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Synthesis of well-defined ADP-Ribosylated biomolecules

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Chapter 1

General Introduction

1.1 Introduction

The human proteome is estimated to have as many as one million different proteins originating from only 20,000 or so protein-coding genes.^{1,2} This is possible because one gene may give rise to a large number of proteins via processes like post-transcriptional splicing and base changes, or post-translational modifications (PTMs). These PTMs are chemical modifications at certain amino acids via one or multiple enzyme catalyzed reactions and are crucial for the functioning of proteins. PTMs play a critical role in the regulation of many cellular processes by influencing protein properties such as protein folding, activity, function and stability. There are over 200 different types of PTMs known to date and the list of modifications is still growing.^{3,4} The five most commonly observed modifications are glycosylation, alkylation, phosphorylation, oxidation and acylation. Glycosyltransferase enzymes are responsible for linking saccharides to proteins and this results predominantly in *N*-glycosylated and *O*-glycosylated proteins. The carboxamide side-chain of asparagine groups is used for *N*-glycosylation whereas *O*-glycosylation occurs on the hydroxyl side-chain of threonine and serine groups. Glycosylation is a reversible PTM and plays an important role in processes like protein-protein interactions, immune responses, cellular stability and signalling. Alkylation is a PTM that can be subdivided into two classes being methylation and prenylation. Methylation typically occurs at the side-chains of lysine or arginine in which lysine can become methylated up to three times and arginine two times. Methylation not only plays a big role in epigenetic regulation but has a wide ranging impact on health and disease in general. A few examples of processes influenced by this PTM are cellular RNA synthesis (histone methylation), postnatal developments, tumorigenesis, inflammation and immune responses. Prenylation involves the transfer of much larger alkyl groups to the side-chain of cysteine and these are either farnesyl (C₁₅) or geranylgeranyl (C₂₀) lipids. These lipids control the subcellular localization of the protein to the membrane and this PTM is irreversible. Protein kinases, the largest class of PTM enzymes, are responsible for phosphorylation of proteins and phosphatase enzymes catalyse the dephosphorylation reaction. The most commonly targeted amino acids are serine, threonine and tyrosine. The introduction of the charged, dianionic phosphate group changes the conformational structure of a protein and has an enormous impact on cell physiology (growth, cell cycle, apoptosis). This PTM has been studied in great depths and is linked to numerous diseases such as Parkinson's (dysregulated phosphorylation), cancer and immune disorders (abnormal phosphorylation of NF-κB cascade) and more specifically lung cancer (phosphorylation of Mcl-1 induced by nicotine). Oxidation of proteins is an irreversible PTM and typically result from excessive oxidative stress in a biological system. This may lead to non-functional proteins which in turn plays a role in many diseases, for example autoimmune diseases and cancer. Finally, acylation covers all PTMs in which an acyl linkage is introduced in the protein and the most commonly

observed acylations are acetylation, myristoylation (C_{14}), palmitoylation (C_{16}) and ubiquitylation. One example of acetylation is the acetylation and deacetylation of lysine residues in the N-terminal tail of histone proteins which is a crucial part of gene regulation. Modification of proteins with a myristoyl or palmitoyl lipid group directs the protein to the membrane interface. Here it is involved in protein-protein interactions, cell trafficking and signal transduction pathways. Ubiquitylation is the transfer of the small ubiquitin protein (8 kDa) to the side-chain of a lysine residue to form a *mono*-ubiquitylated protein. A protein becomes destined for proteosomal degradation when four or more ubiquitin proteins are tandemly linked to form a *poly*-ubiquitylated protein. Ubiquitylation plays a crucial role in almost all cellular processes (e.g. DNA repair, apoptosis, immune response, cell proliferation and differentiation, and stress response) and in many diseases (e.g. neurodegenerative, and cancer).

A protein can undergo one or multiple PTM's resulting in a specific and unique response and functionality.^{5,6} The large number of possible modifications, multitude of possible modification patterns and the highly dynamic nature of most of these PTMs makes it hard to study the effect and function of each individual PTM. Although the continuously increasing sensitivity of mass spectrometers allows for the mapping of the different PTMs, the availability of well-defined molecular structures is often essential to study the biological effect of PTMs. This can be done by either utilizing synthetic, well-defined, oligopeptides modified with the post-translational modification of interest or by utilizing synthetic, full length, post-translational modified proteins.⁷ An organic synthesis approach toward such entities also offers the opportunity to append a marker such as a fluorophore or affinity tag in order to study the effect of the PTM in more detail.

This thesis focuses on a more elusive and considerably less understood PTM, adenosine diphosphate ribosylation (ADP-ribosylation), and describes the synthesis of well-defined substructures corresponding to this PTM. The availability of these molecules allows for a more detailed investigation of the roles and functions of ADP-ribosylation and will contribute to a better understanding of this intriguing PTM.

1.2 ADP-ribosylation

The first observation of ADP-ribosylation, without knowing the exact structure, was by Chambon and co-workers in 1963.⁸ They supplied hen liver nuclear extracts with nicotinamide adenine dinucleotide (NAD^+) and observed the formation of a polyadenylic acid product. The structure was elucidated several years later by various laboratories as being *poly*-ADP-ribose (*poly*-ADPr).⁹⁻¹¹ The formation involves the enzymatic transfer of multiple ADPr molecules to an acceptor protein with the concomitant release of nicotinamide (Figures 1 and 3).^{12,13} The synthesis is catalysed by *mono*-ADP-ribosyl transferase (MART) and *poly*-ADPr polymerase (PARP) enzymes, all of which belong to the PARP family. Therefore, a new nomenclature uses the term ADP-ribosyltransferases (ARTs) to describe all 17 PARP family members.¹⁴ *Mono*- and *poly*-ADP-ribosylation are two distinct types of PTM, having different roles and functions, as will be described below.

1.2.1 *Mono*-ADP-ribosylation

The first step in ADP-ribosylation is covalently linking one ADPr molecule to the side

chain of an amino acid (Figure 1). The side chains of arginine, cysteine, glutamic acid, serine, aspartic acid, asparagine and modified histidine (diphthamide) have been identified as modification sites.^{12,15} *Mono*-ADP-ribosylation is catalysed by *mono*-ARTs or bacterial toxins and usually leads to inactivation of the targeted protein.^{16,17} Bacterial toxins, like clostridial, pertussis, cholera and diphtheria toxins, are involved in the best studied *mono*-ADP-ribosylation reactions and it is shown that this modification plays a critical role in diseases like cholera and botulism. Contrary, both the mechanism and function of mammalian *mono*-ARTs are considerably less well understood and the majority of target proteins and amino acid(s) that are modified, are unknown. Among the various proteins that already have been identified as target are human neutrophil protein 1 (HNP1)¹⁸, karyopherin- β 1 (Kap- β 1)¹⁵, integrin- α 7¹⁹ and glutamate dehydrogenase (GDH)²⁰. *Mono*-ADP-ribosylation of these and other proteins results in a change or inhibition of their function and has impact on the corresponding biological processes. However, the exact interplay between *mono*-ADP-ribosylation of most proteins and these processes remains elusive. The same holds for the enzymes that are responsible for transferring NAD^+ to the target protein and for reversal (hydrolysis) of this modification.

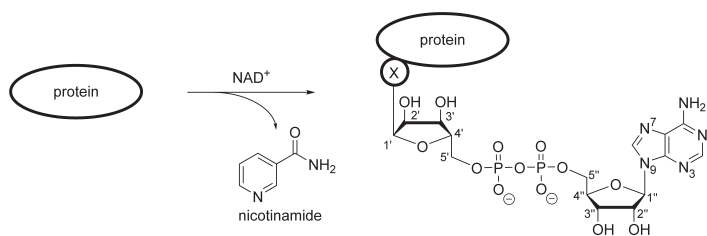


Figure 1. *Mono*-ADP-ribosylation of a protein (X = nucleophilic side chain of target amino acid).

The availability of well-defined substructures of ADPr-modified proteins would most likely help to study the ADP-ribosylation process in more detail. Enzymatic approaches offer little to no control over the ADP-ribosylation reaction with respect to modification site(s), *mono*- or *poly*-ADP-ribosylation and polymer length and polymer branching. Moreover, the low yields are a limiting factor in studying the modification in more detail. The research of this thesis focuses on the development of synthetic routes for well-defined ADP-ribosylated molecules.

The synthesis of *mono*-ADP-ribosylated peptides can assist in the elucidation of the role and function of *mono*-ADP-ribosylation. Up to now, only two syntheses of *mono*-ADP-ribosylated peptides have been reported. The first synthesis is described by the group of Muir and involves the incorporation of an aminoxy or *N*-methylaminoxy functionalized amino acid in a histone H2B peptide fragment (Figure 2B).²¹ The aminoxy functionality is condensed with the aldehyde in the ribose of ADPr at a pH of 4 to 5 to form an unnatural oxime linkage between ADPr and the peptide. The aminoxy peptide **3** gives a mixture of the ring opened ADPr **4a** and closed **4b** while the *N*-methylaminoxy gives exclusively the ring-closed ADPr peptide **6**. Muir and co-workers also showed the benefits of synthesizing ADPr peptides, to study the PTM in more detail, by introducing a biotin group which allowed for binding studies of the ADPr peptides to macro domain mH2A1.1 via streptavidin

pull-down experiments.²¹ The second approach is described by van der Heden-van Noort *et al.* and entails the construction of two *mono*-ADP-ribosylated peptides, the sequences of which are depicted in Figure 2. One sequence was derived from the Rho protein (peptide **1**, Figure 2A) while the other was from the N-terminal tail of histone H2B (peptide **2**, Figure 2A).²² The glycosylation and pyrophosphate formation procedures that were applied will be described later in this chapter.

The work by van der Heden-van Noort *et al.*²² was also the starting point for the work presented in this thesis. Their methodology was optimized with the objective to obtain *mono*-ADPr oligopeptides of larger size and different modification sites (Chapter 3). One of the challenges that is faced in the synthesis of such derivatives is the stereoselective introduction of the challenging α -glycosidic bond (1,2-*cis*), linking the anomeric (1') ribosyl carbon with the side chain of a given amino acid. Another challenge is the formation of the demanding pyrophosphate linkage between adenosine and ribose (chapter 2 and 3). This introductory chapter discusses selected approaches of these chemical transformations.

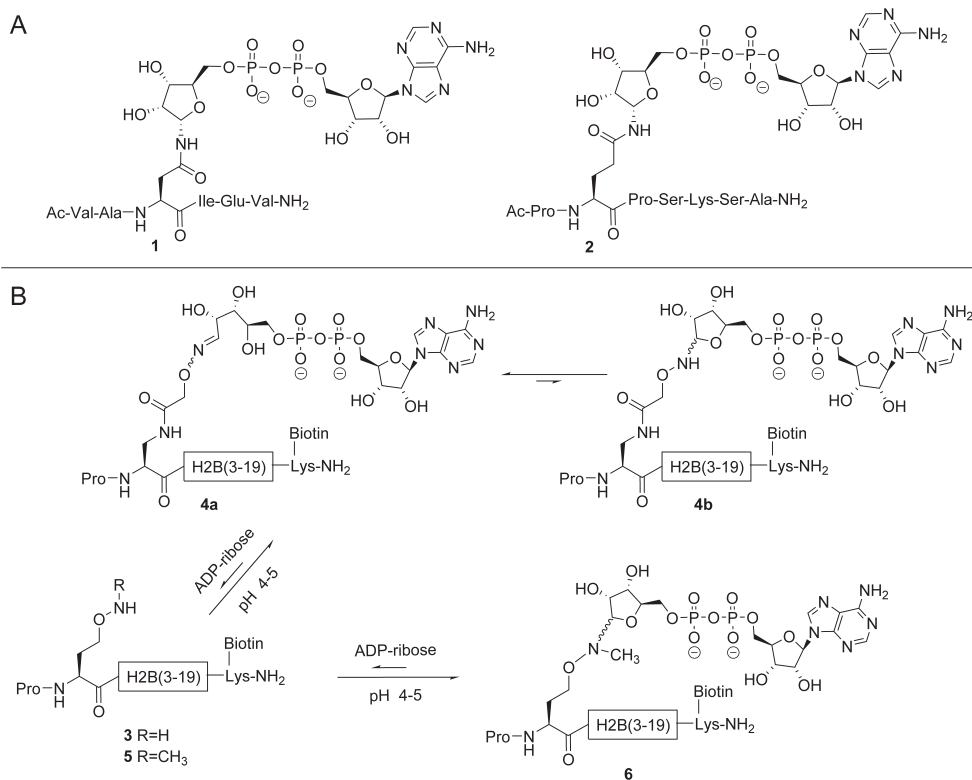


Figure 2. The *mono*-ADPr peptides synthesized by van der Heden-van Noort (A) and the group of Muir (B).

1.2.2 Poly-ADPr

The above described *mono*-ADP-ribosylation also functions as a starting point for *poly*-ADP-ribosylation. Again NAD⁺ is the source for ADPr and the PARP enzymes

can transfer over 200 ADPr molecules to create linear and branched *poly*-ADPr polymers (Figure 3).²³⁻²⁶ Elongation to linear polymers takes place by the formation of an α -glycosidic linkage between the 2'-OH of adenosine and the anomeric ribosyl carbon of donor ADPr. On the other hand branching involves the same reaction but at the 2'-OH of a ribose and is observed once per linear fragment of 20 to 50 ADPr molecules.^{25,27} The highly charged nature, size and potential formation of helicoidal secondary structures of *poly*-ADPr polymers results in rapid cellular responses and influences a variety of cellular processes like DNA repair, cell division and RNA interference.^{28,29} The response has to be fast as the half-life of ADPr polymers is ranging from tens of seconds up to several minutes with the degradation rate depending on the length and branching of the polymer.^{30,31} So far, two enzymes have been identified that efficiently hydrolyze *poly*-ADPr which are *poly*-ADPr-glycohydrolase (PARG) and ADP-ribosylhydrolase 3 (ARH3) with PARG being ten times more active compared to ARH3.³²⁻³⁵ The polymer is degraded in ADPr and *poly*-ADPr fragments which also function as signaling molecules, being involved in processes like apoptosis and cell growth. One of the most intriguing and well-studied processes in which *poly*-ADP-ribosylation plays a crucial role is the repair of single strand breaks (SSB) and double strand breaks (DSB) in DNA. PARP enzymes bind at the site of DNA damage and *poly*-ADP-ribosylate itself as well as the N- and C-terminal tails of histones H1 and H2B. This results in both the relaxation of the chromatin structure, allowing a better access to the breaks, and the recruitment of *poly*-ADPr binding proteins necessary for DNA damage repair.³⁶⁻³⁸

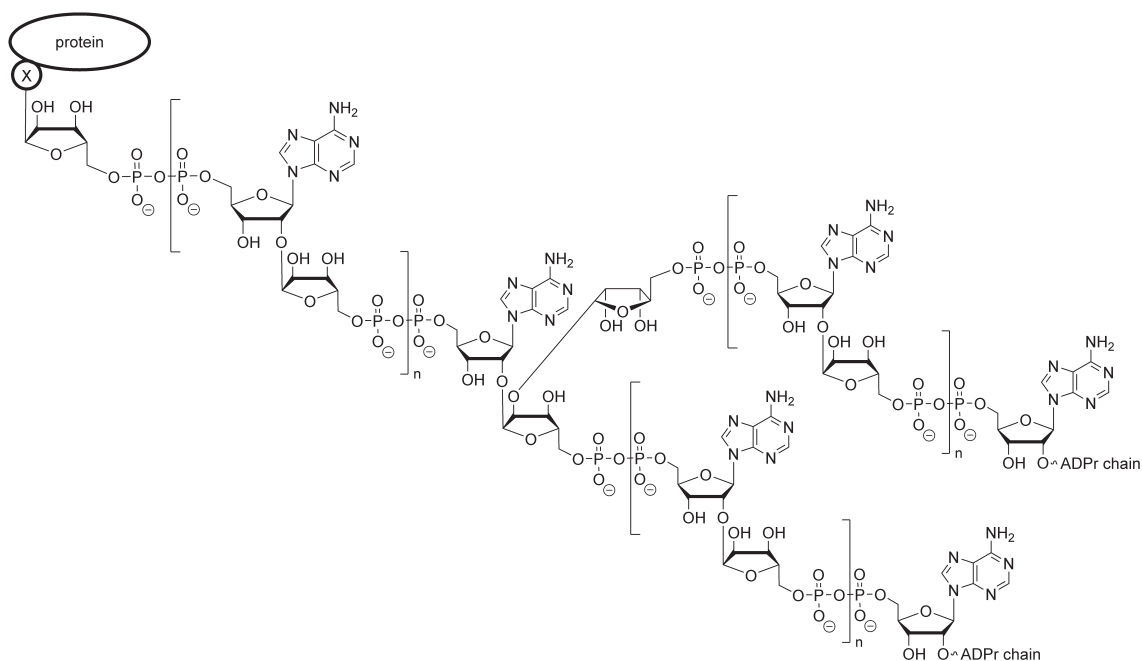


Figure 3. The structure of a *poly*-ADPr polymer linked to a protein.

Although great efforts have been made in elucidating the function and mechanism of *poly*-ADP-ribosylation, many unknowns remain to fully understand this PTM. Both the highly dynamic nature and the variability in length and shape (linear or branched) make a detailed study of this modification difficult. The availability of well-defined *poly*-ADPr constructs would be of great use to gain a better understanding and a synthetic approach would be the most viable way to obtain such constructs. The synthetic challenges and previously reported synthetic efforts towards *poly*-ADPr constructs will be described in this chapter.

1.3 Synthetic strategies towards *mono*- and *poly*-ADPr structures

1.3.1 Ribosylation

The standard approach for the formation of glycosidic linkages relies on a glycosyl donor with a suitable leaving group (i.e. halide, acetimidate, aryl/alkyl-thiol, etc.) at the anomeric center. The anomeric leaving group is activated upon the addition of a specific promotor, thereby allowing a nucleophilic attack of an acceptor molecule via a S_N1 (Figure 4) or S_N2 type mechanism.³⁹ The nature of the protecting groups, anomeric leaving group, promotor, acceptor, solvent and temperature will determine the productivity and stereochemical outcome of a glycosylation.³⁹

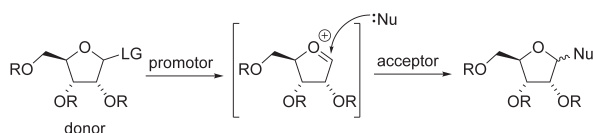


Figure 4. Schematic of a glycosylation reaction proceeding via an oxocarbenium ion intermediate.

The most straightforward glycosidic linkages to form are 1,2-*trans*-linkages. Installation of a participating group, usually an acyl group, at the 2'-position will direct the reaction. The free electron pair of the carbonyl oxygen will form a temporary bond with the anomeric center to create an acyloxonium ion intermediate that blocks the *cis*-side for the acceptor to attack. Contrary, introduction of the 1,2-*cis*-linkages presents a challenge (Figure 5).³⁹

The group of Woerpel extensively studied the C-glycosylating properties of furanoses. By exploring "stripped" furanoses with one or more isolated substituents, they demonstrated that the stereoselectivity of furanosylations is mostly controlled by stereoelectronic effects. Furthermore, a model, explaining the stereochemical outcome of furanosylations, was proposed which proceeds via oxocarbenium ion intermediates (Figure 5).⁴⁰⁻⁴³ Furanose oxocarbenium ions adopt the most stable envelope conformation and the nucleophile can approach the cation either from the inside or the outside of this envelope. Nucleophilic attack from the outside would lead to a transition state with unfavourable eclipsed interactions between

the C-1 and C-2 substituents while inside attack would give favourable staggered interactions. Therefore, nucleophilic attack preferably takes place at the inside of the envelope conformer with the lowest energy, either E_3 or 3E (Figure 5). Careful screening of all possible alkoxy substitution patterns at the C-2, C-3 and C-4/5 position in C-glycosylation reactions of ribose derivatives with allyltrimethylsilane indicated that the E_3 conformer is the most favourable conformation of the ribose derived oxocarbenium ion. The alkoxy substituent at the C-2 position prefers to adopt a pseudoequatorial orientation to stabilize the oxocarbenium ion via hyperconjugation and the C-3 alkoxy group adopts a pseudoaxial position to stabilize the oxocarbenium ion by placing it in closest proximity of the anomeric carbon. The C-3 alkoxy group proved to have the biggest influence on the selectivity in the aforementioned C-glycosylation reactions while the alkyl group at C-4 did not show to influence the selectivity at all.⁴⁰ The validity of the model was confirmed by van Rijssel and van Delft in performing glycosylation reactions on four furanoses (ribose, arabinose, xylose and lyxose) along with quantum mechanical calculations.^{44,45} This revealed a 1,2-*cis*-selectivity for all furanosyl oxocarbenium ions and the potential energy surface maps obtained via the calculations provided detailed information on the preferred orientation of all substituents and on the equilibrium between the E_3 and 3E conformers.

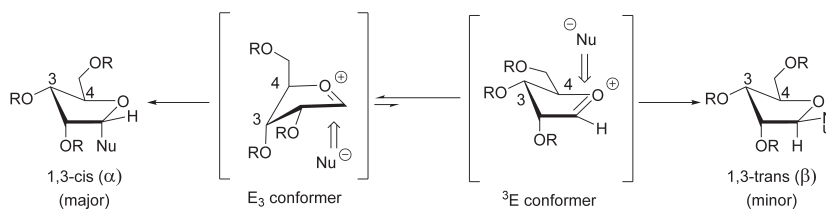


Figure 5. Two-conformer model for the nucleophilic attack of ribosyl oxocarbenium ions.

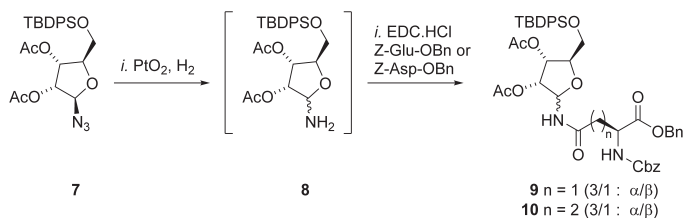
The highly stereoselective outcome for the C-glycosylation and hydride addition reactions is, however, not always observed for other nucleophiles such as O-nucleophiles. This is attributed to the differences in nucleophilicity and steric character of O-nucleophiles, the potential for hydrogen bonding between donor and acceptor, and the reversibility of these type of glycosylations.⁴⁶ These properties makes the synthesis of *mono*- and *poly*-ADPr structures more challenging since these consist of solely α -ribosyl linkages (1,2-*cis*), linking ADPr with either the side-chain of an amino acid or with an ensuing ADPr molecule.

The synthesis of ADPr-peptides requires the availability of suitably protected α -ribosylated amino acid building blocks, of which only a few examples have been described in literature. O-ribosylation of serine was performed with two different donors equipped with either an anomeric bromide or an anomeric fluoride and non-participating benzyl groups at the hydroxyl functionalities.⁴⁷ Both donors generated an anomeric mixture and the bromide donor showed a preference for the α -product (β/α : 0.3) while the fluoride donor gave a slight excess of the β -product (β/α : 1.2). Reduced stereoselectivity was also observed for other O-ribosylation reactions in which a primary alcohol was used as acceptor.⁴⁸ This clearly shows that additional factors play a role in directing the stereoselective

outcome for ribosylation reactions using nucleophiles other than C-nucleophiles.

The synthesis of *N*-ribosidic linkages has only been described for the synthesis of β -linked products and mostly in the framework of nucleoside (i.e. purine and pyrimidine) synthesis with the use of participating groups. A different strategy was chosen by Papini and co-workers to obtain ribosylated asparagine. They started out with ribofuranosyl azide that was reduced with Pd black and hydrogen gas to give the corresponding hemiaminal. Subsequent peptide coupling with activated aspartic acid furnished an anomeric mixture of ribosylated asparagine (56/44; α/β).⁴⁹ The lack of anomeric preference prompted van der Heden-van Noort *et al.* to re-evaluate this synthetic strategy. First, they equipped the ribofuranosyl azide with a different protecting group pattern, namely acetyls at C2' and C3' and TBPDS at C5', in view of orthogonality for the synthesis of *mono*-ADPr peptides as will be discussed later (compound **7**, Scheme 1).²² Careful reduction of the azide using platinum oxide (PtO₂) at 10 °C yielded heminal **8** as a mixture of epimers, which was directly coupled with either activated glutamic acid or aspartic acid to give the anomeric mixtures of ribosylated asparagine **9** (3/1; α/β) and ribosylated glutamine **10** (3/1; α/β). The improved α -selectivity was sufficient to isolate adequate amounts of α -ribosylated glutamine and asparagine for the synthesis of *mono*-ADPr peptides. However, this methodology is limited to the synthesis of *N*-ribosidic linkages and more specifically amide linkages. Therefore, a new methodology is described in chapter 2 that is versatile in choice of nucleophile combined with an excellent α -selectivity.

Scheme 1. Ribosylation of asparagine and glutamine.²²

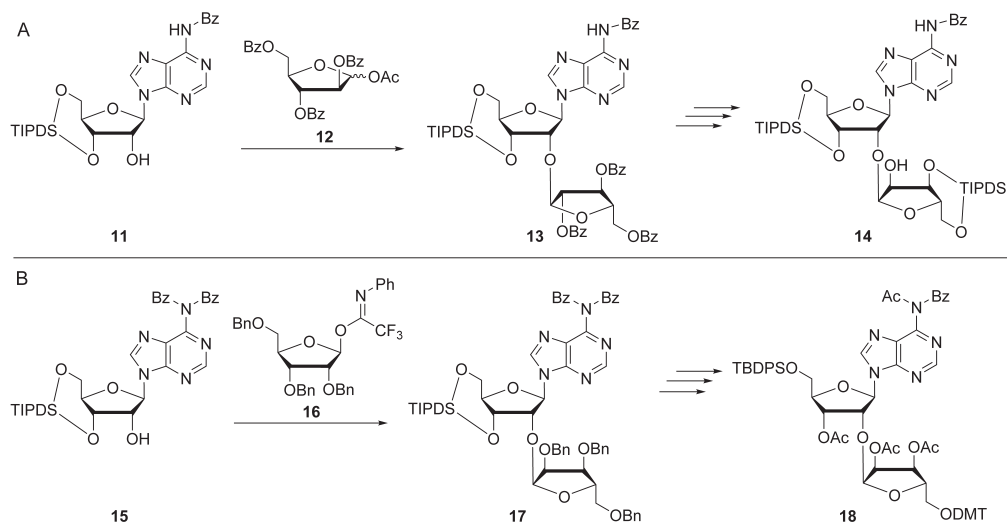


The other challenging α -ribosidic linkages, found in *poly*-ADPr structures, are those between the 2'-OH of adenosine and the anomeric ribosyl carbon of the sequential ADPr units to form linear ADPr polymers. Additionally, branching occurs occasionally at the 2''-OH ribosyl units of the linear polymer introducing an ADPr molecule in a similar fashion as for the linear elongation. These disaccharide nucleoside structures, linking two ribofuranosyl molecules via an *O*-glycosidic bond, are also found in tRNAs.⁵⁰ However, the ribofuranosyl units in tRNAs are linked via β -glycosidic linkages which simplifies their synthesis significantly. The synthesis of α -ribosylated adenosine, required to construct *poly*-ADPr polymers, has only been described by two separate groups. Mikhailov and co-workers exploited the neighbouring group participation effect of benzoylated arabinose **12** in a tin tetrachloride promoted glycosylation reaction with adenosine **11** (Scheme 2A).⁵¹ This yielded exclusively the 1,2-*trans*-linked disaccharide **13** which was transformed in four steps to α -ribofuranosyl adenosine **14** including inversion of the 2' OH functionality via a Robins' oxidation and selective reduction.⁵² The lack of orthogonality in the protecting groups at the 5'-OH functionalities makes this compound impractical

in the synthesis of *poly*-ADPr constructs. This prompted van der Heden-van Noort and co-workers to devise a new synthetic route towards orthogonal protected α -ribofuranosyl adenosine. This more direct approach utilized imidate donor **16**, equipped with non-participating benzyl groups, to directly glycosylate adenosine in an α -selective fashion furnishing α -ribofuranosyl adenosine **17** in only one step (Scheme 2B).⁵³ Several protecting group manipulations yielded fully orthogonal protected α -ribofuranosyl adenosine **18**, suitable for future *poly*-ADPr synthesis.

However, the amounts of building block **18** that were obtained by van der Heden-van Noort *et al.* would not be sufficient to continue towards the actual synthesis of *poly*-ADPr constructs. Upscaling of the synthetic route turned out to be difficult with the glycosylation reaction being the limiting step. Further optimizations of this glycosylation step were not successful and the reaction is apparently limited to a small scale. A new synthesis route would be needed to obtain adequate amounts of orthogonally protected α -ribofuranosyl adenosine for the synthesis of *poly*-ADPr structures and this new route is described in chapter 4. This synthetic route also opened the way to the synthesis of the building block for branched ADPr which is demonstrated in chapter 5.

Scheme 2. Synthesis of α -ribosylated adenosine via a glycosylation reaction of adenosine with; A) 1-O-acetyl-2,3,5-tri-O-benzoyl- β -D-arabinofuranose **12**⁵¹ or B) 1-O-(*N*-phenyl)-2,2,2-trifluoroacetimido-2,3,5-tri-O-benzyl-D-ribofuranose **16**.⁵³

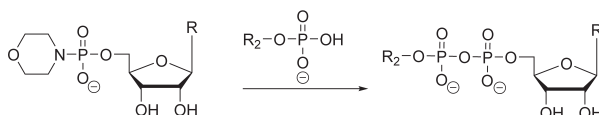


1.3.2 Pyrophosphate bond formation

Besides the glycosidic linkages, the synthesis of asymmetric pyrophosphates is challenging and this holds true even more for multiple pyrophosphates present in *poly*-ADPr structures. The synthesis of a pyrophosphate linkage is in almost all cases achieved by reacting two monophosphate groups of which one of these phosphates has been activated. This approach originates from the ground-breaking work of Khorana and co-workers who use phosphoramidates and later phosphoramidites to

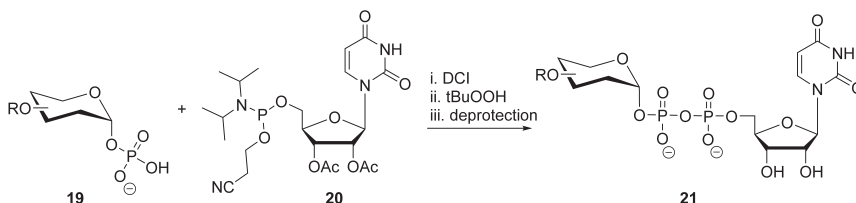
react with a phosphate (monoester) furnishing the pyrophosphate group (Scheme 3).⁵⁴⁻⁵⁶ The Khorana method has become the most popular method and has also been further optimized and/or modified. These changes include the use of a catalyst (e.g. tetrazole)⁵⁷ and other leaving groups (e.g. imidazolidine^{58,59}, methylimidazolidine^{60,61}) instead of morpholidate improving the efficiency and the rate of the pyrophosphate formation.⁶²

Scheme 3. Reaction of a phosphormorpholidate (activated phosphate) with a phosphate to generate a pyrophosphate group.



Another method to activate a phosphate and form an asymmetric pyrophosphate was presented by Meier and co-workers. Inspired by the Ludwig-Eckstein methodology, they used a cyclo-saligenyl phosphate to react with another phosphate furnishing a pyrophosphate linkage (Scheme 5B).^{63,64} A completely different approach was presented by Gold and van Delft *et al.* exploiting the characteristics of phosphoramidite chemistry used in DNA/RNA synthesis.⁶⁵ They reacted uridine phosphoramidite **20** (P^{III}) with glucose phosphate (P^V) **19** to form a P^{III}-P^V intermediate which is transformed into a pyrophosphate (P^V-P^V) **21** via an in-situ oxidation step (Scheme 4). The formation of symmetrical pyrophosphate side products is prevented and the coupling reaction is extremely fast and efficient which is a major advantage over the previously described methodologies that often lack efficiency and/or speed.

Scheme 4. UDP-glucose synthesis via a phosphoramidite-phosphate (P^{III}-P^V) coupling reaction followed by oxidation.⁶⁵

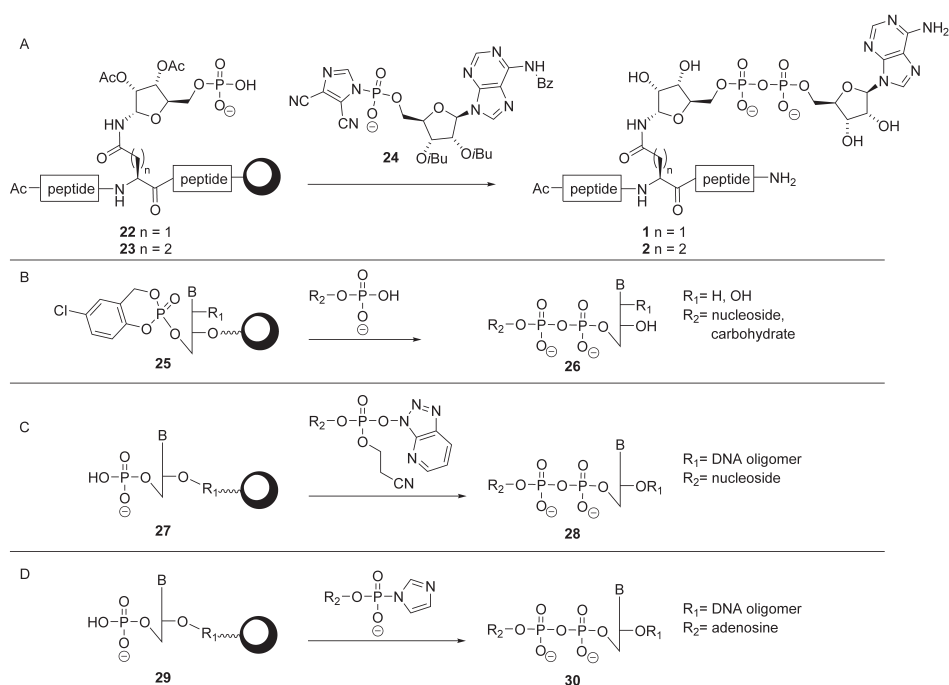


The synthesis of *mono*- and *poly*-ADPr constructs is complex and performing most of the synthetic steps on a solid support would mitigate this complexity slightly. For the formation of asymmetric pyrophosphates on a solid support there is only a limited number of examples in literature. One of these examples is the first successful synthesis of *mono*-ADP-ribosylated peptides by van der Heden-van Noort *et al.*²² The previously described ribosylated amino acids were incorporated in a peptide fragment and phosphorylated on a solid support to give compounds **22** and **23** (Scheme 5A). The two phosphorylated peptides were reacted with adenosine **24** equipped with a dicyanoimidazolidine activated phosphate group at the 5'-OH position. This provided the desired pyrophosphate linkage and with that *mono*-ADPr peptides **1** and **2** (Scheme 5A). Other examples described in the literature for solid phase pyrophosphate

formation have a similar approach in that they also use an activated phosphate to react with a phosphomonoester. The group of Meier extended their cyclo-saligenyl approach to the solid support for the synthesis of nucleoside diphosphate sugars and dinucleoside pyrophosphates with coupling times of four to five days (Scheme 5B).⁶⁶ Piccirilli and co-workers reacted adenosine 5'-phosphorimidazole with immobilized 5'-phosphate DNA oligonucleotides (**29**) of varying length and sequence (Scheme 5D). This approach required elevated temperatures and also reaction times of 48 hours to give acceptable yields. The fastest pyrophosphate formations on solid support were achieved by the group of Sekine using pre-activated phosphoramidite nucleosides.^{67,68} The 5'-phosphoramidite nucleoside is first reacted with 1-hydroxybenzotriazole (HOBt) resulting in an oxidation reaction to give an activated HOBt 5'-phosphotriester of a nucleoside. This activated phosphate is reacted with different 5'-phosphate nucleosides and DNA oligonucleotides and the corresponding pyrophosphate bridge is formed quantitatively in only 10 minutes (Scheme 5C).

Both the method developed by Sekine and co-workers and the solution phase approach devised by Gold and van Delft share the use of phosphoramidite nucleosides and are extremely fast (minutes compared to hours or days) and efficient. Therefore, the Gold-van Delft method was selected, further optimized and successfully applied in the synthesis of *mono*-ADP-ribosylated peptides on solid support as is described in chapter 3.

Scheme 5. Asymmetric pyrophosphate formation on solid support via the reaction of a phosphate with: dicyanoimidazolide phosphate (A), cyclo-saligenyl phosphate (B), HOBt phosphate (C) and imidazolide phosphate (D).



The synthesis of *oligo*-ADPr fragments requires the construction of *oligo*-pyrophosphates and there has only been one method described in literature⁶⁹ prior to the advent of phosphoramidite-phosphate strategy described in this Thesis. This example comprises the synthesis of DNA analogues interconnected via pyrophosphate linkages. However, the previously reported methodology proved to be irreproducible and a detailed study into its viability is described in chapter 6. The application of the phosphoramidite-phosphate strategy in the synthesis of *oligo*-pyrophosphates proved to be successful for both the synthesis of *poly*-ADPr oligomers as well as for thymidine oligonucleotides interconnected via pyrophosphate linkages. This is described in detail in chapter 4 and chapter 6.

1.4 Aim and outline of this thesis

The research described in this Thesis aims at the synthesis of *mono*- and *poly*-ADP-ribosylated constructs. New synthetic methodologies are developed and existing methods are optimized to achieve this. In chapter 2, a new stereoselective glycosylation methodology is demonstrated for the synthesis of α -ribosylated amino acids. All the amino acids used in this ribosylation reaction have been reported as possible ADP-ribosylation sites and for some amino acids an isoster is used for synthesis reasons. The ribosylated amino acids are used in chapter 3 in which the synthesis of *mono*-ADPr peptides is described. Furthermore, the binding affinity of these peptides towards different macrodomain proteins is investigated. The newly developed methodology for the formation of the pyrophosphate linkage in the *mono*-ADPr peptides is extended to the synthesis of *poly*-pyrophosphate linkages, which is demonstrated in chapter 4 and chapter 6. The first part of chapter 4 deals with the synthesis of orthogonally protected 2'-O-ribosyl-adenosine, the building block that is required for the formation of linear *poly*-ADPr oligomers. A new synthetic route is presented for this disaccharide building block that allows for a large scale synthesis, providing sufficient amounts for the preparation of *poly*-ADPr oligomers. The second part of chapter 4 describes the solid phase synthesis of linear *poly*-ADPr oligomers resulting in the formation of a dimeric and trimeric *poly*-ADPr fragment. The first efforts toward the synthesis of branched *poly*-ADPr constructs are described in chapter 5 with the first successful synthesis of the trisaccharide core motif. The synthesis hinges on the methodology set out in chapter 4 for the disaccharide building block and aims for the synthesis of an orthogonally protected building block that could later be incorporated in *poly*-ADPr oligomers. In chapter 6 the wider applicability of the pyrophosphate formation methodology on a solid support is demonstrated. Here, the synthesis of thymidine oligonucleotides interconnected through pyrophosphate linkages is described. Finally, chapter 7 summarizes all the findings and future strategies are discussed for the synthesis of ADPr structures. This involves both the optimization of the presented methodologies as well as an alternative strategy for the synthesis of *mono*-ADPr peptides.

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