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Synthetic Study on ADP-ribosylation

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1 General Introduction

Chemical ADP-ribosylation: mono-ADPr-peptides, oligo-ADP-ribose and isosteres

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1. Introduction

ADP-ribosylation is a post-translational modification (PTM) of proteins that occurs upon enzymatic transfer of ADP-ribosyl moiety from NAD^+ to a nucleophilic side chain of an amino acid of a protein.[1-3] As the result either mono-ADP-ribose (MAR) or poly-ADP-ribose (PAR) becomes grafted to the protein. (Figure 1) Both modifications play an important regulatory role in various physiological and pathological processes.[4] The transfer of PAR to amino acids on protein substrates is catalyzed by four enzymes of the PARP family: PARP1, PARP2, and PARP5a, PARP5b. PAR can exist as a linear or branched polymer. Other PARP family members (PARP3, 4, 6-12, 14-16) transfer only MAR to amino acids on protein substrates. Upon ADP-ribosylation of cellular proteins, either mono- or poly, the posttranslational modification becomes subject to further recognition and processing by proteins that are capable of removing or binding PAR or MAR (Figure 1).[1] Such metabolic variations of ADP-ribosylation status result in a change of the intracellular signaling. Hydrolases such as PARG and enzymes from ARH-family are responsible for the breakdown of PAR and MAR and thus for the reversal of ADP-ribosylation.[5, 6]

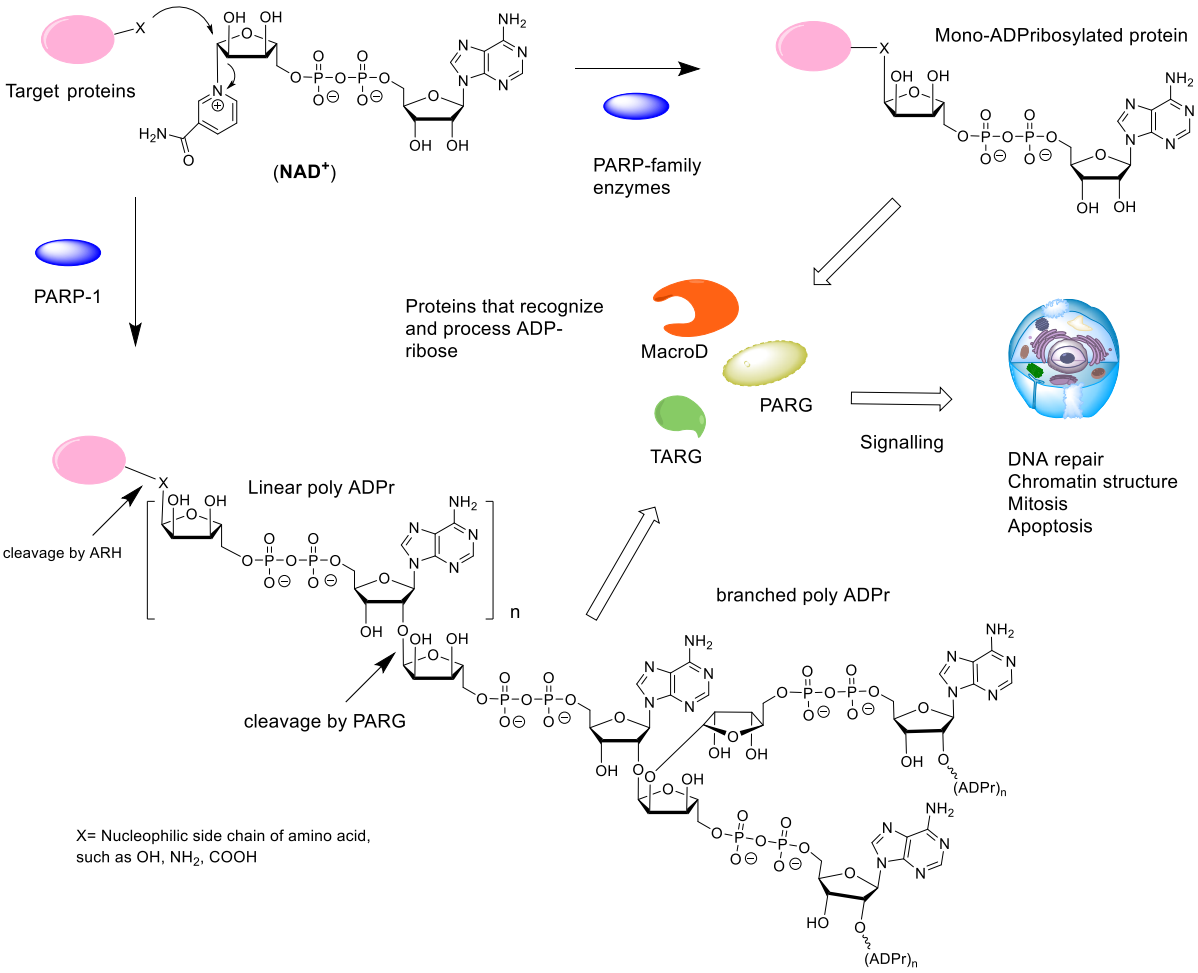


Figure 1. Biosynthesis and metabolism of mono- and poly-ADP-ribosylated proteins

From the point of view of a bioorganic chemist, both mono-ADP-ribosylated (MARylated) and poly-ADP-ribosylated (PARylated) biopolymers (Figure 1) present a significant challenge. Nevertheless, synthetic well-defined ADP-ribosylated proteins or their substructures are useful for the studies that are aimed to elucidation of the biological role of ADP-ribosylation. This Chapter is a review of the synthetic advances towards the synthesis of mono-ADP-ribosylated proteins and oligo-ADP-ribose chains.

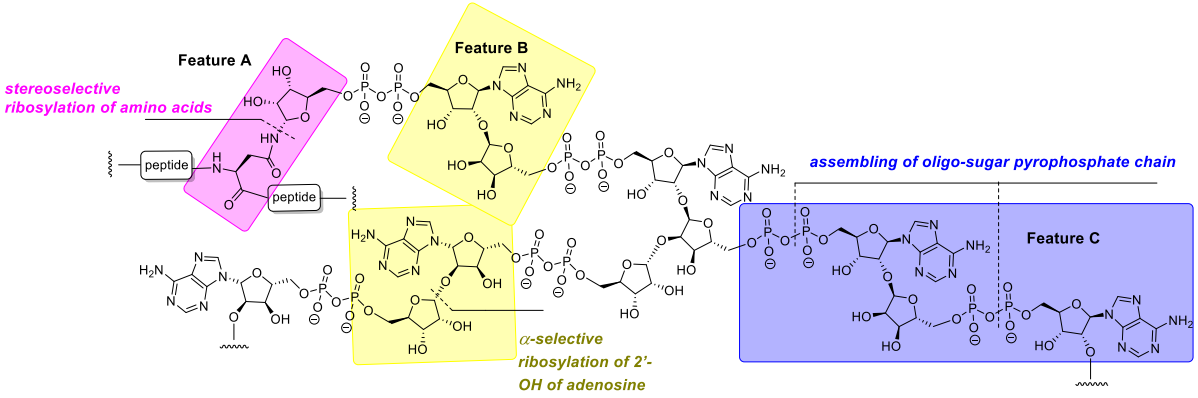


Figure 2. The structure of PAR with its most conspicuous synthetically challenging features

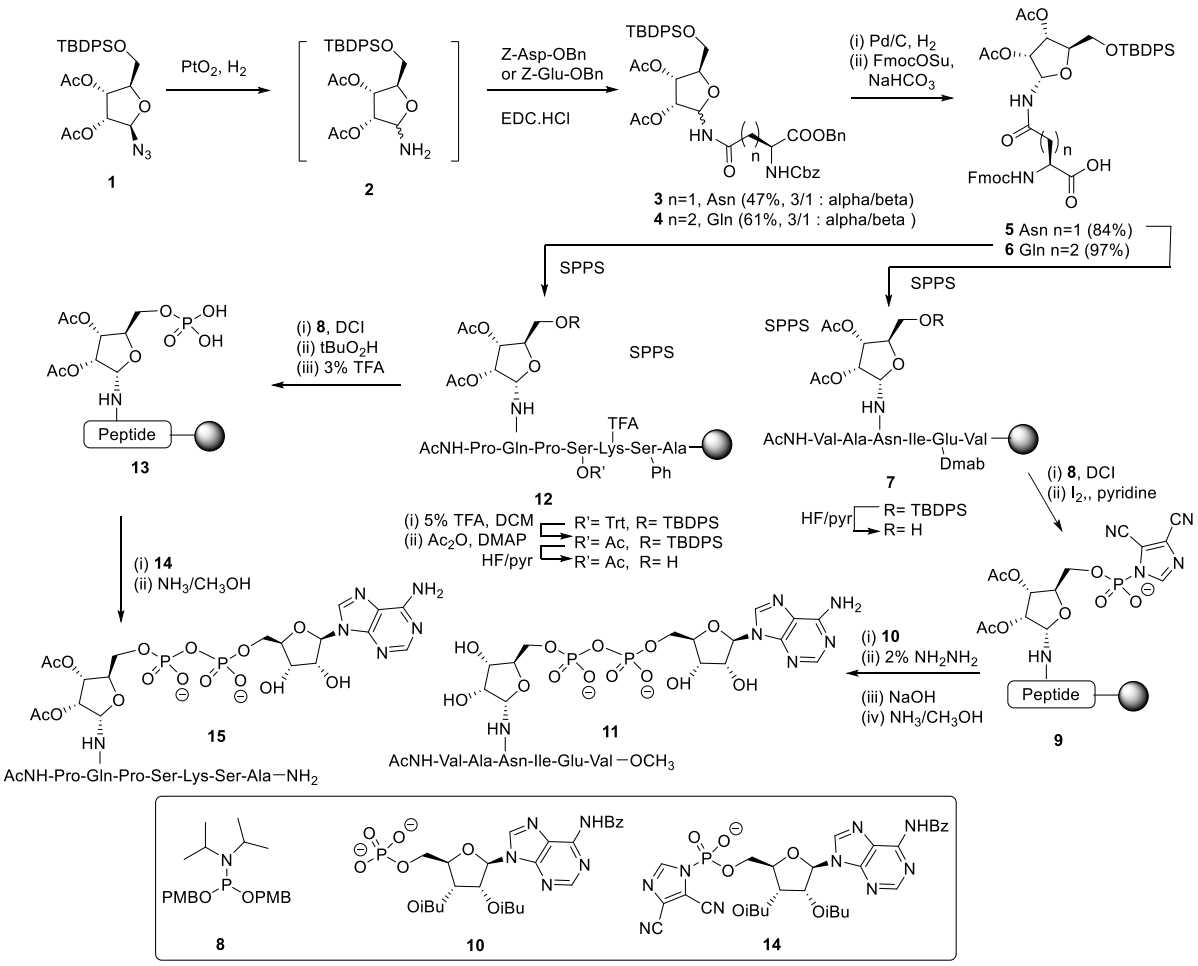
2. Chemical synthesis mono-ADPr-peptides and ADPr-oligomers

The organic synthesis of ADP-ribosylated biomolecules is challenging as the construction of these hybrid structures requires the use of elements from the synthetic chemistry of nucleic acids, oligosaccharides and oligopeptides that are sometimes incompatible. The synthetic challenge is augmented by the necessity to introduce one or even multiple pyrophosphate linkages, which are notoriously difficult to construct efficiently. The following sections describe synthetic approaches to the primary challenges of chemical ADP-ribosylation, that is, ribosylation of side chains of various amino acids (Figure 2, feature A), stereoselective glycosylation of the 2'-OH of adenosine (Figure 2, feature B) and assembling the oligo-sugar pyrophosphate chain of oligo-ADPr (Figure 2, feature C).

2.1 Synthesis of mono-ADP-ribosylated peptides

Mono-ADP-ribosylated proteins play intriguing roles in many cellular processes.[7] An approach to deepen the insight in these processes to a molecular level comprises the design, synthesis and biological evaluation of well-defined synthetic mono-ADP-ribosylated derivatives. Relevant examples of such compounds are mono-ADP-ribosylated oligopeptides,[8-10] as fragments of the naturally occurring proteins. Main challenges in the assembly of these ADP-ribosylated oligopeptides are an efficient procedure for the introduction of the pyrophosphate function and a method for the stereoselective α-ribosylation of the nucleophilic side chains of amino acids. This section is focused on the construction of the α-glycosidic bond that joins the "distal" ribose of the ADPr-moiety and an amino acid side chain in the context of the mono-ADPr-peptide synthesis. The methods that have been developed for the introduction of one pyrophosphate linkage in mono-ADP-ribosylated peptides will be discussed in this Section while the introduction of multiple pyrophosphates in short fragments of poly-ADPr is the subject of in Section 2.2.2. Application of a solid phase approach to mono-ADP-ribosylated oligopeptides is most obvious as a solution phase synthesis would be restricted in terms of length and composition of the oligopeptide. While the introduction of the pyrophosphate moiety is feasible on a solid support,[8, 9] ribosylation of partially protected and immobilized oligopeptides with a protected ribose donor is almost impossible in terms of stereoselectivity and yield. Therefore, attention has been focused on the synthesis of suitably protected ribosylated amino acid building blocks that can be applied in a solid phase peptide synthesis (SPPS). The main hurdle in the synthesis of these ribosylated amino acid building blocks is the difficulty to control the 1,2-cis configuration of the ribosyl anomeric linkage at the glycosylation stage. The sensitivity of this O-glycosidic bond to acid adds another layer of complexity.

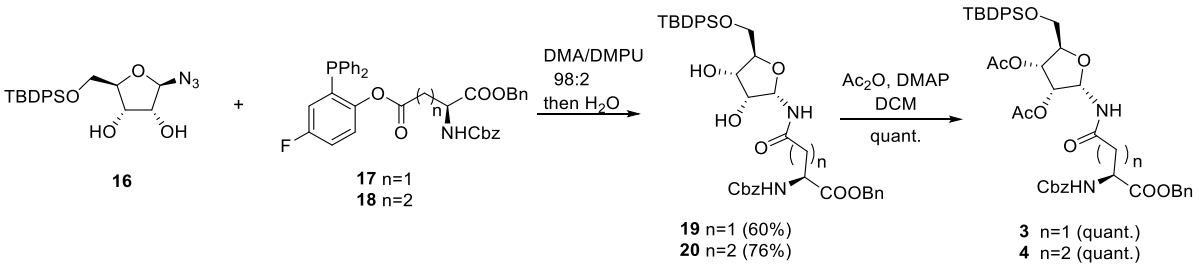
The first reported synthesis of suitably protected α-ribosylated amino acid building blocks and their application in a SPPS assembly of relevant ADP-ribosylated oligopeptides is of van der Heden van Noort *et al*.[8] The choice for Fmoc-based peptide synthesis led to the synthesis of protected α-ribosylated asparagine (Asn) **5** and glutamine (Gln) **6** building blocks (Scheme 1). The route of synthesis started with the reduction of fully protected β-D-ribosylated azide **1** to an epimeric hemiaminal mixture **2**. Subsequently, EDC-mediated coupling with Z-Glu-OBn or Z-Asp-OBn, respectively and silica gel purification gave the individual anomers **3** and **4**. Protective group manipulation provided α-ribosylated Asn (**5**) and Gln (**6**) building blocks with the mutually orthogonal TBDPS and Fmoc protecting groups. Guided by the outcome of a solution phase study, a SPPS was undertaken in which two procedures for the installation of the adenosine diphosphate function were explored. For that purpose, native[11] model peptide **11** containing an ADP-ribosylated Asn residue and peptide **15** originating from the N-terminus of human histone H2B containing an ADP-ribosylated Gln residue were selected. In the latter case Gln was chosen as a stabilized isostere of Glu that was reported to be the natural ADP-ribosylation site.[12] Hexapeptide **7** was obtained via SPPS using a BOP/HOBT Fmoc-based synthesis executed on Tentagel resin equipped with the HMBA linker. Upon removal of the TBDPS group at the 5-OH of the ribose, the immobilized peptide **7** (R=H) was phosphitylated with phosphoramidite **8** under influence of the activator DCI, followed by oxidation using iodine in pyridine to give the activated phosphorimidazolate **9**. Reaction with the protected adenosine phosphate **10** led to the formation of the protected and immobilized target ADP-ribosylated peptide**.** Removal of the Dmab group on Glu and subsequent treatment with ammonia methanol, to affect both removal of the remaining protecting groups and cleavage from the resin, gave after HPLC purification ADP-ribosylated hexapeptide **11**. With the aid of LC-MS analysis of the crude product the C-terminal carboxamide, the ribosyl 5-phosphomonoester and the corresponding H-phosphonate could be identified as side products. It was reasoned that the formation of H-phosphonate could be suppressed by reversal of the procedure for pyrophosphate formation. To this end the phosphate was installed on immobilized peptide (i.e. **13**), while activated phosphorimidazolate (i.e. **14**) was prepared in solution. The assembly of ADP-ribosylated peptide **15** started with the SPPS of heptapeptide **12** according to the same procedure as described for hexapeptide **7**. Protective group manipulation led to **12** (R'= Ac, R= H) having only base labile protecting groups. The phosphate moiety was introduced by phosphitylation with **8** under influence of the activator DCI, oxidation of the intermediate phosphite triester with *t*-BuO_2H and, finally, removal of the *p*-methoxybenzyl groups with TFA to give phosphate monoester **13**. Immobilized **13** was now treated with an excess activated phosphorimidazolate **14** to afford the immobilized and protected precursor of target **15**.



Scheme 1. Synthesis of ADP ribosylated peptide **11** and **15**

Removal of all protecting groups and concomitant cleavage from the resin gave ADP-ribosylated heptapeptide **15**. However, also with this procedure the unwanted formation of the phosphate monoester (from intermediate **13**) and the corresponding H-phosphonate could not be circumvented. In spite of the successful application of the α-ribosylated asparagine **5** and glutamine **6** building blocks in SPPS and the isolation of pure ADP-ribosylated peptides **11** and **15** in reasonable yields, it became apparent that the assembly of ADP-ribosylated peptides would benefit from a more efficient procedure for pyrophosphate formation.

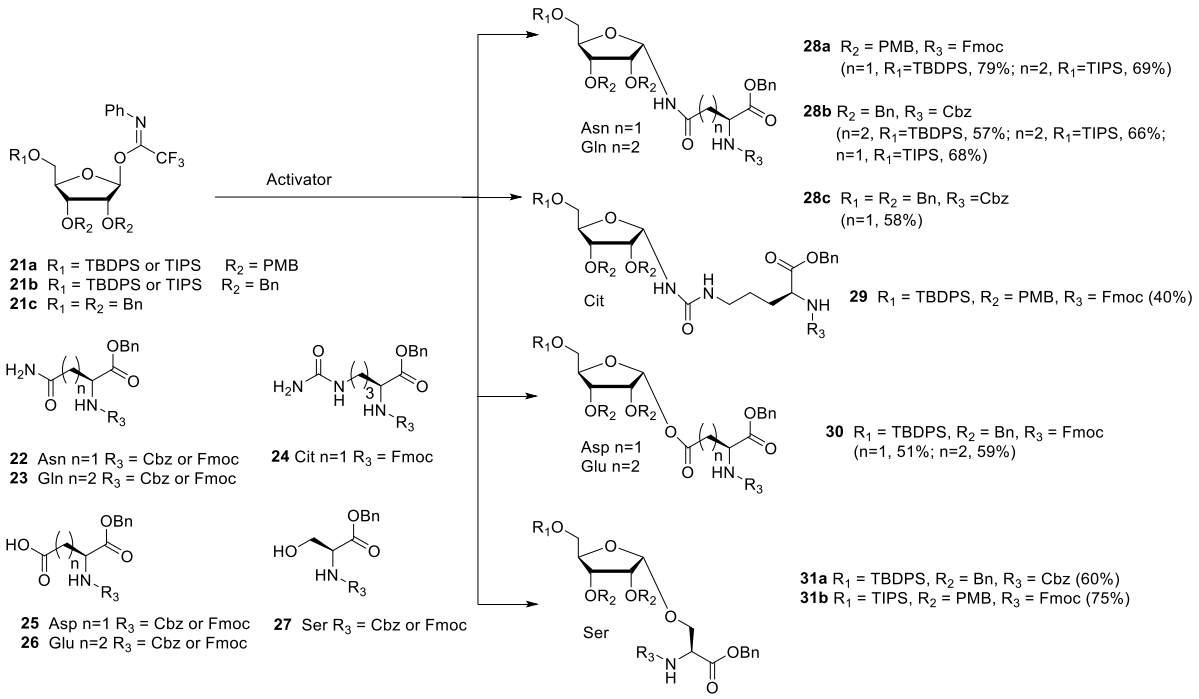
Other authors also undertook the synthesis of protected α-ribosylated asparagine and glutamine building blocks. Thus, Bonache *et al*.[13] have prepared such derivative for the first time, while F. Nisic *et al*.[14, 15] developed a stereoselective synthesis of α- or β-glycofuranosyl amides with the aid of traceless Staudinger ligation of glycofuranosyl azides. Application of this approach for the synthesis of α-N-ribosyl-asparagine/glutamine building blocks is depicted in scheme 2.[16]



Scheme 2. Synthesis of α-ribofuranosyl amides using fluorinated phosphines.

Fluorinated triphenylphosphines functionalized with Z-Asp-OBn (**17**) and Z-Glu-OBn (**18**) were used in ligation reactions with differently protected β-D-ribofuranosyl azides. It turned out that both stereochemistry and productivity of these reactions were dependent on the protection of the hydroxyl groups in the ribose moiety. Protection of the primary 5-OH with the TBDPS group (**16**) produced (Asn) **19** and (Gln) **20** in good yield. Subsequent acetylation of **19** and **20** gave known[8] SPPS building blocks **3** and **4**.

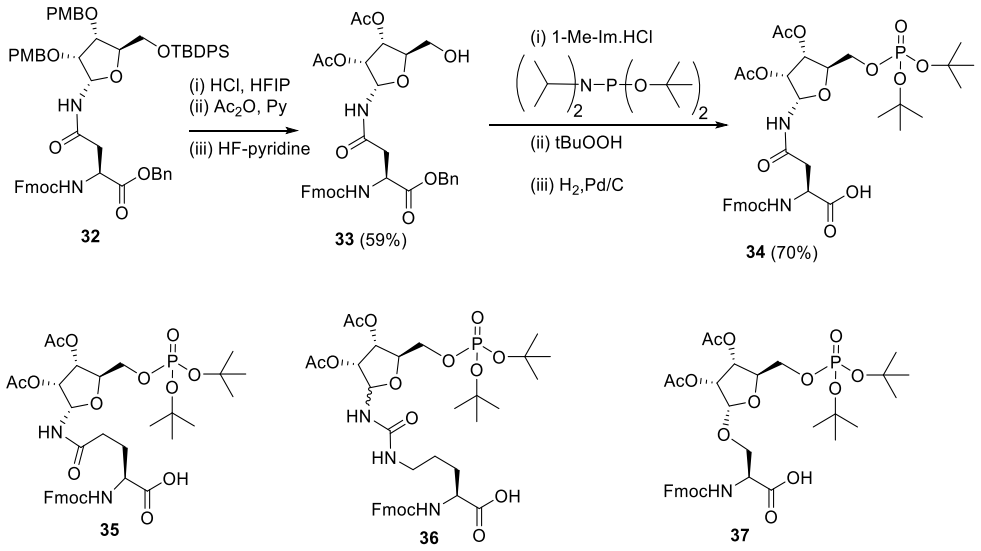
With the aim to broaden the range of the synthetically accessible ribosylated amino acids Kistemaker *et al*.[17] developed an alternative ribosylation method that employed ribosyl donors **21a, b, c** (Scheme 3) with the N-phenyl trifluoroacetimidate leaving group and with non-participating ether protecting groups at the 3- and 2-OH. The latter feature allows the formation of both *O*- and *N*-glycosidic linkages via highly α-selective acid catalyzed glycosylation. Condensation of perbenzylated donor **21c** with Asn acceptor **22** (R_3 = Cbz) under various conditions led to α-product **28c** (n=1). However, these conditions were not transferable to other acceptors (e.g. Glu acceptor **23** (R_3 = Cbz)). It was reasoned that the selectivity of the ribosylation could be improved by replacing the benzyl group at the 5-OH in the ribose by the bulkier TBDPS or TIPS protecting groups to give donor **21b** (R_1 = TBDPS or TIPS). Several activator systems were tested and the results of these tests indicated that TMSOTf and $HClO_4$-SiO_2 were the most favorable activators. Reaction of donor **21b** (R_1 = TBDPS or TIPS) with Asn acceptor **22** (R_3 = Cbz) and Gln acceptor **23** (R_3 = Cbz) gave good to excellent yields of α-products **28b** (n= 1, 2 respectively). Next, this glycosylation protocol was applied to Cbz- and Fmoc-protected glutamic acid (Glu, **26**), aspartic acid (Asp, **25**) and serine (Ser, **27**). Using TMSOTf as activator protected derivatives of ribosylated Asp **30** (n = 1, α/β = 98/2, 51%), ribosylated Glu **30** (n = 2, α/β = 98/2, 59%) and ribosylated Ser **31a** (R_1= TBDPS, α/β = 1/0, 60%) were obtained.



Scheme 3. Trifluoroacetimidate ribosylation of partially protected amino acids.

In order to minimize protecting group manipulations towards the ribosylated amino acid building blocks suitable for SPPS, the benzyl groups at the 2-OH and 3-OH in the ribosyl donor were replaced by acid labile PMB ethers and the Cbz group in the amino acid acceptors was replaced by the Fmoc group. Condensation of imidate donor **21a** (R_1 = TBDPS) with Asn acceptor **22** (R_3 = Fmoc) in DCM under influence of TMSOTf furnished **28a** (n = 1, α/β = 97/3, 79%). A similar condensation using the less nucleophilic citrulline (Cit) acceptor **24** proceeded in a less α-selective manner to give **29** (α/β = 78/22, 40%). The insolubility of Gln acceptor **23** (R_3 = Fmoc) required a change to dioxane/DCM as solvent system and $HClO_4$-SiO_2 as activator to give **28a** (n = 2, α/β = 93/7. 69%). Finally, condensation of Ser acceptor **27** (R_3 = Fmoc) with donor **21a** (R_1 = TIPS) furnished ribosylated Ser **31b** (α/β = 1:0, 75%).

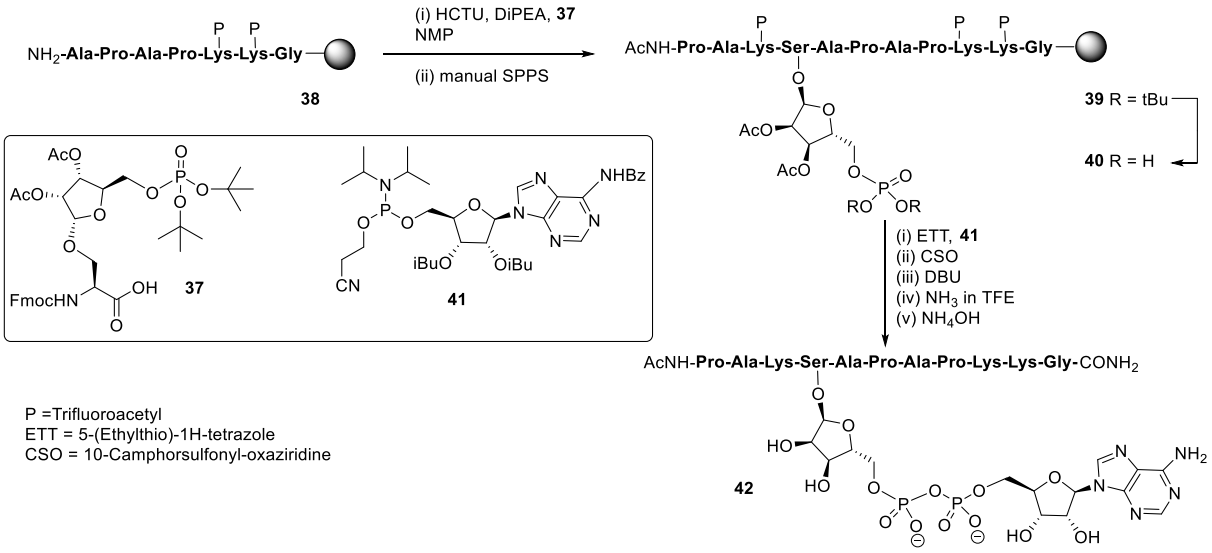
To obtain relevant ADP-ribosylated oligopeptides by SPPS, Kistemaker *et al*.[9, 18] also searched for another procedure for pyrophosphate formation. To this end, the solution phase method for the synthesis of sugar nucleotides, reported by Gold *et al*[19] was adopted. This procedure[9] combines phosphoramidite (P^{III}) with phosphate (P^{V}) chemistry and the adaptation to solid phase procedure required the on-resin formation of phosphomonoester. It was reasoned that this could be circumvented by the development of the protected pre-phosphorylated amino acid building blocks **34**-**37** (Scheme 4). The synthesis of these phosphorylated amino acids is illustrated by the preparation of Asn building block **34**. The PMB groups in fully protected ribosylated Asn **32** were replaced by acetyl groups by acidolysis, followed by acetylation while the 5-OH was unmasked by desilylation to afford **33.** The *tert*-butyl group was selected as an orthogonal phosphate protecting group.



Scheme 4. Ribosylated amino acids with a phosphotriester at the 5-OH.

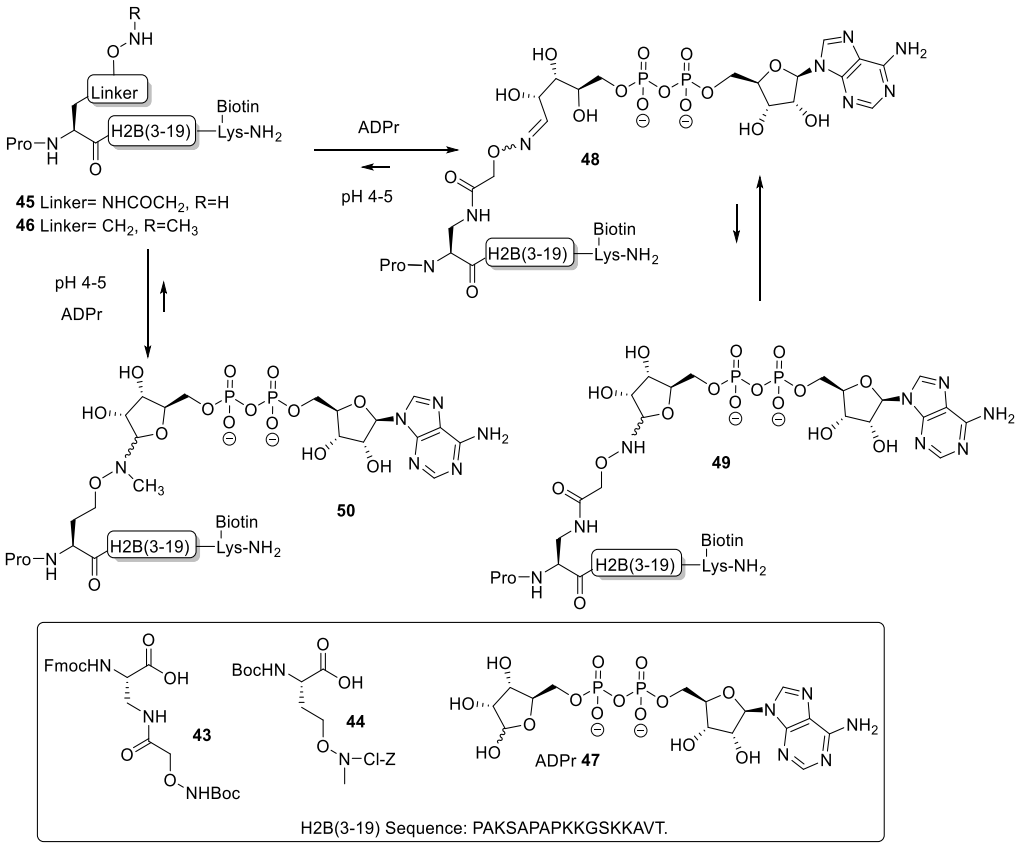
The di-*tert*-butyl phosphate triester was installed with di-*tert*-butyl *N*,*N*-diisopropylphosphoramidite and subsequent oxidation of the intermediate phosphite triester. Finally, hydrogenolysis of the benzyl ester gave the α-configured Asn building block **34** suitable for SPPS. Transferring this procedure to other amino acids showed that the anomeric integrity of Gln **35**, Ser **37** stayed intact while Cit **36** was obtained as an anomeric mixture, which could be separated by column chromatography.

With these building blocks available SPPS could be undertaken and relevant ADP-ribosylated oligopeptide fragments from Histone H2B, RhoA protein and HNP-1 defensin were obtained.[9] The synthesis of Ser-ADPr H2B peptide **42** (Scheme 5) serves as a representative example of the usefulness of this methodology for the preparation of ADP-ribosylated peptides with a native ADP-ribosylation site.[10] SPPS of hendecapeptide **42** was carried on Tentagel resin, equipped with HMBA-linker. First, intermediate immobilized heptapeptide **38** was produced with automated SPPS utilizing Fmoc chemistry and trifluoroacetyl protected lysine residues. Subsequent elongation to phosphoribosylated peptide **39** was done manually using serine phosphotriester **37** and commercially available protected amino acids.



Scheme 5. SPPS of Ser-ADPr hendecapeptide originating from histone H2B.

To allow the pyrophosphate introduction di-*tert*-butyl phosphate protective groups in the immobilized **39** were removed with TFA and DCM, followed by neutralizing with pyridine. Reaction of the obtained phosphate monoester **40** with phosphoramidite **41** under influence of activator ETT was followed by oxidation of the P^{III}-P^{V} intermediate with CSO and, finally, the cyanoethyl group in the pyrophosphate was removed with DBU. Target pyrophosphate **42** was obtained by a two-step procedure. First, cleavage from the resin was attained by treatment with a saturated NH_3 solution in trifluoroethanol. In this way the formation of a carboxylic acid at the C-terminus of the peptide is circumvented and only carboxamide is produced. Addition of NH_4OH effected the removal of all remaining protective groups to give hendecapeptide **42.** This is first reported synthesis of ADP-ribosylated peptide on serine,[10] which has been found to be one of the most widespread ADPr-modification sites in histones, upon DNA damage.[20-23] It is also the first real, naturally occurring ADP-ribosylated oligopeptide as all others are provided with stabilized anomeric ribosyl linkages, that is asparagine (Asn) and glutamine (Gln)[8, 9] as stabilized analogues of aspartic acid (Asp) and glutamic acid (Glu), respectively).

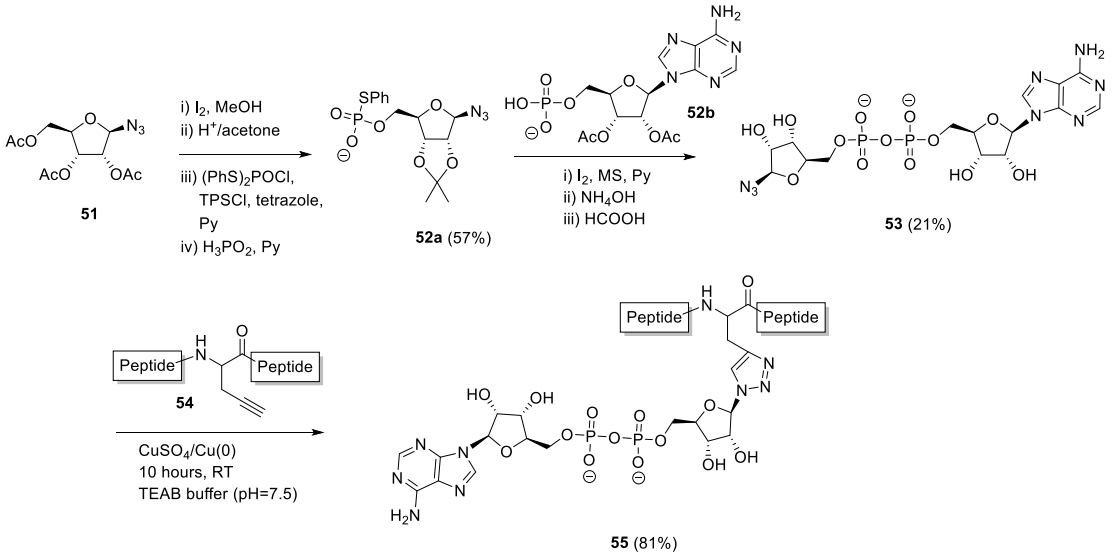


Scheme 6. The aminooxy and N-Methyl aminooxy functionalized peptides **48-50** of the group of Muir.

Moyle and Muir[24] reported the synthesis and biochemical evaluation of stabilized and artificial mono-ADP-ribose conjugated peptides. An N-terminal (3-19) oligopeptide[24] of histone H2B protein with glutamate residue mono-ADP-ribosylated, was selected as a model (Scheme 6). With the aid of manual SPPS on MBHA resin using HBTU/DIPEA, oligopeptides **45** and **46** having either aminooxy or *N*-methyl aminooxy functionality were assembled. The aminooxy-containing building block **43**[25] or *N*-methyl aminooxy containing amino acid **44**[26] was incorporated instead of the glutamic acid at the *N*-terminus. After cleavage from the resin the (N-methyl)aminooxy groups in the oligopeptides **45** and **46** were reacted with the hemiacetal of the ribose moiety in free ADP-ribose **47** producing an ADPr appendage**.** The aminooxy group in **45** led mainly to ring-opened ADPr peptide **48** and a small amount of the ring-closed form **49**, while the N-methyl aminooxy group in **46** gave the ring-closed ADPr peptide **50**, exclusively. By executing the ligation procedure at pH 4.5 the oxime formation is selective, leaving all natural amino acid side-chain functionalities, including those of lysine and arginine residues, intact.

The synthesis of triazole linked peptides using the copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) was reported by Li *et al* (Scheme 7).[27] In order to develop a versatile platform for divergent preparations of ADP-ribosylated peptide, β-N_3-ADPr **53** was prepared, as a precursor for the CuAAC mediated conjugation with alkyne functionalized peptides. Acetylated ribosyl azide **51** was converted into thiophosphate **52a** in 4 steps. The pyrophosphate was introduced by reaction of **52a** and AMP

52b under influence of I_2, providing β-N_3-ADPr **53** after global deprotection. Conjugation of **53** with alkyne-peptide **54** by CuAAC click chemistry furnished triazole linked ADPr-peptide **55** in high yield.



Scheme 7. Synthesis of triazole linked ADPr-peptides by CuAAC chemistry.

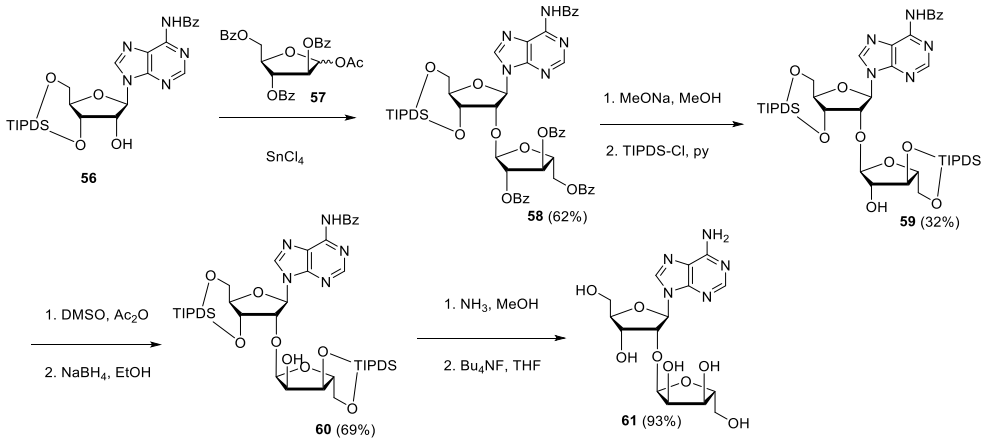
2.2 Synthesis of poly ADPr chain

The organic synthesis of fragments of poly-ADP-ribose (PAR, Figure 1) comprises a repetitive introduction of both an α-glycosidic bond between the ribose and the 2'-OH of adenosine and a pyrophosphate linkage between the primary OHs of the adenosine and the ribose moiety. To acquire ADPr oligomers of a certain length both a solution and a solid phase approach require the design and synthesis of suitably protected and functionalized building blocks. Monomeric building blocks could be envisaged, in which a pyrophosphate moiety is incorporated but those must then act as ADP-ribofuranosyl donors that are suitable for repetitive α-ribosylation of the 2'-OH of the terminal adenosine moiety of the growing PAR-chain. Although, such a method would resemble the biosynthesis of PAR, in which NAD^+ fulfills the role of the ADP-ribofuranosyl donor this approach should be rejected because the repetitive introduction of multiple α-ribosidic bonds in the presence of (anionic) pyrophosphates is almost impossible. Therefore the α-ribosidic bond should be preinstalled in the building block while the pyrophosphate moiety is then repetitively introduced during the assembly of the oligo-ADP-ribose chain. Both syntheses of ADP-ribose oligomers that are reported to date use the latter strategy.[18, 28] The following sections describe the methods that have been developed for the synthesis of 2'-O-ribosylated adenosine building blocks (Section 2.2.1) and the methods of pyrophosphate formation in the framework of the assembly of fragments of poly-ADPr-ribose (Section 2.2.2).

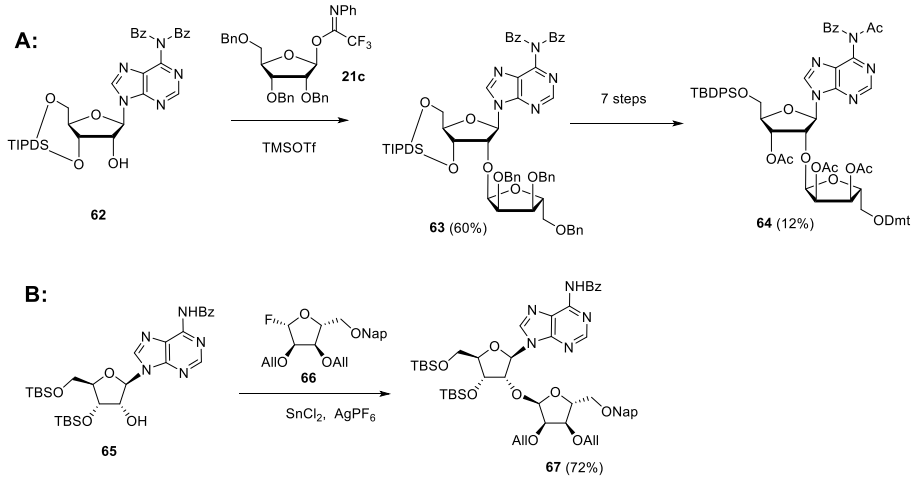
2.2.1 Building blocks synthesis—ribosylated adenosine

In 2008 Mikhailov *et al*[29] reported the first synthesis of a 2'-O-α-D-ribofuranosyladenosine building block (**60**, Scheme 8). The potentially problematic α-ribosylation was circumvented by the use of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-arabinofuranose **57** as a donor. Condensation of donor **57** with adenosine acceptor **56** under the influence of tin tetrachloride afforded, by neighboring group participation, trans-configured disaccharide nucleoside **58**. To arrive to 2'-*O*-α-D-ribofuranosyladenosine building block **60**, the route of synthesis was continued by protective group manipulation and finally by inversion of 2'-OH to give the desired ribo-configuration via an oxidation-reduction sequence. The protective groups in building block **60** were removed to produce 2'-*O*-α-D-ribofuranosyladenosine **61**. The group of Marx[30] applied this method for the preparation of ribosylated adenosine analogues to develop PARP inhibitors.

Although the method of Mikhailov *et al*[29] is robust the route of synthesis to a monomeric building block suitable for the assembly of oligo-ADP-ribose is rather lengthy due to the necessity to invert the 2'-OH position of ribose and the subsequent introduction of orthogonal protective groups. For the synthesis of oligo-ADP-ribose, more direct approaches to attain α-selective glycosylation of adenosine were developed. Van der Heden van Noort *et al*.[30] reported the synthesis of 2'-*O*-α-D-ribosylated adenosine (**64**, Scheme 9A) with TBDPS and Dmt as orthogonal protecting groups on the primary hydroxyl functions of the ribose moieties. The key step is the TMSOTf mediated condensation of (*N*-phenyl)-2,2,2-trifluoroacetimidate donor **21c** and adenosine acceptor **62** to furnish fully protected ribofuranosyl adenosine **63** in an α-selective manner. Subsequent protecting groups manipulation yielded **64**, amenable for the assembly of oligo-ADP-ribose. Recently, Shirinfar *et al*[31] reported the synthesis of a protected phosphorylated ribofuranosyl adenosine building block, using the same glycosylation procedure.

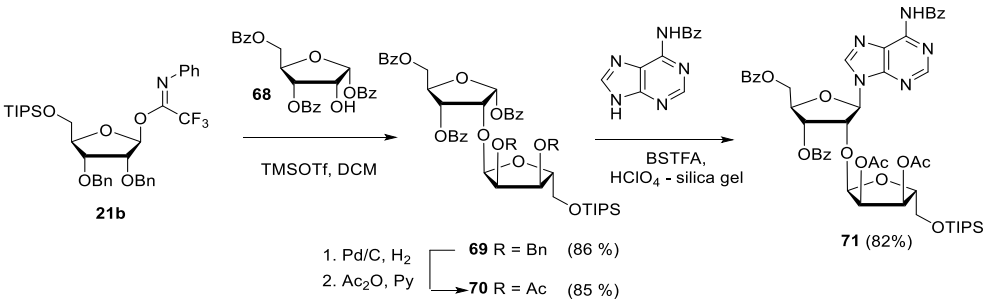


Scheme 8. Synthesis of 2'-O-α-ribosylated adenosine **61** using 1-O-acetyl-2,3,5-tri-O-benzoyl-D-arabinofuranose **57** as the donor



Scheme 9. Synthesis of orthogonally protected ribosylated adenosine with **(A):** 1-*O*-(*N*-phenyl)-2,2,2-trifluoroacetimido-2,3,5-tri-*O*-benzyl-D-ribofuranose **21c** and **(B):** glycosyl fluoride **66** as the donors.

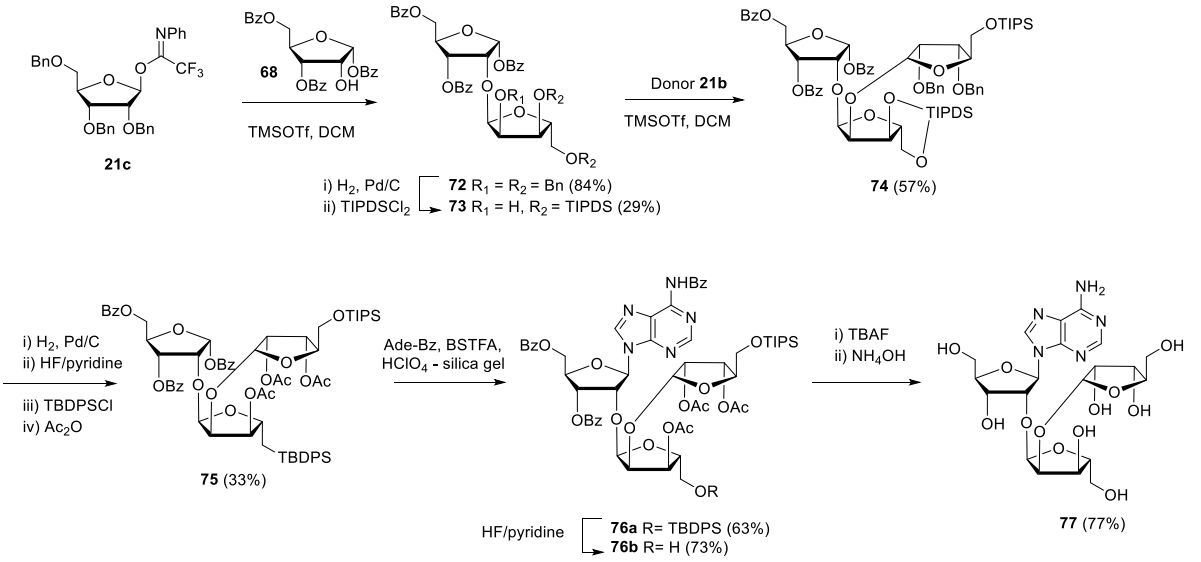
In 2015 Lambrecht *et al*[28] reported the synthesis of orthogonally protected ribosyl adenosine **67** (Scheme 9B) by the α-selective condensation of β-fluoride donor **66**, obtained in six steps from ribose with adenosine acceptor **65**. Crucial for the productivity of this reaction was the use of $AgPF_6/SbCl_2$ as activator combination.



Scheme 10. Synthesis of ribosylated adenosine via Vorbrüggen type glycosylation.

Guided by the need to scale up the process and to acquire sufficient quantities of a suitable ribosyl adenosine building block Kistemaker *et al* [18] developed a new method. Side reactions on the nucleobase often accompany the glycosylation of protected nucleosides limiting the scalability of such synthetic strategies.[32] Therefore it was decided to install the adenine base by a Vorbrüggen reaction after the ribosylation event.[33, 34] As depicted in Scheme 10 reaction of benzylated (*N*-phenyl)-2,2,2-trifluoroacetimidate donor **21b** and commercially available acceptor 1,3,5-tri-*O*-benzoylribose **68** led to the isolation of α-configured disaccharide **69**. After hydrogenolysis and acetylation, **70** was obtained. Vorbrüggen coupling of **70** and Bz-adenine under the influence of immobilized acid ($HClO_4$-SiO_2),

introduced adenine base both regio- and β-stereoselective. Of note is that this approach gives access to large amount of **71**, the precursor of a suitable 2-*O*-ribosylated adenosine building block.



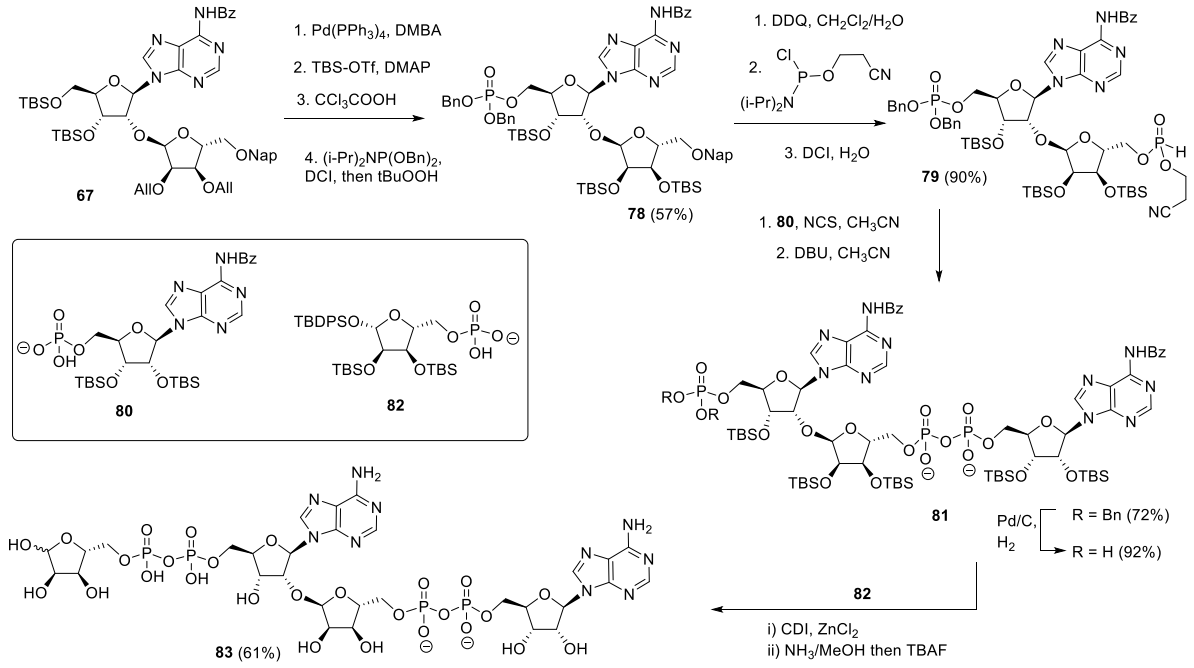
Scheme 11. Synthesis of *O*-α-D-ribofuranosyl-(1'''→2'')-*O*-α-D-ribofuranosyl-(1''→2')-adenosine: the branching point of ADPr-chain

A similar glycosylation strategy was adopted towards the synthesis of the branched ADPr core motif: *O*-α-D-ribofuranosyl-(1'''→2'')-O-α-D-ribofuranosyl-(1''→2')-adenosine **77** which is depicted in Scheme 11.[35] Disaccharide **72** was synthesized from donor **21c** and acceptor **68** as mentioned above. Benzyl groups were removed by hydrogenolysis and 3',5' OH were capped by TIPDS to furnish **73**. Subsequent TMSOTf mediated condensation of **73** with benzylated (*N*-phenyl)-2,2,2-trifluoroacetimidate donor **21b** led to all α configured tri-riboside **74**. After protective group manipulation the adenine base was introduced through the same Vorbrüggen type glycosylation as described above to afford protected **76a**. Careful removal of all protecting groups yielded branched core motif **77** in good yield.

2.2.2 Synthetic approaches to oligo-ADPr

Pyrophosphates are important functional groups in a wide array of naturally occurring compounds and a lot of procedures for the synthesis of pyrophosphates have been reported[19, 36-44]. However, the occurrence of multiple pyrophosphates in one molecule, such as in oligo-ADPr is unprecedented and presents a special challenge. Only two syntheses of short fragments of oligo-ADPr have been published to date. Lambrecht *et al*[28] (Scheme 12) reported a solution phase synthesis of an ADPr dimer, in which they relied on the classic Atherton-Todd chemistry to construct the pyrophosphate bridges.[28] In their

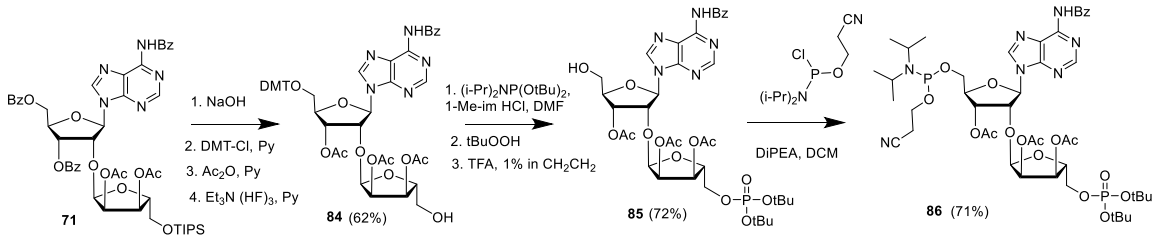
route of synthesis ribosylated adenosine building block **67**, obtained as described above (Scheme 9) was subjected to protective group manipulation to allow the installation of a dibenzyl phosphotriester at the primary OH of adenosine with the aid of phosphoramidite chemistry and subsequent oxidation. The naphthyl ether at the 5'-OH of the ribose moiety in thus obtained **78** was selectively removed and a H-phosphonate diester was introduced with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite and subsequent hydrolysis of the intermediate phosphoramidite to give **79**. Oxidative chlorination of H-phosphonate of **79** with NCS afforded an intermediate chlorophosphate which was condensed with adenosine monophosphate **80** to give after removal of the cyanoethyl group pyrophosphate **81** (R = Bn) in good yield. Hydrogenolysis of the benzyl group afforded terminal phosphate **81** (R = H). Unfortunately, the introduction of the second pyrophosphate with the same method failed. Therefore, silylated ribose monophosphate **82** was treated with CDI and condensation of the resulting phosphorimidazolide with **81** (R = H) gave after removal of all protecting groups target ADP dimer **83** in good yield.



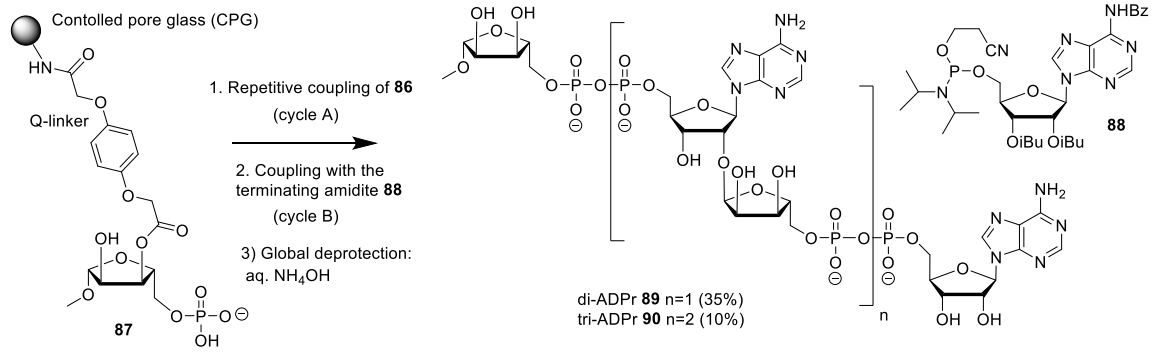
Scheme 12. Synthesis of ADPr dimer **83** by Lambrecht *et al*

Kistemaker *et al.* reported a solid phase synthesis of both an ADPr dimer and trimer (Scheme 14).[18] To be able to introduce multiple pyrophosphates a method to access sugar-nucleotides that was based on the combination of P^{III}-P^{V} chemistry was investigated.[19] This methodology proved to be convenient and expedient not only for the synthesis of mono-ADP-ribosylated peptides (see Scheme 5) but also for the synthesis of various bioorganic pyrophosphate derivatives both in solution[43, 45, 46] and on solid phase.[47, 48] It was expected that this P^{III}-P^{V} method would be uniquely suitable for the repeated pyrophosphorylation on solid phase, not least due to its mild nature and fast kinetics. To be able to

introduce multiple pyrophosphate functions building block **86**, provided with di-*tert*-butyl phosphotriester and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidite was designed. The synthesis of building block **86** (Scheme 13) started with 2-*O*-ribosyladenosine **71** which was obtained in sufficient quantities and good yield as described above (Scheme 10). Protective group manipulation of dimer **71** gave **84** of which the free primary OH in the ribose moiety was provided with a di-*tert*-butyl phosphotriester using standard phosphoramidite chemistry, followed by oxidation of the intermediate phosphite triester. Finally, removal of the DMT group to give **85** and reaction with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite resulted in the isolation of building block **86** that contains both a phosphoramidite and a protected precursor of phosphate monoester.



Scheme 13. Synthesis of ribosylated adenosine building block **86** suitable for solid-phase preparation of oligo-ADPr fragments



Cycle A: 1. **86** (3 eq.), ETT (10 eq.), MeCN, (2x);2. CSO (100 eq.), MeCN, (2x); 3. DBU (100 eq.), DMF, (2x); 4) HCl (5 eq.), HFIP, (4x); 5. pyridine (10% v/v), acetonitrile, (2x).

Cycle B: 1. **88** (3 eq.), ETT (10 eq.), MeCN, (2x); 2. CSO (100 eq.), MeCN, (2x); iii. DBU (100 eq.), DMF, (2x);

Scheme 14. Solid-phase synthesis of ADPr dimer **89** trimer **90**.

The solid phase synthesis of an ADPr dimer **89** and trimer **90** using building block **86** and terminating building block **88** is shown in scheme 14.

Guided by state of the art in automated DNA synthesis, controlled pore glass (CPG)[18] with long alkyl amine chains was used as the solid support while hydroquinone-*O*,*O*'-diacetic acid (Q-linker) was selected as a linker for its improved resistance to DBU that was used to cleave 2-cyanoethyl protections from pyrophosphate. Functionalization of this solid support with protected ribose and introduction of

the phosphate monoester at the primary position gave "initiator" **87**. At this stage, up to two coupling cycles with building block **86** were undertaken. The coupling cycle took one hour and comprises 5-ethylthiotetrazole (ETT) mediated condensation of **80** with the immobilized monophosphate, oxidation of the obtained labile phosphite-phosphate (P^{III}-P^{V}) intermediate by (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine (CSO), removal of the 2-cyanoethyl group in the partially protected pyrophosphate intermediate with DBU. The final unmasking of the di-*tert*-butyl phosphotriester with HCl/HFIP followed by neutralization with pyridine to allow the next elongation with either **86** or terminating building block **88.** The immobilized and partially protected ADPr dimer and trimer were cleaved from the resin and completely deprotected by treatment with aqueous ammonia and purified to give milligram quantities of ADPr dimer **89** and trimer **90.**

3. Aim and outline of this Thesis

Before the starting of the research described in this Thesis, substantial synthetic advances towards mono-ADP-ribosylated proteins and oligo-ADP-ribose chains have already been made as described above. However, the available methodology for the synthesis of ADP-ribosylated molecules remained somewhat limited. Thus, the current method for synthesis of mono-ADP-ribosylated peptides,[8,9] is time-consuming, laborious and sometimes low-yielding, while fully synthetic ADP-ribosylated proteins are not available. A more robust and convenient strategy that allows for the construction of different types of ADP-ribosylated peptides and proteins is very desirable. Concerning ADPr-oligomers, the synthesis of di-ADPr and tri-ADPr has been reported either in solution or on a solid phase. However, longer oligomers have not been reported yet because of the limitation of the current method for pyrophosphate construction. Furthermore, the branched ADPr-oligomers, advanced and complex oligo-ADPr structures, have never been synthesized, making the biological study of this particular part of poly-ADPr-chains even more hampered.

The limitations of the contemporary methods of chemical ADP-ribosylation and a relative scarcity of the well-defined synthetic ADP-ribosylated derivative was an incentive to undertake synthetic studies to further advance the methodologies in the bioorganic chemistry of ADP-ribosylated molecules. This Thesis aims specifically at the developing of new and improved synthetic methodologies and to synthesize advanced mono- or oligo-ADP-ribosylated biomolecules. The target compounds that are described in this Thesis are not only represent a synthetic challenge but also have great value in biology for a better understanding of ADP-ribosylation. The contributions to the chemical ADP-ribosylation that are made through the research described in the chapters of the Thesis are outlined below.

Chapter 2 presents the synthesis and structural analysis of *O*-α-D-ribofuranosyl-(1'''→2'')-*O*-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate), a naturally occurring branched poly-ADPr fragment and the synthesis of its biotinylated derivatives, as valuable tools in searching for new branched PAR binding proteins. **Chapter 3** deals with the solid phase synthesis of deca-pyrophosphate linked thymidine oligomers using a new phosphoramidite building block in which the phosphotriester is protected with Fm-groups. This optimized P(V)-P(III) method for pyrophosphate formation proved to be suitable for the synthesis of oligo-ADPr up to pentamer, as described in **Chapter 4**, accompanying with the first reported α-configured biotinylated ADPr trimer via CuAAC chemistry. As an extension of Chapter 2, **Chapter 5** reports the first total synthesis of the minimal branched poly-ADPr containing three ADPr units. To better understand the binding mechanism between mono-ADP-ribosylated peptides and corresponding proteins, **Chapter 6** describes the synthesis of ADP-ribosylated asparagine and its co-crystal structure with *MacroD2*. In **Chapter 7**, a general approach towards triazole linked mono-ADP-ribosylated peptide is described. The first fully synthetic ADPr-protein has been prepared using CuAAC click chemistry and has been shown to be biologically active. The robust CuAAC click chemistry described in this chapter is also used in Chapter 4 to obtain biotinylated oligo-ADPr. Finally, **Chapter 8** summarizes all the work in this Thesis and discusses future directions in the chemistry of ADP-ribosylated molecules.

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