Synthetic methodology towards ADP-ribosylation related molecular tools

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7 | Summary and Future Prospects

Phosphate esters are ubiquitous functionalities in a wide variety of fundamental biochemical processes, from interlinking the nucleotides in our DNA, to protein regulation and cellular respiration. Therefore, it is not surprising that all four major biomolecules are directly, or indirectly, subjected to various types of phosphorylation reactions. A specific example of a post-translation modification that involves phosphate esters is adenosine diphosphate ribosylation (ADP-ribosylation). This post-translation modification (PTM) is regulated by ADP-ribose transferases that utilize NAD^+ as a glycosyl donor and transfer one (mono) or more (poly) ADP-ribose residues onto a nucleophilic heteroatom of an acceptor biomolecule. In humans, both mono(ADP)ribosylation and poly(ADP)ribosylation are modifications primarily introduced by enzymes of the PARP family. Particularly the PARylation processes of PARP-1, PARP-2 and PARP-3 have become subject of scientific interest due to their roles in DNA damage repair, inflammation, telomere maintenance and regulation of apoptosis. During PARylation, ADP-ribose oligomer grows on a nucleophilic amino acid residue, either in the auto-modification domain of PARP itself or that of another target protein, to form linear or branched chains of poly-ADP-ribose, consisting of up to 200 monomeric units. The produced dendritic ADPR-polymer triggers a conformational change that is essential to the respective biological event, such as the recruitment of DNA repair enzymes. Synthetic organic chemistry has already provided access to structurally well-defined ADP-ribosylated oligopeptides and poly-ADPr fragments, and thereby helped advance our understanding of this important PTM. However, these native constructs contain labile functionalities, such as the pyrophosphate moiety and glycosidic bond, which can constrain both synthesis and biological application due to their susceptible to hydrolytic or enzymatic degradation. An organic chemical approach to address such limitations is to introduce chemical modifications that stabilize specific labile bonds, while retaining similar overall physical and chemical properties. The research described in this Thesis focuses on the development of new molecular tools aimed to facilitate the study of the adenosine diphosphate ribosylation PTM, with an emphasis on stabilizing the aforementioned labile functionalities. In this context new reagents and methodology have been developed for the synthesis of stabilized pyrophosphorylated bioisosteres.

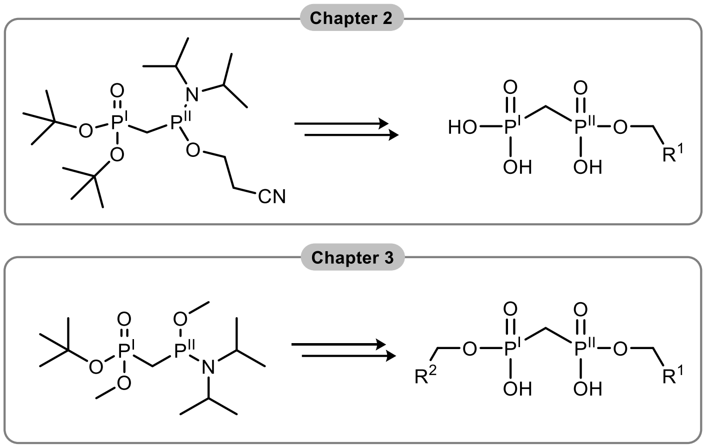
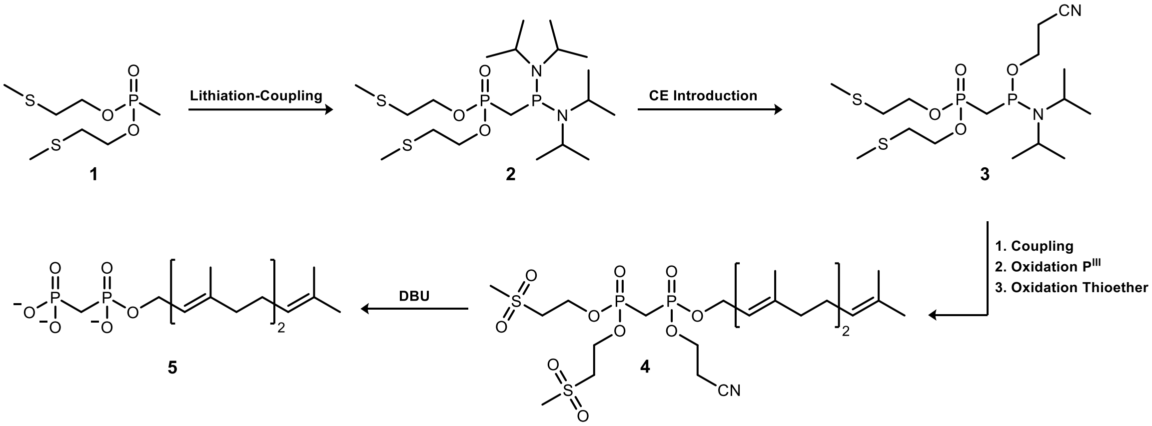


Figure 1: The two primary reagents designed to introduce methylene bisphosphonates in biomolecules described in Chapter 2 and 3.

Chapter 2 discusses a new class of orthogonally protected phosphanylmethylphosphonate reagents that have been developed for the controlled synthesis of methylene bisphosphonate monoesters. Methylene bisphosphonates are stabilized analogues of pyrophosphates in which the oxygen atom, conjoining the two phosphates in the original pyrophosphate, is replaced by a methylene to provide resistance to cleavage. After refinement of reactions conditions, the phosphanylmethylphosphonate reagents became readily accessible through a one-pot procedure by reacting a lithiated methylphosphonate diester with a chlorophosphoramidite. Condensation of the presented reagents with an alcohol of choice through azole-mediated phosphoramidite chemistry, followed by *in situ* oxidation, efficiently provides orthogonally protected methylene bisphosphonate tetraesters. Global deprotection of the acquired tetraester yields the respective terminal methylene bisphosphonate. Using these reagents, several methylene bisphosphonate analogues of biologically relevant nucleoside di- and triphosphates where synthesized. In addition, the method has proven to be compatible with solid-phase chemistry.

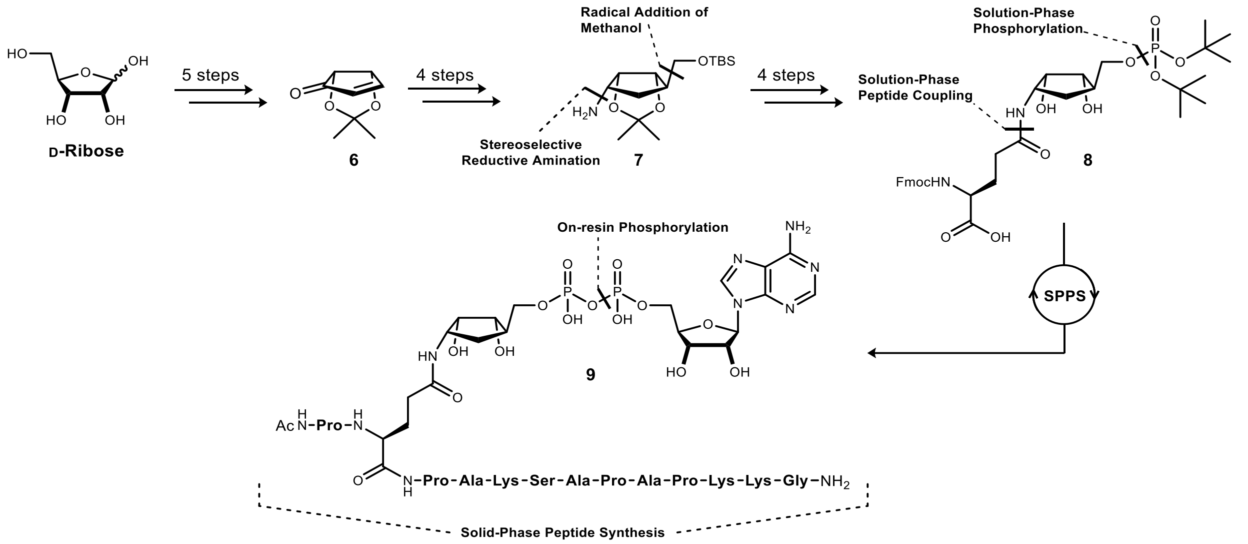
The research described in **Chapter 3** expands on that of Chapter 2, by adopting the concept to the synthesis of unsymmetrical methylene bisphosphonates. This uses a second generation of the reagent, which is protected with two methyl groups and a single *tert*-butyl protective group at the P^V. After installment of the first alcohol, using the phosphoramidite coupling-oxidation sequence, the terminal *tert*-butyl in the methylene bisphosphate tetraester is selectively cleaved. This allows for introduction of a second alcohol, through PyNTP-mediated condensation, yielding the protected methylene bisphosphate with two different alkyl substituents at its termini. Subsequent global deprotection via a two-step-one-pot procedure provides the respective unsymmetrical methylene bisphosphate. The power of this methodology was demonstrated in the synthesis of the methylene bisphosphonate analogues of ADPR and flavin adenine dinucleotide (FAD).

The lithiation conditions used during the assembly of the phosphanylmethylphosphonate reagent prevent the direct installment of base labile protecting groups at the phosphonate end of the molecule. This restriction in orthogonality limits the scope to acid stable substrates, as exemplified by the degradation of the methylene bisphosphonate analogues of farnesyl pyrophosphate and isopentenyl pyrophosphate during *tert*-butyl cleavage (Chapter 2; Scheme 6). A strategy to circumvent this limitation can be envisioned in the implementation of 2-hydroxyethyl methyl sulfide as a “masked” base-labile protecting group. In order to synthesize prenoids the phosphonate could be fitted with two 2-hydroxyethyl methyl sulfide moieties (**1**), then lithiated and reacted with bis(diisopropylamino) chlorophosphoramidite to provide **2**, which in turn would be converted to reagent **3** (Scheme 1). During the phosphanyl coupling-oxidation sequence, an additional oxidation step would be added to fully oxidize the thioether. This could be achieved selectively by treatment with hydrogen peroxide in the presence of cyanuric chloride.[1] The resulting sulfonyl (**4**) is now susceptible to base mediated β-elimination, similarly to the cyanoethyl.



Scheme 1: Proposed variant of the phosphanylmethylphosphonate reagent, which makes use of a “masked” base-labile protecting group 2-hydroxyethyl methyl sulfide. The group becomes base cleavable after oxidation to the corresponding sulfone.

Chapter 4 describes the synthesis of a stabilized analogue of a mono-ADP-ribosylated *N*-terminal peptide of histon H2B, which is a relevant fragment involved in the ADP-ribosylation process triggered by single-strand DNA damage. The Glu-2, corresponding to the native H2B conjugate, is a well-studied site of ADP-ribosylation. Previous attempts at synthesizing the native conjugate were unsuccessful due to the propensity of the anomeric glutamyl ester to migrate. The migration can be circumvented by instalment of glutamine as a glutamate bioisostere, effectively substituting the more stable amide bond for the ester. However, this form of stabilization does not fully exclude epimerization or (acidic) hydrolysis. The intrinsic lability of the glycosidic bond primarily stems from the oxocarbenium resonance enabled by the adjacent ring-oxygen. Therefore, a *carba*-riboside was incorporated as a stabilized replacement of the naturally occurring riboside. A new synthesis towards the α-*carba*-ribosylamine was developed. D-Ribose was converted into a protected 5-iodoriboside and subjected to a tandem fragmentation-vinylation reaction. The diallylic product was ring-closed and oxidized, providing cyclopentenone precursor **6**. Ensuing photochemical addition of methanol, to install the C^5-CH_2OH, efficiently produced the *carba*-ribonolactone core structure. The ketone was converted into a methoxyimine and stereoselectively reduced to obtain the pseudo-anomeric α-configured amine (**7**). Coupling of the amine with a glutamic acid derivative, followed by deprotection and phosphorylation of the C^5-OH afforded final *carba*-ribosylated glutamine building block **8**. Incorporation of the building block into the H2B conjugate fragment was achieved through solid-phase peptide synthesis. On-resin phosphorylation of the *carba*-ribosyl phosphate was used to construct the ADP-pyrophosphate. Successive global deprotection and release from resin provided the target mono-ADP-*carba*-ribosylated H2B conjugate **9** in multi-milligram amounts.



Scheme 2: Summarized synthesis of the mono-ADP-*carba*-ribosylated H2B conjugate described in Chapter 4.

Given the established usefulness of synthetic MARylated oligopeptides as tools for studying the ADP-ribosylation PTM[2], it is not unreasonable to think that improving accessibility to such constructs would benefit the field of biochemistry. In this context shelf-stable pre-ADP-ribosylated amino acids, that are compatible with standard solid phase peptide chemistry, could be envisioned as powerful tools to provide convenient access to a wide variety of MARylated oligopeptides. However, both the anomeric linkage and pyrophosphate bridge presented in native construct would pose incompatibility with SPPS. Chapter 4 presents a solution for the former, while the chemistry described in Chapter 2 and 3 could be adopted to incorporate a methylene bisphosphonate to address the latter. This would require an iteration of the phosphanylmethylphosphonate reagent fitted with protecting groups that are orthogonal with Fmoc-based SPPS (Figure 2). The use of two *p*-methoxybenzyl groups in combination with the masked base-labile protecting group strategy described above is expected to present the desired orthogonality. The resulting reagent **10** is then first to be coupled to an adenosine protected with acid-cleavable groups (**11**). This order of installment would be mandatory due to incompatibility of base-labile MSE group with the integrity of the Fmoc-protection. After basic removal of the MSE, the protected me-ADP can be condensed with the appropriate *carba*-ribosylated asparagine or glutamine (**12**), which itself is easily accessible from **7** (Scheme 2). Final selective removal of the allyl, by $Pd(PPh_3)_4$, would yield the respective pre-ADP-ribosylated amino acid (**13**). Building blocks of this design are expected to be compatible with the standard Fmoc-based SPPS. After incorporation into an oligopeptide of choice, the Boc, PMBs and isopropylidene acetals can be removed by treatment with TFA, with concomitant release from the solid support.

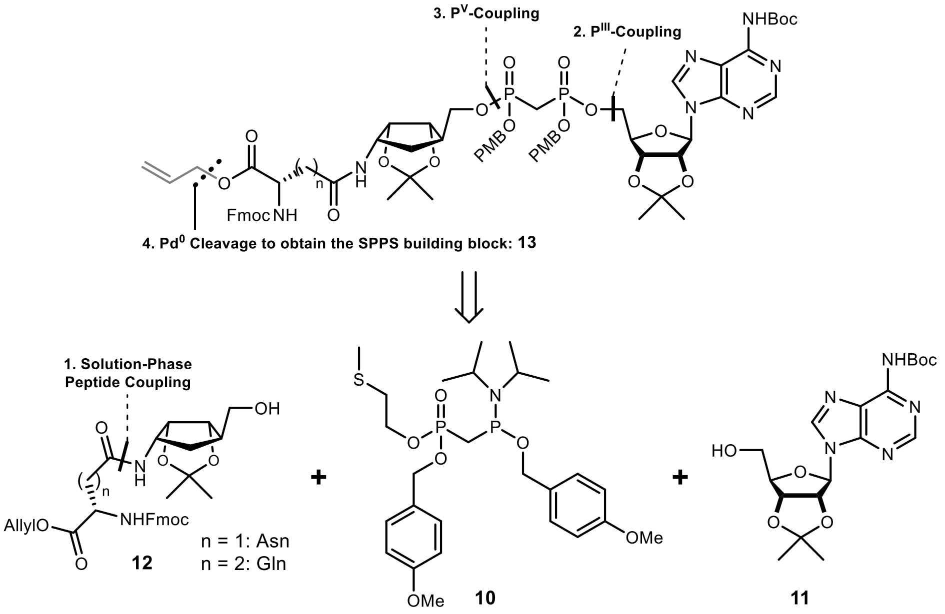


Figure 2: Retrosynthetic analysis of the proposed pre-ADP-ribosylated building block designed for SPPS.

In the same context of facilitating PARylation related research, Kistemaker *et al* developed a synthetic route for the synthesis of structurally well-defined native ADP-ribose oligomers.[3] The methodology employs a pre-phosphorylated disaccharide building block to construct the PAR-polymer via on-resin phosphorylation reactions, similar to that described for compound **9**. An interesting point of continuation would be to combine this disaccharide approach with the methylene bisphosphonate chemistry described in Chapter 2 and Chapter 3. The envisioned building block **14** (Figure 3) could be polymerized onto a resin bound peptide via block synthesis, to result in a methylene bisphosphonate enriched PARylated peptide. First, the disaccharide would have to be functionalized with a methylene bisphosphonate, which is proposed to be done via the phosphanylmethylphosphonate-based methodology. Difficulty arises from finding the correct orthogonality, since the glycosidic bond in 2’-*O*-ribosyladenosine is incompatible with strong acidic conditions. A benzyl at the P^V-position and two masked MSE groups are envisaged as suitable protecting groups for the phosphanylmethylphosphonate reagent (similar to **10** in Figure 2). After installment of the reagent onto the adenosine 5’-*OH*, the benzyl would be cleaved reductively. While adhering to the published route, the 5’-*OH* of the 2’-*O*-ribosyl residue would at this stage be protected with a TIPS-group, which is to be exchanged for a DMT. It is however uncertain if the DMT-group can selectively be installed on the 5’-*OH* in the presence of the methylene phosphoric acid triester. Although it is considered unlikely that the P^V-*OH* would form a stabile bond with the DMT-moiety at all, the difference in stability between a P^V*O-DMT* bond and a 5’-*O-DMT* is expected to be significant, and thus distinguishable. Alternatively, the reagent could be fitted with an allyl instead of the benzyl, which could be removed selectively over the DMT by applying a palladium catalyst. PAR assembly at the C^5-*OH* of an immobilized *carba*-ribosylated peptide is envisioned to be possible by repetitive coupling cycles with building block **14**; each cycle would consist of P^v-coupling, capping and DMT deprotection (Figure 3). In this instance phosphortriester-based chemistry has to be used for the solid-phase phosphorylative elongations, over the more established phosphoramidite approach.[4] Nevertheless, the viability of the phosphortriester approach has thoroughly been demonstrated by the solid-phase synthesis of DNA.[5,6]

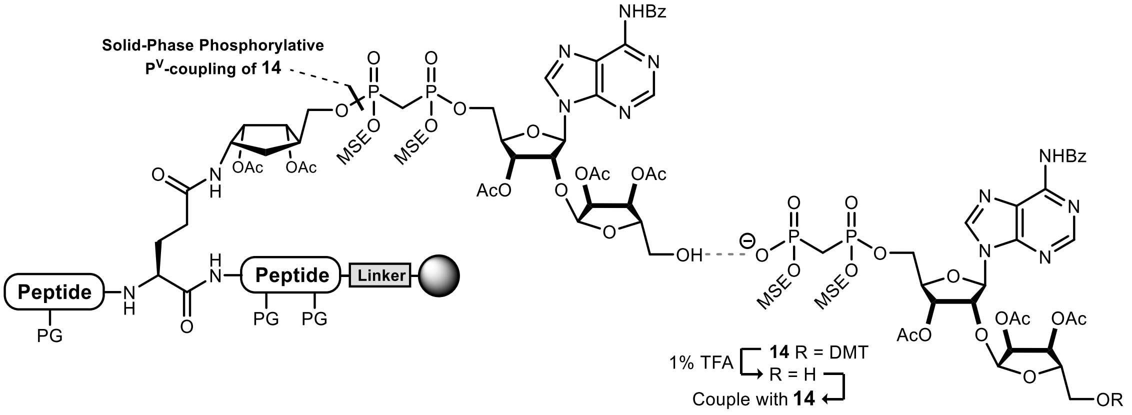


Figure 3: Proposed solid-phase synthesis of a methylene bisphosphonate enriched PARylated peptide. Building block **14** is coupled to the terminal hydroxyl, following DMT deprotection to provide the hydroxyl for the succeeding cycle.

Chapter 5 and Chapter 6 describe the development of new biorthogonal reagents. Bioorthogonal reactions are defined as selective reactions between two artificial moieties that are inert to the diverse spectrum of functional groups that reside in a biological system. These have significantly advanced the field of molecular biology providing tools to study biochemistry in living cells or even complete organisms, enabling the conjugation of functional moieties to proteins or the *in vivo* assembly of molecular tags used for imaging. In the same way, bioorthogonal chemistry has also proven to be a key component in the molecular toolbox used to unravel ADP-ribosylation processes. Various NAD$^+$ analogues functionalized with a bioorthogonal handles have been developed and employed in the mapping of ADP-ribosylation activity. The inverse-electron-demand Diels–Alder (IEDDA) reaction is the most recent addition to bioorthogonal reactions. It involves a Diels–Alder reaction between an electron deficient tetrazine (diene) and strained alkene or alkyne. The main advantage of the IEDDA reaction is that it exhibits the fastest reaction kinetics among bioorthogonal reactions, while the main disadvantages are that both the tetrazine and most reactive dienophiles are relative lipophilic and sterically encumbered. These properties may have a negative effect on both water solubility and the biological response of the functionalized molecules. The contemporary IEDDA dienophiles – norbornene, *trans*-cylcooctene and cyclopropene – use ring strain to acquire the desired reactivity towards tetrazines.

Chapter 5 describes a new compact bioorthogonal ligation handle suitable for IEDDA ligation strategy, based on the *N*-acylazetine core. This moiety was envisioned as the smallest viable structure that could utilize ring-strain in conjunction with the electron-donating properties to increase the dienophilicity. The initial five-step synthesis started by reacting benzhydrylamine with epichlorohydrin, to afford the protected azetidine core. Mesylation of the alcohol, followed by benzhydryl cleavage, yielded 3-mesylazetidine hydrochloride. Application of the former in the ring-opening of glutaric anhydride provided a linker that is sutable to attach the *N*-acylazetine to a biomolecule via an amide bond. In a one-pot procedure, the mesyl-moiety was eliminated by KO*t*Bu, to install the double bond, and the carboxylate was converted into either the pentafluorophenol or *p*-nitrophenol activated ester (PNP). The PNP-tag was used to create a model compound to determine the reaction kinetics with tetrazine. The *N*-acylazetine showed a second-order rate constant of 0.39 ± 0.1 s^{-1} M^{-1}; a value comparable to that of the methylcyclopropene mini-tag. Secondly, the handle was used to functionalize epoxomicin (a broad-spectrum proteasome inhibitor), which in turn was used to successfully label the proteasome through tetrazine ligation. Because the practicality of a ligation handle is highly dependent on its synthetic accessibility, efforts were redirected at optimizing the synthesis. However, several key steps in the original synthesis proved to be constraining to the overall efficiency and scalability. Therefore it was opted to develop a new synthetic route, in which 3-hydroxyazetidine was first *N*-Boc-protected and then tosylated. The tosyl group was selected for it is UV-detectable, has a lower polarity and a better leaving-group capacity. After deprotection of the nitrogen, the resulting azetidine was used to ring-open succinic and glutaric anhydride, yielding the respective four- and five-carbon spacers. Elimination proceeded smoothly at room temperature due to the improved solubility of the tosylated hydroxyazetidine. The thus obtained *N*-acylazetine carboxylate intermediates were converted *in situ* into the corresponding PNP-esters by addition of bis(*p*-nitrophenyl)-carbonate. With this the *N*-azetine tags have become accessible through an efficient four-step synthesis.

Chapter 6 is an extension of the work described in Chapter 5. The research described here delves further into the reaction kinetics, separating the effects of electron induction and ring-strain on the reactivity of the *N*-acylenamines to tetrazines. To this end efforts were made to prepare model *N*-acylazetines, *N*-vinylcarbamates, *N*-vinylureas and a *N*-vinylamide. The *N*-acylazetines were obtained by reacting the four- and five-carbon spacer PNP-esters with morpholine. The *N*-vinylcarbamates were accessed through the Curtius rearrangement of acryl azide. The various attempted synthesis of *N*-vinylureas were unsuccessful due to their instability. Through a comparative study between *N*-acylazetines, *N*-vinylcarbamates and an *N*-vinylamide it was shown that ring-strain has a more significant effect on the rate of the IEDDA cycloaddition of *N*-acylenamines to tetrazines than electron donation.

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