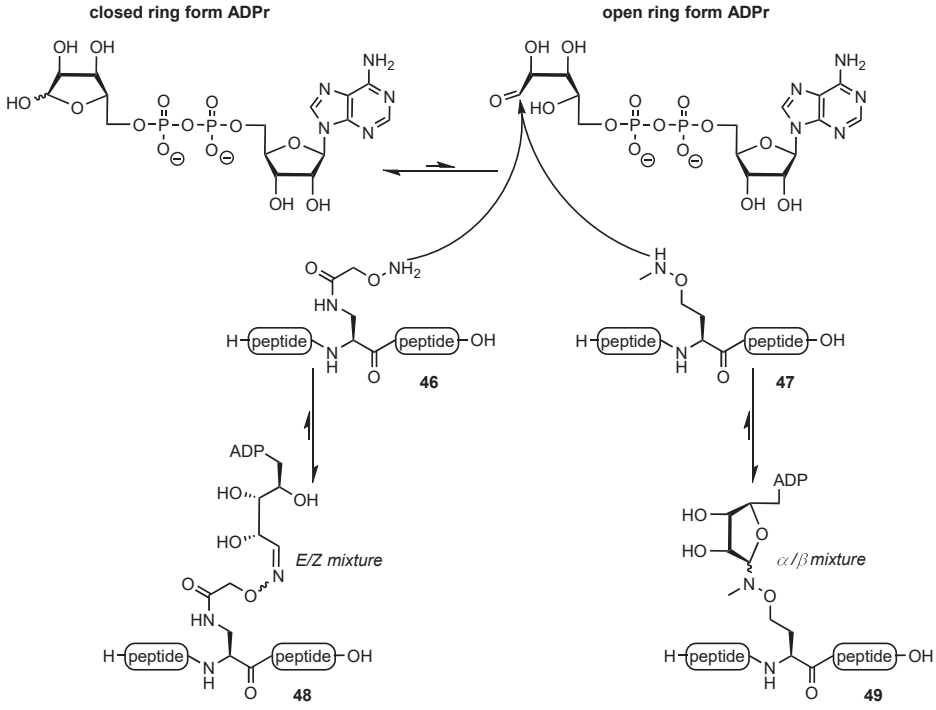


Scheme 5. Synthesis of ADPr building blocks **39**, **42** and **43** and their conjugation to synthetic peptides *via* click chemistry.

The ability to conjugate carbohydrates to peptides through oxime formation[76,77] was applied by the group of Muir to develop a chemoselective ligation method of aminooxy containing peptides with ADPr, producing another mimic of ADP-ribosylated peptides.[78] Oligopeptides corresponding to the *N*-terminus of human histone H2B were assembled with SPPS. The Glu residue in these peptides, a putative site of ADP-ribosylation, was replaced by either a protected aminooxy-containing amino acid or *N*-methyl aminooxy-containing amino acid to give peptides **46** and **47** respectively (Scheme 6). Reaction of ADPr with aminooxy **47** resulted in the formation of an E/Z mixture of the open ring isomer **48**.

On the contrary, reaction of the *N*-methyl aminooxy function in **47** with ADPr, resulted in an anomeric mixture of *N*-ADP-ribosylated peptide **49**. Chemoselectivity was achieved by performing the ligation reaction at slightly acidic pH values (around 4.5) as Lys and Arg are protonated at this pH value and therefore incapable of imine formation.

Some of the prepared ADPr peptides, equipped with a biotin tag, were applied in a pull-down assay with macrodomain containing proteins and it was found that peptide **48** efficiently pulled down mH2A1.1 (a macrodomain containing histone) and is thus able to engage in macrodomain binding. This encouraged a further investigation as to the ability of peptide **48** to bind macrodomains in complex biological systems. Nuclear HeLa cell lysates containing mH2A1.1 were treated with **48** equipped with biotin and a photo-crosslinker. After UV-irradiation to form a covalent bond, **48** was indeed able to pull down mH2A1.1 in a biological system demonstrating the usefulness of such material.



Scheme 6. Synthesis of ADP-ribosylated peptides via the oxime ligation approach. ADP = adenosine diphosphate.

Aim and outline of this Thesis

While in the past decade significant advances have been made towards synthesizing MARylated peptides, most of them however lack the native chemical linkage. Chapter 1 gives a concise summary on the developed methodologies thus far. For instance, MARylated peptides were prepared with Asn and Gln as relative stable Asp and Glu bioisosteres to prevent migration of the anomeric ester linkage to the 2'-OH position of the ribose residue.[48,72] In addition, artificial MARylated peptides, in which the ADPr residue is linked to the peptide via a triazole moiety were obtained via CuAAC, a reaction that is compatible with all natural amino acids.[73–75] Another type of artificial ADPr-peptides is the product of a chemoselective oxime ligation. MARylated peptides were prepared with either E/Z isomeric open chain ribose or an α/β-mixture of ribofuranose *N*-glycoside.

The recent discovery that Ser is the major target of PARP1 in response to DNA damage[79,80] was an incentive to develop an efficient synthetic route to peptides ADP-ribosylated on Ser. Chapter 2 describes the synthesis of phosphoribosylated Ser building blocks that are compatible with modified Fmoc-based SPPS, in which alkali sensitive semi-permanent protections are used to preserve the integrity of the ADPr moiety. With the objective to establish the native configuration of the anomeric centre of the ribose, both α- and β-configured building blocks were synthesized. With these building blocks, ADP-ribosylated peptides with either the α- or the b-epimer incorporated were prepared and used to determine that PARP1 synthesizes Ser-ADPr in an α-selective manner.

Because the alkali sensitive protecting groups are difficult to apply for the common amino acid side chain protections in SPPS, the approach from Chapter 2 is limited. Chapter 3 deals with a new synthetic methodology that addresses these limitations using acid sensitive semi-permanent protections, both for amino acid side chains as well as the 2- and 3-OH of the distal ribose. Moreover, this methodology provides less labor-intensive steps toward the ribosylated Fmoc-building blocks necessary for solid-phase synthesis and gave access not only to Ser-, but also to Thr- and Cys-ADP-ribosylated peptides. With the aid of these peptides the first Cys-ADPr specific hydrolase, the *Streptococcus pyogene* encoded protein *Spy*MacroD, was identified.

Unfortunately, the newly developed strategy proved not to be suitable for the synthesis of ADP-ribosylated peptides modified on Tyr-residues, a problem attributed to the heightened acid-sensitivity of the phenolic Tyr-ADPr glycosidic linkage. A solution was found by combining the strategies described in Chapter 2 and Chapter 3. The resulting

'hybrid' strategy uses alkali sensitive protections for the adenine base and proximal ribose residue from Chapter 2 whilst keeping the acid sensitive amino acid side chain protections from Chapter 3. The hybrid strategy described in Chapter 4 was highly efficient in synthesizing peptides, MARylated on a Tyr-residue.

Although Arg is a canonical target of ARTs, synthetic efforts have thus far been solely aimed at biomimetic Arg-ADPr linkages. Chapter 5 describes the first synthetic strategy capable of synthesizing the native Arg-ADPr linkage. With this newly developed methodology, not only oligopeptides MARylated on Arg but also Ub, ADP-ribosylated on Arg42 have been synthesized. This is the first description of a fully synthetic and functional ADP-ribosylated protein.

Recently, it has also been discovered that nucleic acids with a terminal phosphate monoester can be ADP-ribosylated *in vitro*.[81–86] Not much is known yet about the substrate specificity and which ARTs are responsible for this phenomenon. Chapter 6 is focused on the synthesis of ADP-ribosylated nucleotides in order to provide valuable chemical tools to further the studies on this newly discovered target of ARTs.

Chapter 7 deals with the design and synthesis of potential activity-based probes (ABPs) to target the membrane receptor CD38, which catalyzes the formation of cyclic ADPR from NAD^+. The structures of these ABPs are derived from NAD^+, and bear an epoxide or aziridine as a nucleophilic trap to covalently bind the active site which will allow for activity-based protein profiling (ABPP). In Chapter 8, the development of two photoaffinity probes for PARP1 is described. These probes are equipped with a diazirine containing photo affinity linker to perform photo cross linking of the probes with PARP1. The probes are used on living cells and after treatment of the cells with UV-light, PARP1 is efficiently visualized by in-gel fluorescence or pulled-down for MS-based proteomic studies. The final chapter summarizes all the research results obtained and outlines the directions of the future research.

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