Synthetic Study on ADP-ribosylation

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For my Parents

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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]

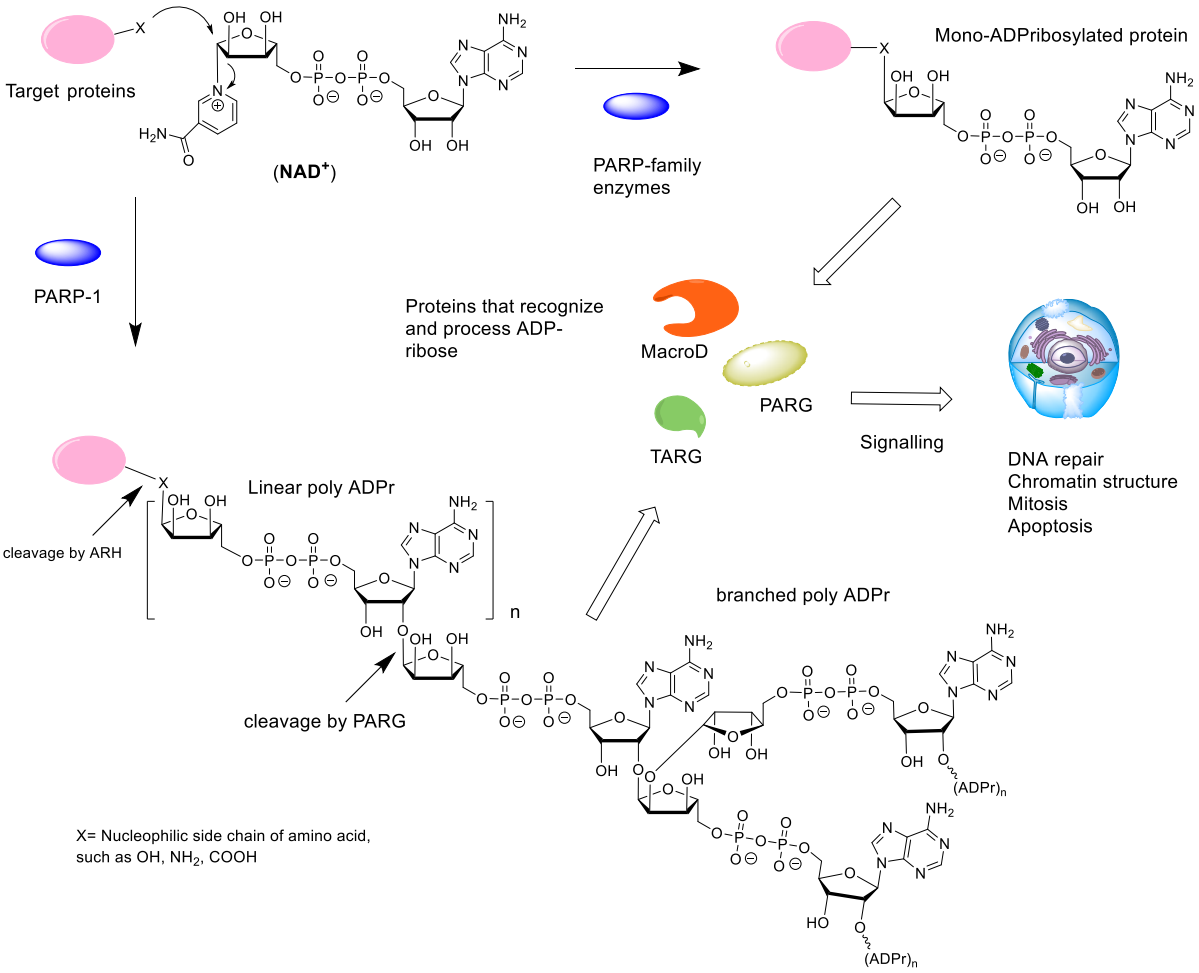


Figure 1. Biosynthesis and metabolism of mono- and poly-ADP-ribosylated proteins

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.

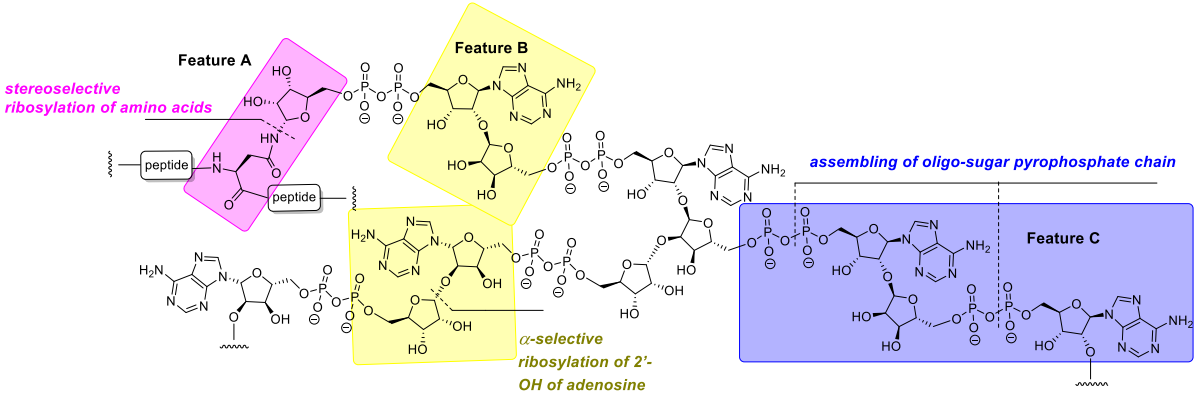


Figure 2. The structure of PAR with its most conspicuous synthetically challenging features

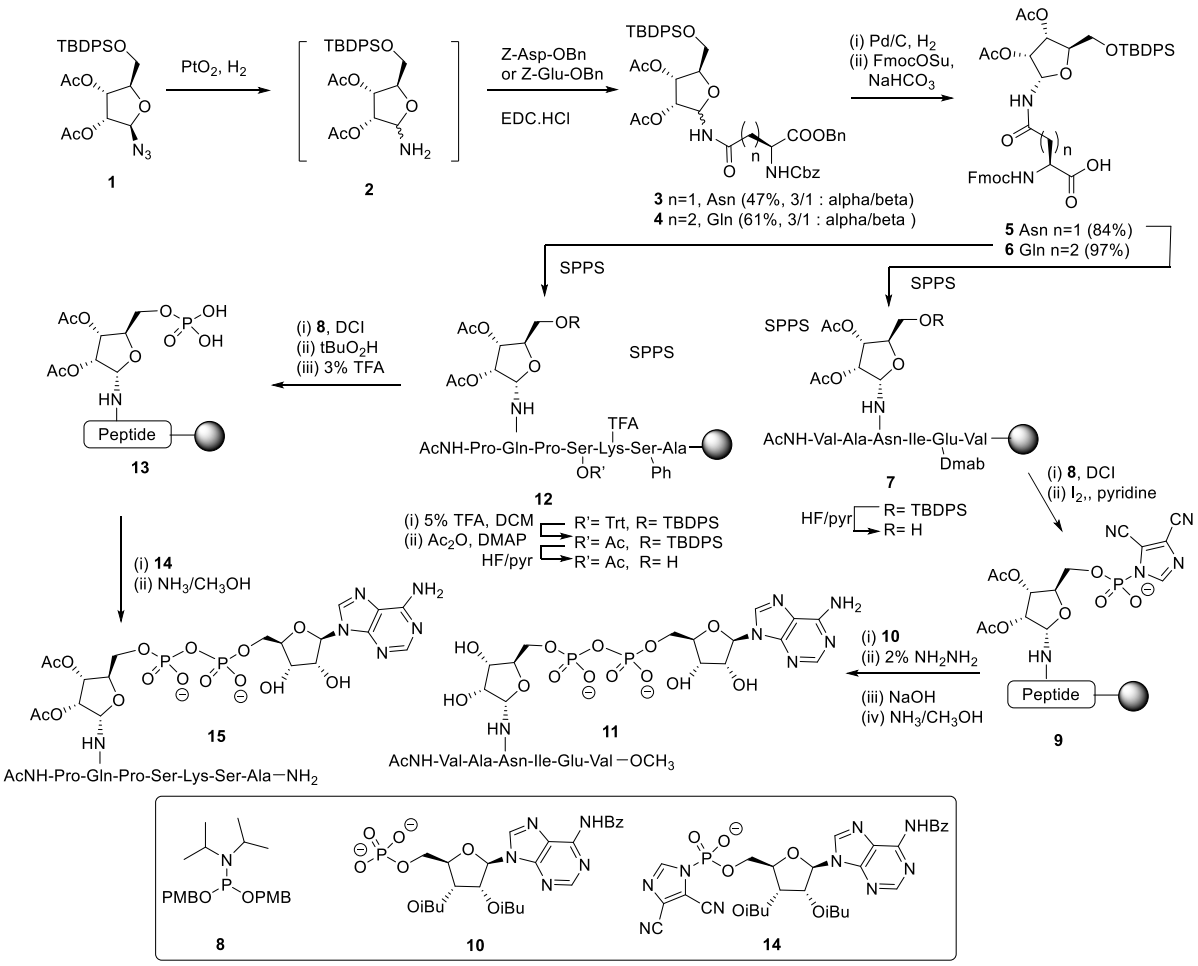
2. Chemical synthesis mono-ADPr-peptides and ADPr-oligomers

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.

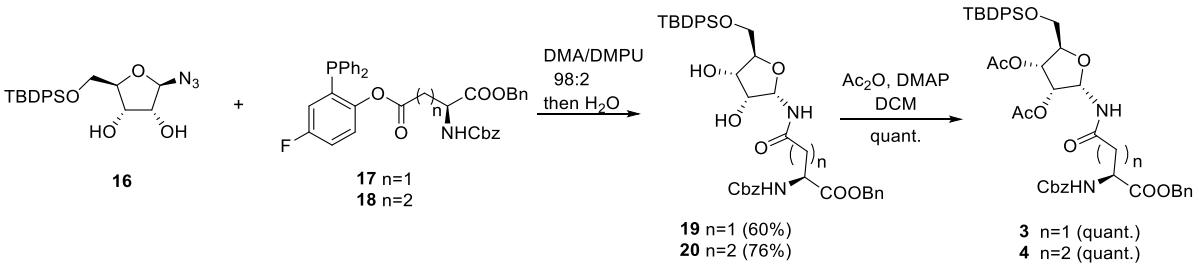
The first reported synthesis of suitably protected α-ribosylated amino acid building blocks and their application in a SPPS assembly of relevant ADP-ribosylated oligopeptides is of van der Heden van Noort *et al*.[8] The choice for Fmoc-based peptide synthesis led to the synthesis of protected α-ribosylated 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.

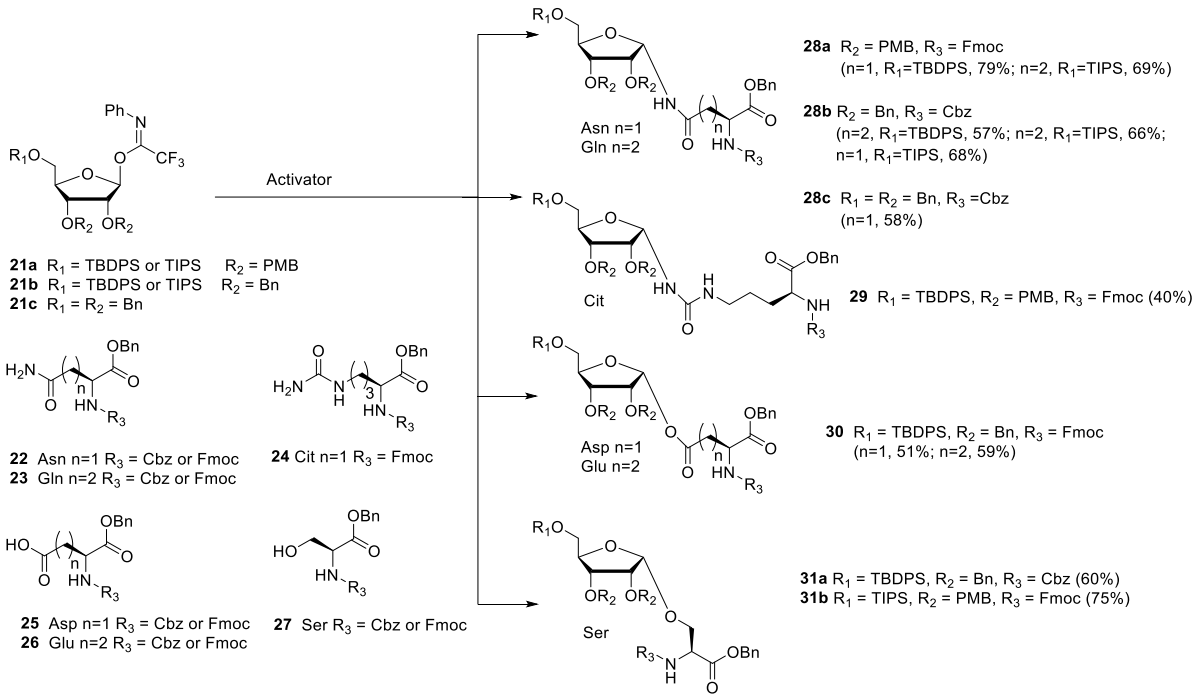
Other authors also undertook the synthesis of protected α-ribosylated asparagine and glutamine building blocks. Thus, Bonache *et al*.[13] have prepared such derivative for the first time, while F. Nisic *et al*.[14, 15] developed a stereoselective synthesis of α- or β-glycofuranosyl amides with the 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**.

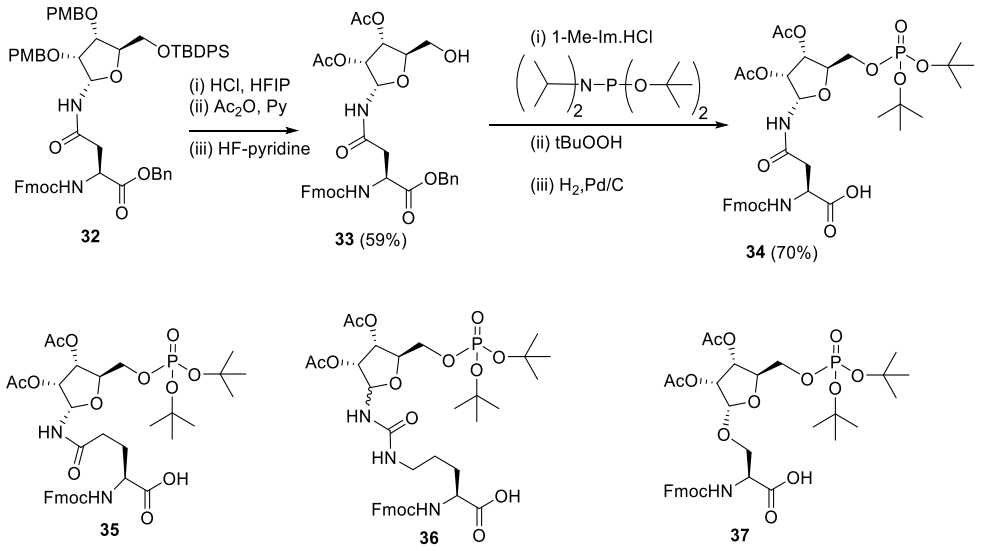
With the aim to broaden the range of the synthetically accessible ribosylated amino acids Kistemaker *et al*.[17] developed an alternative ribosylation method that employed ribosyl donors **21a, b, c** (Scheme 3) with the N-phenyl trifluoroacetimidate leaving group and with non-participating ether protecting 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%).

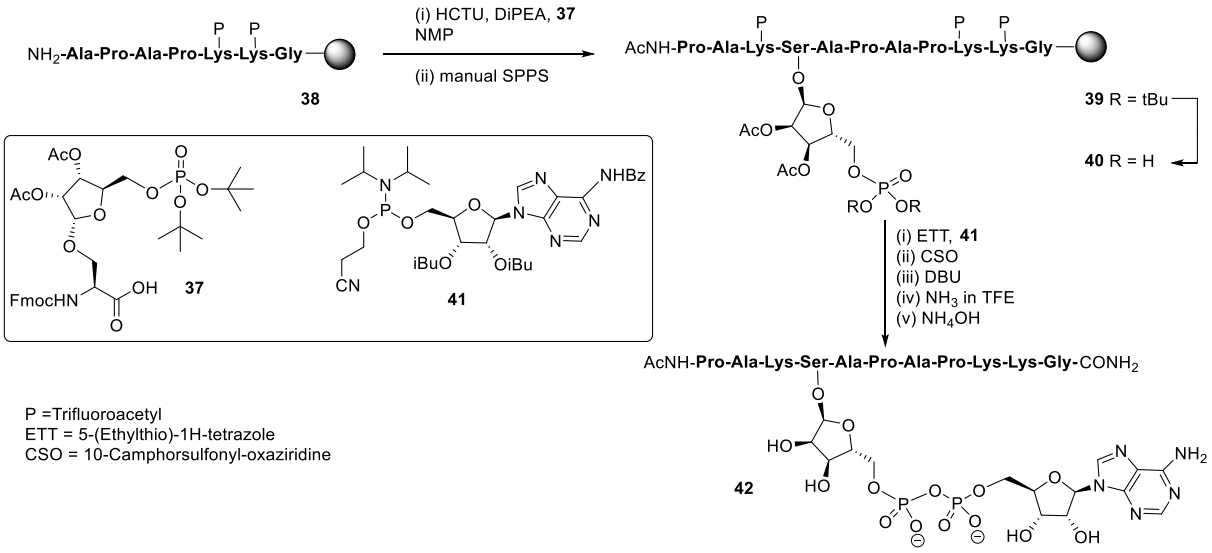
To obtain relevant ADP-ribosylated oligopeptides by SPPS, Kistemaker *et al*.[9, 18] also searched for another procedure for pyrophosphate formation. To this end, the solution phase method for the synthesis of sugar nucleotides, reported by Gold *et al*[19] was adopted. This procedure[9] combines phosphoramidite (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.

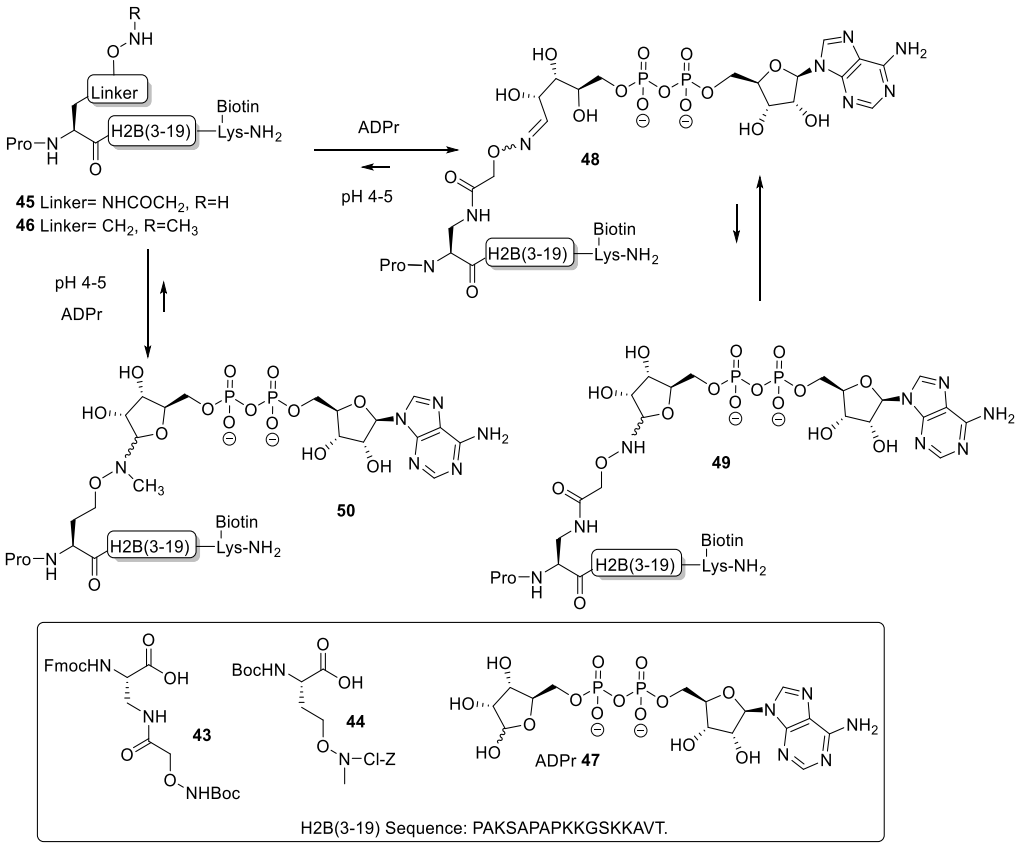
The di-*tert*-butyl phosphate triester was installed with di-*tert*-butyl *N*,*N*-diisopropylphosphoramidite and subsequent oxidation of the intermediate phosphite triester. Finally, hydrogenolysis of the benzyl ester gave the α-configured Asn building block **34** suitable for SPPS. Transferring this 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).

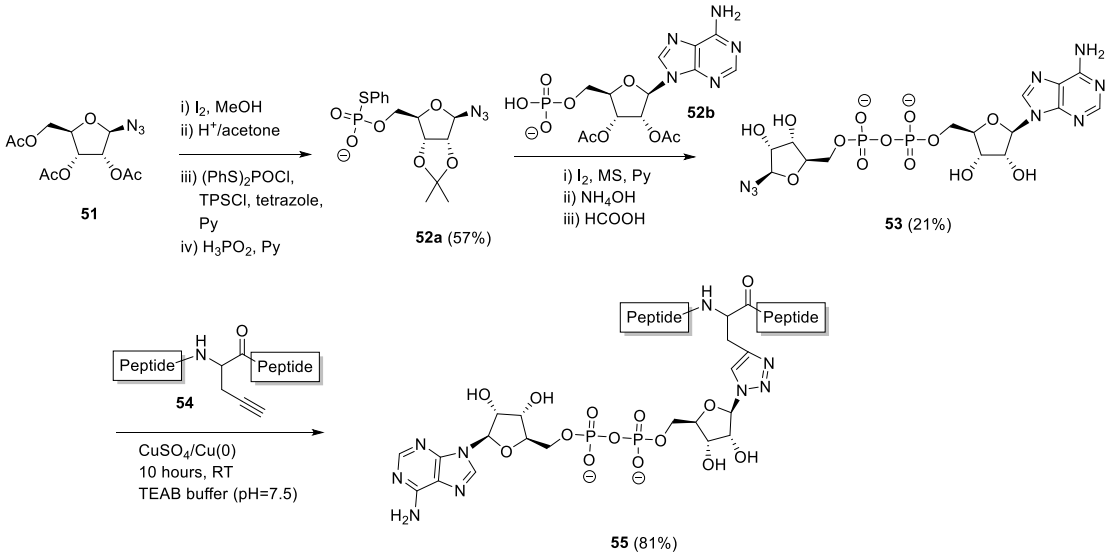


Scheme 6. The aminooxy and N-Methyl aminooxy functionalized peptides **48-50** of the group of Muir.

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.

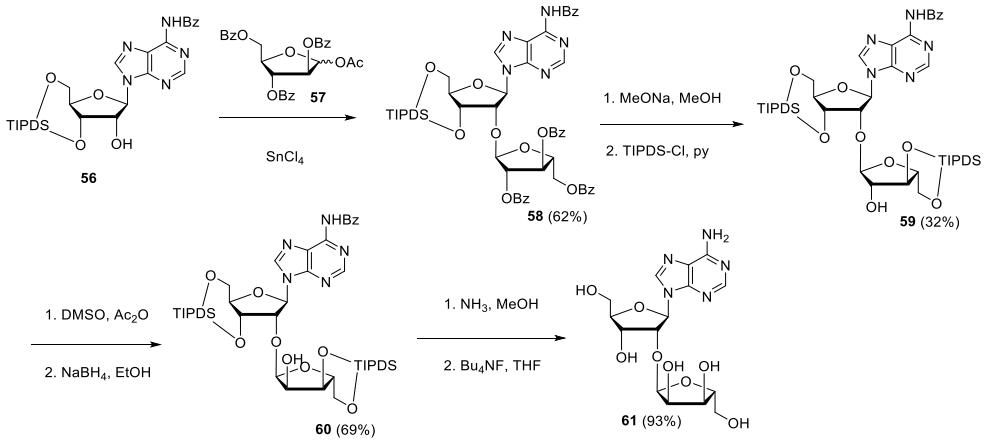
2.2 Synthesis of poly ADPr chain

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).

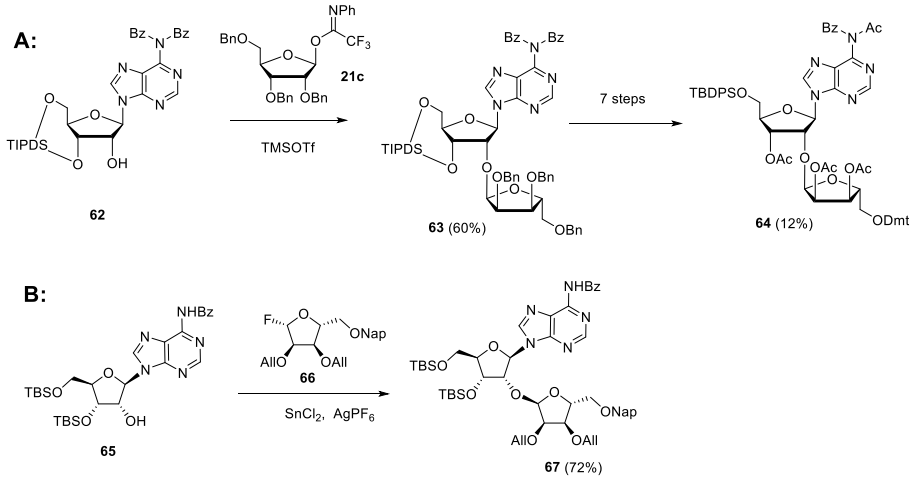
2.2.1 Building blocks synthesis—ribosylated adenosine

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.

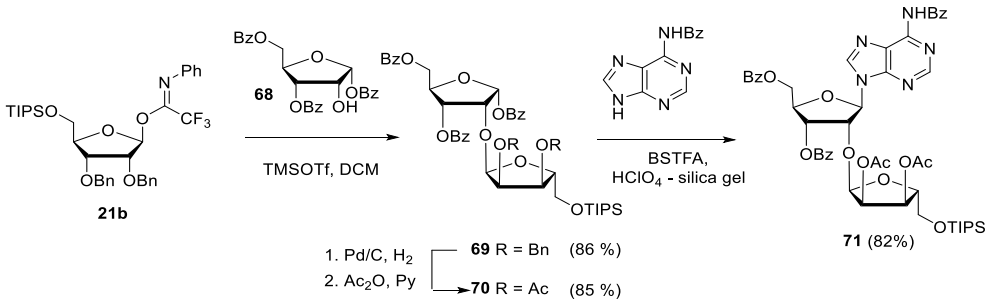


Scheme 8. Synthesis of 2'-O-α-ribosylated adenosine **61** using 1-O-acetyl-2,3,5-tri-O-benzoyl-D-arabinofuranose **57** as the donor



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.

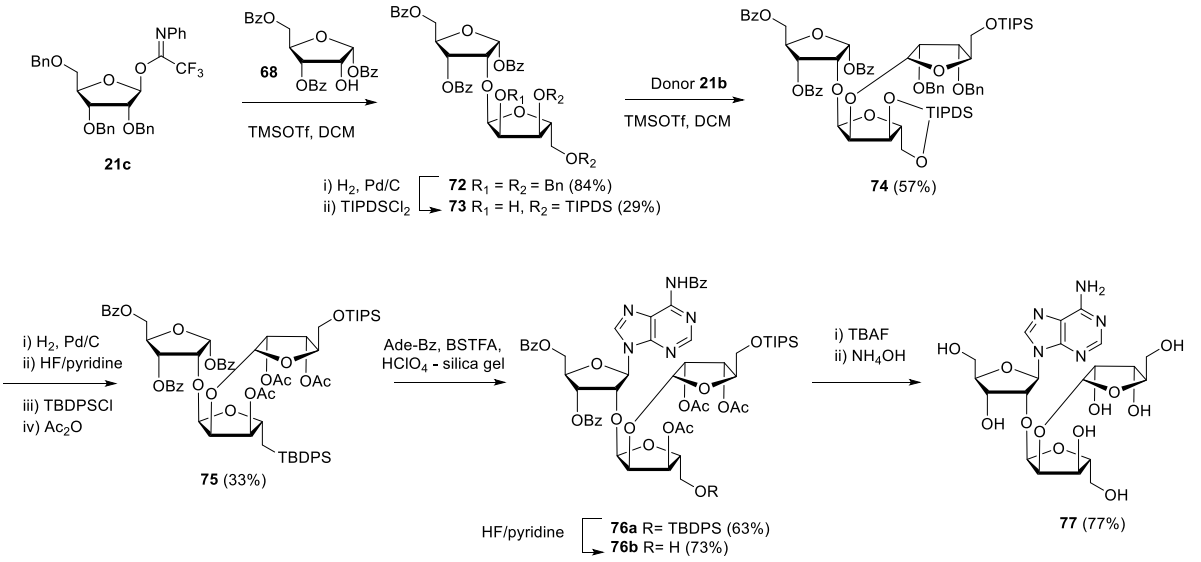
In 2015 Lambrecht *et al*[28] reported the synthesis of orthogonally protected ribosyl adenosine **67** (Scheme 9B) by the α-selective condensation of β-fluoride donor **66**, obtained in six steps from ribose with adenosine acceptor **65**. Crucial for the productivity of this reaction was the use of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



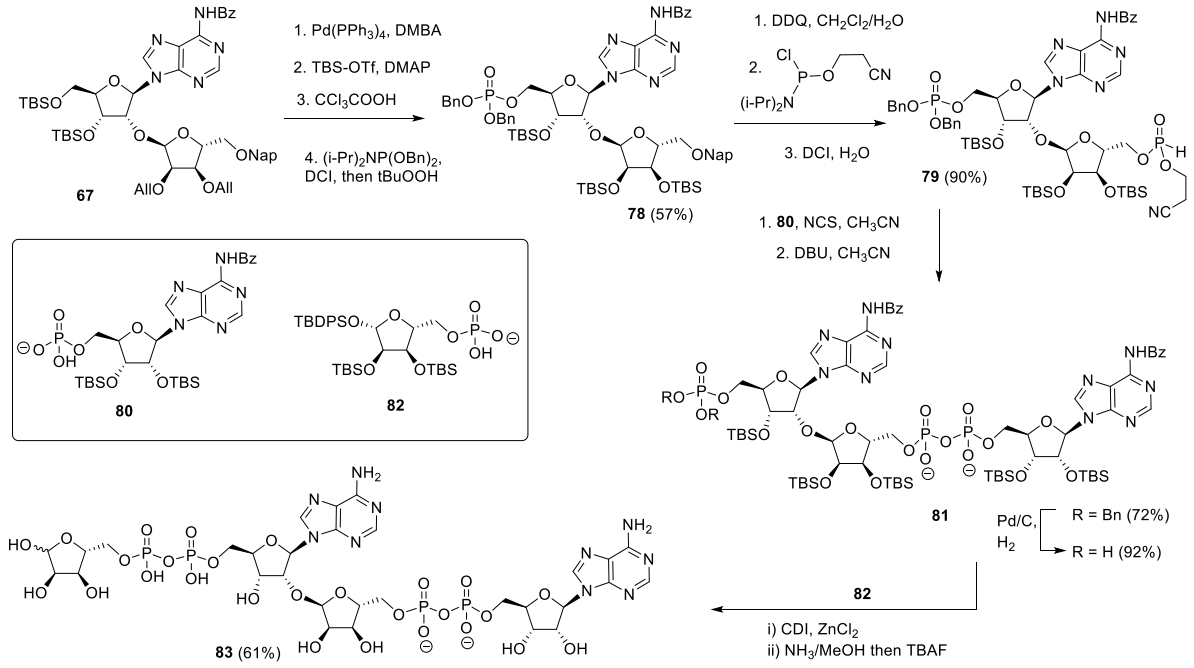
Scheme 11. Synthesis of *O*-α-D-ribofuranosyl-(1'''→2'')-*O*-α-D-ribofuranosyl-(1''→2')-adenosine: the branching point of ADPr-chain

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

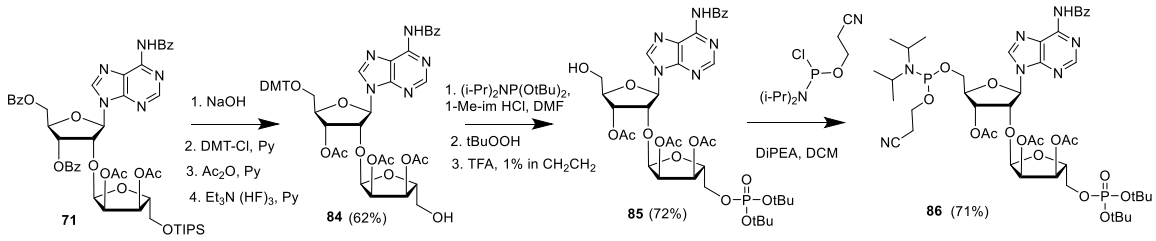
route of synthesis ribosylated adenosine building block **67**, obtained as described above (Scheme 9) was subjected to protective group manipulation to allow the installation of a dibenzyl phosphotriester at the primary OH of adenosine with the aid of phosphoramidite chemistry and subsequent oxidation. 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.



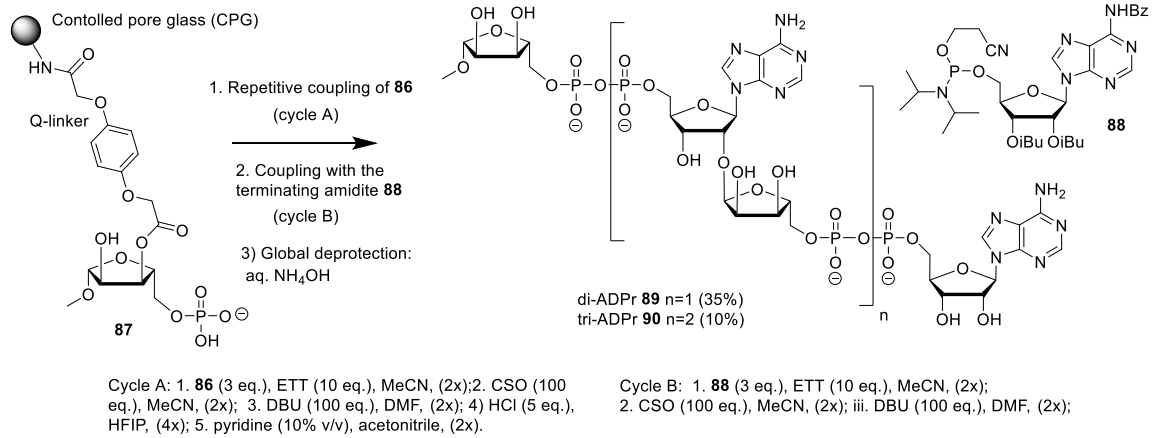
Scheme 12. Synthesis of ADPr dimer **83** by Lambrecht *et al*

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



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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2 Synthesis of a native branched ADPr fragment and its biotinylated derivatives

Part of this chapter has been published:

Liu, Q.; Kistemaker, H. A. V.; Overkleeft, H. S.; van der Marel, G. A.; Filippov, D. V., Synthesis of ribosyl-ribosyl-adenosine-5',5'',5'''(triphosphate)-the naturally occurring branched fragment of poly(ADP ribose). *Chem. Commun.* **2017**, 53 (74), 10255-10258.

Introduction

Poly ADP-ribosylation (PARylation) is an important post-translational modification in which negatively charged ADP-ribose chains are transferred to an acceptor protein using NAD^+ (nicotinamide adenine dinucleotide) as a donor and PARPs (poly ADP ribose polymerases) as the involved enzymes[1] (Figure 1.). PARylation and the resulted polymers (PARs) are involved in many biological events such as DNA repair, transcriptional regulation, cell death and apoptosis.[2] PAR chains can be either linear or branched[3]. Linear PAR can grow to over 200 units in size, with a branching site occurring on average once every 20 to 50 elongation reactions.[1] While the knowledge on linear PAR is steadily growing, less progress is made with the role of branched PAR and its function is still unclear. There are a few reports

on branched PAR after its discovery by Miwa *et al*[3] at the end of the 1970s. For example, the branched and not the linear PAR chains bind most preferably to histones[4] and other nuclear proteins[5]. The branching point is reported not to be the endoglycosidic cleavage site of poly-ADP-ribose glycohydrolase (PARG)[6] which indicates that there might be undiscovered enzymes that specifically recognize the branched PAR structure.[7, 8] In 1981, the chemical structure of the branching point of PAR was established as *O*-α-D-ribofuranosyl-(1'''→2'')-*O*-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate) (Figure 1**, 1**) by Miwa *et al*.[9] They performed an enzymatic synthesis using NAD^+ and calf thymus nuclei, to get PAR *in vitro*. Subsequent hydrolysis of all the pyrophosphate linkages in PAR by treatment with snake venom phosphodiesterase led to the isolation of branched PAR fragment **1**. The configuration of **1** was determined by derivatization and with the aid of physicochemical techniques including gas chromatography, mass spectrometry, and ^{1}H-NMR spectroscopy[9]. Shortly after the structure elucidation, two different groups[10, 11] reported the existence of branched PAR in *vivo,* indicating that the branched PAR fragment made from enzymatic synthesis is indeed the naturally occurring product. Furthermore, enzymatic synthesis is widely applied to simulate in *vivo* conditions and to produce PAR[12-14]. In this respect, the organic synthesis of branched ADPr fragment **1** is a challenging and valuable goal that can confirm this structure elucidation and will support future biological studies.

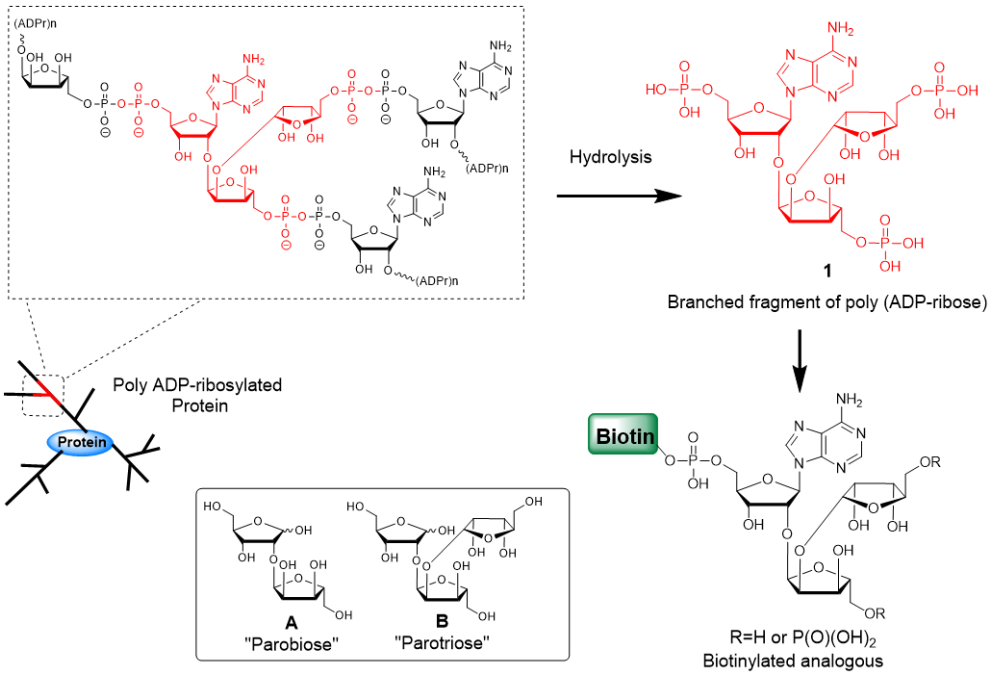
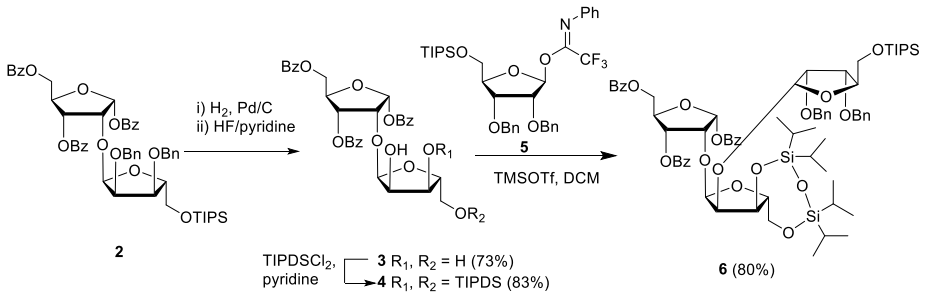


Figure 1. Structure of branched PAR fragment **1** and branched biotinylated analogous. Disaccharide **A** is "parobiose" and trisaccharide **B** is called "parotriose".

Up to now, synthetic methodologies have been developed towards PAR related molecules such as ribosylated amino acids[15], mono-ADP ribosylated peptides[16, 17] and a linear ADP ribose dimer and

trimer.[18] This chapter describes the synthesis of branched ADPr fragment **1** and also its structural analysis in comparison with the enzymatically prepared compound. The synthetic route toward **1** is guided by the earlier reported synthesis of the core motif of branched PAR[19] by adaptation of the protective group strategy and simultaneous introduction of three phosphotriester functions on the 5',5'', 5'''-primary hydroxyls of a suitably protected branched trisaccharide with phosphoramidite chemistry. Furthermore, this chapter describes the use of similar approach to the synthesis of biotinylated derivatives of branched and linear ADPr fragments (Figure 1), which could be valuable tools for searching for new proteins capable to bind branched PAR in proteomic studies.[20]

Results and discussion



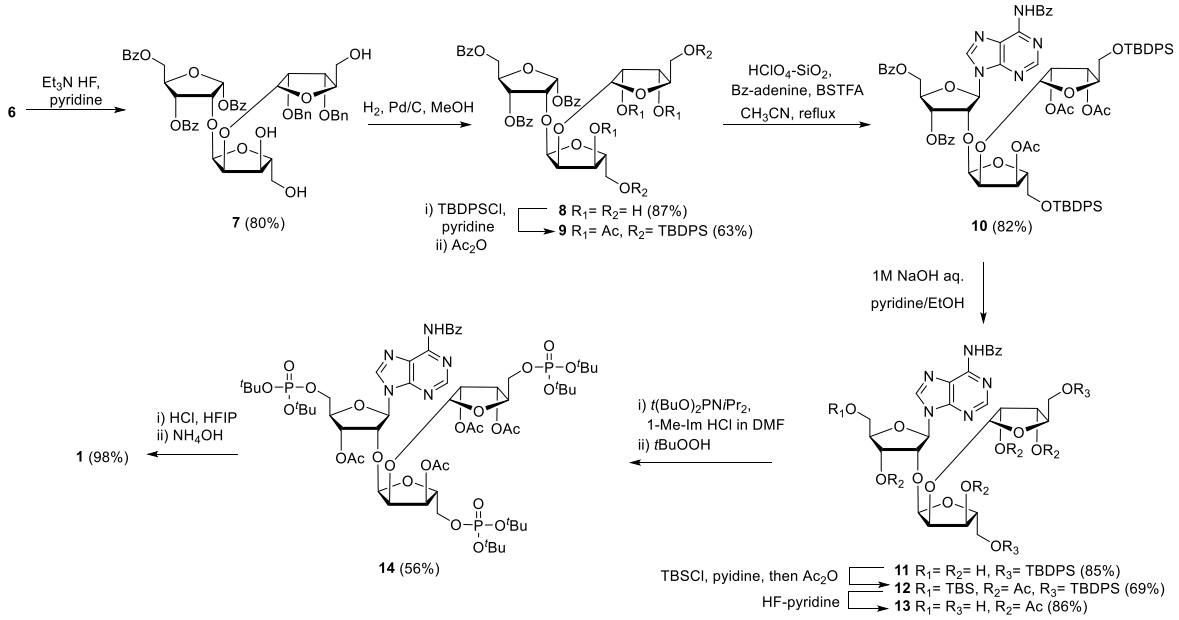
Scheme 1. Synthesis of protected parotriose **6** from parobiose **2**

The first stage of the route to target **1** comprises the preparation of protected parotriose **6**, provided with two challenging 1,2 cis-α-glycosidic linkages[21] (Scheme 1). Hydrogenolysis of the benzyl groups in α-configured and protected parobiose **2**, obtained according to an earlier reported method,[18] was followed by the removal of TIPS (triisopropylsilyl) group with HF·Pyridine to give 2'',3'',5''-OH parobiose **3** in good yield. It is interesting that the presence of the TIPS instead of the benzyl group at the 5'-OH of compound **2,** avoided glycosidic bond cleavage during hydrogenolysis as reported in previous study[19], resulting in a significantly improved yield and making large scale synthesis possible.

The 3'',5''-OH functions in triol **3** were selectively masked with the diol protecting TIPDS group by treatment with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSCl_2$) in pyridine to get alcohol **4**. Coupling of partially protected parobiose **4** with *N*-phenyl trifluoroacetimidate donor **5** afforded the fully protected parotriose **6** with complete α-selectivity and improved yield.[19]

The subsequent introduction of adenine base required a number of protective group manipulations (Scheme 2). The benzyl-protecting groups in **6** had to be replaced because the adenine moiety would complicate hydrogenolysis.[21] However, the removal of benzyl ethers in **6** by Pd/C-catalyzed hydrogenation was tedious (took more than 4 days) and was accompanied by ring opening of the TIPDS group. This side reaction could not be prevented by the use of other catalysts such as $Pd(OH)_2$. Although

the lability of the TIPDS group on the trisaccharides presumably made the hydrogenolysis problematic, selective removal of the TIPDS in **6** could not be attained**.** Therefore, **6** was treated with an excess of $Et_3N{\cdot}HF$ for 24 h to remove all silyl groups. Hydrogenolysis of the thus obtained compound **7** using Pd/C in methanol for 24 h afforded compound **8**, provided with five hydroxyl functions, in high yield. Readjusting the protection by the installation of TBDPS groups on the primary hydroxyls of compound **8** and acetylation of the remaining secondary hydroxyls set the stage for the introduction of N^6-benzolyadenine on the reducing end of parotriose **9**. Vorbrüggen type glycosylation using $HClO_4$-SiO_2 as catalyst and persilylated N^6-benzolyadenine proceeded completely β selective and furnished **10** in high yield.[19] The selective glycosylation on the *N*-9 position and not *N*-3 or *N*-7 was ascertained by UV-spectroscopy. Before three identical phosphate triesters could be installed on the 5',5'', 5'''-primary hydroxyls, protective group manipulation was required to ensure regioselective phosphorylation. Thus, saponification of the acetyl and benzoyl esters with aqueous NaOH in pyridine/ethanol gave intermediate **11,** allowing protection of the remaining free 5'-OH groups with TBDPS groups.



Scheme 2. Synthesis of the branched portion of poly-ADPr **1**

Surprisingly, the reaction of the 5'-OH in **11** with TBDPSCl failed, but fortunately the equally suitable TBS group was introduced successfully using the more reactive TBSCl in pyridine. Subsequent acetylation of this intermediate gave fully protected **12**. After removal of the silyl ethers by HF·pyridine, all primary hydroxyl functions were released to give triol **13**, amenable to the simultaneous introduction of three di-*tert*-butylphosphotriesters. Treatment of **13** with 10 equivalents di-*tert*-butyl-*N*,*N*–diisopropylphosphoramidite using 1-methylimidazole and 1-methylimidazolium chloride as activators under strictly anhydrous conditions[22] and subsequent oxidation of the intermediate

phosphite triesters gave **14** in moderate yield. The low reactivity of 5'-OH of the adenosine moiety as noticed in the silylation of **11** also decreases the yield of the phosphitylation reaction as the formation of target **14** was accompanied by bis-phosphitylated product. In the final stage, the *tert*-butyl groups of the phosphotriester in fully protected **14** were removed by HCl/HFIP in 1 h, followed by ammonolysis of the acyl groups to furnish tris-phosphorylated parotriosyladenine **1** in excellent yield.

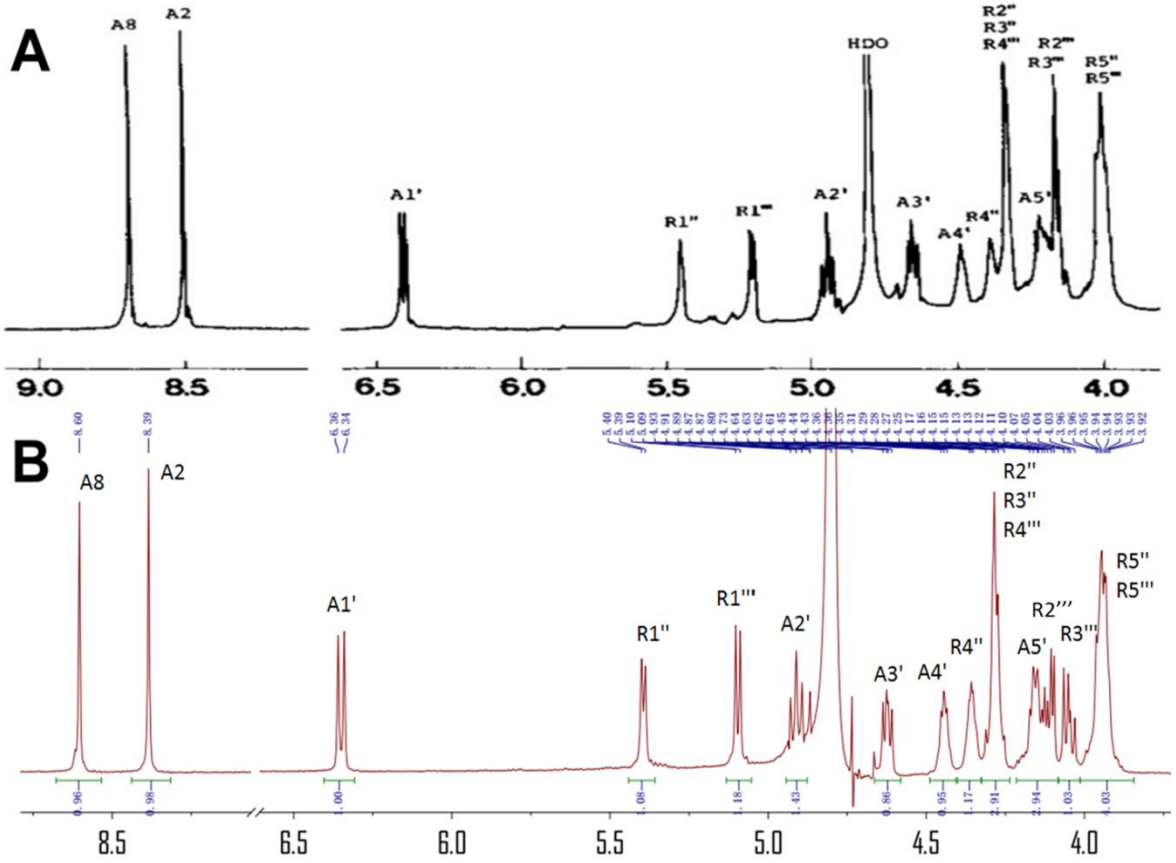
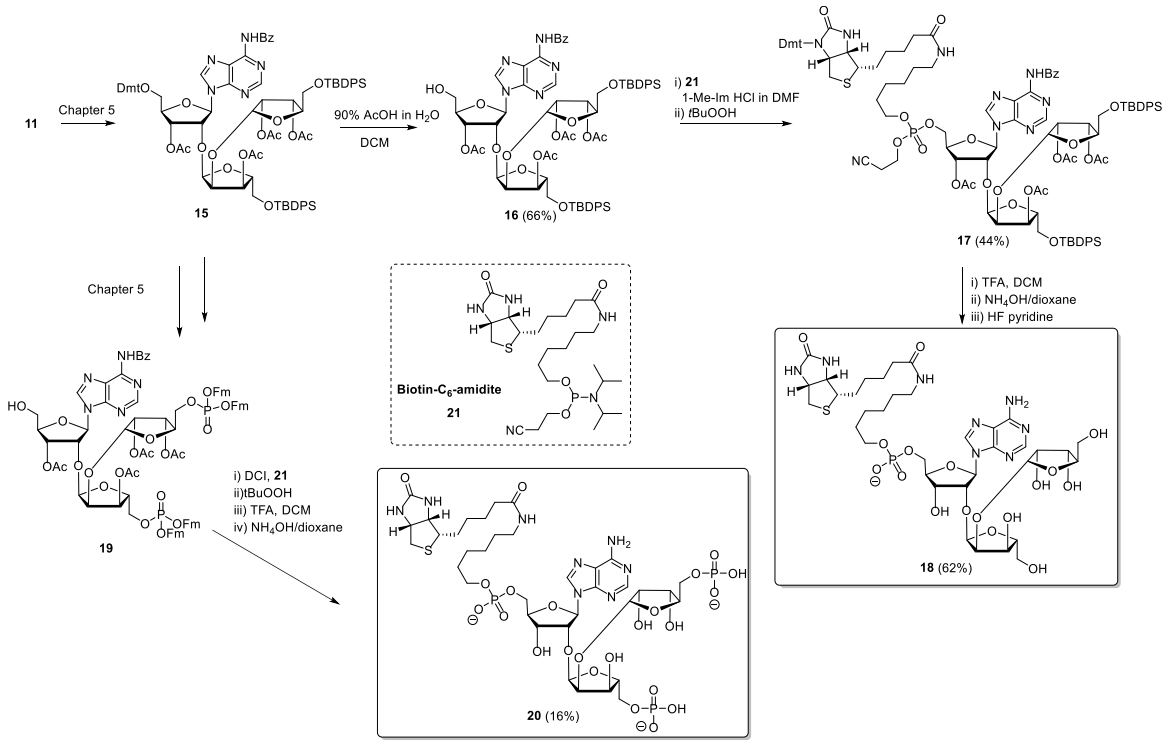


Figure 2. Comparison of ^{1}H-NMR-spectra of the branching portion of PAR. (**A**) Isolated compound **1**, 270 MHz, in D_2O (pH=3) as reported by Miwa et al. (**B**) Synthetic compound **1** from this work, 300 MHz, in CD_3COOD and D_2O (pH=3).

Next, the spectroscopic data of the just obtained target compound **1** were compared with those reported by Miwa[9] for the enzymatically prepared product (Figure 2A). In the first instance, significant differences between the ^{1}H-NMR spectra were observed, that may be attributed to a pH difference of the NMR samples, which in turn may be due to different isolation procedures. The isolation of the synthetic branched ADPr fragment **1** involved global deprotection by ammonia treatment, followed by purification by HW-40 gel filtration using 0.15 M NH_4OAc in H_2O as eluent under essentially neutral conditions. Contrary, Miwa firstly desalted the isolated product by DEAE-cellulose column chromatography, followed by column chromatography on phosphocellulose. By doing this, Miwa

obtained branched ADPr fragment as an acidified sample (pH=3) while the synthetic product occurs in the neutralized form (pH=7) as an ammonium salt. To get a more accurate comparison, the pH of the sample with synthetic fragment **1** in D_2O was reduced from 7 to 3 by adding CD_3COOD. Under these conditions, the NMR spectrum of the synthetic branched ADPr fragment **1** and the enzymatically prepared one of Miwa proved to be virtually identical (Figure 2A vs Figure 2B). A small difference in the multiplicity at R2''' and R3''' could be attributed to the slight difference in the applied field. Overall the chemical shifts of all protons in the synthetic compound **1** are approximately 0.1 ppm upfield from those of enzymatically prepared compound mainly because Miwa used DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as a reference. Taken together, it was concluded that the synthetic branched ADPr fragment and the enzymatically prepared one have the same chemical structure.

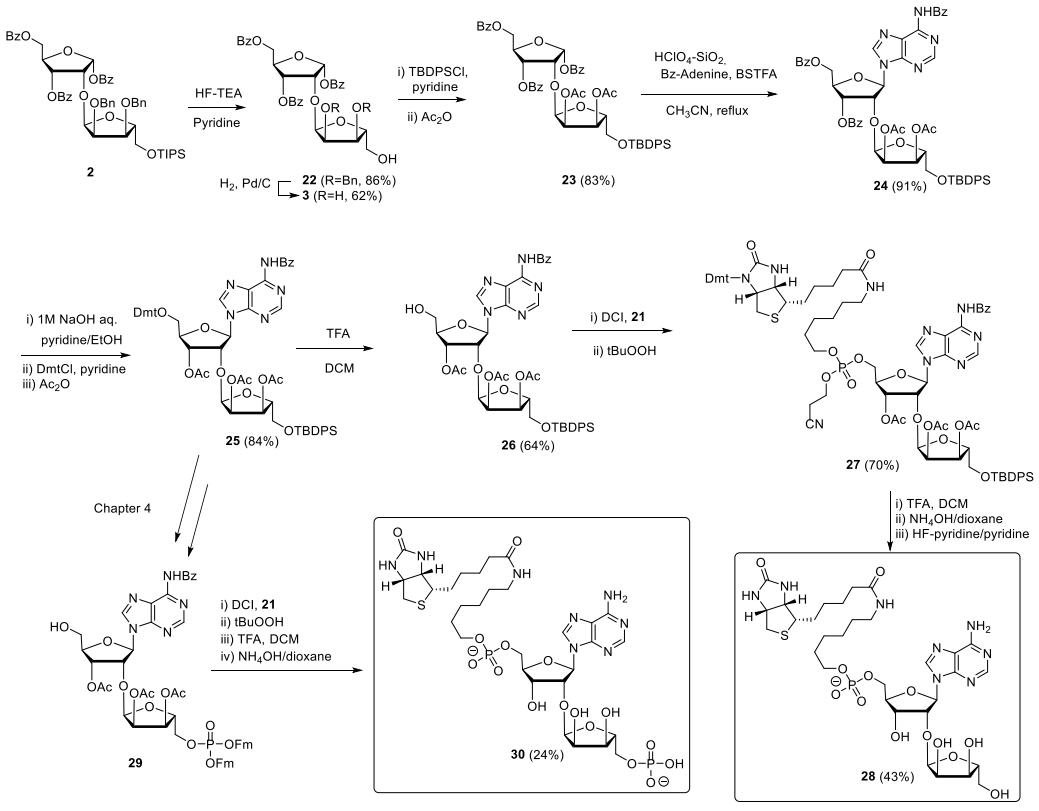


Scheme 3. Synthesis of biotinylated branched ADPr fragment

Biotinylated ADPr derivatives can act as valuable tools in biochemical studies on PAR. Although the synthesis of biotinylated ADPr dimers[8] has been reported, the synthesis of a similar biotinylated branched ADPr fragment is unknown. Recently Chen *et al*[23] reported that the PBZ domain of APLF protein binds to branched PAR by recognizing fragment **1** in the PAR chain, thereby regulating chromatin remodeling in DNA repair. Based on this result, it was reasoned that the search for the yet undiscovered proteins capable of binding to branched PAR would be facilitated by the availability of biotinylated branched ADPr fragments. The successful synthesis approach to compound **1** can be adapted for both the synthesis of biotinylated branched ADPr fragments (**18** and **20**, Scheme 3) and

the biotinylated linear counterparts, as negative controls (**28** and **30**, Scheme 4).

The synthesis of both the phosphorylated (**20**) and unphosphorylated (**18**) biotinylated branch points of PAR started with bis-ribosylated adenosine building block **11** (Scheme 3). Dimethoxytritylation of the 5'-OH in **11** and acetylation of the remaining secondary hydroxyls yielded **15** (details discussion in Chapter 5). Selective removal of the DMT group with 90% AcOH/H_2O in DCM furnished **16,** the liberated hydroxyl of which was amenable for the installation of the biotin moiety by phosphorylation with biotin-C_6-amidite (**21**)[24] under the activation of DCI and subsequent oxidation by *t*-BuOOH to give fully protected **17**. The DMT group in **17** was removed using TFA in DCM, all acetyl esters and the benzamide were cleaved with aqueous NH_4OH and finally HF·pyridine treatment removed the silyl groups to furnish target biotinylated branched ADPr fragment **18** in 62% yield after HW-40 purification. En route to 5'',5'''-phosphorylated biotinylated branched ADPr fragment **20**, compound **15** was desilylated, phosphorylated by coupling with bis (*9H*-fluoren-9-ylmethyl)-diisopropylamidophosphite and subsequently oxidized. Finally, detritylation gave **19** in good yield (see details discussion in Chapter 5). Fluorenylmethyl (Fm) groups[25] were selected to protect both terminal phosphates to achieve the simultaneous removal of the Fm, acetyls and benzoyl groups using NH_4OH treatment. The free hydroxyl in **19** was coupled with Biotin-C_6-amidite **21** under the activation of DCI, followed by oxidation by *t*-BuOOH, as described above for the formation of **17**. The protecting groups were removed by sequential treatment with TFA and aqueous ammonia to yield **20** after RP-HPLC purification.



Scheme 4. Linear biotinylated ADPr fragment.

The synthesis of biotinylated portions of linear PAR (**28** and **30**, Scheme 4) commenced with known protected parobiose **2**[18] and its conversion into **3**. In contrast to the same transformation, described in scheme 1 of this chapter, the TIPS protection in **2** was removed first by the treatment with HF·TEA in pyridine followed by removal of the benzyl groups in the thus obtained **22** by hydrogenolysis with Pd/C and H_2 under high pressure to yield triol **3** after overnight treatment. This reversal of reactions gave a lower yield but the reaction time is markedly reduced from several days to 16 hours. The more acid-resistant TBDPS, instead of the TIPS, was introduced at 5''-OH of **3** and the other secondary alcohols were acetylated to furnish suitably protected parobiose **23**. N^6-benzoyl adenine was installed using the Vorbrüggen glycosylation method,[18] furnishing **24** in excellent yield. The synthetic route was continued by the following one-pot sequence of protecting group manipulations: aqueous NaOH mediated saponification, DMT introduction at 5'-OH and acetylation of the remaining secondary hydroxyls to yield orthogonally protected **25**. To obtain biotinylated linear fragment **28**, building block **25** was successively subjected to TFA mediated detritylation and reaction of the resulting **26** with phosphoramidite **21**, followed by oxidation, as described above, to furnish fully protected **27**. With the aid of the same three-step deprotection protocol as described for the formation of **18**, biotinylated linear fragment **28** was isolated. The final biotinylated iso-ADPr **30** was obtained by subjecting **25** to protective group manipulation and phosphorylation to give **29** (see Chapter 4). Conversion of intermediate **29** into biotinylated target **30** was accomplished using the same method as described for the isolation of **20.**

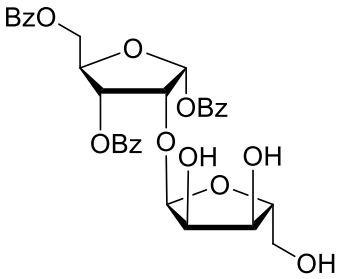
Conclusion

For the first time, O-α-D-ribofuranosyl-(1'''→2'')-O-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate) (**1**), a tris-phosphorylated branched PAR fragment was obtained by organic synthesis. Comparison of the ^{1}H-NMR spectra of this fragment with the naturally occurring product showed the same chemical shifts which means that the structure of **1** was identical to the naturally occurring compound[9] and that the regio- and stereochemistry of branching point of PAR was correctly elucidated by Miwa et al. In addition, key elements of the synthetic methodology presented in this Chapter also give access to biotinylated branched/linear PAR fragments synthesis. These analogous will be a valuable asset for future biological studies toward the discovery of BBPs (branched PAR binding protein) and elucidating biological function of branched ADPr.

Experimental section

General procedure

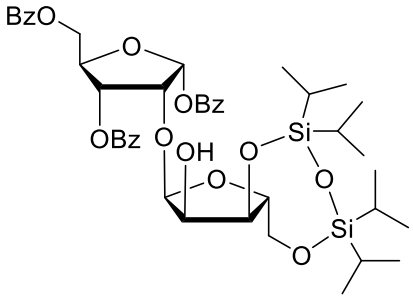
All solvents used were stored over molecular sieves and all reactions were carried out in oven or flame-dried glassware. Unless stated otherwise, all solvents were removed by rotary evaporation under reduced pressure at 40 °C. Reactions were monitored by TLC-analysis using Merk 25 DC plastikfolien 60 F254 with detection by spraying with 20% H_2SO_4 in MeOH or $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ in 10% sulfuric acid, followed by charring at approx. 150°C. LC-MS analysis was performed on a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan) using an analytical Gemini C18 column (Phenomex, 50 x 4.60 mm, 3 micron) in combination with eluents A: H_2O; B: MeCN and C: 1% aq. TFA as the solvent system. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylpthalate (m/z = 391.2842) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were measured on Brüker DPX-300, Brüker AV-400/500/600/850 and all individual signal was assigned using 2D-NMR spectroscopy. Chemical shifts were given in ppm (δ) relative to TMS (0 ppm) or indirectly referenced to H_3PO_4 (0.00 ppm) in D_2O via the solvent residual signal and coupling constants were given in Hz. Infrared (IR) spectra were record on a Shimadzu FT-IR 8300. Optical rotation was measured by MCP 100 Modular Circular Polarimeter using methanol as solvent.



α-1,3,5-Tri-*O*-benzoylparobiose (3)

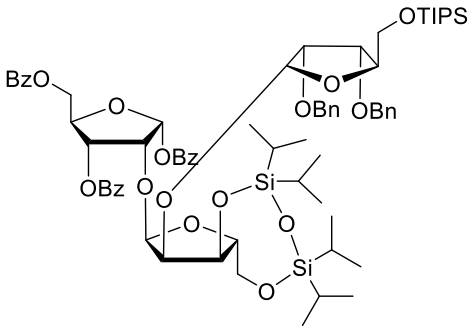
Compound **2** (6.90 g, 7.41 mmol) was dissolved in *t*BuOH/Dioxane/H_2O (120 ml, 4/4/1; v/v/v) and Pd/C (370 mg, 10% loading) was added. H_2 was bubbled through the solution for 72 h. TLC analysis showed an incomplete conversion, therefore, 300 mg Pd/C was added and the reaction was stirred under H_2 for 4 days after which the reaction mixture was filtered over celite, concentrated under reduced pressure and co-evaporated with pyridine (1 x) and toluene (1 x). 60 mL pyridine was added and the mixture was cooled to 0 °C. Et_3N (15.5 mL, 111.0 mmol) and $Et_3N\cdot 3HF$ (18 mL, 111.0 mmol) were added. The mixture was stirred for 18 h at room temperature and quenched by aq. $NaHCO_3$ (sat.). The mixture was extracted with EtOAc (3 x 240 mL) and the combined organic layers were dried over $MgSO_4$. After concentration under reduced pressure, the crude was purified by silica gel chromatography (pentane/EtOAc, 25/75 – 20/80) to obtain **3** as a white foam (3.20 g, 5.38 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 – 8.05 (m, 6H, arom.), 7.62 – 7.57 (m, 3H, arom), 7.49 – 7.46 (m, 2H, arom), 7.42 – 7.36 (m, 4H, arom), 6.76 (d, *J* = 4.2 Hz, 1H, H1'), 5.74 (dd, *J* = 6.3, 2.0 Hz, 1H, H3'), 5.20 (d, *J* = 4.2 Hz, 1H, H1''), 4.88 (td, *J* = 3.7, 1.9 Hz, 1H, H4'), 4.75 (dd, *J* = 6.3, 4.2 Hz, 1H, H2'), 4.63 (AB, *J* = 12.1, 3.8 Hz, 2H, H5'), 4.04 – 3.94 (m, 2H, H2'', H4''), 3.89 – 3.84 (m, 1H, H3'), 3.66 (AB, *J* = 12.1, 3.8 Hz, 1H, H5''), 3.56 (AB, *J* = 12.1, 3.8 Hz, 1H, H5''), 2.70 (d, *J* = 9.6 Hz, 1H, OH), 2.52 (d, *J* = 9.1 Hz, 1H, OH), 1.85 (s, 1H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.84 (CO Bz), 166.14 (CO Bz), 165.93(CO Bz), 133.98, 133.69, 133.60, 130.06, 130.04, 129.80 (arom.), 129.66, 129.51, 129.04 (cq. arom.), 128.74, 128.73, 128.59 (arom.), 102.27 (C1''), 95.31 (C1'), 86.39 (C4''), 82.77 (C4'), 77.48, 77.16 76.84, 75.40(C2'), 72.33(C2''), 72.21 (C3'), 70.53 (C3''), 64.22 (C5'), 62.61 (C5''). IR (film): 3482 (br), 2930, 1717, 1267, 1117, 1093, 1068, 1022, 710 cm^{-1}. HRMS (ESI$^+$) calcd for

$C_{31}H_{30}O_{12}Na$ (M+Na) 617.1629. Found 617.1627. $[\alpha]_D^{20}$ +102.8 (c = 1, in MeOH)



α-1,3,5-Tri-*O*-benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-parobiose (4)

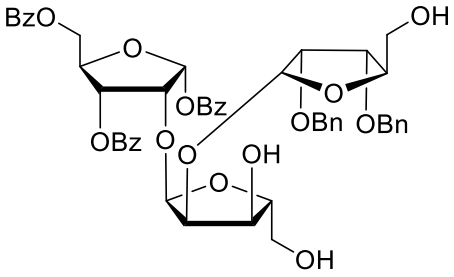
Compound **3** (6.66 g, 11.2 mmol) and imidazole (2.29 g, 33.6 mmol) were co-evaporated with toluene (2 x), dissolved in DCM (66 mL) and then TIPDCl (4.3 mL, 13.4 mmol) was added. The reaction was stirred at room temperature for 15 h and quenched upon the addition of H_2O (200 mL). The mixture was washed by DCM (3 x 100 mL) and the organic layer was dried by $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel chromatography (DCM/acetone, 100/0 – 97/3) to obtain **4** as colorless foam (7.83 g, 9.35 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 – 8.03 (m, 6H, arom.), 7.63 – 7.53 (m, 3H, arom.), 7.48 (t, *J* = 7.6 Hz, 2H, arom), 7.44 – 7.34 (m, 4H, arom.), 6.79 (d, *J* = 4.2 Hz, 1H, H1'), 5.67 (dd, *J* = 6.3, 2.0 Hz, 1H, H3'), 5.19 (d, *J* = 4.0 Hz, 1H, H1''), 4.77 (m, 2H, H2', H4'), 4.65 (AB, *J* = 12.1, 3.5 Hz, 2H, H5'), 4.14 – 4.01 (m, 2H, H2'' H3''), 3.95 – 3.90 (m, 1H, H4''), 3.81 (AB, *J* = 11.8, 3.5 Hz, 1H, H5''), 3.66 (AB, *J* = 11.6, 8.3 Hz, 1H, H5''), 2.85 (d, *J* = 8.2 Hz, 1H, OH), 1.03 (m, 6H, CH_3, TIPDS), 1.00 – 0.83 (m, 18H, CH_3, TIPDS), 0.80 (d, *J* = 7.3 Hz, 2H, CH, TIPDS), 0.73 (d, *J* = 7.2 Hz, 2H, CH, TIPDS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.15, 165.70 (CO Bz), 133.50, 133.48, 133.45, 130.14, 129.97 (arom.), 129.92 (cq. arom.), 129.80 (arom.), 129.63 (cq. arom.), 128.67, 128.57, 128.49 (arom.), 101.93 (C1''), 95.13(C1'), 83.81(C4''), 83.32(C4'), 75.67 (C2'), 71.93 (C3'), 71.03 (C2''), 70.77 (C3''), 64.30 (C5'), 63.39 (C5''), 17.55, 17.49, 17.45, 17.41, 17.06, 16.98, 16.82, 16.68, 13.44, 13.24, 13.01, 12.33 (CH, CH_3, TIPDS).



α-1,3,5-Tri-*O*-benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'',3''-di-*O*-benzyl-5''-*O*-triisopropylsilylparotriose (6)

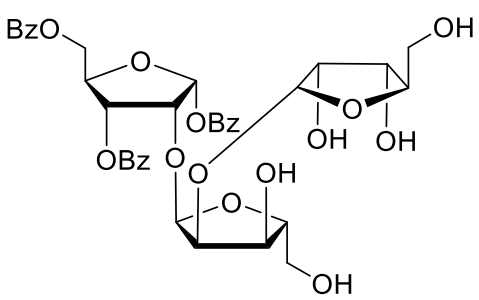
Compounds **4** (7.8 g, 9.32 mmol) and **5** (7.36 g, 11.18 mmol) were co-evaporated with toluene (2 x), 1,4-dioxane (2 x) and DCE (1 x). Dry DCM (150 mL) and freshly activated 4Å molecular sieves were added to the mixture. The mixture was stirred under argon at room temperature for 2 h and then cooled to -78°C. Next, TMSOTf (50 μL, 0.28 mmol) was added, the reaction mixture was stirred at the same temperature for 30 minutes and then was quenched by addition of triethylamine. The reaction mixture was concentrated under reduced pressure and purified by silica gel chromatography (pentane/EtOAc, 70/30 – 50/50) to obtain **6** as a white foam (9.7 g, 7.43 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.6 Hz, 2H, arom.), 8.08 (t, *J* = 7.6 Hz, 4H, arom.), 7.59 – 7.54 (m, 2H, arom), 7.48 (q, *J* = 7.4 Hz, 3H), 7.37 (t, *J* = 7.7 Hz, 2H, arom.), 7.29 – 7.11 (m, 12H, arom.), 6.79 (d, *J* = 4.0 Hz, 1H, H1'), 5.59 (dd, *J* = 6.3, 1.7 Hz, 1H, H3'), 5.36 (d, *J* = 3.4 Hz, 1H, H1''), 5.28 (d, *J* = 3.9 Hz, 1H, H1'''), 4.85 (dd, *J* = 6.2, 4.1 Hz, 1H, H2'), 4.76 – 4.67 (m, 2H, CH_2 Bn, H4'), 4.62 (AB, *J* = 12.0, 3.5 Hz, 1H, H5'), 4.52 (AB, *J* = 12.0, 4.0 Hz, 1H, H5'), 4.49 – 4.36 (m, 2H, CH_2 Bn, H2''), 4.32 (d, *J* = 11.8 Hz, 1H, CH_2 Bn), 4.25 – 4.01 (m, 4H, H3'', H4''', H4'', CH_2 Bn), 3.95 (d, *J* = 11.4 Hz, 1H, CH_2 Bn), 3.83 (AB, *J* = 13.1, 2.3 Hz, 1H, H5''), 3.78 – 3.63 (m, 4H, H5'',H5''', H3'''), 3.43 (dd, *J* = 6.4, 3.9 Hz, 1H, H2'''), 1.11 – 0.94 (m, 42H,

CH_3, TIPS, TIPDS,), 0.93–0.88 (m, 7H, CH, TIPS, TIPDS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.15, 165.99, 165.66 (CO Bz), 138.94, 138.68 (cq. arom.), 133.50, 133.47, 133.30, 130.14 (arom.), 130.04 (cq. arom.), 129.99, 129.93 (arom.), 129.77, 129.68 (cq. arom.), 128.62, 128.55, 128.13, 128.00, 127.85, 127.69, 127.35, 127.16 (arom.), 102.21 (C1''), 101.23 (C1'''), 95.07 (C1'), 83.34 (C4'), 81.18 (C4'''), 81.12 (C4''), 77.36 (C2'''), 75.56 (C3'''), 75.12 (C2'), 73.58 (C2''), 72.35 (C3'), 72.30 (CH_2 Bn), 71.78 (CH_2 Bn), 69.04 (C3''), 64.25 (C5'), 62.61 (C5''), 59.95 (C5'''), 18.07, 17.52, 17.47, 17.42, 17.22, 17.18, 17.09, 16.96, 13.61, 13.13, 12.77, 12.59, 12.03 (CH_3, CH_2, TIPDS, TIPS).



α-1,3,5-Tri-*O*-benzoyl-2'',3''-di-*O*-benzylparotriose (7)

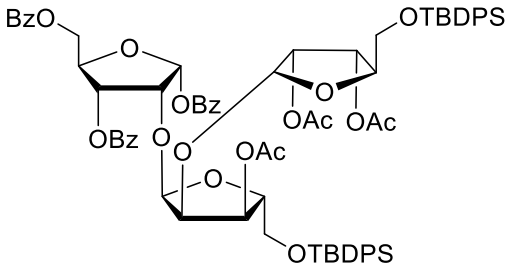
Compound **6** (7.7 g, 5.90 mmol) and 60 mL pyridine were added into a flask and cooled to 0℃. Subsequently, $Et_3N{\cdot}3HF$ (14.5 mL, 88.53 mmol) was added under argon. The reaction was stirred at room temperature for 24 h and then additional 1.5 ml $Et_3N \cdot 3HF$ was added at 0℃. The mixture was stirred for 5 h at room temperature and quenched by addition of aq. $NaHCO_3$ (sat.). 50 mL H_2O was added and the mixture was extracted by EtOAc (3 x 80 mL). The combined organic layers were dried over $MgSO_4$. The mixture was filtered and then concentrated under reduced pressure. Purification by silica gel chromatography (pentane/actone, 70/30 – 50/50) furnished **7** as a white foam (4.3 g, 4.74 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 – 8.04 (m, 6H, arom.), 7.60 – 7.52 (m, 3H,), 7.47 – 7.43 (m, 2H), 7.42 – 7.29 (m, 4H), 7.29 – 7.19 (m, 8H), 7.19 – 7.10 (m, 2H), 6.80 (d, *J* = 4.1 Hz, 1H, H1'), 5.64 (dd, *J* = 6.3, 2.4 Hz, 1H, H3'), 5.37 (d, *J* = 3.6 Hz, 1H, H1''), 5.31 (d, *J* = 3.7 Hz, 1H, H1'''), 4.79 (dd, *J* = 6.3, 4.1 Hz, 1H, H2'), 4.75 (td, *J* = 3.9, 2.4 Hz, 1H, H4'), 4.62 (AB, *J* = 12.0, 3.6 Hz, 1H, H5'), 4.57 (d, *J* = 11.9 Hz, 1H, CH_2, Bn), 4.52 (AB, *J* = 12.0, 4.3 Hz, 1H, H5'), 4.43 – 4.36 (m, 2H, CH_2, Bn), 4.30 (dd, *J* = 5.4, 3.6 Hz, 1H, H2'), 4.10 (q, *J* = 3.3 Hz, 1H, H4''), 4.04 (d, *J* = 11.4 Hz, 1H, CH_2, Bn), 4.01 – 3.90 (m, 2H, H4'', H3''), 3.70 (dd, *J* = 5.9, 3.1 Hz, 1H, H3'''), 3.65 – 3.43 (m, 5H, OH, H2''', H5'', H5'''), 3.36 – 3.30 (m, 1H, H5'''), 1.76 (d, *J* = 6.1 Hz, 1H, OH), 1.53 (dd, *J* = 8.4, 4.8 Hz, 1H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.14 (CO, Bz), 165.70 (CO, Bz), 137.85, 137.73 (cq. arom.), 133.64, 133.61, 133.50, 130.27, 130.10 (arom.), 129.94 (cq. arom), 129.89 (arom.), 129.70, 129.66 (cq. arom.), 128.65, 128.61, 128.57, 128.51, 128.48, 128.46, 128.41, 128.30, 127.98, 127.80, 127.77 (arom.), 102.66 (C1''), 99.56 (C1'''), 95.65 (C1'), 84.40 (C3''), 83.72 (C4''), 82.94 (C4'), 79.44 (C2'''), 76.07 (C3'''), 75.63 (C2'), 72.99 (cq. CH2, Bn), 72.86 (C2''), 72.32 (CH2, Bn), 71.97 (C3'), 70.30 (C4''), 64.19 (C5'), 62.60 (C5'''), 62.03 (C5''). IR (film): 3456 (bs), 2920, 1722, 1267, 1096, 1069, 1024, 712 cm^{-1}. HRMS (ESI^+) calcd for $C_{50}H_{50}O_{16}Na$ (M+Na) 929.2991. Found 929.2999. $[\alpha]_D^{20}$ +98.3 (c = 1, in MeOH)



α-1,3,5-Tri-*O*-benzoylparobiose (8)

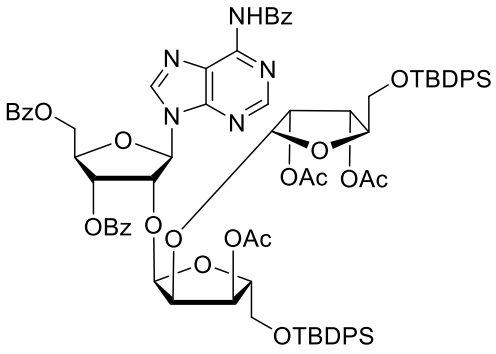
Compound **7** (420 mg, 0.46 mmol) was dissolved in MeOH (10 mL), Pd/C (100mg, 10% loading) and few drops of AcOH were added. The mixture was sonicated under argon for 5 minutes then H_2 was bubbled for 24 h. The reaction was filtered over celite and the residue was concentrated under reduced pressure and purified by silica gel chromatography (DCM/MeOH, 95/5 – 92/8)

to obtain **8** as a white foam (292 mg, 0.40 mmol, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 – 8.00 (m, 6H), 7.62 – 7.52 (m, 3H, arom), 7.49 – 7.33 (m, 6H, arom.), 6.76 (d, *J* = 4.2 Hz, 1H, H1'), 5.69 (dd, *J* = 6.3, 1.7 Hz, 1H, H3'), 5.27 (d, *J* = 3.9 Hz, 1H, H1''), 4.95 (d, *J* = 3.9 Hz, 1H, H1'''), 4.87 – 4.79 (m, 1H, H4'), 4.72 (dd, *J* = 6.3, 4.2 Hz, 1H, H2'), 4.67 – 4.53 (m, 2H, H5'), 4.10 – 4.02 (m, 1H, H2''), 3.96 – 3.93 (m, 3H, H3'', H4''', H4''), 3.73 – 3.35 (m, 7H, H3''', H2''', H5'', H5''', OH), 3.29 (d, *J* = 7.6 Hz, 1H, OH), 3.14 (d, *J* = 9.6 Hz, 1H, OH), 3.04 – 2.98 (m, 2H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.65 (CO, Bz), 166.17 (CO, Bz), 165.92 (CO, Bz), 133.85, 133.76, 133.52, 130.13, 129.76 (arom.), 129.50, 129.47, 129.07 (cq. arom.), 128.65, 128.56 (arom.), 101.41 (C1''), 100.98 (C1'''), 95.33 (C1'), 86.31 (C4''), 85.71 (C4'''), 83.18 (C4'), 75.30 (C2'), 75.21 (C2''), 72.45 (C2'''), 72.14 (C3'), 70.91 (C3''), 70.84 (C3'''), 64.29 (C5'), 62.76 (C5'''), 62.16 (C5''). IR (film): 3466 (bs), 2934, 1717, 1269, 1119, 1094, 1069, 1024, 710 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{36}H_{38}O_{16}Na$ (M+Na) 749.2052. Found 749.2051. $[\alpha]_D^{20}$ +115.8 (c = 1, in MeOH)



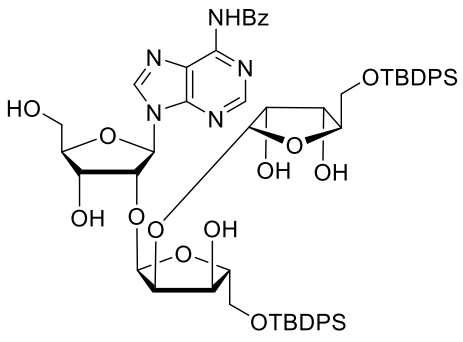
α-1,3,5-Tri-*O*-benzoyl-3'-*O*-acetyl-5'-*O*-tertbutyldiphenylsilyl-2'',3''-di-*O*-acetyl-5''-*O*-tertbutyldiphenylsilylparotriose (9)

Compound **8** (2.7 g, 3.72 mmol) was co-evaporated with pyridine (2 x) and then applied argon. Pyridine (38 mL) and TBDPSCl (4 mL, 15.15 mmol) were added and the mixture was stirred under argon at room temperature for 6 h. Ac_2O (11 mL, 113.4 mmol) was added into the reaction and the mixture was stirred for 16 h after which the reaction was quenched by addition of aq. $NaHCO_3$ (sat.). The mixture was extracted by DCM (3 x 50 mL) and dried by $MgSO_4$ and concentrated under reduced pressure. Purification by silica gel chromatography (pentane/actone, 100/0 – 80/20) obtained **9** as a white foam (3.1 g, 2.33 mmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 – 8.18 (m, 2H, arom.), 8.18 – 8.12 (m, 2H, arom.), 8.10 – 8.05 (m, 2H, arom.), 7.69 – 7.49 (m, 11H, arom.), 7.45 – 7.30 (m, 18H, arom.), 6.80 (d, *J* = 4.3 Hz, 1H, H1'), 5.72 (dd, *J* = 6.3, 1.8 Hz, 1H, H3'), 5.46 (dd, *J* = 6.6, 1.9 Hz, 1H, H3''), 5.39 (dd, *J* = 7.0, 3.2 Hz, 1H, H3'''), 5.32 (d, *J* = 4.2 Hz, 1H, H1''), 5.29 (d, *J* = 4.5 Hz, 1H, H1'''), 4.89 (dd, *J* = 7.0, 4.4 Hz, 1H, H2'''), 4.77 (td, *J* = 3.7, 1.7 Hz, 1H, H4'), 4.72 – 4.56 (m, 3H, H2', H5'), 4.36 (dd, *J* = 6.6, 4.2 Hz, 1H, H2''), 4.09 – 4.06 (m, 2H, H4''', H4''), 3.81 (AB, *J* = 11.2, 2.7 Hz, 1H, H5''), 3.74 – 3.57 (m, 3H, H5'', H5'''), 2.01 (s, 3H, Ac), 1.79 (s, 3H, Ac), 1.64 (s, 3H, Ac), 1.05 (s, 9H, CH_3, TBDPS), 0.97 (s, 9H, CH_3, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.70, 170.09, 169.70 (CO, Ac), 166.17, 166.14, 165.56 (CO, Bz), 135.75, 135.72, 135.70, 135.67, 133.51, 133.49 (arom.), 133.13, 133.09, 133.02, 132.95, 130.25 (cq. arom.), 130.15, 130.12, 129.95, 129.93, 129.89, 129.85, 129.79 (arom.), 129.73 (cq. arom), 128.69, 128.50, 127.92, 127.90, 127.87 (arom.), 101.29 (C1''), 99.52 (C1'''), 95.16 (C1'), 83.73 (C4''), 83.66 (C4'), 83.14 (C4'''), 76.35 (C2'), 74.62 (C2''), 71.75 (C2'''), 71.66 (C3'), 71.26 (C3''), 69.82 (C3'''), 64.44 (C5'), 63.90 (C5''), 63.42 (C5'''), 26.90 (CH_3, TBDPS), 26.85 (CH_3, TBDPS), 20.70 (Ac), 20.39 (Ac), 20.17 (Ac), 19.38 (cq. TBDPS), 19.29 (cq. TBDPS). IR (film): 1728, 1265, 1252, 1112, 1067, 1042, 1026, 709 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{74}H_{80}O_{19}Si_2Na$ (M+Na) 1351.4725. Found 1351.4734. $[\alpha]_D^{20}$ +72.8 (c = 1, in MeOH)



6-*N*-benzoyl-9-(3',5'-di-*O*-benzoyl-3''-*O*-acetyl-5''-*O*-tertbutyldiphenylsilyl-2''',3'''-di-*O*-acetyl-5'''-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (10)

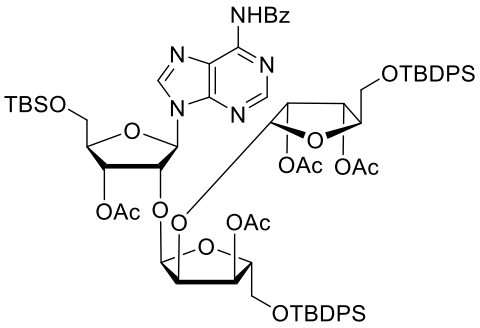
Compound **9** (1.1 g, 0.83 mmol) and N^6-benzoyladenine (0.41 g, 1.71 mmol) were co-evaporated with toluene (2 x), 1,4-dioxane (2 x), MeCN (1 x) and dissolved in dry MeCN (14 mL) under argon. *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (3.2 mL, 12 mmol) was added and the mixture was stirred at room temperature until everything was dissolved. $HClO_4$-SiO_2 (4.3 g, 0.4 mmol/g, 1.71 mmol) was added and the mixture was refluxed for 48 h. Then the reaction was quenched by aq. $NaHCO_3$ (sat.) then filtered. The mixture was extracted with EtOAc (3 x 100 mL), dried by $MgSO_4$ and concentrated under reduced pressure. Purification by silica gel chromatography (pentane/acetone, 100/0 – 85/15 – 75/25 – 70/30) obtained **10** as a white foam (0.99 g, 0.68 mmol, 82%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.04 (s, 1H, NH), 8.68 (s, 1H, H2), 8.41 (s, 1H, H8), 8.08 (tt, *J* = 6.6, 1.4 Hz, 4H, arom.), 8.00 – 7.93 (m, 2H, arom.), 7.65 – 7.45 (m, 13H, arom.), 7.45 – 7.27 (m, 16H, arom.), 6.32 (d, *J* = 4.6 Hz, 1H, H1'), 5.95 (t, *J* = 5.3 Hz, 1H, H3'), 5.70 (t, *J* = 5.0 Hz, 1H, H2'), 5.44 (dd, *J* = 6.9, 2.4 Hz, 1H, H3''), 5.40 (dd, *J* = 7.4, 3.5 Hz, 1H, H3'''), 5.25 (d, *J* = 4.4 Hz, 1H, H1''), 5.17 (d, *J* = 4.3 Hz, 1H, H1'''), 4.94 (dd, *J* = 7.3, 4.4 Hz, 1H, H2'''), 4.89 (AB, *J* = 12.0, 3.8 Hz, 1H, H5'), 4.79 – 4.74 (m, 1H, H4'), 4.70 (AB, *J* = 12.0, 4.9 Hz, 1H, H5'), 4.32 (dd, *J* = 6.9, 4.3 Hz, 1H, H2''), 4.10 (q, *J* = 3.1 Hz, 1H, H4'''), 4.01 (q, *J* = 2.8 Hz, 1H, H4'), 3.78 (AB, *J* = 11.4, 2.7 Hz, 1H, H5'''), 3.70 (AB, *J* = 11.3, 3.2 Hz, 1H, H5'''), 3.58 (AB, *J* = 11.2, 2.8 Hz, 1H, H5''), 3.44 (AB, *J* = 11.2, 3.3 Hz, 1H, H5''), 2.11 (s, 3H, Ac), 2.08 (s, 3H, Ac), 1.68 (s, 3H, Ac), 1.01 (s, 9H, CH_3, TBDPS), 0.96 (s, 9H, CH_3, TBDPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.53, 169.88, 169.79 (CO, Ac), 166.25, 165.35, 164.45 (CO, Bz), 152.87 (CH, C2), 151.35, 149.74 (cq. arom.), 135.61, 135.59 (arom.), 133.71 (aq. arom.), 133.59, 133.44 (arom.), 132.96, 132.91, 132.87 (cq. arom,), 132.82 (arom.), 132.75 (aq. arom.), 129.90, 129.88, 129.85, 129.83 (arom.), 129.57, 129.53 (cq. arom.), 128.92, 128.59, 128.54, 127.88, 127.86, 127.82, 127.81, 127.79 (arom.), 123.92 (cq. arom.), 101.19 (C1''), 98.61 (C1'''), 89.09 (C1'), 83.02 (C4'''), 82.38 (C4''), 80.46 (C4'), 72.98 (C2''), 72.44 (C3'), 71.74 (C2'''), 70.96 (C3''), 69.79 (C3'''), 63.58 (C5'), 63.52 (C5''), 63.13 (C5'''), 26.83, 26.76 (CH_3, Ac), 20.75, 20.66, 20.40 (CH_3, TBDPS), 19.25 (cq. TBDPS). IR (film): 2930, 1728, 1238, 1111, 1069, 1038, 1028, 702 cm^{-1}. HRMS (ESI^+) calcd for $C_{79}H_{84}N_5O_{18}Si_2$ (M+H) 1446.5344. Found 1446.5344. $[\alpha]_D^{20}$ +31.4 (c = 1, in MeOH)



6-*N*-benzoyl-9-(5'',5'''-di-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (11)

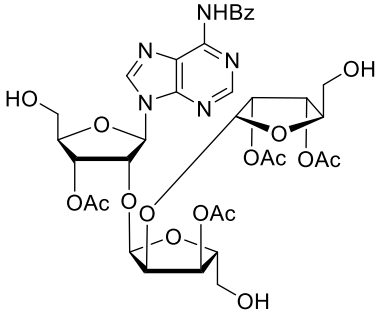
Compound **10** (984 mg, 0.68 mmol) was dissolved in pyridine/EtOH (7 mL; 2/1 v/v), cooled to 0°C after which aqueous NaOH (4.1 mL, 1 M) was slowly added. The reaction mixture was stirred for 2 h at the same temperature after which Amberlite-H^+ was added until pH = 6. The mixture was filtered, concentrated under reduced pressure and purified by silica gel chromatography (DCM/methanol, 100/0 – 97/3 – 95/5) to obtain **11** as a white foam (641 mg, 0.58 mmol, 85%). ^{1}H NMR (400

MHz, $CDCl_3$) δ 9.52 (s, 1H, NH), 8.80 (s, 1H, H2), 8.53 (s, 1H, H8), 8.03 – 7.92 (m, 2H, arom.), 7.62 – 7.59 (m, 8H), 7.56 – 7.49 (m, 1H, arom.), 7.46 – 7.23 (m, 14H, arom.), 6.23 (d, *J* = 7.3 Hz, 1H, H1'), 5.12 (d, *J* = 4.4 Hz, 1H, H1''), 4.99 (d, *J* = 4.0 Hz, 1H, H1'''), 4.94 (dd, *J* = 7.4, 4.7 Hz, 1H, H2'), 4.60 (d, *J* = 4.7 Hz, 1H, H3'), 4.44 (t, *J* = 4.8 Hz, 2H, H2''), 4.39 – 4.22 (m, 5H, H3''', H4', H2''', H3''', H4''), 4.20 (q, *J* = 2.8 Hz, 1H, H4'''), 3.97 (AB, *J* = 13.0, 1.8 Hz, 1H, H5'), 3.77 – 3.65 (m, 5H, H5', H5'', H5'''), 3.73 – 3.63 (m, 4H), 0.99 (s, 9H, TBDPS), 0.98 (s, 9H, TBDPS) ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.04 (CO, Bz), 152.21 (C2), 150.66, 150.30 (cq. arom.), 144.26 (C8), 135.61, 135.59, 135.56 (arom.), 133.58, 133.06 (cq. arom.), 132.91 (arom.), 132.83, 132.65 (cq. arom), 130.00, 129.97, 129.93, 129.86, 128.85, 128.09, 127.91, 127.87, 127.82 (arom.), 124.36 (cq. arom.), 101.94 (C1'''), 101.08 (C1''), 89.48 (C1'), 88.17 (C4'), 86.40 (C4''), 86.19 (C4'''), 80.01 (C2'), 76.99 (C2''), 73.22 (C2'''), 72.92 (C3'), 72.15 (C3''), 71.16 (C3'''), 64.32 (C5'''), 64.13 (C5''), 63.27 (C5'), 26.88 (CH_3, TBDPS), 26.86 (CH_3, TBDPS), 19.26 (cq. TBDPS), 19.23 (cq. TBDPS). IR (film): 3329 (bs), 2930, 2857, 1701, 1612, 1458, 1105, 1072, 1037, 702 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{59}H_{70}N_5O_{13}Si_2$ (M+H) 1112.4503. Found 1112.4511. $[\alpha]_D^{20}$ +48.7 (c = 1, in MeOH)



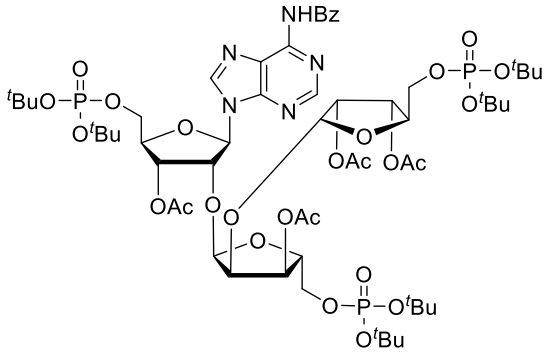
6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-5'-*O*-tertbutyldimethylsilyl-5'',5'''-di-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (12)

Compound **11** (146 mg, 0.13 mmol) was dissolved in dry pyridine (1.3 mL), TBSCl (50 mg, 0.32 mmol) was added and the reaction was stirred for 6 hours at room temperature. TLC showed an incomplete conversion and additional TBSCl (100 mg, 0.66 mmol) was added. The mixture was stirred at room temperature for 5 h after which Ac_2O (0.37 ml, 3.9 mmol) was added. The mixture was stirred at 0°C for 10 h then quenched by aq. $NaHCO_3$ (sat.). 20 mL H_2O was added and the mixture was extracted with DCM (3 x 15 mL), dried over $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography (pentane/actone, 100/0 – 90/10 – 85/15 – 80/20) to obtain **12** as a white foam (133 mg, 0.09 mmol, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (s, 1H, NH), 8.80 (s, 1H, H2), 8.51 (s, 1H, H8), 8.05 – 7.95 (m, 2H, arom.), 7.69 – 7.54 (m, 9H, arom.), 7.50 (t, *J* = 7.6 Hz, 2H, arom.), 7.45 – 7.29 (m, 12H, arom.), 6.33 (d, *J* = 3.8 Hz, 1H, H1'), 5.53 (dd, *J* = 6.8, 2.1 Hz, 1H, H3''), 5.45 (dd, *J* = 7.3, 3.0 Hz, 1H, H3'''), 5.41 (t, *J* = 5.5 Hz, 1H, H3'), 5.34 – 5.32 (m, 2H, H1'', H1'''), 5.10 (t, *J* = 4.6 Hz, 1H, H2'), 5.05 (dd, *J* = 7.1, 4.5 Hz, 1H, H2'''), 4.39 – 4.34 (m, 2H, H2'', H4'), 4.18 (t, *J* = 2.9 Hz, 1H, H4'''), 4.10 – 4.07 (m, 2H, H4'', H5'), 3.93 – 3.68 (m, 5H, H5', H5'', H5'''), 2.14 (s, 3H, Ac), 2.11 (s, 6H, 2Ac), 2.06 (s, 3H, Ac), 1.04 (s, 9H, TBDPS), 1.01 (s, 9H, TBDPS), 0.93 (s, 9H, CH_3, TBS), 0.12 (s, 6H, CH_3, TBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.53, 169.94, 169.71, 169.62 (CO, Ac), 164.61 (CO, Bz), 152.86 (C2), 151.42, 149.58 (cq. arom.), 141.76 (C8), 135.65, 135.62, 135.59 (arom.), 133.83, 133.00, 132.96, 132.83 (cq. arom.), 132.74, 129.89, 129.85, 128.87, 127.93, 127.85, 127.82 (arom), 123.51 (cq. arom.), 100.71 (C1''), 99.37 (C1'''), 88.06 (C1'), 83.30 (C4''), 82.85 (C4', C4'''), 77.99 (C2'), 73.86 (C2''), 71.60 (C2'''), 71.30 (C3''), 71.04 (C3'), 69.97 (C3'''), 63.77 (C5''), 63.34 (C5'''), 62.11 (C5'), 26.82, 26.80 (CH_3, TBDPS), 26.02 (CH_3, TBS), 21.04, 20.86, 20.78, 20.46 (CH_3, Ac), 19.27 (cq. TBDPS), 18.53 (cq. TBS), -5.31, -5.40 ($SiCH_3$, TBS). IR (film): 2951, 2930, 2859, 1746, 1236, 1113, 1043, 702 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{73}H_{92}N_5O_{17}Si_3$ (M+H) 1394.5790. Found 1394.5789. $[\alpha]_D^{20}$ +70.0 (c = 1, in MeOH).



[6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-β-parotriosyl)adenine] (13)

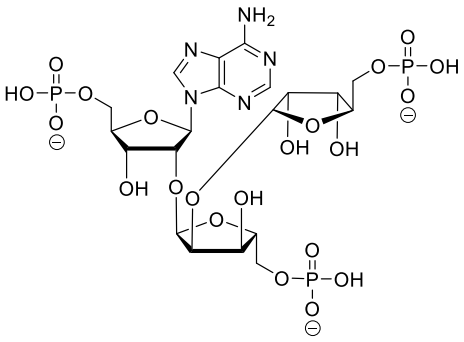
Compound **12** (133 mg, 0.09 mmol) was dissolved in pyridine (1 mL), cooled to 0°C after which HF·pyridine (0.12 mL, 4.3 mmol) was added. The reaction was stirred for 1.5 hours at 0°C after which was quenched by aq. $NaHCO_3$ (sat.) then extracted with EtOAc (4 x 10 mL), dried over $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography (DCM/methanol, 100/0 – 100/1 – 96/4) to obtain **13** as a white foam (62 mg, 77 µmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.54 (s, 1H, NH), 8.64 (s, 1H, H2), 8.59 (s, 1H, H8), 8.02 (d, *J* = 7.4 Hz, 2H, arom.), 7.62 – 7.53 (m, 1H, arom.), 7.49 (t, *J* = 7.6 Hz, 2H, arom.), 6.24 (d, *J* = 10.9 Hz, 1H, OH), 6.10 (d, *J* = 7.8 Hz, 1H, H1'), 5.61 (d, *J* = 5.4 Hz, 1H, H3'), 5.17 (dd, *J* = 7.3, 4.2 Hz, 1H, H3''), 5.12 – 5.08 (m, 2H, H2', H3'''), 4.96 (d, *J* = 4.4 Hz, 1H, H1''), 4.91 (dd, *J* = 7.3, 4.5 Hz, 1H, H2''), 4.68 (d, *J* = 4.2 Hz, 1H, H1'''), 4.24 (s, 1H, H4'), 4.03 – 3.98 (m, 3H, H4'', H2''', H4'''), 3.91 (AB, *J* = 12.6 Hz, 1H, H5'), 3.82 – 3.48 (m, 5H, H5', H5'', H5'''), 3.41 (bs, 1H, OH), 2.99 (bs, 1H, OH), 2.14 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.06 (s, 3H, Ac). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.56, 170.03, 169.71 (CO, Ac), 165.25 (CO, Bz), 152.04 (C2), 150.72, 150.52 (cq. arom.), 144.26 (C8), 133.28 (cq, arom), 133.06, 128.90, 128.21 (arom.), 124.72 (cq. arom.), 101.58 (C1'''), 98.42 (C1''), 89.08 (C1'), 86.54 (C4'), 82.24 (C4''), 82.09 (C4'''), 77.68 (C2'), 73.89 (C3'), 72.12 (C2''), 71.44 (C2'''), 70.41 (C3''), 69.68 (C3'''), 62.79 (C5'), 61.79 (C5''), 61.54 (C5'''), 21.15, 20.97, 20.76, 20.72 (CH_3, Ac). IR (film): 3352 (bs), 2932, 1738, 1612, 1584, 1456, 1369, 1238, 1092, 1043 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{35}H_{42}N_5O_{17}$ (M+H) 804.2570. Found 804.2573. $[\alpha]_D^{20}$ +78.3 (c = 1, in MeOH)



6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-5',5'',5'''-tri-*O*-(ditertbutylphosphoryl)-β-parotriosyl)adenine (14)

1-Methyl-imidazole·HCl (200 mg, 1.68 mmol) and 1-methyl-imidazole (88 µL, 1.1 mmol) were co-evaporated with dry CH_3CN (3 x), then N_2 was applied. To this mixture, freshly activated molecular sieves and dry DMF (0.9 mL) were added and the activator solution was stirred at room temperature for 2 hours under N_2. Next, compound **13** (73 mg, 91 µmol) was co-evaporated with dry 1,4-dioxane (3 x) and mixed with the activator solution, after which di-tert-butyl-*N*,*N*-diisopropylphosphoramidite (0.28 mL, 0.9 mmol) was added and the reaction was stirred at room temperature for 1 hour. Then *t*BuOOH in decane (0.56 mL, 5.5 M, 3.08 mmol) was added at 0°C and the reaction mixture was stirred for 1 hour at room temperature. The reaction was quenched by aq. $NaHCO_3$ (sat.) and extracted with EtOAc (3 x 10 mL), dried over $MgSO_4$, concentrated under reduced pressure. Purification by silica gel chromatography (DCM/MeOH, 100/0 – 95/5) then LH-20 gel filtration (DCM/methanol, 50/50) obtained **14** as a white foam (70 mg, 51 µmol, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.18 (s, 1H, NH), 8.81 (s, 1H, H2), 8.45 (s, 1H, H8), 8.10 – 7.99 (m, 2H, arom.), 7.67 – 7.58 (m, 1H, arom.), 7.53 (dd, *J* = 8.3, 6.7 Hz, 2H, arom.), 6.26 (d, *J* = 4.7 Hz, 1H, H1'), 5.50 (t, *J* = 5.1 Hz, 1H, H3'), 5.37 (dd, *J* = 7.1, 2.8 Hz, 1H, H3''), 5.33 – 5.26 (m, 1H, H2'), 5.23 (dd, *J* =

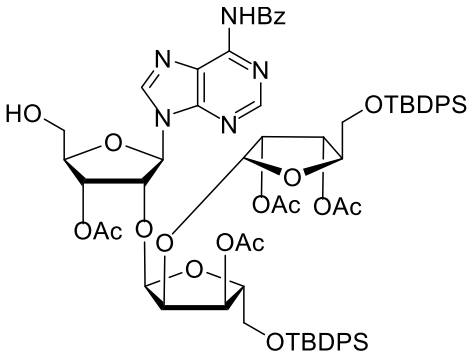
7.4, 3.6 Hz, 1H, H3'''), 5.19 (d, *J* = 4.3 Hz, 1H, H1''), 5.17 (d, *J* = 4.4 Hz, 1H, H1'''), 4.86 (dd, *J* = 7.4, 4.4 Hz, 1H, H2'''), 4.44 (tt, *J* = 4.2, 2.1 Hz, 1H, H4'), 4.38 – 4.33 (m, 1H, H5'), 4.26 – 4.18 (m, 4H, H5', H4'', H4''', H2''), 4.15 – 3.97 (m, 4H, H5'', H5'''), 2.17 (s, 3H, Ac), 2.14 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.07 (s, 3H, Ac), 1.56 – 1.39 (m, 54H, *t*Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.46, 169.70, 169.56, 169.49 (CO, Ac), 164.61 (CO, Bz), 152.88 (C2), 151.45, 149.79 (cq. arom.), 142.46 (C8), 133.82 (cq. arom.), 132.85, 128.96, 127.99 (arom.), 123.80 (cq. arom.), 100.58 (C1''), 98.85 (C1'''), 88.10 (C1'), 83.22, 83.17, 83.15, 83.10, 82.96, 82.92, 82.90, 82.85, 82.82 (cq. *t*Bu), 81.20, 81.12, 81.04, 80.86, 80.77 (C4', C4'', C4'''), 77.02, 72.94 (C2''), 71.51 (C3'), 71.23 (C2'''), 70.48 (C3''), 69.56 (C3'''), 65.85, 65.79 (C5'''), 65.55, 65.50 (C5''), 65.03, 64.97 (C5'), 29.97, 29.93, 29.88 (CH_3, *t*Bu), 21.00, 20.97, 20.76, 20.52 (CH_3, Ac). ^{31}P NMR (162 MHz, $CDCl_3$) δ -9.85, -9.99, -10.04. IR (film): 2980, 1746, 1371, 1244, 1040, 997.2 cm^{-1}. HRMS (ESI^+) calcd for $C_{59}H_{92}N_5O_{26}P_3$ (M+H) 1379.5243. Found 1380.5339. $[\alpha]_D^{20}$ +41.1 (c = 1, in MeOH)



_O_-α-D-ribofuranosyl-(1'''→2'')-_O_-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate)
[Parotriosyladenine-5',5'',5'''-tri-_O_-phosphate] (1)

Compound **14** (20 mg, 14.5 µmol) was dissolved in HFIP (0.6 mL), concentrated HCl was added (7.2 µL, 87 µmol) and the reation mixture was stirred at room temperature for 1 h and ^{31}P-NMR spectroscopy showed complete cleavage of the tert-butyl groups. 80 µL NH_4OH (35%) was added to quench the reaction and concentrated under reduced pressure. Co-evaporating the residue with 1,4-dioxane (3 x) then 2 mL NH_4OH (35%) was added and the mixture was stirred at room temperature for 3 days. LCMS showed complete reaction and then concentrated under reduced pressure. The residue was purified by HW-40 gel filtration (0.15 M, NH_4OAc in Miliq H_2O). Repeated lyophilization obtained **1** as a white solid (11.0 mg, 14.2 µmol, 98%). ^{1}H NMR (400 MHz, D_2O) δ 8.60 (s, 1H, H8), 8.26 (s, 1H, H2), 6.27 (d, *J* = 6.3 Hz, 1H, H1'), 5.36 (d, *J* = 3.8 Hz, 1H, H1''), 4.98 (d, *J* = 4.4 Hz, 1H, H1'''), 4.93 (dd, *J* = 6.3, 5.1 Hz, 1H, H2'), 4.60 (dd, *J* = 5.1, 3.0 Hz, 1H, H3'), 4.39 – 4.38 (m, 1H, H4'), 4.35 – 4.30 (m, 1H, H4''), 4.29 – 4.19 (m, 3H, H2'', H3'', H4'''), 4.05 (dd, *J* = 6.2, 3.0 Hz, 1H, H3'''), 4.02 – 4.00 (m, 2H, H5'), 3.95 (dd, *J* = 6.3, 4.3 Hz, 1H, H2'''), 3.86 – 3.74 (m, 4H, H5'', H5'''). ^{13}C NMR (101 MHz, D_2O) δ 155.66 (cq. arom. C6), 153.00 (cq. arom. C2), 149.11 (cq. arom. C4), 140.29 (cq. arom. C8), 118.61 (cq. arom. C5), 101.46 (C1'''), 101.12 (C1''), 85.25 (C1'), 85.17 (C4'), 84.46 (C4''), 84.22 (C4'''), 80.25 (C2'), 75.55 (C2''), 71.29 (C2'''), 70.71 (C3'), 69.95 (C3''), 69.78 (C3'''), 63.90 (C5'', C5'''), 63.76 (C5'). ^{31}P NMR (162 MHz, D_2O) δ 3.53, 3.48, 3.46. IR (film): 3180 (bs), 1686, 1647, 1420, 1034, 930, 795, 783, 719 cm^{-1}. HRMS (ESI^+) calcd for $C_{20}H_{33}N_5O_{21}P_3$ (M+H) 772.0875. Found 772.0874. $[\alpha]_D^{20}$ +29.6 (c = 1, in MeOH)

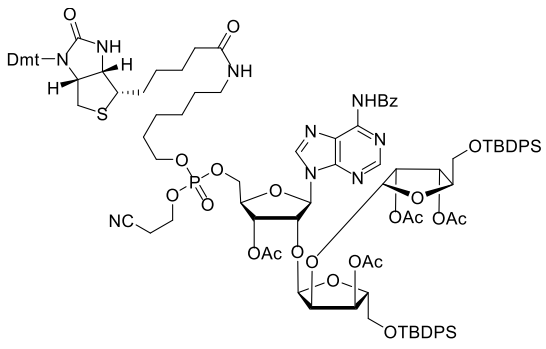
Synthesis of compound **15** will be described in Chapter 5



6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-5'',5'''-di-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (16)

Compound **15** (65mg, 41 µmol), DCM (0.4 mL) and AcOH (0.8 mL, 90% in H_2O) were added into a flask. The reaction was stirred for 3 hours after which it was quenched with aq. $NaHCO_3$ (sat.), extracted with DCM (3 x), dried (Na_2SO_4) concentrated and purified by silica gel column chromatography (DCM/acetone, 100/0 – 75/15) to furnish **16** as a white foam (34 mg, 27 µmol, 66 %).

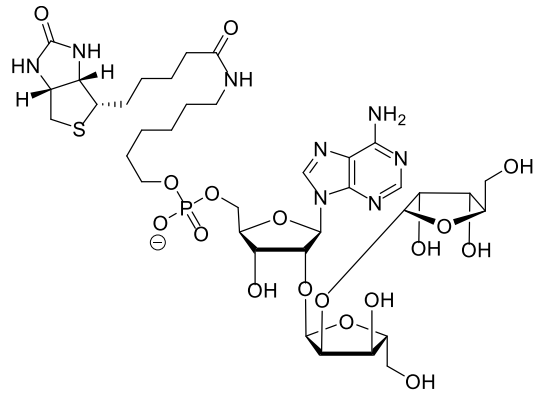
^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.07 (s, 1H, NH), 8.86 (s, 1H, H2), 8.59 (s, 1H, H8), 7.99 – 7.91 (m, 2H, Ar), 7.67 – 7.54 (m, 9H, Ar), 7.54 – 7.47 (m, 2H, Ar), 7.44 – 7.23 (m, 12H, Ar), 6.38 – 6.26 (m, 1H, OH), 6.14 (d, *J* = 7.9 Hz, 1H, H1'), 5.66 (d, *J* = 5.5 Hz, 1H, H3'), 5.42 (ddd, *J* = 14.8, 7.3, 3.5 Hz, 2H, H3'', H3'''), 5.17 (dd, *J* = 7.9, 5.5 Hz, 1H, H2'), 5.06 (d, *J* = 4.3 Hz, 1H, H1'''), 5.01 (dd, *J* = 7.4, 4.3 Hz, 1H, H2'''), 4.82 (d, *J* = 4.5 Hz, 1H, H1''), 4.34 – 4.25 (m, 2H, H4', H2''), 4.10 (q, *J* = 3.2 Hz, 1H, H4'''), 4.05 – 3.98 (m, 2H, H4'', H5'), 3.88 (t, *J* = 12.3 Hz, 1H, H5'), 3.81 – 3.66 (m, 4H, H5''', H5''), 2.22 (s, 3H, Ac), 2.15 – 2.14 (m, 6H, Ac), 2.10 (s, 3H, Ac), 1.03 (s, 9H, TBDPS), 0.96 (s, 9H, TBDPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.54, 170.08, 169.71, 169.65 (CO Ac), 164.28 (CO Bz), 150.54, 150.37 (Cq. Ar), 135.71, 135.69, 135.68, 135.66 (Ar), 133.68, 133.06 (Cq. Ar), 133.02 (Ar), 132.95, 132.91, 132.86 (Cq. Ar), 129.99, 129.95, 129.06, 127.94, 127.92, 127.89, 127.84 (Ar), 124.54 (Cq. Ar), 101.20 (C1''), 98.35 (C1'''), 89.84 (C1'), 87.20 (C4'), 82.51 (C4''), 82.24 (C4'''), 77.55 (C2'), 74.46 (C3'), 72.33 (C2''), 71.80 (C2'''), 70.97 (C3''), 69.74 (C3'''), 63.63 (C5''), 63.12 (C5'''), 63.01 (C5'), 26.94, 26.87 (CH_3 TBDPS), 21.19, 21.07, 20.86, 20.83 (CH_3 Ac), 19.33, 19.29 (Cq. TBDPS). IR (film): 2931, 1743, 1739, 1609, 1447, 1427, 1235, 1227, 1112, 1039, 701 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{67}H_{77}N_5O_{17}Si_2$ (M+H) 1280.4926. Found 1280.4965. $[\alpha]_D^{20}$ +56.9 (c = 1, in Methanol)



6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-5'-O-{6-[(2-Cyanoethoxy)phosphoryl]-[1-N-(4,4'-dimethoxytrityl)biotinyl]aminohexane}-5'',5'''-di-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (17)

1-Methyl-imidazole·HCl (19 mg, 0.16 mmol) and 1-methyl-imidazole (9 µL, 0.11 mmol) were co-evaporated with dry CH_3CN (3 x), then N_2 was applied. To this mixture, freshly activated molecular sieves and dry DMF (0.5 mL) were added and the activator solution was stirred at room temperature for 2 hours under N_2. Next, compound **16** (34 mg, 27 µmol) was co-evaporated with dry 1,4-dioxane (3 x) after which the activator solution above was added. Subsequently, biotin-C_6-phosphoramidite **21** (46 mg, in 0.5 mL ACN, 54 µmol) was added and the reaction was stirred at room temperature for 20 minutes. *t*BuOOH in decane (29 µL, 5.5 M, 0.16 mmol) was added at 0 °C and the reaction mixture was stirred for 1 hour at room temperature. The reaction was quenched by aq. $NaHCO_3$ (sat.), extracted with DCM (3 x), dried ($MgSO_4$), concentrated, purified by LH-20 gel filtration (DCM/methanol, 50/50) to obtain **17** as a white foam (25 mg, 12 µmol, 44 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.37 (d, *J* = 17.4

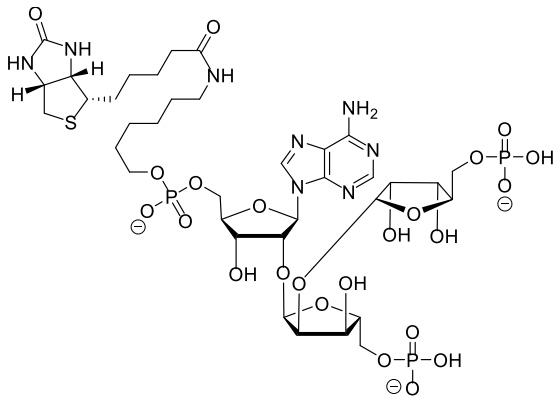
Hz, 1H, NH Ade), 8.79 (s, 1H, H2), 8.44 (d, *J* = 3.2 Hz, 1H, H8), 7.99 (d, *J* = 17.4 Hz, 2H, Ar), 7.72 – 7.08 (m, 27H, Ar), 6.79 (dd, *J* = 8.9, 2.0 Hz, 4H, Ar DMT), 6.27 (d, *J* = 4.1 Hz, 1H, H1'), 6.05 (d, *J* = 6.3 Hz, 1H, NH Biotin), 5.60 – 5.38 (m, 4H, H3', H3'', H3'''), 5.30 – 5.23 (m, 3H, H2', H1'', H1'''), 5.01 (dt, *J* = 7.4, 2.4 Hz, 1H, H2''), 4.56 – 4.29 (m, 6H, H4', H5', H2''', CH Biotin), 4.27 – 3.98 (m, 6H, H4'', H4''', $CH_2OP=O$), 3.90 – 3.62 (m, 10H, CH_3 DMT, H5'', H5'''), 3.29 – 3.00 (m, 3H, CHS, CH_2S), 2.68 (dt, *J* = 9.0, 6.1 Hz, 2H, *CH*$_2$NH), 2.42 (AB, *J* = 13.0 Hz, 1H, *CH*$_2$CONH), 2.26 (AB, *J* = 13.0, 5.5 Hz, 1H, *CH*$_2$CONH), 2.22 – 1.96 (m, 12H, CH_3 Ac), 1.74 – 1.17 (m, 14H, CH_2), 1.03 – 0.99 (m, 18H, CH_3 TBDPS). ^{31}P NMR (162 MHz, $CDCl_3$) δ -1.11, -1.15. IR (film): 3250, 2931, 1743, 1695, 1447, 1428, 1363, 1237, 1113, 1035, 1006, 737, 703 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{86}H_{110}N_9O_{22}PSSi_2$ (M+2H)/2 869.8376 (-DMT). Found 869.8372. $[\alpha]_D^{20}$ +30.7 (c = 1, in $CHCl_3$)



9-(5'-O-(6-O-biotinylaminohexany)-phosphoryl)-β-parotriosyladenine (18)

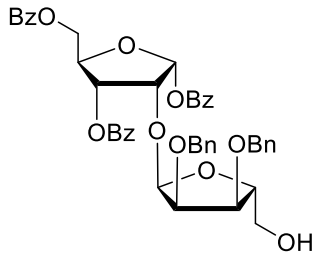
Compound **17** (25 mg, 12 µmol), DCM (0.5 mL) and TFA (2 µL, 24 µmol) were added into the flask and the reaction was stirred at room temperature for 30 minutes. TLC showed no complete reaction and more TFA (3 µL, 36 µmol) was added. 1 hour later the reaction was quenched by NH_4OH (160 µL) and concentrated. To the residue, NH_4OH (0.4 mL) and 1,4-dioxane (0.4 mL) were added and stirred for 24 hours at room temperature. The reaction was concentrated, co-evaporated with 1,4-dioxane (2 x), toluene (3 x) and pyridine (2 x). The residue was dissolved in pyridine (0.2 mL) and HF·pyridine (56 µL, 70% HF pyridine solution, 2.16 mmol base on HF) was added at 0 ℃. The mixture was stirred for 16 hours after which it was quenched by NH_4OH, concentrated, purified by HW-40 gel filtration [25% ACN in aqueous NH_4OAc (0.15 M)]. Repeated lyophilization obtained **18** as a white solid (6.96 mg, 7.43 µmol, 62 %). ^{1}H NMR (400 MHz, Deuterium Oxide) δ 8.54 (s, 1H, H2), 8.29 (s, 1H, H8), 6.28 (d, *J* = 6.2 Hz, 1H, H1'), 5.38 (d, *J* = 3.5 Hz, 1H, H1''), 5.02 – 4.93 (m, 2H, H2', H1'''), 4.62 (dd, *J* = 5.1, 3.0 Hz, 1H, H3'), 4.54 (dd, *J* = 8.0, 4.8 Hz, 1H, CH Biotin), 4.39 (t, *J* = 2.8 Hz, 1H, H4'), 4.34 (dd, *J* = 8.0, 4.5 Hz, 1H, CH Biotin), 4.20 (dt, *J* = 17.2, 2.6 Hz, 3H, H2'', H3'', H4''), 4.14 – 4.02 (m, 3H, H4''', H5'), 3.94 (dd, *J* = 6.3, 3.2 Hz, 1H, H3'''), 3.86 (dd, *J* = 6.3, 4.3 Hz, 1H, H2'''), 3.75 – 3.54 (m, 6H, H5'', H5''', *CH*$_2$OP=O), 3.21 (dt, *J* = 9.8, 5.3 Hz, 1H, CHS), 3.11 – 2.87 (m, 3H, $CONHCH_2$, CH_2S), 2.71 (d, *J* = 13.0 Hz, 1H, CH_2S), 2.17 (t, *J* = 7.1 Hz, 2H, CH_2CONH), 1.75 – 1.00 (m, 14H, CH_2). ^{13}C NMR (101 MHz, D_2O) δ 176.77 (CO Biotin), 154.88, 149.43 (Cq. Ar), 101.63 (C1'''), 101.33 (C1''), 85.76 (C1'), 85.72 (C4''), 85.43 (C4'''), 85.02, 84.93 (C4'), 79.88 (C2'), 75.86 (C2''), 71.92 (C2'''), 70.99 (C3'), 70.23 (C3''), 69.93 (C3'''), 66.57 (C5''), 65.17 (C5'), 62.39 (CH Biotin), 61.74 ($CH_2OP=O$), 61.71 (C5'''), 60.55 (CH Biotin), 55.73 (CHS), 40.02 (CONH*CH*$_2$), 39.46 (CH_2S), 35.78 (*CH*$_2$CONH), 29.99, 29.91, 28.49, 28.13, 27.98, 26.04, 25.53, 24.88 (CH_2). ^{31}P NMR (162 MHz, D_2O) δ 1.04. HRMS (ESI$^+$) calcd for $C_{36}H_{58}N_8O_{17}PS$ (M+H) 937.3373. Found 937.3372.

Synthesis of compound **19** is described in Chapter 5



9-(5'-O-(6-O-biotinylaminohexany)-phosphoryl-5'',5'''-di-*O*-phosphoryl)-β-parotriosyladenine (20)

Compound **19** (20 mg, 12 µmol), DCI (96 µL, 0.25 M in ACN, 24 µmol) and freshly flame-dried 3Å molecular sieves were added into a flask and the mixture was stirred under N_2 at room temperature. **21** (20 mg, 24 µmol) was added and it was stirred for 1 hour. *t*BuOOH in decane (11 µL, 5.5 M, 60 µmol) was added at 0 °C and the reaction mixture was stirred for 1 hour at room temperature. The reaction was quenched by aq. $NaHCO_3$ (sat.), extracted with DCM (3 x), dried (Na_2SO_4) concentrated and purified by LH-20 gel filtration (DCM/methanol, 50/50). The fractions containing product were collected and concentrated. To the residue, DCM (0.2 mL) and TFA (1.8 µL, 24 µmol) were added and the mixture was stirred for 1 hour after which it was quenched by NH_4OH. The mixture was concentrated, added 1,4-dioxane (0.4 mL) and NH_4OH (0.4 mL). The reaction was stirred for 24 hours and concentrated. HPLC purification (0 - 30%, A: 25mM NH_4OAc/H_2O B: ACN) and repeated lyophilization yielded **20** (2.10 mg, 1.92 µmol, 16 %) as a white solid. ^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.54 (s, 1H, H2), 8.32 (s, 1H, H8), 6.29 (d, *J* = 5.7 Hz, 1H, H1'), 5.38 (d, *J* = 3.9 Hz, 1H, H1''), 5.05 (d, *J* = 4.4 Hz, 1H, H1'''), 4.96 (t, *J* = 5.5 Hz, 1H, H2'), 4.61 (dd, *J* = 5.2, 3.6 Hz, 1H, H3'), 4.53 (dd, *J* = 8.0, 4.9 Hz, 1H, CH Biotin), 4.40 – 4.36 (m, 1H, H4'), 4.36 – 4.29 (m, 2H, H4''', CH Biotin), 4.28 – 4.20 (m, 3H, H2'', H3'', H4''), 4.10 – 4.02 (m, 3H, H5', H3'''), 3.98 (dd, *J* = 6.3, 4.4 Hz, 1H, H2'''), 3.95 – 3.84 (m, 4H, H5'', H5'''), 3.73 – 3.60 (m, 2H, CH_2OP=O), 3.21 (ddd, *J* = 9.8, 5.7, 4.5 Hz, 1H, CHS), 3.09 – 2.96 (m, 2H, CONH*CH*$_2$), 2.91 (dd, *J* = 13.1, 5.0 Hz, 1H, CH_2S), 2.70 (d, *J* = 13.0 Hz, 1H, CH_2S), 2.17 (t, *J* = 7.1 Hz, 2H, *CH*$_2$CONH), 1.69 – 1.38 (m, 6H, CH_2), 1.29 (dtd, *J* = 14.5, 7.5, 3.4 Hz, 4H, CH_2), 1.15 – 1.02 (m, 4H, CH_2). ^{13}C NMR (151 MHz, D_2O) δ 177.44 (CO), 166.24 (CO), 154.37 (C4), 150.82 (C8), 149.83 (C6), 141.92 (C2), 119.62 (C5), 102.43 (C1'''), 102.02 (C1''), 86.87 (C1'), 85.44, 85.38 (C4'), 85.06, 85.00 (C4'''), 84.98, 84.92 (C4''), 80.52 (C2'), 76.48 (C2''), 72.41 (C2'''), 71.26 (C3'), 70.80 (C3''), 70.63 (C3'''), 67.21, 67.18 (*CH*$_2$OP=O), 65.67, 65.63, 65.62, 65.59 (C5', C5'', C5'''), 63.00 (CH Biotin), 61.15 (CH Biotin), 56.33 (CHS), 40.62 (CONH*CH*$_2$), 40.08 (CONH*CH*$_2$), 36.38 (CH_2S), 30.58, 30.54, 29.09, 28.73, 28.58, 26.65, 26.14, 25.50 (CH_2). ^{31}P NMR (202 MHz, D_2O) δ 1.08, 1.00. HRMS (ESI$^+$) calcd for $C_{36}H_{60}N_8O_{23}P_3S$ (M+H) 1097.2699. Found 1097.2716.



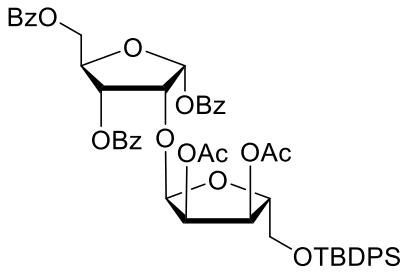
α-1,3,5-Tri-*O*-benzoyl-2',3'-di-*O*-benzyl-parobiose (22)

Compound **2** (3 g, 3.22 mmol), pyridine (15 mL) and TEA (6.7 mL) were added into a flask and cooled to 0°C. TEA·3HF was added and the reaction was stirred at room temperature for 24 h. The reaction was quenched by additioin of aq. $NaHCO_3$ (sat.). 50 mL H_2O was added and the mixture was extracted by EtOAc (3 x). The combined organic layers were dried ($MgSO_4$), filtered and concentrated. Purification by silica gel chromatography (pentane/EtOAc, 80/20 – 60/40) furnished **22** as a white foam (2.14 g, mmol, 86 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.20 – 7.95 (m, 6H, arom.), 7.61 – 7.38 (m, 5H, arom.), 7.32 – 6.99 (m, 14H, arom.), 6.81 (t, *J* = 3.4 Hz, 1H, H1'), 5.69 (dd, *J* = 6.4, 2.0 Hz, 1H, H4'), 5.34 – 5.22 (m,

1H, H1''), 4.82 – 4.53 (m, 5H, H3', H2', H5', CH_2 Bn), 4.46 (d, *J* = 12.0 Hz, 1H, CH_2 Bn), 4.34 (dd, *J* = 15.8, 12.0 Hz, 2H, CH_2 Bn), 4.18 – 4.08 (m, 1H, H4''), 3.89 – 3.78 (m, 2H, H3'', H2''), 3.64 (AB, *J* = 12.0, 3.0 Hz, 1H, H5''), 3.43 (AB, *J* = 12.0 Hz, 1H, H5''), 1.96 (s, 1H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.37, 166.11, 165.77 (CO Bz), 138.17, 138.05 (Cq. arom.), 133.36, 133.24, 130.15, 130.12 (arom.), 129.90 (Cq. arom.), 129.76(arom.), 129.64 (Cq. arom.), 128.57, 128.42, 128.33, 128.25, 128.16, 127.69, 127.54, 127.39, 127.30 (arom.), 102.27 (C1''), 95.13 (C1'), 83.36 (C4''), 82.51 (C3'), 78.07 (C2''), 75.60, 75.51 (C3'', C2'), 72.64, 72.28 (CH_2 Bn), 72.07 (C4'), 64.33 (C5'), 62.06 (C5''). IR (film): 1733, 1715, 1268, 1091, 1069, 1018, 1011, 710, 697 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{45}H_{42}O_{12}Na$ (M+Na) 797.2568. Found 797.2569. $[\alpha]_D^{20}$ +82.3 (c = 1, in Methanol)

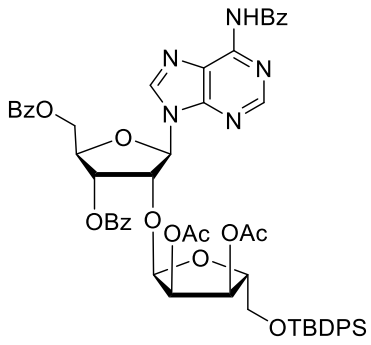
Compound 3 (from compound **22**)

Compound **22** (2.14 g, mmol), MeOH (13 mL), Pd/C (107 mg, 10% loading), and few drops of AcOH were added into reactor, then H_2 replaced air residue 3 times. The mixture was stirred under 88 mbar H_2 for 16 h and filtered over celite. The residue was concentrated, purified by silica gel column chromatography (pentane/EtOAc, 70/30 - 30/70) to obtain **3** as a white foam (1.02 g, mmol, 62%).



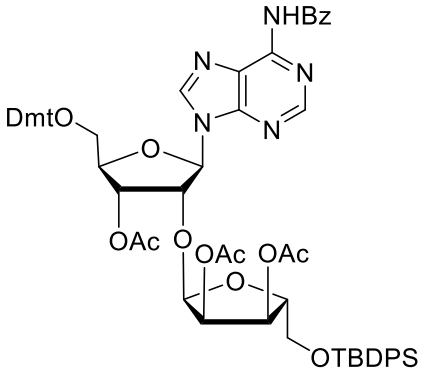
α-1,3,5-Tri-*O*-benzoyl-2',3'-di-*O*-acetyl-5'-*O*-tertbutyldiphenylsilyl-parobiose (23)

Compound **3** (0.95 g, 1.59 mmol), pyridine (16 mL) and TBDPSCl (0.5 mL, 1.92 mmol) were added into a flask and the mixture was stirred at room temperature for 16 h and TLC showed incompletely conversion. Another portion of TBDPSCl was added. 5 h later, Ac_2O was added and the reaction was stirred at room temperature for 3 h after which the reaction was quenched by aq. $NaHCO_3$ (sat.). The mixture was extracted by DCM (3 x) and dried ($MgSO_4$). The mixture was filtered, concentrated, purified by silica gel chromatography (pentane/acetone, 95/5 – 90/10 – 80/20) to furnish **23** as a white foam (1.22 g, mmol, 83 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.16 (ddt, *J* = 10.8, 7.2, 1.4 Hz, 4H, arom.), 8.10 – 8.02 (m, 2H, arom.), 7.66 – 7.52 (m, 7H, arom.), 7.46 – 7.30 (m, 12H, arom.), 6.82 (d, *J* = 4.3 Hz, 1H, H1'), 5.75 (dd, *J* = 6.4, 1.7 Hz, 1H, H3'), 5.46 (d, *J* = 4.5 Hz, 1H, H1'), 5.38 (dd, *J* = 7.0, 2.5 Hz, 1H, H3''), 4.99 (dd, *J* = 7.0, 4.6 Hz, 1H, H2''), 4.82 (td, *J* = 3.7, 1.7 Hz, 1H, H4'), 4.72 – 4.57 (m, 3H, H2', H5'), 4.10 (q, *J* = 2.8 Hz, 1H, H4''), 3.75 – 3.60 (m, 2H, H5''), 1.62 (s, 3H, CH_3 Ac), 1.44 (s, 3H, CH_3 Ac), 1.01 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.36, 169.75 (CO Ac), 166.18, 165.91, 165.14 (CO Bz), 135.72, 135.66, 133.52, 133.48 (arom.), 132.94, 132.88 (Cq. arom.), 130.19, 130.15 (arom.), 130.00 (Cq. arom.), 129.93, 129.90, 129.77 (arom.), 129.60 (Cq. arom.), 128.71, 128.47, 128.44, 127.89, 127.88 (arom.), 101.10 (C1''), 95.17 (C1'), 83.47 (C4'), 83.32 (C4''), 75.94 (C2'), 71.74 (C3'), 71.47 (C2''), 70.03 (C3''), 64.38 (C5'), 63.54 (C5''), 26.83 (CH_3 TBDPS), 19.84, 19.83 (CH_3 Ac), 19.27 (Cq. TBDPS). IR (film): 1721, 1715, 1451, 1265, 1249, 1222, 1111, 1104, 1067, 1024, 956, 701 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{51}H_{52}O_{14}SiNa$ (M+Na) 939.3019. Found 939.3020. $[\alpha]_D^{20}$ +82.9 (c = 1, in $CHCl_3$)



6-*N*-benzoyl-9-(3',5'-Tri-*O*-benzoyl-2'',3''-di-*O*-acetyl-5''-*O*-tertbutyldiphenylsilyl-β-parobiosyl) adenine (24)

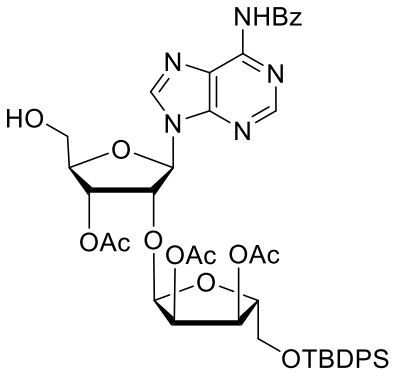
Compound **23** (1.16 g, 1.26 mmol) and N^6-benzoyladenine (452 mg, 1.89 mmol) were co-evaporated with dry 1,4-dioxane and dissolved in dry ACN. *N*,*O*-bis(trimethylsilyl)trifluorioacetamide (BSTFA) (1.69 mL, 6.30 mmol) was added and the mixture was stirred at room temperature until everything was dissolved. $HClO_4$-SiO_2 was added and the mixture was refluxed for 16 h after which was quenched by aq. $NaHCO_3$ (sat.). The filtrate was extracted by DCM and dried by $MgSO_4$. The mixture was filtered and concentrated and purified by silica gel chromatography (pentane/acetone, 100/0 – 80/20 – 70/30) furnished **24** as a white foam (1.19 g, 1.15 mmol, 91%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.07 (s, 1H, NH), 8.68 (s, 1H, H2), 8.17 (s, 1H, H8), 8.10 (ddd, *J* = 11.4, 8.4, 1.4 Hz, 4H, arom.), 8.05 – 7.97 (m, 2H, arom.), 7.64 – 7.48 (m, 9H, arom.), 7.48 – 7.29 (m, 10H, arom.), 6.27 (d, *J* = 5.5 Hz, 1H, H1'), 5.93 (dd, *J* = 5.4, 4.0 Hz, 1H, H3'), 5.53 (t, *J* = 5.5 Hz, 1H, H2'), 5.44 – 5.29 (m, 2H, H1'', H3''), 4.96 – 4.78 (m, 2H, H2''', H5'), 4.78 – 4.59 (m, 2H, H4', H5'), 4.00 (q, *J* = 2.8 Hz, 1H, H4''), 3.61 (AB, *J* = 11.4, 2.6 Hz, 1H, H5''), 3.50 (AB, *J* = 11.3, 3.1 Hz, 1H, H5''), 1.87 (s, 3H, CH_3 Ac), 1.77 (s, 3H, CH_3 Ac), 0.98 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.29, 169.67 (CO Ac), 166.21, 165.36, 164.60 (CO Bz), 152.99 (arom.), 151.73, 149.80 (Cq. arom.), 142.03, 135.64, 135.60, 133.71 (arom.), 133.64 (Cq. arom.), 133.56, 132.92 (arom.), 132.89, 132.76 (Cq. arom.), 129.94, 129.90, 129.87, 129.79 (arom.), 129.48, 129.37 (Cq. arom.), 128.97, 128.69, 128.62, 127.94, 127.85, 127.83 (arom.), 123.82 (Cq. arom.), 101.60 (C1''), 87.80 (C1'), 83.13 (C4''), 80.93 (C4'), 77.77 (C2'), 72.45 (C3'), 71.62 (C2''), 70.02 (C3''), 63.67 (C5'), 63.25 (C5''), 26.78 (CH_3 TBDPS), 20.39, 20.35 (CH_3 Ac), 19.22 (Cq TBDPS). IR (film): 2938, 1722, 1609, 1583, 1451, 1263, 1244, 1111, 1069, 1026, 700 cm^{-1}. HRMS (ESI^+) calcd for $C_{56}H_{56}N_5O_{13}Si$ (M+H) 1034.3638. Found 1034.3646. $[\alpha]_D^{20}$ +8.1 (c = 1, in Methanol)



6-*N*-benzoyl-9-(3',2'',3''-tris-*O*-acetyl-5'-*O*-dimethoxyltrityl-5''-*O*-tertbutyldiphenylsilyl-β-parobiosyl) adenine (25)

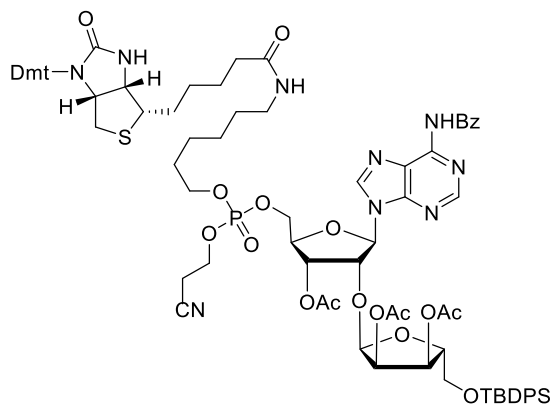
Compound **24** (196 mg, 0.19 mmol) was dissolved in pyridine/EtOH (2.1 mL; 2/1, v/v), cooled to 0°C after which aq. NaOH (1.1 mL, 1 M) was slowly added. The reaction was stirred for 1.5 hours at same temperature after which Amblite-H^+ was added in portions until pH=6. The mixture was filtered, concentrated under reduced pressure and co-evaporated with toluene (2 x), pyridine (2 x) and dissolved in pyridine (1.9 mL). 4,4'-dimethoxyltrityl chloride (DmtCl, 77 mg, 0.23 mmol) was added. The mixture was stirred for 16 h and TLC showed incomplete conversion after which the reaction was concentrated under reduced pressure. Pyridine and DmtCl (192 mg, 0.57 mmol) were added into the residue and the mixture was stirred for 1 h. The reaction was cooled to 0°C and Ac_2O (0.36 mL, 3.79 mmol) was added. The mixture was stirred at 0 °C for 2.5 h and quenched by aq. $NaHCO_3$ (sat.). DCM extracted (3 x) the mixture and the organic layers were combined and dried ($MgSO_4$), filtered, concentrated and purified by silica gel column chromatography (pentane/EtOAc/acetone, 90/10/0 – 70/30/0 – 80/0/20 – 70/0/30) to obtain **25** as a colorless oil (182 mg, 0.16 mmol, 84%). ^{1}H NMR (400

MHz, Chloroform-*d*) δ 9.05 (s, 1H, NH), 8.75 (s, 1H, H2), 8.16 (s, 1H, H8), 8.06 – 7.94 (m, 2H, arom.), 7.71 – 7.15 (m, 22H, arom.), 6.88 – 6.74 (m, 4H, DMT), 6.27 (d, *J* = 5.9 Hz, 1H, H1'), 5.58 (dd, *J* = 5.2, 3.6 Hz, 1H, H3'), 5.45 (dd, *J* = 7.1, 2.8 Hz, 1H, H3''), 5.37 (d, *J* = 4.6 Hz, 1H, H1''), 5.23 (t, *J* = 5.5 Hz, 1H, H2'), 4.94 (dd, *J* = 7.0, 4.6 Hz, 1H, H2''), 4.34 (d, *J* = 3.5 Hz, 1H, H4'), 4.14 (d, *J* = 2.8 Hz, 1H, H4''), 3.83 – 3.68 (m, 8H, OMe DMT, H5''), 3.51 (AB, *J* = 10.7, 3.5 Hz, 2H, H5'), 2.10 (d, *J* = 1.0 Hz, 6H, CH_3 Ac), 1.85 (s, 3H, CH_3 Ac), 1.02 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.33, 169.74, 169.65 (CO Ac), 164.59 (CO Bz), 158.76, 151.96, 149.69, 144.42 (Cq. arom.), 135.68, 135.65 (arom.), 135.42, 133.79, 132.93 (Cq. arom.), 132.91 (arom.), 132.73 (Cq. arom.), 130.24, 130.22, 129.97, 129.92, 129.00, 128.29, 128.09, 127.93, 127.91, 127.23 (arom.), 123.35 (Cq. arom.), 113.36 (arom.), 101.61 (C1''), 87.06 (Cq. DMT), 86.49 (C1'), 83.14 (C4''), 82.66 (C4'), 78.49 (C2'), 72.36 (C3'), 71.62 (C2''), 70.25 (C3''), 63.50 (C5''), 63.03 (C5'), 55.32 (OMe DMT), 26.82 (CH_3 TBDPS), 20.96, 20.95, 20.34 (CH_3 Ac), 19.28 (Cq. TBDPS). IR (film): 2935, 1743, 1739, 1506, 1244, 1219, 1092, 1030, 703 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{65}H_{68}N_5O_{14}Si$ (M+H) 1170.4527. Found 1170.4518. $[\alpha]_D^{20}$ -135.0 (c = 1, in Methanol)



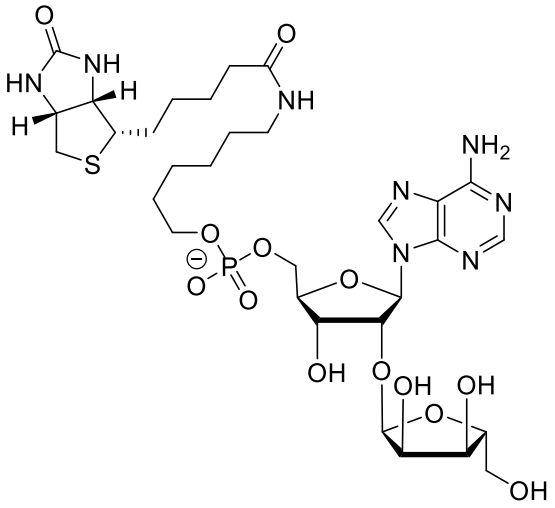
6-*N*-benzoyl-9-(3',2'',3''-tris-*O*-acetyl-5''-*O*-tertbutyldiphenylsilyl-β-parobiosyl) adenine (26)

Compound **25** (71 mg, 61 µmol), DCM (0.61 mL) and TFA (9.3 µL, 122 µmol) were added into a flask and the mixture was stirred at room temperature for 1 hour. TLC showed complete conversion and the reaction was quenched by aq. $NaHCO_3$ (sat.). DCM extracted (3 x) the mixture and the organic layers were combined, dried ($MgSO_4$), filtered, concentrated and purified by silica gel column chromatography (DCM/acetone, 95/5 – 90/10 – 85/15) to furnish **26** as a white foam (34 mg, 39 µmol, 64 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.08 (s, 1H, NH), 8.82 (s, 1H, H2), 8.11 (s, 1H, H8), 8.05 – 7.94 (m, 2H, arom.), 7.66 – 7.48 (m, 7H, arom.), 7.46 – 7.30 (m, 6H, arom.), 6.11 (d, *J* = 11.3 Hz, 1H, OH), 6.02 (d, *J* = 7.8 Hz, 1H, H1'), 5.68 (d, *J* = 5.3 Hz, 1H, H, H3'), 5.37 (dd, *J* = 7.0, 2.9 Hz, 1H, H3''), 5.14 (dd, *J* = 7.8, 5.3 Hz, 1H, H2'), 5.09 (d, *J* = 4.7 Hz, 1H, H1''), 4.92 (dd, *J* = 7.0, 4.7 Hz, 1H, H2''), 4.32 (q, *J* = 1.4 Hz, 1H, H4'), 4.06 – 3.94 (m, 2H, H4'', H5'), 3.87 (t, *J* = 11.8 Hz, 1H, H5'), 3.76 – 3.62 (m, 2H, H5''), 2.15 (s, 3H, CH_3 Ac), 2.13 (s, 3H, CH_3 Ac), 1.97 (s, 3H, CH_3 Ac), 0.99 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.24, 169.65, 169.53 (CO Ac), 164.46 (CO Bz), 150.58, 150.51 (Cq. arom.), 135.66 (arom.), 133.50 (Cq. arom.), 133.12 (arom.), 132.85, 132.69 (Cq. arom.), 129.98, 129.95, 129.07, 127.98, 127.92, 127.89 (arom.), 124.64 (Cq. arom.), 101.23 (C1''), 89.80 (C1'), 86.89 (C4'), 83.09 (C4''), 77.61 (C2'), 73.78 (C3'), 71.58 (C2''), 70.24 (C3''), 63.43 (C5''), 62.89 (C5'), 26.83 (CH_3 TBDPS), 21.03, 20.94, 20.44 (CH_3 Ac), 19.24 (Cq. TBDPS). IR (film): 2931, 2857, 1743, 1609, 1584, 1456, 1360, 1235, 1113, 1048, 703 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{44}H_{50}N_5O_{12}Si$ (M+H) 868.3220. Found 868.3230. $[\alpha]_D^{20}$ +5.6 (c = 1, in $CHCl_3$)



6-*N*-benzoyl-9-(3',2'',3''-tri-*O*-acetyl-5'-*O*-{6-[(2-cyanoethoxy)phosphoryl]-[1-*N*-(4,4'-dimethoxytrityl)biotinyl]aminohexanyl}-5''-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (27)

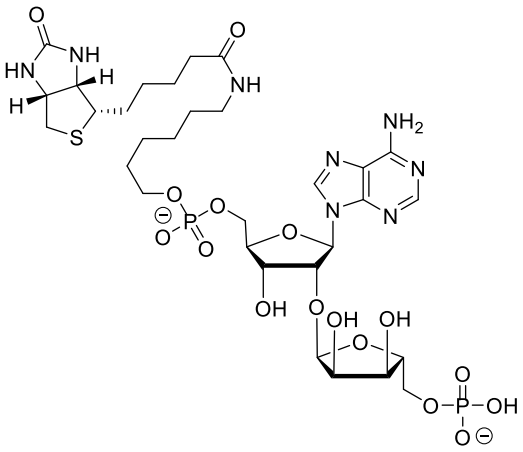
1-Methyl-imidazole·HCl (36 mg, 0.30 mmol) and 1-methyl-imidazole (16 µL, 0.20 mmol) were co-evaporated with dry CH_3CN (3 x), then N_2 was applied. To this mixture, freshly activated molecular sieves and dry DMF (0.5 mL) were added and the activator solution was stirred at room temperature for 2 hours under N_2. Next, compound **26** (29 mg, 33 µmol) was co-evaporated with dry 1,4-dioxane (3 x) after which the activator solution above was added. Subsequently, **21** (113 mg, in 1 mL ACN, 0.9 mmol) was added and the reaction was stirred at room temperature for 20 minutes. *t*BuOOH in decane (60 µL, 5.5 M, 0.33 mmol) was added at 0 ℃ and the reaction mixture was stirred for 1 hour at room temperature. The reaction was quenched by aq. $NaHCO_3$ (sat.), extracted with EtOAc (3 x), dried ($MgSO_4$), concentrated, purified by LH-20 gel filtration (DCM/methanol, 50/50) and silica gel chromatography (DCM/MeOH, 100/0 – 97/3) to obtain **27** as a white foam (38 mg, 23 µmol, 70%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.47 (d, *J* = 14.8 Hz, 1H, NH), 8.80 (d, *J* = 1.7 Hz, 1H, H2), 8.31 (d, *J* = 1.2 Hz, 1H, H8), 8.06 – 7.92 (m, 2H, arom.), 7.68 – 7.54 (m, 5H, arom.), 7.52 – 7.33 (m, 8H, arom.), 7.33 – 7.21 (m, 5H, arom.), 7.21 – 7.07 (m, 4H, arom.), 6.86 – 6.71 (m, 4H, aom.), 6.21 (dd, *J* = 5.1, 3.9 Hz, 1H, H1'), 6.02 (d, *J* = 18.0 Hz, 1H, NH Biotin), 5.59 (d, *J* = 9.7 Hz, 1H, NH Biotin), 5.54 – 5.41 (m, 2H, H3', H3''), 5.36 (dd, *J* = 4.6, 1.9 Hz, 1H, H1''), 5.18 – 5.06 (m, 1H, H2'), 4.95 (dd, *J* = 7.1, 4.6 Hz, 1H, H2''), 4.51 – 4.27 (m, 5H, CH Biotin, H4', H5'), 4.25 – 4.00 (m, 5H, CH_2OP=O, H4''), 3.85 – 3.66 (m, 9H, OMe DMT, H5''), 3.25 – 3.03 (m, 3H, CONH*CH₂*, CH Biotin), 2.68 (dt, *J* = 12.3, 5.9 Hz, 2H, CH_2OCN), 2.46 – 2.37 (m, 1H, CH_2S), 2.25 (AB, *J* = 13.1, 5.7 Hz, 1H, CH_2S), 2.18 – 2.02 (m, 8H, CH_3 Ac, *CH₂*CONH), 1.91 (d, *J* = 3.1 Hz, 3H, CH_3 Ac), 1.73 – 1.19 (m, 14H, CH_2), 1.02 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.99, 172.96, 170.29, 169.80, 169.79, 169.74, 169.72 (CO), 165.06, 165.02, 161.60, 158.45, 151.93, 150.09, 143.86, 135.92, 135.84 (Cq. arom.), 135.69, 135.65 (Cq. arom.), 133.39, 133.37 (Cq. arom.), 132.92 (arom.), 132.85, 132.74 (Cq. arom.), 131.39, 130.00, 129.97, 129.81, 128.90, 128.19, 128.17, 127.92, 127.90, 127.64, 127.00 (arom.), 124.07, 124.02, 116.81, 116.74 (Cq. arom.), 112.90 (arom.), 101.52 (C1''), 87.41, 87.28 (C1'), 83.13 (C4''), 81.17, 81.09 (C4'), 78.00 (C2'), 72.79 (CN), 71.55 (C2''), 71.05 (C3'), 70.21 (C3''), 68.71, 68.64 (CH_2OP=O), 66.45, 66.39, 66.33, 66.28 (C5'), 65.53, 65.52 (CH Biotin), 63.49 (C5''), 62.32, 62.27, 62.23 (CH_2OP=O), 59.77, 59.75 (CH Biotin), 55.33 (OMe DMT), 54.33, 54.32 (*CH*CH_2 Biotin), 39.28, 39.26 (CONH*CH₂*), 39.09, 39.05 (CH_2S), 36.05, 36.03, 35.30, 34.50, 33.82, 30.98, 29.92, 29.86, 29.80, 29.28, 29.22, 28.70, 28.30, 28.28 (CH_2), 26.83 (CH_3 TBDPS), 26.06, 26.00, 25.54, 25.49, 24.85, 24.81 (CH_2), 20.95, 20.87, 20.40 (CH_3 Ac), 19.80, 19.73 (CH_2CN), 19.27 (Cq. arom.). ^{31}P NMR (122 MHz, $CDCl_3$) δ -1.56, -1.67. IR (film): 2930, 1743, 1700, 1695, 1653, 1616, 1507, 1456, 1290, 1238, 1181, 1034, 1030, 703 cm^{-1}. HRMS (ESI^+) calcd for $C_{84}H_{99}N_9O_{19}PSSi$ (M+H) 1628.6279. Found 1628.6376. $[\alpha]_D^{20}$ +32.5 (c = 1, in $CHCl_3$)



9-[5'-*O*-(6-*O*-biotinylaminohexany)-phosphoryl-β-parobiosyl]adenine (28)

Compound **27** (14 mg, 8.60 µmol), DCM (0.4 mL) and TFA (4 µL, 51.60 µmol) were added into the flask and the reaction was stirred at room temperature for 1 hour. TLC showed no complete reaction and more TFA (2 µL, 25.80 µmol) was added. 1 hour later the reaction was quenched by NH_4OH (55 µL) and concentrated. To the residue, NH_4OH (0.4 mL) and 1,4-dioxane (0.4 mL) were added and stirred for 24 hours at room temperature. The reaction was concentrated, co-evaporated with 1,4-dioxane (2 x), toluene (3 x) and pyridine (2 x). The residue was dissolved in pyridine (0.4 mL) and HF·pyridine (60 µL, 70% HF pyridine solution, 2.31 mmol base on HF) was added at 0 ℃. The mixture was stirred for 16 hours after which it was quenched by NH_4OH, concentrated, purified by HW-40 gel filtration [25% ACN in aqueous NH_4OAc (0.15 M)]. Repeated lyophilization obtained **28** as a white solid (3.01 mg, 3.74 µmol, 43%). ^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.62 (d, *J* = 2.7 Hz, 1H, H2), 8.41 (d, *J* = 3.3 Hz, 1H, H8), 6.34 (d, *J* = 5.2 Hz, 1H, H1'), 5.28 (d, *J* = 4.3 Hz, 1H, H1''), 4.96 (t, *J* = 5.2 Hz, 1H, H2'), 4.67 (t, *J* = 4.5 Hz, 1H, H3'), 4.60 (dd, *J* = 8.0, 4.9 Hz, 1H, CH Biotin), 4.45 (q, *J* = 3.0 Hz, 1H, H4'), 4.41 (dd, *J* = 8.0, 4.4 Hz, 1H, CH Biotin), 4.25 (q, *J* = 3.5 Hz, 1H, H4''), 4.19 – 4.06 (m, 4H, H5', H2'', H3''), 3.83 – 3.71 (m, 3H, H5'', CH_2OP=O), 3.67 (AB, *J* = 12.4, 4.6 Hz, 1H, H5''), 3.29 (dt, *J* = 9.7, 5.1 Hz, 1H, CHS), 3.16 – 3.06 (m, 2H, CONH*CH_2*), 2.98 (AB, *J* = 13.0, 4.9 Hz, 1H, CH_2S), 2.77 (d, *J* = 13.1 Hz, 1H, CH_2S), 2.24 (t, *J* = 7.7 Hz, 2H, *CH_2*CONH), 1.76 – 1.48 (m, 6H, CH_2), 1.45 – 1.32 (m, 4H, CH_2), 1.25 – 1.14 (m, 4H, CH_2). ^{13}C NMR (214 MHz, D_2O) δ 177.39 (CO), 166.21 (CO), 153.32 (C4), 149.60 (C6), 119.61 (C5), 102.68 (C1''), 87.34 (C1'), 86.41 (C4''), 85.35, 85.31 (C4'), 80.26 (C2'), 72.45 (C2''), 71.34 (C3'), 70.69 (C3''), 67.20, 67.17 (CH_2OP=O), 65.51, 65.49 (C5'), 62.98 (CH Biotin), 62.38 (C5''), 61.13 (CH Biotin), 56.30 (CHS), 40.58 (CH_2S), 40.05 (CONH*CH_2*), 36.37 (*CH_2*CONH), 30.58, 30.54, 29.08, 28.71, 28.56, 26.62, 26.10, 25.51 (CH_2). ^{31}P NMR (122 MHz, D_2O) δ -0.33. HRMS (ESI^+) calcd for $C_{31}H_{50}N_8O_{13}PS$ (M+H) 805.2950. Found 805.2972.

Synthesis of compound **29** will be described in Chapter 4



9-[5'-O-(6-O-biotinylaminohexanyl)-phosphoryl-5''-*O*-phosphoryl-β-parotriosyl]adenine (30)

Compound **29** (11 mg, 10 µmol), DCI (0.12 mL, 0.25 M in ACN, 30 µmol) and freshly flame-dried 3Å molecular sieves were added into a flask and the mixture was stirred under N_2 at room temperature. **21** (34 mg, 40 µmol) was added and it was stirred for 1 hour. *t*BuOOH in decane (9 µL, 5.5 M, 50 µmol) was added at 0 ℃ and the reaction mixture was stirred for 1 hour at room temperature. The reaction was quenched by aq. $NaHCO_3$ (sat.), extracted with DCM (3 x), dried (Na_2SO_4) concentrated and purified by LH-20 gel filtration (DCM/methanol, 50/50). The fractions containing product were collected and concentrated. To the residue, DCM (0.4 mL) and TFA (1.6 µL, 20 µmol) were added and the mixture was stirred for 1 hour after which it was quenched by NH_4OH. The mixture was concentrated, added 1,4-dioxane (0.4 mL) and NH_4OH (0.4 mL). The reaction was stirred for 24 hours and concentrated. HPLC purification (0 - 30%, A: 25mM NH_4OAc/H_2O B: ACN) and repeated lyophilization yielded **30** (2.1 mg, 2.37 µmol, 24%) as a white solid. ^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.51 (s, 1H, H2), 8.30 (s, 1H, H8), 6.27 (d, *J* = 5.1 Hz, 1H, H1'), 5.25 (d, *J* = 4.3 Hz, 1H, H1''), 4.91 (t, *J* = 5.2 Hz, 1H, H2'), 4.62 (dd, *J* = 5.2, 4.1 Hz, 1H, H3'), 4.54 (ddd, *J* = 8.0, 5.0, 0.8 Hz, 1H, CH Biotin), 4.38 (t, *J* = 3.5 Hz, 1H, H4'), 4.34 (dd, *J* = 8.0, 4.5 Hz, 1H, CH Biotin), 4.32 (tt, *J* = 3.8, 1.9 Hz, 1H, H4''), 4.18 – 4.13 (m, 2H, H2'', H5'), 4.11 – 4.04 (m, 2H, H5', H3''), 3.93 (AB, *J* = 11.5, 6.0, 3.8 Hz, 1H, H5''), 3.89 (AB, *J* = 11.4, 5.0, 3.5 Hz, 1H, H5''), 3.71 – 3.63 (m, 2H, CH_2OP=O), 3.22 (ddd, *J* = 9.7, 5.7, 4.5 Hz, 1H, CHS), 3.03 (ddt, *J* = 40.2, 13.4, 6.7 Hz, 2H, CONH*CH*$_2$), 2.91 (dd, *J* = 13.1, 5.0 Hz, 1H, CH_2S), 2.71 (d, *J* = 13.0 Hz, 1H, CH_2S), 2.21 – 2.14 (m, 2H, *CH*$_2$CONH), 1.69 – 1.45 (m, 4H, CH_2), 1.42 (p, *J* = 6.8 Hz, 2H, CH_2), 1.33 – 1.27 (m, 4H, CH_2), 1.17 – 1.02 (m, 4H, CH_2). ^{13}C NMR (214 MHz, D_2O) δ 177.41 (CO), 166.23 (CO), 154.93 (C4), 149.79 (C6), 119.63 (C5), 102.74 (C1''), 87.15 (C1'), 85.29, 85.25 (C4'), 85.14, 85.10 (C4''), 80.12 (C2'), 72.31 (C2''), 71.19 (C3'), 70.78 (C3''), 67.19, 67.16 (CH_2OP=O), 65.69, 65.67 (C5''), 65.50, 65.48 (C5'), 62.99, 61.14 (CH Biotin), 56.31 (CHS), 40.60 (CH_2S), 40.07 (CONH*CH*$_2$), 36.37 (*CH*$_2$CONH), 30.56, 30.52, 29.08, 28.71, 28.56, 26.64, 26.12, 25.49 (CH_2). ^{31}P NMR (162 MHz, D_2O) δ 1.10, 0.97. HRMS (ESI$^+$) calcd for $C_{31}H_{51}N_8O_{16}P_2S$ (M+H) 885.2613. Found 885.2628.

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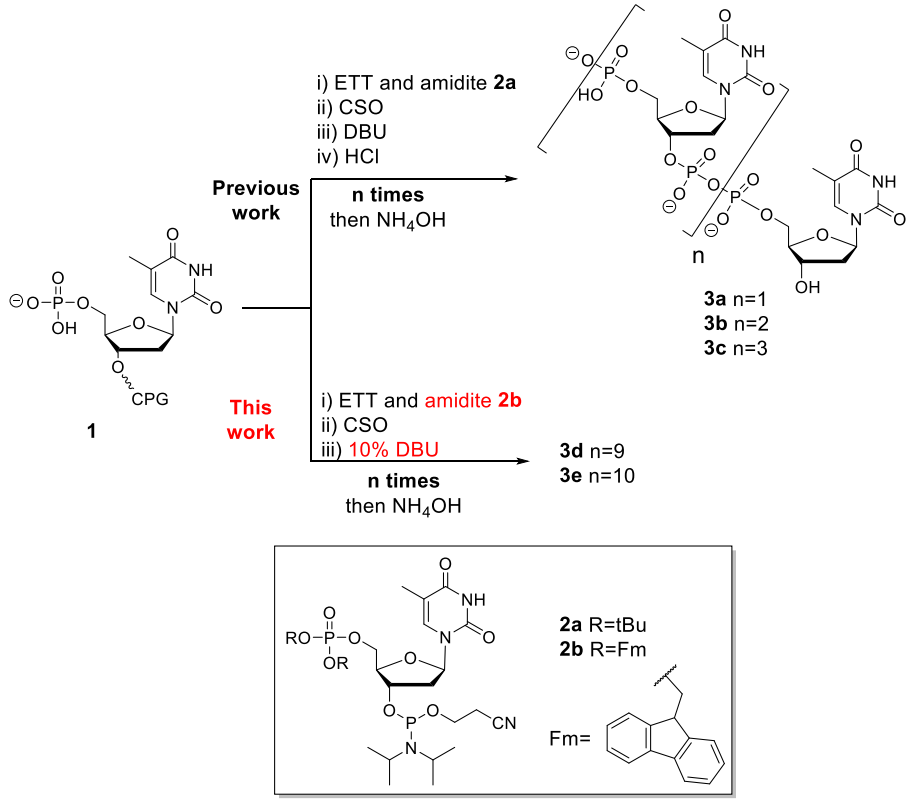
3 Synthesis of 3',5'-pyrophosphate-linked thymidine oligomers

Introduction

Pyrophosphate functions can be present in many biomolecules, ranging from lipids and inositols to nucleic acids, proteins and carbohydrates and these pyrophosphate containing molecules play an important role in a variety of biological processes.[1-4] Their broad occurrence in nature and the synthetic challenge that pyrophosphates represent have led to a row of synthetic methods for the construction of pyrophosphate esters.[5-7] This is illustrated by the continuous appearance of new or modified procedures for the synthesis of nucleoside diphosphate sugars, that are donors in the glycosyltransferase mediated biosynthesis of carbohydrates and glycoproteins. Gold *et al*[5] reported P(V)-P(III) coupling method for the formation of pyrophosphate linkages and prepared three UDP-N-acetylglucosamine derivatives. This procedure was not only adopted for the synthesis of other sugar-nucleotides[8] and glycolipids[9] but also to the synthesis of a cADPR antagonist[10] and ADP-ribosylated oligopeptides.[11, 12] Whereas these molecules contain one pyrophosphate function, poly-ADP-ribose is a polymer of repeating pyrophosphates and therefor the assembly of fragments of poly-ADP-ribose presents an additional challenge. Using the P(V)-P(III) methodology, Kistemaker *et al* reported the first synthesis of an ADPr trimer that was successfully applied in a biophysical study.[13, 14] In a comparative model study the same authors prepared pyrophosphate-linked thymidine oligomers, up to a length of a tetramer.[9] Guided by a hypothesis in prebiotic chemistry that pyrophosphate-linked DNA oligomers have played a role in the origin of oligonucleotides, Anderson and Krishnamurthyused used the same P(V)-P(III) methodology for the synthesis of pyrophosphate-linked thymidine oligomers and dertermined their binding to DNA and RNA.[10] Despite the impressive achievements attained in the

above mentioned syntheses, it is clear that the length and the associated quality of ADP-ribose oligomers and pyrophosphate-linked thymidine oligomers are restricted. It is therefore necessary to optimize the P(V)-P(III) methodology of pyrophosphate synthesis.



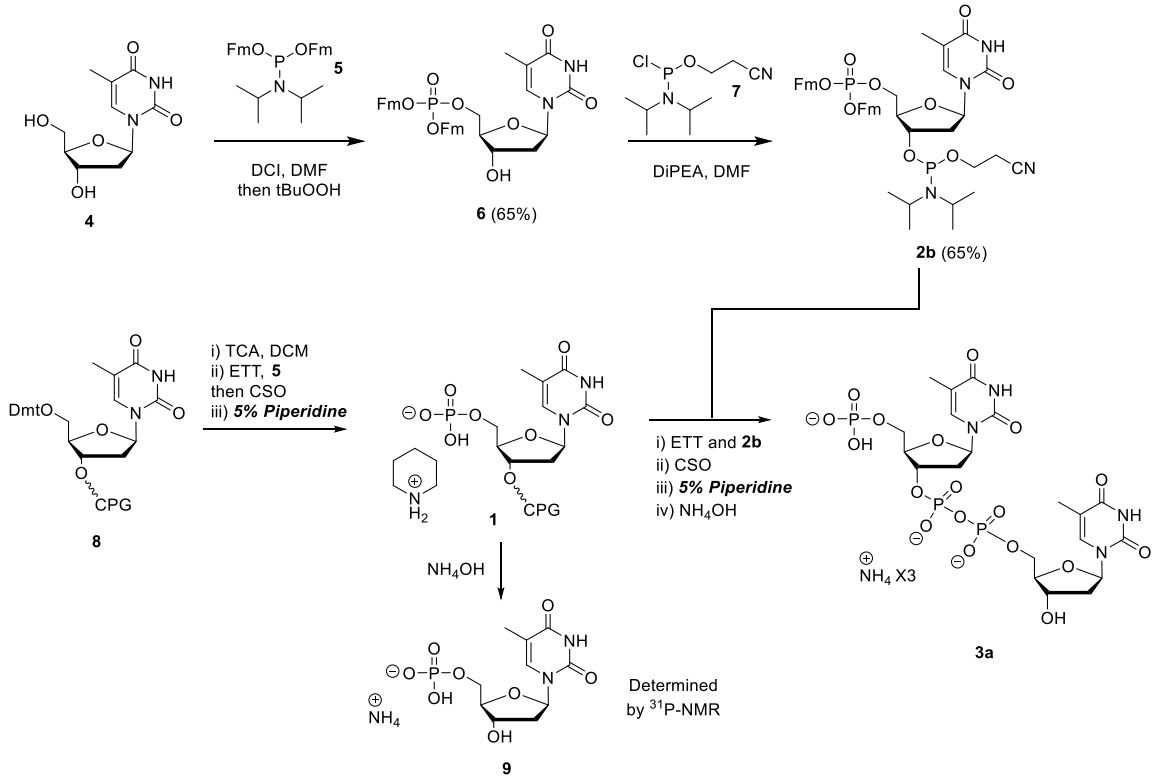
Scheme 1. Previous work and this work on the synthesis of pyrophosphate linked thymindine oligomers.

On the basis of the accessibility of the building blocks, the optimization of the synthesis of pyrophosphate-linked thymidine oligomers is first executed. The reported solid phase synthesis[7, 8, 15] used CPG immobilized 5'-phosphothymidine **1** together with an elongation cycle entailing the following steps. The coupling of monophosphate (e.g. **1**) with the phosphoramidite **2a** under influence of activator ETT that generates an intermediate with a P(V)-P(III) bond was followed by oxidation of the P(III) center with CSO to provide a P(V)-P(V) pyrophosphate bond. The subsequent removal of the cyanoethyl (CE) group with DBU and the deprotection of *tert*-butyl groups at the terminal phosphotriester with a catalytical amount of HCl in HFIP[11, 13, 16] allow the next elongation cycle (Scheme 1). The formation of the side products and shorter oligomers, found in the crude mixture at the end of the synthesis, is possibly caused by the repeated acidic removal of *tert*-butyl groups in the fourth step of the elongation cycle (Scheme 1).[15] The amount of side products increases with the length of the oligomers (such as decamer) making the purification of longer oligomers impossible.[8] Based on these considerations, replacement of the *t*Bu group by the base labile fluorenylmethyl (Fm)[17, 18] group that

can be easily removed using DBU or piperidine will improve the synthesis and making the longer pyrophosphate-linked thymidine oligomers available.

This Chapter describes the synthesis and application of the new phosphoramidite building block **2b** with a Fm protected 5'-phosphotriester for the assembly of pyrophosphate-linked thymidine oligomers (Scheme 1). The implementation of building block **2b** reduces the number of steps in the coupling cycle from four to three: phosphitylation, oxidation and base deprotection. The usefulness of building block **2b** for the synthesis of molecules with multi-pyrophosphates is demonstrated by the synthesis of pyrophosphate linked thymidine decamer.

Results and discussion



Scheme 2. Synthesis of key phosphoramidite monomer **2b** and oligonucleotide **3a**

The synthesis of the key phosphoramidite building blocks **2b** commenced with selective phosphitylation of thymidine **4** with known $(FmO)_2PN(iPr)_2$ (**5**)[13, 14, 15] using DCI as an activator, followed by oxidation of *t*BuOOH of the intermediate phosphite triester to give **6** (Scheme 2). The di-phosphorylated byproduct was also observed and could be separated by column chromatography. The ensuing introduction of cyanoethyl phosphoramidite on 3'-OH using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite **7** in DCM/DMF and TEA as a base failed.[13] TLC and LC-MS analysis showed the presence of side-products, originating from the cleavage of one of the Fm groups by TEA.

Switching to the more hindered Hünig's base (DIPEA) went without Fm cleavage. Of note is that **2b** is sensitive to both mild base and acid because of the presence of a phosphoramidite and the Fm groups, making normal TEA neutralized silica gel column unsuitable for purification. Therefore, a special neutralized silica gel column was used for the purification of **2b** (see experimental section). Additionally, the H-phosphonate generated from the hydrolysis of excessive amount of reagent **7** could not be easily separated (around 14 ppm on ^{31}P-NMR). This byproduct was also observed in previous studies and did not interfere with the pyrophosphate coupling.[13, 15]

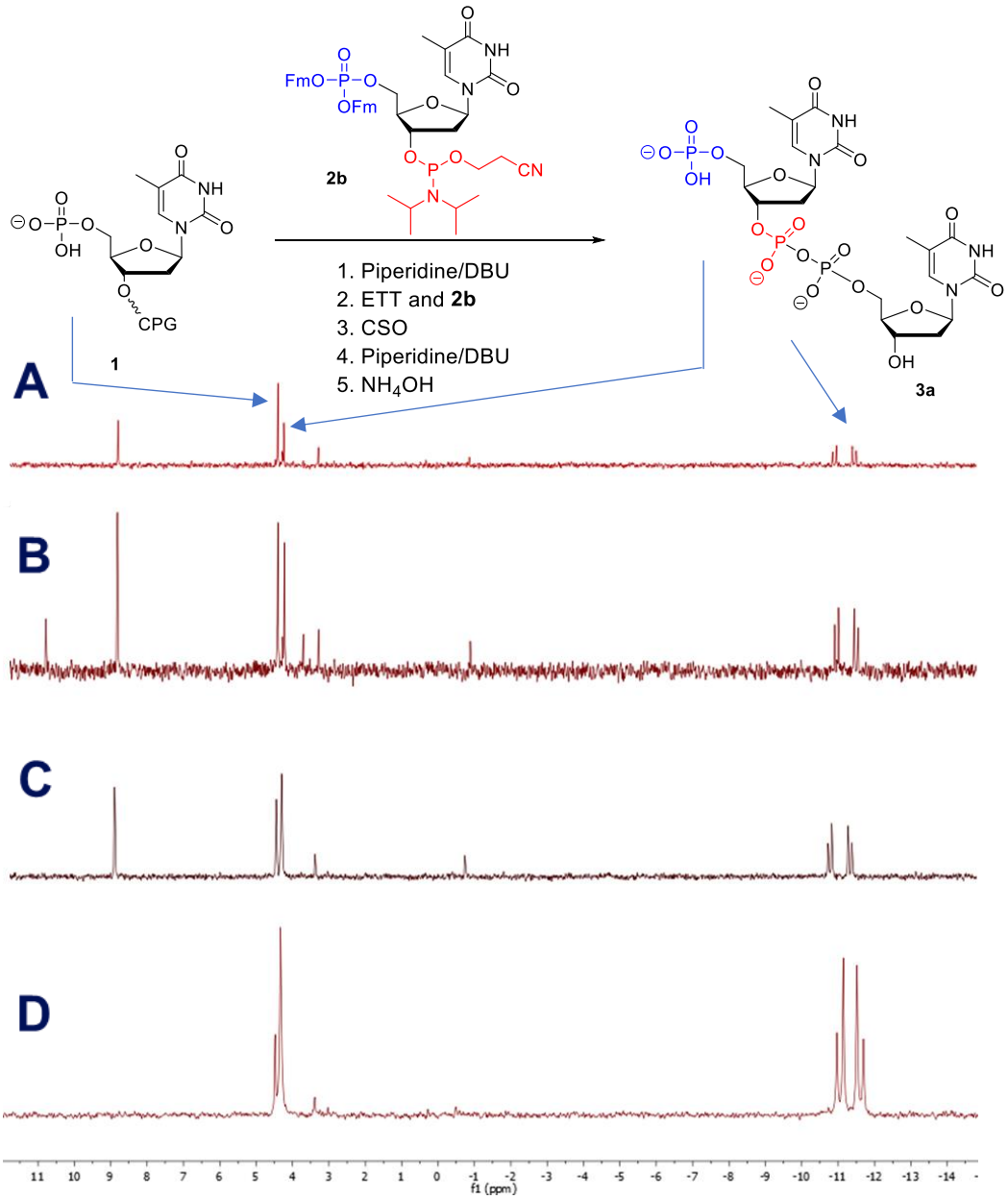
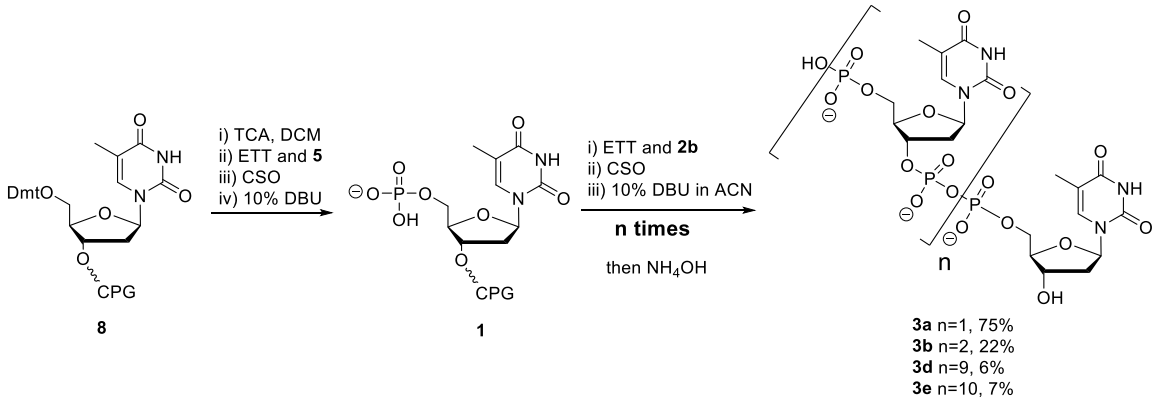


Figure 1. ^{31}P-NMR spectrums of the crude sample of pTppT cleaved from the solid phase. **A**: without step 1, step 4 = 5% piperidine in DMF; **B**: Step 1 = step 4 = 5% piperidine in DMF; **C**: Step 1 = step 4 = 20% piperidine in DMF; **D**: Step 1 = step 4 = 5% DBU in DMF

To test the viability of building block **2b**, the solid phase synthesis of monomer **3a**, having one pyrophosphate moiety, was undertaken (Scheme 2). First, commercially available 3'-dT CPG **8** was detritylated with TCA in DCM and converted into phosphate monoester **1** by treatment with phosphoramidite **5** and activator ETT, followed by oxidation of the intermediate phosphite triester by CSO and finally removal of both Fm groups in the obtained phosphate triester with 5% piperidine[19] in DMF (10 min). A small amount of resin **1** was cleaved by NH_4OH, obtaining **9** for characterization by ^{31}P-NMR. Then, resin **1** was transformed to the pyrophosphate linked nucleotide **3a** via 4 steps: a) P(V)-P(III) bond formation by coupling with **2b** under influence of ETT; b) CSO mediated oxidation; c) removal of the CE and both terminal Fm groups by piperidine (10 min); d) NH_4OH treatment to cleave **3a** from resin. The crude **3a** was analyzed by ^{31}P-NMR spectroscopy and spectrum A (Figure 1) shows two doublets at around -11 ppm which indicated the successful construction of the pyrophosphate bond. The phosphate monoester of **3a** appeared at about 4.3 ppm, accompanying with a bigger unknown peak at around 4.5 ppm which could be ascribed to the starting material, that is cleaved from CPG. It is assumed that the incomplete conversion from **1** to **3a** resulted from the incomplete Fm deprotection of the 5' phosphate of **1**, as mono Fm protected **1** would be inert in the P(V)-P(III) coupling step. Thus, additional piperidine treatment (5% in DMF, step 1) for 10 minutes before the pyrophosphates coupling showed that less amount of **9** was generated, originating from starting material **1** (Spectra B). Switching to a higher concentration of piperidine (20% in DMF) resulted in a better conversion (Spectra C). Next DBU, a stronger base (5% in DMF, 10 minutes) was used, furnishing **3a** as the dominant product with only a small amount of remaining starting material. A prolongation of the DBU treatment to 30 minutes gave, however the same result. It is notable that the DBU treatment did not cleave **1** from the resin, indicating the use of DBU is safe with respect to the preservation of the integrity of the linker. These data collectively showed that Fm protection of the intermediate phosphate triester and the ensuing DBU mediated Fm deprotection is suitable for the projected P(V)-P(III) coupling procedure to pyrophosphate formation on solid support.



Scheme 3. The synthesis of thymidine pyrophosphate oligomer

At this stage the effort was made to prepare an oligomer synthesis using an automated oligonucleotide synthesizer. The synthesis of immobilized phosphomonoester **1** was repeated with a few adaptations. After detritylation of resin **8** using TCA, the coupling with amidite **5** and activator ETT was repeated three times, ensuring the full conversion of all the hydroxyl groups (Scheme 3). After CSO oxidation, the Fm groups were cleaved by 10% DBU, to accomplish a complete deprotection. Next, phosphomonoester **1** was elongated with one pyrophosphate via above mentioned elongation cycle of three steps: phosphitylation (ETT and **2b**), oxidation (CSO), and deprotection (10% DBU). Repetition of this procedure n times should introduce n immobilized pyrophosphate linkages that upon NH_4OH treatment would result in the cleavage from the resin of the target pyrophosphate-linked thymidine oligomers, the quality of which can be established by anion exchange HPLC (Figure 2). First monomer **3a** (n=1) and dimer **3b** (n=2), were prepared and the crude mixtures were analyzed by HPLC. Purification resulted in the isolation of monomer **3a** (n=1) and dimer **3b** (n=2) in 75% yield and 22% yield respectively. Encouraged by this result the synthesis of decamer **3e** (n=10) was undertaken. Surprisingly, analytical anion exchange HPLC showed not only the presence of target decamer **3e** (n=10) but also shorter oligomers ranging from dimer to nonamer (Figure 2). Purification using anion exchange column chromatography gave pure decamer **3e** and nonamer **3d** in 7% and 6% yield respectively, which was an improvement compared with earlier synthesis.[8] The generation of these shorter oligomers during decamer synthesis could be attributed to an incomplete P(V)-P(III) coupling, thus unreacted P(V) species could still react with **2b** in the next cycle, generating n-1 oligomers. In the standard DNA oligomer synthesis, 5'-OH was capped by acetyls to suppress the side reaction, however, in our case, capping a 5'-phosphomonoester is difficult. An additional source of the shorter fragments might be an unwanted reaction of the nucleophilic pyrophosphates present in the growing chain with the incoming **2b**. Such a side reaction would lead to branched triphosphate upon the oxidation that would break under the alkaline conditions in the final deprotection step and generate the truncated sequences.

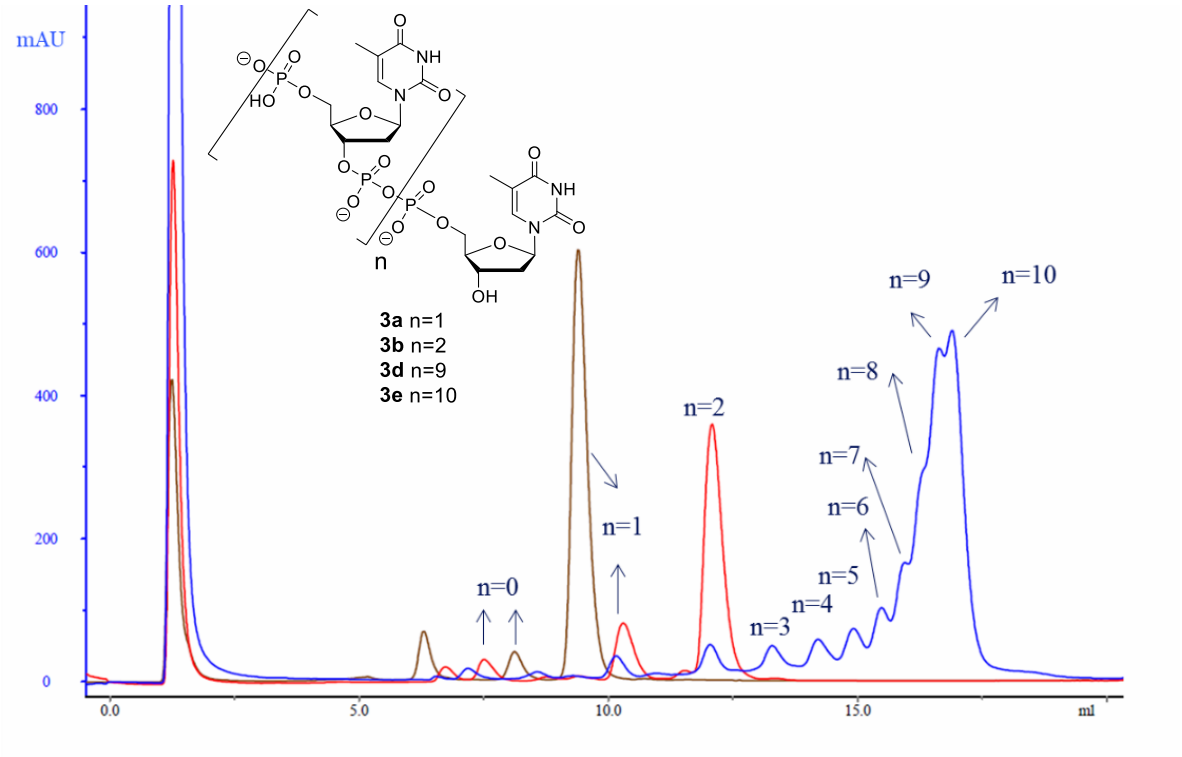


Figure 2. Analytical IE-HPLC traces of crude **3a** (brown), **3b** (red) and **3e** (blue) taken with UV detection. Column: DNAPac PA-100, 4*250 mm. Gradient: pH 7 buffer, 0%-70%. In 20 min. A: 10 Mm NaOAc, 10 mM $NaClO_4$; B:10mM NaOAc 500mM $NaClO_4$

Conclusion

This Chapter describes the design and synthesis of the new Fm protected phosphoramidite **2b** and its application in the solid phase synthesis of 3',5'-pyrophosphate-linked thymidine oligomers. Application of the base labile fluorenylmethyl (Fm) as a phosphate protecting group avoid intermediate acid treatment of the immobilized 3',5'-pyrophosphate-linked thymidine oligomers, thereby preventing possible acid mediated pyrophosphate cleavage. Optimization of the deprotection conditions of Fm groups to 10% DBU in ACN and its subsequent implementation in the solid phase synthesis led to the successful isolation of the 3' 5'-pyrophosphates linked thymidine decamer **3e**. The use of fluorenylmethyl (Fm) instead of the *t*Bu group improved the synthesis of 3',5'-pyrophosphate-linked thymidine oligomers, but was also accompanied by the formation of substantial amounts of shorter oligomers. Although it must be investigated whether this side reaction(s) can be suppressed, the improvement of method described here is sufficient to evaluate its applicability to the synthesis of the biologically relevant ADPr oligomers.

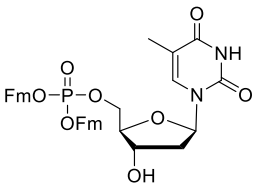
Acknowledgement

Nico Meeuwenoord is kindly acknowledged for his help in oligomer synthesis and final product purification.

Experimental section

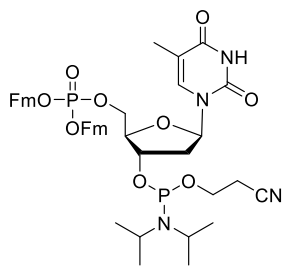
General procedure

All chemicals and solvents were used as described in Chapter 2. TLC, NMR, LCMS, anion exchange, HRMS, IR, optical rotation facilities were used as described in Chapter 2. dT-CPG **8** was purchased from Sigma-Aldrich. The synthesis of compound **5** will be discussed in Chapter 4. The solid phase synthesis of the thymidine oligomers was performed on a Mermade-6 oligonucleotide synthesizer (Bioautomation corporation).



5'-O-(di-O-flourenylmethyl)-phosphoryl-2'-deoxylthymidine (6)

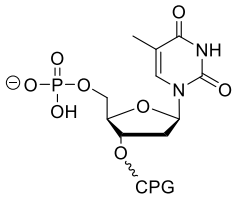
DCI (12 mL, 3.0 mmol) and thymidine **4** (242 mg, 1.0 mmol) were coevaporated with ACN (3 x) and dissolved in DMF (7 mL) with 3 Å molecular sieves under N_2. To this solution, $(FmO)_2PN(iPr)_2$ **5** (625 mg, 1.2 mmol, in 3 mL DMF) was added and the reaction was stirred for 30 minutes. The reaction mixture was cooled to 0 °C and *t*BuOOH (0.55 mL, 3 mmol, 5.5 M in nonane) was added. The mixture was allowed to reach to room temperature and was stirred for 1 hour and quenched by aq. $NaHCO_3$ (sat.). EtOAc extracted this mixture (1 x) and the organic layer was extracted with H_2O (1 x) and brine (1 x) and dried ($MgSO_4$). The mixture was filtered, concentrated and purified by silica gel column chromatography (DCM : MeOH = 99/1 – 98/2 – 95/5) to furnish **6** (439 mg, 0.65 mmol, 65%) and 3',5'-diphosphorylated byproduct (100 mg, 0.09 mmol, 9%) as a white foam. ^{1}H NMR (500 MHz, Methanol-*d*$_4$) δ 7.71 (dddt, *J* = 14.8, 12.0, 7.6, 1.0 Hz, 4H, arom.), 7.54 – 7.17 (m, 12H, arom.), 7.11 (q, *J* = 1.2 Hz, 1H, H6), 6.15 (dd, *J* = 7.8, 6.1 Hz, 1H, H1'), 4.37 – 4.26 (m, 4H, CH_2 Fm), 4.12 (dt, *J* = 6.2, 3.0 Hz, 1H, H3'), 4.05 (q, *J* = 6.1 Hz, 2H, CH Fm), 3.85 – 3.66 (m, 3H, H4', H5'), 2.11 (AB, *J* = 13.7, 6.1, 3.0 Hz, 1H, H2'), 1.82 (AB, *J* = 13.9, 7.8, 6.3 Hz, 1H, H2'), 1.48 (d, *J* = 1.2 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, MeOD) δ 164.02 (C4), 149.98 (C2), 142.21, 142.19, 142.16, 140.74, 140.69, 140.66 (Cq. arom.), 134.89 (C6), 126.90, 126.88, 126.87, 126.85, 126.10, 126.09, 123.81, 123.79, 123.74, 123.69, 119.00, 118.94, 118.91 (arom.), 109.59 (C5), 84.14 (C1'), 84.02, 83.96 (C4'), 69.78 (C3'), 68.22, 68.18, 68.14, 68.09 (CH_2 Fm), 66.26, 66.21 (C5'), 46.99 (CH Fm), 38.74 (C2'), 10.37 (CH_3). ^{31}P NMR (202 MHz, MeOD) δ -2.05. IR (film): 3065, 1686, 1450, 1274, 1104, 1017, 758, 741, 554, 515 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{38}H_{36}N_2O_8P$ (M+H) 679.2204. Found 679.2201. $[\alpha]_D^{20}$ +2.6 (c = 1, in DCM)



3'-*O*-(*N*,*N*-diisopropylamino-2-cyanoethylphosphinyl)-5'-O-(di-O-flourenylmethyl)-phosphoryl-2'-deoxylthymidine (2b)

Compound **6** (900 mg, 1.33 mmol), DMF (13 mL), DIPEA (0.69 mL, 3.98 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite **7** (0.33 mL, 1.46 mmol) were added into the flask under N_2. The reaction was stirred at room temperature for 10 minutes after which was quenched by 0.15 mL EtOH. Excessive amount of EtOAc was added and the mixture was washed with aq. $NaHCO_3$ (sat.) (2 x), H_2O (1 x) and brine (1 x). The organic layer

was dried (Na_2SO_4) and filtered. The filtration was co-evaporated with toluene (1 x) then purified by automatic column (pentane/EA, 100/0 – 50/50 – 45/65) to furnish **2b** as a white foam (760 mg, 0.87 mmol, 65%). **Note**: Careful wash was needed for the work-up because the DIPEA in the reaction could cleave the Fm group. Automatic column was performed on Biotage Isolera Specktra Four machine using High-quality IRR silica gel column (40-63 µm). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.76 – 7.63 (m, 4H, arom.), 7.55 – 7.13 (m, 13H, arom.), 6.26 (ddd, *J* = 8.1, 5.8, 4.8 Hz, 1H, H1'), 4.40 (ddq, *J* = 9.3, 6.0, 2.8 Hz, 1H, H3'), 4.35 – 3.43 (m, 11H, H4', H5', CH_2 Fm, CH Fm, CH *i*Pr), 2.78 – 2.70 (m, 2H, CH_2 OCE), 2.57 (dt, *J* = 19.8, 6.2 Hz, 2H, CH_2 OCE), 2.33 (AB, *J* = 42.7, 13.7, 5.9, 2.6 Hz, 1H, H2'), 1.90 (AB, *J* = 19.9, 14.0, 8.2, 6.4 Hz, 1H, H2'), 1.64 (s, 3H, CH_3), 1.18 – 1.06 (m, 12H, *i*Pr). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.80, 163.78 (C4), 150.43, 150.37 (C2), 142.84, 142.82, 142.77, 141.45, 141.42, 141.38, 141.35 (Cq. arom.), 135.00 (C6), 127.99, 127.96, 127.14, 127.12, 124.95, 124.93, 124.86, 124.81, 120.12, 120.08, 120.04 (arom.), 117.67, 117.63, 116.98 (CN), 111.34, 111.29 (C5), 84.73 (C1'), 84.15, 84.12, 84.09, 84.06, 83.82, 83.77, 83.76, 83.71 (C4'), 73.28, 73.15, 73.01 (C3'), 69.34, 69.33, 69.30, 69.21, 69.17 (CH_2 Fm), 66.75, 66.71, 66.58, 66.54 (C5'), 58.20, 58.16, 58.13, 58.07, 57.98 (CH_2 OCE), 47.90, 47.84 (CH Fm), 43.34, 43.24 (CH *i*Pr), 39.38, 39.34, 39.32 (C2'), 24.61, 24.55, 24.46 (CH3 *i*Pr), 20.39, 20.34, 20.32, 20.26, 20.11, 20.05 (CH_2 OCE), 12.30 (CH_3). ^{31}P NMR (202 MHz, $CDCl_3$) δ 149.17, 148.84, 14.17 (H-phosphonate), -1.30, -1.34. IR (film): 2967, 1688, 1450, 1365, 1275, 1183, 1021, 914, 515 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{41}H_{39}N_3O_{10}P_2Na$ ([H-phosphonate]+Na) 818.2003. Found 818.1996. $[\alpha]_D^{20}$ +6.6 (c = 1, in DCM)



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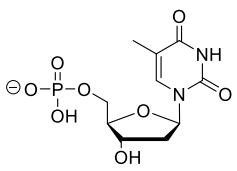
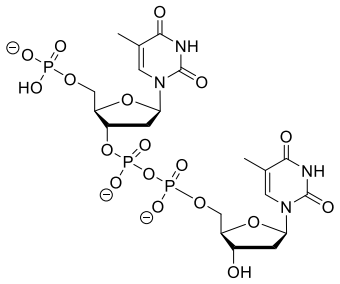


9

5'-O-Phosphoryl-dT-CPG (1) and 5'-O-phosphoryl-dT (9)

To a 20 mL reaction syringe with filter frit was added **8** (2.0 g, 60 µmol, 30 µmol/g). Dichloroacetic acid (5 %, v/v, in DCM) was added repeatedly until no yellow color was observed. The resin was extensively washed with DCM (3 x), ACN (5 x) under N_2. DCI (0.96 mL, 0.24 mmol, 0.25M in ACN) and $(FmO)_2PN(iPr)_2$ **5** (0.48 mL, 0.24 mmol, 0.5 M in ACN) were added into the resin and the mixture was shaken under N_2 for 10 minutes and drained. Repeat this coupling 1 more time and the resin was drained and washed with ACN (5 x). 3.6 mL (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) (0.5 M in ACN) was added and the mixture was shaken for 30 minutes under N_2. The resin was drained, washed with ACN (3 x), DCM (3 x) and drained under N_2. Piperidine (8.3 mL, 4.2 mmol, 5% in DMF) was added and the mixture was shaken for 10 minutes. The resin was drained, washed with ACN (3 x) and dried under reduced pressure to obtain resin **1**. A test sample (20 mg) was added into a 2 mL reaction syringe and 1mL NH_4OH (35%) was added to cleave the molecule from resin. 1 hour later the filtration was concentrated and analyzed by ^{31}P-NMR in D_2O. ^{31}P NMR (162 MHz, D_2O): δ 4.45 (s)



pTppT (3a)

To a 5 mL reaction syringe with filter frit, **1** (200 mg, 10 µmol) was added, washed with ACN (3 x) under N_2. 1 mL DBU (5%, v/v, in ACN) was added and it was shaken for 10 minutes (2 x) to remove Fm groups on 5-phosphate after which was drained and washed with ACN (5 x). Then ETT (0.20 mL, 50 µmol, 0.25 M in ACN) and **2b** (0.1 mL, 20 µmol, 0.2M in ACN) were added into the resin and the mixture was shaken for 10 minutes (2 x). The mixture was drained and the resin was washed with ACN (5 x) under N_2. The intermediate phosphate-phosphite was oxidized with (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) solution (1 mL, 0.5 mmol, 0.5 M in ACN) for 5 minutes (2 x) and washed with ACN (5 x). DBU solution (1 mL, 5%, v/v, in ACN) was added into the syringe and was shaken for 20 minutes to remove Fm groups on 5'-phosphate and CE group after which was drained and washed with ACN (5 x) under N_2. The resin was treated with NH_4OH (35%) overnight to cleave the product from the resin. The mixture was filtered and the filtration was concentrated. The crude material was purified by anion exchange and desalting to obtain **3a** (3.02 mg, 4.3 µmol, 43%) and pT (0.73 mg, 2.3 µmol, 23%) as white solid.

Column: Resource 15Q (10 mm x 10 cm).

Gradient: 40% - 65%. (A: 10 mM NaOH, B: 10 mM NaOH and 1 M NaCl)

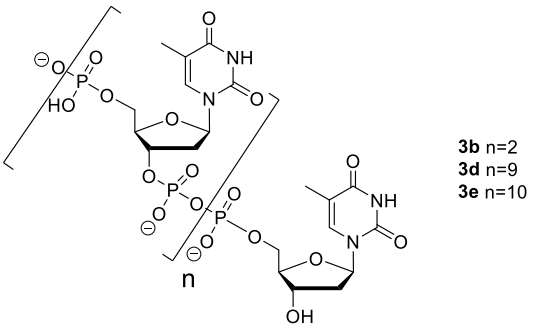
Desalting: 0.15M NH_4HCO_3

Compound 9

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.70 (q, *J* = 1.1 Hz, 1H, H6), 6.23 (dd, *J* = 7.8, 6.3 Hz, 1H, H1'), 4.46 (dt, *J* = 6.3, 3.2 Hz, 1H, H3'), 4.02 (qd, *J* = 3.8, 1.3 Hz, 1H, H4'), 3.85 (dd, *J* = 4.9, 3.8 Hz, 2H, H5'), 2.34 – 2.15 (m, 2H, H2'), 1.81 (d, *J* = 1.2 Hz, 3H, CH_3). ^{31}P NMR (162 MHz, D_2O): δ 4.45 (s)

Compound 3a

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.74 – 7.54 (m, 2H, H6), 6.30 – 6.12 (m, 2H, H1'), 4.52 (s, 1H, H3'), 4.49 (td, *J* = 4.7, 2.8 Hz, 1H, H3'), 4.35 – 4.22 (m, 1H, H4'), 4.14 – 4.00 (m, 3H, H4', H5'), 3.94 (dt, *J* = 5.0, 2.5 Hz, 2H, H5'), 2.45 (AB, *J* = 14.1, 5.8, 1.6 Hz, 1H, H2'), 2.35 – 2.19 (m, 3H, H2'), 1.79 (dd, *J* = 4.1, 1.2 Hz, 6H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ 1.38 (phosphate), -11.00, -11.11, -11.72, -11.83 (pyrophosphate).



3b n=2
3d n=9
3e n=10

pTppTppT (3b), pT(ppT)$_9$ (3d) and pt(ppT)$_{10}$ (3e)

200 mg **8** (10 µmol) was added into a reaction column of a Mermade 6 oligonucleotide synthesizer, TCA solution (2 mL, 3%, m/v, in DCM) was added into the resin and drained (5 x). The resin was washed with ACN (5 x) after which ETT (600 uL, 0.25 M in ACN) and **5** (300 uL, 0.1 M in ACN) were added into the resin and the mixture was left to stand for 5 minutes and drained (3 x). The resin was rinsed by with ACN (3 x). The intermediate phosphite was oxidized with CSO solution (2 mL, 0.5 M in ACN) for 5 minutes (2 x). The resin was drained and washed with ACN (3 x). DBU solution (2 mL,

10%, v/v, in ACN) was added into the resin and was left to stand for 5 minutes (4 x) to remove Fm groups on 5'-phosphate after which was drained and washed with ACN (5 x). Next, pyrophosphate coupling cycle was performed (n times):

ETT (600 uL, 0.25 M in ACN) and **2b** (300 uL, 0.1 M in ACN) were added into the resin and the mixture was left to stand for 5 minutes, drained (2 x). The resin was rinsed by ACN (3x). The intermediate phosphate-phosphite was oxidized with CSO solution (2 mL, 0.5 M in MeCN) for 5 minutes (2 x). The resin was drained and washed with ACN (3 x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the resin and was left to stand for 5 minutes (4 x) to remove Fm groups on 5'-phosphate and CE group after which was drained and washed with ACN (3 x).

After the completion of the cycle above (n times), the resin was transferred to a tube and treated with 10 mL NH_4OH (35%). The mixture was stirred overnight in a sealed condition, filtered and concentrated. The crude was purified by the same anion exchange chromatography and desalting mentioned above to obtain **3a-e** as white solid. For HRMS measurement, **3e** and **3d** were dissolved in MiliQ H**2**O (1 mg/mL) and added into a reaction syringe containing Dowex resin (NH_4^+ form). The mixture was shaken for 1 hour and filtered. The fieltration was used for HRMS mesurment.

n = 1, **3a** (5.69 mg, 7.52 µmol, 75%)

n = 2, **3b** (2.63 mg, 2.24 µmol, 22%)

n = 10, **3e** (3.15 mg, 0.70 µmol, 7%)

n = 9, **3d** (2.54 mg, 0.62 µmol, 6%)

3b

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.80 – 7.53 (m, 3H, H6), 6.35 – 6.11 (m, 3H, H1'), 4.88 (t, *J* = 6.5 Hz, 2H, H3'), 4.51 (p, *J* = 5.0, 4.4 Hz, 1H, H3'), 4.29 (d, *J* = 8.0 Hz, 2H, H4'), 4.17 – 4.05 (m, 6H, H4', H5'), 3.96 (d, *J* = 3.6 Hz, 1H, H5'), 2.46 (ddd, *J* = 15.1, 10.1, 5.9 Hz, 1H, H2'), 2.32 – 2.20 (m, 5H, H2'), 1.85 – 1.75 (m, 9H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ 0.52 (phosphate), -11.63, -11.74, -11.78, -11.88, -12.36, -12.41, -12.46, -12.52 (pyrophosphates).

3d

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.76 – 7.47 (m, 10H, H6), 6.21 (dtd, *J* = 11.5, 7.9, 5.6 Hz, 10H, H1'), 4.94 – 4.77 (m, 9H, H3'), 4.50 (td, *J* = 4.9, 3.1 Hz, 1H, H3'), 4.29 (dt, *J* = 10.2, 2.9 Hz, 9H, H4'), 4.20 – 4.04 (m, 19H, H4', H5'), 4.00 (q, *J* = 3.6 Hz, 2H, H5'), 2.59 – 2.34 (m, 9H, H2'), 2.34 – 2.17 (m, 11H, H2'), 1.92 – 1.70 (m, 30H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ -0.11 (phosphate), -11.65, -11.75, -11.79, -11.89, -12.39, -12.43, -12.48, -12.54 (pyrophosphates). HRMS (ESI^+) calcd for $C_{100}H_{147}N_{21}O_{98}P_{19}$ ($M+3NH_4^+$)/3 1266.0721. Found 1266.0717.

3e

^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.64 (d, *J* = 27.5 Hz, 11H, H6), 6.27 – 6.19 (m, 11H, H1'), 4.88 (s, 10H, H3'), 4.58 – 4.46 (m, 1H, H3'), 4.30 (s, 10H, H4'), 4.12 (s, 21H, H4', H5'), 4.01 (s, 2H, H5'), 2.50 – 2.46 (m,10H, H2'), 2.29 - 2.25 (m, 12H, H2'), 1.83 – 1.80 (m, 33H, CH_3). ^{31}P NMR (202 MHz, D_2O) δ -0.06 (phosphate), -11.62, -11.73, -11.76, -11.84, -11.88, -12.37, -12.40, -12.47, -12.51 (pyrophosphates). HRMS (ESI^+) calcd for $C_{110}H_{167}N_{25}O_{108}P_{21}$ ($M+3NH_4^+$)/3 1394.0762. Found 1394.0764.

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4 Synthesis of well-defined linear ADPr oligomers and biotinylated derivatives thereof

Introduction

ADP-ribosylation is a post-translational modification that plays a crucial role in various important cellular events.[1] ADP-ribosylation is catalyzed by a family of PARP enzymes, mediating the transfer of a single ADPr moiety from NAD^+ to the nucleophilic side chains of specific amino acids in target proteins.[2] Some of these enzymes can also add additional ADPr units, generating poly ADP ribose (PAR) chains up to a length of hundreds of ADP ribose units.[3] Poly ADP-ribosylation is involved in several processes including, regulation of chromatin structure, apoptosis-inducing factor (AIF)-dependent cell death and DNA repair. Several types of proteins participate in PARP dependent pathways, such as "readers" (e.g. macrodomain proteins, WWE domain and PBZ domains), that recognize and bind with PAR and "erasers" (e.g. PARG and ARH1/3), that hydrolyze PAR.[2-4] It can be assumed that most of these readers and erasers only interact with relatively small parts of the PAR chain. Since the interaction at a molecular level between PAR and the associated proteins or enzymes is still elusive, the availability of ADPr oligomers is of prime importance. Although enzymatically prepared PAR fragments have been widely applied for biological experiments, the enzymatic synthesis suffers from low yields, inhomogeneous samples and insufficient quantities, in particular concerning short oligomers (2-10 units).[5-7]

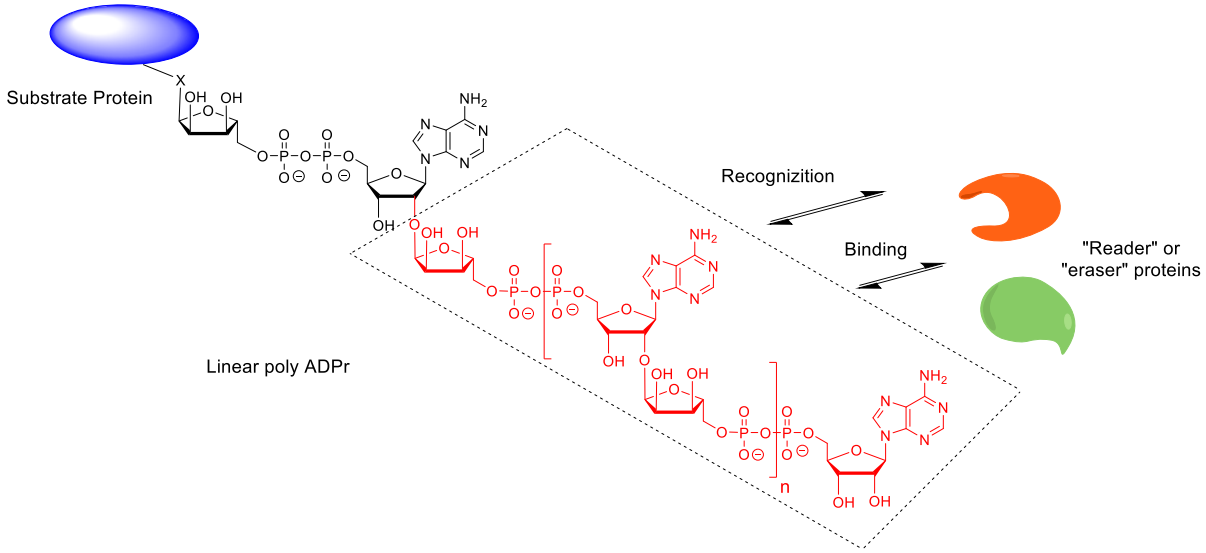


Figure 1. The structure poly-ADPr and “reader”/"eraser” proteins.

Chemical synthesis of well-defined ADPr oligomers is, therefore, a relevant alternative to tackle the problems of insufficient purity and limited supply, mentioned above.[8-10] The group of Hergenrother presented a solution phase synthesis of ADPr dimers by which a PAR–protein binding assay was performed and the first human PARG substrate-enzyme co-crystal structure was elucidated. Biotinylated or fluorescently labeled derivatives of di-ADPr have been prepared as well.[10] In Leiden, Kistemaker *et al.*[8] developed a solid phase synthesis approach, culminating in the isolation of an ADPr trimer. Ensuing experiments showed that the ADPr trimer, in contrast to the dimer and monomer, binds and activates a chromatin remodeler protein ALC1 which is involved in oncogenesis.[9] These encouraging breakthroughs demonstrate the potential of synthetic oligo-ADPr for the elucidation of the interaction of relevant proteins with PAR in a length-dependent manner.[11] It is to be expected that the availability of such well-defined ADPr fragments will contribute to future insights in oncology,[9, 12] neurodegeneration[13] and inflammation.[14] The development of methods for the synthesis of ADP-ribose oligomers and derivatives thereof (such as biotinylated and fluorescently labelled) is a valuable approach to obtain molecular tools for both protein binding assays[10, 15] and proteomics studies.[16-19]

This Chapter describes a solid phase synthesis of a series of linear ADPr oligomers (varying in length from dimer to pentamer), using a new protecting group strategy. This procedure builds upon the method described in Chapter 3 and involves the application of base-labile fluorenylmethyl (Fm) group as the temporary protection to avoid repetitive exposure of the growing ADPr-chain to acidic conditions that were used in an original method for the solid phase synthesis of ADPr-chains developed by Kistemaker *et al.*[8] In addition, ADPr mono-, di-, and trimer, provided with a propargyl handle, were prepared via the Fm-based chemistry and subsequently functionalized via a click reaction to furnish the corresponding biotinylated ADPr oligomers.

Results and discussion

The synthesis of ADPr oligomers is highly demanding due to the presence of both a 1,2-cis-α-ribosylated adenosine and an anionic pyrophosphate in the repeating unit. Both moieties are not only difficult to construct but also possess a certain degree of acid lability (Figure 2). Kistemaker *et al.* has reported a solid phase synthesis of an ADPr dimer and trimer by a combination of P(III) and P(V) chemistry, where in the key step a phosphomonoester at the ribose reacted with a phosphoramidite at the adenosine residue.[8, 20] Prior to the formation of this phosphite-phosphate intermediate the *tBu* protecting groups of the ribose phosphotriester should be released to give the phosphomonoester. We reasoned that the repeated acidic deprotection of the *tBu* groups exerts an adverse effect on the

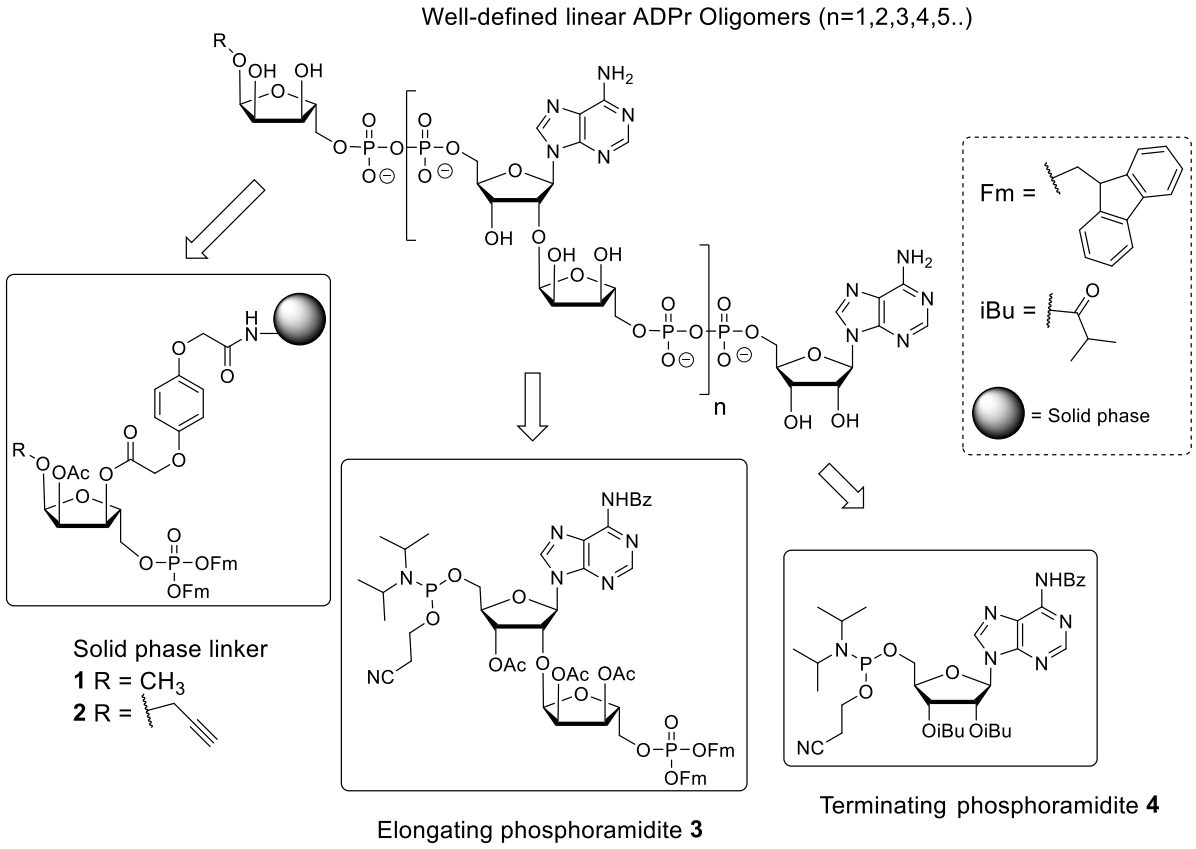
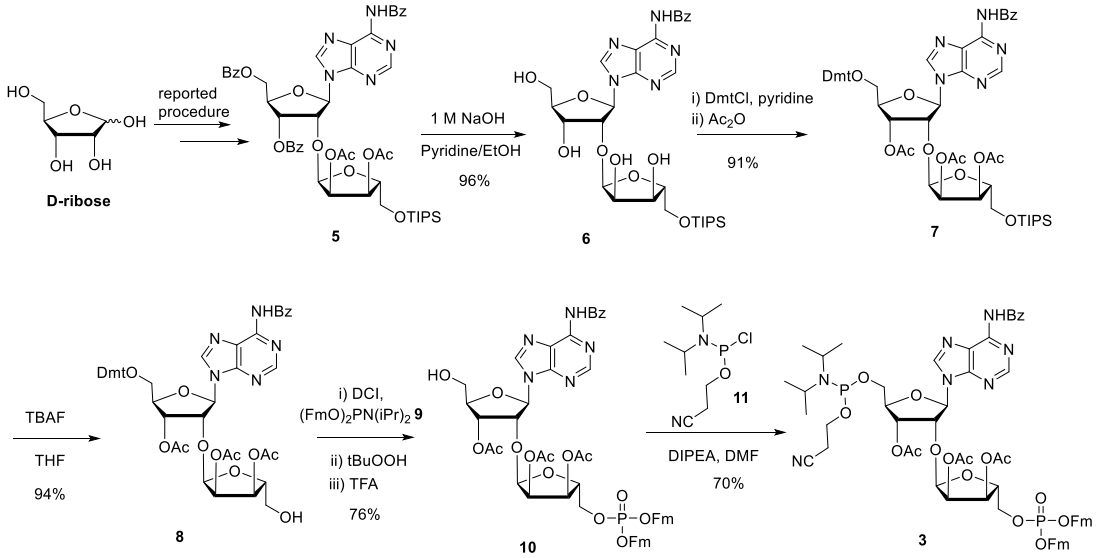


Figure 2. Retrosynthetic analysis of linear ADPr oligomers.

maximal length of oligomer, that can be reached. On the basis of these considerations a synthetic route with only base labile protecting groups was designed. The *tBu* groups in the phosphotriester precursor were replaced by the base labile fluorenylmethyl (Fm) protecting groups, leading to key building block **3** for repeated introduction of the pyrophosphate function.[21, 22] Furthermore, the first α-configured 1-*O*-methyl or 1-*O*-propargyl phosphorylated ribose residue was immobilized via the alkali labile Q linker on either LCAA-CPG or on Tenta Gel N (TG) resin (**1** or **2**).[8, 10, 23, 24] The hydroxyl groups of **1** and **2** and

both the hydroxyl groups and exocyclic amine in terminal phosphoramidite **4** are all protected with alkali sensitive protective groups.

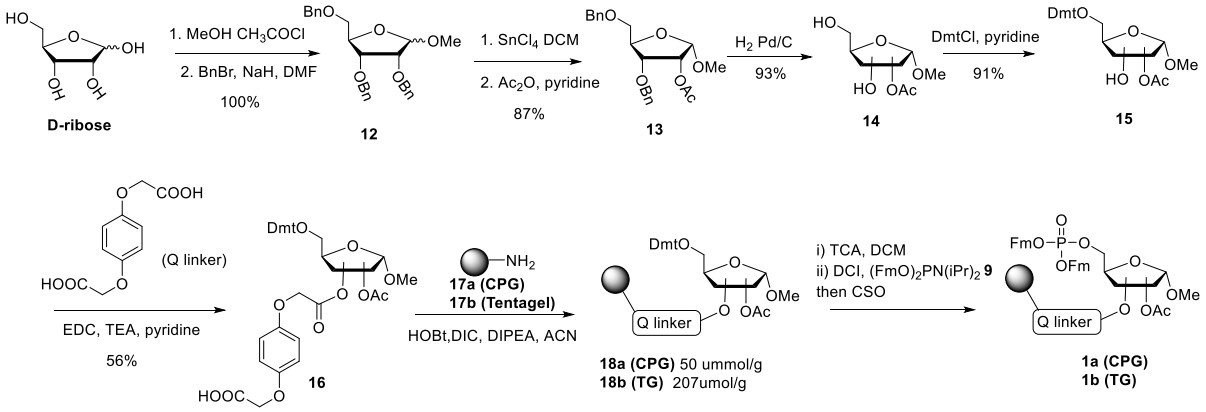
The route of synthesis to key building block **3** is outlined in Scheme 1 and commences with the construction of 1,2-cis-α-ribosylated nucleoside **5** by adoption of the method reported by Kistemaker *et al.*[8] Of note, this method is characterized by the post-glycosylation introduction of the adenine base via a Vorbrüggen coupling and can be performed on a scale that gives access to substantial amount of **5**.



Scheme 1. Synthesis of key repeating phosphoramidite **3**.

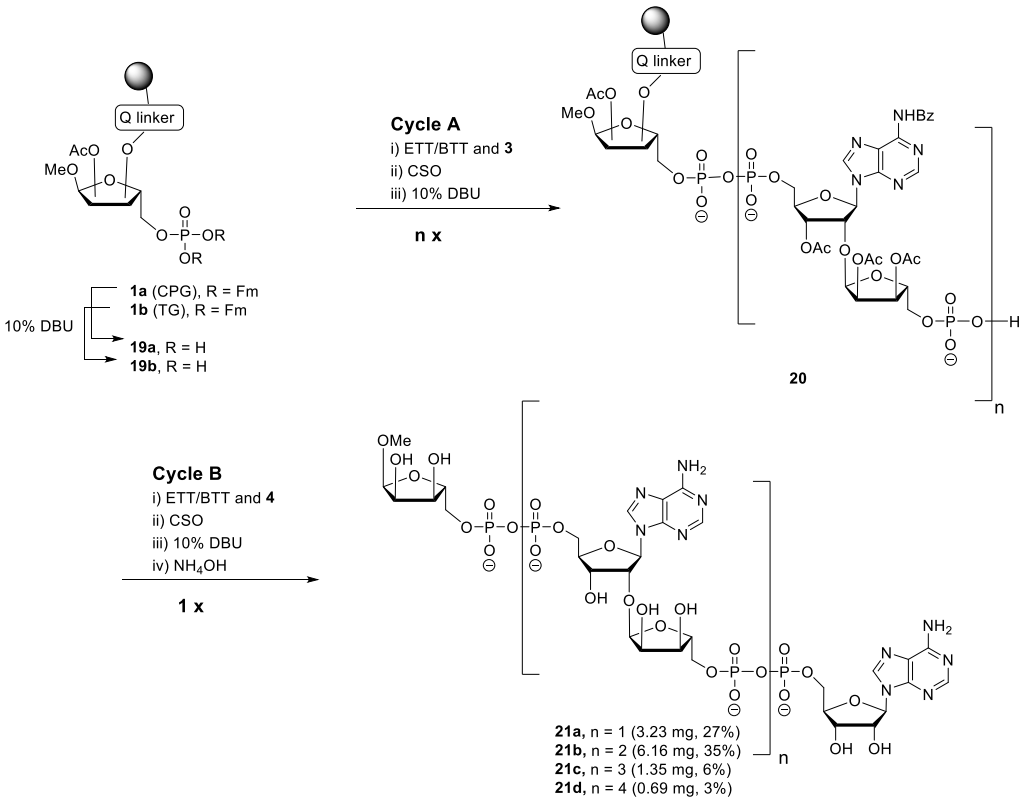
The protecting group manipulations *en route* to orthogonally protected intermediate **7** entail a saponification of the benzoyl and acetyl esters in **5** with aqueous NaOH (1 M) to give after column chromatography tetraol **6** in an excellent yield (96%). Subsequent selective dimethoxytritylation of the primary hydroxyl followed by acetylation of the remaining secondary hydroxyls gave **7** in high yield and in sufficient amount (2.3 mmol). This adaptation of the earlier described one pot 3-step procedure (saponification, DMT introduction and acetylation) turned out to be more favorable.[8] The bis-Fm triester is introduced by the removal of 5''-TIPS group of **7** with TBAF in THF, followed by the treatment of the liberated primary OH in **8** with bis-(*9H*-fluoren-9-ylmethyl)-diisopropylamidophosphite **9**[25, 26] in the presence of 4,5-dicyanoimidazole (DCI), *in situ* oxidation using *t*BuOOH and, finally, careful work-up to remove the excessive DCI. The obtained crude phosphotriester was treated with TFA in DCM to selectively remove DMT, furnishing **10** in 76% yield (3 steps). The phosphoramidite functionality was introduced using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite **11** in the presence of DIPEA in DMF to provide key phosphoramidite **3** in a good yield. This reaction also requires a careful work-up procedure for the removal of DIPEA, which is capable to cleave one of the base labile Fm groups. In

addition, the simultaneous occurrence of an acid labile (phosphoramidite) and a base labile (Fm) group in compound **3** requires column chromatography with high-quality IRR silica gel (see experimental section) instead of the traditional TEA-neutralized one.



Scheme 2. Synthesis of the solid supports **1a** and **1b** with a ribose phosphotriester immobilized via Q linker.

The projected solid phase synthesis of ADPr oligomers requires a solid support to which the first protected ribose phosphotriester is connected via the base labile Q-linker (Scheme 2). To this end, D-ribose was converted into 1-*O*-methyl-2,3,5-tris-O-benzyl-ribofuranoside (**12**) in high yield, which was isolated as a mixture of anomers. The 2-*O*-benzyl in **12** was selectively removed with $SnCl_4$/DCM[27] and the released hydroxyl was acetylated, after which the pure α-anomer **13** could be isolated. The isomerization of β- to the desired α-anomer can be explained by the opening and closing of the furanose ring under Lewis acidic ($SnCl_4$) conditions. Pd/C catalyzed hydrogenolysis of the benzyl groups in **13** was accompanied by acetyl migration to give **14** as a mixture of regio-isomers. After protection of the primary OH with the DMT group to give **15**, the Q-linker was introduced[8] with EDC/TEA in pyridine. The obtained acid **16** was used to functionalize both LCAA-CPG **17a** and TentaGel N (TG) resin **17b** to yield **18a** and **18b** respectively with desired loadings (50 µmol/g for **18a** and 207 µmol/g for **18b**). Apart from the usual CPG support, the TG resin was selected on the basis of its polystyrene matrix grafted with a polyethylene glycol (PEG) linker. Finally, the Fm-protected phosphotriester was installed by the following reaction sequence. At first, the DMT group was removed with TCA in DCM, then phosphitylation of the primary OH with phosphoramidite **9** and oxidation by CSO were performed to give either CPG **1a** or TG **1b**. The successful introduction of phosphate function was confirmed by ^{31}P-NMR analysis of the mixture obtained by NH_4OH treatment of the supports **1a** and **1b**.



Scheme 3. Synthesis of linear ADPr oligomers

At this stage, the functionalized solid supports (**1a**, **1b**) and elongating phosphoramidite (**3**) were available, and the terminating phosphoramidite (**4**) was prepared as well according to a known procedure.[8]

To test the feasibility and efficiency of the pyrophosphate formation via the proposed method, the synthesis of an ADPr dimer was undertaken in a fritted syringe via manual couplings (Scheme 3). The influence of the solid support was determined by the application of both CPG **1a** and TG **1b** supports. Removal of the Fm groups of **1a**/**b** by DBU treatment (10% in ACN, 15 min x 2) gave phosphomonoester (**19a**/**b**) suitable for pyrophosphate coupling. Guided by the protocol of Kistemaker *et al*,[8] the following revised 3-step elongation cycle A was executed: a) treatment of immobilized phosphate monoester **19a**/**b** with phosphoramidite **3** using 5-(ethylthio)-1*H*-tetrazole (ETT) as activator (10 min x 2); b) oxidation of the phosphate-phosphite (P^V-P^{III}) to a phosphate-phosphate (P^V-P^V) bridge with CSO (5 min x 2); c) treatment the immobilized and partly protected pyrophosphate with DBU (10% in ACN, 15 min x 2) to simultaneously remove both the cyanoethyl (CE) group on the pyrophosphate and the Fm groups on the terminal phosphate. Completion of the first elongation cycle gave the immobilized phosphomonoester **20** (n = 1), ready for the introduction of the second pyrophosphate. To obtain the ADPr dimer **21a** the synthesis was continued with similar cycle B in which phosphoramidite **4** was used in "step a" to form the phosphate-phosphite (P^V-P^{III}) intermediate. After completion of cycle B, the

resin was treated with NH_4OH to remove all the protecting groups and to release the target ADPr dimer from the resin. Subsequent anion exchange chromatography purification yielded 0.32 mg (3%) and 3.23 mg (27%) of dimer **21a** from CPG and TG resin respectively. This result suggests that for this manual oligo-ADPr synthesis TG resin is more preferable than traditional CPG resin.[8, 28] In both syntheses, we found 1-*O*-methyl mono-ADPr side product which may be attributed to an inefficient pyrophosphate coupling of the cycle A.

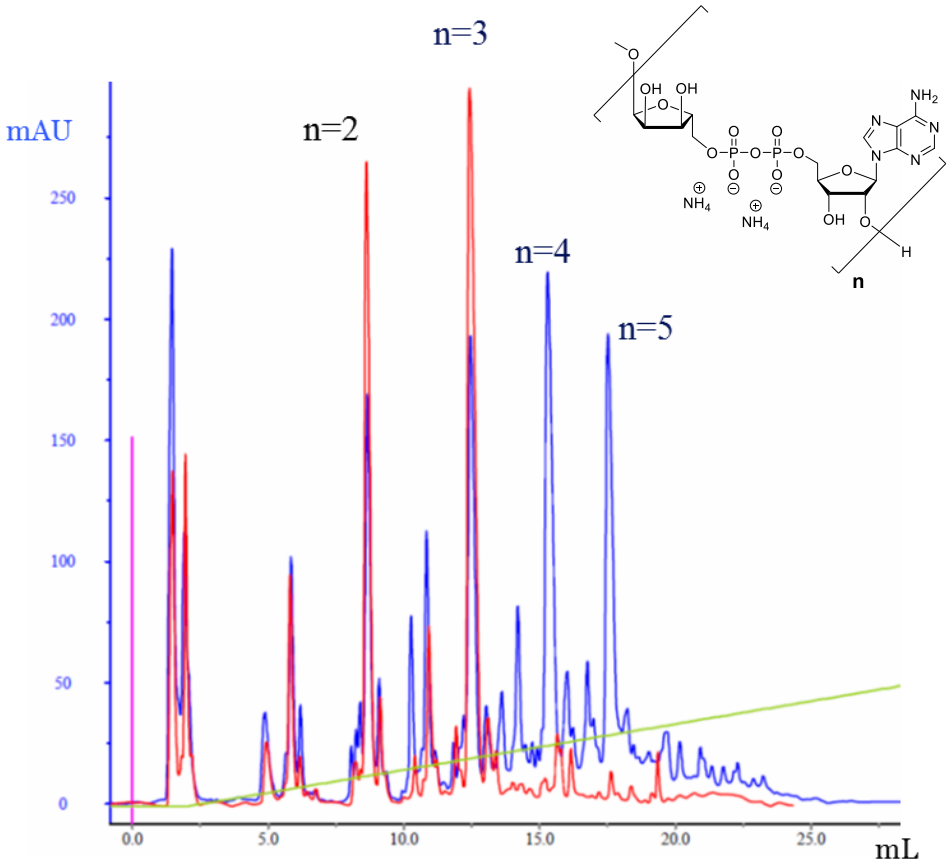
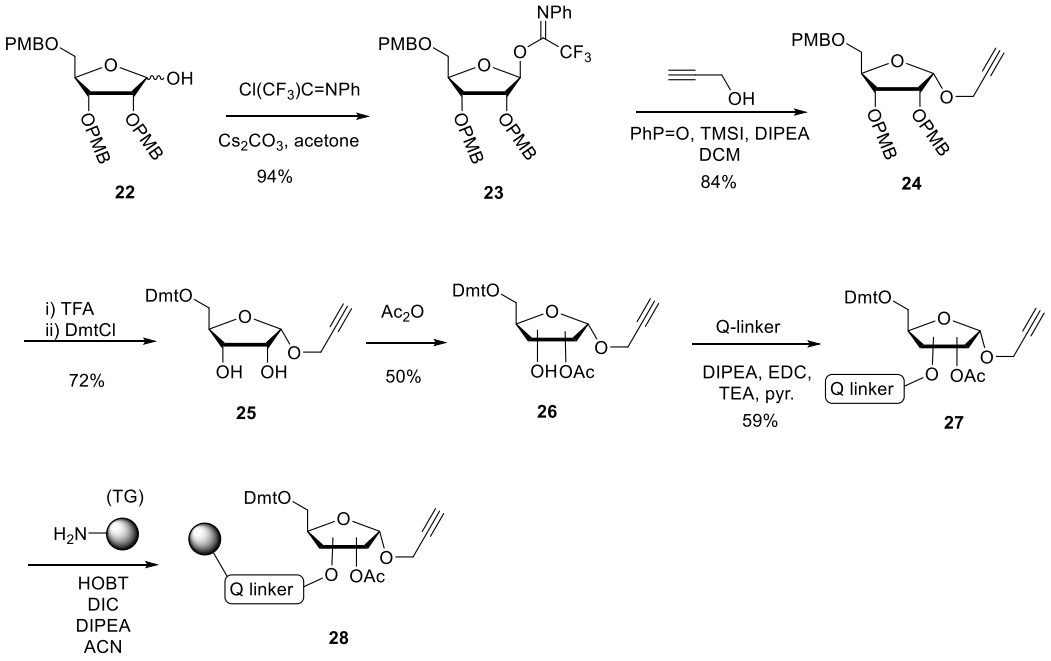


Figure 3. Analytical anion exchange chromatography[a] UV spectra for the crude sample of the synthesis of ADPr trimer (red) and pentamer (blue)

a Column: DNAPac PA-100, 4*250 mm. Gradient: NH_4OAc, 0%-20%. In 10 column volume.

The successful manual synthesis of dimer **21a** was an incentive to explore the synthesis of ADPr oligomers using an automated DNA synthesizer. On the basis of the above experiment, TG resin was chosen for synthesis while other pilot studies showed that 5-(benzylthio)-1*H*-tetrazole (BTT)[29, 30] is a better activator than ETT and an elongated DBU treatment time (10 min x 4) is necessary to completely cleave the Fm groups (Scheme 3). With these adaptations, an ADPr trimer was successfully synthesized on a DNA synthesizer (Figure 3, red line) with 35% yield, which is higher than the method in which *tBu*

groups were used for phosphate monoester protection.[8] Next, a more ambitious pentamer synthesis was performed using same method. After cleavage from resin, crude sample was analyzed by anion exchange chromatography showing the target pentamer peak together with tetramer and trimer peaks (Figure 3, blue line). Purification by anion exchange chromatography and gel filtration furnished the individual ADPr trimer **21b** (1.18 mg), tetramer **21c** (1.35 mg) and pentamer **21d** (0.69 mg), respectively. The unequalled synthesis of the tetra- and pentamer was followed by an attempt to assemble a decamer. However, the crude reaction mixture showed substantial side-products which made isolation of pure oligomers impossible. The formation of side-products and the shorter oligomers could be attributed to the incomplete coupling of cycle A and possibly the absence of a capping procedure.



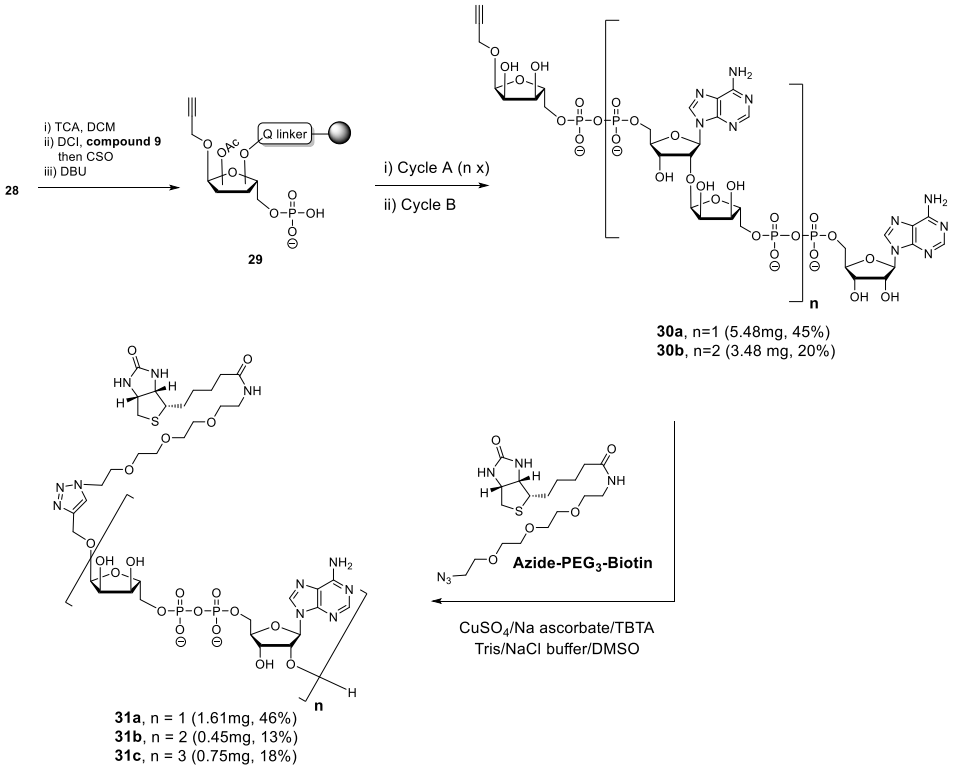
Scheme 4. Synthesis of resin **28** with an immobilized propargyl functionalized ribose triester

Next, the attention was directed to the synthesis of biotinylated ADPr oligomers by the synthesis of TG resin **28** on which a ribose with a propargyl group at the anomeric position is immobilized (Scheme 4). In this way, ADPr oligomers with a terminal alkyne can be prepared, which after solid phase synthesis can be functionalized with labels and handles (such as biotin) via copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. The synthesis of resin **28** started with the conversion of known 2,3,5-tri-*O*-*p*-methoxylbenzyl-ribofuranose **22**[31] into imidate donor **23**, using $Cl(CF_3)C=NPh$, Cs_2CO_3 in acetone (Scheme 4). Coupling of donor **23** with propargyl alcohol under the influence of TMSOTf/DCM gave **24** as an anomeric mixture ($\alpha : \beta = 1.35 : 1$). Fortunately, activation of **23** with a combination of TMSI and $Ph_3P=O$ (6 equivalents), as reported for the introduction of α-glucosidic

linkages, yielded solely α product **24** in high yield.[32, 33] In this ribosylation reaction DIPEA was added to prevent PMB cleavage.[31,34, 35] Subsequent protective group manipulation comprised the removal of the PMB groups in **24** by TFA assisted acidolysis, the regioselective tritylation of the primary hydroxyl function and, finally, acetylation of either the 2- or 3-OH in **25** with Ac_2O (0.9 equivalent) to give ribofuranoside **26**. Resin **28** was prepared by the installation of the Q-linker on either the 2- or 3-OH of **26** to give **27** that was subsequently coupled to TG resin (loading: 165 µmol/g) using the same procedures as described for the conversion of **15** into supports **1a**/**1b** (Scheme 2).

The assembly of biotinylated ADPr oligomers is depicted in scheme 5 and starts with the solid phase synthesis of propargylated ADPr dimer **30a** and trimer **30b**, using the same procedures and elongation cycles as used for the above described ADPr oligomers **21a**-**d** (Scheme 3). Fully protected resin **28** was detritylated, phosphitylated with reagent **9**, oxidized to the corresponding phosphotriester and finally treated with DBU to yield 1-*O*-propargyl-α-phosphoriboside resin **29**, which is suitable for oligomer synthesis. The solid phase procedures toward propargylated ADPr dimer **30a** and trimer **30b** used cycle A, one and two times respectively (n = 1 or 2) and ended each synthesis with cycle B. After purification by anion exchange chromatography, ADPr dimer **30a** and trimer **30b** were isolated in reasonable yield and quantity.

The thus prepared propargylated mono ADPr,[24] dimer **30a** and trimer **30b** were conjugated to commercially available azide-PEG_3-biotin, using a modified copper(I)-catalyzed alkyne-azide



Scheme 5. Assembly of biotinylated ADPr oligomers via copper(I)-catalyzed cycloaddition. The cycles A and B are the same as in scheme 4.

cycloaddition method (catalytic cocktail: $CuSO_4$/Na ascorbate/TBTA). The cycloaddition was monitored by LC-MS analysis, which showed of complete consumption of propargyl oligomers after 1 hour. HPLC purification yielded biotinylated mono-, di- and tri-ADPr (**31a-c**), structures and purity of which were confirmed by ^{1}H-NMR, ^{31}P-NMR, LC-MS and HRMS.

Conclusion

A new solid phase procedure towards well-defined linear ADPr oligomers is discussed. The novelty of the procedure entails the application of base sensitive temporary and permanent protective groups. In particular, protection of the intermediate phosphate monoester, that has to be liberated repeatedly, with fluorenylmethyl (Fm) groups facilitated the construction of multiple pyrophosphates linkages. To put the solid phase procedure into practice, a highly advanced Fm protected elongating phosphoramidite (**3)** was synthesized in good yield and the resin was provided with the alkali labile Q-linker. ADPr oligomers up to a pentamer were assembled in sufficient quantities. A similar procedure resulted in a propargylated ADPr dimer and trimer allowing the formation of the corresponding biotinylated-ADPr oligomers. The latter will be used as valuable tools for future protein binding and proteomics studies.

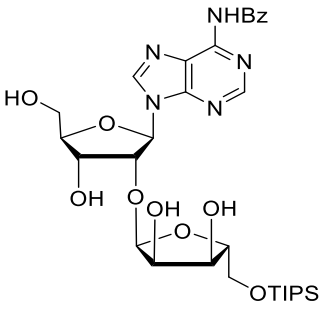
Acknowledgments

Nico Meeuwenoord is kindly acknowledged for his help in oligomer synthesis and final product purification. Stanley Tsui is kindly acknowledged for part of building block synthesis.

Experimental section

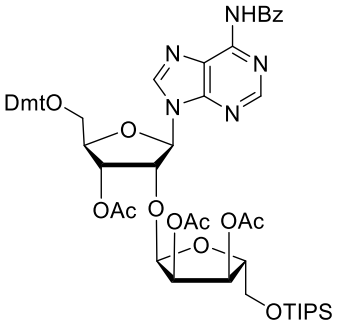
General procedure

All chemicals were used as received unless stated otherwise. All solvents used in reaction (including solid phase synthesis) were dried over 3Å molecular sieves. Solvents removal by rotary evaporation was under reduced pressure at 40 ℃. TLC, NMR, LCMS, anion exchange, HRMS, IR, optical rotation facilities were used as described in Chapter 2. Azide-PEG_3-biotin and LCAA-CPG resin were purchased from Sigma-Aldrich. Tenta Gel N resin was purchased from Rapp Polymere.



N^6-benzoyl-9-(5-O-triisopropylsilyl-β-parobiosyl) adenine (6)

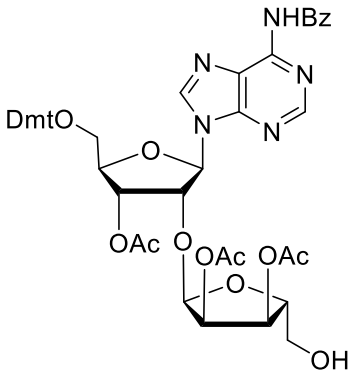
To a round-bottom flask, compound **5** (2.48 g, 2.63 mmol), pyridine (18 mL) and EtOH (9 mL) were added in sequence. The mixture was cooled to 0 °C after which aqueous NaOH (15.78 mL, 1 M solution) was slowly added. The reaction was stirred for 1 h at 0 °C after which Amberlite-H^+ was added until pH = 6. The mixture was filtered, concentrated and purified by silica gel column chromatography (DCM/MeOH, 100/0 – 100/3 – 100/5) to obtain **6** as a white foam (1.66 g, 2.52 mmol, 96%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.69 (s, 1H, NH), 8.69 (s, 1H, H2), 8.32 (s, 1H, H8), 8.00 (d, *J* = 7.1 Hz, 2H, arom), 7.57 (t, *J* = 7.4 Hz, 1H, arom.), 7.47 (t, *J* = 7.7 Hz, 2H, arom.), 6.11 (d, *J* = 7.0 Hz, 1H, H1'), 5.78 (brs, 1H, OH), 5.01 (d, *J* = 4.1 Hz, 1H, H1''), 4.95 (dd, *J* = 7.1, 4.6 Hz, 1H, H2'), 4.76-4.41 (m, 4H, H3', OH x 3), 4.29 (s, 1H, H4'), 4.23-4.19 (m, 3H, H2'', H3'', H4''), 3.93-3.67 (m, 4H, H5', H5''), 0.98-0.97 (m, 21H, TIPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.29 (CO, Bz), 150.84, 150.20, 133.44 (Cq. arom.), 133.04, 128.88, 128.23 (arom.), 124.07 (cq, arom.), 101.73 (C1''), 89.09 (C1'), 87.81 (C4'), 86.67 (C4''), 79.39 (C2'), 73.35 (C2''), 72.69 (C3'), 71.72 (C3''), 63.95 (C5''), 63.06 (C5'), 18.03, 18.01, 11.92 (TIPS). IR (film): 3337, 2942, 2866, 1704, 1614, 1584, 1459, 1252, 1093, 1042, 883, 709, 686 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{31}H_{46}N_5O_9Si$ (M+H) 660.3059. Found 660.3061. $[\alpha]_D^{20}$ +5.2 (c = 1, in DCM)



N^6-benzoyl-9-(3',2'',3''-tris-*O*-acetyl-5'-*O*-dimethoxyltrityl-5''-*O*-triisopropylsilyl-β-parobiosyl) adenine (7)

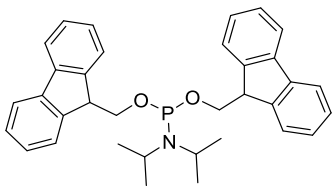
Compound **6** (1.66 g, 2.52 mmol) was co-evaporated with pyridine (1 x), then N_2 was applied. Dry pyridine (12 mL) and 4,4'-dimethoxyltrityl chloride (DMTCl, 1.36 g, 4.01 mmol) was added into the flask. 40 minutes later, TLC showed complete conversion and the mixture was cool to 0°C after which Ac_2O (1.42 mL, 15 mmol) was added. The mixture was stir at 0°C for 5 h and was quenched by aq. $NaHCO_3$ (sat.). DCM extracted (3 x) the mixture and the organic layers were combined and dried ($MgSO_4$). The mixture was filtered, concentrated and purified by silica gel column chromatography (pentane/acetone, 100/0 – 90/10 – 85/15 – 80/20 – 70/30) to obtain **7** as a white foam (2.50 g, 2.30 mmol, 91%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.37 (s, 1H, NH), 8.70 (s, 1H, H2), 8.17 (s, 1H, H8), 8.01(d, *J* = 7.3 Hz, 2H, arom.), 7.58 – 7.51 (m, 1H, arom. Bz), 7.47 – 7.43 (m, 4H, arom. Bz, DMT), 7.33 – 7.19 (m, 7H, DMT), 6.81 (d, *J* = 9 Hz, 4H, arom. DMT), 6.26 (d, *J* = 6.0 Hz, 1H, H1'), 5.62 (dd, *J* = 5.2, 3.6 Hz, 1H, H3'), 5.38 (dd, *J* = 7.0, 2.7 Hz, 1H, H3''), 5.35 (d, *J* = 4.7 Hz, 1H, H1''), 5.26 (t, *J* = 5.6 Hz, 1H, H2'), 4.86 (dd, *J* = 7.0, 4.6 Hz, 1H, H2''), 4.35 (q, *J* = 3.5 Hz, 1H, H4'), 4.15 (q, *J* = 2.8 Hz, 1H, H4''), 3.86 (AB, ddd, *J* = 11.0, 2.9 Hz, 2H, H5''), 3.76 – 3.75 (m, 6H, CH3, DMT), 3.52 (AB, *J* = 10.6, 3.5 Hz, 2H, H5'), 2.16 (s, 3H, Ac), 2.11 (s, 3H, Ac), 1.84 (s, 3H, Ac), 1.07 – 1.03 (m, 21H, TIPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.13, 169.49, 169.40 (CO, Ac), 164.65 (CO, Bz), 158.57 (Cq. arom.), 152.68 (CH, C2), 151.84 (C4), 149.63 (C6), 144.26 (Cq. arom.), 141.38 (CH, C8), 135.26 (Cq. arom.), 133.59, 132.61, 130.04, 128.68, 128.10, 127.84, 127.82, 126.98 (arom.), 123.41 (C5), 113.15 (Cq. arom.),

101.23 (C1''), 86.83 (Cq, DMT), 86.34 (C1'), 83.24 (C4''), 82.49 (C4'), 77.90 (C2'), 72.20 (C3'), 71.45 (C2''), 70.06 (C3''), 63.10 (C5''), 62.86 (C5'), 55.09 (CH_3, DMT), 20.77, 20.73, 20.10 (CH3, Ac), 17.79, 17.76, 11.76 (TIPS).



N^6-benzoyl-9-(3',2'',3''-tris-*O*-acetyl-5'-*O*-dimethoxyltrityl-β-parobiosyl) adenine (8)

Compound **7** (2.50 g, 2.30 mmol), dry THF (23 mL) and TBAF (tetrabutylammonium fluoride solution 1.0 M in THF, 4.60 mL, 4.60 mmol) was added into a flask and the mixture was stirred for 24 h. Excessive amount of EtOAc was added and the mixture was washed by H_2O (2 x) and brine (2 x). The organic layer was dried ($MgSO_4$), filtered, concentrated and purified by silica gel column chromatography (DCM/methanol, 100/0 – 99/1 –99/2) to obtain **8** as a white foam (2.01g, 2.16 mmol, 94%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.40 (s, 1H, NH), 8.72 (s, 1H, H2), 8.20 (s, 1H, H8), 8.04 (d, *J* = 7.1 Hz, 2H, arom. Bz), 7.63 – 7.54 (m, 1H, arom. Bz), 7.54 – 7.46 (m, 2H, arom. Bz), 7.45 – 7.36 (m, 2H, arom. DMT), 7.36 – 7.15 (m, 7H, arom. DMT), 6.81 (d, *J* = 9.0 Hz, 4H, arom. DMT), 6.24 (d, *J* = 5.9 Hz, 1H, H1'), 5.55 (dd, *J* = 5.2, 3.7 Hz, 1H, H3'), 5.33 (d, *J* = 4.6 Hz, 1H, H1''), 5.25 – 5.16 (m, 2H, H2'), 4.78 (dd, *J* = 7.3, 4.5 Hz, 1H, H3''), 4.33 (q, *J* = 3.6 Hz, 1H, H4'), 4.12 (q, *J* = 3.4 Hz, 1H, H4''), 3.82 – 3.66 (m, 8H, CH_3 DMT, H5''), 3.50 (AB, *J* = 10.7, 3.7 Hz, 2H, H5'), 2.86 (s, 1H, OH), 2.13 (s, 3H, CH_3 Ac), 2.10 (s, 3H, CH_3 Ac), 1.83 (s, 3H, CH_3 Ac). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.43, 169.86, 169.70(CO, Ac), 158.67 (Cq. arom.), 151.82 (C4), 149.78 (C6), 144.34 (Cq. arom.), 135.43, 133.68 (Cq. arom), 132.85, 130.17, 128.87, 128.21, 128.03, 127.15 (arom.), 123.24 (C5), 113.30 (arom.), 101.32 (C1''), 86.96 (Cq. DMT), 86.42 (C1'), 82.68 (C4''), 82.50 (C4'), 78.23 (C2'), 72.12 (C3'), 71.24 (C2''), 69.72 (C3''), 62.95 (C5'), 61.93 (C5''), 55.31 (CH_3, DMT), 20.95, 20.82, 20.23 (CH_3 Ac). IR (film): 2931, 1743, 1734, 1609, 1583, 1508, 1448, 1247, 1227, 1178, 1091, 1030 cm^{-1}. HRMS (ESI^+) calcd for $C_{49}H_{50}N_5O_{14}$ (M+H) 932.3349. Found 932.3369. $[\alpha]_D^{20}$ +28.6 (c = 1, in $CHCl_3$)

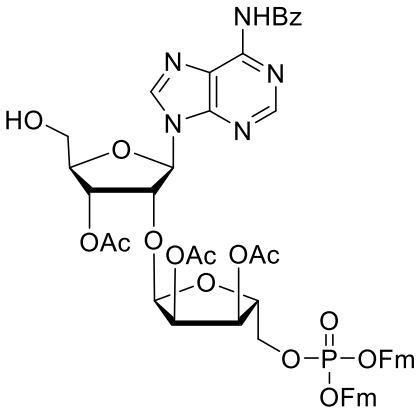


Bis(*9H*-fluoren-9-ylmethyl)-diisopropylamidophosphite (9)

Note: The title compound was made according to reported procedure with some modifications.[25] TLC used for this reaction was pre-run in 5% Et_3N in pentane. $CDCl_3$ used for NMR analysis was filtered through a layer of basic aluminum oxide before use.

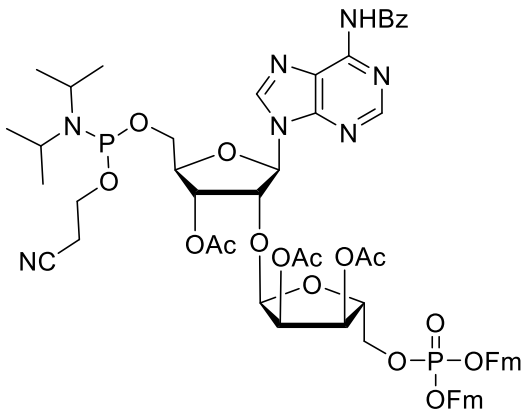
Diisopropylaminophosphodichloridite (2.28 g, 11.3 mmol) was added to a 0 °C solution of 9-fluorenylmethanol (4.44 g, 22.6 mmol) and EtN*iPr*$_2$ (5.88 mL, 41.6 mmol) in THF (25 mL). The mixture was stirred for 1 h at room temperature, then quenched by 1M pH 7 phosphate buffer (200 mL). The mixture was extracted with EtOAc (200 mL x 2), dried (Na_2SO_4), filtered and concentrated. The residue was further purified by flash silica gel column chromatography (prepared with 1% Et_3N in pentane and eluting with pentane/ethyl acetate/Et_3N 100:5:1) to give the **9** as a light yellow oil (4.10 g, 7.9 mmol, 70%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.77 (ddt, *J* = 8.3, 7.5, 1.0 Hz, 4H, arom.), 7.67 (ddq, *J* = 11.9, 7.5, 0.9 Hz, 4H, arom.), 7.47 – 7.36 (m, 4H, arom.), 7.36 – 7.24 (m, 4H, arom.), 4.21 (t, *J* = 7.1 Hz, 2H, CH Fm), 4.03 (dt, *J* = 9.9, 6.8 Hz, 2H, CH_2 Fm), 3.84 (dt, *J* = 9.9, 7.3 Hz, 2H, CH_2 Fm), 3.68 (dp, *J* = 10.1, 6.8 Hz, 2H, NC*H*(CH_3)$_2$), 1.19 (d, *J* = 6.8 Hz, 12H, NCH*(CH$_3$)$_2$*). ^{13}C

NMR (126 MHz, $CDCl_3$) δ 145.07, 144.79, 141.50, 141.39 (Cq. arom.), 127.53, 127.49, 126.96, 126.94, 125.58, 125.34, 119.96, 119.89 (arom.), 66.11, 65.97 (CH_2 Fm), 49.35, 49.29 (CH Fm), 43.22, 43.12 (N*CH*(CH_3)$_2$), 24.78, 24.73 (NCH*(CH$_3$)$_2$*). ^{31}P NMR (202 MHz, $CDCl_3$) δ 146.72.



N^6-benzoyl-9-(3',2'',3'''-tris-*O*-acetyl-5''-*O*-(di-flourenylphosphoryl)-β-parobiosyl)adenine (10)

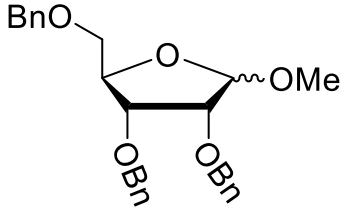
Compound **8** (1.99 g, 2.14 mmol), DCI activator (4,5-dicyanoimidazole solution 0.25 M in ACN, 17 mL, 4.28 mmol) and freshly activated 3Å molecular sieves were added in to flask. **9** (0.2 M in ACN, 16 mL, 3.21 mmol) were added into the mixture and the reaction was stirred for 10 minutes at room temperature after which *tBu*OOH (5.5 M in decane, 3.89 mL, 21.40 mmol) was added at 0°C. The reaction was stirred at same temperature for 30 minutes and quenched by aq. $NaHCO_3$ (sat.). The mixture was filtered and EtOAc was added to the filtration. The mixture was washed by H_2O (1 x) and brine (2 x) and the organic layer was dried (Na_2SO_4), filtered, concentrated and co-evaporated with toluene (3 x). To the residue, DCM (28 mL) and TFA (0.41 mL, 5.35 mmol) were added and the reaction was stirred for 10 minutes at room temperature after which was quenched by aq. $NaHCO_3$ (sat.). DCM extracted (2 x) the mixture and the organic layers are combined and washed by H_2O (1 x) and brine (1 x). The organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by silica gel column chromatography (DCM/methanol, 100/0 – 100/1 – 100/2 – 100/3) to obtain **10** as a white foam (1.72 g, 1.62 mmol, 76%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.08 (s, 1H, NH), 8.78 (s, 1H, H2), 8.05 (s, 1H, H8), 8.03 – 7.99 (m, 2H, arom. Bz), 7.68 (ddd, *J* = 15.0, 7.6, 3.5 Hz, 4H, arom.), 7.64 – 7.59 (m, 1H, arom.), 7.56 – 7.41 (m, 6H), 7.39 – 7.19 (m, 8H), 6.00 (d, *J* = 11.5 Hz, 1H, 5'-OH), 5.96 (d, *J* = 7.8 Hz, 1H, H1'), 5.64 (d, *J* = 5.2 Hz, 1H, H3'), 5.13 (dd, *J* = 7.8, 5.2 Hz, 1H, H2'), 5.05 (dd, *J* = 7.3, 4.1 Hz, 1H, H3''), 4.95 (d, *J* = 4.6 Hz, 1H, H1''), 4.62 (dd, *J* = 7.3, 4.6 Hz, 1H, H2''), 4.31 (d, *J* = 1.8 Hz, 1H, H4'), 4.28 – 4.14 (m, 4H, CH_2 Fm), 4.12 – 4.03 (m, 3H, H4'', CH Fm), 4.00 – 3.80 (m, 4H, H5', H5''), 2.15 (s, 3H, CH_3 Ac), 2.09 (s, 3H, CH_3 Ac), 1.96 (s, 3H, CH_3 Ac). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.02, 169.62, 169.35(CO, Ac), 164.48(CO, Bz), 152.47(CH, C2), 150.66, 150.59, 143.16, 143.12, 143.09, 143.00, 141.50, 141.46, 133.54(Cq. arom.), 133.11, 129.07, 128.05, 128.03, 127.99, 127.26, 125.25, 125.24, 125.21(arom.), 124.73 (Cq, arom.), 120.13, 120.12, 120.10, 120.09 (arom.), 101.12 (C1''), 89.59 (C1'), 86.74 (C4'), 80.40 (C4''), 77.77 (C2'), 73.75 (C3'), 70.99 (C2''), 69.51, 69.46 (CH_2 Fm), 69.30 (C3''), 66.26 (C5'), 62.90 (C5'), 47.99, 47.93 (CH Fm), 21.04, 20.78, 20.40 (CH_3 Ac). ^{31}P NMR (202 MHz, $CDCl_3$) δ -1.21. IR (film): 2924, 1743, 1609, 1507, 1452, 1448, 1229, 1219, 1078, 1030, 830, 740 cm^{-1}. HRMS (ESI^+) calcd for $C_{56}H_{53}N_5O_{15}P$ (M+H) 1066.3270. Found 1066.3298. $[\alpha]_D^{20}$ +5.8 (c = 1, in $CHCl_3$)



N^6-benzoyl-9-(3',2''3'''-tris-*O*-acetyl-5'-*O*-(*N*,*N*-diisopropylamino-*O*-cyanoethyl)phosphoramidite)-5''-*O*-(di-flourenylphosphoryl)-β-parobiosyl)adenine (3)

Compound **10** (1.38 g, 1.30 mmol), DMF (13 mL), DIPEA (0.56 mL, 3.24 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite **11** (0.32 mL, 1.43 mmol) were added into the flask and stirred at room temperature for 15 minutes. Methanol (0.2 mL) was added to quench the excessive phosphorimidite after which excessive amount EtOAc was added and the mixture was washed by aq. $NaHCO_3$ (sat. 1 x), H_2O (1 x) and brine (2 x). The organic layer was dried (Na_2SO_4) and filtered. The filtration was co-evaporated with toluene (1 x) then purified by automatic column (pentane/EtOAc, 20/80 – 0/100) to furnish **3** as a white foam (1.15 g, 0.91 mmol, 70%). **Note**: Careful wash was needed for the work-up because the DIPEA in the reaction could cleave the Fm group. Automatic column was performed on Biotage Isolera Specktra Four machine using High-quality IRR silica gel column (40-63 µm). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.16 (s, 1H, NH), 8.79 (d, *J* = 7.6 Hz, 1H, H2), 8.45 (d, *J* = 8.5 Hz, 1H, H8), 8.10 – 7.93 (m, 2H, arom.), 7.76 – 7.18 (m, 19H, arom.), 6.37 – 6.21 (m, 1H, H1'), 5.48 (ddd, *J* = 20.8, 5.1, 3.2 Hz, 1H, H3'), 5.24 (d, *J* = 4.5 Hz, 1H, H1''), 5.11 (ddd, *J* = 7.5, 6.0, 3.7 Hz, 1H, H3''), 4.94 (dt, *J* = 6.3, 4.6 Hz, 1H, H2'), 4.65 (ddd, *J* = 10.9, 7.3, 4.5 Hz, 1H, H2''), 4.35 (dd, *J* = 3.1, 1.7 Hz, 1H, H4'), 4.29 – 3.72 (m, 12H, Fm, H5', H5'', O*CH*$_2$CH$_2$CN), 3.67 – 3.44 (m, 2H, *CH*(CH$_3$)$_2$), 2.71 – 2.59 (m, 2H, *CH*$_2$CN), 2.15 (d, *J* = 8.8 Hz, 3H, CH$_3$ Ac), 2.09 (s, 3H, CH$_3$ Ac), 1.83 (d, *J* = 9.3 Hz, 2H, CH$_3$ Ac), 1.20-1.14 (m, 12H, CH*(CH$_3$)$_2$*). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.09, 170.08, 169.77, 169.73, 169.41, 169.38 (CO Ac), 164.67, 164.64 (CO Bz), 151.99, 151.89 (C4), 149.69 (C6), 143.05, 143.03, 142.96, 142.94, 141.42, 141.40, 133.79 (Cq. arom.), 132.84, 132.82, 128.94, 128.91, 127.94, 127.19, 125.13, 125.10 (arom.), 123.19, 123.07 (C5), 120.06, 120.03 (arom.), 117.98, 117.76 (CN), 101.59 (C1''), 86.19, 85.84 (C1'), 83.13, 83.06, 82.82, 82.75 (C4'), 80.50, 80.43 (C4''), 79.46, 79.43 (C2'), 72.39, 72.17(C3'), 70.80, 70.77 (C2''), 69.41, 69.37 (CH_2 Fm), 69.29, 69.26 (C3''), 66.41, 66.37, 66.32 (C5''), 62.76, 62.66, 62.62, 62.54 (C5'), 58.80, 58.66, 58.63, 58.50 (O*CH2*CH$_2$CN), 47.92, 47.90, 47.86, 47.83 (CH Fm), 43.29, 43.24, 43.19, 43.15 ((CH$_3$)$_2$*CH*N), 24.78, 24.72(*(CH$_3$)$_2$*CHN), 20.95, 20.88, 20.74 (CH$_3$ Ac), 20.47, 20.41 (OCH$_2$*CH$_2$*CN), 20.19, 20.16(CH$_3$ Ac). ^{31}P NMR (202 MHz, $CDCl_3$) δ 150.02, 149.62, 14.79 (H-phosphate), -1.05, -1.07. IR (film): 2968, 1744, 1609, 1451, 1236, 1074, 1025, 981, 741 cm^{-1}.

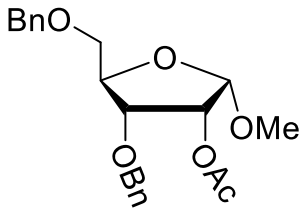
HRMS (ESI$^+$) calcd for $C_{59}H_{57}N_6O_{17}P_2$ ([H-phosphonate]+H) 1183.3250. Found 1183.3246. $[\alpha]_D^{20}$ +13.9 (c = 1, in DCM)



1-*O*-Methyl-2,3,5-tris-*O*-benzyl-αβ-D-ribofuranoside (12)

D-Ribose (5 g, 33.30 mmol), methanol (120 mL) and acyl chloride (0.62 mL, 10.99 mmol) were added into a flask and the reaction was stirred at room temperature for 5 hours after which was quenched by $NaHCO_3$ (6 g). The mixture was filtered and concentrated. The residue was then co-evaporated with toluene (3 x) and DMF (160 mL) was added into the flask. The mixture

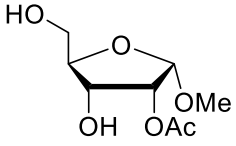
was cooled down to 0 °C then NaH (5.4 g, 166.5 mmol, 60% in mineral oil) was added. After gas generation was ceased, BnBr (15 mL, 166.5 mmol) was added in 3 portions over 10 min. The mixture was allowed to warm up to room temperature carefully and stirred for 16 h. MeOH (15 mL) was added to quench the reaction and H_2O and EtOAc were added. The water layer was washed with EtOAc then all the organic layers were combined and dried ($MgSO_4$). The mixture was filtered, concentrated and purified by silica gel column chromatography (pentane/EtOAc, 19/1 – 2/1) to obtain **12** as a colorless oil (14.5 g, 33.30 mmol, 100%). Spectroscopic data was identical with the reported same compound.[36] ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.44 – 7.12 (m, 20H, arom. αβ), 4.91 (d, *J* = 1.2 Hz, 1H, H1-β), 4.87 (d, *J* = 4.3 Hz, 0.3H, H1-α), 4.69 – 4.39 (m, 8H, CH_2-Bn-αβ), 4.35 (ddd, *J* = 7.1, 5.8, 3.7 Hz, 1H, H4-β), 4.27 – 4.21 (m, 0.3H, H4-α), 4.02 (dd, *J* = 7.1, 4.7 Hz, 1H, H3-β), 3.86 – 3.80 (m, 1.3H, H2-β, H3-α), 3.77 (dd, *J* = 6.8, 4.3 Hz, 0.3H, H2-α), 3.60 (AB, *J* = 10.6, 3.8 Hz, 1H, H5-β), 3.51 (AB, *J* = 10.6, 5.8 Hz, 1H, H5-β), 3.46 (s, 1H, OMe-α), 3.40 (AB, *J* = 10.4, 4.1 Hz, 0.3H, H5-α), 3.34 (AB, *J* = 10.4, 4.2 Hz, 0.3H, H5-α), 3.30 (s, 3H, OMe-β). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.37, 138.31, 137.98, 137.89, 137.87 (Cq. arom. αβ), 128.44, 128.41, 128.39, 128.35, 128.34, 128.31, 128.05, 127.99, 127.95, 127.91, 127.84, 127.80, 127.71, 127.69, 127.67, 127.63, 127.61, 127.54 (arom. αβ), 106.40 (C1-β), 102.53 (C1-α), 82.15(C4-α), 80.51 (C4-β), 79.75 (C2-β), 78.44 (C3-β), 77.86 (C2-α), 75.03 (C3-α), 73.48, 73.19, 72.47, 72.45, 72.35, 72.34 (CH_2 Bn-αβ), 71.37 (C5-β), 70.19 (C5-α), 55.57 (OMe-α), 55.09 (OMe-β).



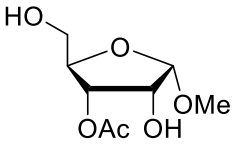
1-*O*-Methyl-2-*O*-acetyl-3,5-di-*O*-benzyl-α-D-ribofuranoside (13)

Compound **12** (8.3 g, 19.10 mmol) and DCM (95 mL) were added into a flask after which the solution was cooled down to 0 °C. $SnCl_4$ (19.1 mL, 19.1 mmol, 1M solution in DCM) was added to the reaction and the mixture was stirred at 4 °C for 16 hours. The reaction was quenched by aq. $NaHCO_3$ (sat.) and filtered. The organic filtration was washed by H_2O (1 x), brine (1 x) and dried ($MgSO_4$). The mixture was filtered, concentrated and co-evaporated with toluene (3 x). The residue was re-dissolved in pyridine (95 mL), added DMAP (117 mg, 0.96 mmol) and acetic anhydride (18.0 mL, 191.0 mmol). The reaction was stirred at room temperature for 3 hours after which was quenched by aq. $NaHCO_3$ (sat.). EtOAc was added to extract the mixture and the organic layer was further washed by H_2O (1 x) and brine (1 x). The organic layer was dried ($MgSO_4$), filtered and concentrated. The residue was purified by silica gel column chromatography (pentane/EtOAc, 95/5 – 80/20 – 70/30) to obtain **13** as a colorless oil (6.4 g, 16.57 mmol, 87 %). ^{1}H NMR (500 MHz, Chloroform-d) δ 7.40 – 7.22 (m, 10H), 5.12 (d, *J* = 4.5 Hz, 1H, H1), 4.91 (dd, *J* = 7.1, 4.5 Hz, 1H, H2), 4.68 (d, *J* = 12.4 Hz, 1H, C*H*H Bn), 4.53 (d, *J* = 12.1 Hz, 1H, C*H*H Bn), 4.47 (dd, *J* = 12.3, 10.4 Hz, 2H, 2xC*H*H Bn), 4.21 (d, J = 4.0 Hz, 1H, H4), 4.04 (dd, *J* = 7.1, 4.2 Hz, 1H, H3), 3.51 – 3.43 (m, 4H, OMe, H5), 3.33 (AB, *J* = 10.5, 4.2 Hz, 1H, H5), 2.20 (s, 3H, Ac). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.22 (CO Ac), 137.75, 137.74 (Cq. arom.), 128.21, 128.17, 127.97, 127.67, 127.50 (arom.), 101.64 (C1), 81.36 (C4), 75.01 (C3), 73.23, 72.90 (CH_2 Bn), 71.99 (C2), 69.25 (C5), 55.40 (OMe), 20.61 (Me Ac). IR (film): 2928, 1740, 1453, 1372, 1238, 1124, 1096, 1065, 1027, 739, 698 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{22}H_{26}O_6Na$ (M+Na) 409.1622. Found 409.1622. $[\alpha]_D^{20}$ +95.0 (c = 1, in DCM)

1-*O*-Methyl-2-*O*-acetyl-α-D-ribofuranoside (14A)/



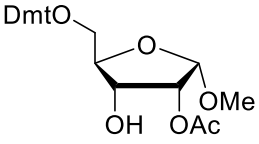
14A



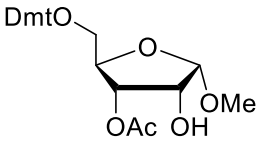
14B

1-*O*-Methyl-3-*O*-acetyl-α-D-ribofuranoside (14B)

Compound **13** (1.24 g, 3.21 mmol) was dissolved in tBuOH/Dioxane/H_2O (12 mL, 4/4/1; v/v/v). Pd/C (124 mg, 10 wt % Pd) were added and H_2 bubbled through the mixture for 24 hours at room temperature after which the reaction mixture was filtered over celite. The filtration was concentrated under reduced pressure and co-evaporated with toluene (1 x). The residue was purified by silica gel column chromatography (DCM/methanol, 100/2 – 100/5) to obtain **14** as a colorless oil (615 mg, 2.98 mmol, 93 %). (80% 2-OAC product and 20% 3-OAc product) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.10 (d, *J* = 4.1 Hz, 1H, H1-A), 4.96 (dd, *J* = 7.2, 3.3 Hz, 0.25H, H3-B), 4.92 (d, *J* = 4.6 Hz, 0.25H, H1-B), 4.75 (dd, *J* = 6.3, 4.1 Hz, 1H, H2-A), 4.19 (td, *J* = 7.2, 6.8, 3.5 Hz, 1H, H3-AB), 4.13 (td, *J* = 3.6, 2.4 Hz, 1H, H4-A), 4.05 (q, *J* = 3.4 Hz, 0.25H, H4-B), 3.81 – 3.73 (m, 1.5H, H5-A, H5-B), 3.69 (AB, *J* = 11.5, 3.4 Hz, 1H, H5-A), 3.45 (s, 0.75H, OMe-B), 3.41 (s, 3H, OMe-A), 2.84 (d, *J* = 9.6 Hz, 1H, 3-OH-A), 2.74 (d, *J* = 10.8 Hz, 0.25H, 3-OH-B), 2.40 (bs, 1.25H, 5-OH-AB), 2.15 (s, 3H, Ac-A), 2.11 (s, 0.75H, Ac-B). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.92, 170.47 (CO, Ac), 102.57 (C1-B), 101.96 (C1-A), 86.03 (C4-A), 83.07 (C4-B), 73.24 (C2-A), 71.49 (C3-B), 71.35 (C2-B), 70.04 (C3-A), 62.65 (C5-A), 62.51 (C5-B), 55.57 (OMe-B), 55.37 (OMe-A), 21.00 (CH_3 Ac-B), 20.77 (CH_3 Ac-A). IR (film): 3444, 2932, 1735, 1374, 1234, 1081, 1028, 964, 899, 5002, 607, 479 cm^{-1}. HRMS (ESI$^+$) calcd for $C_8H_{14}O_6Na$ (M+Na) 229.0683. Found 229.0685. $[\alpha]_D^{20}$ +112.3 (c = 1, in DCM)



15A



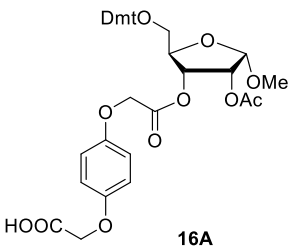
15B

1-*O*-Methyl-2-*O*-acetyl-5-O-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (15A) /

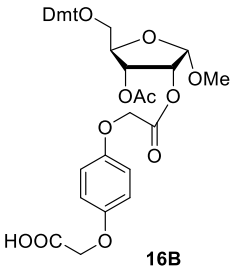
1-*O*-Methyl-3-*O*-acetyl-5-O-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (15B)

Compound **14** (584 mg, 2.83 mmol), 4,4'-dimethoxyltrityl chloride (DMTCl, 1.01 g, 2.98 mmol), and pyridine were added into a flask and the solution was stirred for 16 hours at room temperature after which was concentrated. The residue was dissolved in EtOAc and washed by aq. $NaHCO_3$ (sat.). The organic layer was dried ($MgSO_4$), filtered and concentrated. The residue was purified by silica gel column chromatography (pentane/EtOAc, 100/0 – 90/10 – 80/20 – 60/40) to obtain **15** as a light yellow foam (1.31 g, 2.58 mmol, 91 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.46 – 7.42 (m, 2H, DMT arom.), 7.38 – 7.16 (m, 7H, DMT arom.), 6.88 – 6.80 (m, 4H, DMT arom.), 5.22 (d, *J* = 4.1 Hz, 0.25H, H1-B), 5.16 (dd, *J* = 7.0, 2.8 Hz, 0.75H, H3-A), 5.06 – 5.04 (m, 1H, H1-A, H2-B), 4.44 (dd, *J* = 7.0, 4.7 Hz, 0.75H, H2-A), 4.27 – 4.24 (m, 0.5H, H3-B, H4-B), 4.16 (q, *J* = 3.3 Hz, 0.75H, H4-A), 3.79 (s, 6H, OMe DMT), 3.51 (s, 2.25H, OMe-A), 3.47 (s, 0.75H, OMe-B), 3.39 – 3.33 (m, 1H, H5), 3.25 – 3.16 (m, 1H, H5), 2.72 (s, 1H, OH), 2.20 (s, 0.75H, Ac-B), 2.10 (s, 2.25H, Ac-A). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.44(CO Ac-A), 170.30 (CO Ac-B), 158.55, 144.83, 144.69, 136.03, 135.96, 135.87, 135.76 (Cq. arom.), 130.40, 130.13, 128.22, 127.91, 126.87, 113.21 (arom.), 102.59 (C1-A), 101.94(C1-B), 86.27, 86.26 (Cq. DMT), 85.50 (C4-B), 81.97 (C4-A), 73.34 (C2-B), 72.08 (C3-A), 71.42 (C2-A), 70.73 (C3-B), 63.68 (C5-A), 63.67 (C5-B), 55.70 (OMe), 55.34 (OMe DMT-B), 55.25 (OMe DMT-A), 21.01 (CH_3 Ac-A), 20.85 (CH_3 Ac-B).

IR (film): 3507, 2933, 2837, 1741, 1608, 1509, 1446, 1300, 1248, 1177, 1077, 1035, 830, 596 cm^{-1}. HRMS (ESI^+) calcd for $C_{29}H_{32}O_8Na$ (M+Na) 531.1989. Found 531.1992. $[\alpha]_D^{20}$ +53.1 (c = 1, in DCM)



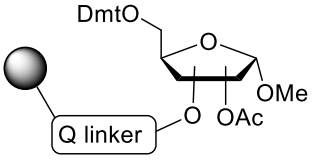
16A



16B

1-*O*-Methyl-2-*O*-acetyl-3-*O*-hydroquinone-*O,O'*-diacetylhemiester-5-O-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (16A) / 1-*O*-Methyl-2-*O*-hydroquinone-*O,O'*-diacetylhemiester-5-*O*-(4,4'-di-methoxyltrityl)-3-*O*-acetyl-α-D-ribofuranoside (16B)

Compound **15** (1.22 g, 2.40 mmol) was dissolved in pyridine (12 mL). DMAP (29 mg, 0.24 mmol), EDC (446 mg, 2.88 mmol), Et_3N (0.24 mL, 1.73 mmol) and hydroquinone-O,O'-diacetic acid (Q-linker) (650 mg, 2.88 mmol) were added and the reaction was stirred at room temperature for 16 hours. The reaction mixture was concentrated, diluted with $CHCl_3$ and washed with H_2O. The water layer was extracted with $CHCl_3$ and the combined organic layers were dried ($MgSO_4$), concentrated and purified by silica gel chromatography neutralized with 1% Et_3N (DCM/methanol, 100/0 – 99/1 – 95/5 – 90/10) to obtain **16** as a white foam (962 mg, 1.34 mmol, 56%). ^1H NMR (500 MHz, Chloroform-*d*) δ 7.38 – 7.32 (m, 2H, arom.), 7.27 – 7.18 (m, 6H, arom.), 7.17 – 7.10 (m, 1H, arom.), 6.85 – 6.73 (m, 8H, arom.), 5.35 (dq, *J* = 7.5, 2.6 Hz, 1/3H, H3-B), 5.28 – 5.22 (m, 2/3H, H3-A), 5.19 (dd, *J* = 5.4, 3.7 Hz, 4/3H, H1-A, H2-A), 5.17 – 5.12 (m, 2/3H, H1-B, H2-B), 4.63 – 4.48 (m, 2H, CO*CH*$_2$O), 4.39 – 4.37 (m, 2H, *CH*$_2$COOH), 4.15 (q, *J* = 3.4 Hz, 1H, H4), 3.72 – 3.71 (m, 6H, OMe DMT), 3.38 (s, 3H, OMe), 3.33 – 3.29 (m, 1H, H5), 3.20 – 3.13 (m, 1H, H5), 2.01 (s, 1H, CH_3 Ac-B), 1.97 (s, 2H, CH_3 Ac-A). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.30(COOH-A), 174.28 (COOH-B), 170.49 (CO Ac-A), 170.03(CO Ac-B), 168.82(CH_2*CO*O-B), 168.45(CH_2*CO*O-A), 158.66, 158.65, 153.83, 152.15, 152.13, 144.75, 136.00, 135.96, 135.85, 135.78 (Cq. arom.), 130.21, 130.19, 128.29, 128.01, 126.98, 126.97, 116.00, 115.91, 115.71, 113.31 (arom.), 101.66 (C1-B), 101.64 (C1-A), 86.47 (Cq. DMT-B), 86.43(Cq. DMT-A), 81.21 (C4-B), 81.03 (C4-A), 71.78 (C2-A), 71.64 (C3-B), 71.42 (C2-B), 70.60 (C3-A), 67.62 (*CH*$_2$COOH), 66.25 (*CH*$_2$COO-B), 66.05 (*CH*$_2$COO-A), 63.45 (C5-B), 63.41 (C5-A), 55.88 (OMe-A), 55.76 (OMe-B), 55.36 (OMe DMT), 20.93 (CH_3 Ac-A), 20.75 (CH_3 Ac-B). IR (film): 2934, 1738, 1607, 1507, 1445, 1246, 1178, 1073, 1032, 828cm^{-1}. HRMS (ESI^+) calcd for $C_{39}H_{40}O_{13}Na$ (M+Na) 739.2361. Found 739.2364. $[\alpha]_D^{20}$ +33.0 (c = 1, in DCM)



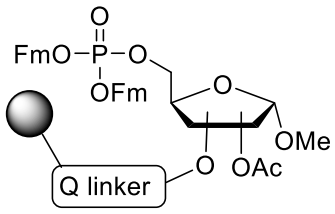
18a (CPG)
18b (TG)

1-*O*-Methyl-2-*O*-acetyl-3-*O*-Q-CPG-5-*O*-(4,4'-di-methoxyltrityl)-α-ribofuranoside/ 1-*O*-Methyl-2-*O*-Q-CPG-3-*O*-acetyl-5-*O*-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (18a, CPG)

1-*O*-Methyl-2-*O*-acetyl-3-*O*-Q-TG-5-*O*-(4,4'-di-methoxyltrityl)-α-ribofuranoside/ 1-*O*-Methyl-2-*O*-Q-TG-3-*O*-acetyl-5-*O*-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (18b, TG)

To a 20 mL reaction syringe with filter frit was added LCAA-CPG (3 g, 0.20 mmol) or Tentagel N NH2 (800 mg, 0.20 mmol), MeCN (12 mL), compound **16** (430 mg, 0.6 mmol), HOBT (12 mg, 0.08 mmol), DIC (0.28 mL, 1.8 mmol) and DIPEA (0.52 mL, 3 mmol). The mixture was shaken at room temperature for 16 hours. The reaction mixture was drained and the CPG/Tentagel was washed with ACN (2 x), DMF (2 x) and DCM (3 x) under N_2. The remaining

unmodified amine groups were capped by adding a mixture of CAP 1 (6 mL) and CAP 2 (6 mL). The mixture was shaken for 2 hours, drained and washed with DMF (3 x) and DCM (3 x) under N_2. The CPG/Tentagel was dried under reduced pressure and the loading was determined by trityl analysis at 503 nm. The loadings for **18a** (CPG) and **18b** (TG) are 50 µmol/g and 207 µmmol/g respectively.



1a (CPG)
1b (TG)

1-*O*-Methyl-2-*O*-acetyl-3-*O*-Q-CPG-5-*O*-(di-*O*-fluorenylmethylphosphoryl)-α-D-ribofuranoside/ 1-*O*-Methyl-2-*O*-Q-CPG-3-*O*-acetyl-5-*O*-(di-*O*-fluorenylmethylphosphoryl)-α-D-ribofuranoside (1a, CPG)

1-*O*-Methyl-2-*O*-acetyl-3-*O*-Q-TG-5-*O*-(di-*O*-fluorenylmethylphosphoryl)-α-D-ribofuranoside/ 1-*O*-Methyl-2-*O*-Q-TG-3-*O*-acetyl-5-*O*-(di-*O*-fluorenylmethylphosphoryl)-α-D-ribofuranoside (1b, TG)

To a 20 mL reaction syringe with filter frit was added **18a** (3 g) or **18b** (900 mg). Dichloroacetic acid (5 %, v/v, in DCM) was added repeatedly until no yellow color was observed. The resin was extensively washed with DCM (3 x), ACN (5 x) under N_2. ETT (0.25M in ACN, 12 eq) and **9** (0.2 M in ACN, 4 eq) were added into the resin and the mixture was shaken under N_2 for 10 minutes and drained. Repeat this coupling 1 more time and the resin was drained and washed with ACN (5 x). 9 mL (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) (0.5 M in ACN) was added and the mixture was shaken for 30 minutes under N_2. The resin was drained, washed with ACN (5 x) under N_2 and dried under reduced pressure to obtain 1a or 1b which was stored at 4 °C before use. A test sample of **1a** (60 mg) or **1b** (20 mg) was added into a 2 mL reaction syringe. To this syringe was added 1 mL DBU solution (10%, v/v, in ACN) and was shaken for 10 min to remove Fm groups on 5-phosphate after which was drained and washed with ACN (3 x). Treatment with 1mL NH_4OH (35%) for 1 hour to cleave the product from resin and the filtration was concentrated. ^{31}P NMR (162 MHz, D_2O): **1a** δ 5.28 (s); **1b** δ 5.30 (s)

1-*O*-methyl-α-ADPr dimer (21a, manual synthesis)

To a 5 mL reaction syringe with filter frit, **1a** (200 mg, 10 µmol) or **1b** (50 mg, 10 µmol) was added, washed with ACN (3 x) under N_2. 2 mL DBU solution (10%, v/v, in ACN) and was shaken for 15 min (two times) to remove Fm groups on 5-phosphate after which was drained and washed with ACN (5 x) to give **19a/b**. Then:

Cycle A: ETT (0.48 mL, 0.25 M in ACN) and **3** (0.4 mL, 0.2M in ACN) were added into the resin and the mixture was shaken for 10 minutes, drained and followed by a second addition of ETT and **3**. The mixture was drained and the resin was washed with ACN (5 x) under N_2. The intermediate phosphate-phosphite was oxidized with (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) solution (2 mL, 0.5 M in ACN) for 5 minutes (2 x) and washed with ACN (5 x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the syringe and was shaken for 15 min (2 x) to remove Fm groups on 5'-phosphate and CE group after which was drained and washed with ACN (5 x) under N_2.

Cycle B: ETT (0.48 mL, 0.25 M in ACN) and **4** (0.4 mL, 0.2M in ACN) were added to the resin and the mixture was shaken for 10 minutes, drained and followed by a second addition of ETT and **4**. The mixture was drained and the resin was washed with ACN (5 x) under N_2. The intermediate phosphate-phosphite was oxidized with (1S)-

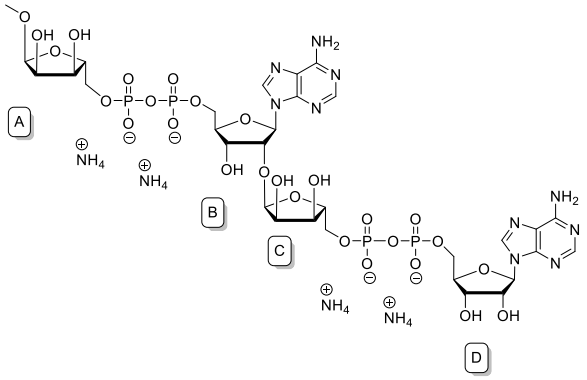
(+)-(10-camphorsulfonyl)-oxaziridine (CSO) solution (2 mL, 0.5 M in ACN) for 5 minutes (2 x) and washed with ACN (5 x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the syringe and was shaken for 10 min to remove CE group after which the syringe was drained and the resin was washed with ACN (5 x) under N_2. The resin was treated with NH_4OH (35%) overnight to cleave the product from the resin and remove all the protecting groups. The mixture was filtered and the filtrate was concentrated.

The crude material was purified by anion exchange to obtain ADPr dimer **21a** (0.32 mg, 0.27 µmol, 3%, from CPG; 3.23 mg, 2.73 µmol, 27%, from TG) as a white solid.

Column: Resource Q 6mL.

Gradient: 30% - 70%. (A: 10 mM NH_4OAc, B: 1 M NH_4OAc)

Dimer **21a**:



^{1}H NMR (500 MHz, Deuterium Oxide) δ 8.47 (s, 2H, H2), 8.27 (d, *J* = 2.7 Hz, 2H, H8), 6.19 (d, *J* = 3.1 Hz, 1H, H1-B), 5.98 (d, *J* = 5.9 Hz, 1H, H1-D), 5.30 (d, *J* = 4.3 Hz, 1H, H1-C), 4.93 – 4.90 (m, 1H, H1-A), 4.69 – 4.63 (m, 1H, H2-D), 4.55 (dd, *J* = 5.3, 3.1 Hz, 1H, H2-B), 4.53 – 4.43 (m, 2H, H3-B, H3-D), 4.39 – 4.25 (m, 4H, H4-BCD, H5-B), 4.25 – 4.15 (m, 6H, H2-A, H3-A, H4-A, H5-B, H5-D), 4.15 – 4.10 (m, 2H, H2-C, H3-C), 4.03 – 4.00 (m, 4H, H5-A, H5-C), 3.36 (s, 3H, OMe). ^{13}C NMR (126 MHz, D_2O) δ 152.43, 152.30 (C4), 148.37, 147.92 (C6), 118.40, 118.19 (C5), 103.20 (C1-A), 101.24 (C1-C), 87.27 (C1-D), 87.09 (C1-B), 84.19, 84.15, 84.13 (C4-C, C4-D), 83.17, 83.10, 83.02 (C4-B, C4-A), 78.87 (C2-B), 74.57 (C2-D), 71.33 (C2-A), 70.78 (C2-C), 70.49 (C3-D), 69.78 (C3-A), 69.61 (C3-C), 68.72 (C3-B), 65.59, 65.55 (C5-A, C5-C), 65.23, 65.21 (C5-D), 64.29, 64.25 (C5-B), 55.40 (OMe). ^{31}P NMR (202 MHz, D_2O) δ -11.08, -11.18, -11.26, -11.32, -11.37, -11.44, -11.54. LC-MS: Rt = 2.98 min. 0-50% NH_4OAc. ESI MS+ calc. 1115.2 found 1115.2 $[M+1]^+$. HRMS (ESI^+) calcd for $C_{31}H_{47}N_{10}O_{27}P_4$ (M+H) 1115.1557. Found 1115.1558.

1-*O*-methyl-α-ADPr trimer 21b (DNA synthesizer)

200 mg **1b** was added into a 5 mL reaction syringe with filter frit and the resin was washed with ACN (5 x) under N_2. 3 mL DBU solution (10%, v/v, in ACN) was added into the syringe and was shaken for 20 minutes to remove Fm groups on 5-phosphate after which was drained. The DBU treatment was repeated for another 20 minutes. The resin was washed with ACN (5 x) and dried under reduced pressure to remove traceless water before use. 50 mg resin from above was transferred into a reaction column of a Mermade 6 oligonucleotide synthesizer and the complete synthesis was performed under an argon atmosphere. For trimer synthesis, Cycle A was performed 2 times and Cycle B was performed 1 time.

Cycle A: The resin was rinsed with ACN (3 x) and drained. BTT (600 uL, 0.25 M in ACN) and LIU-78 (300 uL, 0.1M in ACN) were added into the resin and the mixture was left to stand for 10 minutes, drained. Repeat this coupling for two more times. The resin was rinsed by with ACN (3 x). The intermediate phosphate-phosphite was oxidized with (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) solution (2 mL, 0.5 M in MeCN) for 5 minutes (2 x). The resin was drained and washed with ACN (3x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the resin and was left to stand for 10 minutes (4 x) to remove Fm groups on 5'-phosphate and CE group after which was drained and washed with ACN (3 x).

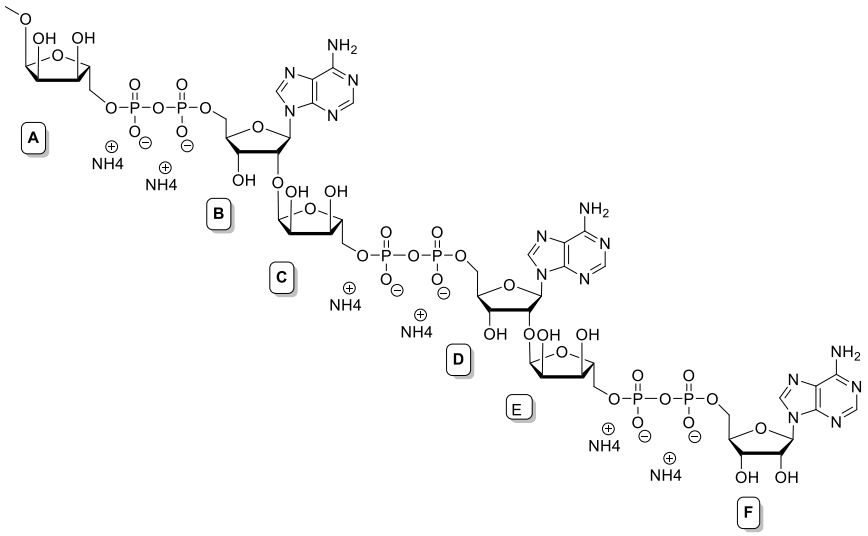
Cycle B: The resin was rinsed with ACN (3x) and drained. BTT (600 uL, 0.25 M in ACN) and **4** (300 uL, 0.1M in ACN) were added into the resin and the mixture was left to stand for 10 minutes, drained. Repeat this coupling for two more times. The resin was rinsed by with ACN (3x). The intermediate phosphate-phosphite was oxidized with CSO solution (2 mL, 0.5 M in MeCN) for 5 minutes (2 x). The resin was drained and washed with ACN (3 x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the resin and was left to stand for 10 minutes to remove CE group after which was drained and washed with ACN (3 x).

The resin was transferred to a tube and treated with 10 mL NH_4OH (35%). The mixture was stirred overnight in a sealed condition, filtered and concentrated. The crude was purified by anion exchange to obtain ADPr trimer **21b** (6.16 mg, 3.51 µmol, 35%) and ADPr dimer **21a** (4.5 mg, 3.81 µmol, 38%) as white solid.

Column: Resource Q 6mL.

Gradient: 30% - 70%. (A: 10 mM NH_4OAc, B: 1 M NH_4OAc)

Trimer **21b**:



^{1}H NMR (500 MHz, Deuterium Oxide) δ 8.36 – 8.24 (m, 3H, H2), 8.09 – 7.97 (m, 3H, H8), 6.13 (d, *J* = 3.2 Hz, 1H, H1-B), 5.97 (d, *J* = 3.1 Hz, 1H, H1-D), 5.94 (d, *J* = 5.9 Hz, 1H, H1-F), 5.30 (d, *J* = 4.2 Hz, 1H, H1-C), 5.19 (d, *J* = 4.1 Hz, 1H, H1-E), 4.85 – 4.82 (m, 1H, H1-A), 4.63 (t, *J* = 5.5 Hz, 1H, H2-F), 4.57 (dd, *J* = 5.3, 3.3 Hz, 1H, H2-B), 4.54 (t, *J* = 5.7 Hz, 1H, H3-B), 4.48 – 4.40 (m, 3H, H2-D, H3-F, H3-D), 4.34 – 3.94 (m, 24H), 3.33 (s, 3H, OMe). ^{13}C NMR (214 MHz, D_2O) δ 155.66, 155.55, 155.45 (C4), 149.44, 149.14, 148.84 (C6), 119.28, 119.11, 119.00 (C5), 104.13 (C1-A), 102.35 (C1-E), 102.24 (C1-C), 87.87 (C1-F), 87.71 (C1-D), 87.40 (C1-B), 85.18, 85.14, 85.10, 84.82, 84.78, 84.09, 84.05, 84.01, 83.96, 83.81, 83.78 (C4-ABCDEF), 80.26 (C2-D), 79.69 (C2-B), 75.38 (C2-F), 72.33, 72.30, 71.70, 71.41,

70.82, 70.81, 70.80, 70.55, 70.05, 69.87 (the rest C2, C3), 66.50, 66.19, 65.51 (C5-ABCDEF), 56.33, 56.32 (OMe). ^{31}P NMR (202 MHz, D_2O) δ -10.45, -10.51, -10.55, -10.61, -10.67, -10.69, -10.71, -10.78, -10.80, -10.82. LC-MS: Rt = 2.96 min. 0-50% NH_4OAc. ESI MS+ calc. 1656.2 found 1656.2 $[M+1]^+$. HRMS (ESI^+) calcd for $C_{46}H_{68}N_{15}O_{40}P_6$ (M+H) 1656.2168. Found 1656.2196.

1-*O*-methyl-α-ADPr pentamer/tetramer/trimer (21b-d, from pentamer synthesis)

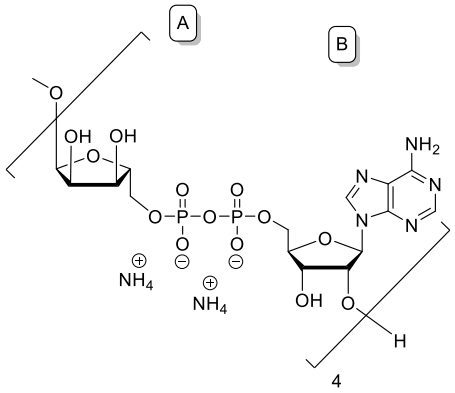
21b-d was synthesized according to the similar procedure as that of **21a**. Cycle A was performed 4 times and cycle B was performed 1 time after which the resin was transferred into a tube and treated with NH_4OH (35%) overnight in a sealed condition. The mixture was filtered, concentrated under reduced pressure and purified by anion exchange and gel filtration to obtain ADPr trimer **21b** (1.18 mg, 0.71 μmol, 7%), tetramer **21c** (1.35 mg, 0.61 μmol, 6%) and pentamer **21d** (0.69 mg, 0.25 μmol, 3%) as white solid.

Anion exchange column: Resource Q 6mL. Gradient: 50% - 80%. (A: 10 mM NH_4OAc, B: 1 M NH_4OAc)

Gel filtration gradient: 20% ACN in 0.15 M aq. NH_4HCO_3.

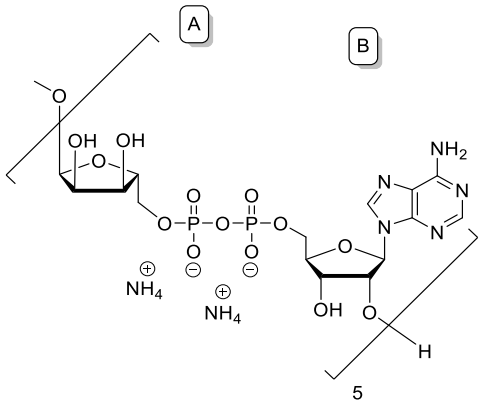
Trimer **21b**: Identical data with manual synthesis.

Tetramer **21c**:

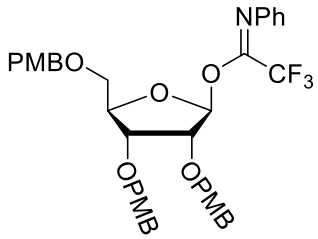


^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.39 (s, 1H), 8.38 (s, 1H), 8.32 (s, 1H), 8.31 (s, 1H, H2-ade), 8.10 (d, *J* = 1.2 Hz, 1H), 8.08 (d, *J* = 1.2 Hz, 1H), 8.06 (d, *J* = 1.1 Hz, 1H), 8.02 (d, *J* = 1.1 Hz, 1H, H8-ade), 6.17 (d, *J* = 3.5 Hz, 1H, H1-B), 6.03 (d, *J* = 3.0 Hz, 2H, H1-B), 5.99 (d, *J* = 5.8 Hz, 1H, H1-B), 5.34 (d, *J* = 4.3 Hz, 1H, H1-A), 5.27 (t, *J* = 4.5 Hz, 2H, H1-A), 4.92 – 4.89 (m, 1H, H1-A), 4.66 – 4.65 (m, 2H), 4.58 (t, *J* = 5.7 Hz, 1H), 4.55 (dd, *J* = 5.3, 3.8 Hz, 1H), 4.52 (t, *J* = 5.5 Hz, 1H), 4.50 – 4.45 (m, 3H), 4.39 – 4.01 (m, 32H), 3.38 (d, *J* = 1.0 Hz, 3H, OMe). ^{31}P NMR (202 MHz, D_2O) δ -11.10, -11.21, -11.24, -11.31, -11.34, -11.43, -11.47. LC-MS: Rt = 2.83 min. 0-50% NH_4OAc. ESI MS+ calc. 1099.1 found 1099.8 $[M+2]^+$. HRMS (ESI^+) calcd for $C_{61}H_{90}N_{20}O_{53}P_8$ (M+2H)/2 1099.1426. Found 1099.1446.

Pentamer **21d**:



^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.38 (s, 1H), 8.33 (s, 1H), 8.31 – 8.26 (m, 3H, H2-ade), 8.12 (s, 1H), 8.02 (d, *J* = 3.7 Hz, 2H), 7.98 (d, *J* = 5.0 Hz, 2H, H8-ade), 6.19 (d, *J* = 3.8 Hz, 1H, H1-B), 6.08 – 6.05 (m, 2H, H1-B), 6.04 (t, *J* = 1.8 Hz, 1H, H1-B), 5.98 (d, *J* = 5.9 Hz, 1H, H1-B), 5.31 (d, *J* = 4.2 Hz, 1H, H1-A), 5.26 (d, *J* = 3.6 Hz, 1H, H1-A), 5.24 (s, 1H, H1-A), 5.22 (d, *J* = 4.2 Hz, 1H, H1-A), 4.87 (d, *J* = 2.5 Hz, 1H, H1-A), 4.65 (dt, *J* = 10.4, 4.9 Hz, 2H), 4.59 (t, *J* = 5.5 Hz, 1H), 4.52 (d, *J* = 3.3 Hz, 5H), 4.49 – 4.46 (m, 1H), 4.39 – 3.98 (m, 41H), 3.37 (s, 3H, OMe). ^{31}P NMR (202 MHz, D_2O) δ -11.10, -11.10, -11.12, -11.21, -11.25, -11.31, -11.36, -11.45. LC-MS: Rt = 2.84 min. 0-50% NH_4OAc. ESI MS+ calc. 913.4 found 913.1 $[M+3]^+$. HRMS (ESI^+) calcd for $C_{61}H_{90}N_{20}O_{53}P_8$ (M+2H)/2 1369.6732. Found 1369.6742.



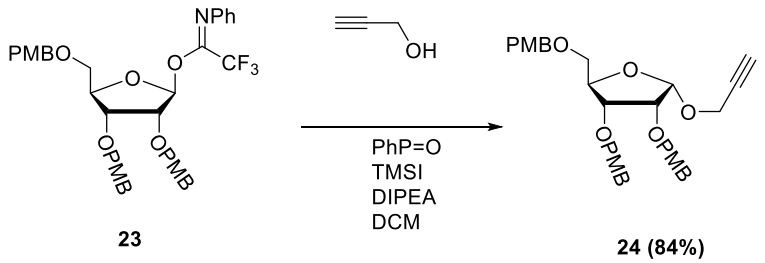
1-*O*-((*N*-Phenyl)-2,2,2-trifluoroacetimido)-2,3,5-tri-*O*-*p*-methoxylbenzyl-β-D-ribofuranose (23)

Compund **22**[31] (9.3 g, 18.23 mmol) was dissolved in acetone (93 mL). Cs_2CO_3 (8.89 g, 27.34 mmol,) and 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (3.23 mL, 20.05 mmol) were added and the reaction mixture was stirred at room temperature for 3 hours. After filtered over celite, the solvent was removed and the residue was purified using silica gel column chromatography neutralized with 1% Et_3N (Pentane/EA, 100/0 – 90/10 – 80/20) to afford the title compound as a light yellow oil (11.67 g, 17.13 mmol, 94 %).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.35 – 7.04 (m, 9H, arom.), 6.89 – 6.75 (m, 8H, arom), 6.27 (s, 1H, H1), 4.77 – 4.31 (m, 7H, CH_2 PMB, H4), 4.08 (t, *J* = 6.1 Hz, 1H, H3), 4.01 (d, *J* = 4.6 Hz, 1H, H2), 3.82 – 3.76 (m, 9H, OMe), 3.68 – 3.65 (m, 1H, H5), 3.54 (AB, *J* = 11.2, 5.2 Hz, 1H, H5). ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.60, 159.54, 159.28, 143.97, 130.42 (Cq. arom.), 129.96 (arom.), 129.76 (Cq. arom.),, 129.66 (arom.), 129.58 (arom.), 129.53 (Cq. arom.), 129.34, 129.32, 128.85, 113.96, 113.92, 113.84, 113.80 (arom.), 102.65 (C1), 82.34 (C4), 78.17 (C2), 77.05 (C3), 73.27, 73.04, 72.42, 72.22, 72.01 (CH_2 PMB-αβ), 69.89, 69.54 (C5-αβ), 55.40, 55.39 (OMe, αβ). IR (film): 2935, 2837, 1709, 1612, 1512, 1302, 1246, 1205, 1156, 1110, 1033, 819, 755, 695, 515 cm^{-1}. HRMS (ESI^+) calcd for $C_{29}H_{34}O_8Na$ (M+Na) 533.2146. Found 533.2147. $[\alpha]_D^{20}$ +41.6 (c = 1, in DCM)

1-*O*-Propargyl-2,3,5-tri-*O*-*p*-methoxylbenzyl-α-D-ribofuranose (24)

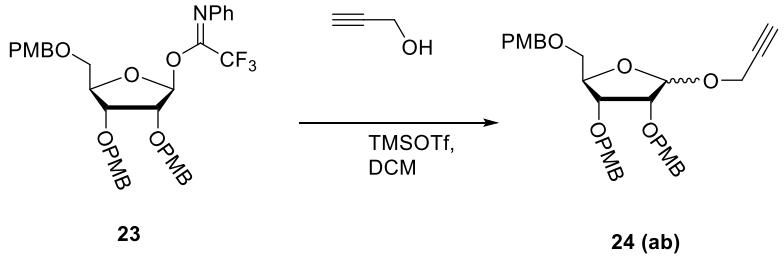
Method 1:



23 **24 (84%)**

Compound **23** (3.07 g, 4.50 mmol) were added into a flask and co-evaporated with toluene (3 x). $Ph_3P=O$ (7.51 g, 27 mmol), DCM (45 mL), freshly flame-dried 3Å molecular sieves, DIPEA (0.78 mL, 4.50 mmol) and propargyl alcohol (0.52 mL, 9.00 mmol) were added into the flask contain **23** after which it was cooled down to 0 °C. TMSI (0.64 mL, 4.5 mmol) was then added into the reaction under N_2 and the mixture was stirred for 1 hour before it was quenched by aq. $Na_2S_2O_3$ (sat.). The reaction was diluted with DCM and the organic layer was washed with H_2O (1 x) and brine (1 x), dried ($MgSO_4$), filtered and concentrated. Silica gel chromatography purification (pentane/EtOAc, 100/0 – 80/20) afforded **24** as a light yellow oil (2.07 g, 3.78 mmol, 84%).

Method 2:



23 **24 (ab)**

Compound **23** (1.64 g, 2.41 mmol), propargyl alcohol (0.14 mL, 2.41 mmol, **23** and propargyl alcohol were co-evaporated with dioxane for 2 times), DCM (24 mL) and freshly flame-dried 3Å molecular sieves were added into a flask and stirred under N_2 for 1 hour. The mixture was cooled down to -78 °C and TMSOTf (8.7 µL, 48 µmol) was added. The reaction was stirred at same temperature for 20 minutes after which it was quenched by addition of excessive amount of TEA and concentrated. Silica gel chromatography purification (pentane/EtOAc, 95/5 – 75/25) afforded **24** (α and β anomer) as a light yellow oil. (α-anomer: 272 mg, 0.50 mmol, 21%; β-anomer: 205 mg, 0.37 mmol, 15%).

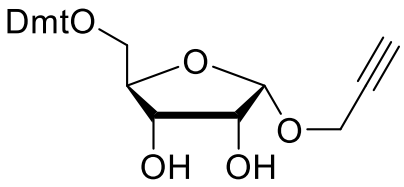
α anomer:

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.31 – 7.24 (m, 2H), 7.24 – 7.18 (m, 2H), 7.17 – 7.09 (m, 2H), 6.91 – 6.78 (m, 6H, arom. PMB), 5.31 (dd, *J* = 2.9, 1.4 Hz, 1H, H1), 4.66 – 4.31 (m, 8H, CH_2 PMB, O*CH*$_2$CCH), 4.20 (td, *J* = 4.1, 2.2 Hz, 1H, H4), 3.85 – 3.74 (m, 11H, H2, H3, OMe), 3.37 (AB, *J* = 10.5, 4.0 Hz, 1H, H5), 3.31 (AB, *J* = 10.5, 4.2 Hz, 1H, H5), 2.39 (t, *J* = 2.4 Hz, 1H, OCH_2C*CH*). ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.37, 159.28, 159.24, 130.40, 130.05, 129.93 (Cq. arom.), 129.86, 129.74, 129.33, 113.80, 113.70 (arom.), 98.89 (C1), 82.56 (C4), 79.67 (Cq. CH_2*C*CH), 77.22 (C2), 74.77 (C3), 74.32 (CH_2C*CH*), 73.15, 71.99, 71.87 (CH2 PMB), 69.75 (C5), 55.33 (CH3 PMB), 54.24 (O*CH*$_2$CCH). IR (film): 3281, 2910, 1611, 1585, 1512, 1247, 1032, 819, 750 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{32}H_{40}O_8N$ (M+NH_4) 566.2748. Found 566.2745. $[\alpha]_D^{20}$ +68.6 (c = 1, in DCM)

β anomer:

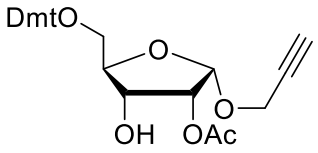
^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.35 – 7.13 (m, 6H), 6.92 – 6.77 (m, 6H, arom. PMB), 5.16 (s, 1H, H1), 4.71 – 4.26 (m, 7H, CH_2 PMB, H4), 4.16 (dd, *J* = 3.4, 2.4 Hz, 1H, O*CH*$_2$CCH), 4.01 (dd, *J* = 7.5, 4.7 Hz, 1H, H3), 3.86 (d, *J* =

4.7 Hz, 1H, H2), 3.82 – 3.71 (m, 9H, OMe PMB), 3.57 (AB, *J* = 10.7, 3.4 Hz, 1H, H5), 3.44 (AB, *J* = 10.7, 5.7 Hz, 1H, H5), 2.39 (t, *J* = 2.4 Hz, 1H, $OCH_2C\mathit{CH}$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.43, 159.36, 159.18, 130.43, 129.96, 129.90 (Cq. arom.), 129.76, 129.56, 129.25, 113.89, 113.80, 113.77 (arom.), 103.34 (C1), 80.83 (C4), 79.28 (Cq. $CH_2\mathit{C}CH$), 79.21 (C2), 77.82 (C3), 74.55 ($CH_2C\mathit{CH}$), 72.81, 72.11, 72.05 (CH_2 PMB), 70.70 (C5), 55.34 (CH_3 PMB), 55.32 (CH_3 PMB), 54.07 ($O\mathit{CH_2}CCH$). IR (film): 3281, 2931, 2836, 1611, 1585, 1512, 1301, 1244, 1173, 1094, 1030, 818, 516cm^{-1}. HRMS (ESI$^+$) calcd for $C_{32}H_{40}O_8N$ (M+NH_4) 566.2748. Found 566.2747. $[\alpha]_D^{20}$ +17.4 (c = 1, in DCM)

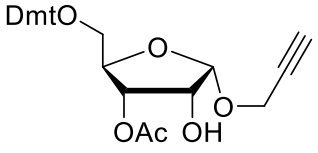


1-*O*-Propargyl-*O*-(4,4'-di-methoxyltrityl)-α-D-ribofuranose (25)

Compound **24** (2.07 g, 3.78 mmol), DCM (37 mL) and TFA (1.74 mL, 22.68 mmol) were added into the flask and the mixture was stirred for 1 hour after which it was diluted with toluene and concentrated. The residue was co-evaporated with toluene (2 x), pyridine (2 x) before pyridine (25 mL) and DMTCl (2.56 g, 7.56 mml) was added. The reaction was stirred for 1 hour and quenched by addition of aq. $NaHCO_3$ (sat.). DCM extracted the mixture (3 x) and the organic layers were combined, dried ($MgSO_4$), filtered and concentrated. Silica gel chromatography purification (silica gel was neutralized with 2% TEA in pentane before use, pentane/EtOAc, 100/0 – 70/30 – 50/50) afforded **25** as a colorless oil. (1.34 g, 2.73 mmol, 72%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.47 – 7.37 (m, 2H), 7.36 – 7.15 (m, 9H, arom. DMT), 6.87 – 6.78 (m, 4H, DMT), 5.32 (d, *J* = 4.5 Hz, 1H, H1), 4.44 – 4.35 (m, 2H, $O\mathit{CH_2}CCH$), 4.35 – 4.28 (m, 1H, H2), 4.17 (td, *J* = 3.6, 2.3 Hz, 1H, H4), 4.02 (dd, *J* = 4.8, 2.9 Hz, 1H, H3), 3.78 (s, 6H, OMe DMT), 3.34 (AB, *J* = 10.2, 3.6 Hz, 1H, H5), 3.14 (AB, *J* = 10.2, 3.7 Hz, 1H, H5), 2.95 (d, *J* = 9.5 Hz, 1H, 2-OH), 2.55 (d, *J* = 8.0 Hz, 1H, 3-OH), 2.46 (t, *J* = 2.4 Hz, 1H, $CH_2C\mathit{CH}$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.58, 144.81, 136.03, 135.84 (Cq. arom.), 130.16, 130.14, 128.21, 127.97, 126.92, 113.26, 113.25 (arom.), 100.42 (C1), 86.27 (Cq. DMT), 85.05 (C4), 79.00 (Cq. $OCH_2\mathit{C}CH$), 75.10 ($CH_2C\mathit{CH}$), 72.27 (C2), 71.54 (C3), 63.74 (C5), 55.31 (OMe DMT), 54.89 ($O\mathit{CH_2}CCH$). IR (film): 3467, 3284, 2931, 2835, 1608, 1445, 1248, 1176, 1088, 1032, 828, 754 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{29}H_{30}O_7Na$ (M+Na) 513.1884. Found 513.1882. $[\alpha]_D^{20}$ +65.9 (c = 1, in DCM)



26A

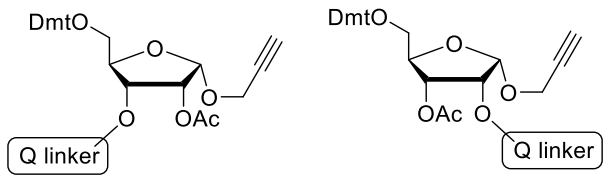


26B

1-*O*-Propargyl-2-*O*-acetyl-5-O-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (26A) / 1-*O*-Propargyl-3-*O*-acetyl-5-O-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (26B)

Compound **25** (1.04 g, 2.12 mmol) and pyridine (10 mL) were added into a flask after which it was cooled down to 0 °C. Ac_2O (0.18 mL, 1.91 mmol) was added to the flask slowly and the mixture was stirred overnight at room temperature. The mixture was then concentrated and diluted with EtOAc and H_2O. The aqueous layer was separated washed with EtOAc (1 x). All organic layers are combined, dried ($MgSO_4$), filtered and concentrated. Silica gel chromatography purification (silica gel was neutralized with 2% TEA in pentane before use, pentane/EtOAc, 90/10 – 80/20 – 50/50) afforded **26** (A+B) as a colorless oil. (568 mg, 1.07 mmol, 50%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.46 – 7.37 (m, 2H), 7.36 – 7.24 (m, 6H), 7.24 – 7.17 (m, 1H), 6.86 – 6.79 (m, 4H, arom. DMT), 5.52 (d, *J* = 4.1 Hz, 0.25H, H1-B), 5.35 (d, *J* = 4.7 Hz, 0.75H, H1-A), 5.15 (dd, *J* = 7.0, 2.6 Hz, 0.75H, H3-A), 5.08 (dd, *J* = 5.9, 4.2 Hz, 0.25H, H3-B), 4.48 (ddd, *J* = 11.4, 7.0, 4.6 Hz, 0.75H, H2-A), 4.43 – 4.31 (m, 2H, O*CH*$_2$CCH), 4.29 – 4.21 (m, 0.5H, H3-B, H4-B), 4.17 – 4.11 (m, 0.75H, H4-A), 3.79 (s, 6H, OMe DMT), 3.38 – 3.32 (m, 1H, H5-AB), 3.23 – 3.14 (m, 1H, H5-AB), 2.69 (d, *J* = 10.4 Hz, 0.25H, OH-B), 2.63 (d, *J* = 11.2 Hz, 0.75H, OH-A), 2.46 (q, *J* = 2.5 Hz, 1H, CH$_2$C*CH*), 2.20 (s, 0.75H, OMe-B), 2.09 (s, 2.25H, OMe-A). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.48 (CO Ac), 158.63, 144.84, 144.72, 136.06, 135.99, 135.90, 135.79 (Cq. arom.), 130.24, 130.20, 128.31, 128.29, 128.01, 127.99, 126.97, 113.29 (arom.), 99.78 (C1-A), 99.43 (C1-B), 86.38 (OCH$_2$*C*CH), 86.01 (C4-B), 82.72 (C4-A), 79.21 (Cq. arom.), 74.99 (OCH$_2$C*CH*-B), 74.84 (OCH$_2$C*CH*-A), 73.24 (C2-B), 72.04 (C3-A), 71.67 (C2-A), 70.72 (C3-B), 63.65 (C5), 55.36 (OMe DMT), 54.78 (O*CH*$_2$CCH-A), 54.67 (O*CH*$_2$CCH-B), 21.09 (Me Ac-A), 20.92 (Me Ac-B). IR (film): 3283, 2932, 1741, 1608, 1509, 1301, 1249, 1177, 1084, 830, 751 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{31}H_{32}O_8Na$ (M+Na) 555.1989. Found 555.1991. $[\alpha]_D^{20}$ +65.0 (c = 1, in DCM)

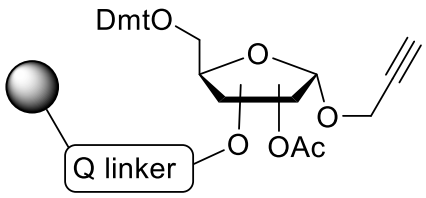


27A **27B**

1-*O*-Propargyl-2-*O*-acetyl-3-*O*-hydroquinone-*O,O*'-diacetylhemiester-5-O-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (27A) /

1-*O*-Propargyl-2-*O*-hydroquinone-*O,O*'-diacetylhemiester-5-O-(4,4'-di-methoxyltrityl)-3-*O*-acetyl-α-D-ribofuranoside (27B)

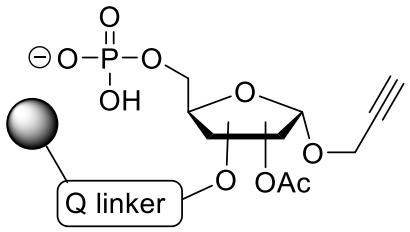
Compound **26** (544 mg, 1.02 mmol) was dissolved in pyridine (5.5 mL). DMAP (12 mg, 0.10 mmol), EDC (235 mg, 1.23 mmol), Et_3N (103 uL, 0.74 mmol) and hydroquinone-O,O'-diacetic acid (Q-linker) (277 mg, 1.23 mmol) were added and the reaction was stirred at room temperature for 16 hours. The reaction mixture was concentrated, diluted with $CHCl_3$ and washed with H_2O. The water layer was extracted with $CHCl_3$ and the combined organic layers were dried ($MgSO_4$), concentrated under reduced pressure and purified by silica gel chromatography (silica gel was neutralized with 2% TEA in pentane before use, then DCM/methanol, 100/0 – 99/1 – 95/5 – 90/10) to obtain **27** as a white foam (445 mg, 0.60 mmol, 59%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.47 – 7.36 (m, 2H), 7.36 – 7.24 (m, 6H), 7.24 – 7.17 (m, 1H), 6.94 – 6.78 (m, 8H, arom.), 5.62 – 5.53 (m, 1H, H1), 5.44 (dd, *J* = 6.8, 2.6 Hz, 0.25H, H2-B), 5.36 – 5.25 (m, 1.75H, H2-A, H3), 4.71 – 4.57 (m, 2H, CH2 Q linker), 4.48 (d, *J* = 7.0 Hz, 2H, CH_2 Q linker), 4.33 (t, *J* = 2.2 Hz, 2H, O*CH*$_2$CCH), 4.25 (dd, *J* = 4.5, 2.7 Hz, 1H, H4), 3.78 (s, 6H, DMT), 3.38 (AB, *J* = 10.2, 3.2 Hz, 1H, H5), 3.28 – 3.18 (m, 1H, H5), 2.46 (dt, *J* = 13.0, 2.4 Hz, 1H, OCH$_2$C*CH*), 2.07 – 2.02 (m, 3H, Ac). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.87 (CO-Q), 170.49 (CO Ac-A), 169.91 (CO Ac-B), 168.80 (CO-Q-B), 168.39 (CO-Q-A), 158.64, 153.60, 152.28, 152.23, 144.68, 135.93, 135.76 (Cq. arom.), 130.20, 130.17, 128.26, 128.02, 126.98, 116.01, 115.97, 115.72, 113.30 (arom.), 98.74 (H1-B), 98.65 (H1-A), 86.45 (Cq. DMT), 81.74 (C4-B), 81.56 (C4-A), 79.03 (OCH$_2$*C*CH), 75.01 (OCH$_2$C*CH*-A), 74.80 (OCH$_2$C*CH*-B), 71.63 (C2-A), 71.29 (C2-B), 70.50 (C3), 67.27 (CH2-Q), 66.19 (CH2-Q-B), 66.04 (CH2-Q), 63.32 (C5), 55.35 (OMe DMT), 54.86 (O*CH*$_2$CCH-A), 54.78 (O*CH*$_2$CCH-B), 20.87 (Ac-A), 20.71 (Ac-B). IR (film): 2933, 1742, 1608, 1508, 1249, 1180, 1033, 829, 597 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{41}H_{40}O_{11}Na$ (M+Na) 931.2463. Found 731.2444. $[\alpha]_D^{20}$ +53.2 (c = 1, in DCM)



1-*O*-Propargyl-2-*O*-acetyl-3-*O*-Q-TG-5-*O*-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside /

1-*O*-Propargyl-2-*O*-Q-TG-3-*O*-acetyl-5-*O*-(4,4'-di-methoxyltrityl)-α-D-ribofuranoside (28)

To a 20 mL reaction syringe with filter frit was added Tenta Gel N resin (700 mg, 0.17 mmol), ACN (5 mL), compound **27** (392 mg, 0.53 mmol), HOBT (11 mg, 0.08 mmol), DIC (0.25 mL, 1.59 mmol) and DIPEA (0.46 mL, 2.65 mmol). The mixture was shaken at room temperature for 16 hours. The reaction mixture was drained and the Tentagel was washed with ACN (2 x), DMF (2 x) and DCM (3 x) under N_2. The remaining unmodified amine groups were capped by adding a mixture of CAP 1 (6 mL) and CAP 2 (6 mL). The mixture was shaken for 2 hours, drained and washed with DMF (3 x) and DCM (3 x) under N_2. The Tentagel was dried under reduced pressure and the loading was determined by trityl analysis at 503 nm. The loading for **28** is 165 µmol/g.



1-*O*-Propargyl-2-*O*-acetyl-3-*O*-Q-TG-5-*O*-phosphoryl-α-D-ribofuranoside/

1-*O*-Propargyl-2-*O*-Q-TG-3-*O*-acetyl-5-*O*-phosphoryl-α-D-ribofuranoside (29)

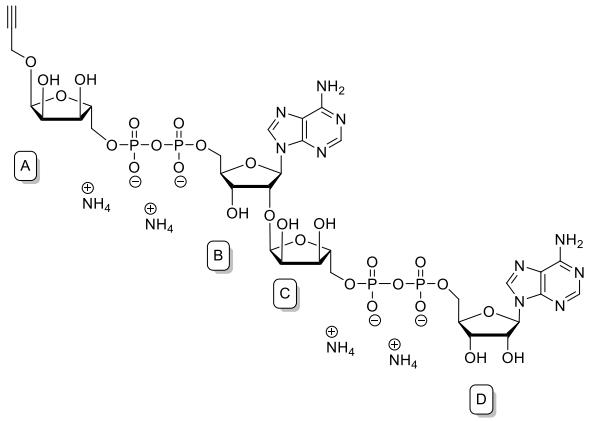
To a 20 mL reaction syringe with filter frit was added **28** (700 mg). Dichloroacetic acid (5 %, v/v, in DCM) was added repeatedly until no yellow color was observed. The resin was extensively washed with DCM (3 x), ACN (5 x) under N_2. ETT (5.54 mL, 0.25M in ACN, 12 eq) and **9** (2.31 mL, 0.2 M in ACN, 4 eq) were added into the resin and the mixture was shaken under N_2 for 10 minutes and drained. Repeat this coupling 1 more time and the resin was drained and washed with ACN (5 x). 9 mL (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) (0.5 M in ACN) was added and the mixture was shaken for 30 minutes under N_2. The resin was drained, washed with ACN (5 x) under N_2 and dried under reduced pressure to obtain Fm protected initiator. A test sample of it (30 mg) was added into a 2 mL reaction syringe. To this syringe was added 1 mL DBU solution (10%, v/v, in ACN) and was shaken for 10 minutes to remove Fm groups on 5-phosphate after which was drained and washed with ACN (3 x). Treatment with 1mL NH_4OH (35%) for 1 hour to cleave the product from resin and the filtration was concentrated. ^{31}P NMR (162 MHz, D_2O) showed single peak at 4.58 ppm which suggest the Fm protected phosphate was successfully introduced. 450 mg Fm protected resin was added into a 20 mL reaction syringe with filter frit and the resin was washed with ACN (5 x) under N_2. DBU solution (5 mL, 10%, v/v, in ACN) was added into the syringe and was shaken for 25 minutes to remove Fm groups after which it was drained. The DBU treatment was repeated for another 25 minutes. The resin was washed with ACN (5 x) and dried under reduced pressure furnished initiator **29**.

1-*O*-Propargyl-α-ADPr dimer (30a) and 1-*O*-Propargyl-α-ADPr trimer (30b)

61 mg (10 µmol) initiator resin **29** was transferred into a reaction column of a Mermade 6 oligonucleotide synthesizer and the complete synthesis was performed under an argon atmosphere.

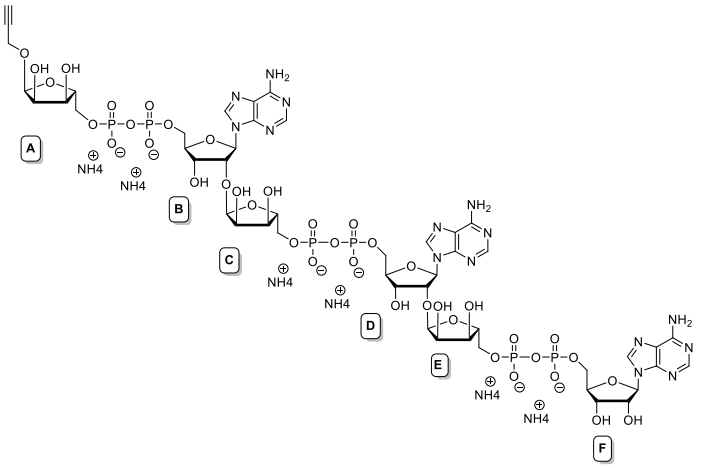
For **30a** synthesis, Cycle A was performed once and Cycle B was performed once, yielding **30a** (5.48 mg, 4.54 µmol, 45%) after anion exchange chromatography purification (20% - 70%; A: 10 mM NH_4OAc, B: 1 M NH_4OAc). For **30b** synthesis, Cycle A was performed twice and Cycle B was performed once, yielding **30b** (3.48 mg, 1.95 µmol, 20%) and **30a** (2.44 mg, 2.02 µmol, 20%) after anion exchange chromatography purification (25% - 75%, A: 10 mM NH_4OAc, B: 1 M NH_4OAc).

30a:



^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.41 (s, 1H, H2), 8.38 (s, 1H, H2), 8.17 (s, 1H, H8), 8.15 (s, 1H, H8), 6.19 (d, *J* = 3.6 Hz, 1H, H1-B), 6.01 (d, *J* = 5.8 Hz, 1H, H1-D), 5.29 (d, *J* = 4.3 Hz, 1H, H1-C), 5.14 (d, *J* = 4.2 Hz, 1H, H1-A), 4.67 (t, *J* = 5.5 Hz, 1H, H2-D), 4.64 (dd, *J* = 5.3, 3.7 Hz, 1H, H2-B), 4.59 (t, *J* = 5.6 Hz, 1H, H3-B), 4.48 (dd, *J* = 5.2, 3.5 Hz, 1H, H3-D), 4.40 – 4.35 (m, 3H, H4-BCD), 4.33 – 3.99 (m, 15H, H4-A, H5-ABCD, H2-BC, H3-BC, CH_2-prop.), 2.82 (t, *J* = 2.4 Hz, 1H, CH-prop.). ^{13}C NMR (214 MHz, D_2O) δ 155.91, 155.87 (C4), 153.23, 153.12 (C8), 149.60, 149.26 (C6), 119.36, 119.23 (C5), 102.37, 102.34 (C1-A), 101.58, 101.55 (C1-C), 87.96, 87.95 (C1-B), 87.46, 87.44 (C1-D), 85.14, 85.12, 85.10, 85.08, 84.84, 84.81, 84.59, 84.56, 84.55, 84.52, 84.01, 83.97 (C4-ABCD), 80.03 (Cq. Prop.), 79.90 (H2-B), 76.61, 76.57 (CH-prop.), 75.41 (C2-D), 72.32, 71.84, 71.39, 70.78, 70.42, 70.12 (C2-AC, C3-ABCD), 66.59, 66.57, 66.48, 66.45, 66.20, 66.18, 65.55, 65.51 (C5-ABCD), 55.79, 55.77 (CH_2-prop.). ^{31}P NMR (202 MHz, D_2O) δ -10.35, -10.45, -10.52, -10.60, -10.62, -10.68, -10.78. LC-MS: Rt = 3.02 min. 0-50% NH_4OAc. ESI MS+ calc. 1139.2 found 1139.2 $[M+1]^+$. HRMS (ESI^+) calcd for $C_{33}H_{47}N_{10}O_{27}P_4$ (M+H) 1139.1557. Found 1139.1566.

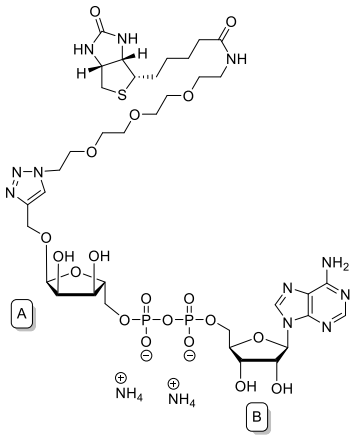
30b:



^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.43 (s, 1H, H2), 8.42 (s, 1H, H2), 8.36 (s, 1H, H2), 8.18 (s, 1H, H8), 8.16 (s, 1H, H8), 8.13 (s, 1H, H8), 6.18 (d, *J* = 3.2 Hz, 1H, H1-B), 6.04 (d, *J* = 3.4 Hz, 1H, H1-D), 6.00 (d, *J* = 5.9 Hz, 1H, H1-F), 5.34 (d, *J* = 4.3 Hz, 1H, H1-C), 5.26 (d, *J* = 4.3 Hz, 1H, H1-E), 5.17 (d, *J* = 4.3 Hz, 1H, H1-A), 4.68 (t, *J* = 5.5 Hz, 1H, H2-F), 4.60 (dt, *J* = 4.4, 2.2 Hz, 1H, H2-B), 4.56 (t, *J* = 5.8 Hz, 1H, H3-B), 4.52 (dd, *J* = 5.2, 3.4 Hz, 1H, H2-D), 4.51 – 4.46 (m, 2H, H3-DF), 4.41 – 3.99 (m, 24H, H2-ACE, H3-ACE, H4-ABCDEF, H5-ABCDEF, CH_2-prop.), 2.83 (t, *J* = 2.4 Hz, 1H, CH-prop.). ^{13}C NMR (214 MHz, D_2O) δ 154.45, 154.35, 154.28 (C4), 149.36, 148.95, 148.76 (C6), 119.29, 119.14, 119.03 (C5), 102.30 (C1-C), 102.18 (C1-E), 101.58 (C1-A), 88.08 (C1-F), 87.87 (C1-D), 87.75 (C1-B), 85.14, 85.12, 85.10, 85.09, 84.92, 84.88, 84.59, 84.55, 84.11, 84.07, 83.88, 83.84 (C4-ABCDEF), 80.19 (C2-D), 80.04 (Cq. prop.), 79.75 (C2-B), 76.59 (CH, prop.), 75.42 (C2-F), 72.31, 72.30, 71.86, 71.38, 70.80, 70.77, 70.41, 69.96, 69.72 (C2-ACE, C3-ABCDEF), 66.55, 66.52, 66.49, 66.47, 66.19, 66.17, 65.46, 65.44, 65.36, 65.34 (C5-ABCDEF), 55.78 (CH_2-prop.). ^{31}P NMR (202 MHz, D_2O) δ -10.44, -10.47, -10.50, -10.54, -10.56, -10.63, -10.72. LC-MS: Rt = 2.96 min. 0-50% NH_4OAc. ESI MS+ calc. 1680.2 found 1680.1 $[M+1]^+$. HRMS (ESI^+) calcd for $C_{48}H_{69}N_{15}O_{40}P_6$ (M+2H)/2 840.6120. Found 840.6115.

Biotin-PEG$_3$-mono-ADPr (**31a)**

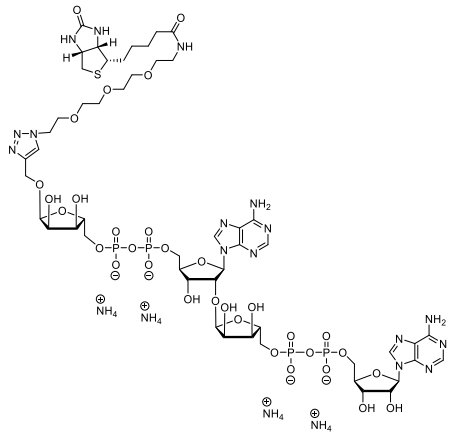
α-1-O-propargyl-mono ADPr[24](200 uL, 1 eq, 10 mg/mL in H_2O) was added to azide-PEG_3-biotin (125 uL, 2 eq, 25 mg/mL in ACN). Subsequently, 500 uL buffer (20 mM TRIS/150 mM NaCl, pH 7.6) was added to the mixture. Next, 120 uL click cocktail was added (1:1:1 v/v/v, CuSO4 (26 mg/mL in water): Sodium Ascorbate (120 mg/mL in water): TBTA ligand (52 mg/mL in DMSO)) after which 100 uL DMSO was added to increase solubility. The mixture was shaken for 30 minutes at room temperature followed using LC-MS analysis. The reaction should be finished in 1 hour (determined by total conversion of propargy-ADPr) and was quenched using 30 uL EDTA (0.5 M). The mixture was purified by HPLC (0% - 30%, A:25 mM NH_4OAc in H_2O, B: ACN) to furnish **31a** (1.61 mg, 1.55 µnol, 46%) as white solid after repeated lyophilization.



^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.55 (s, 1H, H2), 8.27 (s, 1H, H8), 8.02 (s, 1H, triazole), 6.15 (d, *J* = 6.0 Hz, 1H, H1-B), 5.13 – 5.06 (m, 1H, H1-A), 4.65 – 4.60 (m, 3H, CH2-PEG, C*H*H-triazole), 4.58 (ddd, *J* = 8.0, 5.1, 0.8 Hz, 1H, CH-biotin), 4.54 (dd, *J* = 5.1, 3.5 Hz, 1H, H3-B), 4.40 (ddt, *J* = 12.4, 8.0, 3.8 Hz, 2H, H4-B, CH-biotin), 4.26 – 4.21 (m, 2H, H5-B), 4.20 (dq, *J* = 3.8, 2.1, 1.5 Hz, 1H, H4-A), 4.17 – 4.12 (m, 2H, H2-A, H3-A), 4.07 – 4.01 (m, 2H, H5-A), 3.97 (dd, *J* = 5.6, 4.5 Hz, 2H, CH2-PEG), 3.67 – 3.57 (m, 10H, CH2-PEG), 3.37 (dd, *J* = 6.0, 4.8 Hz, 2H, CH2-PEG), 3.28 (ddd, *J* = 9.6, 5.7, 4.5 Hz, 1H, CH-S), 2.96 (AB, *J* = 13.1, 5.0 Hz, 1H, CH2-S), 2.79 – 2.71 (m, 1H, CH2-S), 2.25 (t, *J* = 7.3 Hz, 2H, CH2-biotin), 1.74 – 1.51 (m, 4H, CH2-biotin), 1.41 – 1.33 (m, 2H, CH2-biotin). ^{13}C NMR (214 MHz, D_2O) δ 178.13 (CO, biotin), 166.55 (CONH), 156.27 (C4), 150.29 (Cq. triazole), 145.16 (C6), 126.47 (CH, triazole), 119.81 (C5), 102.69 (C1-A), 88.12 (C1-B), 85.26, 85.22 (C4-B), 84.59, 84.55 (C4-A), 75.59 (C2-B), 72.22 (C2-A), 71.66 (C3-B), 70.89 (PEG), 70.83 (PEG), 70.75 (C3-A), 70.71, 70.64, 70.07, 69.99, 69.97, 69.95 (PEG), 66.84, 66.82 (C5-A), 66.43, 66.41 (C5-B), 63.28 (CH-biotin), 61.68 (PEG), 61.46 (CH-biotin), 56.54 (CH-S), 51.23 (CH2-trizole), 40.90 (CH2-S), 40.14 (PEG), 36.66, 29.07, 28.88, 26.34 (CH2-biotin). ^{31}P NMR (202 MHz, D_2O) δ -11.14, -11.24, -11.34, -11.44. LC-MS: Rt = 4.81 min. 0-50% NH_4OAc. ESI MS+ calc. 1042.3 found 1042.3 $[M+1]^+$. HRMS (ESI^+) calcd for $C_{36}H_{58}N_{11}O_{19}P_2S$ (M+H) 1042.3101. Found 1042.3111.

Biotin-PEG$_3$-di-ADPr (31b)

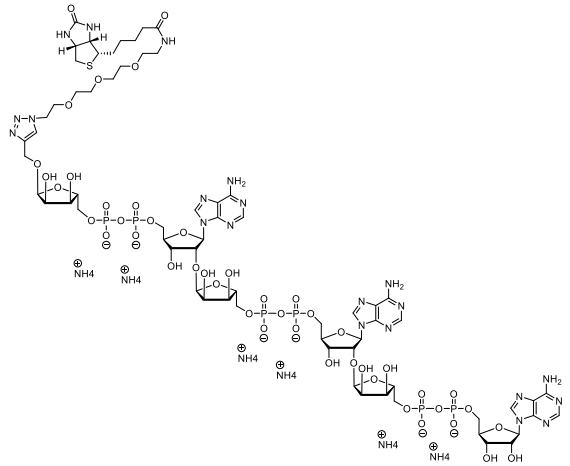
31b was synthesized using same procedure as **31a** but using 244 uL propargy di-ADPr **30a**. Purification by HPLC (0% - 30%, A:25 mM NH_4OAc in H_2O, B: ACN) to furnish **31b** (0.45 mg, 0.27 µmol, 13%) as white solid after repeated lyophilization.



^{1}H NMR (500 MHz, Deuterium Oxide) δ 8.41 (s, 1H, H2), 8.38 (s, 1H, H2), 8.15 (s, 1H, H8), 8.14 (s, 1H, H8), 8.01 (s, 1H, CH-triazole), 6.18 (d, *J* = 3.6 Hz, 1H, H1-B), 6.01 (d, *J* = 5.9 Hz, 1H, H1-D), 5.27 (d, *J* = 4.2 Hz, 1H, H1-C), 5.13 – 5.07 (m, 1H, H1-A), 4.70 – 4.56 (m, 6H), 4.51 – 4.47 (m, 1H), 4.41 – 3.93 (m, 18H), 3.62 (ddd, *J* = 15.5, 8.3, 4.6 Hz, 10H, PEG), 3.37 (t, *J* = 5.3 Hz, 2H, PEG), 3.27 (dt, *J* = 9.8, 5.2 Hz, 1H, CH-S), 2.96 (AB, *J* = 13.0, 5.0 Hz, 1H, CH_2-S), 2.79 – 2.72 (m, 1H, CH_2-S), 2.24 (t, *J* = 7.3 Hz, 2H), 1.83 – 1.31 (m, 6H, CH_2-biotin). ^{31}P NMR (202 MHz, D_2O) δ -11.11, -11.21, -11.29, -11.34, -11.37, -11.45, -11.48. LC-MS: Rt = 4.53 min. 0-50% NH_4OAc. ESI MS+ calc. 1583.4 found 1583.3 $[M+1]^+$. HRMS (ESI^+) calcd for $C_{51}H_{80}N_{16}O_{32}P_4S$ (M+2H)/2 792.1892. Found 792.1889.

Biotin-PEG$_3$-tri-ADPr (31c)

31c was synthesized using same procedure as **31a** but using 300 uL propargyl tri-ADPr **30b**. Purification by HPLC (0% - 30%, A:25 mM NH_4OAc in H_2O, B: ACN) to furnish **31c** (0.69 mg, 0.31 µmol, 18%) as white solid after repeated lyophilization.



^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.38 (s, 1H, H2), 8.35 (s, 1H, H2), 8.29 (s, 1H, H2), 8.09 (s, 1H, H8), 8.05 (s, 1H, H8), 8.04 (s, 1H, H8), 8.00 (s, 1H, CH-triazole), 6.18 (d, *J* = 3.7 Hz, 1H, H1-B), 6.03 (d, *J* = 3.6 Hz, 1H, H1-D), 5.99 (d, *J* = 5.9 Hz, 1H, H1-F), 5.31 (d, *J* = 4.3 Hz, 1H, H1-C), 5.21 (d, *J* = 4.2 Hz, 1H, H1-E), 5.11 – 5.07 (m, 1H, H1-A), 4.67 (t, *J* = 5.5 Hz, 2H), 4.64 – 4.54 (m, 8H), 4.53 – 4.46 (m, 4H), 4.40 – 3.92 (m, 36H), 3.68 – 3.55 (m, 10H, PEG), 3.37 (t, *J* = 5.3 Hz, 2H, PEG), 3.26 (dt, *J* = 9.7, 5.1 Hz, 1H, CH-S), 2.95 (dd, *J* = 13.1, 5.0 Hz, 1H, CH_2-S), 2.78 – 2.71 (m, 1H, CH_2-S), 2.24 (t, *J* = 7.3 Hz, 2H), 1.72 – 1.30 (m, 6H, CH_2-biotin). ^{31}P NMR (202 MHz, D_2O) δ -11.12, -11.14, -11.22, -11.24, -11.29, -11.34, -11.38, -11.41, -11.51. LC-MS: Rt = 5.74 min. 0-20% NH_4OAc. ESI MS+ calc. 1062.7 found 1063.4 $[M+2]^+$. HRMS (ESI^+) calcd for $C_{66}H_{101}N_{21}O_{45}P_6S$ (M+2H)/2 1062.7198. Found 1062.7216.

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5 Total synthesis of branched ADP-ribose trimer

Introduction

ADP-ribosylation is an important post-translational modification, that plays a key role in many cellular events such as DNA repair, apoptosis, chromatin regulation.[1-3] This reversible modification arises through a covalent attachment, catalyzed by the enzymes of the PARP family, of mono-ADP ribose (MAR) and poly-ADP ribose (PAR) chains to the nucleophilic side chains of specific amino acids in target proteins. Hydrolases, such as PARG, catalyze the reverse reaction by removing MAR and PAR from these proteins. PAR, termed "the third nucleic acid", can exist as a linear or branched polymer.[4] Although it has been known for more than 40 years, the biological function and binding module of branched PAR remains elusive.[4-7] Few studies disclose some unique biological characteristics of the branched polymer such as a tighter binding with a histone[8, 9] and a higher resistance to PARG than its linear counterpart.[10] Recently, Chen *et al*[11] reported, that PBZ (PAR-binding zinc finger) domain of APLF (aprataxin polynucleotide-kinase-like factor), involved in DNA damage response, recognizes the branched PAR chain. However, the protein binding details at a molecular level are missing partly due to the lack of a structurally defined branched ADPr-chains that could serve as molecular tools. To date, almost all reported PAR-binding proteins/domains recognize only mono- or di-ADPr fragments,[12-14] suggesting that unknown proteins/domains which specifically recognize and bind branched tri-ADPr core might exist. The availability of well-defined branched PAR oligomers would facilitate such investigation and will contribute to a) the discovery of new branched PAR binding proteins;[11] b) the elucidation of the

binding mechanism via co-crystallization with proteins like APLF or PARG;[15] c) a better understanding of the bio-function of branched PAR. Branched core oligomer **1** (Figure 1), the smallest branched PAR, comprises three full ADPr units, making it a perfect tool for biological study.

Guided by the previous synthetic approaches towards linear oligo-ADPr (Chapter 4) and the core nucleotide of the branched ADPr[16, 17] (Chapter 2), the first total synthesis of minimal branched ADPr structure **1** is reported in this Chapter.

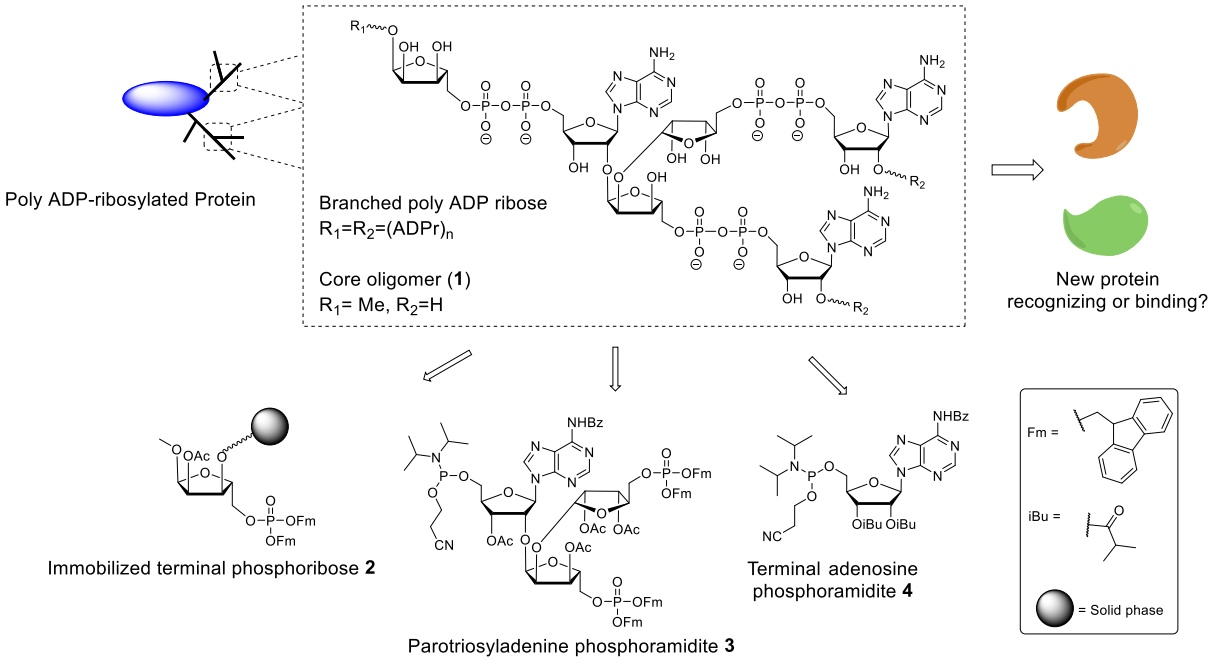
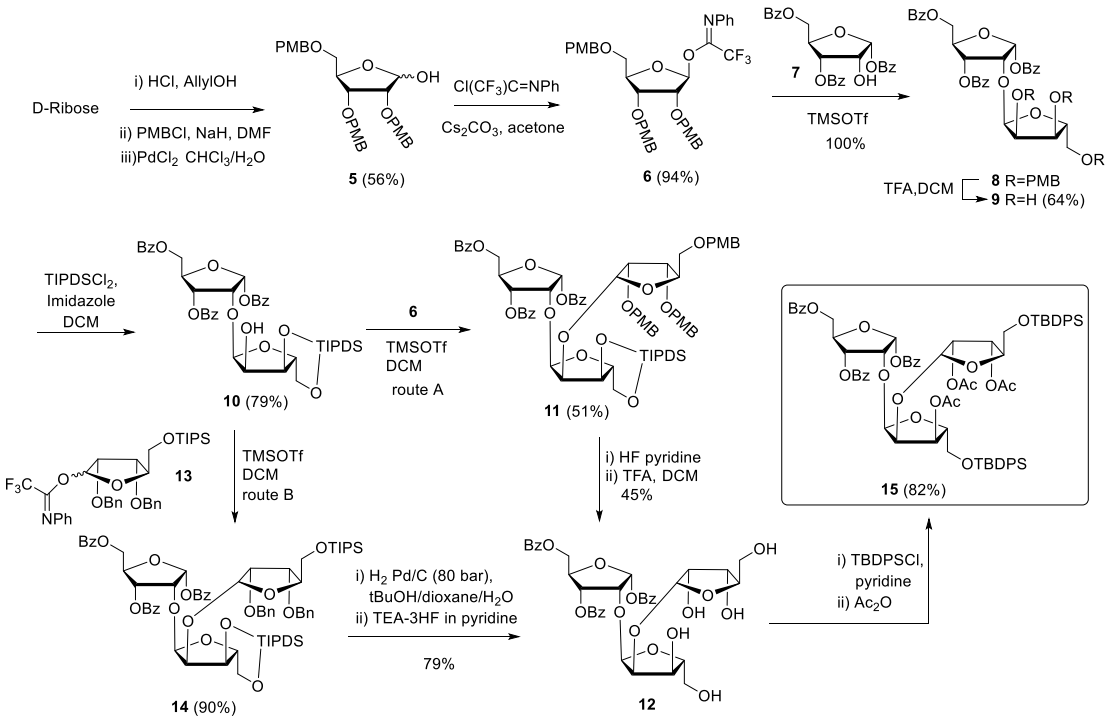


Figure 1. The branched ADP-ribose trimer and its retrosynthetic analysis

Results and discussion

The three anionic pyrophosphates functions in target ADPr-trimer **1** ensure that a solid phase approach is preferred over a solution phase approach. Retrosynthetically this means that oligomer **1** can be prepared with the aid of three components (Figure 1): (a) immobilized terminal phosphoribose **2**; (b) orthogonally protected phosphoramidite **3** of parotriosyl adenine and (c) known terminal adenosine phosphoramidite **4**.[18-21] The successful solid phase synthesis of linear ADPr oligomers, using a bis (9-fluorenyl)methyl (Fm) protected phosphoramidite (Chapter 4), was an incentive to use a similar strategy for the construction of branched oligomers using building blocks compounds **2**, **3** and **4** with solely base labile protecting groups. Notable in the synthetic scheme is the advanced key phosphoramidite **3** which should enable the first pyrophosphate construction. Not only the construction of phosphoramidite **3** is challenging but also its application in the P(III)-P(V) method for the first pyrophosphate introduction. An additional challenge represents the use of phosphoramidite

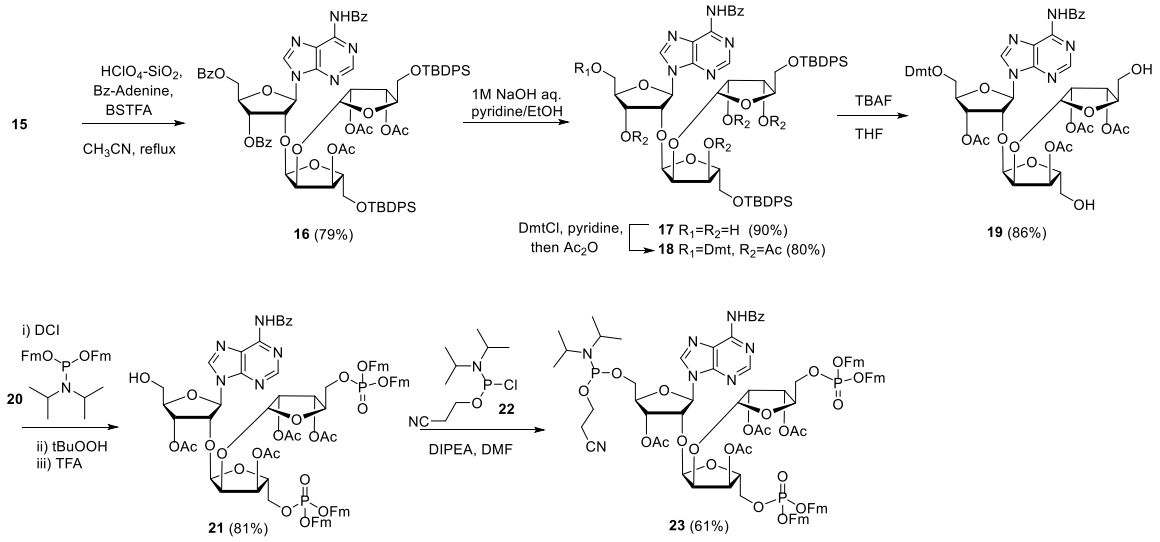
4 for the simultaneous introduction of two pyrophosphates in a molecule already provided with one pyrophosphate.



Scheme 1. Synthesis of protected parotriose **15**

The synthesis of immobilized terminal phosphoribose **2** and known terminal adenosine phosphoramidite **4** are described in Chapter 4. The synthesis of phosphoramidite **3** of a hyperglycosylated nucleoside with orthogonal protecting groups is depicted in Scheme 1 and 2. The key hurdle in the synthesis of **3** is the conversion of D-ribose into protected parotriose **15**, having two 1,2 cis-α-ribosidic bonds which are difficult to introduce (Scheme 1). Our previous method has enabled the selective construction of this core motif[16, 17] however the applied benzyl protective groups were difficult to remove. To circumvent this, PMB (p-methoxybenzyl) protected donor **6** was devised here in order to avoid hydrogenolysis and to preserve the α-selectivity. Firstly, D-ribose was allylated, per-methoxylbenzylated and finally de-allylated using $PdCl_2$ as a catalyst,[22, 23] to yield 2,3,5-tri-*O*-*p*-methoxylbenzyl-D-ribofuranose **5**. Conversion of **5** into the corresponding imidate donor **6** with 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride and Cs_2CO_3 in acetone proceeded in good yield. Gratifyingly, the first TMSOTf mediated glycosylation using donor **6** and known acceptor **7**[19] (Chapter 4) furnished disaccharide **8** in high yield and excellent stereoselectivity (only α product). Subsequent rapid acidolysis using TFA removed all PMBs affording **9**. An attempted deprotection of the PMBs with DDQ resulted in a lower yield due to the formation of a 2,3-methoxybenzylidene side product. Replacement of the Bn groups by the PMB groups in the synthesis of key disaccharide **9** avoids hydrogenolysis while the overall

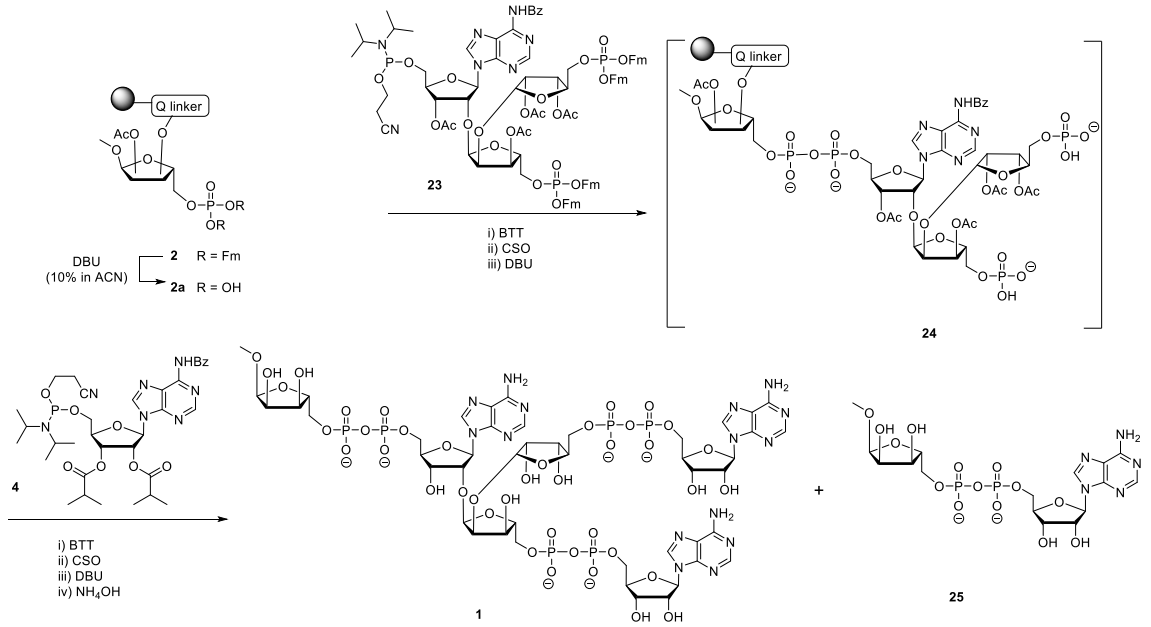
yield is slightly improved.[16] Next, the 3,5-OH was silylated to give TIPDS protected **10**, allowing a selective glycosylation on 2-OH. Condensation of donor **6** and acceptor **10** using the same TMSOTf activation conditions as applied for the synthesis of **8** gave trisaccharide **11** with excellent stereoselectivity (only α product) but in a moderate yield. Subsequently, all PMBs and silyl group need to be removed for upcoming protecting groups manipulation. Unfortunately, pentol **12** was obtained in only 45% yield after HF-pyridine mediated desilylation and TFA assisted removal of the PMBs. Switching the order of deprotection sequence does not give a better yield. The low yield could be attributed to acidic cleavage of one or both glycosidic bonds. To avoid the problematic acidolysis, known donor **13** (Chapter **2**)[16, 21] was used for the trisaccharide construction (route B). Condensation of donor **13** with acceptor **10** using the same TMSOTf/DCM condition gave trisaccharide **14** in a good yield and with excellent α stereoselectivity. Pd/C catalyzed high pressured (80 bar) hydrogenolysis followed by TEA/HF mediated desilylation produced **12** in 79% yield, compared with 45% from route A. Apparently, route B is more suitable for the large scale synthesis of **12**. Next, the protective group manipulation could be continued by selective silylation of both primary OHs in pentol **12** followed by acetylating of all secondary OHs to furnish protected parotriose **15** in 82% yield.



Scheme 2. Synthesis of phosphoramidite **23**

Now, the stage was set for the introduction of N^6-benzoylated adenine at the anomeric position of **15** via a Vorbrüggen type glycosylation catalyzed by $HClO_4$-SiO_2 to afford protected parotrosyl adenine **16** (Scheme 2).[16] The introduction of the two phosphotriesters and the phosphoramidite was attained by the following synthetic manipulations: 1M aq. NaOH was used for saponification of all acetyl and benzoyl esters to give **17** in a high yield. The released 5'-OH in **17** should be provided with cyanoethyl phosphoramidite whereas the Fm-protected phosphate triesters should be installed on the other two

primary positions, temporarily protected with TBDPS groups. Dimethoxytritylation of 5'-OH in **17**, followed by acetylation of the remaining secondary alcohols in the same reaction vessel gave **18**. Two silyl groups in **18** were carefully removed by TBAF to liberate terminal 5''-OH and 5'''-OH (**19**), allowing access to a high yielding single-operation cascade: a) DCI mediated phosphitylation of these OHs with Fm phosphoramidite **20**b) oxidation of the resulting phosphites to phosphate triesters by *t*BuOOH and c) treatment with stoichiometrical TFA to rapidly remove the DMT to obtain **21**. Finally, key phosphoramidite **23** was obtained by treatment of alcohol **21** with standard aminophosphorochloridite **22** and DIPEA in DMF. It is essential to use a neutralized silica gel column for the purification of **23** as it shares the base- and acid-sensitivity with its previously reported linear counterpart (Chapter 4). Eventually, starting from D-ribose, the advanced phosphoramidite **23** was prepared via the 14 step-sequence in sufficient amount (0.36 mmol) for the purposes of the solid phase synthesis of branched ADPr-oligomers.

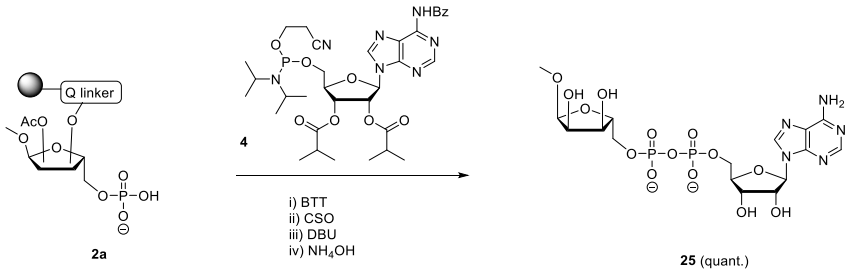


Scheme 3. Synthesis of branched core oligo-ADPr **1**

With sufficient amounts of branched amidite **23** at the disposal, the solid phase synthesis of target branched tri-ADPr **1**, could be undertaking on an automated oligonucleotide synthesizer (Scheme 3). The solid phase mediated introduction of pyrophosphate moieties using P(V)-P(III) chemistry requires, besides a suitable phosphoramidite, the availability of an immobilized phosphate monoester. Immobilized terminal ribose **2** was prepared by functionalization of Tentagel N, a solid support specifically intended for nucleotide synthesis, with a properly protected 5-O-phosphate of ribose equipped with Q linker, as described in Chapter 4. Removal of Fm groups in **2** by DBU treatment (10% in ACN) furnished immobilized phosphomonoester **2a**. The first pyrophosphate was introduced by the following three step procedure: a) BTT assisted P(V)-P(III) coupling of **2a** and phosphoramidite **23**, b)

CSO oxidation of resulting phosphite to phosphate c) DBU (10% in ACN) mediated cleavage of both cyanoethyl (CE) and terminal Fm groups. The produced immobilized intermediate **24** has two phosphate monoester function allowing the simultaneous introduction of the next two pyrophosphates by the same three step P(V)-P(III) coupling cycle using terminal phosphoramidite **4**. Final treatment of the obtained intermediate with aqueous NH_4OH removed all the protecting groups and cleaved the target molecule from solid support.

Analysis of the crude reaction mixture by anion exchange column chromatography and LC-MS showed target branched ADPr **1** together with mono ADPr **25** as a side product. Surprisingly, intermediate **24** (in which the protecting groups and Q-linker are lacking) could not be detected by LC-MS. This outcome suggests that the double pyrophosphate formation using amidite **4** was complete while the formation of monomer **25** probably results from the incomplete pyrophosphate coupling in the first step. Final anion exchange chromatography purification led to the isolation of 0.68 mg of target branched ADPr **1** and 2.83 mg of ADP-ribose (**25**). With a goal to suppress the formation of side product **25**, the use of CPG resin[21, 24] instead of Tentagel was attempted but did not show a better result, as evidenced by the analysis with anion exchange HPLC and ^{31}P-NMR. In addition, increasing the excess of amidite **23** or elongating coupling time of the reaction of **23** with **2a** also did not have a benefical effect. Finally, to estimate the reactivity of the immobilized phosphate monoester 2a, it was coupled with phosphoramidite **4** (Scheme 4), yielding only monomer **25** as gauged by LCMS and ^{31}P-NMR, while unreacted 1-methyl-5-phosphoribose side-product could not be detected. These data collectively show that the reactivity of phosphoramidite **23** for pyrophosphate formation is much lower than that of normal phosphoramidite such as **4**. The bulky phosphate triesters on **23,** containing four Fm groups might affect the reactivity and thereby the rate of the reaction of the phosphoramidite on immobilized phosphate monoester **2a**.



Scheme 4. Direct coupling between **2a** and **4**

Conclusion

In conclusion, this chapter describes the first total synthesis of the branched ADPr **1** that contains three ADPr units. The novel key phosphoramidite reagent **23**, functionalized with two phosphate

triesters was synthesized in 14 steps starting from D-ribose. The three pyrophoshates were introduced on solid support using P(V)-P(III) chemistry, a procedure which allowed the simultaneous introduction of two pyrophoshates. Although the yield of branched ADPr oligomer is low, sufficient amounts can be obtained for implementation in biochemical assays such as protein binding and co-crystallization with interesting target proteins such as PARG and APLF. The developed modular synthesis route also allows for the future modification of branched poly ADPr molecules, for example, clickable propargyl branched ADPr-oligomers.

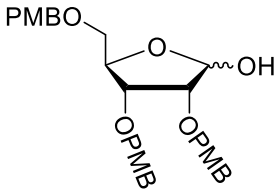
Acknowledgments

Nico Meeuwenoord is kindly acknowledged for his help in oligomer synthesis and final product purification.

Experimental section

General procedure

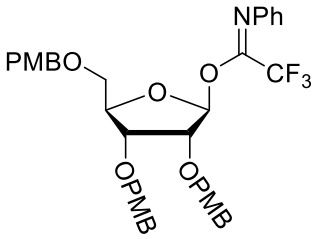
All chemicals were used as received unless stated otherwise. All solvents used in the reaction (including solid phase synthesis) were dried over 3Å molecular sieves. Solvents removal by rotary evaporation was under reduced pressure at 40°C. TLC, NMR, LCMS, anion exchange, HRMS, IR, optical rotation facilities were used as described in Chapter 2.



2,3,5-tri-*O*-*p*-methoxylbenzyl-D-ribofuranose (5)

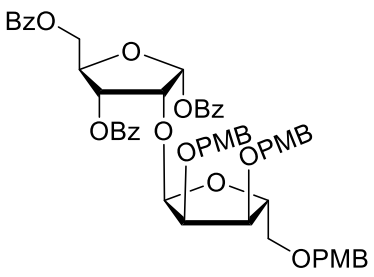
D-Ribose (6.0 g, 40 mmol), allyl alcohol (100 mL) and acetyl chloride (2.0mL, 28 mmol) were added into a flask and the mixture was stirred for 2.5 hours after which was quenched by $NaHCO_3$ solid and filtered over celite. The filtration was concentrated under reduced pressure and co-evaporated with toluene (2 x). To the residue, DMF (100 mL) was added and cooled down to 0 °C. NaH (60% in mineral oil, 6.4 g, 160 mmol) was added in 3 portions. After hydrogen generation ceased, 4-methoxybenzyl chloride (19 mL, 14 mmol) was added dropwise at 0°C under N_2. The mixture was stirred overnight after which was quenched by aqueous saturated NH_4Cl. EA and H_2O were added into the mixture and the organic layer was washed additionally by H_2O (2 x) and brine (1 x) and dried by $MgSO_4$. The mixture was concentrated under reduced pressure. To the residue, $CHCl_3$ (120 mL), H_2O (80 mL) and $PdCl_2$ (2.12 g, 12 mmol) were added and the mixture was vigorously stirred at 50 °C under O_2 atmosphere for 48 hours after which the mixture was concentrated under reduced pressure. EA (50 mL) and saturated aqueous $NaHCO_3$ (50 mL) were added into the residue and the organic layer was separated, dried by $MgSO_4$, filtered and concentrated under reduced pressure. Silica gel column chromatography (pentane/EA, 100/0 – 90/10 – 70/30) furnished **5** as light brown oil (11.44 g, 22.42 mmol, 56 %, α-product: 60%, β-product: 40%). ^{1}H-NMR and ^{13}C-NMR showed identical date with reported **5**.[22] ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.35 –

7.10 (m, 6H, arom.), 6.94 – 6.73 (m, 6H, arom.), 5.27 – 5.28 (m, 1H, H1), 4.67 – 4.37 (m, 5.6H, CH_2 PMB), 4.32 – 4.24 (m, 1.4H, H4, C*H*H PMB), 4.19 – 4.15 (m, 1.4H, OH, H3-β), 3.95 – 3.89 (m, 1.2H, H3-α, H2-β), 3.83 – 3.77 (m, 9.4H, OMe, H2-β), 3.61 (AB, *J* = 10.3, 2.8 Hz, 0.4H, H5-β), 3.47 – 3.37 (m, 1.6H, H5-αβ). ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.55, 159.50, 159.49, 159.48, 159.37, 130.12, 130.02, 129.98 (cq. arom.), 129.82, 129.75 (arom.), 129.71 (Cq. arom.), 129.69, 129.68, 129.56 (arom.), 129.51(Cq. arom.), 129.34, 114.00, 113.97, 113.93, 113.91, 113.90 (arom.), 100.52 (C1-β), 96.38 (C1-α), 81.10 (C4-α), 81.08 (C4-β), 80.55 (C2-β), 77.48 (C2-α), 77.36 (C3-α), 76.89 (C3-β), 73.27, 72.48, 72.20, 72.15, 72.03 (CH2 PMB), 69.82 (C5-α), 69.16 (C5-β), 55.40 (OMe-α), 55.38 (OMe-β).



1-*O*-((*N*-Phenyl)-2,2,2-trifluoroacetimido)-2,3,4-tri-*O*-*p*-methoxylbenzyl-D-ribofuranose (6)

Compound **5** (9.3 g, 18.23 mmol) was dissolved in acetone (93 mL). Cs_2CO_3 (8.89 g, 27.34 mmol,) and 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (3.23 mL, 20.05 mmol) were added and the reaction mixture was stirred at room temperature for 3 hours. After filtered over celite, the solvent was removed and the residue was purified using silica gel column chromatography neutralized with 1% Et_3N (Pentane/EA, 100/0 – 90/10 – 80/20) to afford the title compound as a light yellow oil (11.67 g, 17.13 mmol, 94 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.35 – 7.04 (m, 9H, arom.), 6.89 – 6.75 (m, 8H, arom), 6.27 (s, 1H, H1), 4.77 – 4.31 (m, 7H, CH_2 PMB, H4), 4.08 (t, *J* = 6.1 Hz, 1H, H3), 4.01 (d, *J* = 4.6 Hz, 1H, H2), 3.82 – 3.76 (m, 9H, OMe), 3.68 – 3.65 (m, 1H, H5), 3.54 (AB, *J* = 11.2, 5.2 Hz, 1H, H5). ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.60, 159.54, 159.28, 143.97, 130.42 (Cq. arom.), 129.96 (arom.), 129.76 (Cq. arom.),, 129.66 (arom.), 129.58 (arom.), 129.53 (Cq. arom.), 129.34, 129.32, 128.85, 113.96, 113.92, 113.84, 113.80 (arom.), 102.65 (C1), 82.34 (C4), 78.17 (C2), 77.05 (C3), 73.27, 73.04, 72.42, 72.22, 72.01 (CH_2 PMB-αβ), 69.89, 69.54 (C5-αβ), 55.40, 55.39 (OMe, αβ). IR (film): 2935, 2837, 1709, 1612, 1512, 1302, 1246, 1205, 1156, 1110, 1033, 819, 755, 695, 515 cm^{-1}. HRMS (ESI^+) calcd for $C_{29}H_{34}O_8Na$ (M+Na) 533.2146. Found 533.2147. $[\alpha]_D^{20}$ +41.6 (c = 1, in DCM)



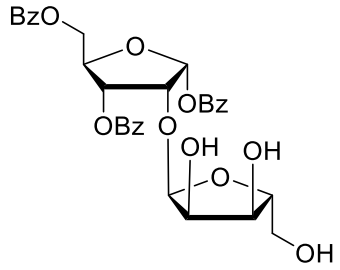
α-1,3,5-Tri-*O*-benzoyl-2',3',5'-tri-*O*-*p*-methoxylbenzyl-parobiose (8)

Compound **6** (5.9 g, 8.66 mmol) and α-D-ribofuranose 1,3,5-tribenzoate **7** (3.64 g, 7.87 mmol) were co-evaporated with toluene (1 x), 1,4-dioxane (2 x) and DCE (1 x), dissolved in dry DCM and stirred with freshly activated 3 Å molecular sieves at room temperature for 1 hour under N_2 to remove traces of water. The solution was then cooled to -78 ℃ and TMSOTf (28 µL, 0.16 mmol) was added to the reaction mixture. The reaction was being stirred at the same temperature for 10 minutes after which it was quenched by the addition of triethylamine. The reaction mixture was concentrated under reduced pressure and purified by silica gel chromatography (DCM/EtOAc, 97/3 – 95/5) to afford **8** as a white foam (7.51 g, 7.87 mmol, 100%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.17 – 8.14 (m, 4H, arom.), 8.10 – 8.02 (m, 2H, arom.), 7.58 – 7.46 (m, 3H, arom.), 7.44 – 7.40 (dd, *J* = 8.2, 7.0 Hz, 2H, arom.), 7.33 – 7.19 (m, 4H, arom.), 7.14 – 7.07 (m, 2H, arom.), 7.03 – 6.94 (m, 4H, arom.), 6.83 – 6.80 (m, 2H, arom., H1'), 6.69 – 6.58 (m, 4H, arom.), 5.68 (dd, *J* = 6.4, 1.9 Hz, 1H, H3'),

5.29 (d, *J* = 4.0 Hz, 1H, H1''), 4.77 – 4.70 (m, 2H, H4', H2'), 4.70 – 4.55 (m, 2H, H5'), 4.52 – 4.16 (m, 7H, CH_2 PMB, H4''), 3.89 – 3.79 (m, 2H, H2'', H3''), 3.79 (s, 3H, OMe), 3.73 (s, 3H, OMe), 3.72 (s, 3H, OMe), 3.42 (AB, *J* = 10.8, 3.3 Hz, 1H, H5''), 3.31 (AB, *J* = 10.8, 3.7 Hz, 1H, H5'').

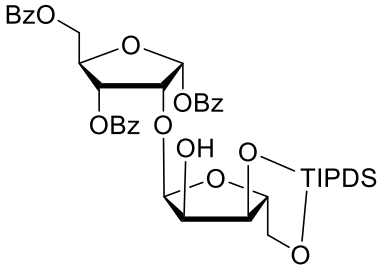
^{13}C NMR (101 MHz, $CDCl_3$) δ 166.32, 166.12, 165.76 (CO Bz), 159.19, 158.99, 158.96 (Cq. arom.), 133.24, 133.16 (Cq. arom.), 130.33 (arom.), 130.18 (Cq. arom.), 130.04, 130.00, 129.87 (arom.), 129.75 (Cq. arom.), 129.64 (arom.), 129.29, 129.22, 128.99, 128.53, 128.35, 128.31, 113.73, 113.53, 113.50 (arom.), 102.01 (C1''), 95.12 (C1'), 83.38 (C4'), 81.67 (C4''), 77.60 (C2''), 75.44 (C2'), 75.27 (C3''), 72.98 (CH_2 PMB), 72.24 (C3'), 72.07 (CH_2 PMB), 71.70 (CH_2 PMB), 69.05 (C5''), 64.35 (C5'), 55.25, 55.22, 55.21 (OMe). IR (film): 2934, 1721, 1612, 1513, 1451, 1266, 1248, 1175, 1111, 1068, 1026, 820, 710, 516 cm^{-1}. HRMS (ESI^+) calcd for $C_{55}H_{54}O_{15}Na$ (M+Na) 977.3355. Found 977.3357. $[\alpha]_D^{20}$ +84.0 (c = 1, in DCM)



α-1,3,5-tri-*O*-benzoylparobiose (9)

Compound **8** (7.0 g, 7.34 mmol), DCM (60 mL) and TFA (3.37 mL, 44 mmol) were added into a flask and the reaction was stirred for 90 minutes after which was quenched by saturated aqueous $NaHCO_3$. DCM extracted the mixture (4 x) and the organic layers were combined, dried ($MgSO_4$) and filtered. The filtration was concentrated under reduced pressure and purified by silica gel chromatography (DCM/acetone, 100/1 – 100/7 – 90/10 – 80/20) to furnish **9** as a white foam (3.22 g, 5.42 mmol, 74%).

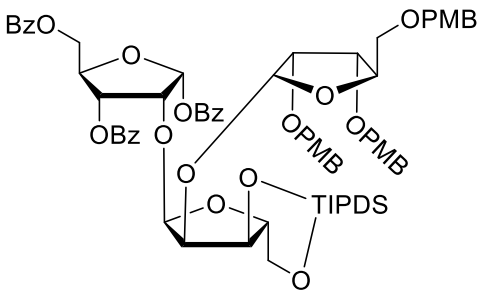
^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.13 – 8.03 (m, 6H, arom.), 7.63 – 7.55 (m, 3H, arom.), 7.47 (t, *J* = 7.8 Hz, 2H, arom.), 7.43 – 7.33 (m, 4H, arom.), 6.76 (d, *J* = 4.2 Hz, 1H, H1'), 5.74 (dd, *J* = 6.3, 2.0 Hz, 1H, H3'), 5.19 (d, *J* = 4.2 Hz, 1H, H1''), 4.87 (td, *J* = 3.8, 1.9 Hz, 1H, H4'), 4.75 (dd, *J* = 6.3, 4.2 Hz, 1H, H2'), 4.63 (AB, *J* = 12.1, 3.8 Hz, 2H, H5'), 4.00 – 3.97 (m, 2H, H2'', H4''), 3.86 (s, 1H, H3'), 3.64 (AB, *J* = 12.2, 3.1 Hz, 1H, H5''), 3.55 (AB, *J* = 12.1, 3.9 Hz, 1H, H5''), 2.73 (s, 1H, OH), 2.56 (s, 1H, OH), 2.04 (s, 1H, OH). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.82, 166.14, 165.92 (CO Bz), 133.94, 133.66, 133.57, 130.04, 130.03, 129.79 (arom.), 129.66, 129.51, 129.05 (Cq. arom.), 128.71, 128.57 (arom.), 102.26 (C1''), 95.31 (C1'), 86.38 (C4''), 82.77 (C4'), 75.38 (C2'), 72.30 (C2''), 72.20 (C3'), 70.51 (C3''), 64.21 (C5'), 62.56 (C5'').



α-1,3,5-tri-*O*-benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-parobiose (10)

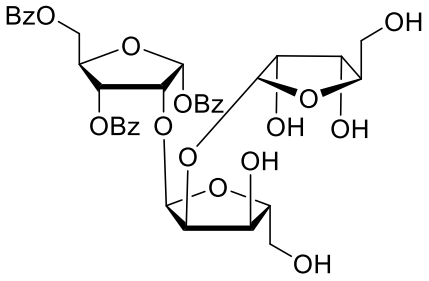
Compound **9** (2.48 g, 4.17 mmol) and imidazole (852 mg, 12.52 mmol) were co-evaporated with toluene (2 x), dissolved in DCM (41 mL) and then $TIPDSCl_2$ (1.6 mL, 5.01 mmol) was added. The reaction was stirred at room temperature for 16 hours and quenched upon the addition of H_2O (200 mL). The mixture was washed by DCM (3 x) and the organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was purified by silica gel chromatography (DCM/acetone, 100/0 – 97/3) to obtain **10**[16] as colorless foam (2.75 g, 3.29 mmol, 79%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.20 – 8.02 (m, 6H, arom.), 7.65 – 7.53 (m, 3H, arom.), 7.53 – 7.44 (m, 2H, arom.),

7.44 – 7.35 (m, 4H, arom.), 6.79 (d, *J* = 4.2 Hz, 1H, H1'), 5.67 (dd, *J* = 6.4, 2.1 Hz, 1H, H3'), 5.19 (d, *J* = 4.2 Hz, 1H, H1''), 4.81 – 4.73 (m, 2H, H2', H4'), 4.73 – 4.58 (m, 2H, H5'), 4.12 – 4.05 (ddd, *J* = 19.8, 7.7, 4.6 Hz, 2H, H2'', H3''), 3.93 (ddd, *J* = 8.4, 5.0, 3.5 Hz, 1H, H4''), 3.82 (dd, *J* = 11.7, 3.6 Hz, 1H, H5''), 3.66 (AB, *J* = 11.7, 8.3 Hz, 1H, H5''), 2.84 (d, *J* = 8.6 Hz, 1H, OH), 1.13 – 0.83 (m, 24H, CH_3 TBDPS), 0.80 (d, *J* = 7.3 Hz, 2H, CH TIPDS), 0.74 (d, *J* = 7.3 Hz, 2H, CH TIPDS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.15, 165.70 (CO Bz), 133.49, 133.46, 133.43, 130.16, 129.99, 129.82 (arom.), 129.71 (Cq. arom.), 128.67, 128.57, 128.49 (arom.), 101.96 (C1''), 95.14 (C1'), 83.87 (C4''), 83.32 (C4'), 75.67 (C2'), 71.95 (C3'), 71.08 (C2''), 70.84 (C3''), 64.31 (C5'), 63.50 (C5''), 17.55, 17.49, 17.46, 17.41, 17.07, 16.98, 16.83, 16.69, 13.46, 13.27, 13.04, 12.38 (CH, CH_3 TIPDS).



α-1,3,5-tri-*O*-benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'',3'',5''-di-*O*-*p*-methoxylbenzyl-parobiose (11)

Compound **10** (1.43 g, 1.71 mmol) and **6** (1.40 g, 2.05 mmol) were co-evaporated with toluene (2 x), 1,4-dioxane (2 x) and DCE (1 x), dissolved in dry DCM and stirred with freshly activated 3 Å molecular sieves at room temperature for 1 hour under N_2 to remove traces of water. The solution was then cooled to -78 °C and TMSOTf (10 µL, 0.05 mmol) was added to the reaction mixture. The reaction was stirred at the same temperature for 30 minutes after which it was quenched by the addition of triethylamine. The reaction mixture was concentrated under reduced pressure and purified by silica gel chromatography (pentane/EtOAc, 100/0 – 90/10 – 80/20) to afford **11** as a white foam (1.16 g, 0.87 mmol, 51%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.18 – 8.11 (m, 2H, arom.), 8.07 (d, *J* = 6.9 Hz, 4H, arom.), 7.61 – 7.51 (m, 2H, arom.), 7.47 (td, *J* = 7.6, 3.9 Hz, 3H, arom.), 7.35 (t, *J* = 7.8 Hz, 2H, arom.), 7.27 – 7.16 (m, 4H, arom.), 7.12 – 7.02 (m, 4H, arom.), 6.90 – 6.67 (m, 7H, arom. H1'), 5.65 (dd, *J* = 6.2, 1.8 Hz, 1H, H3'), 5.38 (d, *J* = 3.5 Hz, 1H, H1''), 5.32 (d, *J* = 3.8 Hz, 1H, H1'''), 4.85 (dd, *J* = 6.2, 4.1 Hz, 1H, H2'), 4.75 – 4.60 (m, 3H, H4', H5', C*H*H PMB), 4.54 (AB, *J* = 12.0, 4.0 Hz, 1H, H5'), 4.48 – 4.45 (m, 2H, H2'', C*H*H PMB), 4.34 (dd, *J* = 32.6, 11.5 Hz, 2H, C*H*2, PMB), 4.26 – 4.00 (m, 4H, H4''', H3'', C*H*H PMB, H4''), 3.89 – 3.67 (m, 12H, H5'', C*H*H PMB, OMe PMB), 3.61 – 3.47 (m, 2H, H3''', H5'''), 3.47 – 3.33 (m, 2H, H2''', H5'''), 1.12 – 0.80 (m, 28H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.04, 165.87, 165.59 (CO Bz), 159.14, 159.05, 158.84 (Cq. arom.), 133.45, 133.39, 133.24 (arom.), 131.05, 130.43, 130.38 (Cq. arom.), 130.08 (arom.), 129.92 (Cq. arom.), 129.88, 129.81 (arom.), 129.65, 129.57, 129.43, 129.26, 129.21, 128.55, 128.52, 128.48, 113.73, 113.70, 113.66, 113.61, 113.49, 113.32 (arom.), 102.31 (C1''), 101.30 (C1'''), 94.98 (C1'), 83.35 (C4'), 80.89 (C4''), 79.10 (C4'''), 76.30 (C2'''), 75.51 (C3'''), 75.31 (C2'), 73.24 (C2''), 72.96 (CH2 PMB), 72.28 (C3'), 72.07, 71.10 (CH2 PMB), 68.92 (C3''), 68.73 (C5'''), 64.20 (C5'), 59.82 (C5''), 55.23, 55.21, 55.16 (OMe), 17.42, 17.37, 17.32, 17.17, 17.08, 17.05, 16.94 (CH_3 TIPDS), 13.48, 13.03, 12.67, 12.51 (CH TIPDS). IR (film): 2944, 2867, 1724, 1613, 1514, 1266, 1248, 1112, 1035, 711cm^{-1}. HRMS (ESI$^+$) calcd for $C_{72}H_{92}NO_{20}Si_2$ (M+NH_4) 1346.5746. Found 1346.5753. $[\alpha]_D^{20}$ +77.6 (c = 1, in DCM)

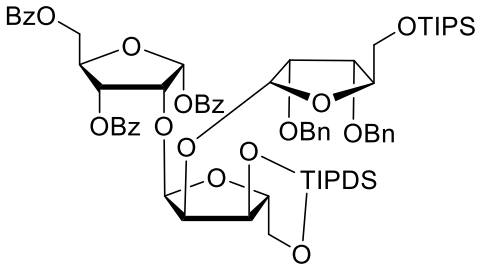


α-1,3,5-tri-*O*-benzoyl-2'',3''-di-*O*-benzylparotriose (12)

The procedure from **11**:

Compound **11** (430 mg, 0.32 mmol) and pyridine (3 mL) were added into a flask and the reaction was cooled to 0°C. Subsequently, HF-pyridine (0.25 mL, 9.62 mmol) was added at the same temperature under N_2. The reaction was stirred at room temperature for 3 hours and quenched by the addition of aqueous saturated $NaHCO_3$. The mixture was extracted by DCM (3 x) and the organic layers are combined and dried ($MgSO_4$). The mixture was filtered, concentrated under reduced pressure and co-evaporated with toluene (3 x). To the residue, DCM (3 mL) and TFA (0.25 mL, 3.27 mmol) were added and the reaction was stirred for 20 minutes after which was quenched by addition of aqueous saturated $NaHCO_3$. The mixture was extracted by DCM (3 x), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was purified by silica gel chromatography (DCM/Methanol, 100/0 – 99/1 – 97/3 – 95/5 – 96/4) to obtain **12** as a white foam (106 mg, 0.15 mmol, 47%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.17 – 7.97 (m, 6H, arom.), 7.63 – 7.50 (m, 3H. arom.), 7.50 – 7.32 (m, 6H, arom.), 6.76 (d, *J* = 4.1 Hz, 1H, H1'), 5.69 (dd, *J* = 6.3, 1.7 Hz, 1H, H3'), 5.27 (d, *J* = 4.0 Hz, 1H, H1''), 4.95 (d, *J* = 4.0 Hz, 1H, H1'''), 4.82 (td, *J* = 4.1, 1.6 Hz, 1H, H4'), 4.72 (dd, *J* = 6.3, 4.2 Hz, 1H, H2'), 4.61 (AB, *J* = 12.0, 4.1 Hz, 2H, H5'), 4.07 (dd, *J* = 5.8, 4.0 Hz, 1H, H2''), 3.98 – 3.93 (m, 3H, H3'', H4''', H4''), 3.72 – 3.35 (m, 7H, H3''', H2''', H5'', H5''', OH), 3.30 (s, 1H, OH), 3.22 – 3.08 (m, 1H, OH), 3.00 – 2.94 (m, 2H, OH). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.67, 166.20, 165.96 (CO Bz), 133.85, 133.77, 133.52, 130.16, 129.79 (arom.), 129.56, 129.54, 129.14 (Cq. arom.), 128.67, 128.58, (arom.) 101.44 (C1''), 101.05 (C1'''), 95.37 (C1'), 86.33 (C4''), 85.75 (C4'''), 83.21 (C4'), 75.34 (C2'), 75.29 (C2''), 72.46 (C2'''), 72.16 (C3'), 70.91 (C3''), 70.85 (C3'''), 64.31 (C5'), 62.78 (C5'''), 62.19 (C5'').

Procedure from **14** to **12**



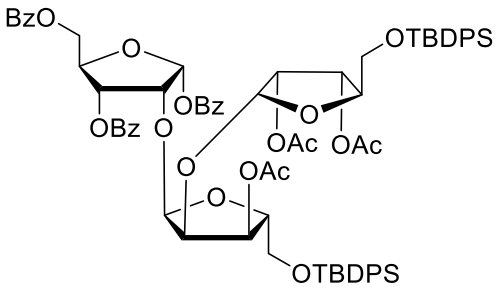
α-1,3,5-tri-*O*-benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'',3''-di-*O*-benzyl-5''-*O*-triisopropylsilylparotriose (14)

Compound **14** was synthesized according to our previously reported procedure with minor modification.[16] Compound **10** (4.7 g, 5.61 mmol) and **13** (5.17 g, 7.86 mmol) were co-evaporated with toluene (1 x), 1,4-dioxane (2 x) and DCE (1 x). Dry DCM (94 mL) and freshly activated 3Å molecular sieves were added to the mixture. The reaction was stirred under N_2 at room temperature for 2 hours and then cooled to -78 °C. Next, TMSOTf (30 uL, 0.17 mmol) was added, the reaction mixture was stirred at the same temperature for 15 minutes and then was quenched by addition of triethylamine. The reaction mixture was concentrated under reduced pressure and purified by silica gel chromatography (pentane/EA, 100/0 – 100/3 – 90/10) to obtain **14** as a white foam (6.58 g, 5.04 mmol, 90%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.17 – 8.12 (m, 2H, arom.), 8.08 (ddd, *J* = 11.8, 8.4, 1.4 Hz, 4H, arom.), 7.59 – 7.53 (m, 2H, arom.), 7.52 – 7.43 (m, 3H, arom.), 7.36 (t, *J* = 7.8 Hz, 2H, arom.), 7.29 – 7.14 (m, 12H, arom.), 6.79 (d, *J* = 4.1 Hz, 1H, H1'), 5.60 (dd, *J* = 6.3, 1.9 Hz, 1H, H3'), 5.36 (d, *J* = 3.5 Hz, 1H, H1''), 5.27 (d, *J* = 3.8 Hz, 1H, H1'''), 4.84 (dd, *J* = 6.3, 4.1 Hz, 1H, H2'), 4.76 – 4.66 (m, 2H, CH_2 Bn, H4'), 4.62 (AB, *J* = 12.0,

3.4 Hz, 1H, H5'), 4.52 (AB, *J* = 12.0, 4.0 Hz, 1H, H5'), 4.46 – 4.43 (m, 1H, C*H*H Bn), 4.40 (dd, *J* = 5.3, 3.5 Hz, 1H, H2''), 4.32 (d, *J* = 11.8 Hz, 1H, CH_2, Bn), 4.22 – 4.14 (m, 2H, H3'', H4'''), 4.04 (dt, *J* = 8.8, 2.5 Hz, 1H, H4''), 3.97 (d, *J* = 11.4 Hz, 1H, C*H*H Bn), 3.82 (AB, *J* = 13.0, 2.4 Hz, 1H, H5''), 3.78 – 3.64 (m, 4H, H5'', H5''', H3'''), 3.44 (dd, *J* = 6.4, 3.8 Hz, 1H, C*H*H Bn), 1.13 – 0.85 (m, 49H, TIPS, TIPDS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.14, 165.99, 165.64 (CO Bz), 138.98, 138.71 (Cq. arom.), 133.48, 133.45, 133.28, 130.14 (arom.), 130.06 (Cq. arom.), 130.00, 129.92 (arom.), 129.80, 129.70 (Cq. arom.), 128.61, 128.55, 128.53, 128.12, 127.99, 127.84, 127.68, 127.34, 127.14 (arom.), 102.22 (C1''), 101.25 (C1'''), 95.08 (C1'), 83.33 (C4'), 81.18 (C4'''), 81.16 (C4''), 77.38 (C2'''), 75.61 (C3'''), 75.14 (C2'), 73.61 (C2''), 72.34 (C3'), 72.30 (CH_2, Bn), 71.78 (CH_2, Bn), 69.09 (C3'''), 64.25 (C5'), 62.64 (C5''), 60.02 (C5'''), 18.06, 17.51, 17.46, 17.41, 17.40, 17.21, 17.16, 17.08, 16.95, 13.60, 13.13, 12.78, 12.60, 12.03 (CH_3, CH, TIPDS, TIPS).

α-1,3,5-tri-*O*-benzoyl-2'',3''-di-*O*-benzylparotriose (12)

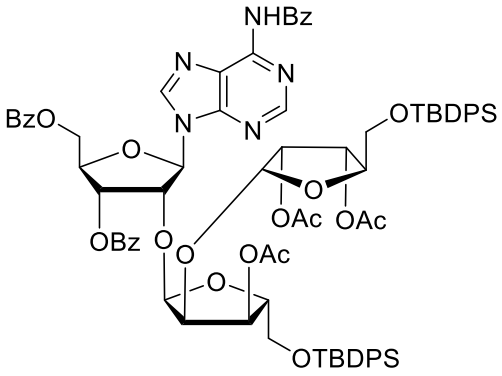
Compound **14** (3.21 g, 2.46 mmol) was dissolved in *t*BuOH/dioxane/H_2O (50 mL, 4/4/1; v/v/v), Pd/C (500 mg, 10% loading) and one drop of AcOH were added. The mixture was sonicated under N_2 for 10 minutes then was transferred into an autoclave. The reaction in the autoclave was stirred for 16 hours under 80 bar of H_2 after which was filtered and concentrated under reduced pressure. The residue was co-evaporated with toluene (3 x) and pyridine (3 x). To the intermediate, pyridine (12 mL), triethylamine (5.14 mL, 36.91 mmol) and $Et_3N{\cdot}3HF$ (6.02 mL, 36.91 mmol) were added successively under 0 °C. The reaction was allowed to warm up to room temperature and stirred for 16 hours after which was quenched carefully by addition of aqueous saturated $NaHCO_3$. DCM extracted (3x) the mixture and dried over $MgSO_4$. Purification by silica gel chromatography (DCM/Methanol, 100/0 – 95/5 – 90/10) to obtain **12**[16] as white foam (1.41 g, 1.94 mmol, 79%).



α-1,3,5-tri-*O*-benzoyl-3'-*O*-acetyl-5'-*O*-tertbutyldiphenylsilyl-2'',3''-di-*O*-acetyl-5''-*O*-tertbutyldiphenylsilylparotriose (15)

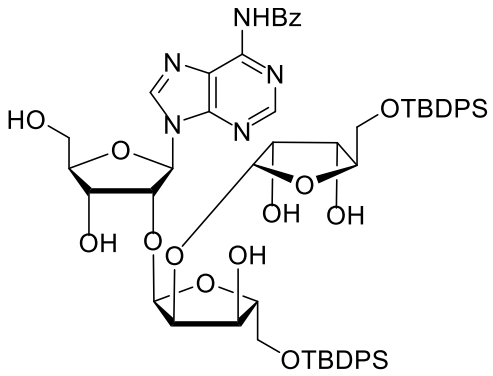
Compound **12** (2.36 g, 3.25 mmol) was co-evaporated with pyridine (1 x) and then N_2 was applied. Pyridine (32 mL) and TBDPSCl (2.54 mL, 9.75 mmol) were added and the mixture was stirred under N_2 at room temperature for 16 hours. Ac_2O (9.2 mL, 97.5 mmol) was added into the reaction and the mixture was stirred for 6 hours after which was quenched by addition of aqueous saturated $NaHCO_3$. The mixture was extracted by DCM (3 x), dried ($MgSO_4$) and concentrated under reduced pressure. Purification by silica gel chromatography (pentane/actone, 100/0 – 90/10 – 80/20) furnished **15**[16] as a white foam (3.54 g, 2.66 mmol, 82%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.23 – 8.17 (m, 2H, arom.), 8.17 – 8.13 (m, 2H, arom.), 8.11 – 8.04 (m, 2H, arom.), 7.65 (dq, *J* = 6.5, 1.5 Hz, 4H, arom.), 7.63 – 7.50 (m, 7H, arom.), 7.45 – 7.30 (m, 18H, arom.), 6.80 (d, *J* = 4.3 Hz, 1H, H1'), 5.72 (dd, *J* = 6.3, 1.8 Hz, 1H, H3'), 5.46 (dd, *J* = 6.6, 1.9 Hz, 1H, H3''), 5.39 (dd, *J* = 7.0, 3.2 Hz, 1H, H3'''), 5.31 (d, *J* = 4.2 Hz, 1H, H1''), 5.29 (d, *J* = 4.4 Hz, 1H, H1'''), 4.88 (dd, *J* = 7.0, 4.4 Hz, 1H, H2'''), 4.76 (td, *J* = 3.8, 1.8 Hz, 1H, H4'), 4.71 – 4.58 (m, 3H, H2', H5'), 4.35 (dd, *J* = 6.6, 4.2 Hz, 1H, H2'''), 4.09 – 4.06 (m, 2H, H4''', H4''), 3.81 (AB, *J* = 11.2, 2.8 Hz, 1H, H5''), 3.69 (AB, *J* = 11.2, 3.1 Hz, 1H, H5''), 3.67 – 3.58 (m, 2H, H5'''), 2.01 (s,

3H, Ac), 1.78 (s, 3H, Ac), 1.63 (s, 3H, Ac), 1.05 (s, 9H, CH_3 TBDPS), 0.97 (s, 10H, CH_3, TBDPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.70, 170.10, 169.70 (CO, Ac), 166.18, 166.15, 165.57 (CO, Bz), 135.76, 135.74, 135.72, 135.68, 133.51, 133.49 (arom.), 133.16, 133.11, 133.04, 132.98, 130.28 (Cq. arom.), 130.17, 130.13 (arom.), 129.99 (Cq. arom.), 129.96, 129.94, 129.90, 129.86, 129.81 (arom.), 129.75 (Cq. arom.), 128.70, 128.51, 127.93, 127.91, 127.90, 127.88 (arom.), 101.29 (C1''), 99.53 (C1'''), 95.17 (C1'), 83.74 (C4''), 83.67 (C4'), 83.14 (C4'''), 76.35 (C2'), 74.63 (C2''), 71.76 (C2'''), 71.67 (C3'), 71.26 (C3''), 69.84 (C3'''), 64.45 (C5'), 63.92 (C5''), 63.43 (C5'''), 26.91, 26.86 (CH_3 TBDPS), 20.70, 20.39, 20.17 (Ac), 19.38, 19.30 (Cq. TBDPS).



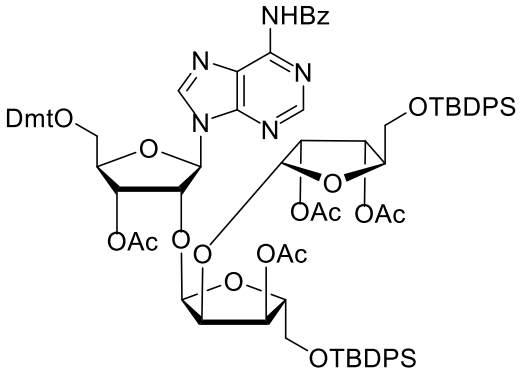
6-*N*-benzoyl-9-(3',5'-di-*O*-benzoyl-3''-*O*-acetyl-5''-*O*-tertbutyldiphenylsilyl-2''',3'''-di-*O*-acetyl-5'''-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (16)

Compound **15** (1.93 g, 1.48 mmol) and N^6-benzoyladenine (0.71 g, 2.97 mmol) were co-evaporated with 1,4-dioxane (2 x), ACN (1 x) and dissolved in dry ACN (24 mL) under N_2. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (5.56 mL, 20.76 mmol) was added and the mixture was stirred at room temperature for 5 minutes. $HClO_4$-SiO_2 (7.4 g, 0.4 mmol/g, 2.96 mmol) was added and the mixture was refluxed for 16 hours. The reaction was quenched by aqueous saturated $NaHCO_3$ then filtered. The mixture was extracted with EtOAc (3 x), dried ($MgSO_4$) and concentrated under reduced pressure. Purification by silica gel chromatography (pentane/acetone, 100/0 – 85/15 – 80/20) gave **16**[16] as a white foam (1.82 g, 1.26 mmol, 85%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.99 (s, 1H, NH), 8.68 (d, *J* = 1.8 Hz, 1H, H2), 8.40 (d, *J* = 1.9 Hz, 1H, H8), 8.07 (tt, *J* = 6.6, 1.6 Hz, 4H, arom.), 8.01 – 7.93 (m, 2H, arom.), 7.65 – 7.47 (m, 13H, arom.), 7.46 – 7.25 (m, 16H, arom.), 6.32 (dd, *J* = 4.7, 1.8 Hz, 1H, H1'), 5.94 (td, *J* = 5.4, 1.8 Hz, 1H, H3'), 5.68 (t, *J* = 5.0 Hz, 1H, H2'), 5.43 (dt, *J* = 6.9, 2.2 Hz, 1H, H3''), 5.41 – 5.37 (m, 1H, H3'''), 5.24 (dd, *J* = 4.4, 1.8 Hz, 1H, H1''), 5.16 (dd, *J* = 4.5, 1.8 Hz, 1H, H1'''), 4.93 (ddd, *J* = 7.2, 4.3, 1.7 Hz, 1H, H2'''), 4.91 – 4.85 (m, 1H, H5'), 4.75 (q, *J* = 4.6 Hz, 1H, H4'), 4.70 (AB, *J* = 11.9, 5.0 Hz, 1H, H5'), 4.31 (ddd, *J* = 6.8, 4.3, 1.8 Hz, 1H, H2''), 4.09 (t, *J* = 2.9 Hz, 1H, H4'''), 4.00 (q, *J* = 2.7 Hz, 1H, H4'), 3.79 – 3.76 (m, 1H, H5'''), 3.71 – 3.68 (m, 1H, H5'''), 3.59 – 3.56 (m, 1H, H5''), 3.45 – 3.41 (m, 1H, H5''), 2.10 (s, 3H, Ac), 2.07 (s, 3H, Ac), 1.68 (s, 3H, Ac), 1.01 (s, 9H, CH_3 TBDPS), 0.95 (s, 9H, TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.57, 169.93, 169.83 (CO Ac), 166.30, 165.40, 164.49 (CO Bz), 152.94 (CH C2), 151.41 (Cq. arom.), 149.95 (CH C8), 149.80 (Cq. arom), 136.09, 135.67 (Cq. arom), 133.79 (Cq. arom), 133.63, 133.49 (arom.), 133.03, 132.98, 132.94 (Cq. arom.), 132.87 (arom.), 132.82 (Cq. arom.), 129.93, 129.90, 129.88 (aom.), 129.63, 129.60 (Cq. arom.), 128.97, 128.64, 128.59, 127.93, 127.91, 127.89 (arom.), 123.95 (Cq. arom.), 123.86 (arom.), 101.26 (C1''), 98.72 (C1'''), 89.14 (C1'), 83.11 (C4'''), 82.45 (C4''), 80.52 (C4'), 77.48 (C2'), 73.09 (C2''), 72.49 (C3'), 71.79 (C2'''), 71.02 (C3''), 69.86 (C3'''), 63.62 (C5'), 63.57 (C5''), 63.19 (C5'''), 26.87 (CH_3 TBDPS), 26.81 (CH_3 TBDPS), 20.77, 20.68, 20.43 (CH_3 Ac), 19.29, 19.26 (Cq. TBDPS).



6-*N*-benzoyl-9-(5'',5'''-di-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (17)

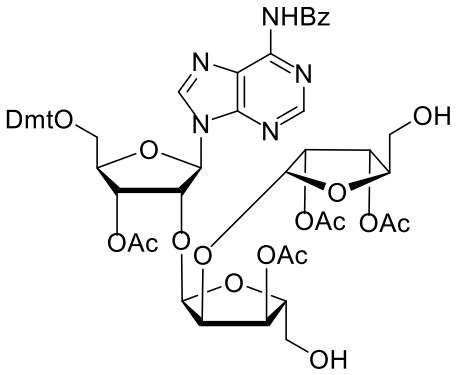
Compound **16** (1.82 g, 1.26 mmol) was dissolved in pyridine/EtOH (12.6 mL; 2/1 v/v), cooled to 0 °C after which aqueous NaOH (7.56 mL, 1 M solution) was slowly added. The reaction mixture was stirred for 2 hours at the same temperature after which Amberlite-H^+ was added until pH = 6. The mixture was filtered, concentrated under reduced pressure and purified by silica gel chromatography (DCM/methanol, 100/0 – 97/3 – 95/5) to obtain **17**[16] as a white foam (1.26 g, 1.13 mmol, 90%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.31 (s, 1H, NH), 8.82 (s, 1H, H2), 8.39 (s, 1H, H8), 8.02 – 7.95 (m, 2H, arom.), 7.67 – 7.54 (m, 8H, arom.), 7.48 (t, *J* = 7.8 Hz, 2H, arom.), 7.44 – 7.28 (m, 13H, arom.), 6.15 (d, *J* = 7.5 Hz, 1H, H1'), 6.07 (br, 1H, OH), 5.09 (d, *J* = 4.5 Hz, 1H, H1''), 5.00 (d, *J* = 4.0 Hz, 1H, H1'''), 4.94 (dd, *J* = 7.6, 4.6 Hz, 1H, H2'), 4.60 (d, *J* = 4.7 Hz, 1H, H3'), 4.41 (t, *J* = 4.8 Hz, 1H, H2''), 4.39 – 4.33 (m, 2H, H3''', H4'), 4.31 – 4.19 (m, 4H, H2''', H3''', H4'', H4'''), 4.00 (AB, *J* = 13.0, 1.8 Hz, 1H, H5'), 3.78 (d, *J* = 12.8 Hz, 1H, H5'), 3.75 – 3.64 (m, 4H, H5'', H5'''), 1.00 (s, 9H, TBDPS), 1.00 (s, 9H, TBDPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.86 (CO Bz), 150.75, 150.45 (Cq. arom.), 135.69, 135.68, 135.66, 135.64 (arom.), 133.61, 133.09 (Cq. arom.), 133.05 (arom.), 132.89, 132.87, 132.71 (arom.), 130.09, 130.06, 130.04, 129.97, 129.00, 128.11, 127.99, 127.99, 127.97, 127.91 (arom.), 124.66 (Cq. arom.), 102.17 (C1'''), 101.26 (C1''), 89.74 (C1'), 88.39 (C4'), 86.56 (C4''), 86.18 (C4'''), 80.15 (C2'), 73.13 (C2'''), 73.01 (C3'), 72.10 (C3''), 71.34 (C3'''), 64.36 (C5'''), 64.19 (C5''), 63.47 (C5'), 26.95 (CH3 TBDPS), 26.93 (CH_3 TBDPS), 19.34 (Cq. TBDPS), 19.30 (Cq. TBDPS).



6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-5'-*O*-dimethoxyltrityl-5'',5'''-di-*O*-tertbutyldiphenylsilyl-β-parotriosyl)adenine (18)

Compound **17** (1.26 g, 1.13 mmol) was co-evaporated with pyridine (1 x), then N_2 was applied. Dry pyridine (5.6 mL) and 4,4'-dimethoxyltrityl chloride (DMTCl, 612 mg, 1.81 mmol) was added into the flask. 1 hour later, TLC showed incomplete conversion (DCM:methanol = 9.5:0.5 as eluent) and additional DMTCl (153 mg, 0.45 mmol) was added. The reaction was stirred for 1 hour after which it was cooled down to 0°C. Ac_2O (2.13 mL, 22.6 mmol) was added to the reaction flask. The mixture was stirred at 0°C for 5 h after which was quenched by aqueous saturated $NaHCO_3$. DCM extracted (3 x) the mixture and the organic layers were combined, dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification by silica gel column chromatography (pentane/acetone, 100/0 – 90/10 – 85/15 – 80/20 – 70/30) furnished **18** as a white foam (1.43 g, 0.90 mmol, 80%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.97 (s, 1H, NH), 8.67 (s, 1H, H2), 8.38 (s, 1H, H8), 8.03 – 7.94 (m, 2H, arom.), 7.68 – 7.56 (m, 9H, arom.), 7.56 – 7.48 (m, 2H, arom.), 7.45 – 7.17 (m, 21H, arom.), 6.82 – 6.75 (m, 4H, DMT), 6.27 (d, *J* = 4.3 Hz, 1H, H1'), 5.57 – 5.47 (m, 2H, H3', H3''), 5.42 (dd, *J* = 7.3, 3.3 Hz, 1H, H3'''), 5.35 (t, *J* = 4.9 Hz, 1H, H2'), 5.27 (d, *J* = 4.4 Hz, 1H, H1''),

5.25 (d, *J* = 4.4 Hz, 1H, H1’’’), 4.99 (dd, *J* = 7.3, 4.4 Hz, 1H, H2’’’), 4.41 (q, *J* = 4.3 Hz, 1H, H4’), 4.35 (dd, *J* = 6.8, 4.3 Hz, 1H, H2’’), 4.15 (q, *J* = 3.1 Hz, 1H, H4’’’), 4.10 (q, *J* = 2.9 Hz, 1H, H4’’), 3.85 – 3.65 (m, 10H, OMe DMT, H5’’’, H5’’), 3.64 – 3.53 (m, 2H, H5’), 3.46 (AB, *J* = 10.6, 4.5 Hz, 1H, H5’), 2.13 (s, 3H, CH_3 Ac), 2.08 (s, 6H, CH_3 Ac), 2.05 (s, 3H, CH_3 Ac), 1.03 (s, 10H, CH_3, TBDPS), 0.99 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.59, 169.92, 169.72, 169.69 (CO Ac), 164.52 (CO Bz), 158.66, 158.64, 151.44, 149.62, 144.49 (Cq. arom.), 135.68, 135.65 (arom.), 135.58, 133.83, 133.03, 132.96, 132.85, 132.84 (Cq. arom.), 130.21, 130.14, 129.95, 129.92, 129.90, 128.96, 128.27, 127.99, 127.93, 127.90, 127.88, 127.85, 127.08 (arom.), 123.69 (Cq. arom.), 113.26 (arom.), 100.79 (C1’’), 99.15 (C1’’’), 88.41 (C1’), 86.75 (Cq. DMT), 83.19 (C4’’), 82.72 (C4’’’), 81.98 (C4’), 77.23 (C2’), 73.58 (C2’’), 71.92 (C3’), 71.67 (C2’’), 71.26 (C3’’), 69.91 (C3’’’), 63.79 (C5’’), 63.29 (C5’’’), 62.66 (C5’), 55.31 (OMe DMT), 26.86, 26.82 (CH_3 TBDPS), 21.08, 20.96, 20.82, 20.55 (CH_3 Ac), 19.29, 19.29 (Cq. TBDPS). IR (film): 2935, 1743, 1739, 1507, 1245, 1241, 1236, 1233, 1227, 1223, 1178, 1175, 1113, 1107, 1092, 1037, 1030, 703 cm^{-1}. HRMS (ESI^+) calcd for $C_{88}H_{96}N_5O_{19}Si_2$ (M+H) 1582.6311. Found 1582.6273. $[\alpha]_D^{20}$ +56.9 (c = 1, in $CHCl_3$)

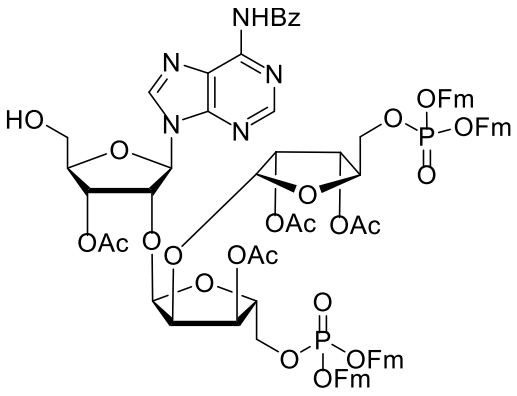


6-*N*-benzoyl-9-(3’,3’’2’’’,3’’’-tetra-*O*-acetyl-5’-*O*-dimethoxyltrityl-β-parotriosyl)adenine (19)

18 (1.43 g, 0.90 mmol), dry THF (9 mL) and TBAF (tetrabutylammonium fluoride solution 1.0 M in THF, 2.7 mL, 2.7 mmol) was added into a flask and the mixture was stirred for 16 hours at room temperature. Excess of EtOAc was added and the mixture was washed by H_2O (2 x) and brine (1 x). The organic layer was dried by $MgSO_4$. The mixture was filtered, concentrated under reduced pressure and purified by silica gel column chromatography (DCM/methanol, 100/0 – 100/1 – 100/2 – 100/3) to obtain **19** as a white foam (0.86 g, 0.78 mmol, 87%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.31 (s, 1H, NH), 8.61 (s, 1H, H2), 8.38 (s, 1H, H8), 8.08 – 7.99 (m, 2H, arom.), 7.63 – 7.55 (m, 1H, arom.), 7.55 – 7.47 (m, 2H, arom.), 7.45 – 7.40 (m, 2H, arom.), 7.36 – 7.28 (m, 4H, arom.), 7.28 – 7.18 (m, 3H, arom.), 6.79 (dd, *J* = 9.0, 3.0 Hz, 4H, DMT arom.), 6.24 (d, *J* = 5.6 Hz, 1H, H1’), 5.57 (dd, *J* = 5.4, 3.9 Hz, 1H, H3’), 5.45 (t, *J* = 5.5 Hz, 1H, H2’), 5.24 (dd, *J* = 7.3, 3.4 Hz, 1H, H3’’), 5.15 (dd, *J* = 7.3, 4.0 Hz, 1H, H3’’’), 5.11 (d, *J* = 4.3 Hz, 1H, H1’’), 5.03 (d, *J* = 4.4 Hz, 1H, H1’’’), 4.84 (dd, *J* = 7.3, 4.4 Hz, 1H, H2’’’), 4.37 (d, *J* = 4.0 Hz, 1H, H4’), 4.13 – 4.10 (m, 2H, H2’’, H4’’), 4.05 (q, *J* = 3.2 Hz, 1H, H4’’’), 3.81 – 3.61 (m, 10H, OMe DMT, H5’’, H5’’’), 3.56 (AB, *J* = 10.6, 3.9 Hz, 1H, H5’), 3.46 (AB, *J* = 10.6, 4.6 Hz, 1H, H5’), 3.07 (bs, 1H, OH), 2.53 (bs, 1H, OH), 2.14 (s, 3H, CH_3 Ac), 2.10 (s, 3H, CH_3 Ac), 2.08 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.64, 170.16, 169.77, 169.73 (CO Ac), 165.07 (CO Bz), 158.64 (Cq. arom.), 152.75 (C2), 151.68, 149.74, 144.49, 135.65, 135.62, 133.55 (Cq. arom.), 132.95, 130.22, 130.19, 128.94, 128.28, 128.09, 127.97, 127.07 (arom.), 123.84 (Cq. arom), 113.24 (arom.), 101.14 (C1’’), 98.77 (C1’’’), 87.69 (C1’), 86.78 (Cq. arom.), 82.83 (C4’’), 82.56 (C4’’’), 82.39 (C4’), 76.93 (C2’), 73.04 (C2’’), 72.19 (C3’), 71.36 (C2’’’), 70.60 (C3’’), 69.74 (C3’’’), 62.97 (C5’), 62.24 (C5’’), 61.80 (C5’’’), 55.33 (OMe DMT), 21.03, 21.02, 20.77, 20.55 (CH_3 Ac).

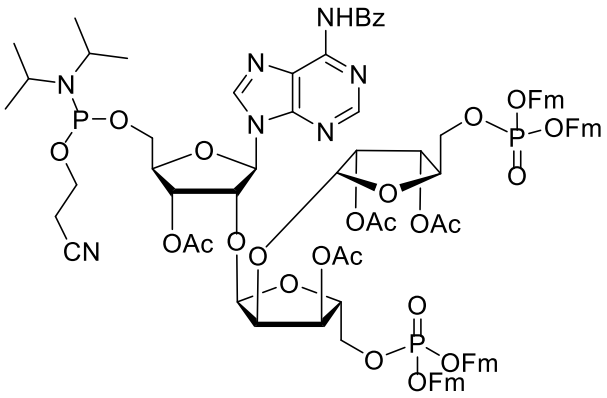
IR (film): 2935, 1739, 1734, 1730, 1609, 1607, 1583, 1507, 1456, 1448, 1369, 1238, 1227, 1224, 1176, 1090, 1030, 829, 734, 705 cm^{-1}. HRMS (ESI^+) calcd for $C_{56}H_{60}N_5O_{19}$ (M+H) 1106.3877. Found 1106.3896. $[\alpha]_D^{20}$ +68.0 (c = 1, in

$CHCl_3$)



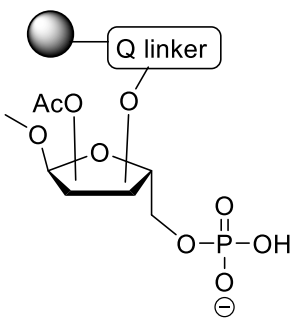
6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-5'',5'''-di-*O*-(di-fluorenyl)-β-parotriosyl)adenine (21)

Compound **19** (0.86 g, 0.78 mmol), DCI activator (4,5-dicyanoimidazole solution 0.25 M in ACN, 12.48 mL, 3.12 mmol) and freshly activated 3Å molecular sieves were added into flask. Compound **20** (0.2 M in ACN, 11.7 mL, 2.34 mmol) was added into the mixture and the reaction was stirred for 10 minutes at room temperature after which *tBu*OOH (5.5 M in decane, 1.42 mL, 7.80 mmol) was added at 0 ℃. The reaction was stirred at the same temperature for 45 minutes and quenched by aqueous saturated $NaHCO_3$. The mixture was filtered and excessive amount of EtOAc was added to the filtration. The organic layer was washed by H_2O (1 x) and brine (2 x) and was dried (Na_2SO_4). The mixture was filtered, concentrated under reduced pressure and co-evaporated with toluene (3 x). To the residue, DCM (10.4 mL) and TFA (0.15 mL, 1.95 mmol) were added and the reaction was stirred for 10 minutes at room temperature after which was quenched aqueous saturated $NaHCO_3$. DCM extracted (2 x) the mixture and the organic layers were combined and washed by H_2O (1 x) and brine (1 x). The organic layers were dried ($NaSO_4$), filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (DCM/methanol, 100/0 – 100/1 – 100/2 – 100/3) to obtain **21** as a white foam (1.01 g, 0.60 mmol, 77%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.07 (s, 1H, NH), 8.78 (s, 1H, H2), 8.55 (s, 1H, H8), 7.95 – 7.89 (m, 2H, arom.), 7.72 – 7.61 (m, 8H, arom.), 7.56 – 7.51 (m, 1H, arom.), 7.48 – 7.14 (m, 26H), 6.24 (dd, *J* = 11.9, 2.4 Hz, 1H, OH), 6.12 (d, *J* = 8.0 Hz, 1H, H1'), 5.66 (d, *J* = 5.4 Hz, 1H, H3'), 5.15 (dd, *J* = 8.0, 5.4 Hz, 1H, H2'), 5.10 – 5.06 (m, 2H, H3'', H3'''), 4.94 (d, *J* = 4.4 Hz, 1H, H1''), 4.84 (dd, *J* = 7.7, 4.4 Hz, 1H, H2''), 4.70 (d, *J* = 4.3 Hz, 1H, H1'''), 4.31 – 3.80 (m, 22H, CH/CH2 Fm, H4', H2''', H4'', H4''', H5', H5'', H5'''), 2.19 (s, 3H, CH_3 Ac), 2.14 (s, 3H, CH_3 Ac), 2.08 (s, 3H, CH_3 Ac), 2.05 (s, 3H, CH_3 Ac). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.21, 169.82, 169.55, 169.46 (CO Ac), 164.41 (CO Bz), 152.31 (C2), 150.59, 150.36 (Cq. arom.), 143.11, 143.07, 143.00, 142.91, 141.43, 141.39, 133.50 (Cq. arom.), 132.87, 128.89, 127.95, 127.20, 127.17, 127.15, 127.13, 125.16, 125.13, 125.10 (arom.), 124.61 (Cq. arom.), 120.11, 120.08, 120.05 (arom.), 101.26 (C1'''), 98.15 (C1''), 89.28 (C1'), 86.88 (C4'), 79.97, 79.90 (C4'''), 79.50, 79.44 (C4''), 77.69 (C2'), 74.23 (C3'), 71.45 (C2'''), 71.08 (C2'), 69.85 (C3'''), 69.48, 69.43, 69.37 (CH_2 Fm), 69.06 (C3''), 66.32, 66.29 (C5''), 66.09, 66.05 (C5'''), 62.95 (C5'), 47.95, 47.93, 47.89, 47.87, 47.83 (CH Fm), 21.15, 20.81, 20.69, 20.65 (CH_3 Ac). ^{31}P NMR (202 MHz, $CDCl_3$) δ -1.15, -1.20. IR (film): 2931, 1743, 1739, 1582, 1451, 1448, 1238, 1234, 1103, 1067, 1016, 991, 759, 740 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{91}H_{84}N_5O_{23}P_2$ (M+H) 1676.5027. Found 1676.5161. $[\alpha]_D^{20}$ +38.3 (c = 1, in $CHCl_3$)



6-*N*-benzoyl-9-(3',3''2''',3'''-tetra-*O*-acetyl-5'-*O*-(*N*,*N*-diisopropylamino-*O*-cyanoethyl)phosphoramidite)-5'',5'''-di-*O*-(di-flourenyl)-β-parotriosyl)adenine (23)

Compound **21** (1.01 g, 0.60 mmol), DMF (6 mL), DIPEA (0.21 mL, 1.2 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite **22** (0.15 mL, 0.66 mmol) were added into the flask under N_2. The reaction was stirred at room temperature for 10 minutes after which was quenched by 0.3 mL methanol. Excessive amount of EtOAc was added and the mixture was washed with aqueous saturated $NaHCO_3$ (2 x), H_2O (1 x) and brine (1 x). The organic layer was dried (Na_2SO_4) and filtered. The filtration was co-evaporated with toluene (1 x) then purified by automatic column (DCM/acetone, 100/0 – 90/10 – 80/20) to furnish **23** as a white foam (684 mg, 0.36 mmol, 60%). **Note**: Careful wash was needed for the work-up because the DIPEA in the reaction could cleave the Fm group. Automatic column was performed on Biotage Isolera Specktra Four machine using High-quality IRR silica gel column (40-63 µm). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.39 – 9.15 (m, 1H, NH), 8.72 (d, *J* = 7.4 Hz, 1H, H2), 8.50 (d, *J* = 10.7 Hz, 1H, H8), 8.01 (d, *J* = 7.7 Hz, 2H, arom.), 7.68 – 7.13 (m, 35H), 6.24 (dd, *J* = 9.8, 4.8 Hz, 1H, H1'), 5.49 (dt, *J* = 15.0, 5.0 Hz, 1H, H3'), 5.20 (ddt, *J* = 13.5, 7.6, 3.9 Hz, 3H, H1'', H2', H3''), 5.14 – 5.05 (m, 2H, H1''', H3'''), 4.79 (td, *J* = 7.8, 4.4 Hz, 1H, H2'''), 4.40 (q, *J* = 4.1 Hz, 1H, H4'), 4.41 – 3.75 (m, 23H, CH_2/CH Fm, H4'', H4''', H2'', H5', H5'', H5''', O*C*H_2CH_2CN), 3.59 (dddd, *J* = 13.7, 9.5, 6.8, 2.5 Hz, 2H, $(CH_3)_2$*CH*), 2.72 (td, *J* = 6.2, 2.7 Hz, 1H, C*H*HCN), 2.66 (t, *J* = 6.3 Hz, 1H, C*H*HCN), 2.59 (dq, *J* = 6.2, 3.7, 3.0 Hz, 1H, C*H*HCN), 2.15 – 2.06 (m, 12H, CH_3 Ac), 1.22 – 1.10 (m, 12H, *(*CH_3*)*$_2$CHN). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.16, 170.15, 169.54, 169.52, 169.51, 169.47, 169.27, 169.23 (CO Ac), 164.62, 164.59 (CO Bz), 151.53, 151.43, 149.67, 142.99, 142.98, 142.95, 142.88, 142.87, 142.81, 141.27, 141.25, 141.24, 133.60, 132.58, 128.68, 128.66, 127.93, 127.84, 127.79, 127.04, 125.04, 125.02, 124.99, 124.94 (arom.), 123.82, 123.77 (Cq. arom.), 119.98, 119.97, 119.92, 119.88 (arom.), 117.86, 117.74 (CN), 100.70 (C1''), 98.80 (C1'''), 87.77, 87.48 (C1'), 82.25, 82.18, 82.07, 82.00 (C4'), 80.41, 80.35 (C4''), 80.30, 80.23 (C4'''), 77.64, 77.59 (C2'), 72.67 (C2''), 71.62, 71.52 (C3'), 70.81, 70.76 (C2'''), 69.84, 69.81 (C3''), 69.29, 69.24 (CH_2 Fm), 69.11 (C3'''), 66.41, 66.40, 66.37 (C5''), 66.19, 66.15 (C5'''), 62.13, 62.00, 61.87, 61.75 (C5'), 58.67, 58.62, 58.51, 58.45, 58.13, 58.08 (O*C*H_2CH_2CN), 47.81, 47.74 (CH Fm), 43.15, 43.12, 43.06, 43.02 ($(CH_3)_2$*CH*N), 24.65, 24.62, 24.59, 24.57, 24.54, 24.52 (*(*CH_3*)*$_2$CHN), 20.82, 20.76, 20.72, 20.49 (CH_3 Ac), 20.35, 20.30 (*C*H_2CN), 20.27 (CH_3 Ac). ^{31}P NMR (202 MHz, $CDCl_3$) δ 149.11, 148.96, 14.18 (H-phosphonate), -1.60, -1.62, -1.70, -1.73. IR (film): 2969, 1743, 1698, 1609, 1581, 1511, 1451, 1367, 1238, 1158, 1017, 984, 759, 742 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{94}H_{87}N_6O_{25}P_3$ ([H-phosphonate]+H) 1793.5007. Found 1793.5032. $[\alpha]_D^{20}$ +34.8 (c = 1, in DCM)



1-*O*-methyl-2-*O*-Q-Tentagel-3-*O*-acetyl-5-*O*-phosphate-α-D-ribofuranoside/1-*O*-methyl-2-*O*-acetyl-3-*O*-Q-Tentagel-5-*O*-phosphate-α-D-ribofuranoside (2a)

200 mg resin **2** was added into a 5 mL reaction syringe with filter frit and the resin was washed with ACN (5 x) under N_2. 3 mL DBU solution (10%, v/v, in ACN) was added into the syringe and was shaken for 20 minutes to remove Fm groups on 5-phosphate after which was drained. The DBU treatment was repeated for another 20 minutes. The resin was washed with ACN (5 x) and dried under reduced pressure to remove traces of water before use to furnish resin **2a**.

1-*O*-Methyl-α-branched core oligo-ADPr (1) and 1-*O*-methyl-α-mono ADPr (25)

50 mg (10 μmol) resin **2a** was transferred into a reaction column of a Mermade 6 oligonucleotide synthesizer and the complete synthesis was performed under an argon atmosphere.

Cycle A was performed 1 time and Cycle B was performed 1 time.

Cycle A:

The resin was rinsed with ACN (3 x) and drained. 5-(Benzylthio)-1*H*-tetrazole (BTT) (480 uL, 0.25 M in ACN) and **23** (400 uL, 0.1 M in ACN) were added into the resin and the mixture was left to stand for 10 minutes, drained. Repeat this coupling for two more times. The resin was rinsed with ACN (3 x). The intermediate phosphate-phosphite was oxidized with (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) solution (2 mL, 0.5 M in ACN) for 5 minutes (2 x). The resin was drained and washed with ACN (3x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the resin and was left to stand for 10 minutes (4 x) after which was drained and washed with ACN (3 x).

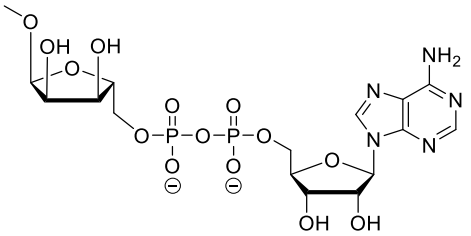
Cycle B:

The resin was rinsed with ACN (3 x) and drained. BTT (480 uL, 0.25 M in ACN) and **4** (400 uL, 0.1M in ACN) were added into the resin and the mixture was left to stand for 10 minutes, drained. Repeat this coupling for another 3 times. The resin was rinsed by with ACN (3 x). The intermediate phosphate-phosphite was oxidized with (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) solution (2 mL, 0.5 M in ACN) for 5 minutes (2 x). The resin was drained and washed with ACN (3 x). DBU solution (2 mL, 10%, v/v, in ACN) was added into the resin and was left to stand for 10 minutes after which was drained and washed with ACN (3 x).

After cycle A and B, the resin was transferred to a tube and treated with 10 mL NH_4OH (35%). The tube was sealed and stirred overnight, filtered and concentrated under reduced pressure. The crude was purified by anion exchange to obtain branched core oligomer **1** 0.68 mg (0.43 mmol, 4%) and mono-ADPr **25** 2.83 mg (4.94 mmol, 50%) as white solid.

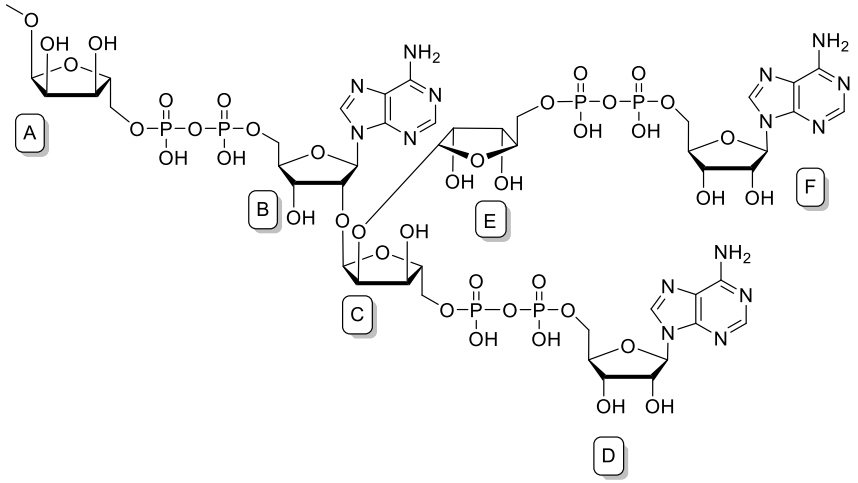
Column: Resource Q 6mL.

Gradient: 25% - 75%. (A: 10 mM NH_4OAc, B: 1 M NH_4OAc)



1-*O*-methyl-α-ADP-riboside (25)

^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.53 (s, 1H, H2), 8.28 (s, 1H, H8), 6.15 (d, *J* = 5.9 Hz, 1H, H1'), 4.54 (d, *J* = 4.5 Hz, 2H, H3'), 4.40 (d, *J* = 2.9 Hz, 1H, H4'), 4.23 (t, *J* = 4.0 Hz, 2H, H5'), 4.18 (s, 1H, H4''), 4.13 – 4.12 (m, 2H, H2'', H3''), 4.01 (t, *J* = 4.7 Hz, 2H, H5''), 3.38 (s, 3H, OMe). ^{13}C NMR (214 MHz, D_2O) δ 155.72 (C4), 152.95 (C8), 149.21 (C6), 118.70 (C5), 103.26 (C1''), 86.83 (C1'), 83.99, 83.95 (C4'), 83.23, 83.18 (C4''), 74.27 (C2'), 70.83 (C2''), 70.43 (C3'), 69.68 (C3''), 65.62, 65.60 (C5''), 65.22, 65.20 (C5'), 55.46 (OMe). ^{31}P NMR (202 MHz, D_2O) δ -10.47, -10.57, -10.68, -10.78. LC-MS: Rt = 3.57 min. 0-50% NH_4OAc. ESI MS+ calc. 574.1 found 574.1 $[M+1]^+$. HRMS (ESI^+) calcd for $C_{16}H_{26}N_5O_{14}P_2$ (M+H) 574.0946. Found 574.0949.



1-*O*-Methyl-α-branched tri-ADP riboside (1)

^{1}H NMR (850 MHz, Deuterium Oxide) δ 8.35 (s, 2H, H2), 8.20 (s, 1H, H2), 8.06 – 8.05 (m, 2H, H8), 7.99 (s, 1H, H8), 6.01 (d, *J* = 4.6 Hz, 1H, H1-B), 5.92 (d, *J* = 11.4 Hz, 2H, H1-DF), 5.13 – 5.06 (m, 1H, H1-C), 4.94 (d, *J* = 4.3 Hz, 1H, H1-E), 4.83 (t, *J* = 2.3 Hz, 1H, H1-A), 4.60 (t, *J* = 5.3 Hz, 1H, H2-D), 4.58 – 4.56 (m, 2H, H2-BF), 4.46 (t, *J* = 5.0 Hz, 1H, H3-B), 4.42 (t, *J* = 4.5 Hz, 1H, H3-F), 4.39 (s, 1H, H3-D), 4.30 – 3.93 (m, 24H, the rest H2, H3, H5), 3.30 (s, 3H, OMe). ^{13}C NMR (214 MHz, D_2O) δ 119.06, 119.05 (C5), 104.15 (C1-A), 101.98 (C1-E), 101.08 (C1-C), 88.07 (C1-D), 87.91 (C1-F), 87.25 (C1-B), 85.04, 84.76, 84.69, 84.67, 84.09, 84.06 (C4-ABDCEF), 80.02, 80.00, 75.61, 75.44, 75.34, 72.44, 71.72, 71.30, 71.14, 70.56, 70.53, 70.48, 70.26 (C2, C3-ABCDEF), 66.52, 66.39, 66.33, 66.18, 66.09, 65.67 (C5-ABDCEF), 56.36 (OMe). ^{31}P NMR (202 MHz, D_2O) δ -11.04, -11.11, -11.14, -11.17, -11.19, -11.24, -11.33, -11.43. LC-MS: Rt = 3.54 min. 0-50% NH_4OAc. ESI MS+ calc. 1656.2 found 1656.3 $[M+1]^+$.
HRMS (ESI^+) calcd for $C_{46}H_{68}N_{15}O_{40}P_6$ (M+H) 1656.2168. Found 1656.2171.

Procedure for direct coupling between **2a** and **4 :**

50 mg (10 µmol) resin **2a** was transferred into a reaction column of a Mermade 6 oligonucleotide synthesizer

and the complete synthesis was performed under an argon atmosphere. Cycle B was performed 1 time. Then the resin was transferred to a tube and treated with 10 mL NH_4OH (35%). The mixture was stirred overnight in a sealed condition, filtered and concentrated under reduced pressure. The crude was analyzed by LC-MS and ^{31}P-NMR to confirm the complete conversion from **2a** to **25**.

Supporting Data:

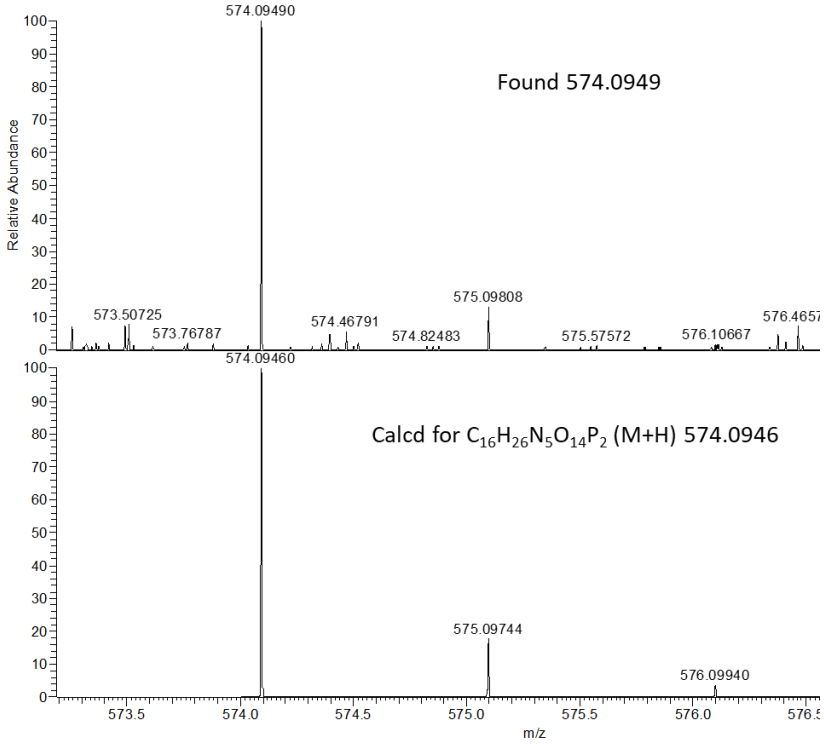


Figure 1S. HRMS data of mono ADPr **25**

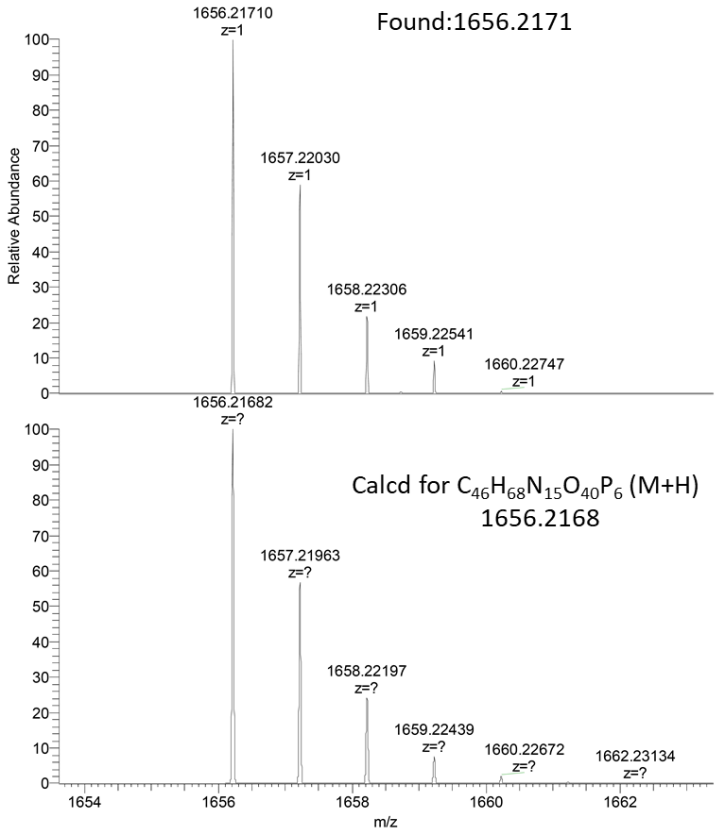


Figure 2S. HRMS data of branched tri-ADPr **1**

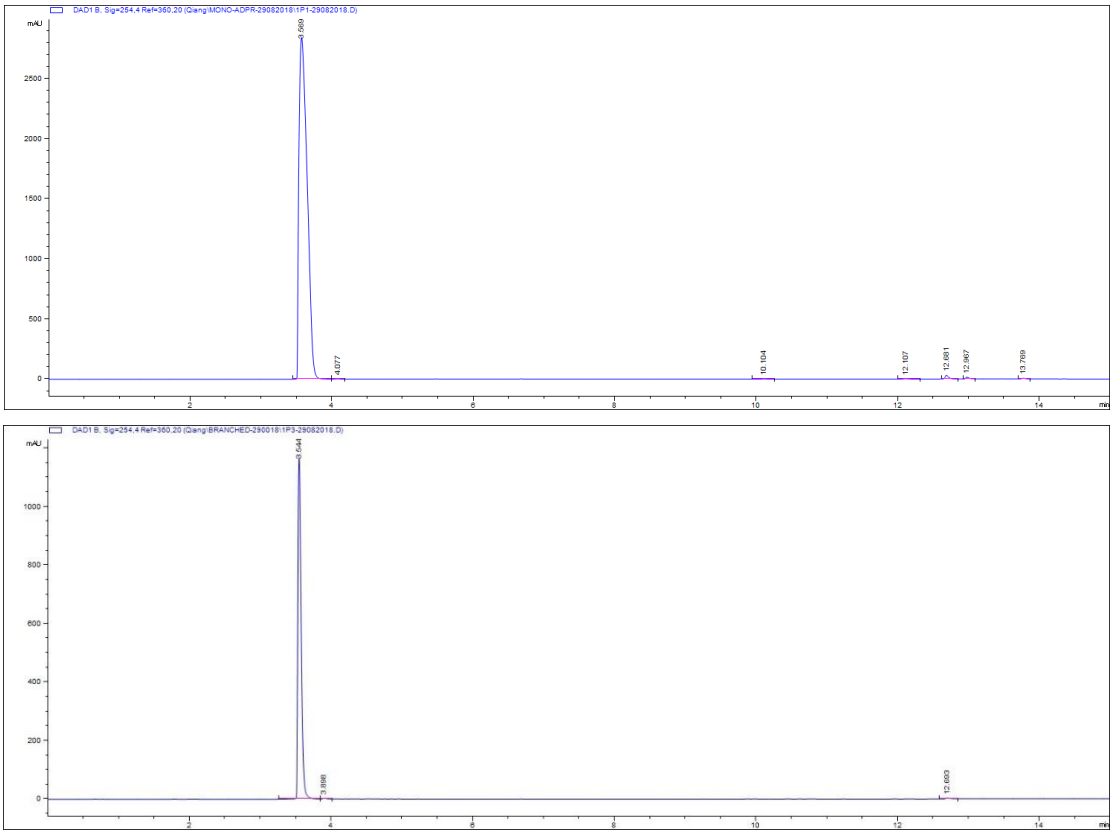


Figure 3S. UV spectra of LC-MS analysis of ADP-ribose **25** (upper) and branched ADPr **1** (lower)

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6 Synthesis of ADP-ribosylated asparagine as a stabilized isostere of ADPr-Asp for structural studies of macrodomains

Introduction

Post-translational modification (PTM) of proteins by ADP-ribosylation of specific amino acids regulates many cellular pathways that are critical for genome stability, including DNA repair, chromatin structure, mitosis and apoptosis.[1, 2] ADP-ribosylation has attracted therapeutic interest since its dysregulation appears to be linked to diseases such as cancers, diabetes, neurodegenerative disorders, ischemia, and inflammatory disorders.[3-5] ADP-ribosylation is a dynamic and reversible process, which is modulated through interplay between enzymes that covalently introduce the post-translational modifications and the enzymes that reverse these reactions. To date, nearly all nucleophilic amino acid side chains have been reported as targets of ADP-ribosylation including, aspartic acid, glutamic acid, arginine, lysine and most recently serine.[6] However, the origin of the selectivity and the mechanisms belonging to ADP-ribosylation are not completely understood. Macrodomains[7] are high-affinity ADP-ribose binding modules that exist in many important ADPr related proteins like PARPs, PARG and macroH2A 1.1. To investigate the interaction of ADP-ribosylated proteins and macrodomains, synthetic ADP-ribosylated oligopeptides have proven to be powerful tools to gather this information.[8-10] ADPr-Asp is considered to be one of the most widespread modification sites. The synthesis of ADPr-Asp containing oligopeptides is restricted by the lability of the ester linkage and its tendency to migrate from the anomeric center to 2-OH of ribose (Figure 1). One possible way to tackle this problem is to stabilize the glycosidic bond by applying ADPr-Asn, having an amide instead of an ester bond. ADPr-Asn

is not only a more stable isostere of ADPr-Asp, ADPr-Asn itself is also considered as an ADP-ribosylation site,[11] making ADPr-Asn oligopeptides interesting targets for studying the binding with macrodomains.

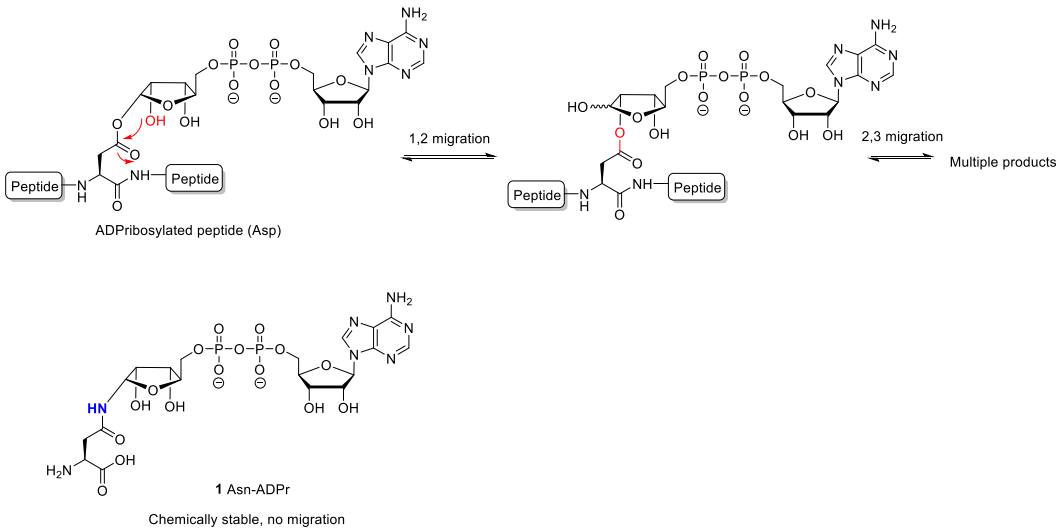
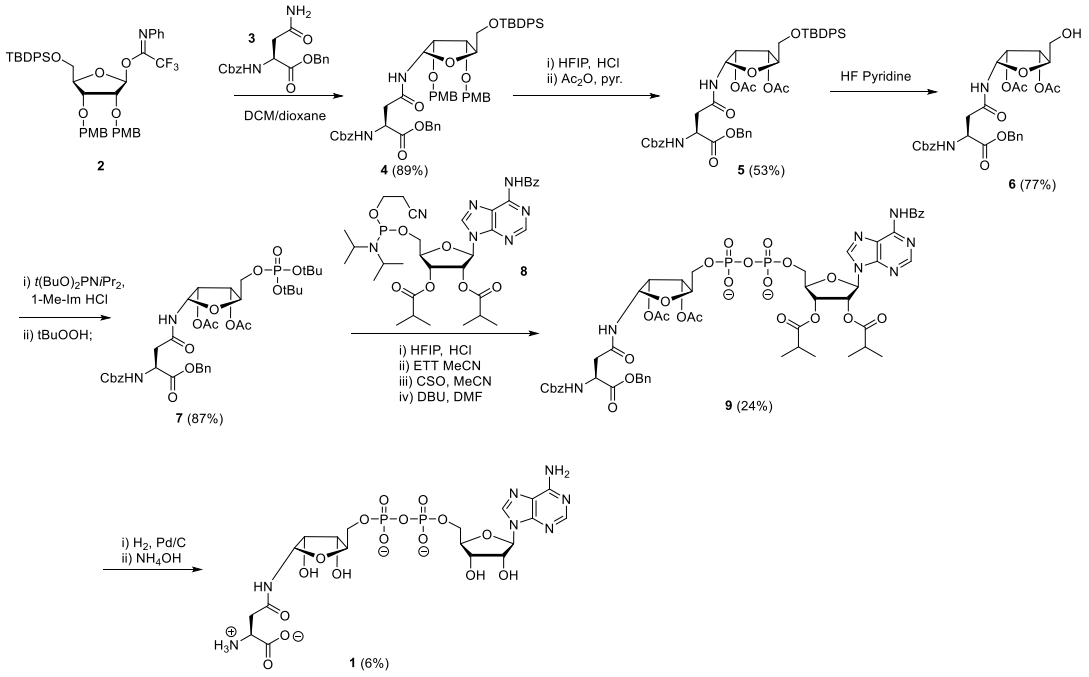


Figure 1. 1,2 migration mechanism of Asp-ADPr peptides and the structure of Asn-ADPr **1**

This Chapter describes the synthesis of Asn-ADPr **1** and the crystal structure of Methanobrevibacter oralis macrodomain (MorMOD) in the presence of Asn-ADPr **1**.

Results and discussion

The synthesis of Asn-ADPr **1**, started with the preparation of an orthogonally protected ribosylated Asn building block and was followed by the introduction of pyrophosphate at the 5-OH of the ribose moiety using P(V)-P(III) chemistry (Scheme 1).[10, 12] Coupling of known trifluoroacetimidate ribofuranose donor **2**[13] with Cbz-Asn-Bn acceptor **3** under influence of TBSOTf as activator in a mixture of DCM and 1,4-dioxane proceeded in complete α-stereoselective fashion to furnish the desired asparagine derivative **4** in a good yield. The two PMB groups on the ribose residue of **4**, required to obtain the α-product, had to be replaced to facilitate the removal of all the protecting groups in the ultimate stage of the synthesis.



Scheme 1. Synthesis of Asn-ADPr **1**

As described in Chapter 5, a treatment of **4** with DDQ under buffered conditions led to the unwanted formation of 4-methoxylbenzylidene acetal. In contrast, subjection of **4** to a solution of 0.1 equivalent HCl in HFIP (hexafluoro-2-propanol)[10, 14] removed both PMBs, and subsequent acetylation of the diol furnished α-product **5**. This acidolysis was accompanied by minimal epimerization to the β-product that was separated by column chromatography. Next, the 5-OH in **5** was liberated by HF-pyridine mediated desilylation to get **6**. Compound **6** was converted into phosphotriester **7** in a high yield by treatment with *tert*-butyl protected phosphoramidite [(*t*BuO)$_2$PN*i*Pr$_2$] and activator 1-methylimidazolium chloride[15] followed by oxidation with *t*BuOOH. Both *t*Bu protecting groups in triester **7** were rapidly cleaved by the same HCl/HFIP method, resulting in the corresponding phosphate monoester, as determined by ^{31}P-NMR. To install the pyrophosphate linkage, the obtained crude phosphomonoester was coupled with phosphoramidite **8** [P(III)] under the activation of ETT [5-(Ethylthio)-1H-tetrazole], followed by oxidation mediated by CSO [(1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine]. Subsequent DBU treatment removed the cyanoethyl group to furnish partially protected pyrophosphate **9** which, was purified by column chromatography and LH-20 gel filtration. Unfortunately, purified **9** showed broad peaks in both ^{1}H-NMR and ^{31}P-NMR spectra (broad peak at about -12 ppm in ^{31}P-NMR) from which an exact structure could not be ascertained. Luckily, LC-MS data showed one single peak (Rt 7.27 min) with desired mass (experimental section) indicating that the right product was obtained. It was hypothesized that metal ions in the LH-20 column might chelate with **9**, resulting in the broad NMR signals. The complete removal of the Cbz and Bn, groups was achieved by Pd/C catalyzed hydrogenolysis of **9** for 48 hours, as monitored by LC-MS analysis. Of note, adenine did

not hamper hydrogenolysis and stayed intact. In addition, no obvious aspartimide side-product was generated as reported previously.[10] The acetyl and benzoyl groups in obtained crude intermediate were removed by treatment with aqueous NH_4OH for 24 hours to give **1**, as determined by LC-MS. Purification of **1** via reverse phase column chromatography proved to be difficult by its hydrophilic nature and the accompanying short retention time. Target ADPr-Asn **1** was purified by HW-40 gel filtration to remove most of the salts and byproducts, followed by ion exchange chromatography. Although 0.6 mg (0.89 µmol) Asn-ADPr **1** was isolated, ^{1}H-NMR analysis showed the presence of 20% β-anomer. The formation of this inseparable epimer probably occurred during acidic deprotection of the *t*Bu groups.

With the synthetic sample of ADPr-Asn available, a co-crystallization experiment was attempted to demonstrate the usefulness of synthetic ADPr-amino acids for the studies on structural biology of ADP-ribose processing enzymes.

As macrodomaine of the MacroD-type are efficient hydrolases of aspartate/glutamate linked ADP-ribose, the ADPr-Asn (**1**) was considered as hydrolysis-resistant and a regiochemically defined substrate analogue for this class of enzymes. Its usability was demonstrated by co-crystallization experiments with the zinc-dependent macrodomain *Mor*MOD from *Methanobrevibacter oralis* (Figure 2a,c). The crystals showed full occupancy of the ligand binding site with the ADP-ribose moiety adopting a conformation highly similar to the previously solved structure of human MacroD2 (r.m.s.d. of 0.636 Å over 145 C^{α}; Figure 2b,d). However, addition of the asparagine moiety to the ADPr revealed, amongst others, two key features related to the catalytic behavior of this sub-class of macrodomains. First, even though the crystallization solution contained an α/β mixture, only the α-anomer can be observed in the electron density. This selectivity can be explained by the tight packing around the C1'' position, which precludes the binding of the β-anomer (Figure 2c). This finding strongly suggests that macrodomains show stereoselectivity similar to the previous reported one of the (ADP-ribosyl)hydrolase family.[9, 15] Second, the crystyl structure reveals an interaction of asparagine $O^{\delta 1}$ with the catalytic zinc ion in the pre-catalytic state. This indicates that the native *O*-glycosidic bond is broken by the interaction with the zinc ion. It can be inferred from these observations that the two reaction intermediates are a de-modified aspartate residue, which can transiently form a coordination bond with the zinc ion, and a ribose oxocarbenium ion that can react with a highly coordinated water molecule coordinated at the β-face of the distal ribose (Figure 2e).

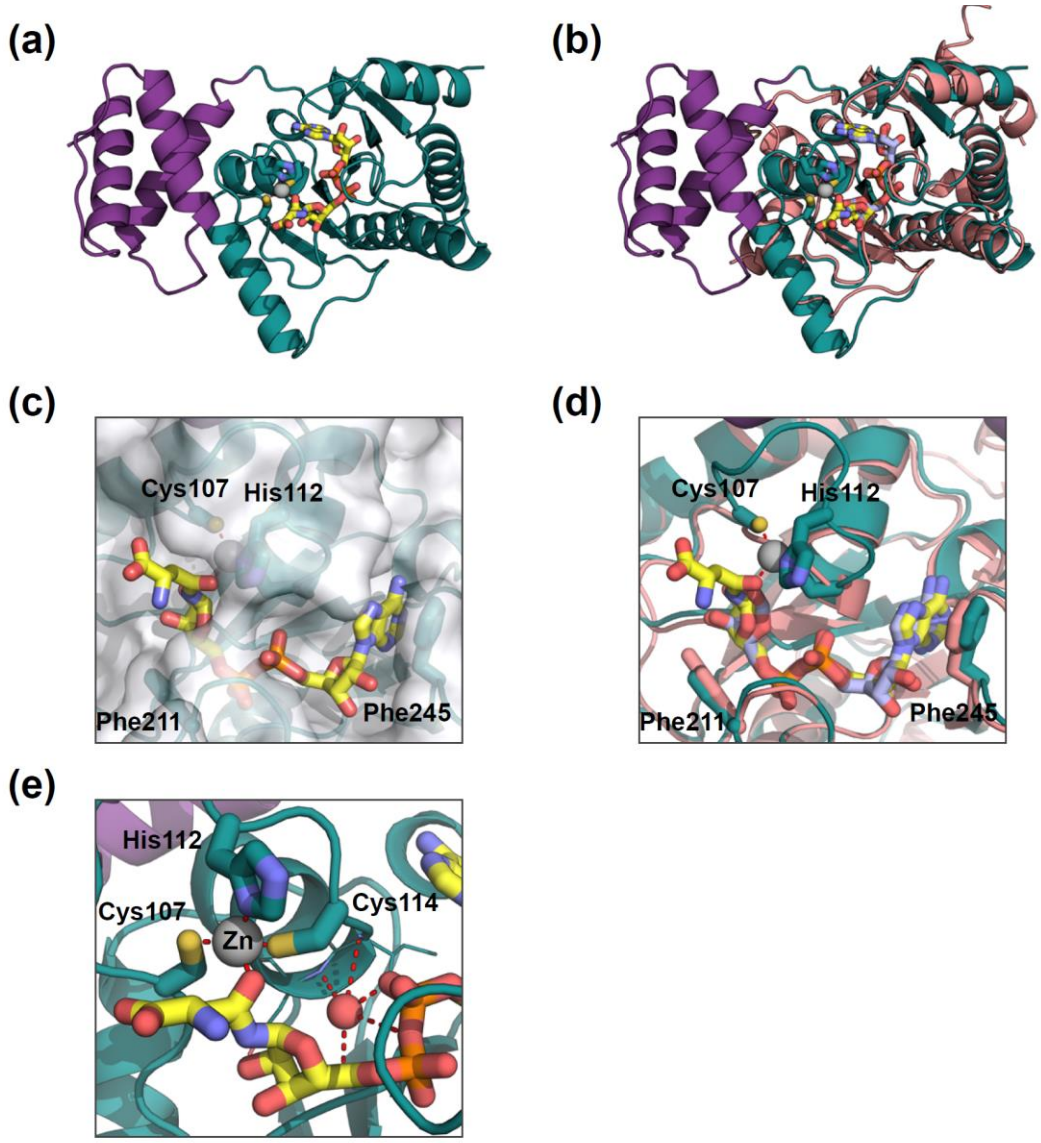


Figure 2. Asn-ADPr as substrate analogue in the crystallisation of *Methanobrevibacter oralis* macrodomain (*Mor*MOD): **(a)** Overall structure of *Mor*MOD in ribbon representation. The classical macrodomain fold is teal, N-terminal extension in purple, the catalytic zinc ion in grey and Asn- ADPr in yellow. **(b)** Structural comparison of *Mor*MOD:Asn-ADPr and human MacroD2:ADPr (PDB: 4IQY) complexes. The *Mor*MOD structure is colored as in (a), MacroD2 in light red and ADPr in light blue. **(c)** Ribbon-surface representation of the Asn-ADPr binding cleaved. Phe245 contributes to the binding of the adenosine moiety via pi-stacking interaction and Phe211 supports orientation of the distal ribose in the active site. The zinc coordinating residues (C-H-C motif) are given in stick representation. **(d)** Comparison of Asn-ADPr (*Mor*MOD) and ADPr (MacroD2) coordination reveals highly similar binding of both molecules in the respective binding sites. Note, MacroD2 is a zinc-independent MacroD-type hydrolase and the zinc containing loop is replaced by a short tetra-glycine motif. Indicated residue numbers are given for *Mor*MOD. **(e)** Close up of distal ribose coordination showing the interaction of asparagine $O^{\delta 1}$ with the catalytic zinc. The highly ordered water molecule involved in the catalytic cycle is shown as red sphere.

Conclusion

In summary, we have successfully synthesized ADPr-Asn, which is a stable mimics of ADPr-Asp for

protein binding studies. Co-crystallization with *Methanobrevibacter oralis* macrodomain (*Mor*MOD) gave new insights into substrate selectivity of macrodomains and catalytic mechanism of their zinc-dependent sub-class.

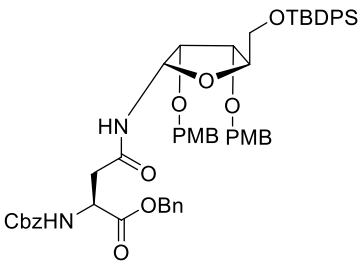
Acknowledgment

We thank Johannes G. M. Rack in the laboratory of Ivan Ahel (Sir William Dunn School of Pathology, University of Oxford) for the crystal structure data.

Experimental section

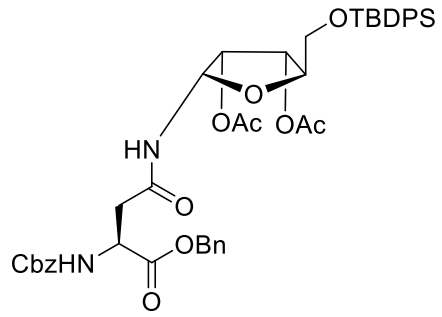
General procedure

All chemicals were used as received unless stated otherwise. All solvents used in reaction (including solid phase synthesis) were dried over 3Å molecular sieves. Solvents removal by rotary evaporation was under reduced pressure at 40 °C. TLC, NMR, LCMS, anion exchange, HRMS, IR, optical rotation facilities were used as described in Chapter 2.



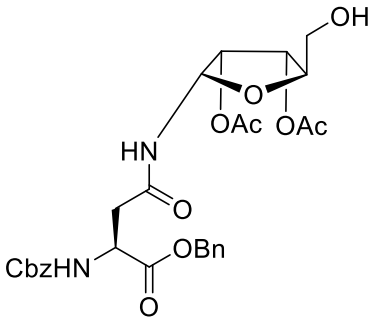
N^{γ}-(2,3-di-*O*-(4-methoxylbenzyl)-5-*O*-*tert*-butyldiphenylsilyl-α-D-ribosyl)-N^{α}-benzyloxycarbonyl asparagine benzyl ester (4)

The trifluoroacetimidate donor (**2**) (2.30 g, 2.88 mmol) and Cbz-Asn-OBn (0.85g, 2.40 mmol) were co-evaporated with 1,4-dioxane (3 x), dissolved in dry DCM/1,4-dioxane (24 mL, 1/1; v/v) and stirred with freshly activated 3 Å molecular sieves at room temperature for 1 hour under N_2 to remove traces of water. The reaction mixture was cooled to -10 °C and TBSOTf (33 µL, 0.14 mmol) was added to the reaction mixture. The mixture was allowed to reach room temperature and stirred for 30 minutes. The reaction was quenched by the addition of triethylamine (0.2 mL), filtered through a pad of celite and concentrated. The mixture was purified by silica gel chromatography (Pentane/EtOAc, 80/20 – 70/30 – 60/40) to obtain **4** as a white foam (2.0 g, 2.14 mmol, 89%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.67 – 7.51 (m, 4H, arom.), 7.47 – 7.13 (m, 20H, arom.), 6.96 (d, *J* = 9.2 Hz, 1H, NH), 6.84 (ddd, *J* = 12.1, 6.1, 2.5 Hz, 4H, arom.), 6.05 (d, *J* = 8.8 Hz, 1H, NH), 5.82 (dd, *J* = 9.2, 5.4 Hz, 1H, H1'), 5.25 – 5.00 (m, 4H, CH_2 PMB), 4.65 – 4.57 (m, 2H, CH Asn, CH_2 Bn), 4.54 – 4.40 (m, 3H, CH_2 Bn), 4.19 – 4.00 (m, 3H, H2, H3, H4), 3.84 – 3.71 (m, 6H, OMe PMB), 3.55 (d, *J* = 3.7 Hz, 2H, H5), 2.96 (AB, *J* = 16.1, 4.4 Hz, 1H, CH_2 Asn), 2.68 (dd, *J* = 16.2, 4.4 Hz, 1H, CH_2 Asn), 0.97 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.99, 170.18, 159.61, 159.56 (CO), 135.74, 135.63 (arom.), 133.19, 132.81 (Cq. arom.), 130.31, 129.98 (arom.), 129.86 (Cq. arom.), 129.65, 129.55 (arom.), 129.42 (Cq. arom.), 128.66, 128.64, 128.34, 128.30, 128.19, 128.09, 127.93, 114.10, 114.07 (arom.), 83.20 (C4), 78.73 (C1), 77.69 (C3), 76.67 (C2), 72.42, 72.37 (CH_2 Bn), 67.57, 67.08 (CH_2 PMB), 64.15 (C5), 55.43 (OMe PMB), 50.85 (CH Asn), 38.15 (CH_2 Asn), 26.91 (CH_3 TBDPS), 19.29 (Cq. TBDPS). IR (film): 2939, 1723, 1612, 1511, 1507, 1302, 1247, 1212, 1173, 1113, 1030, 822, 701 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{56}H_{63}N_2O_{11}Si$ (M+H) 967.4196. Found 967.4201. $[\alpha]_D^{20}$ +25.1 (c = 1, in MeOH)



N^{γ}-(2,3-di-*O*-acetyl-5-*O*-*tert*-butyldiphenylsilyl-α-D-ribosyl)-N^{α}-benzyloxycarbonyl asparagine benzyl ester (5)

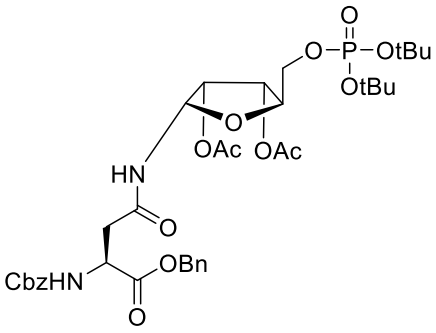
HCl solution (0.74 mL, 0.1M in HFIP) was added into HFIP (15 mL) then the solution was added into **4** (720 mg, 0.74 mmol). The reaction was stirred for 25 minutes at room temperature and quenched with pyridine (0.2 mL). The mixture was co-evaporated under reduced pressure with toluene (3x) and dissolved in pyridine (7.5 mL). The mixture was cooled to 0 °C and Ac_2O (1.4 mL, 20 eq.) was added and the reaction was stirred for 16 hours at room temperature. The reaction mixture was concentrated in *vacuo*, dissolved in DCM and extracted with aq. $NaHCO_3$ (sat.). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography(Pentane/EtOAc, 100/0 – 80/20 – 70/30 – 60/40) to obtain **5** as a white foam (318 mg, 0.39 mmol, 53%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.67 (ddd, *J* = 7.9, 3.9, 1.8 Hz, 4H, arom.), 7.46 – 7.35 (m, 6H, arom.), 7.35 – 7.25 (m, 10H, arom.), 6.42 (d, *J* = 9.5 Hz, 1H, NH), 6.07 (dd, *J* = 9.5, 5.6 Hz, 1H, H1), 5.99 (d, *J* = 8.2 Hz, 1H, NH), 5.56 (dd, *J* = 5.3, 2.7 Hz, 1H, H3'), 5.50 (t, *J* = 5.5 Hz, 1H, H2'), 5.24 – 5.13 (m, 2H, CH_2 Cbz), 5.09 (s, 2H, CH_2 Bn), 4.63 (dt, *J* = 8.6, 4.6 Hz, 1H, CH Asn), 4.09 (dd, *J* = 5.1, 2.3 Hz, 1H, H4), 3.71 (d, *J* = 2.9 Hz, 2H, H5), 3.03 (AB, *J* = 16.0, 4.6 Hz, 1H, CH_2 Asn), 2.88 – 2.76 (m, 1H, CH_2 Asn), 2.13 – 1.97 (m, 6H, CH_3 Ac), 1.06 (s, 9H, CH_3 TBDPS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.74, 169.74, 169.48, 169.17, 156.26 (CO), 136.08 (Cq. arom.), 135.72, 135.66 (arom.), 135.29, 132.83, 132.54 (Cq. arom.), 129.95, 129.91, 128.63, 128.60, 128.43, 128.29, 128.27, 128.07, 127.90 (arom.), 82.34 (C4), 78.66 (C1), 72.56 (C3), 70.11 (C2), 67.69 (CH_2 Bn), 67.13 (CH_2 Cbz), 63.74 (C5), 50.86 (CH Asn), 38.25 (CH_2 Asn), 26.80 (CH_3 TBDPS), 20.86, 20.50 (CH_3 Ac), 19.20 (Cq. TBDPS).



N^{γ}-(2,3-di-*O*-acetyl-α-D-ribosyl)-N^{α}-benzyloxycarbonyl asparagine benzyl ester (6)

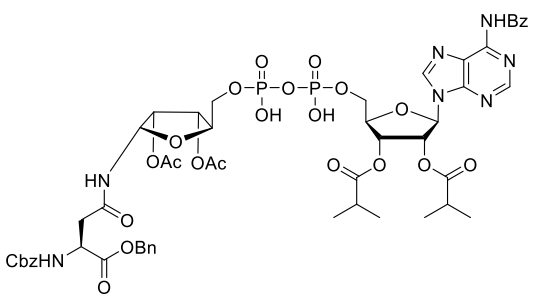
Compounds **5** (318 mg, 0.39mmol) were dissolved in pyridine (4 mL) and HF·Pyridine (0.15 mL, 5.89 mmol) was added. The reaction was stirred for 2.5 hour at 0°C and TLC showed incomplete reversion. Additional HF·Pyridine (0.15 mL, 5.89 mmol) was added and 2 hours later the reaction was quenched by aq. $NaHCO_3$ (sat.). The reaction mixture was extracted with EtOAc, the organic layer dried over $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography (Pentane/Actone, 75/25 – 60/40 – 70/30 – 60/40) to obtain **6** as a white foam (173 mg, 0.30 mmol, 77%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.39 – 7.20 (m, 10H, arom.), 6.63 (d, *J* = 9.5 Hz, 1H, NH), 6.09 (d, *J* = 8.2 Hz, 1H, NH), 6.01 (dd, *J* = 9.5, 4.8 Hz, 1H, H1), 5.37 – 5.31 (m, 2H, H2, H3), 5.23 – 4.98 (m, 4H, CH_2 Bn, Cbz), 4.60 (dt, *J* = 8.2, 4.9 Hz, 1H, CH Asn), 4.07 (q, *J* = 3.2 Hz, 1H, H4), 3.74 (AB, *J* = 12.4, 2.9 Hz, 1H, H5), 3.59 (AB, *J* = 12.5, 3.4 Hz, 1H, H5), 2.99 (AB, *J* = 16.1, 5.2 Hz, 1H, CH_2 Asn), 2.81 (AB, *J* = 16.1, 4.7 Hz, 2H, CH_2 Asn), 2.07 (s, 6H, CH_3 Ac), 1.25 (s, 1H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.86, 170.36, 169.74, 169.36, 156.32 (CO), 136.14, 135.32 (Cq arom.), 128.65, 128.61, 128.47, 128.29, 128.26, 128.09 (arom.), 81.47 (C4), 78.64 (C1), 71.53 (C3), 70.47 (C2), 67.67 (CH_2 Bn), 67.13 (CH_2 Cbz), 61.89 (C5), 50.84 (CH Asn), 38.12 (CH_2 Asn), 20.75, 20.55 (CH_3 Ac). IR (film): 3364,

1734, 1715, 1507, 1374, 1238, 1214, 1194, 1179, 1043, 1028 cm^{-1}. HRMS (ESI^+) calcd for $C_{28}H_{32}N_2O_{11}$ (M+H) 573.2079. Found 573.2077. $[\alpha]_D^{20}$ +47.1 (c = 1, in $CHCl_3$)



N^{γ}-(2,3-di-*O*-acetyl-5-*O*-(di-*tert*-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-benzyloxycarbonyl asparagine benzyl ester (7)

Firstly, Co-evaporating 1-methylimidazolium chloride (162 mg, 1.36 mmol) and 1-methyl-imidazole (72 µL, 0.91 mmol) with dry CH_3CN (3 x), then N_2 was applied. To the mixture, freshly activated molecular sieves and dry DMF (2.3 mL) was added and the activator solution was stirred at room temperature for 1 hours under N_2. Secondly, co-evaporating **6** (130 mg, 0.23 mmol) with dry 1,4-dioxane (3 x), then activator solution was added after which Di-tert-butyl-N,N-diisopropylphosphoramidite (0.11 mL, 0.34 mmol) was added and the reaction was stirred at room temperature for 0.5 hour. Then *t*BuOOH in decane (0.41 mL, 5.5 M, 2.27 mmol) was added at 0°C and the reaction mixture was stirred for 1 hour a room temperature. The reaction was quenched upon addtion of aq. $NaHCO_3$ (sat.) and extracted with EtOAc (3x), dried over $MgSO_4$, concentrated under reduced pressure. Purification by silica gel chromatography (Pentane/Actone, 100/0 – 80/20 – 75/25) to obtain **7** as a colorless oil (153 mg, 0.20 mmol, 87%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.48 – 7.16 (m, 10H, arom.), 6.43 (d, *J* = 9.4 Hz, 1H, NH), 6.02 – 5.96 (m, 2H, H1, NH), 5.39 (dd, *J* = 5.3, 3.5 Hz, 1H, H3), 5.30 (t, *J* = 5.4 Hz, 1H, H2), 5.25 – 5.04 (m, 4H, CH_2 Cbz, Bn), 4.61 (dt, *J* = 8.7, 4.6 Hz, 1H, CH Asn), 4.23 – 4.13 (m, 1H, H4), 4.04 (dd, *J* = 5.6, 3.3 Hz, 2H, H5), 3.01 (AB, *J* = 16.1, 4.6 Hz, 1H, CH_2 Asn), 2.81 (dd, *J* = 16.1, 4.6 Hz, 1H, CH_2 Asn), 2.10 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac), 1.48 (s, 18H, CH_3 *t*Bu). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.65, 169.70, 169.34, 168.99, 156.20 (CO), 136.04, 135.24 (Cq. arom.), 128.60, 128.55, 128.41, 128.24, 128.21, 128.02 (arom.), 83.00, 82.98, 82.93, 82.91 (Cq. *t*Bu), 80.06, 79.98 (C4), 78.52 (C1), 71.89 (C3), 69.73 (C2), 67.63 (CH_2 Cbz), 67.08 (CH_2 Bn), 65.75, 65.69 (C5), 50.76 (CH Asn), 38.15 (CH_2 Asn), 29.84, 29.80 (CH_3 *t*Bu), 20.71, 20.42 (CH_3 Ac). ^{31}P NMR (162 MHz, $CDCl_3$) δ -9.93. HRMS (ESI^+) calcd for $C_{36}H_{49}N_2O_{14}PNa$ (M+Na) 787.2814. Found 787.2809.

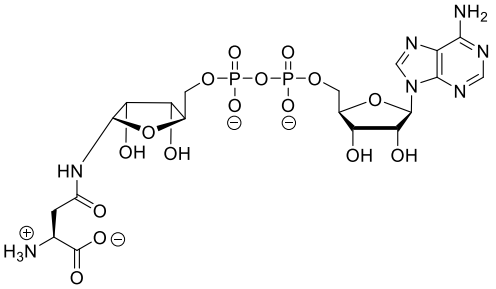


N^{γ}-(N^6-benzoyl-2'',3''-di-*O*-acetyl-2'3'-di-O-isobutyryl-adenosine diphosphate-α-D-ribosyl)-N^{α}-benzyloxycarbonyl asparagine benzyl ester (9)

1 mL HCl solution (0.1M in HFIP) was added into HFIP (1 mL) then the solution was added into **7** (70 mg, 0.09 mmol). The reaction was stirred for 30 minutes at room temperature and quenched with TEA (0.13 mL). The mixture was concentrated under reduced pressure and followed by co-evaporation with toluene (2x), pyridine/H_2O (9/1; v/v) (1x), pyridine (1x) and toluene (5x). ETT (2.19 mL, 0.25 M in solution, 0.55 mmol) in CH_3CN and molecular sieves were added and the mixture was left to stand for 16 hours under N_2 atmosphere. Compound **8** was co-evaporated with dioxane (1x), CH_3CN (3x), dissolved in CH_3CN (1 mL) and added to the mixture of **7** and ETT. The reaction was stirred for 10 minutes and analyzed with ^{31}P-NMR spectroscopy. CSO (2 mL, 0.5 M, 1.0 mmol) in

CH_3CN was added and after stirring for 20 minutes analyzed with ^{31}P-NMR. Dry DBU (2 mL, 0.5 M, 1.0 mmol) in DMF was added and after stirring for 5 minutes pyridinium chloride (116 mg, 1.0 mmol) was added to quench the reaction. The mixture was filtrated and washed by MeOH and then concentrated. Purification by silica gel chromatography (DCM/MeOH, 100/0 – 95/5 –90/0 – 85/15) and then LH-20 (DCM/MeOH, 50/50) to get to obtain **9** as a colorless oil (27 mg, 22 µmol, 24%). ^{31}P NMR (162 MHz, $CDCl_3$) δ br. -12.77. LC-MS: Rt = 7.27 min. 10-90% TFA. ESI MS+ calc. 1226.3 found 1226.1 $[M+1]^+$.

***N*$^{\gamma}$-(adenosine diphosphate-α-D-ribosyl)-asparagine (1)**



Compound **9** (20 mg, 16 µmol) was added into a flask and then *t*BuOH/Dioxane/H_2O (2 mL, 4:4:1; v/v), Pd/C (25 mg, 10% loading) and few drops of AcOH were added. The mixture was sonicated for 5 minutes under N_2. H_2 was bubbled for 48 hours and LC-MS showed completely conversion. The reaction was filtrated over celite and washed by MeOH. Concentrated the mixture under reduced pressure and then co-evaporated with dioxane (2 x) then dissolved in aq. NH_4OH (2 mL). The reaction was stirred for 24 h after which concentrated under reduced pressure. Purification by HW-40 (NH_4OAc in H_2O, 0.15M), ion exchange (10 mmol – 0.5 mmol NH_4OAc in H_2O) and repeat lyophilization obtained **1** as a white solid (0.6 mg, 0.89 µmol, 6%). ^1H NMR (400 MHz, Deuterium Oxide) δ 8.47 (d, *J* = 2.8 Hz, 1H, H2), 8.22 (s, 1H, H8), 6.11 (d, *J* = 5.9 Hz, 1H, H1'), 5.70 – 5.59 (m, 1H, H1''), 4.74 (t, *J* = 5.6 Hz, 1H, H2'), 4.50 (dd, *J* = 5.2, 3.5 Hz, 1H, H3'), 4.36 (t, *J* = 2.8 Hz, 1H, H4'), 4.19 (td, *J* = 4.6, 4.1, 2.3 Hz, 3H, H2'', H5'), 4.11 – 3.89 (m, 4H, H3'', H4'', H5''), 3.02 (AB, *J* = 17.2, 3.9 Hz, 1H, CH_2 Asn), 2.81 (AB, *J* = 17.2, 9.4 Hz, 1H, CH_2 Asn). ^{13}C NMR (101 MHz, D_2O) δ 86.74 (C1'), 83.91, 83.83 (C4'), 80.63 (C4''), 80.19 (C1''), 74.18 (C2'), 70.59 (C3'), 70.33 (C2''), 69.94 (C3''), 65.32 (C5'), 65.17 (C5''), 51.04 (CH Asn), 35.42 (CH_2 Asn). ^{31}P NMR (202 MHz, D_2O) δ -10.35, -10.45, -10.60, -10.71. HRMS (ESI^+) calcd for $C_{19}H_{30}N_7O_{16}P_2$ (M+H) 674.1219. Found 674.1238. LC-MS: Rt = 2.80 min. 0 - 50% NH_4OAc. ESI MS+ calc. 674.1 found 674.1 $[M+1]^+$.

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7 A general approach towards triazole-linked adenosine diphosphate ribosylated peptides and proteins

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Introduction

Regulation of protein activity is controlled by post-translational modifications (PTMs) that are installed on specific side-chain functionalities of amino acids in the involved protein. Simple PTMs, such as acetylation, methylation, and phosphorylation, have been subject of a large amount of studies, and the focus of PTM research is shifting to more complex PTMs. One of these PTMs is called adenosine diphosphate ribosylation (ADP-ribosylation), a modification in which a specific nucleophilic side chain in the target protein displaces β-oriented nicotinamide from NAD^+ under the agency of an ADPr-transferase (ART) resulting in an α-oriented glycosidic linkage to the protein.[1] Mono-ADP-ribosylation is not only a PTM effected by bacterial toxins and the starting point for poly-ADP-ribosylation but also a regulatory modification in its own right. Mono-ADP-ribosylation is reported to take place on a variety of amino acid side chains, including arginine (Figure 1), glutamic acid, aspartic acid, asparagine, and cysteine, but recently it was pointed out that serine might be the main point of attachment for ADP-ribosylation.[2, 3] Research in the field of protein ADP-ribosylation benefits greatly from ADP-ribosylated

molecular tools. One way to obtain such tools in sufficient quantities is through chemical synthesis. Methods towards naturally occurring mono-ADP-ribosylated oligopeptides, ADPr oligomers, and NAD^+-analogues have been reported and employed in studying ADP-ribosyl hydrolase affinity,[4, 5] inhibition of ADP-ribosylating toxins, finding substrate proteins for poly ADPr polymerases,[6] and determining the structure of poly ADP ribose glycohydrolases.[7] Such ADPr peptides and related substances are valuable for the interrogation of the complex biology that underlies this PTM.[8, 9] In the chemical synthesis of peptides and proteins, most commonly an acidic step to remove protective groups is employed. Such conditions, however, may cause either epimerization at the anomeric center of ribose or complete loss of the ADPr-moiety. Mild alkaline conditions, carry the risk of degradation of the β-substituted amino acids and are clearly incompatible with the esters of ADP-ribosylated Glu and ADP-ribosylated Asp. The reported synthesis of ADPr amino acids and peptides so far have been carefully tuned to minimize those risks and incorporation of ADPr amino acids asks for a modified protective group strategy in most cases.[8, 10] To prevent the need for highly specialized methods to prepare these amino acid-ribose conjugates we propose a general strategy that would allow a post-synthetic introduction of the ADPr moiety to a peptide or protein of interest.

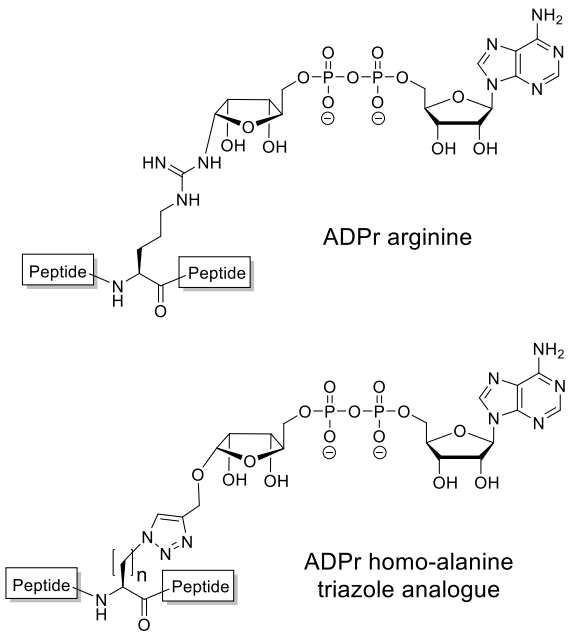
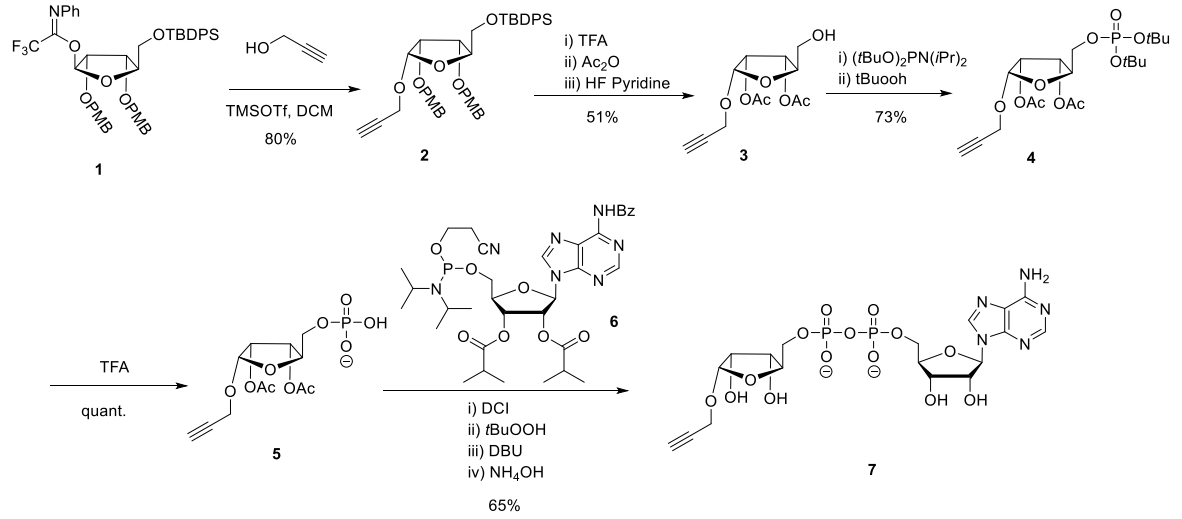


Figure 1. Structure of α-linked ADPr arginine and ADPr triazole analogue linkages.

In this Chapter, an ADPr triazole analogue was selected as a relevant replacement for ADPr amino acids in peptides (Figure 1). Oligopeptides with an azide incorporated can be obtained by standard solid-phase peptide synthesis (SPPS) using an azido–alanine or azido–homoalanine building block at the site of the modification. The azido-oligopeptides, thus obtained, could be "clicked" with α-1-*O*-propargyl-ADPr (ADPr-pr, **7** in Scheme 1) via a conventional copper catalyzed azide–alkyne cycloaddition (CuAAC), furnishing a triazole linked ADP-ribosylated peptides as bioisosteres of the

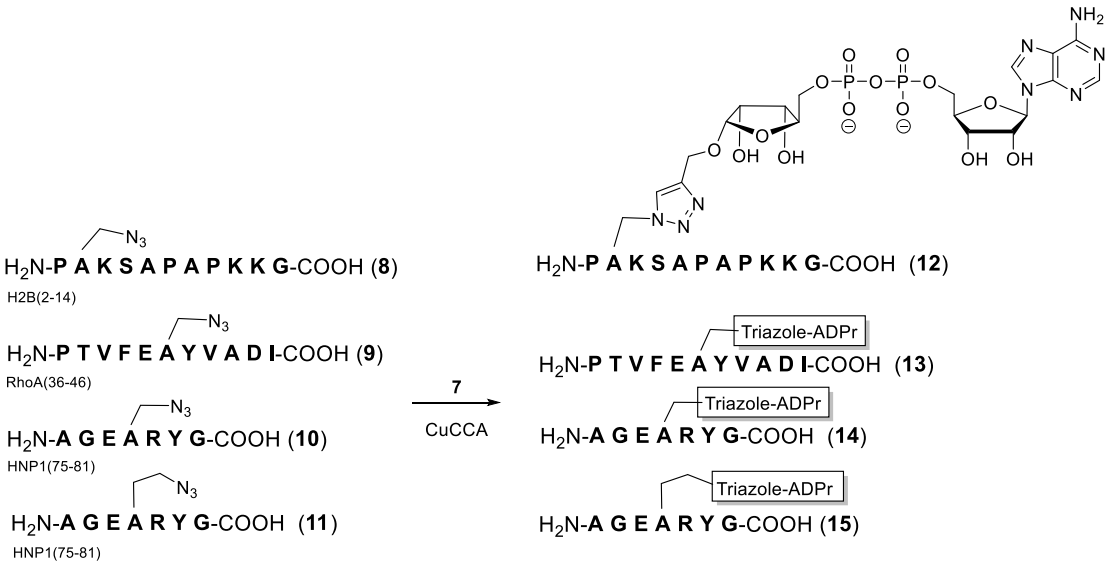
native molecules. Furthermore, this strategy was also applied to the synthesis of ADP-ribosylated protein mimics in which ADPr-pr was selectively conjugated to a synthetic ubiquitin at a desired site. Importantly, the synthetic ADPr-ubiquitin was found to possess similar bioactivity as the natural counterpart in our auto-ubiquitination assay, highlighting the broad application of this method in the biological interrogation of ADPr biology.



Scheme 1. Synthesis of 1-*O*-propargyl-ADPr **7**

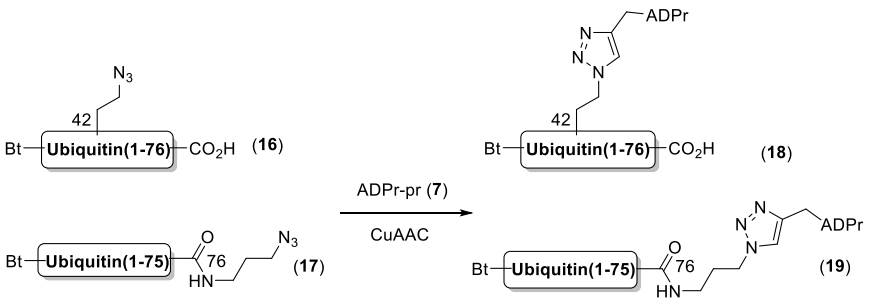
Results and discussion

The synthesis of ADPr-pr **7** started with the condensation of propargyl alcohol with trifluoroacetimidate ribofuranose donor **1**[10] (Scheme 1, see also Chapter 6) to yield an anomeric mixture **2** (α:β, 71:29). In contrast to two previously reported syntheses that show the preparation of either β-O-alkyne or β-azide containing ADPr analogues, the biologically relevant α-anomer could be isolated[7, 11] in this work. Protective group manipulations to introduce acetyls on the 2- and 3-hydroxyls allowed alkaline deprotection in the final stage of the synthesis instead of acid treatment, preventing possible degradation of the ADPr-pr moiety. Phosphitylation of the primary alcohol and subsequent oxidation furnished crucial phosphotriester intermediate **4**. Removal of the *t*Bu groups set the stage for the installation of the pyrophosphate moiety using adaptation of the procedure of Gold *et al*.[4, 12] Reaction of phosphomonoester **5** with suitably protected adenosine phosphoramidite **6**[13] using dicyanoimidazole (DCI) as activator and oxidation of the intermediate P(V)-P(III) species was followed by a two-step alkaline deprotection procedure. Purification using size exclusion chromatography gave access to ADPr-pr **7** in a quantity of 100 mg. Formation of the pyrophosphate and subsequent deprotection proceeded in an overall isolated yield of 65%, which compares favorably to other approaches.[8, 11, 14]



Scheme 2. CuAAC reaction towards ADPr peptides

To assess the viability of ADPr-pr building block **7** in the projected cycloaddition, three peptides derived from mono-ADP-ribosylated proteins were prepared; namely Histone H2B (2–14) (Scheme 2, compound **8**), RhoA (36–46) (compound **9**) and human neutrophil defensin 1; HNP1 (75–81) (compound **10**). In the selected peptides, Gln3, Asn50, and Arg78, respectively, were substituted, for β-azidoalanine to allow conjugation by CuAAC. After completion of the SPPS, the immobilized oligopeptides were treated with a cleavage mixture consisting of 90.5% trifluoroacetic acid (TFA), 5% water, 2.5% phenol and 2% triisopropylsilane to globally remove the protective groups and cleave the peptide from the resin. RP-HPLC purification yielded target peptides **8**–**10** that were used in the CuAAC reaction with ADPr-pr **7** in 20 mm tris(hydroxymethyl)aminomethane/150 mm NaCl buffer at pH 7.6 in the presence of 10 mm $CuSO_4$, 60 mm sodium ascorbate, and 10 mm tris triazole ligand.[15] Since these peptides and ADPr-pr **7** dissolved readily in this buffer, the click reaction proceeded efficiently and quickly (within minutes to one hour), in contrast to a previous study.[11] To mimic the length of the Arg-ADPr linkage more closely, HNP1-peptide **11** was prepared, in which the Arg was replaced not with β-azidoalanine but with azidohomoalanine. Again CuAAC to ADPr-pr proceeded uneventfully, yielding **15**.



Scheme 3. CuAAC reaction towards Ub-ADPr conjugates

Having established an efficient procedure to prepare ADPr oligopeptides, it was investigated whether this method could be expanded for the preparation of ADPr proteins. Ubiquitin (Ub), a 76 amino acid residue long post-translational modifier itself, has recently been found to be modified with ADPr on different positions. This cross-talk between ADP-ribosylation and ubiquitination is reported to have a regulatory effect on the DNA repair mechanism, where low levels of NAD^+ lead to ubiquitination of histone protein H4, but high levels of NAD^+ lead to ADP-ribosylation of Gly76; the C-terminus of Ub.[16] Other studies show that Arg42 of Ub is ADP-ribosylated by a family of effector proteins originating from *Legionella pneumophila*, the pathogen causing Legionnaires' disease.[17-19] These SidE effectors are the first reported class of enzymes that are able to ubiquitinate target proteins independently of the normally employed enzymatic cascade of E1, E2, and E3 enzymes, utilizing Ub-ADPr as crucial intermediate. Using their unique properties, SidE proteins can hijack the host cells Ub pool and use it to the advantage of pathogene. In analogy to peptide **11**, Ub mutant **16**, in which Arg42 has been replaced by azidohomoalanine, was prepared using the linear SPPS approach of El Oualid *et al.*[20] Gly76 modified Ub **17** was prepared by first synthesizing Ub75 on trityl resin followed by treatment with mild acid (20% hexafluoroisopropanol in dichloromethane). In this step, the peptide was liberated from the solid support while leaving all side chain protecting groups in place. Activation of the free C-terminal carboxylic acid and coupling of 3-azido-1-propanamine followed by strong acid treatment and RP-HPLC purification yielded azide modified Ub **17**. Copper-catalyzed click reaction with ADPr-pr building block **7**, followed by dialysis to remove traces of excess ADPr-pr and click reagents and finally size exclusion chromatography, gave easy access to ADP-ribosylated ubiquitin analogues **18** and **19**, respectively (Scheme 3).

Of note is that this procedure does not require the use of RP-HPLC purification after the introduction of the ADPr moiety. To assess whether the artificial triazole linkage is tolerated and this method indeed results in useful ADPr protein analogues, Ub-ADPr **18** was compared to Arg42 Ub-ADPr from natural sources (Ub-ADPr wt). Both Ub-ADPr **18** and Ub-ADPr wt were efficiently recognized by an ADPr antibody in western blot (Figure 2A, lower panel), a first indication that the triazole analogue does not differ too far from its natural counterpart. One of the properties of *Legionella* effector SdeA is its auto-ubiquitination behavior, an effect that is not fully understood so far, but is reported for all four SidE family members. Comparison of the ability of recombinant SdeA to use **18** in an auto-ubiquitination assay showed indeed that SdeA is modified with Ub multiple times (see Figure 2A,B). Although at a reduced rate compared to Ub-ADPr wt, artificial **18** was processed by SdeA and significant auto-ubiquitination takes place. A control experiment using non-ADP-ribosylated wild type Ub shows no auto-ubiquitination of SdeA (Figure 2C). These results further confirmed that Ub-ADPr conjugate **18** functions similar to Ub-ADPr wt.

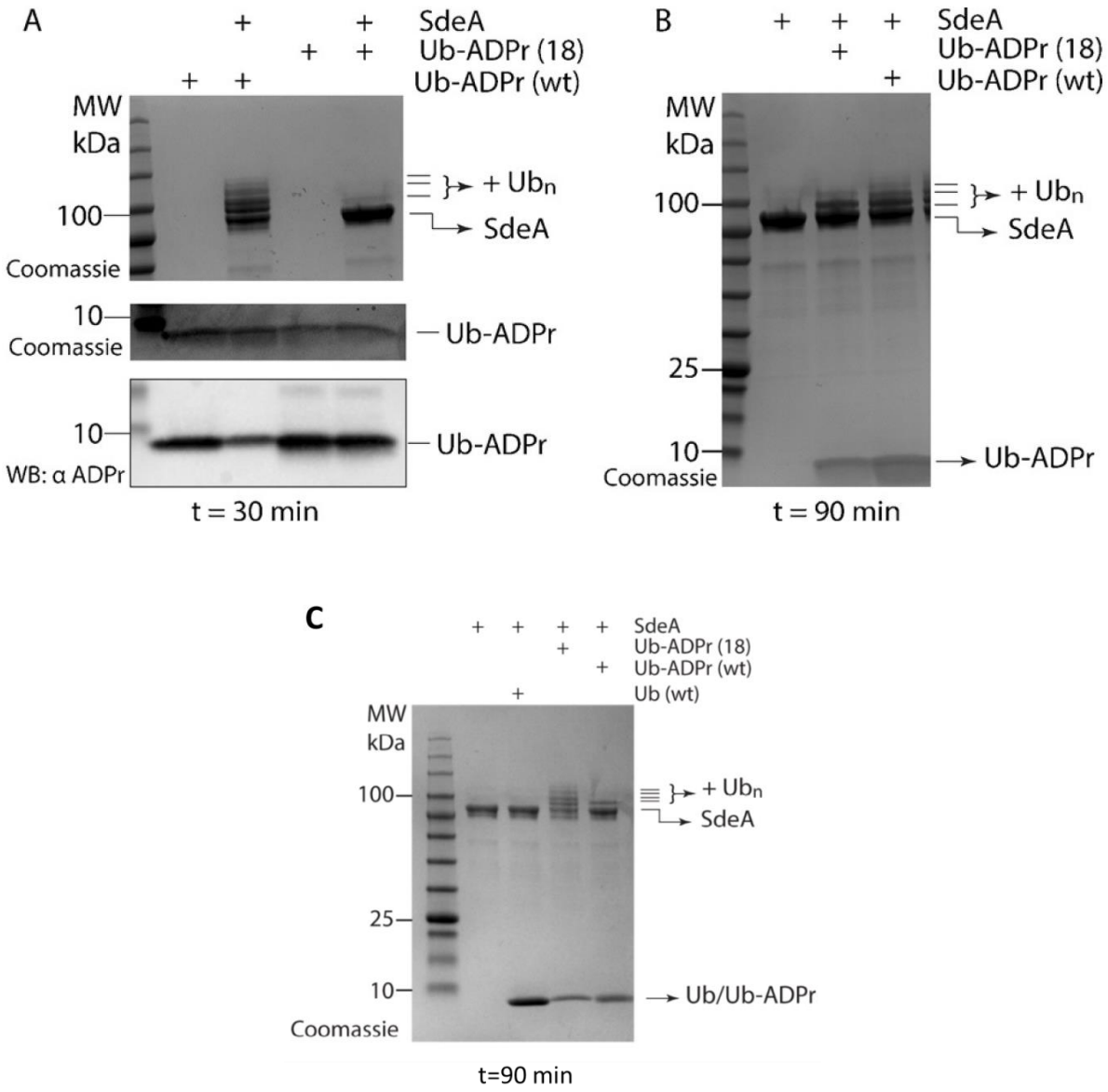


Figure 2. **A**/**B**: Comparison of Ub-ADPr (wt) and Ub-ADPr analogue (**18**) processing by SdeA at 30 min and 90 min. **C**: Control experiment of Ub-ADPr (wt), Ub-ADPr (**18**) and Ub (wt) processing by SdeA at 90 min.

Conclusion

This chapter presents the design and synthesis of propargylated ADP-ribose **7** suitable to take part in an efficient cycloaddition with oligopeptides and proteins having an azide at a predetermined position. In this way, oligopeptides and proteins carrying an analogue of the mono ADP-ribose post-translational modification are made available. Four ADPr-oligopeptide conjugates derived from known

ADP-ribosylated proteins were prepared efficiently. Furthermore, two analogues of ADPr ubiquitin, shown to play a role in Legionnaires' disease and DNA repair, were prepared using the same CuAAC chemistry. The effectiveness of these reactions and subsequent purifications provided an easy entry to this interesting class of post-translationally modified proteins. Triazole-containing Ub-ADPr **18** was shown to be recognized in western blot and accepted by SdeA in an auto-ubiquitination assay, indicating that this method provides a useful platform for the biological interrogation of ADPr biology.

Acknowledgement

Mengjie Shen is kindly acknowledged for the help in the lyophilization of ADPr-peptide.

Experimental Section

General procedure for synthesis

General reagents were obtained from Sigma Aldrich, Fluka and Acros and used as received. Solvents were purchased from BIOSOLVE or Aldrich. Peptide synthesis reagents were purchased from Novabiochem. TLC, NMR, LCMS, anion exchange, gel filtration, HRMS, IR, optical rotation facilities were used as described in Chapter 2.

Bioassay

Purification of SdeA (193-998) was performed as previously described.[19] For *in vitro* ubiquitination reaction, 2 µg of purified SdeA was incubated with 4 µg of either Ub wt, Ub-ADPr wt or Ub-ADPr **18** in 30 µL reaction buffer containing 50 mM Tris, 50 mM NaCl at pH 7.5. Reaction components were incubated at 37 °C for indicated duration. Reaction was stopped by adding SDS loading buffer and samples analysed by Coommassie staining and western-blot detection of Ub variants using anti-pan ADP-ribose binding reagent (Millipore, catalogue number: MABE1016).

Solid phase peptide synthesis

SPPS was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 25 µmol scale, using fourfold excess of amino acids relative to pre-loaded Fmoc amino acid Wang type resin (0.2 mmol/g, Applied Biosystems®) or pre-loaded Fmoc amino acid trityl resin (0.2 mmol/g, Rapp Polymere GmbH). The Ub (mutant) peptide sequences were synthesized on resin following the procedures as described before.[20] Peptides were treated with TFA/TIS/H_2O/Phenol for 2.5 hours followed by precipitation from Et_2O/Pentane and dissolved in lyophilization mixture (MQ: CH3CN: Formic acid 65/25/ 10, v:v:v) and lyophilized. Bt-PEG2-Ub_{75} was cleaved of the trityl resin using hexafluoroisopropanol in DCM (1:4 v/v) for 2 times 20 minutes and filtered. The flow through was collected and concentrated *in vacuo*, followed by coevaporation with dichloroethane (3 x) to remove residual HFIP.

Subsequently the protected peptide was dissolved in DCM and reacted with PyBOP, DiPEA and 3-azido-1-propanamine for 16 hours. The reaction was concentrated *in vacuo* and treated with TFA/TIS/H_2O/Phenol for 2.5 hours. The peptide was precipitated from Et_2O/pentane and subsequently purified using RP-HPLC.

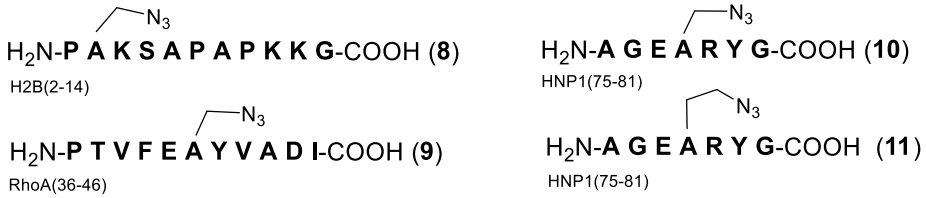
RP-HPLC purifications

A) Waters preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 µm OBD (30 x 150 mm) column at a flowrate of 37.5 mL/min using 3 mobile phases: A: MQ, B: CH_3CN and C: 1% TFA in MQ. Gradient: 20 -> 45% B, 5% C.

B) Shimadzu semi-preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 µm OBD (10 x 150 mm) column at a flowrate of 6.5 mL/min. using 2 mobile phases: A: MQ + 0.05% FA, B: CH_3CN + 0.05 % FA. Gradient: 0 -> 15% B.

C) Gilson preparative RP-HPLC system, equipped with Phenomenex Gemini (10 x 250 mm) column at a flowrate of 5 mL/min, using 2 mobile phases: A: 50 mM NH_4OAc in MQ, B: CAN.

Purification and analytical data of peptides



Azido peptides (**8** – **10**) were dissolved in 0.1% Formic Acid in MQ and purified using RP-HPLC. Pure fractions were pooled and lyophilized. Azidohomoalanine-HNP1 peptide **(11)** was used crude in the CuAAC reaction after cleavage from the resin followed by lyophilization.

Histone H2B(2-14): P[A*]PAKSAPAPKKG ([A*]: Azidoalanine) – Compound (8)

LC-MS: Rt = 0.41 min., ESI MS^+ (amu) calcd: 1260.47, found 1261.05 $[M+H]^+$, 630.86 $[M+2H]^{2+}$, 420.91 $[M+3H]^{3+}$, 315.92 $[M+4H]^{4+}$.

RhoA (36-46): PTVFE[A*]YVADI ([A*]: Azidoalanine) – Compound (9)

LC-MS: Rt = 2.14 min., ESI MS^+ (amu) calcd: 1265.39, found 1266.02 $[M+H]^+$, 633.31 $[M+2H]^{2+}$

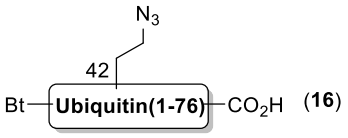
HNP1 (75-81): AGE[A*]RYG ([A*]: Azidoalanine) – Compound (10)

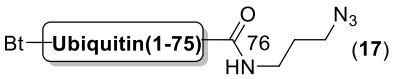
LC-MS: Rt = 0.55 min., ESI MS^+ (amu) calcd: 763.77, found 764.29 $[M+H]^+$, 382.66 $[M+2H]^{2+}$

HNP1 (75-81): AGE[A*]RYG ([A*]: Azidohomoalanine) – Compound (11)

LC-MS: Rt = 4.84 min., ESI MS^+ (amu) calcd: 777.78, found 778.3 $[M+H]^+$

Purification and analytical data of ubiquitin azide mutants





The crude Ub mutant was taken up in a minimal amount of warm DMSO and diluted in warm MilliQ while the final DMSO concentration was kept as low as possible (<10%). Next, the peptide was purified by preparative RP-HPLC (A). Pure fractions were pooled and lyophilized.

Biotin-PEG2-(R42Azidohomoalanine) Ub_{76} - Compound (16)

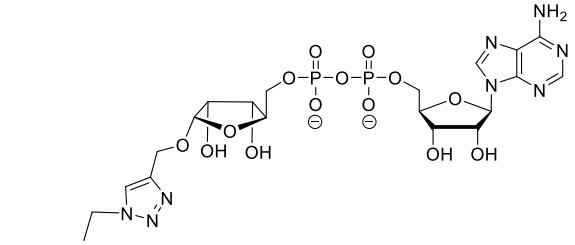
LC-MS: Rt = 2.07 min., ESI MS^+ (amu) calcd: 8888.26, found 8889.00 (deconv.)

Biotin-PEG2-(G76Azidopropanamine)Ub_{76} - Compound (17)

LC-MS: Rt = 1.92 min., ESI MS^+ (amu) calcd: 8943.34, found 8943.00 (deconv.)

General procedure for CuAAC reactions:

150 µL ADPr-propargyl (1.5 eq, 10 mg/mL in DMSO) was added to azido modified peptide (1.0 eq.) and subsequently added to 500 µL buffer (20 mM TRIS/150 mM NaCl, pH 7.6). To this was added 60 µL of freshly prepared click-mixture (1:1:1 v/v/v, $CuSO_4$ (26 mg/mL in water): Sodium Ascorbate (120 mg/mL in water): TBTA ligand (52 mg/mL in CH_3CN)). The reaction was shaken for 30 min at room temperature or 37°C and followed using LC-MS analysis. Once the azide starting material was fully converted to the ADPr-conjugate the reaction was quenched using 15 µL EDTA (0.5 M). The reaction was then purified by gel filtration, lyophilized and purified by RP-HPLC purification.



Histone H2B(2-14)-ADPr: P[A*]PAKSAPAPKKG ([A*]: triazolyl ADPr) – Compound (12)

The product was synthesized using the general procedure. HPLC purification using (C) with gradient: 4-10% B, in 12 min. The combined fractions were lyophilized to yield the title compound as a white solid (1.51 mg, 0.81 µmol, yield: 25%). ^{1}H NMR (500 MHz, D_2O) δ 8.41 (s, 1H, 2H), 8.13 (s, 1H, 8H), 7.90 (s, 1H, triazole), 6.02 (d, *J* = 6.0 Hz, 1H, H1'-ade), 4.96 (d, *J* = 4.3 Hz, 1H, H1''-rib). ^{31}P NMR (202 MHz, D_2O) δ: -10.65. LC-MS: Rt = 4.12 min.

ESI MS+ (amu) calcd: 1857.8, found 1858.5 $[M+H]^{+,}$ 929.5 $[M+2H]^{2+}$. HRMS: $[C_{73}H_{118}N_{24}O_{29}P_2 + 2H]^{2+}$: found 929.4082, calc. 929.4064, $[C_{73}H_{118}N_{24}O_{29}P_2 + 3H]^{3+}$: found 619.9447, calc. 619.402, $[C_{73}H_{118}N_{24}O_{29}P_2 + 4H]^{4+}$: found 465.2092, calc. 465.2071, $[C_{73}H_{118}N_{24}O_{29}P_2 + 5H]^{5+}$: found 372.3672, calc. 372.3649.

RhoA (36-46)-ADPr: PTVFE[A*]YVADI ([A*]: triazolyl ADPr) – Compound (13)

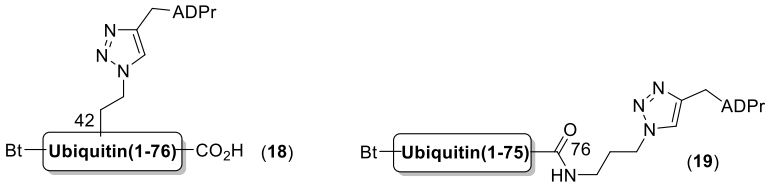
The product was synthesized using the general procedure. HPLC purification using (C) with gradient: 13-19% B, in 12 min. The combined fractions were lyophilized to yield the title compound as a white solid (0.4 mg, 0.21 µmol, yield: 3%). ^{1}H NMR (500 MHz, D_2O) δ 8.46 (s, 1H,2H), 8.17 (s, 1H, 8H), 7.89 (s, 1H, triazole), 7.24 – 7.13 (m, 3H, arom. Phe), 7.11 (dd, *J* = 6.8, 1.8 Hz, 2H, arom. Phe), 7.02 – 6.95 (m, 2H, arom. Tyr), 6.76 – 6.67 (m, 2H, arom. Tyr), 6.06 (d, *J* = 5.8 Hz, 1H, H1'-ade), 4.99 (d, *J* = 3.9 Hz, 1H, H1''-rib). ^{31}P NMR (202 MHz, D_2O) δ: -10.48, -10.60, -10.63, -10.76. LC-MS: Rt = 7.34 min. ESI MS+ (amu) calcd: 1862.8, found 1863.4 $[M+H]^{+,}$ 932.3 $[M+2H]^{2+}$. HRMS: $[C_{76}H_{109}N_{19}O_{32}P_2 + 2H]^{2+}$: found 931.8559, calc. 931.8548, $[C_{76}H_{109}N_{19}O_{32}P_2 + 3H]^{3+}$: found 621.5708, calc. 621.5732.

HNP1 (75-81)-ADPr: AGE[A*]RYG ([A*]: triazolyl ADPr) – Compound (14)

The product was synthesized using the general procedure. HPLC purification using (C) with gradient: 2-8% B, in 12 min. The combined fractions were lyophilized to yield the title compound as a white solid (2.36 mg, 1.73 µmol, yield: 53%). ^{1}H NMR (600 MHz, D_2O) δ 8.46 (s, 1H, 2H), 8.18 (s, 1H, 8H), 7.85 (s, 1H, triazole), 7.09 – 7.00 (m, 2H, arom. Tyr), 6.76 – 6.68 (m, 2H, arom. Tyr), 6.07 (d, *J* = 5.8 Hz, 1H, H1'-ade), 5.05 (d, *J* = 4.4 Hz, 1H, H1''-rib). ^{31}P NMR (202 MHz, D_2O) δ : -10.47, -10.58, -10.66, -10.76. LC-MS: Rt = 4.30 min. ESI MS+ (amu) calcd: 1361.1, found 1361.3 $[M+H]^{+,}$ 681.3 $[M+2H]^{2+}$.HRMS: $[C_{48}H_{70}N_{18}O_{25}P_2 + H]^{+}$: found 1361.4315, calc. 1361.4313, $[C_{48}H_{70}N_{18}O_{25}P_2 + 2H]^{2+}$: found 681.2223, calc. 681.2195, $[C_{48}H_{70}N_{18}O_{25}P_2 + 3H]^{3+}$: found 454.8186, calc. 454.8166.

HNP1 (75-81)-ADPr: AGE[A*]RYG ([A*]: triazolyl ADPr) – Compound (15)

The product was synthesized using the general procedure and purified with gel filtration. The combined fractions were lyophilized to yield the title compound as a white solid (3.13 mg, 2.28 µmol, yield: 76%). ^{1}H NMR (500 MHz, D_2O) δ: 8.55 (s, 1H, 2H), 8.25 (s, 1H, 8H), 7.89 (s, 1H, triazole), 7.08 (d, *J* = 8.4 Hz, 2H, arom. Tyr), 6.75 – 6.68 (m, 2H, arom. Tyr), 6.12 (d, *J* = 5.7 Hz, 1H, H1'-ade), 5.09 (d, *J* = 3.9 Hz, 1H, H1''-rib). ^{31}P NMR (202 MHz, D_2O) δ: -10.43, -10.53, -10.66, -10.76. LC-MS: Rt = 4.36 min. ESI MS+ (amu) calcd: 1375.2, found 1375.3 $[M+H]^{+,}$ 688.3 $[M+2H]^{2+}$. HRMS: $[C_{49}H_{72}N_{18}O_{25}P_2 + 2H]^{2+}$: found 688.2247, calc. 688.2274.



Biotin-PEG2-(R42*) Ub_{76} (R42*: triazolyl ADPr) - Compound (18)

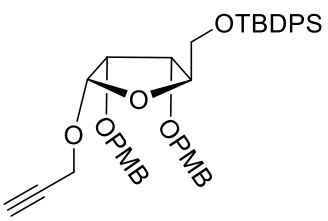
Ubiquitin **16** (5.1 mg), 1.0 eq. was dissolved in 45 uL DMSO and subsequently added to 510 uL buffer (20 mM TRIS/150 mM NaCl, pH 7.6). To this was added 45uL of freshly prepared click-mixture (1:1:1 v/v/v, $CuSO_4$ (26 mg/mL in water): Sodium Ascorbate (120 mg/mL in water): TBTA ligand (52 mg/mL in acetonitrile)) and pH was

adjusted to 7.4 before addition of 60 uL ADPr-propargyl 2.4 eq. (13 mg/mL in DMSO). The reaction was shaken for 90 min at room temperature and followed using LC-MS analysis. Once the azide starting material was fully converted to the ADPr-conjugate the reaction was quenched using 10 uL EDTA (0.5 M). Dialysis using a 3.5-5 kD MWCO dialysis device removed traces of excess ADPr-propargyl reagent as well as copper/ligand/ascorbate from the reaction, followed by size exclusion chromatography resulted in the title compound (4.6 mg, 0.48 umol, 84%. Calculated based on SDS-page /Coomassie stain comparison with standard curve of wt Ub) . LC-MS: Rt = 2.00 min., ESI MS+ (amu) calcd: 9485.36, found 9486.00. HRMS: $[C_{411}H_{675}N_{113}O_{137}P_2S + 7H]^{7+}$: found 1355.9868, calc. 1355.9886, $[C_{411}H_{675}N_{113}O_{137}P_2S + 8H]^{8+}$ found 1186.6187, calc. 1186.6161, $[C_{411}H_{675}N_{113}O_{137}P_2S + 9H]^{9+}$: found 1054.8846, calc. 1054.8818, $[C_{411}H_{675}N_{113}O_{137}P_2S + 10H]^{10+}$: found 949.4930, calc. 949.4944, $[C_{411}H_{675}N_{113}O_{137}P_2S + 11H]^{11+}$: found 863.2679, calc. 863.2684, $[C_{411}H_{675}N_{113}O_{137}P_2S + 12H]^{12+}$: found 791.4152, calc. 791.4133, $[C_{411}H_{675}N_{113}O_{137}P_2S + 13H]^{13+}$: found 730.6146, calc. 730.6129.

Biotin-PEG2-(G76*)Ub_{76} (G76*: triazolyl ADPr) - Compound (19)

Ubiquitin **17** (3.8 mg), 1.0 eq. was dissolved in 50 uL DMSO and subsequently added to 250 uL buffer (20 mM TRIS/150 mM NaCl, pH 7.6). To this was added 45 uL of freshly prepared click-mixture (1:1:1 v/v/v, $CuSO_4$ (26 mg/mL in water): Sodium Ascorbate (120 mg/mL in water): TBTA ligand (52 mg/mL in acetonitrile)) and pH was adjusted to 7.4 before addition of 40 uL ADPr-propargyl 2.4 eq. (13 mg/mL in DMSO). The reaction was shaken for 30 min at room temperature and followed using LC-MS analysis. Once the azide starting material was fully converted to the ADPr-conjugate the reaction was quenched using 15 uL EDTA (0.5 M). Dialysis using a 3.5-5 kD MWCO dialysis device removed traces of excess ADPr-propargyl reagent as well as copper/ligand/ascorbate from the reaction, followed by size exclusion chromatography resulted in the title compound (3.2 mg, 0.34 umol, 80%). Calculated based on SDS-page /Coomassie stain comparison with standard curve of wt Ub). LC-MS: Rt = 1.96 min., ESI MS+ (amu) calcd: 9540.44 , found 9541.00. HRMS: $[C_{414}H_{684}N_{116}O_{135}P_2S + 7H]^{7+}$: found 1363.8558, calc. 1363.8586, $[C_{414}H_{684}N_{116}O_{135}P_2S + 8H]^{8+}$ found 1193.5070, calc. 1193.5023, $[C_{414}H_{684}N_{116}O_{135}P_2S + 9H]^{9+}$: found 1061.0068, calc. 1061.0029, $[C_{414}H_{684}N_{116}O_{135}P_2S + 10H]^{10+}$: found 955.0046, calc. 955.0034, $[C_{414}H_{684}N_{116}O_{135}P_2S + 11H]^{11+}$: found 868.2764, calc. 868.2766, $[C_{414}H_{684}N_{116}O_{135}P_2S + 12H]^{12+}$: found 796.0090, calc. 796.0042, $C_{414}H_{684}N_{116}O_{135}P_2S + 13H]^{13+}$: found 734.8512, calc. 734.8506.

Synthesis of α-propargyl-ADPr (7)



1-*O*-propargyl-2,3-bis-*O*-(4-methoxybenzyl)-5-*O*-tert-butyldiphenylsilyl-α,β-D-ibofuranoside (2)

The trifluoroacetimidate donor **1** (2.15 g, 2.75 mmol), described previously,[10] and propargyl alcohol (160 µL, 2.75 mmol) were co-evaporated with 1,4-dioxane (3 x) and dissolved in dry DCM (30 mL) along with freshly activated 3Å molecular sieves. The reaction mixture was stirred at room temperature for 1 hour under argon to remove traces of water. The reaction mixture was cooled to -78 °C, TMSOTf (10 µL, 55 µmol) was added and the reaction mixture was stirred at -78 °C for 30 minutes. The reaction was quenched by the addition of triethylamine,

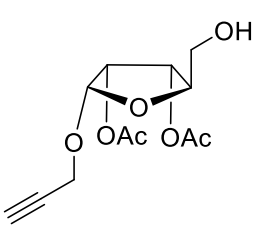
filtered, concentrated *in vacuo* and purified by silica gel chromatography (Pentane/EtOAc, 90/10 – 85/15) to obtain the title compound with the two anomers separated (β-anomer: 419 mg, 0.63 mmol; α-anomer: 1.04 g, 1.57 mmol; α/β=71/29; 80% in total yield).

β-anomer:

^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.66 (d, *J* = 6.9 Hz, 4H, arom. TBDPS), 7.46 – 7.32 (m, 6H, arom. TBDPS), 7.29 (d, *J* = 8.5 Hz, 2H, arom. PMB), 7.18 (d, *J* = 8.5 Hz, 2H, arom. PMB), 6.87 (d, *J* = 8.6 Hz, 2H, arom. PMB), 6.81 (d, *J* = 8.6 Hz, 2H, arom. PMB), 5.22 (s, 1H, H1'), 4.58 (AB, *J* = 36.4, 11.7 Hz, 2H, CH_2 PMB), 4.39 (AB, *J* = 43.3, 11.4 Hz, 2H, CH_2 PMB), 4.26 – 4.23 (m, 1H, H4'), 4.19 – 4.11 (m, 3H, H3', C*H2*CCH), 3.89 (d, *J* = 4.5 Hz, 1H, H2'), 3.85 (AB, *J* = 11.3, 3.3 Hz, 1H, H5'), 3.80 (s, 3H, CH_3 PMB), 3.78 (s, 3H, CH_3 PMB), 3.69 (AB, *J* = 11.3, 4.1 Hz, 1H, H5'), 2.38 (t, *J* = 2.4 Hz, 1H, CH2CC*H*), 1.03 (s, 9H, *t*Bu TBDPS). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 159.48, 159.38 (Cq. Arom.), 135.73, 135.70 (arom.), 133.55, 133.52, 130.08, 129.98 (Cq. Arom.), 129.84, 129.80, 129.75, 129.53, 127.81, 127.80, 113.96, 113.87 (arom.), 103.17 (C1'), 82.39 (C4'), 79.45 (C2'), 79.32 (CH_2*C*CH), 77.07 (C3'), 74.60 (CH_2C*C*H), 72.18, 72.07 (CH_2 PMB), 64.01 (C5'), 55.40, 55.38 (CH_3 PMB), 54.26 (*C*H_2CCH), 26.95 (*t*Bu TBDPS), 19.40 (Cq. *t*Bu TBDPS).

α-anomer:

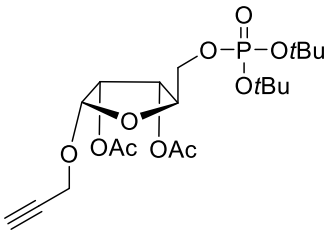
^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.59 (d, *J* = 7.8 Hz, 2H, arom. TBDPS), 7.54 (d, *J* = 7.8 Hz, 2H, arom. TBDPS), 7.43 – 7.21 (m, 10H, arom. TBDPS, PMB), 6.85 (d, *J* = 8.5 Hz, 2H, arom. PMB), 6.81 (d, *J* = 8.5 Hz, 2H, arom. PMB), 5.31 (d, *J* = 4.3 Hz, 1H, H1'), 4.65 (AB, *J* = 12.2, 7.5 Hz, 2H, CH_2 PMB), 4.57 (d, *J* = 12.0 Hz, 1H, CH_2 PMB), 4.51 (d, *J* = 12.4 Hz, 1H, CH_2 PMB), 4.39 (d, *J* = 2.3 Hz, 2H, C*H2*CCH), 4.15 – 4.12 (m, 1H, H4'), 3.96 (dd, *J* = 6.5, 2.7 Hz, 1H, H3'), 3.86 (dd, *J* = 6.5, 4.4 Hz, 1H, H2'), 3.78 (s, 3H, CH_3 PMB), 3.77 (s, 3H, CH_3 PMB), 3.60 (AB, *J* = 11.2, 3.4 Hz, 1H, H5'), 3.50 (AB, *J* = 11.1, 3.1 Hz, 1H, H5'), 2.40 (t, *J* = 2.2 Hz, 1H, CH2CC*H*), 0.94 (s, 9H, *t*Bu TBDPS). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 159.39, 159.23 (Cq. Arom.), 135.69, 135.62 (arom.), 133.28, 133.14, 130.52, 129.97 (Cq. Arom.), 129.84, 129.78, 129.71, 129.68, 127.79, 127.75, 113.89, 113.76 (arom.), 98.93 (C1'), 84.13 (C4'), 79.84 (CH_2*C*CH), 77.71 (C2'), 74.85 (C3'), 74.23 (CH_2C*C*H), 72.19, 71.98 (CH_2 PMB), 64.12 (C5'), 55.32 (CH_3 PMB), 54.22 (*C*H_2*C*CH), 26.85 (*t*Bu TBDPS), 19.26 (Cq. *t*Bu TBDPS). HRMS: $[C_{40}H_{46}O_7Si + Na]^+$: 689.2902 found, 689.2902 calculated.



1-*O*-propargyl-2,3-bis-*O*-acetyl-α-D-ribofuranoside (3)

Compound **2** (660 mg, 0.99 mmol) was dissolved in DCM (20 mL) and TFA (0.6 mL) was added. The reaction was stirred at room temperature for 20 minutes and co-evaporated with toluene. The crude intermediate was dissolved in pyridine (5 mL) and acetic anhydride (1.9 mL) and DMAP (cat.) were added. The reaction was stirred at room temperature for 1 hour, diluted with DCM and washed with sat. aq. $NaHCO_3$. The organic layer was dried ($MgSO_4$), concentrated and co-evaporated with pyridine (2x). The crude intermediate was dissolved in pyridine (5 mL) and HF·pyridine (1 mL) was added. The reaction was stirred at room temperature for 1 hour and carefully quenched with sat. aq. $NaHCO_3$. The mixture was extracted with DCM, dried ($MgSO_4$) and concentrated under reduced pressure. Column chromatography (Pentane/EtOAc, 60/40 – 50/50) yielded the title compound as a white foam (138 mg, 0.57 mmol, 58% over 3 steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.50 (d, *J* = 4.5 Hz, 1H, H1'), 5.24 (dd, *J* = 7.3, 3.4 Hz, 1H, H3'), 5.03 (dd, *J* = 7.3, 4.6 Hz, 1H, H2'), 4.34 (dd, *J* = 33.8, 2.4 Hz, 2H, C*H2*CCH), 4.18 (q, *J* = 3.3 Hz, 1H, H4'), 3.85 (AB, *J* = 12.1, 3.1 Hz, 1H, H5'), 3.81 (AB, *J* = 12.1, 3.4 Hz, 1H, H5'), 2.53 (s, 1H, OH), 2.47 (t, *J*

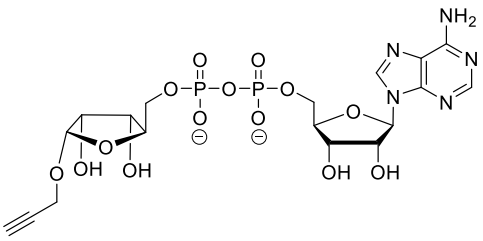
= 2.3 Hz, 1H, CH_2CCH), 2.14 (s, 3H, CH_3 Ac), 2.14 (s, 3H, CH_3 Ac). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 170.90, 170.04 (CO Ac), 98.55 (C1'), 83.07 (C4'), 78.78 (CH2*C*CH), 74.98 (CH_2CCH), 71.12 (C3'), 70.02 (C2'), 62.10 (C5'), 54.57 (CH_2CCH), 20.93, 20.62 (CH_3 Ac).



1-*O*-propargyl-2,3-di-*O*-acetyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribofuranoside (4)

Compound **3** (136 mg, 0.50 mmol) and pyridinium chloride (230 mg, 2 mmol) were co-evaporated with pyridine (3x) and dissolved in pyridine (5 mL) under an atmosphere of argon. Di-*tert*-butyl-*N,N*-diisopropylphosphoramidite (0.23 mL, 0.75 mmol) was added and the reaction was stirred at room temperature for 15 minutes. Then *t*BuOOH (5.5 M in nonane) (0.7 mL, 3.75 mmol) was added and the reaction mixture was stirred for 30 minutes. The reaction was quenched upon addtion of aq. $NaHCO_3$ (sat.), extracted with DCM, dried ($MgSO_4$) and concentrated *in vacuo*. Column chromatography (pentane/EtOAc, 60/40 – 50/50) yielded the title compound as a white foam (172 mg, 0.37 mmol, 74%).

^1H NMR (500 MHz, $CDCl_3$) δ: 5.49 (d, *J* = 4.5 Hz, 1H, H1'), 5.27 (dd, *J* = 7.2, 3.2 Hz, 1H, H3'), 5.05 (dd, *J* = 7.2, 4.6 Hz, 1H, H2'), 4.33 (d, *J* = 2.2 Hz, 2H, C*H2*CCH), 4.27 – 4.25 (m, 1H, H4'), 4.18 – 4.15 (m, 2H, H5'), 2.42 (t, *J* = 2.3 Hz, 1H, CH2CC*H*), 2.13 (s, 6H, CH_3 Ac), 1.49 (s, 18H, CH_3 *t*Bu). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 170.47, 169.71 (CO Ac), 98.53 (C1'), 82.85, 82.81, 82.79, 82.75 (Cq. *t*Bu), 81.15, 81.08 (C4'), 78.75 (CH_2CCH), 74.71 (CH_2CCH), 70.82 (C2'), 69.91 (C3'), 65.80, 65.76 (C5'), 54.57 (CH_2CCH), 29.83, 29.82, 29.80, 29.79 (CH_3 *t*Bu), 20.81, 20.54 (CH_3 Ac). ^{31}P NMR (202 MHz, $CDCl_3$) δ: -9.23. HRMS: $[C_{20}H_{33}O_{10}P + H]^+$: 465.1885 found, 465.1884 calculated.



α-1-*O*-propargyl-ADPr (7)

Compound **5** (120 mg, 0.26 mmol) was dissolved in DCM (5.7 mL) and TFA (0.3 mL) was added. The reaction was stirred at room temperature for 15 minutes, co-evaporated with toluene and pyridine (2x). The intermediate phosphate was analysed by ^{31}P NMR to confirm the complete removal of both *t*Bu groups (^{31}P-NMR (121 MHz, D_6-actone) δ: -0.14). Intermediate phosphate and dicyanoimidazole (77 mg, 0.65 mmol) were co-evaporated with ACN (3x) and dissolved in dry ACN (2mL). Adenosine amidite **6** (277 mg, 0.39 mmol) was added to the reaction mixture, stirred at room temperature for 15 minutes and *t*BuOOH (5.5 M in nonane) (0.25 mL, 1.38 mmol) was added. The reaction was stirred for 30 minutes, DBU (0.2 mL, 1.4 mmol) was added and the reaction was stirred for an additional 30 minutes. Aq. NH_4OH (35%) (5 mL) was added to the reaction mixture, the reaction was stirred for 16 hours and concentrated under reduced pressure. Size exclusion chromatography followed by lyophilization yielded the title compound as a white powder (100 mg, 0.17 mmol, 65%). ^1H NMR (500 MHz, D_2O) δ: 8.42 (s, 1H, H2), 8.15 (s, 1H, H8), 6.05 (d, *J* = 5.5 Hz, 1H, H1'-ade), 5.04 (d, *J* = 4.0 Hz, 1H, H1'-rib), 4.67 (t, *J* = 5.5 Hz, 1H, H2'-ade), 4.44 (dd, *J* = 5.0, 4.5 Hz, 1H, H3'-ade), 4.32 – 4.29 (m, 1H, H4'-ade), 4.21 – 4.13 (m, 4H, H5'-ade, C*H2*CCH), 4.12 – 4.10 (m, 1H, H4'-rib), 4.10 – 4.04 (m, 2H, H2'-rib, H3'-rib), 3.93 – 3.91 (m, 2H, H5'-rib), 2.73 – 2.72 (m, 1H, CH_2CCH). ^{13}C NMR (126 MHz, D_2O) δ: 155.49 (C4), 149.03 (C6), 118.59 (C5), 100.62

(C1'-rib), 86.89 (C1'-ade), 83.85, 83.78, 83.66, 83.59 (C4'-rib, C4'-ade), 79.07 ($CH_2\mathit{C}CH$), 74.29 (C2'-ade), 70.92 (C3'-ade), 70.32 (C2'-rib), 69.48 (C3'-rib), 65.57, 65.54 (C5'-rib), 65.17, 65.13 (C5'-ade), 54.84 ($\mathit{C}H_2\mathit{C}CH$). ^{31}P NMR (202 MHz, D_2O) δ: -10.46, -10.57, -10.65, -10.76. HRMS: $[C_{18}H_{25}N_5O_{14}P_2 + H]^+$: 598.0939 found, 598.0946 calculated.

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8 Summary and future prospects

This Thesis focuses on the design and synthesis of ADP-ribosylated compounds that can be applied in biological studies. **Chapter 1** reviews the majority of the synthetic work in the field of ADP-ribosylation which includes methods toward ADP-ribosylated amino acids, ADP-ribosylated peptides/proteins, linear and branched ADPr oligomers. The target molecules of the following experimental chapters are outlined in Figure 1.

Synthetic mono-ADP-ribosylated oligopeptides are useful tools to investigate biological ADP-ribosylation events at a molecular level. Although several ADP-ribosylated oligopeptides have been synthesized previously,[1-3] synthetic mono-ADP-ribosylated amino acids are relatively scarce. **Chapter 6** deals with the synthesis of α-configured Asn-ADPr **1**, a more stable bioisostere of ADPr-Asp and the structure elucidation of the co-crystals of Asn-ADPr **1** with a macrodomain of a bacterial protein. The presented synthesis of **1**, using suitably protected ribose donors and P(V)-P(III) chemistry for the introduction of the pyrophosphate can be transferred to other mono-ADP-ribosylated amino acids. A general procedure to prepare analogs of mono-ADPr peptides and even a protein (**2**) is the subject of **Chapter 7**. A newly designed propargyl-ADPr building block reacted efficiently via CuAAC reaction with an oligopeptide having either an azidoalanine or an azidohomoalanine residue, to afford several triazole linked mono-ADPr peptides. In a similar way, triazole linked ubiquitin was generated, which is the first reported artificial ADPr-protein. Importantly, the synthetic ADPr-Ub possessed similar bioactivity as the native counterpart in the auto-ubiquitination assay, suggesting a potential application of triazole linked mono-ADPr-peptides/proteins in biological studies.

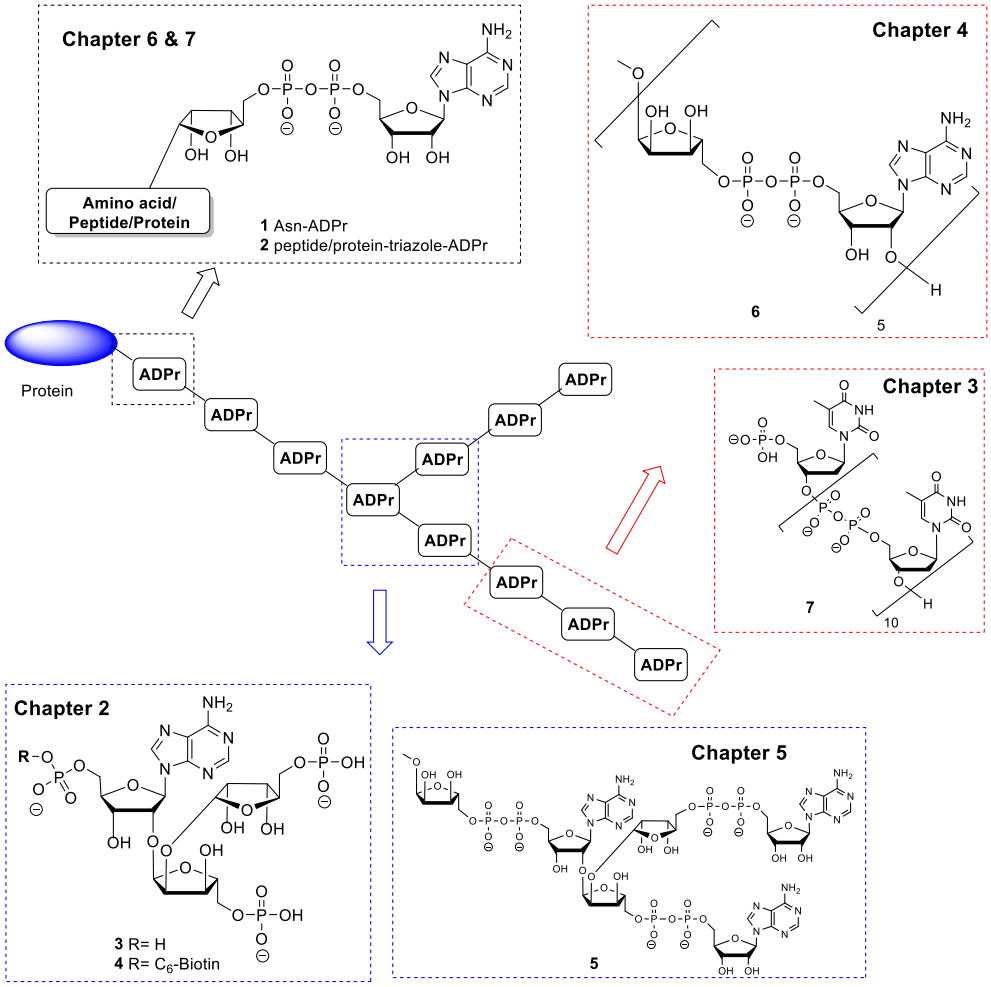


Figure 1. Target ADPr molecules of this thesis

Although it was discovered 40 years ago, branched poly-ADPr (PAR) has always been an enigma in the ADPr biology.[4, 5] This Thesis describes the synthesis of various branched fragments of PAR able for application in biological studies to investigate the function of the branch points in the structure of PAR. **Chapter 2** deals with the synthesis of branched fragment **3** (Figure 1) with two sequential 1,2-cis glycosylations, Vorbrüggen coupling to install the adenine and phosphorylation of three primary alcohols as key transformations. ^{1}H-NMR comparison of the obtained synthetic compound with its enzymatically obtained natural counterpart showed identical structure, indicating that the regio- and stereochemistry of branch point of PAR was correctly elucidated by Miwa.[4] Furthermore, this synthetic methodology was also utilized for the synthesis of biotinylated linear or branched PAR fragments like **4**, as valuable tools for future discovery of proteins that bind to branched PAR. An even more ambitious synthetic target, branched ADPr trimer **5**, containing three full ADPr units (Figure 1), is described in **Chapter 5**. The introduction of the three pyrophosphate linkages with P(V)-P(III) chemistry, as described in Chapter 2, is adapted for solid-phase synthesis. A highly advanced branched

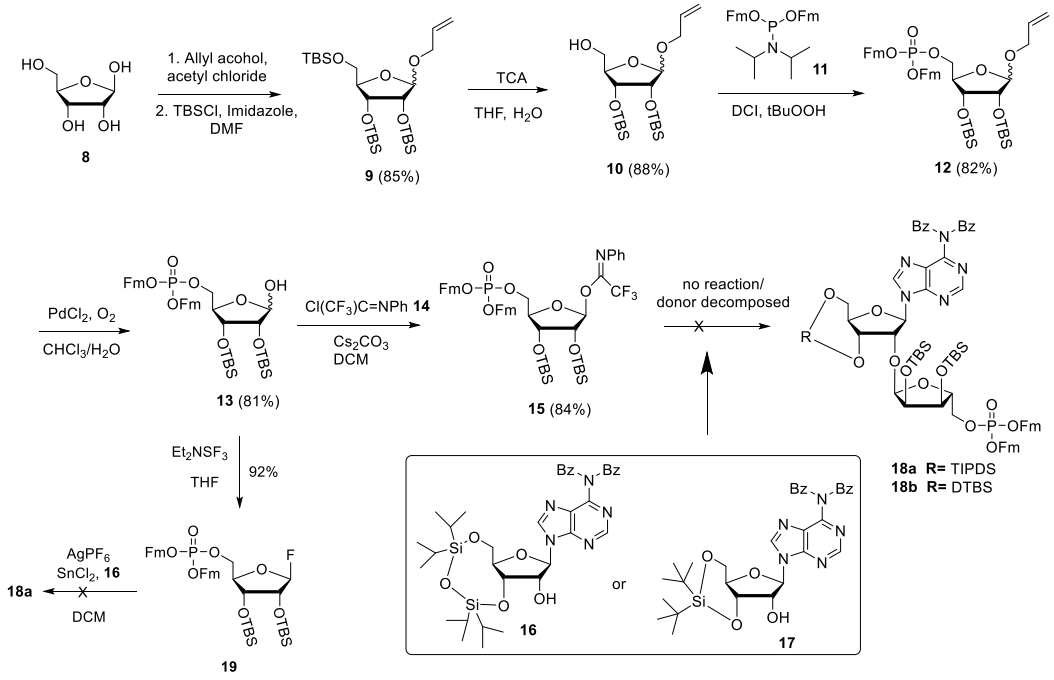
phosphoramidite with two phosphotriesters was synthesized in 14 steps and applied for the introduction of the first pyrophosphate. The two remaining pyrophosphates were then simultaneously installed with the aid of an adenosine phosphoramidite. The binding of branched compounds **4** and **5** with "reader" proteins of interest[6] is presently under investigation. The modular synthesis of **5** is a valuable asset for future synthesis of other branched ADPr.

Poly-ADPr plays a pivotal role in various physiological and pathological processes such as DNA repair, apoptosis, inflammation and neurodegeneration disease.[7-10] Synthetic ADPr oligomers would facilitate a better understanding of the involved processes at a molecular level and eventually contribute to the discovery of new drug targets.[11, 12] The challenges in the synthesis of ADPr oligomers are many. The difficulties begin with the preparation of advanced building blocks in sufficient quantities with the stereoselective ribosylation and regioselective adenine introduction as key steps. On top of this comes the notoriously difficult construction of multiple pyrophosphate linkages that are intrinsic labile and the anionic properties of the intermediates and target ADPr oligomers that restrict its assembly on the solid phase and hamper the monitoring of the involved reactions. The synthetic procedure to the pyrophosphate linkages, that is used throughout the research described in this thesis, is performed with P(V)-P(III) chemistry. **Chapter 3** describes the solid-phase synthesis of 3',5'-pyrophosphate-linked thymidine oligomers, using a new phosphoramidite thymidine building block with an Fm protected 5'-phosphotriester. The successful construction of 3',5'-pyrophosphate-linked thymidine decamer was an incentive to expand the Fm-based chemistry towards the preparation of ADPr oligomers, described in **Chapter 4**, which describes the first synthesis of ADPr pentamer.

Toward an alternative synthesis of the phosphoramidite building block for the assembly of ADPr oligomers

The solid phase assembly of linear ADPr oligomers, such as described in Chapter 4, requires the repeated use of a building block, the synthesis of which is rather lengthy and time-consuming.[13] The number of steps toward this building block can potentially be reduced by changing the applied protecting groups. The use of benzyl groups enables selective 1,2-cis ribosylation but also requires intermediate removal by hydrogenolysis which proved to be troublesome and tedious on a number of occasions. Besides, the introduction of adenine moiety in a late stage by Vorbrüggen-type reaction also increases the number of protective group manipulations.[13, 14] To circumvent these issues an alternative route of synthesis was explored with the condensation of *N*-phenyl-trifluoroacetimidate donor **15** and adenosine acceptor **16** or **17** as a key step (Scheme 1). In this way the route of synthesis could be shortened by avoiding both the use of benzyl groups and the Vorbrüggen coupling. The synthesis of donor **15** started with Fischer glycosylation of ribose **8** with allyl alcohol, followed by

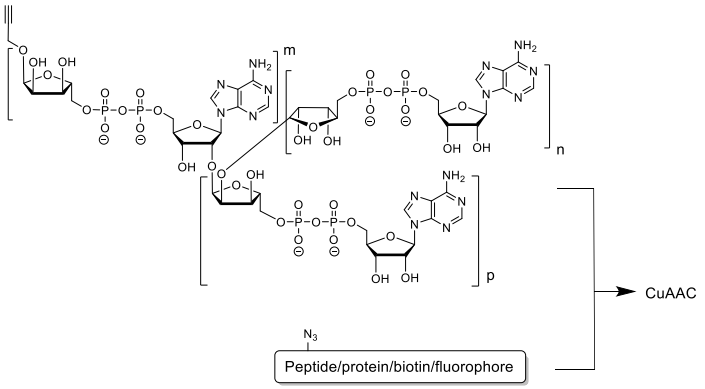
silylation of the remaining hydroxyls to obtain **9** in good yield. TBS group on the primary OH in **9** was selectively removed with TCA in THF/H_2O,[12] allowing for the phosphitylation of **10** and subsequent oxidation to give phosphotriester **12**. $PdCl_2$ catalyzed deallylation (see Chapter 5) yielded hemiacetal **13** which could be used for the synthesis of various donors. The introduction of 2,2,2-trifluoro-*N*-phenylacetimidoyl group with Cs_2CO_3 in acetone failed due to the concomitant cleavage of Fm groups.[15, 16] Fortunately the use of DCM to suppress the solubility of Cs_2CO_3, furnished *N*-phenyl-trifluoroacetimidate donor **15** in good yield. The attempted couplings of **15** with adenosine acceptors **16** and **17** to get desired α-ribosylated adenosine **18,** using activators, like TMSOTf, DTBS(OTf)$_2$, TfOH and Tf_2NH, have failed. The low reactivity of these adenosine acceptors could hamper glycosylation, as the glycosylation between **15** and other acceptors was successful determined by TLCMS.



Scheme 1. Toward an alternative synthetic route to key linear phosphoramidite

Inspired by the report of Lambrecht *et al*[12] who coupled a ribosyl fluoride with an adenosine acceptor under activation of $AgPF_6/SnCl_2$, donor **19** was prepared in good yield from alcohol **13** using Et_2NSF_3 in THF (Scheme 1). However, the subsequent coupling of **16** with **19** using the same procedure was unsuccessful. The difficulties with the direct ribosylation of adenosine acceptor described above testify for the low reactivity of 2'-OH of adenosine in glycosylation reactions. Other methods like gold-catalyzed glycosylation using *O*-alkynylbenzoates as donors should be attempted in the future.[17] Once the glycosylation is successful, **18a**/**b** could be easily converted into the phosphoramidite building block for the assembly of ADPr oligomers.[13]

Conjugation of oligo-ADPr with peptides, proteins and molecular probes



Scheme 2. Proposed CuAAC click chemistry of ADPr oligomer (branched or linear) with biomolecules and molecular probes relevant for research in biology

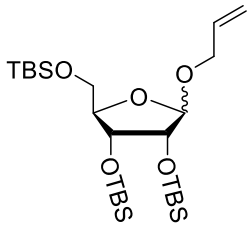
Using the methods described in **Chapter 4** and **Chapter 5**, more complicated synthetic targets like branched (m, n, p ≥ 1) or linear ADPr oligomers (n = 0, m, p ≥ 2) with a propargyl at the anomeric center could be envisaged (Scheme 2). These precious oligomers could be further conjugated via CuAAC chemistry with either natural substrates (like peptides or proteins) or tags (such as biotin or fluorophorescent labels), containing an azide functionality (see **Chapter 7**). With these ADPr conjugates in hand, many biological experiments are possible, such as the use of biotinylated branched ADPr oligomers for searching corresponding binding proteins[6] and the use of a series of linear ADPr oligomers, varying in length, to study the binding of these oligomers with "reader" and "eraser" proteins.[18-20]

Acknowledgement

Liming Wang, Zhen Wang, Dr. Sizhe Li and Yongzhen Zhang are kindly acknowledged for the nice discussion on glycosylation reactions in this chapter.

Experimental section

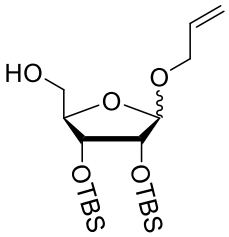
The general procedure is the same as described in Chapter 2.



1-*O*-Allyl-2,3,5-tri-*O*-tert-butyldimethylsilyl-α,β-D-ribofuranoside (9)

D-Ribose **8** (3 g, 20 mmol), allyl alcohol (50 mL) and acetyl chloride (1 mL, 14 mmol) were added into the flask and stirred at room temperature for 2 hours before it was quenched upon addition of pyridine (2.5 mL). The mixture was concentrated, co-evaporated with toluene (2 x). To the residue, DMF (30 mL), imidazole (6.8 g, 100 mmol) and TBSCl (34.65 mL, 100 mmol) was added and stirred at room temperature for 16 hours after which the reaction was quenched by addition of H_2O (50 mL) and Et_2O (50 mL). The organic layer was

separated, washed with additional H_2O (2 x) and brine (1 x), dried ($MgSO_4$), filtered, concentrated and purified by silica gel chromatography (pentane/EtOAc, 100/0 – 100/2) to afford **9** as a colorless oil (9.01 g, 16.93 mmol, 85%). ^{1}H NMR (500 MHz, chloroform-*d*) δ 5.97 – 5.81 (m, 1H, *CH*=CH_2), 5.37 – 5.07 (m, 2H, CH=*CH_2*), 4.92 (d, *J* = 4.1 Hz, 0.25H, H1-α), 4.82 (d, *J* = 1.6 Hz, 0.75H, H1-β), 4.32 – 3.88 (m, 5H, H3, H2, O*CH_2*CH=CH_2, H4), 3.82 – 3.58 (m, 2H, H5), 0.96 – 0.82 (m, 27H, TBS), 0.14 – 0.00 (m, 18H, TBS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 135.12, 134.56 (*CH*=CH_2), 117.19, 116.14 (CH=*CH_2*), 106.31, 101.99 (C1), 85.12, 83.22 (C4), 76.69, 74.10 (C2), 71.66, 71.54 (C3), 68.77, 68.56 (O*CH_2*CH=CH_2), 63.29, 63.02 (C5), 26.17, 26.15, 26.06, 26.04, 25.97 (CH_3, TBS), 18.64, 18.61, 18.49, 18.34, 18.27, 18.22 (Cq. TBS), -4.12, -4.21, -4.30, -4.34, -4.44, -4.47, -4.72, -4.77, -5.09, -5.19, -5.26, -5.34 ($SiCH_3$, TBS). IR (film): 2953, 2929, 2896, 2857, 1472, 1463, 1252, 1129, 1004, 835, 775 cm^{-1}. HRMS (ESI^+) calcd for $C_{26}H_{56}O_5Si_3Na$ (M+Na) 555.3328. Found 555.3327. $[\alpha]_D^{20}$ +9.2 (c = 1, in DCM)



1-*O*-Allyl-2,3-di-*O*-tert-butyldimethylsilyl-α,β-D-ribofuranoside (10)

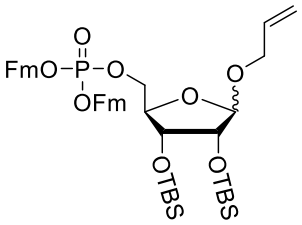
TCA solution (12.8 g, 79 mmol, in 6 mL H_2O) was added dropwise to a flask containing **9** (909 mg, 1.71 mmol) and THF (24 mL) at 0°C. After complete addition, the reaction was stirred at 0°C for 2 hours after which it was quenched by aq. $NaHCO_3$ (sat.). DCM extracted this mixture (3 x) and the organic layers are combined, dried ($MgSO_4$), filtered, concentrated, and purified by silica gel chromatography (pentane/EtOAc, 100/0 – 90/10) to afford **10** (α+β) as a colorless oil. (α anomer: 510 mg, 1.23 mmol, 72%; β anomer: 117 mg, 0.28 mmol, 16%)

α anomer:

^{1}H NMR (500 MHz, chloroform-*d*) δ 5.90 (dddd, *J* = 17.2, 10.6, 5.9, 4.9 Hz, 1H, *CH*=CH_2), 5.31 (dq, *J* = 17.2, 1.8 Hz, 1H, CH=*CH_2*), 5.13 (dq, *J* = 10.4, 1.6 Hz, 1H, CH=*CH_2*), 4.96 (d, *J* = 3.8 Hz, 1H, H1), 4.27 (ddt, *J* = 13.0, 4.9, 1.6 Hz, 1H, O*CH_2*CH=CH_2), 4.15 – 3.91 (m, 4H, O*CH_2*CH=CH_2, H4, H3, H2), 3.80 (AB, *J* = 12.0 Hz, 1H, H5), 3.58 (AB, *J* = 11.6, 3.3 Hz, 1H, H5), 1.89 (s, 1H, OH), 0.90 (d, *J* = 10.6 Hz, 18H, TBS), 0.11 – 0.02 (m, 12H, TBS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 134.71 (*CH*=CH_2), 116.66 (CH=*CH_2*), 102.10 (C1), 83.78 (C4), 74.05 (C2), 71.19 (C3), 68.97 (O*CH_2*CH=CH_2), 62.04 (C5), 26.12, 26.02 (CH_3, TBS), 18.60, 18.28 (Cq. TBS), -4.11, -4.19, -4.45, -4.80 ($SiCH_3$, TBS). HRMS (ESI^+) calcd for $C_{20}H_{42}O_5Si_2Na$ (M+Na) 441.2463. Found 441.2469.

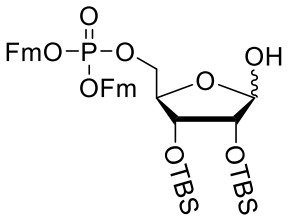
β anomer:

^{1}H NMR (500 MHz, chloroform-*d*) δ 5.90 (dddd, *J* = 17.2, 10.4, 6.1, 5.2 Hz, 1H, *CH*=CH_2), 5.34 – 5.17 (m, 2H, CH=*CH_2*), 4.81 (s, 1H, H1), 4.27 (dd, *J* = 7.5, 4.1 Hz, 1H, H3), 4.20 (AB, *J* = 13.0, 5.3, 1.5 Hz, 1H, O*CH_2*CH=CH_2), 4.09 – 3.98 (m, 2H, O*CH_2*CH=CH_2, H4), 3.94 (d, *J* = 4.1 Hz, 1H, H2), 3.82 (AB, *J* = 12.1, 2.5 Hz, 1H, H5), 3.56 (AB, *J* = 12.0 Hz, 1H, H5), 1.87 (s, 1H, OH), 0.94 – 0.83 (m, 18H, TBS), 0.12 – 0.06 (m, 12H, TBS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 134.14 (*CH*=CH_2), 117.86 (CH=*CH_2*), 106.77 (C1), 82.79 (C4), 76.90 (C2), 70.95 (C3), 69.12 (O*CH_2*CH=CH_2), 61.61 (C5), 26.00, 25.92 (CH_3, TBS), 18.25, 18.21 (Cq. TBS), -4.12, -4.42, -4.46, -4.90 ($SiCH_3$, TBS). IR (film): 2929, 2858, 1472, 1253, 1161, 834, 774cm^{-1}. HRMS (ESI^+) calcd for $C_{20}H_{42}O_5Si_2Na$ (M+Na) 441.2463. Found 441.2465. $[\alpha]_D^{20}$ -2.1 (c = 1, in DCM)



1-*O*-Allyl-2,3-di-*O*-tert-butyldimethylsilyl-5-O-(di-O-flourenylmethyl)-phosphoryl-α,β-D-ribofuranoside (12)

Compound **10** (4.72 g, 11.29 mmol), DCI activator (4,5-dicyanoimidazole solution 0.25 M in ACN, 90 mL, 22.58 mmol) and freshly activated 3Å molecular sieves were added in to the flask. 8 mL DMF was added into the flask and then **11** (0.4 M in ACN, 31 mL, 12.42 mmol) were added. The reaction was stirred for 10 minutes at r.t. after which *t*BuOOH (5.5 M in decane, 16.42 mL, 90.32 mmol) was added at 0°C. The reaction was stirred at same temperature for 30 minutes and quenched by aq. $NaHCO_3$ (sat.). The mixture was filtered and EtOAc was added to the filtration. The mixture was washed by H_2O (1 x) and brine (2 x) and the organic layer was dried (Na_2SO_4), filtered, concentrated, and purified by silica gel column chromatography (pentane/acetone, 100/5 – 100/15) to obtain **12** (α+β mixture) as a colorless oil (7.82 g, 9.25 mmol, 82%). ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.78 – 7.67 (m, 4H), 7.60 – 7.50 (m, 4H), 7.44 – 7.31 (m, 4H), 7.31 – 7.21 (m, 4H, Ar), 5.76 (dddd, *J* = 17.0, 10.4, 6.3, 5.0 Hz, 1H, *CH*=CH_2), 5.23 – 5.00 (m, 2H, CH=*CH_2*), 4.81 – 4.69 (m, 1H, H1), 4.35 – 3.73 (m, 13H, H2, H3, H4, H5, CH-Fm, CH2-Fm, O*CH_2*CH=CH_2), 0.94 – 0.79 (m, 18H, TBS), 0.11 – -0.04 (m, 12H, TBS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 143.30, 143.25, 141.48, 141.46 (Cq, Ar), 134.57, 134.15, 128.05, 128.02, 127.97, 127.24, 125.40, 125.37, 125.33, 125.31, 125.27, 120.17, 120.13, 120.12, 120.09, 120.08 (Ar), 117.49 (β, CH=*CH_2*), 116.51 (α, CH=*CH_2*), 106.08 (H1- β), 101.93 (H1-α), 81.57 (H4-α), 80.39 (H4-β), 76.38 (H2-β), 73.59 (H2-α), 71.80 (H3-β), 71.13 (H3-α), 69.51, 69.49, 69.47, 69.44 (CH_2-Fm), 68.98 (O*CH_2*CH=CH_2, α), 68.25 (O*CH_2*CH=CH_2, β), 67.75, 67.71 (C5-β), 66.87, 66.82 (C5-α), 48.10, 48.03 (CH-Fm), 26.09, 25.99 (TBS, α), 25.96, 25.90 (TBS, β), 18.20, 18.10 (Cq. TBS), -4.09, -4.19, -4.37, -4.49, -4.82, -4.90 ($SiCH_3$, αβ). ^{31}P NMR (202 MHz, $CDCl_3$) δ -0.85 (β), -0.88 (α). IR (film): 2928, 2893, 2856, 1450, 1254, 1127, 1076, 1014, 991, 837, 776, 756 cm^{-1}. HRMS (ESI$^+$) calcd for $C_{48}H_{63}O_8PSi_2Na$ (M+Na) 877.3691. Found 877.3695. $[\alpha]_D^{20}$ +10.3 (c = 1, in DCM)

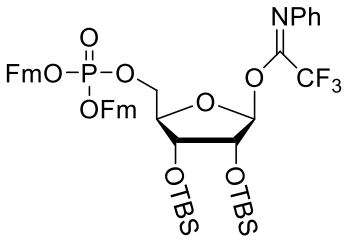


2,3-di-*O*-Tert-butyldimethylsilyl-5-O-(di-O-flourenylmethyl)-phosphoryl-α,β-D-ribofuranoside (13)

Compound **12** (440 mg, 0.51 mmol), $CHCl_3/H_2O$ (v/v, 2.1 mL/1.4 mL) and $PdCl_2$ (18 mg, 0.10 mmol) were added into a flask and the mixture was stirred vigorously under O_2 at 45°C for 48 hours after which it was quenched by $NaHCO_3$ (aq. sat.). DCM extracted the mixture (2 x) and the organic layers are combined, dried ($MgSO_4$), filtered, concentrated. Purification by silica gel column chromatography (pentane/acetone, 100/5 – 100/10) afforded **13** (α+β mixture) as a white foam (341 mg, 0.42 mmol, 82%).

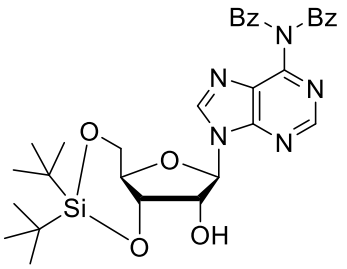
^{1}H NMR (500 MHz, chloroform-*d*) δ 7.79 – 7.64 (m, 4H, Ar), 7.62 – 7.46 (m, 4H, Ar), 7.45 – 7.21 (m, 8H, Ar), 5.16 – 5.08 (m, 0.35H, H1-β), 5.03 (dd, *J* = 11.5, 4.1 Hz, 0.65H, H1-α), 4.63 (d, *J* = 4.4 Hz, 0.35H, OH-β), 4.51 – 4.32 (m, 0.7H, CH_2-Fm-β, H3-β), 4.32 – 4.06 (m, 7H, CH2-Fm-αβ, CH-Fm-αβ, H4-α, OH-α, H5-β), 4.06 – 3.97 (m, 1.35H, H4-β, H3-α, H5-β), 3.95 – 3.90 (m, 1H, H2), 3.87 (dd, *J* = 5.9, 4.4 Hz, 1.3H, H5-α), 0.96 – 0.81 (m, 18H, CH_3, TBS), 0.15 – 0.00 (m, 12H, $SiCH_3$, TBS). ^{13}C NMR (126 MHz, $CDCl_3$) δ 143.08, 143.05, 141.46, 141.44 (Cq. Ar), 128.00, 127.95, 127.94, 127.91, 127.22, 127.18, 127.16, 125.27, 125.17, 125.14, 125.13, 125.11, 120.14, 120.09, 120.07, 120.05, 120.02 (Ar), 102.71 (C1-β), 97.70 (C1-α), 82.73, 82.66 (C4-α), 80.00, 79.97 (C4-αβ), 77.25 (C2-β), 73.52 (C3-α),

72.49 (C2-α), 70.60, 70.02 (C3-β), 69.97, 69.56, 69.51, 69.47, 69.44, 69.43, 69.39 (CH_2-Fm), 67.37, 67.32 (C5-β), 66.79, 66.74 (C5-α), 48.09, 48.03, 48.00, 47.99, 47.94, 47.92, 47.89, 47.83 (CH-Fm), 25.93, 25.89, 25.81 (CH_3, TBS), 18.34, 18.18, 18.09, 18.04 (Cq. TBS), -4.17, -4.38, -4.52, -4.57, -4.61, -4.68, -4.96, -5.03 ($SiCH_3$, TBS). ^{31}P NMR (202 MHz, $CDCl_3$) δ -0.85 (β), -0.91 (α). IR (film): 2952, 2929, 2893, 1471, 1450, 1253, 1077, 1014, 837, 740, 515 cm^{-1}. HRMS (ESI^+) calcd for $C_{45}H_{59}O_8PSi_2Na$ (M+Na) 837.3378. Found 837.3381. $[\alpha]_D^{20}$ +24.1 (c = 1, in DCM)



1-*O*-((*N*-Phenyl)-2,2,2-trifluoroacetimido)-2,3-di-*O*-tert-butyldimethylsilyl-5-*O*-(di-*O*-flourenylmethyl)-phosphoryl-α,β-D-ribofuranoside (15)

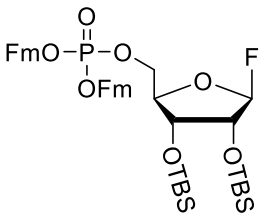
Compound **13** (1.43 g, 1.76 mmol), Cs_2CO_3 (858 mg, 2.63 mmol), DCM (20 mL) and $Cl(CF_3)C$=NPh **14** (0.37 mL, 2.28 mmol) were added into a flask and the mixture was stirred under N_2 for 16 hours. TLC (pentane/acetone=9/1) showed incomplete conversion and more **14** (0.37 mL, 2.28 mmol) was added. The reaction was stirred for 24 hour after which it was filtered, extracted with aq. $NaHCO_3$ (sat. 2 x) and brine (1 x). The organic layer is dried ($MgSO_4$), filtered and concentrated. Purification by silica gel (neutral spherical silica gel is used) column chromatography (pentane/acetone, 98/2 – 90/10) to obtain **15** (α+β mixture) as a colorless oil (1.46 g, 1.48 mmol, 84%). ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.83 – 7.68 (m, 4H), 7.64 – 7.51 (m, 4H), 7.47 – 7.34 (m, 4H), 7.33 – 7.24 (m, 6H), 7.16 – 7.08 (m, 1H), 6.81 (d, *J* = 7.7 Hz, 2H, Ar), 6.11 (s, 1H, H1), 4.41 – 4.02 (m, 11H, H2, H3, H4, H5, Fm), 0.93 (s, 18H, CH_3, TBS), 0.26 – 0.03 (m, 12H, $SiCH_3$, TBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.65, 143.15, 143.13, 141.39, 141.36 (Cq. Ar), 128.76, 127.87, 127.85, 127.14, 127.11, 125.27, 125.22, 125.17, 124.24, 120.01, 119.99, 119.97, 119.95, 119.50 (Ar), 103.64 (C1), 81.67 (C4), 81.58 (C2), 75.51 (C3), 70.95, 69.46, 69.40, 69.33 (CH_2-Fm), 66.51, 66.46 (C5), 47.98, 47.96, 47.90, 47.89 (CH-Fm), 25.83, 25.72 (CH_3, TBS), 18.06, 18.03 (Cq. TBS), -4.23, -4.51, -4.75, -5.03 ($SiCH_3$, TBS). IR (film): 2930, 2857, 1717, 1599, 1450, 1259, 1208, 1160, 1014, 939, 867, 741 cm^{-1}. HRMS (ESI^+) calcd for $C_{53}H_{63}F_3NO_8PSi_2Na$ (M+Na) 1008.3674. Found 1008.3668. $[\alpha]_D^{20}$ +21.8 (c = 1, in DCM)



N^6-Dibenzoyl-3',5'-di-tert-butylsily-adenosine (17)

N^6-Dibenzoyl adenosine (200 mg, 0.42 mmol), pyridine (2 mL) and TEA (175 uL, 1.26 mmol) were added into a flask and the mixture was cooled down to 0°C after which $DTBS(OTf)_2$ (0.21 mL, 0.63 mmol) was added. The reaction was stirred at room temperature for 20 minutes before it was quenched by $NaHCO_3$ (aq. sat.). DCM (2 x) extracted the mixture and the organic layers were combined, dried (Na_2SO_4), filtered and concentrated. Purification by silica gel column chromatography (DCM/MeOH, 100/0 – 100/5) afforded **17** as a white foam (231 mg, 0.38 mmol, 90%). ^{1}H NMR (400 MHz, Acetonitrile-d_3) δ 8.63 (s, 1H, H2), 8.10 (s, 1H, H8), 7.92 – 7.81 (m, 4H, Ar), 7.53 – 7.43 (m, 2H, Ar), 7.36 (t, *J* = 7.7 Hz, 4H, Ar), 6.02 (s, 1H, H1), 4.85 (dd, *J* = 9.0, 5.0 Hz, 1H, H2), 4.71 (d, *J* = 5.0 Hz, 1H, H4), 4.47 (dd, *J* = 8.4, 4.4 Hz, 1H, H3), 4.20 – 4.01 (m, 2H, H5), 2.88 (s, 1H, OH), 1.11 (s, 9H, C(CH_3)$_3$), 1.06 (s, 9H, C(CH_3)$_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.38 (CO, Bz), 152.47 (Ar), 152.32, 152.20 (Cq. Ar), 143.88 (Ar), 134.09 (Cq. Ar), 133.18, 129.59, 128.89 (Ar), 128.16 (Cq. Ar), 91.10 (C1), 75.71 (C4), 75.26 (C2), 73.88 (C3), 67.47 (C5), 27.49, 27.34 (C(CH_3)$_3$), 22.88, 20.50

(Cq, *C*(CH_3)$_3$). IR (film): 2935, 2860, 1702, 1598, 1576, 1236, 980, 825, 734 cm^{-1}. HRMS (ESI^+) calcd for $C_{32}H_{38}N_5O_6Si$ (M+H) 616.2586. Found 616.2583. $[\alpha]_D^{20}$ -49.2 (c = 1, in DCM)



1-Fluro-2,3-di-*O*-tert-butyldimethylsilyl-5-O-(di-O-flourenylmethyl)-phosphoryl-β-D-ribofuranoside (19)

Compound **13** (300 mg, 0.37 mmol) was dissolved in THF (2 mL) in a flask and the solution was cooled down to -70°C after which it was added diethylaminosulfur trifluoride (DAST, 58 uL, 0.44 mmol). The reaction was stirred at room temperature for 45 minutes after which it was quenched by $NaHCO_3$ (aq. sat.) at 0°C. The mixture was extracted by EtOAc and the organic layer was washed with H_2O (1 x), brine (1 x), dried, filtered and concentrated. Purification by silica gel column chromatography (pentane/EtOAc, 100/0 – 100/15) to obtain **19** as a colorless oil (277 mg, 0.34 mmol, 92%). ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.73 (t, *J* = 7.4 Hz, 4H, Ar), 7.56 (dddd, *J* = 8.5, 7.5, 5.5, 1.0 Hz, 4H, Ar), 7.46 – 7.18 (m, 8H, Ar), 5.45 (d, *J* = 64.3 Hz, 1H, H1), 4.46 – 3.95 (m, 11H, H2, H3, H4, H5, Fm), 0.90 (d, *J* = 10.0 Hz, 18H, CH_3, TBS), 0.18 – 0.04 (m, 12H, $SiCH_3$, TBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.37, 143.30, 143.27, 143.26, 141.47, 141.46, 141.44 (Cq. Ar), 127.94, 127.93, 127.22, 127.21, 127.20, 125.40, 125.36, 125.35, 120.09, 120.08, 120.05 (Ar), 114.80, 112.55 (C1), 81.59, 81.56, 81.51, 81.49 (C4), 75.66, 75.37 (C2), 70.07, 70.05 (C3), 69.58, 69.52 (CH_2-Fm), 65.92, 65.87 (C5), 48.05, 47.97 (CH-Fm), 25.90, 25.79 (CH_3, TBS), 18.15, 18.08 (Cq. TBS), -4.15, -4.48, -4.56, -5.01 ($SiCH_3$, TBS). IR (film): 2929, 2857, 1463, 1259, 1170, 1105, 1011, 838, 756 cm^{-1}. HRMS (ESI^+) calcd for $C_{45}H_{58}FO_7PSi_2Na$ (M+Na) 839.3335. Found 839.3337. $[\alpha]_D^{20}$ +23.6 (c = 1, in DCM)

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Chinese Summary

中文总结

本论文的研究方向为二磷酸腺苷核糖基化（ADP-ribosylation）相关分子的有机合成及生物学应用。二磷酸腺苷核糖基化是蛋白质转录后修饰（post-translational modification）的一种，其结果是在酶的催化下，将单二磷酸腺苷核糖（mono ADP-ribose, or MAR）或聚二磷酸腺苷核糖（poly ADP-ribose, PAR）转移到蛋白质的亲核性氨基酸侧链上。这种修饰在许多细胞生理过程和病理机制中发挥着重要作用，像 DNA 修复，细胞凋亡，转录调节，肿瘤产生，神经退行和炎症等。鉴于一系列关于二磷酸腺苷核糖基化的生物难题仍未解决，化学合成相关工具分子具有极大的应用价值。本文主要描述各种单或寡居二磷酸腺苷核糖基化分子（mono or oligo ADP-ribosylated molecule）的合成。

第一章描述了基于有机合成方向二磷酸腺苷核糖基化的研究进展（Chemical ADP-ribosylation）。本章节从介绍二磷酸腺苷核糖基化的生物学意义入手，着重介绍了合成这种生物聚合物的难度所在：1. 立体选择性地在多种氨基酸侧链上构建 α 糖苷键，2. 2’位腺苷的选择性糖基化，3. 多个焦磷酸键的构建。本章随后介绍了近年来有机合成（单）二磷酸腺苷核糖基化多肽和寡聚二磷酸腺苷核糖的研究进展。

第二章描述了分枝状二磷酸腺苷核糖片段及其生物素化衍生物的合成。本章上半部分描述了天然分枝状二磷酸腺苷核糖片段核糖基-核糖基-腺苷三磷酸的有机合成并与 1981 年 Miwa 报道的酶合成的相同化合物具有相同的核磁共振图谱，间接证明了 Miwa 解析的化学构型的正确性。下半部分描述了生物素标记的分枝状和直连状二磷酸腺苷核糖片段的有机合成，为以后探究分枝状聚合二磷酸腺苷核糖结合蛋白的发现打下基础。

第三章探究了多个焦磷酸键构建的方法。焦磷酸键在生物学中具有重要意义而其化学合成非常困难。本章节采用新型碱敏感的芴甲基（Fm）作为磷酸的保护基，通过固相上的 P(V)-P(III) 策略成功合成了 3’,5’焦磷酸键相连的脱氧胸腺嘧啶核苷寡聚物，包括十聚体。这种合成方法为后来的寡居二磷酸腺苷核糖（oligo ADPr）的合成提供重要参考。

第四章描述了一种固相合成寡居二磷酸腺苷核糖（oligo ADPr）的方法。创新之处是采用上一章节的芴甲基保护基策略，将合成的复杂的直连状亚磷酰胺用于基于 P(V)-P(III) 策略的寡居二磷酸腺苷核糖的固相合成，最后首次成功合成了二磷酸腺苷核糖的五聚体。此外，采用相似方法，合成了生物素化的寡居二磷酸腺苷核糖，可以用于未来蛋白质组学研究。

第五章描述了分枝状寡居二磷酸腺苷核糖的全合成。我们首先合成了复杂的含有两个磷酸三酯的分枝状的亚磷酰胺，并将它用于固相合成。在完成第一个焦磷酸键构建后，端基的芴甲基（Fm）被选择性脱保护，进而与腺苷亚磷酰胺缩合同时得到第二和第三个焦磷酸键。此分子对于研究分枝状二磷酸腺苷核糖结合蛋白具有极大价值。

第六章描述了二磷酸腺苷核糖基化天冬酰胺（ADPr-Asn）的合成，及其作为二磷酸腺苷核糖基化天冬氨酸(ADPr-Asp)的稳定化电子等排体用于 Macrodomains 的结构研究。天冬氨酸是二磷酸腺苷核糖基化的常见位点， 然而酯糖苷键容易发生迁移，不适于生物结构研究。采用结构相近，但不易迁移的酰胺键，如天冬酰胺，可以很好的解决这一问题。在本章中，我们首先合成了二磷酸腺苷核糖基化天冬酰胺，并用其作为底物与 *Methanobrevibacter oralis macrodomain* (MorMOD)进行共结晶，得到其蛋白质结合和催化的生物数据。

第七章描述了一种合成三氮唑连接的二磷酸腺苷核糖基化多肽和蛋白质的方法。由于传统的二磷酸腺苷核糖基化多肽合成时间周期长，产率低，我们采用点击化学（click chemistry）的策略，分别合成带有炔丙基的二磷酸腺苷核糖和带有叠氮基的多肽或蛋白质，将两者用铜催化的炔-叠氮环加成的反应进行拼合得到了三氮唑连接的二磷酸腺苷核糖基化多肽或蛋白质（泛素）。生物活性数据表明，合成的三氮唑连接的二磷酸腺苷核糖基化的泛素具有与天然产物相似的生物活性，表明该方法为研究二磷酸腺苷核糖基化生物学提供了一个重要的化学平台。

第八章总结了本论文的所有章节并且对未来二磷酸腺苷核糖基化的化学合成进行简要展望。

List of publications

- A solid-phase synthesis method for the assembly of linear and branched ADPr oligomers

  Manuscript in preparation

- Synthesis of 3',5'-pyrophosphate-linked thymidine oligomers

  Manuscript in preparation

- **Liu, Q.**; van der Marel, G. A.; Filippov, D. V., Chemical ADP-ribosylation: mono-ADPr-peptides and oligo-ADP-ribose. *Org. Biomol. Chem.* **2019**, 17 (22), 5460-5474. (*Review*)

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Curriculum Vitae

Qiang Liu was born on 30th September 1989 in Jinan, Shandong province, China. In 2008, he was enrolled as a bachelor student at Jining Medical Unversity in China where he studied pharmaceutical science and obtained his bachelor degree in 2012. Successively, he started master study with obtained "Second-class Scholarship for Graduate Student" at Sichuan University where he was trained as a medicinal chemist under the supervision of prof. Yong Deng, focusing on the design, synthesis and biological evaluation of anti-Alzheimers agents. In 2015, he obtained his master degree successfully and part of his work has been published in *Bioorganic & Medicinal Chemistry*.

In September 2015, thanks to the funding from Chinese Scholarship Council (CSC), he went abroad to Leiden University in the Netherlands to start his PhD study under the supervision of prof. Gijs van der Marel and Dr. Dmitri Filippov. His PhD project focused on the synthetic study on ADP-ribosylated molecules. These molecules have been sent to several top ADPr groups in USA, UK, Germany, Spain, and the Netherlands for further use in biological experiments. Part of his work has been orally presented at CHAINS symposium (2018). In addition, posters on part of the PhD research were presented at EUROCARB XX (2019), Dutch Peptide Symposium (2017 and 2018), The EMBO Meeting (2016) in Germany and other meetings in Netherlands. Part of his PhD work has been published in *Angew Chem Int Ed, Chem Commun,* and *Org Biomol Chem*.