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

Citation
Voorneveld, J. (2022, September 8). *Synthetic peptides, nucleic acids and molecular probes to study ADP-Ribosylation*. Retrieved from https://hdl.handle.net/1887/3455319

Version: Publisher's Version
License: Leiden University Non-exclusive license
Downloaded from: https://hdl.handle.net/1887/3455319

Note: To cite this publication please use the final published version (if applicable).

Chapter 9

Summary and Future Prospects

Summary and Future Prospects

Although tremendous efforts have led to a significant improvement of synthetic methodologies to obtain ADP-ribosylated peptide fragments, work prior to that presented in this Thesis has solely led to ADPr-peptides where the natural aspartic acid (Asp) or glutamic acid (Glu) ADPr residues were substituted with asparagine (Asn) and glutamine (Gln) ADPr.[1,2] In the case of arginine (Arg), Arg- to citrulline-ADPr[2] or triazoles served as isosteric replacements for the native ADPr linkage.[3] The synthetic efforts mentioned above are discussed in more dept in Chapter 1. The work presented in this Thesis is mostly focused on synthesizing native ADP-ribosylated compounds. This final chapter will summarize the results and outline interesting and promising lines of research for future explorations.

Chapter 2

Chapter 2 is focused on synthesizing and investigating the native chemical structure of serine-(Ser) ADPr.[4] This modification was recently discovered and it turned out that serine is the main acceptor of ADP-ribosylation in the DNA damage response pathway.[5,6] Up to now it is generally believed that ARTs transfer NAD^+ in an α-fashion. However, for the formation of Ser-ADPr, a previously unencountered complex of PARP1 with HPF1 is formed,[7,8] giving rise to the question if the α-selectivity, which is observed for other acceptors, persists for Ser-ADPr. Therefore, two phosphoribosylated Ser building blocks were prepared with either the α (**1**)- or β (**2**)-configuration (Figure 1). These were then used to synthesize *mono*-ADP-ribosylated (MARylated) peptides containing an α- or β-glycosidic linkage between the ribosyl moiety of ADPr and the Ser side chain using a modified SPPS methodology.[2,4] It was hypothesized that substrate recognition by the human ADPr hydrolase 3 (hARH3), which is specific for Ser-ADPr,[9] would be selective for the native epimer. Treatment of α- and β-ADP-ribosylated peptides **3** and **4** with ARH3 indeed revealed α-Ser-ADPr to be the native epimer as α-linked Ser-ADPr was efficiently hydrolyzed whereas the β-isomer stayed intact.

After confirming that the Ser side chain is ADP-ribosylated in an α-selective fashion by the PARP1-HPF1 complex, the chemical stability of this modification is of particular interest. Ser-ADP-ribosylated peptide **3** was treated with reagents commonly encountered in the field of biochemistry and proteomics of ADP-ribosylation[10,11] i.e. aqueous acid (0.1 M TFA), alkali (0.1 M NaOH) and NH_2OH (0.5 M). It turned out that Ser-ADPr undergoes β-elimination when exposed to NaOH with over 50% breakdown into dehydroalanine and free ADPr after 5 hours.

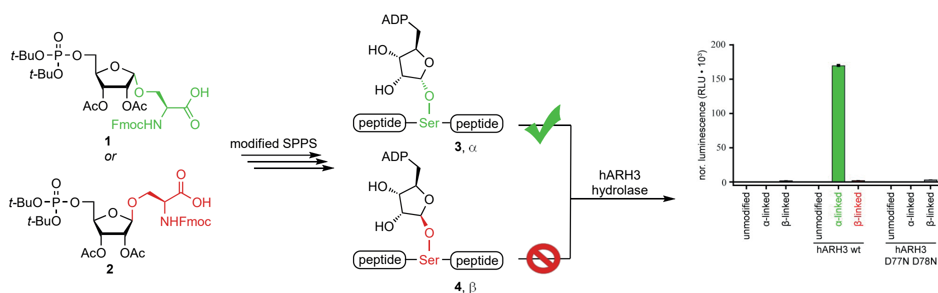
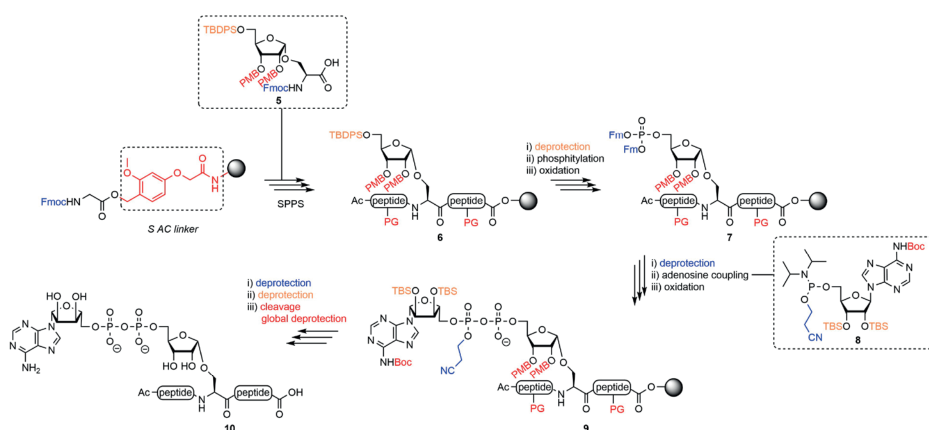


Figure 1. Schematic overview of Chapter 2.

Chapter 3

The development of a new methodology to synthesize ADP-ribosylated peptides is described in Chapter 3 and outlined in Scheme 1. The improvement this methodology brings about is two-fold: i) it eliminates the need for extensive protecting group manipulations during the solution phase synthesis of orthogonally protected, ribosylated amino acid building blocks like Ser **5** and ii) it harmonizes the protecting group patterns of the ADPr-moiety with that of the amino acids commonly applied in SPPS. For instance, Fmoc-Ser(Trt)-OH and Fmoc-Thr(Trt)-OH) are not compatible with the solid-phase strategy described in Chapter 2. Pursuing sequences with a Ser or Thr flanking the modification site, the trityl (Trt) protecting group of the hydroxyls in these amino acids must be replaced by an acetyl to allow further processing of the incorporated *t*Bu-protected phosphotriester. With the new methodology, acid sensitive protecting groups on amino acid side chains, can be kept throughout the entire synthetic route.



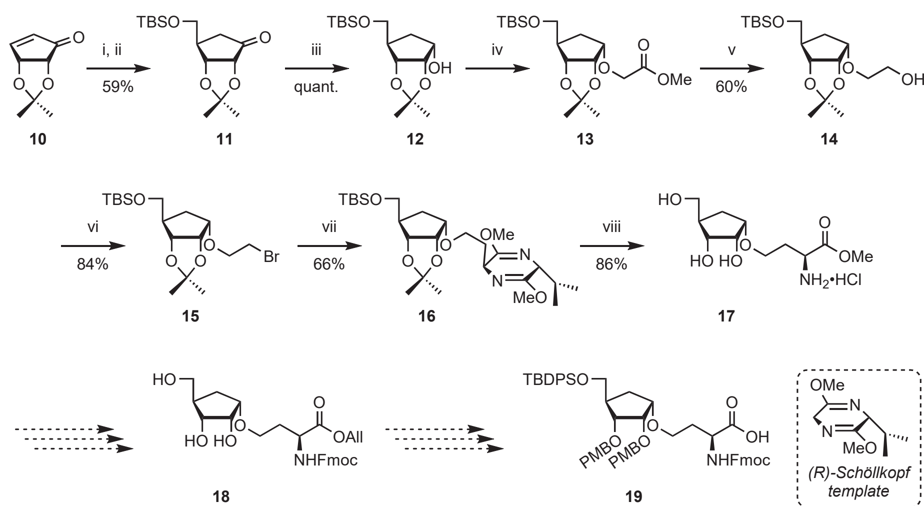
Scheme 1. Outline of the synthetic strategy developed in Chapter 3 to synthesize MARylated peptides.

With this newly developed strategy, a range of Ser-, Thr- and Cys-ADPr peptides have been synthesized and with these peptides a previously unknown hydrolase selective for *S*-ADPr (Cys) was discovered.

After demonstrating the effectiveness of the newly devised synthetic methodology, its scope can further be broadened by the synthesis of stabilized ADP-ribosylated analogues which can be used for antibody generation. The employment of ADPr specific antibodies has been explored in the past but was unproductive due to the inherent lability of the pyrophosphate moiety *in vivo* upon immunization of rabbits.[12–15] Furthermore, any crystal structures obtained from ARH3 with synthetic MARylated Ser-ADPr peptides are inherently derived from a catalytically inactive, mutated ARH3. The isosteres of ADP-ribosylated peptides which contain chemically stabilized mimics of the glycosidic linkage and pyrophosphate moieties can provide for a useful tool for *in vivo* studies and structural biology.

Stabilized mimic of the glycosidic ribosyl-Ser linkage in Ser-ADPr

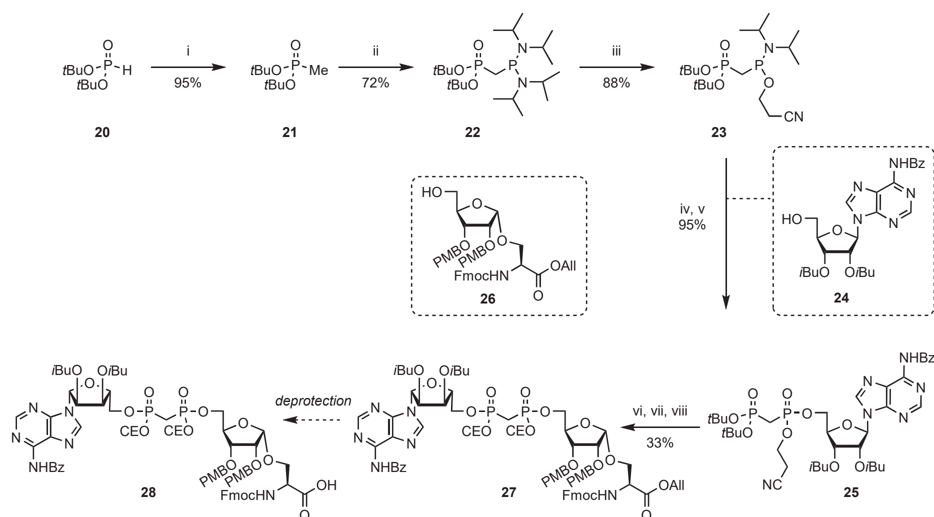
Stabilization of the glycosidic linkage by replacing the endocyclic oxygen with a methylene group to furnish *carba*-ribosyl Ser-ADPr will likely inhibit the hydrolytic activity of ARH3 towards this substrate. Synthetic routes to furnish *carba*-ribosyl precursors such as **10** (Scheme 2) are available in literature[16,17] and provide a starting point for the synthesis of *carba*-ribosyl homoserine **17**. This building block is stabilized in two ways: i) the Ser side chain is elongated with one additional methylene group, minimizing the possibility of elimination reactions described in Chapter 2[4] and ii) the endocyclic oxygen is replaced with a methylene group, rendering it unreactive towards ARH3 hydrolysis. The proposed synthetic route commences with known *carba*-ribose precursor **10** which is subjected to a 1,4-photoaddition with methanol, followed by silylation of the formed primary alcohol to yield **11** in 59% yield over two steps. Stereoselective reduction of ketone **11** forms α-*carba*-ribose **12**. Alkylation of the alcohol with methyl bromoacetate followed by complete reduction of the ester functionality furnished **14**. To allow decoration of the *carba*-ribose with Schöllkopf's template, alkyl bromide **15** was prepared. The efficiency of introduction of the template was enhanced by the addition of Ag_2CO_3 and hexamethyl phosphoramide (HMPA) to furnish **16** in 66% yield. Subsequent acidolysis of the bislactim with HCl and concomitant cleavage of the silyl ether and isopropylidene protecting groups, resulted in the isolation of homoserine derivative **17**. The proposed ensuing protecting group manipulation entails saponification of the methyl ester, protection of the amine with Fmoc and introduction of the allyl ester, leading to the conversion of **17** into **18**. Finally, introduction of earlier applied protecting groups would provide building block **19**, suitable for the same Fmoc based SPPS methodology to synthesize MARylated peptides as described in Chapter 3. With the aid of **19**, peptides analogous to Ser-ADPr but with a stabilized glycosidic linkage and not susceptible to β-elimination are within reach.



Scheme 2. Synthesis of *carba*-ribosylated homoserine building block compatible with Fmoc-based SPPS. Reagents and conditions: i) MeOH, Ph_2CO, *h*v, 60 °C. ii) TBS-Cl, imidazole, DCM. iii) $NaBH_4$, MeOH, 0 °C. iv) methyl bromoacetate, NaH, 15-crown-5, TBAI, THF. v) $LiAlH_4$, Et_2O, 0 °C. vi) Br_2, imidazole, PPh_3, 0 °C. vii) (*R*) — Schöllkopf template, *n*-BuLi, Ag_2CO_3, HMPA, THF, -78 °C. viii) aq. HCl, THF.

Stabilized isostere of the pyrophosphate moiety in Ser-ADPr

To obviate pyrophosphate cleavage in biological systems, methylene bisphosphonates analogues have been employed for pyrophosphate containing biomolecules.[18–21] In the field of ADPr research, such ADP-ribose analogues has been used to generate antibodies against ADPr but no specificity could be obtained thus far.[12] To overcome the notoriously difficult synthesis of non-symmetric methylene bisphosphonates, Engelsma *et al.*[22] developed phosphanylmethylphosphonate reagents that can be utilized in P^{III} – P^{V} chemistry and applied to the preparation of adenosine bisphosphonate ribose (ABPr). By employing the key reagent **23** using this methodology, Ser-conjugated ABPr **28** (Scheme 3) can be synthesized. The first step in this route comprises DCI mediated coupling of protected adenosine derivative **24** with **23** and subsequent oxidation with *t*BuOOH to give **25** in 96% yield. To introduce the ribosylated Ser moiety, both *t*Bu groups were removed with an HCl/HFIP cocktail.[23] Next, a double, PyNTP (3-nitro-1,2,4-triazol-1-yl-tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate) mediated condensation step starting with a stoichiometric amount of 2-cyanoethanol followed by ribosyl building block **26** (easily prepared via synthons described in Chapter 3) furnished **27**. Cleavage of the allyl protecting groups will allow the isolation of ADP-ribosylated Ser isostere **28**, ready for Fmoc-based SPPS. Synthetic peptides, modified with the bisphosphonate stabilized bridge, are resistant to enzymatic turnover or spontaneous hydrolysis during immunization of animals and thus, possibly, Ser-ADPr specific antibodies can be generated.

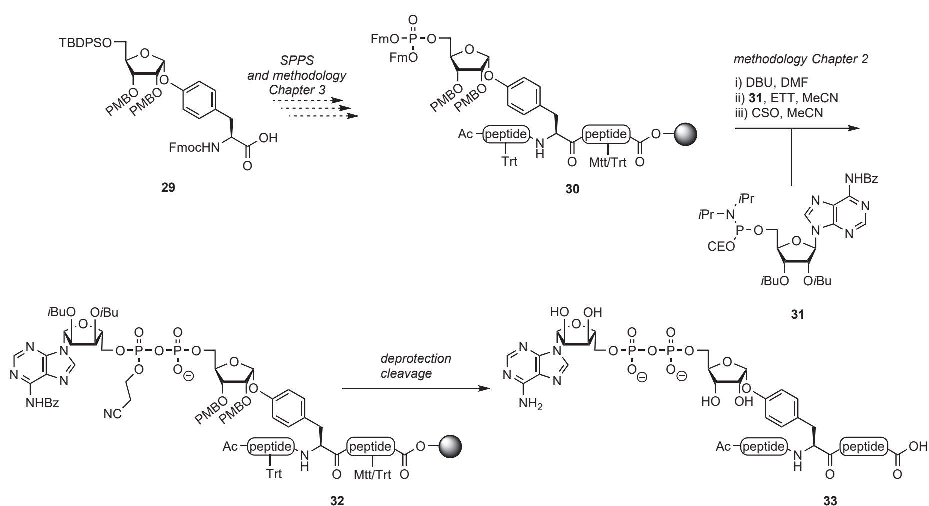


Scheme 3. Synthesis of Ser-ADPr building block **27**, stabilized with a methylene bisphosphonate bridge. Reagents and conditions: i) MeI, NaH, MeCN. 0 — 50 °C. ii) LDA, THF, -78 °C, then ((*i*Pr)$_2$N)$_2$PCl. iii) 2-cyanoethanol, DCI, DCM. iv) **24**, DCI, MeCN, v) *t*BuOOH, nonane. vi) HCl, HFIP. vii) 2-cyanoethanol, PyNTP, MeCN. viii) **26**, PyNTP, MeCN.

Chapter 4

In Chapter 4 the strategy developed for the synthesis of Ser-, Thr- and Cys-ADPr peptides, was explored for Tyr-ADPr peptides. However, it turned out that the phenolic glycosidic linkage was cleaved when exposed to 10% TFA in DCM, which is required to remove the Boc protecting group of the adenine moiety at the end of the synthesis. Therefore, a strategy was invented that combined the approach presented in Chapter 3 with the one from Chapter 2. This alteration led to a methodology that allowed the efficient synthesis of Tyr-ADPr peptides. Such a 'hybrid' approach for the synthesis of Tyr-ADPr peptide making use of acid sensitive protections for the peptide and base sensitive ones for adenosine is described in detail in Chapter 4 and summarized here in Scheme 4.

First, building block **29** was prepared and used in SPPS as described in Chapter 3 to furnish intermediate immobilized peptide **30**. Next, the pyrophosphate moiety was installed using reagent **31**, described in Chapter 2, to furnish peptide **32**. Finally, a sequence of deprotection reactions which does include a treatment with TFA but in significantly lower concentrations (2.5% in DCM as opposed to 10%). Said conditions proved to be compatible with the phenolic glycosidic linkage yielding Tyr-ADPr peptides **33**. Biological studies with these peptides further demonstrated the permissiveness of ARH3 with respect to its substrates and revealed the unsuspected hydrolytic activity of PARG and ARH1 towards Tyr-ADPr.

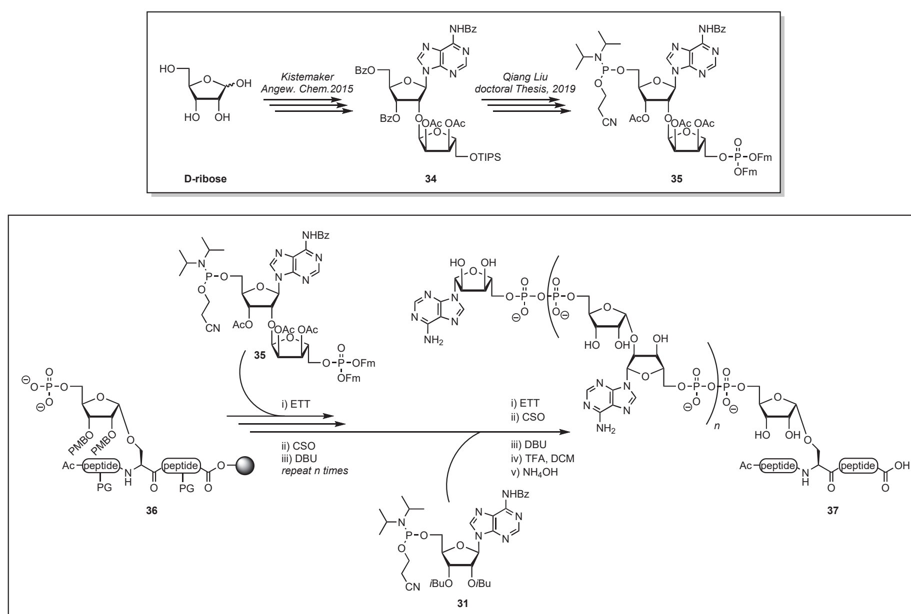


Scheme 4. Outline of the 'hybrid' strategy for Tyr-ADP-ribosylated peptides as described in Chapter 4.

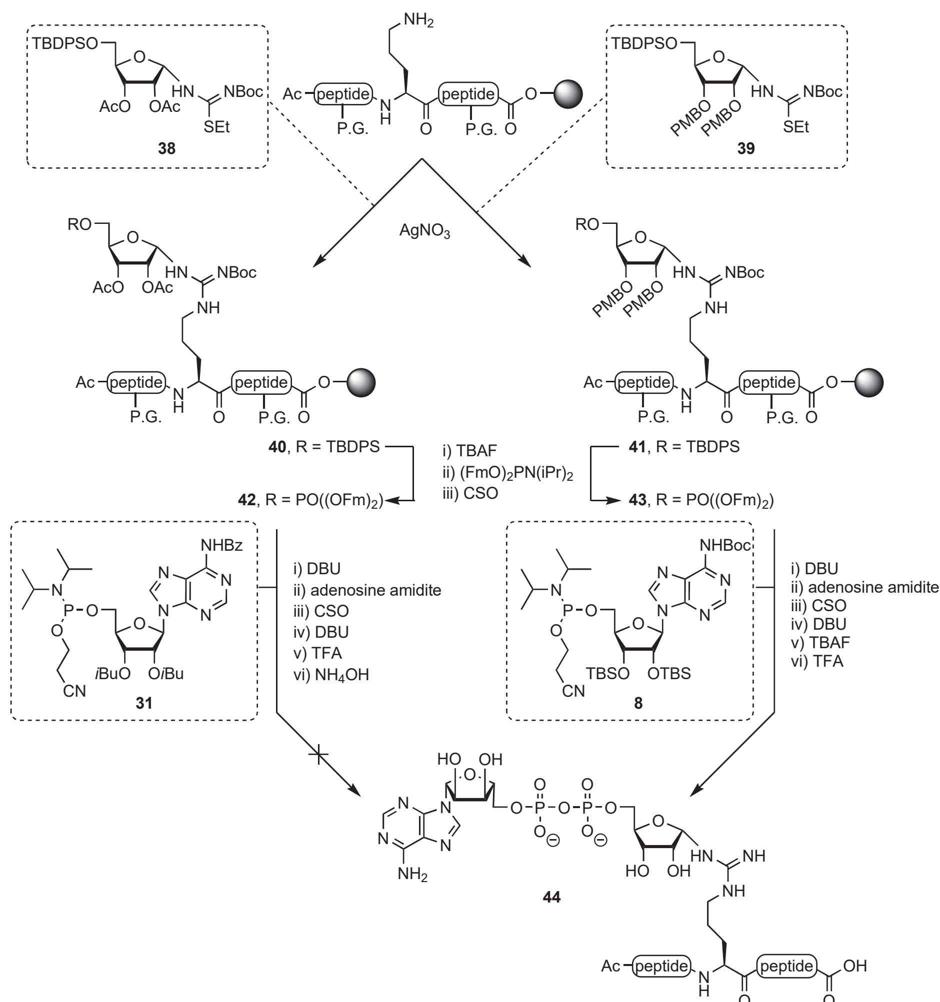
Beyond the thus assembled *O*-MARylated peptides, the next challenge can be found in PARylated peptides with a well-defined length of the PAR-chain. The synthesis of linear ADPr oligomers has been developed independently from the synthesis of MARylated peptides. To synthesize PAR chains on-resin, Kistemaker *et al*. designed a bifunctional disaccharide building block[24] containing a phosphoramidite and a phosphotriester. This methodology was refined by Liu *et al*.[25] by the design and application of disaccharide **35** (Scheme 5). It is proposed here to bring the strategies for the synthesis of ADP-ribosylated peptides and PAR-chains together. When bifunctional building block **35** is implemented in the synthetic strategy of MARylated peptides, described in Chapter 3, the coupling cycle can potentially be repeated *n* times starting from immobilized phosphomonoester **36**. When the desired chain length has been reached, adenosine phosphoramidite **31** can be used to terminate the PAR chain. Cleavage of the partially protected product from the resin followed by saponification of all remaining protecting groups will result in ADP-ribosylated peptides with a well-defined oligo-ADPr-chain, structures that remain inaccessible thus far.

Chapter 5

Having synthesized *O*- and *S*-ADPr peptides, one major native ADP-ribosylation site still remained synthetically inaccessible, namely Arg-ADPr. Chapter 5 focuses on developing a strategy to obtain the first fully synthetic peptides with this modification. Although an *N*-glycosidic linkage of the distal ribose to the Asn, Gln and Cit side chain could be built via glycosylation as reported by Kistemaker *et al.*[2] and as described in Chapters 2, 3 and 4 for the *O*- and S-glycosidic linkages, a different approach was opted for ADPr-Arg. Rather than glycosylation, a guanidyl riboside was appended at the side chain of ornithine using building blocks **38** or **39** (Scheme 6). As was demonstrated for Ser-, Thr-, Cys-, and Tyr-ADPr peptides, their glycosidic linkages show various reactivity towards basic, acidic, oxidative or nucleophilic conditions. Therefore, both the strategy described in Chapter 3 and the 'hybrid' strategy from Chapter 4 were investigated for their potential to synthesize Arg-ADPr peptides (Scheme 6). Indeed, the Arg-ADPr linkage proved labile towards nucleophilic bases such as aqueous ammonia which rendered the 'hybrid' strategy unsuitable for generating Arg-ADPr peptides. However, the ADP-ribosylated guanidyl group proved acid stable which not only allowed for the synthesis of Arg-ADPr peptides but also ubiquitin, MARylated on Arg42. This is the first report of a fully synthetic protein bearing ADPr on Arg.



Scheme 5. Synthesis of ADP-ribosylated peptides using a combination of the strategy for MARylated peptides presented in Chapter 3 and the work of Kistemaker[24] and Liu *et al.*[25]

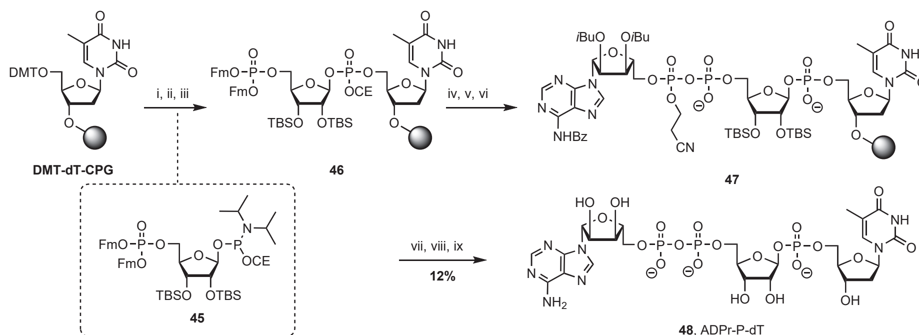


Scheme 6. Synthetic strategies described in Chapter 5 to synthesize Arg-ADP-ribosylated peptides.

Chapter 6

Apart from the ADP-ribosylation of specific amino acids in certain proteins, nucleic acids bearing a terminal phosphomonoester can undergo ADP-ribosylation.[26,27] Chapter 6 is dedicated to developing a methodology for the synthesis of ADP-ribosylated nucleotides, potentially suitable for an automated solid-phase oligonucleotide synthesis. Chapter 6 describes a novel, bifunctional ribosyl building block **45** (Scheme 7) for the synthesis of nucleotides, the 5'-phosphate of which is linked to ADP-ribose. The strategy was first explored in solution resulting in ADPr with a glycosidic linkage to AMP. After the successful solution-phase synthesis, the compatibility of ribosyl building block **45** with conventional

solid phase nucleotide synthesis was tested. After optimization of the chemistry, ADPr-P-dT **48** was manually synthesized on resin. This unprecedented synthesis of ADP-ribosylated nucleotide paves the way for automated ADPr oligonucleotide synthesis.

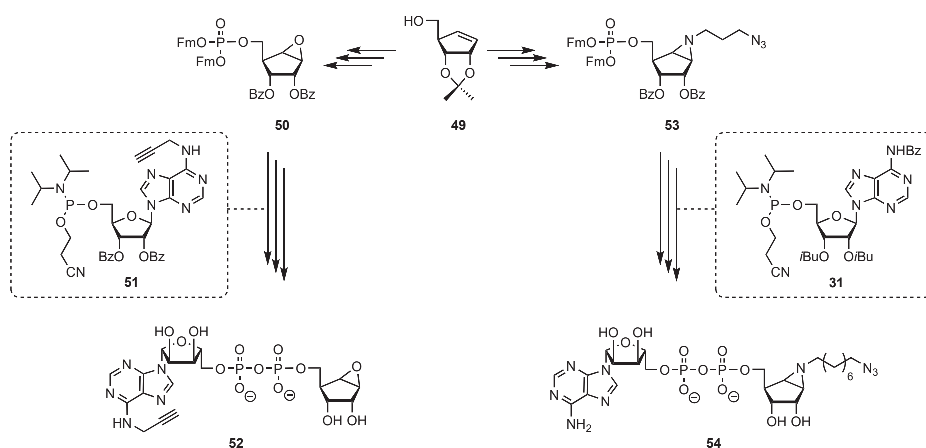


Scheme 7. Solid phase synthesis of ADPr 5'-linked to a nucleotide. Reagents and conditions: i) DCA, DCM. ii) **45**, activator 42®, MeCN. iii) CSO, MeCN. iv) DBU, MeCN. v) **31**, activator 42®, MeCN. vi) CSO, MeCN. vii) DBU, MeCN. viii) NH_4OH, H_2O. ix) TEA·3HF, TEA, NMP.

9

Chapter 7

Cluster of differentiation 38 (CD38) is a transmembrane protein responsible for the conversion of NAD^+ to free ADPr and cyclic ADPr (cADPr). Chapter 7 describes the synthesis of two potential activity-based probes (ABPs) for CD38 that utilize an electrophilic trap in the form of either an epoxide or aziridine to covalently bind with the catalytic Glu-residue in the active site of CD38. Similar mechanism-based probes employing epoxides and/or aziridines as electrophilic trap have been developed successfully for a variety of glycosidases.[28–31] The starting point of the synthetic route towards ABPs **52** and **54** is cyclopentene **49** (Scheme 8). In case of ABP **52**, precursor epoxide **50** bears an orthogonally protected 5'-phosphotriester. After its conversion into a phosphomonoester, the ribosyl moiety is suitable for a P^{III} – P^{V} coupling step with appropriately protected adenosine phosphoramidite **51**. The adenosine in ABP **52** is functionalized with a propargyl group on the exocyclic amine, enabling the installation of a reporter group via CuAAC for in-gel fluorescence or MS/MS proteomics. A similar route was pursued to obtain ABP **54**, the aziridine moiety of which is decorated with an azide containing linker for CuAAC ligation and thus previously described adenosine phosphoramidite **31** was ideally suited to synthesize ABP **54**. After the P^{III} – P^{V} coupling step, final removal of all protecting groups (i.e., *i*Bu and Bz) and HPLC purification, both ABP probes **52** and **54** were isolated in sufficient amounts to evaluate their binding affinity with CD38.



Scheme 8. Synthesis of potential ABPs **52** and **54** for CD38 as described in Chapter 7.

Chapter 8

Chapter 8 deals with the development of two affinity-based probes for PARP1 (ARTD1) that are based on the clinical and FDA approved PARP1 inhibitor olaparib. The photo-affinity based probes were designed by functionalization of olaparib derivatives with diazirine-alkyne linkers and are displayed in Figure 2, **55** and **56**. The probes were easily synthesized by HCTU mediated coupling of readily available olaparib derivatives with diazirine-alkyne containing linkers. The probes were incubated with living Raji cells enabling the non-covalent binding of the recognition element (olaparib 'part') of the probes with PARP1. Subjection of the cells to UV-irradiation activates the diazirine allowing carbene formation which then inserts itself in the peptide backbone, covalently labeling the inhibited protein. After cell lysis and denaturation, the lysate was labelled using CuAAC to introduce the reporter group. An azide containing fluorophore was used to allow in-gel fluorescence analysis whereas ligation with biotin allowed streptavidin affinity enrichment and pull-down of the labelled proteins. Analysis of the isolated proteins using an MS/MS based proteomics procedure showed that especially **56** was able to effectively target PARP1 in living cells. The ability of **56** to covalently bind PARP1 was also corroborated by in-gel fluorescence analysis. In addition, target validation was performed by Western-Blot analysis confirming the observed fluorescent band to be PARP1.

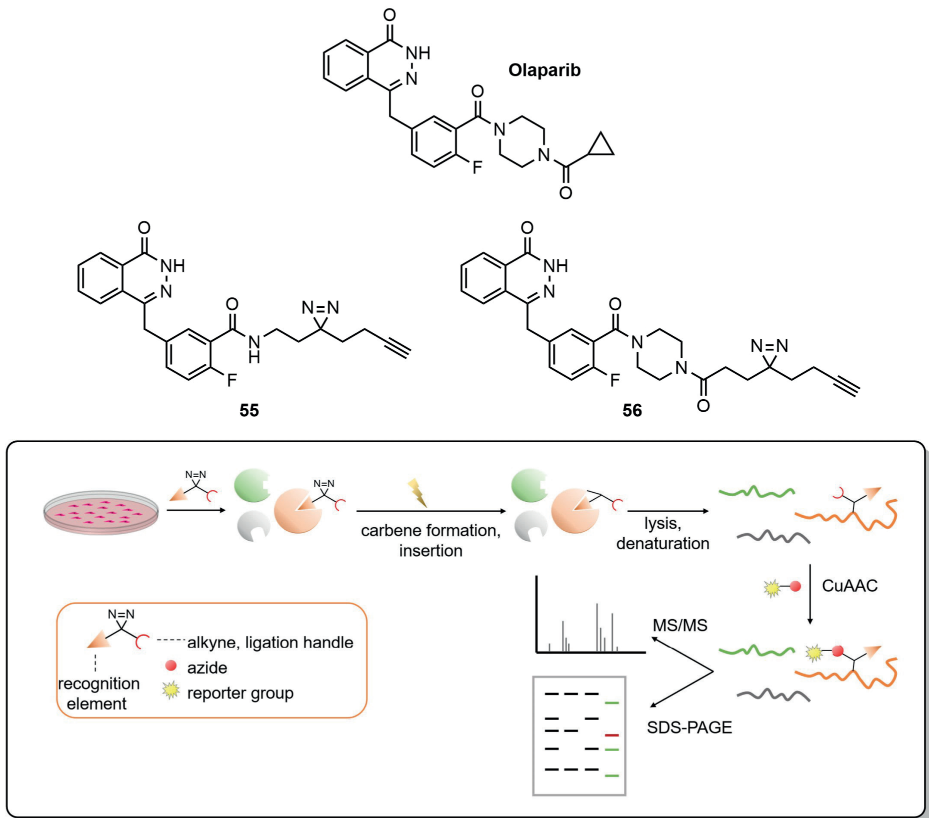


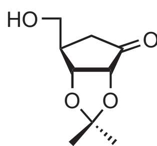
Figure 2. AfBPs **55** and **56** and schematic workflow of the proteomics performed with these probes.

Experimental section

General synthetic procedures

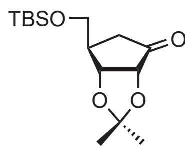
All reagents were used as received unless stated otherwise. Solvents used in synthesis were dried and stored over 4Å molecular sieves, except for MeOH and MeCN which were stored over 3Å molecular sieves. Triethylamine (TEA) and diisopropylethylamine (DIPEA) were stored over KOH pellets. Column chromatography was performed on silica gel 60 Å (40-63 µm, Macherey-Nagel). TLC analysis was performed on Macherey-Nagel aluminum sheets (silica gel 60 F_{254}). TLC was used to visualize compounds by UV at wavelength 254 nm and by spraying with either cerium molybdate spray (25 g/L $(NH_4)_6Mo_7O_{24}$, 10 g/L $(NH_4)_4Ce(SO_4)_4 \cdot H_2O$ in 10% H_2SO_4 water solution) or $KMnO_4$ spray (20 g/L $KMnO_4$ and 10 g/L K_2CO_3 in water) followed by charring at c.a. 250 °C. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Nucleodur C18 Gravity 3 µm 50 x 4.60 mm column (detection at 200-600 nm) coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI or coupled to a Thermo LCQ Fleet Ion mass spectrometer with ESI. The method used was 10→90% 13.5 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: 10% to 90% MeCN; 8.5→ 11 min: 90% MeCN; 11→13.5 min: 10% MeCN) or 0→50% 13.5 min. NMR spectra were recorded on a Bruker AV-400, AV-500 or AV-600 NMR. Chemical shifts (δ) are given in ppm relative to tetramethyl silane as internal standard. Coupling constants (*J*) are given in Hz. All given ^{13}C-APT spectra are proton decoupled.

(2*R*,3*R*,4*R*)-2,3-O-isopropylidene-4-hydroxymethyl-cyclopentanone



A chromatography column was charged with enone **10** (1.23 g, 8.0 mmol) and benzophenone (262 mg, 1.44 mmol, 0.18 eq.) in anhydrous methanol (508 mL, 0.016 M) and purged with nitrogen gas for 1 hour, followed by additional purging with argon for 1 hour. The colorless solution was irradiated at 365 and 366 nm utilizing a Spectroline ENF-260C/FE (6 watts) and CAMAG 4 (14 watts) lamp. After irradiating overnight, the solution became yellow and the methanol was evaporated under reduced pressure to provide a crude amber residue. Purification by silica gel column chromatography (20 -> 60% EtOAc in pentane) furnished the title compound as a colorless oil (900 mg, 4.83 mmol, 60%). **Rf:** 0.22 (40% EtOAc in pentane). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 4.72 (d, 1H, *J* = 5.4 Hz, H-2), 4.29 (dt, 1H, *J* = 5.5, 1.4 Hz, H-3), 3.83 (dd, 1H, *J* = 10.3, 3.0 Hz, H-5), 3.69 (dd, 1H, *J* = 10.3, 3.6 Hz, H-5'), 2.82 (br. s, 1H, 5-OH), 2.74 (ddd, 1H, *J* = 18.4, 9.1, 1.0 Hz, H-6), 2.57 – 2.51 (m, 1H, H-4), 2.17 (dq, 1H, *J* = 18.5, 1.5 Hz, H-6'). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 214.7 (C=O), 111.3 (Cq), 81.4 (C-2), 78.9 (C-3), 64.0 (C-5), 38.9 (C-4), 37.2 (C-6), 26.7, 24.7 (Me).

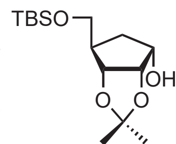
(2*R*,3*R*,4*R*)-2,3-O-isopropylidene-4-[((tert-butyldimethylsilyl)oxy)methyl]cyclopentanone (11)



Enone **10** (325 mg, 1.75 mmol) and imidazole (178 mg, 2.62 mmol, 1.5 eq.) were co-evaporated with anhydrous 1,4-dioxane (5 mL) and dissolved in anhydrous DCM (13.6 mL, 0.13 M) after which tert-butyldimethylsilyl chloride (50 wt. % in toluene, 0.73 mL, 2.1 mmol, 1.2 eq.) was added. The suspension was stirred for 4 hours, quenched by the addition of methanol (1 mL) and diluted with H_2O. The organic layer was separated and the aqueous layer extracted twice with DCM. The resulting organic layers were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography (0 -> 20% EtOAc in pentane) afforded the title compound as a colorless oil (516 mg, 1.72 mmol, 98%). **Rf:** 0.86 (10% EtOAc in pentane). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 4.66 (d, 1H, *J* = 5.4 Hz, H-2), 4.24 (dd, 1H, *J* = 5.4, 1.4 Hz, H-3), 3.84 (dd, 1H, *J* = 9.8, 2.3 Hz, H-5), 3.64 (dd, 1H, *J* = 9.8, 2.8 Hz, H-5'), 2.74 (dd, 1H, *J* = 18.1, 9.1 Hz, H-6), 2.55 – 2.50 (m, 1H, H-4), 2.09 (dq, 1H, *J* = 18.1, 1.4 Hz, H-6'), 1.44 (s,

3H, CH_3 Me), 1.36 (s, 3H, CH_3 Me), 0.86 (s, 9H, CH_3 *t*Bu), 0.05 (s, 3H, CH_3 SiMe), 0.03 (s, 3H, CH_3 SiMe). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 212.9 (C=O), 111.1 (Cq), 82.0 (C-2), 79.2 (C-3), 65.4 (C-5), 39.2 (C-4), 37.3 (C-6), 26.9 (Me), 25.9 (*t*Bu), 24.7 (Me), 18.30 (Cq tBu), -5.6, -5.7 (SiMe).

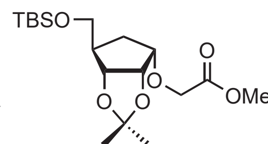
(1*S*,2*S*,3*R*,4*R*)-2,3-O-isopropylidene-4-[((tert-butyldimethylsilyl)oxy)methyl]-cyclopentanol (12)



Compound **11** (360 mg, 1.2 mmol) was dissolved in methanol (36 mL, 0.03 M) and cooled to 0 °C after which sodium borohydride (55 mg, 1.45 mmol, 1.2 eq.) was added. After stirring for 15 minutes, the cooling bath was removed and the mixture stirred for another 15 minutes. The reaction was quenched by the addition of acetone (5 mL) and concentrated to dryness. The resulting orange residue was taken up in EtOAc and washed with sat. aq. NaCl. The aqueous layer was extracted twice with EtOAc and the organic layers were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography (10 -> 20% EtOAc in pentane) afforded the title compound as a viscous colorless oil (362 mg, 1.2 mmol, quant.). **Rf:** 0.62 (10% EtOAc in pentane. **^{1}H NMR** (500 MHz, $CDCl_3$): δ 4.51 (dd, 1H, *J* = 5.9, 1.3 Hz, H-3), 4.45 (t, 1H, *J* = 5.7 Hz, H-2), 4.22 (td, 1H, *J* = 7.6, 5.6 Hz, H-1), 3.62 (dd, 1H, *J* = 10.1, 4.5 Hz, H-5), 3.50 (dd, 1H, *J* = 10.1, 4.6 Hz, H-5'), 2.47 (br. s, 1H, 1-OH), 2.23 – 2.17 (m, 1H, H-4), 1.90 – 1.84 (m, 2H, H-6), 1.51 (s, 3H, CH_3 Me), 1.37 (s, 3H, CH_3 Me), 0.90 (s, 9H, CH_3 tBu), 0.05 (s, 6H, CH_3 SiMe). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 111.0 (Cq), 83.1 (C-3), 79.8 (C-2), 72.0 (C-1), 64.7 (C-5), 44.0 (C-4), 35.7 (C-6), 26.3 (Me), 26.0 (*t*Bu), 24.3 (Me), 18.3 (Cq *t*Bu), -5.5 (SiMe).

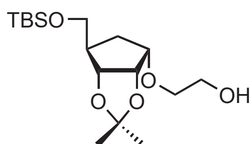
9

(1*S*,2*S*,3*R*,4*R*)-1-*O*-(2-methoxy-2-oxoethoxy)-2,3-*O*-isopropylidene-4- [((*tert*butyldimethylsilyl)oxy) methyl]-cyclopentane (13)



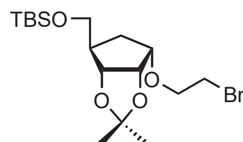
A 50 mL round bottom flask was flame-dried under vacuum, placed under an argon atmosphere and charged with cyclopentanol **12** (1.51 g, 5.0 mmol), which was subsequently co-evaporated thrice with anhydrous 1,4-dioxane. The residue was dissolved in anhydrous THF (25 mL, 0.2 M) after which sodium hydride (60 wt. % in mineral oil, 600 mg, 15 mmol, 3 eq.) and 15-crown-5 (1.2 mL, 6.0 mmol, 1.2 eq.) were added. The solution was stirred for 30 minutes until bubbling seized followed by the addition of TBABr (359 mg, 1.0 mmol, 0.2 eq.) and methyl bromoacetate (1.4 mL, 15 mmol, 3 eq.). The reaction was stirred for 9 days after which it was quenched by the careful addition of sat. aq. NH_4Cl (5 mL), diluted with H_2O and the aqueous layer was extracted thrice with Et_2O. The organic layers were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (5 -> 20% EtOAc in pentane) afforded the title compound as a colorless oil and an inseparable mixture with impurities and starting material (1.8 g, 4.81 mmol, 96%). **R_f:** 0.67 (20% EtOAc in pentane). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 4.56 (t, 1H, *J* = 5.2 Hz, H-2), 4.41 (dd, 1H, *J* = 5.5, 1.2 Hz, H-3), 4.22 - 415 (m, 2H, *J* = 16.6 Hz, CH_2COOMe), 4.07 (ddd, 1H, *J* = 9.8, 6.6, 4.9 Hz, H-1), 3.72 (s, 3H, CH_3 COOMe), 3.57 (dd, 1H, *J* = 10.0, 4.4 Hz, H-5), 3.47 (dd, *J* = 10.0, 4.0 Hz, H-5'), 2.15 – 2.07 (m, 2H, H-4, H-6), 1.83 (dd, 1H, *J* = 10.9, 6.5 Hz, H-6'), 1.48 (s, 3H, CH_3 Me), 1.31 (s, 3H, CH_3 Me), 0.86 (s, 9H, CH_3 *t*Bu), 0.01 (s, 6H, CH_3 SiMe). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.0 (C=O COOMe), 110.9 (Cq), 82.7 (C-3), 80.4 (C-1), 79.1 (C-2), 67.3 (C_s CH_2COOMe), 65.1 (C-5), 51.8 (COOMe), 44.1 (C-4), 31.6 (C-6), 26.5 (Me), 25.9 (*t*Bu), 24.5 (Me), 18.2 (Cq *t*Bu), -5.6 (SiMe). **LC-MS** (10 -> 90% B in A): Rt = 8.71.

(1*S*,2*S*,3*R*,4*R*)-1-*O*-hydroxyethoxy-2,3-*O*-isopropylidene-4-[((*tert*-butyldimethyl silyl)oxy)methyl]-cyclopentane (14)



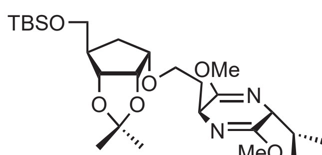
To an ice-cooled solution of crude methyl ester **13 (**1.8 mg,4.81 mmol) in anhydrous Et_2O (48.1 mL, 0.1 M) was added $LiAlH_4$ (456 mg, 12.0 mmol, 2.5 eq.). The suspension was stirred for 2 hours, quenched by the dropwise addition of sat. aq. NH_4Cl (5 mL) and stirred while gradually warming to room temperature. The mixture was diluted with 1 M aq. HCl, extracted six times with Et_2O and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by column chromatography (20 -> 40% EtOAc in pentane) to afford the title compound as a colorless oil (1.03 g, 2.98 mmol, 60% over 2 steps) and recovered starting material **2** (359 mg, 1.19 mmol, 24%). $\mathbf{R_f}$**:** 0.17 (20% EtOAc in pentane). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 4.55 (t, 1H, *J* = 5.3 Hz, H-2), 4.47 (d, 1H, *J* = 5.6 Hz, H-3), 4.01 (ddd, 1H, *J* = 10.4, 6.7, 5.0 Hz, H-1), 3.79 – 3.66 (m, 3H, H-7, H-8 CH_2CH_2OH), 3.63 – 3.57 (m, 2H, H-5, H-7’), 3.49 (dd, 1H, *J* = 10.0, 4.6 Hz, H-5’), 3.01 (br. s, 1H, 8-OH), 2.15 (dt, 1H, *J* = 9.0, 4.7 Hz, H-4), 2.06 (ddd, 1H, *J* = 12.5, 10.4, 8.4 Hz, H-6), 1.83 (dd, 1H, *J* = 12.4, 6.7 Hz, H-6’), 1.50 (s, 3H, CH_3 Me), 1.33 (s, 3H, CH_3 Me), 0.89 (s, 9H, CH_3 *t*Bu), 0.04 (s, 6H, CH_3 SiMe). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 110.9 (Cq), 83.2 (C-3), 80.4 (C-1), 78.8 (C-2), 71.8 (C-7), 65.1 (C-5), 62.0 (C-8), 43.7 (C-4), 32.3 (C-6), 26.4 (Me), 26.0 (*t*Bu), 24.6 (Me), 18.3 (Cq *t*Bu), -5.5 (SiMe).

(1*S*,2*S*,3*R*,4*R*)-1-*O*-bromoethoxy-2,3-*O*-isopropylidene-4-[((*tert*-butyldimethylsilyl)oxy)methyl]-cyclopentane (15)



Ethoxy alcohol **14** (520 mg, 1.5 mmol) was co-evaporated thrice with anhydrous 1,4-dioxane, dissolved in anhydrous DCM (15 mL, 0.1 M) and cooled to 0 °C. To this cooled solution imidazole (204 mg, 3.0 mmol, 2 eq.), PPh_3 (789 mg, 3.0 mmol, 2 eq.) and Br_2 (154 μL, 3.0 mmol 2.0 eq.) were added consecutively. After stirring for 1 hour, TLC indicated complete conversion and the solution was concentrated to dryness. The residue was taken up in Et_2O, washed with sat. aq. $NaHCO_3$ and sat. aq. $Na_2S_2O_3$ and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography (0 -> 10% EtOAc in pentane) afforded the title compound as a colorless oil (513 mg, 1.25 mmol, 84%). $\mathbf{R_f}$**:** 0.81 (5% EtOAc in pentane). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 4.55 (t, 1H, *J* = 5.2 Hz, H-2), 4.45 (d, 1H, *J* = 5.5 Hz, H-3), 4.04 (ddd, 1H, *J* = 10.8, 6.6, 4.8 Hz, H-1), 3.92 (dt, 1H, *J* = 10.7, 6.7 Hz, H-7 OCH_2CH_2), 3.79 (dt, 1H, *J* = 10.7, 6.9 Hz, H-7’ OCH_2CH_2), 3.61 (dd, 1H, *J* = 10.0, 4.6 Hz, H-5), 3.54 – 3.47 (m, 3H, H-5’, H-8 CH_2CH_2Br), 2.20 – 2.06 (m, 2H, H-4, H-6), 1.83 (dd, 1H, *J* = 12.2, 6.5 Hz, H-6), 1.52 (s, 3H, CH_3 Me), 1.35 (s, 3H, CH_3 Me), 0.92 (s, 9H, CH_3 *t*Bu), 0.07 (s, 6H, CH_3 SiMe). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 111.0 (Cq), 82.8 (C-3), 80.6 (C-1), 79.4 (C-2), 70.2 (C-7), 65.2 (C-5), 44.2 (C-4), 31.9 (C-6), 30.3 (C-8), 26.6 (Me), 26.0 (*t*Bu), 24.7 (Me), 18.3 (Cq *t*Bu), -5.4 (SiMe).

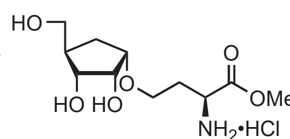
(1*S*,2*S*,3*R*,4*R*)-1-*O*-ethyl-((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5- dihydro pyrazine)-2,3-*O*-isopropylidene-4-[((*tert*-butyldimethylsilyl)oxy) methyl] cyclopentane (16)



A 5 mL pear shaped flask was flame-dried and subsequently charged with (*R*)-Schöllkopf reagent (112.6 μL, 0.629 mmol, 1.05 eq.) under inert atmosphere. THF (0.5 mL) was added and the solution was cooled to -78 °C after which *n*-BuLi (2.5 M in hexanes, 251.3 μL, 1.05 eq.) was added dropwise over the duration of 3 minutes and the mixture stirred for an additional 30 minutes. A solution of co-evaporated ethoxybromide **15** (245 mg, 0.598 mmol, 1.0 eq.) in THF/HMPA (v/v; 5/2, 0.7 mL, 1.45 mL total, 0.43 M) was added to the yellow aza-enolate solution. Next, Ag_2CO_3 (165 mg, 0.598

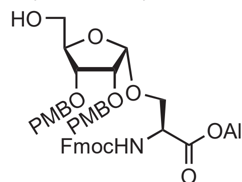
mmol, 1.0 eq.) was added and the mixture was stirred overnight while allowed to gradually warm to room temperature. The reaction was quenched by the addition of sat. aq. NH_4Cl (5 mL) and filtered over a pad of Celite. The filtrate was diluted with sat. aq. NH_4Cl, extracted thrice with Et_2O and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (2.5 -> 20% EtOAc in pentane) furnished an inseparable diastereomeric mixture of title dihydropyrazine **6** as a yellow oil (201 mg, 0.392 mmol, 66%, dr = 1/2.9). **R_f:** 0.32 (10% EtOAc in pentane). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 4.59 – 4.48 (m, 1H, H-2), 4.48 – 4.37 (m, 1H, 3-H), 4.08 (d, *J* = 7.6 Hz, 1H), 4.07 (dt, 1H, *J* = 7.6, 4.2 Hz, H-1), 3.95 – 3.87 (m, 3H, C^1-C*H*HCH$_2$, 2 x C*H* bislactim), 3.86 (s, 3H, OMe), 3.66 (s, 3H, OMe), 3.66 – 3.64 (m, 1H, C^1-C*H*HCH$_2$), 3.62 – 3.54 (m, 2H, H-5, C^1-CH$_2$C*H*H), 3.51 – 3.46 (m, 2H, H-5, C^1-CH$_2$C*H*H), 2.27 – 2.17 (m, 1H, C*H*(CH_3)$_2$ isopropyl), 2.13 – 1.98 (m, 1H, H-4), 1.96 – 1.83 (m, 1H, H-6), 1.78 – 1.73 (m, 1H, H-6), 1.49 (s, 3H, CH_3 Me), 1.31 (s, 3H, CH_3 Me), 1.04 (d, 1H, *J* = 6.9 Hz, CH_3 isopropyl), 0.89 (s, 9H, CH_3 *t*Bu), 0.70 (d, 1H, *J* = 6.9 Hz, CH_3 isopropyl), 0.05 (s, 6H, CH_3 SiMe). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 163.94, 163.85, 163.6, 163.1, 111.1, 110.9, 110.8, 67.4, 66.8, 65.3, 65.23, 65.21, 64.8, 60.9, 36.02, 35.8, 34.4, 31.6, 31.5, 18.3, -5.40. **LC-MS** (10 -> 90% B in A): Rt = 9.44 and 9.83.

(1*S*,2*S*,3*R*,4*R*)-1-*O*-(*R*)-isothreonine-2,3-*O*-isopropylidene-4-[((*tert*-butyldimethyl silyl)oxy)methyl]-cyclopentane (17)



Compound **16** (0.300 mmol, 154 mg, dr = 1/2.9) was dissolved in THF (3 mL) after which a freshly prepared solution of 0.25 M aq. HCl (2.4 mL, 0.6 mmol, 2.0 eq., 5.4 mL total, 0.056 M) was added. The mixture was stirred at room temperature for 3 days, whereafter LC-MS indicated complete hydrolysis and the mixture was subsequently washed with Et_2O. The resulting aqueous layer was concentrated to dryness and co-evaporated thrice with anhydrous toluene (3 x 5 mL) to furnish the title compound as a 1:1 mixture with D-valine methyl ester, an amorphous colorless solid, as the corresponding hydrochloride salts (204 mg, 0.259 mmol, 86%). **^{1}H NMR** (500 MHz, D_2O): δ 4.28 (dt, *J* = 6.9, 3.7 Hz, 1H), 4.06 (td, *J* = 7.0, 3.8 Hz, 1H), 4.03 – 3.96 (m, 2H), 3.84 (dq, *J* = 12.7, 5.7, 5.0 Hz, 2H), 3.80 – 3.73 (m, 9H), 3.73 – 3.65 (m, 2H), 3.65 – 3.53 (m, 3H), 3.52 – 3.45 (m, 2H), 2.33 – 2.12 (m, 6H), 1.94 – 1.77 (m, 2H), 1.67 – 1.50 (m, 2H), 0.99 – 0.96 (m, 6H). **^{13}C NMR** (126 MHz, D_2O): δ 172.1, 171.9, 80.9, 80.8, 80.8, 75.7, 74.8, 74.7, 74.6, 74.2, 74.1, 74.1, 72.5, 67.3, 67.1, 67.0, 65.1, 65.0, 65.0, 60.0, 55.3, 55.3, 55.1, 53.9, 53.5, 46.1, 46.1, 46.0, 33.4, 31.0, 31.0, 30.9, 30.8, 30.6, 19.0, 18.7. **LC-MS** (10 -> 90% B in A): Rt = 0.765.

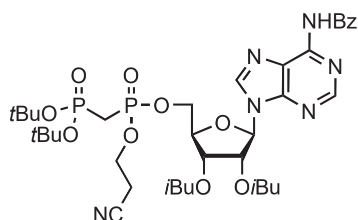
$O^β$-(2,3-di-*O*-*para*-methoxybenzyl-β-D-ribosyl)-$N^α$- fluorenylmethoxycarbonyl serine allyl ester (26)



1-*O*-(2,3-bis-*O*-(4-methoxybenzyl)-5-*O*-((*tert*-butyl)-diphenylsilyl)-α-D-ribosyl)-*N*-fluorenylmethoxycarbonyl serine allyl ester (see compound **25** in Chapter 3, 250 mg, 0.255 mmol) was dissolved in anhydrous pyridine (2.56 mL), after which HF:pyr.(70 wt.%, 0.256 mL) was added. After stirring for 5 hours, TLC indicated full consumption of the starting material and the mixture was quenched by the addition of sat. aq. $NaHCO_3$ and the aqueous phase was extracted thrice with DCM. The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (4 -> 10% acetone in DCM) afforded the title compound as an off-white wax (180 mg, 0.243 mmol, 95%). **R_f:** 0.26 (5% acetone in DCM). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 7.73 (dd, 2H, *J* = 7.5, 2.0 Hz, Fmoc arom.), 7.57 (dd, 2H, *J* = 7.6, 4.1 Hz, Fmoc arom.), 7.36 (td, 2H, *J* = 7.5, 3.5 Hz, Fmoc arom.), 7.30 – 7.21 (m, 6H, Fmoc arom. + PMB arom.), 6.89 – 6.78 (m, 4H, PMB arom), 6.45 (d, 1H, *J* = 9.2 Hz, NH), 5.87 (ddq, 1H, *J* = 17.2, 12.0, 6.0 Hz, C*H*CH$_2$ allyl), 5.30 (dd, 1H, *J* = 17.2, 1.5 Hz, CHCH_{2a} allyl), 5.18 (dd, 1H, *J* = 10.5, 1.3 Hz, CHCH_{2b} allyl), 4.93 (d, 1H, *J* = 4.0 Hz, H-1), 4.69

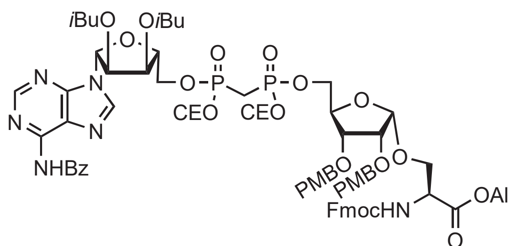
– 4.37 (m, 8H, CH_2CH allylic +2x CH_2 PMB + CH Ser + CH_{2a} Fmoc), 4.26 (dd, 1H, *J* = 10.5, 7.6 Hz, CH_{2b} Fmoc), 4.19 – 4.12 (m, 3H, H-4, CH, Fmoc), 3.92 (dd, 1H, *J* = 10.5, 3.2 Hz, CH_2), 3.87 (dd, 1H, *J* = 6.4, 3.5 Hz, H-3), 3.78 (s, 3H, OCH_3 PMB), 3.77 – 3.74 (m, 2H, H-2, 5-OH),3.73 (s, 3H, OCH_3 PMB), 3.66 (d, 1H, *J* = 11.6 Hz, H-5), 3.46 (d, 1H, *J* = 12.3 Hz, H-5'). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 170.3 (C=O allyl ester), 159.5, 159.4 (Cq PMB), 156.5 (C=O carbamate), 144.2, 143.9, 141.4, 141.3 (Fmoc), 131.8 (*C*HCH_2 allyl), 130.3, 129.9, 129.8, 129.6 (PMB), 127.8, 127.7, 127.2, 127.1, 125.5, 125.3, 120.02, 120.00 (Fmoc), 118.6 (CH*C*H_2 allyl), 113.9, 113.8 (PMB), 101.6 (C-1), 83.6 (C-4), 78.4 (C-2), 75.0 (C-3), 72.5, 72.3 (*C*H_2 PMB), 68.1 (CH_2), 67.1 (*C*H_2 Fmoc), 66.2 (*C*H_2CH allylic), 62.8 (C-5), 55.4, 55.3 (OMe PMB), 54.6 (CH Ser), 47.2 (*C*H Fmoc).

N^6-benzoyl-2',3'-di-*O*-isobutyryl-(R_P/S_P)-5'-*O*-di-*tert*-butyl(((2-cyanoethoxy)phosphoryl) methyl) phosphonate adenosine (25)



A 25 mL round bottom flask was flame-dried under vacuum, placed under an argon atmosphere, charged with adenosine **24** (1.74 g, 3.41 mmol, 1.1 eq.) and phosphanyl methylphosphonate **23**[22] (1.27 g, 3.10 mmol) co-evaporated with anhydrous acetonitrile and the residue was dissolved in anhydrous acetonitrile (15.5 mL, 0.2 M). DCI (549 mg, 4.65 mmol, 1.5 eq.) was added and stirring commenced until ^{31}P NMR indicated complete phosphonitylation (δ 172.92) after 30 minutes. *t*BuOOH (5.5 M in nonane, 1.13 mL, 6.19 mmol, 2.0 eq.) was added and stirring commenced for an additional 15 minutes, whereafter ^{31}P NMR showed complete conversion to the phosphonate (δ 20.88). The mixture was diluted with EtOAc and the organic layer was washed with H_2O, sat. aq. $NaHCO_3$ and sat. aq. NaCl respectively. The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (20 -> 40% acetone in DCM) afforded the title compound as a mixture of R_P/S_P diastereomers, as a crystalline white foam (2.45 g, 2.93 mmol, 95%). **R_f:** 0.25 (30% acetone in DCM). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 9.24 (s, 2H, 6-NH), 8.81 (s, 1H, H-2), 8.80 (s, 1H, H-2), 8.50 (s, 1H, H-8), 8.41 (s, 1H, H-8), 8.06 – 7.99 (m, 4H, *o*-Bz), 7.93, 7.66 – 7.59 (m, 2H, *p*-Bz), 7.56 – 7.50 (m, 4H, *m*-Bz), 6.33 (d, 1H, *J* = 6.0 Hz, H-1'), 6.29 (d, 1H, *J* = 5.7 Hz, H-1'), 5.95 (t, 1H, *J* = 5.7 Hz, H-2'), 5.88 (t, 1H, *J* = 5.9 Hz, H-2'), 5.73 (dd, 1H, *J* = 5.7, 3.9 Hz, H-3'), 5.69 (dd, 1H, *J* = 5.7, 3.9 Hz, H-3'), 4.56 – 4.43 (m, 6H, H-4', H-5'), 4.40 – 4.26 (m, 4H, OC$H_2$$CH_2$CN), 2.85 – 2.69 (m, 4H, OCH_2CH_2CN), 2.69 – 2.61 (m, 2H, $CH(Me)_2$ *i*Bu), 2.59 – 2.44 (m, 6H, P-CH*H*-P, $CH(Me)_2$ *i*Bu), 1.55 – 1.49 (m, 36H, CH_3 ^{t}Bu), 1.25 – 1.21 (m, 12H, CH_3), 1.17 – 1.07 (m, 12H. **^{13}C-APT NMR** (126 MHz, $CDCl_3$): δ 175.9, 175.8, 175.52, 175.51 (C=O *i*Bu), 165.04, 165.03 (C=O Bz), 151.92 (C-2), 151.86 (C-6), 149.72 (C-4), 149.70 (C-8), 133.42 (*ipso*-Bz), 133.09 (*p*-Bz), 129.02 (*m*-Bz), 127.99 (*p*-Bz), 123.47, 123.36 (C-5), 116.92, 116.82 (Cq CH_2CH_2CN), 86.4, 86.0 (C-1), 84.61, 84.58, 84.54, 84.51, 84.47, 84.42 (Cq *t*Bu), 81.93, 81.88, 81.7, 81.6 (C-4'), 73.2, 73.1 (C-2'), 70.42, 70.35 (C-3'), 65.50, 65.45, 65.18, 65.13 (C-5'), 61.34, 61.30 (*C*$H_2$$CH_2$CN), 33.86, 33.84, 33.70, 33.68 (CH *i*Bu), 30.71, 30.41 (P-*C*-P), 30.39, 30.36, 30.33 (*t*Bu), 29.59, 29.42, 28.51, 28.30 (P-*C*-P), 19.96, 19.95, 19.91, 19.89 (CH_2CH_2CN), 18.97, 18.87, 18.84, 18.72 (CH_3 *i*Bu). **^{31}P NMR** (202 MHz, $CDCl_3$): δ 23.32 (d, *J* = 8.1 Hz, CH_2P(O)OCNE), 22.98 (d, *J* = 8.1 Hz, CH_2P(O)OCNE), 9.16 (d, *J* = 8.2 Hz, $(tBuO)_2P(O)CH_2$), 9.06 (d, *J* = 7.9 Hz, $(tBuO)_2P(O)CH_2$). **LC-MS** (30 -> 70% B in A): Rt = 7.25.

(R_p/S_p)-Ser-ABP ribosylated derivative (27)



To a solution of (di-*tert*-butyl)methylene bisphosphonate adenosine **25** (500 mg, 0.600 mmol) in hexafluoro isopropanol (1.43 mL, 0.42 M) was added a 6 M HCl 1,4-dioxane:H_2O (1:1 ; v/v, 116.4 µL, 0.7 mmol, 1.2 eq.). Reaction progress was gauged with ^{31}P NMR, which indicated complete acidolysis of the di-*tert*-butyl phosphonate moiety into the corresponding deprotected phosphonate (δ 16.37) after 4 hours. The mixture was subsequently diluted with 1,4-dioxane:toluene (1:1; v/v, 20 mL) and concentrated *in vacuo*. The co-evaporation process was repeated thrice to remove all traces of hexafluoro isopropanol and furnished deprotected methylene bisphosphonate adenosine as a crystalline white solid (433 mg, 0.599 mmol, quant.), which was used without further purification.

The resulting bisphosphonate adenosine was aliquoted and 73 mg (0.1 mmol) was co-evaporated thrice with anhydrous acetonitrile and dissolved in anhydrous acetonitrile (1 mL, 0.1 M). 2-Cyanoethanol (7.2 µL, 0.105 mmol, 1.05 eq.), anhydrous TEA (27.9 µL,0.2 mmol, 2.0 eq.) and anhydrous 2,6-lutidine (116 µL, 1.0 mmol, 10 eq.) and PyNTP (150 mg, 0.3 mmol, 3.0 eq.) were added successively and the resulting solution was stirred for 5 hours. Subsequently, ribosylated serine **26** (78 mg, 0.105 mmol, 1.05 eq.), which was co-evaporated thrice in anhydrous acetonitrile prior to use, as a solution in anhydrous acetonitrile (1 mL) and PyNTP (150 mg, 0.3 mmol, 3.0 eq.) were added. After stirring overnight, the mixture was quenched by the addition of 1 M NaOAc in MeOH (1 mL), stirred for 30 minutes and subsequently diluted with DCM (20 mL). The organic layer was washed with 1 M aq. HCl, dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (0 -> 5% acetone in DCM) furnished crude title ADPr derivative **27** as a colorless residue. A second purification by column chromatography (0 -> 5% acetone in DCM) to remove residual traces of hexafluorophosphate afforded the title compound as a colorless wax (49 mg, 0.033 mmol, 33%). **^{31}P NMR** (202 MHz, $CDCl_3$): δ 26.97, 25.51, 25.15, 25.00. **LC-MS** (10 -> 90% B in A): Rt = 9.22 and 9.38.

References

[1] G. J. van der Heden van Noort, M. G. van der Horst, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov, *J. Am. Chem. Soc.* **2010**, *132*, 5236–5240.

[2] H. A. V Kistemaker, A. P. Nardozza, H. S. Overkleeft, G. A. van der Marel, A. G. Ladurner, D. V. Filippov, *Angew. Chemie - Int. Ed.* **2016**, *55*, 10634–10638.

[3] Q. Liu, H. A. V. Kistemaker, S. Bhogaraju, I. Dikic, H. S. Overkleeft, G. A. van der Marel, H. Ovaa, G. J. van der Heden van Noort, D. V. Filippov, *Angew. Chemie - Int. Ed.* **2018**, *57*, 1659–1662.

[4] J. Voorneveld, J. G. M. Rack, I. Ahel, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov, *Org. Lett.* **2018**, *20*, 4140–4143.

[5] O. Leidecker, J. J. Bonfiglio, T. Colby, Q. Zhang, I. Atanassov, R. Zaja, L. Palazzo, A. Stockum, I. Ahel, I. Matic, *Nat. Chem. Biol.* **2016**, *12*, 998–1000.

[6] L. Palazzo, O. Leidecker, E. Prokhorova, H. Dauben, I. Matic, I. Ahel, *Elife* **2018**, *7*, 1–12.

[7] I. Gibbs-Seymour, P. Fontana, J. G. M. Rack, I. Ahel, *Mol. Cell* **2016**, *62*, 432–442.

[8] J. J. Bonfiglio, P. Fontana, Q. Zhang, T. Colby, I. Gibbs-Seymour, I. Atanassov, E. Bartlett, R. Zaja, I. Ahel, I. Matic, *Mol. Cell* **2017**, *0*, 1–9.

[9] P. Fontana, J. J. Bonfiglio, L. Palazzo, E. Bartlett, I. Matic, I. Ahel, *Elife* **2017**, *6*, 1–20.

[10] J. Moss, N. J. Oppenheimer, R. E. West, S. J. Stanley, *Biochemistry* **1986**, *25*, 5408–5414.

[11] Y. Zhang, J. Wang, M. Ding, Y. Yu, *Nat. Methods* **2013**, *10*, 981–984.

[12] T. Meyer, H. Hilz, *Eur. J. Biochem.* **1986**, *155*, 157–165.

[13] H. Osago, M. Terashima, N. Hara, K. Yamada, M. Tsuchiya, *J. Biochem. Biophys. Methods* **2008**, *70*, 1014–1019.

[14] K. D. Siegmund, F. Klink, *FEBS Lett.* **1992**, *312*, 139–142.

[15] B. Eide, P. Gierschik, A. Spiegel, *Biochemistry* **1986**, *25*, 6711–6715.

[16] S. Mashhood Ali, K. Ramesh, R. T. Borchardt, *Tetrahedron Lett.* **1990**, *31*, 1509–1512.

[17] H. Ovaa, B. Lastdrager, J. D. C. Codée, G. A. van der Marel, H. S. Overkleeft, J. H. van Boom, *J. Chem. Soc. Perkin Trans. 1* **2002**, 2370–2377.

[18] T. C. Myers, K. Nakamura, J. W. Flesher, *J. Am. Chem. Soc.* **1963**, *85*, 3292–3295.

[19] L. M. Yates, D. Fiedler, *ACS Chem. Biol.* **2016**, *11*, 1066–1073.

[20] V. S. Borodkin, M. A. J. Ferguson, A. V. Nikolaev, *Tetrahedron Lett.* **2004**, *45*, 857–862.

[21] V. E. Marquez, C. K. H. Tseng, G. Gebeyehu, D. A. Cooney, G. S. Ahluwalia, J. A. Kelley, M. Dalai, R. W. Fuller, Y. A. Wilson, D. G. Johns, *J. Med. Chem.* **1986**, *29*, 1726–1731.

[22] S. B. Engelsma, N. J. Meeuwenoord, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov, *Angew. Chemie - Int. Ed.* **2017**, *56*, 2955–2959.

[23] A. G. Volbeda, H. A. V. Kistemaker, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov, J. D. C. Codée, *J. Org. Chem.* **2015**, *80*, 8796–8806.

[24] H. A. V Kistemaker, L. N. Lameijer, N. J. Meeuwenoord, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov, *Angew. Chemie - Int. Ed.* **2015**, *54*, 4915–4918.

[25] Q. Liu, Ridderprint B.V., Leiden, **2019**, pp. 62–92.

[26] E. Matta, A. Kiribayeva, B. Khassenov, B. T. Matkarimov, A. A. Ishchenko, *Sci. Rep.* **2020**, *10*, 1–11.

[27] D. Munnur, I. Ahel, *FEBS J.* **2017**, *284*, 4002–4016.

[28] N. Li, H. S. Overkleeft, B. I. Florea, *Curr. Opin. Chem. Biol.* **2012**, *16*, 227–233.

[29] M. D. Witte, M. T. C. Walvoort, K. Li, W. W. Kallemeijn, W. E. Donker-Koopman, R. G. Boot, J. M. F. G. Aerts, J. D. C. Codée, G. A. van der Marel, H. S. Overkleeft, *ChemBioChem* **2011**, *12*, 1263–1269.

[30] K. Y. Li, J. Jiang, M. D. Witte, W. W. Kallemeijn, H. van den Elst, C. S. Wong, S. D. Chander, S. Hoogendoorn, T. J. M. Beenakker, J. D. C. Codée, J. M. F. G. Aerts, G. A. van der Marel, H. S. Overkleeft, *European J. Org. Chem.* **2014**, *2014*, 6030–6043.
[31] W. W. Kallemeijn, K.-Y. Li, M. D. Witte, A. R. A. Marques, J. Aten, S. Scheij, J. Jiang, L. I. Willems, T. M. Voorn-Brouwer, C. P. A. A. van Roomen, R. Ottenhoff, R. G. Boot, H. van den Elst, M. T. C. Walvoort, B. I. Florea, J. D. C. Codée, G. A. van der Marel, J. M. F. G. Aerts, H. S. Overkleeft, *Angew. Chemie Int. Ed.* **2012**, *51*, 12529–12533.