Synthetic Study on ADP-ribosylation

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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.

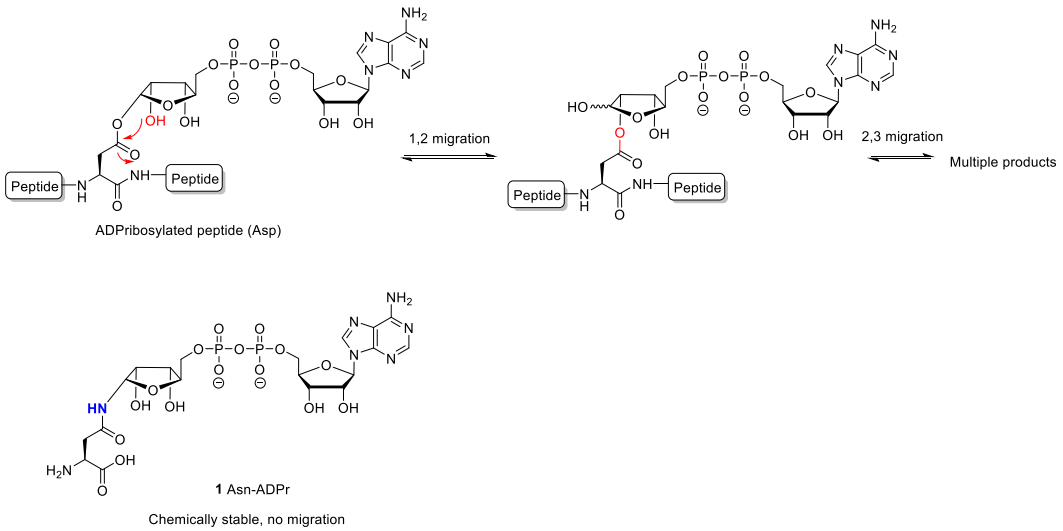
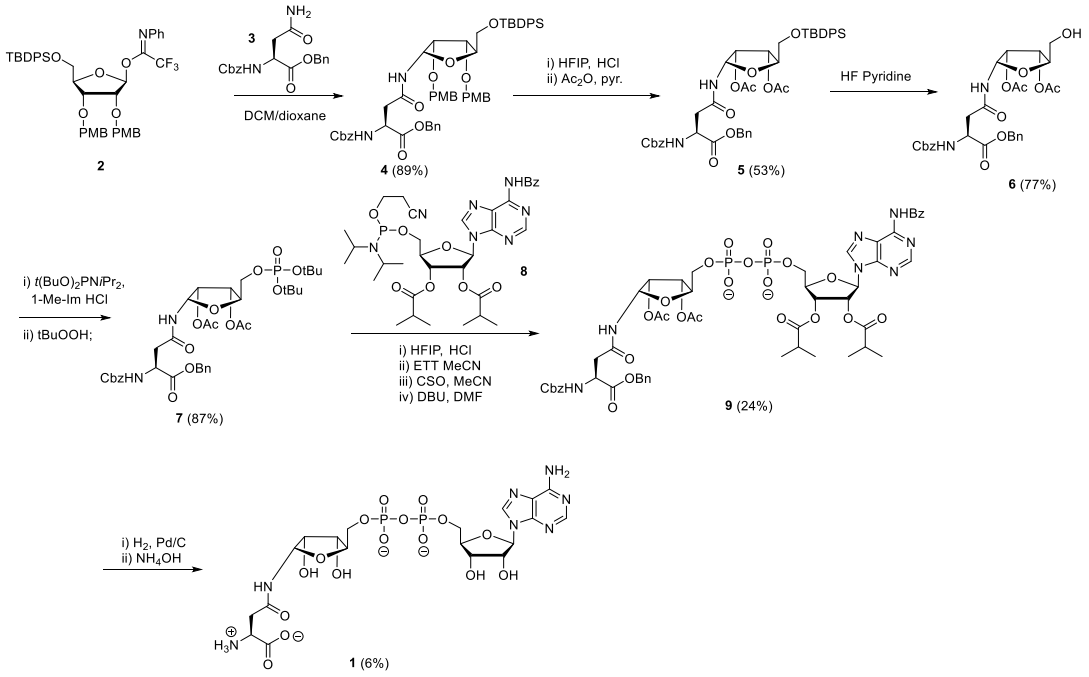


Figure 1. 1,2 migration mechanism of Asp-ADPr peptides and the structure of Asn-ADPr **1**

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).

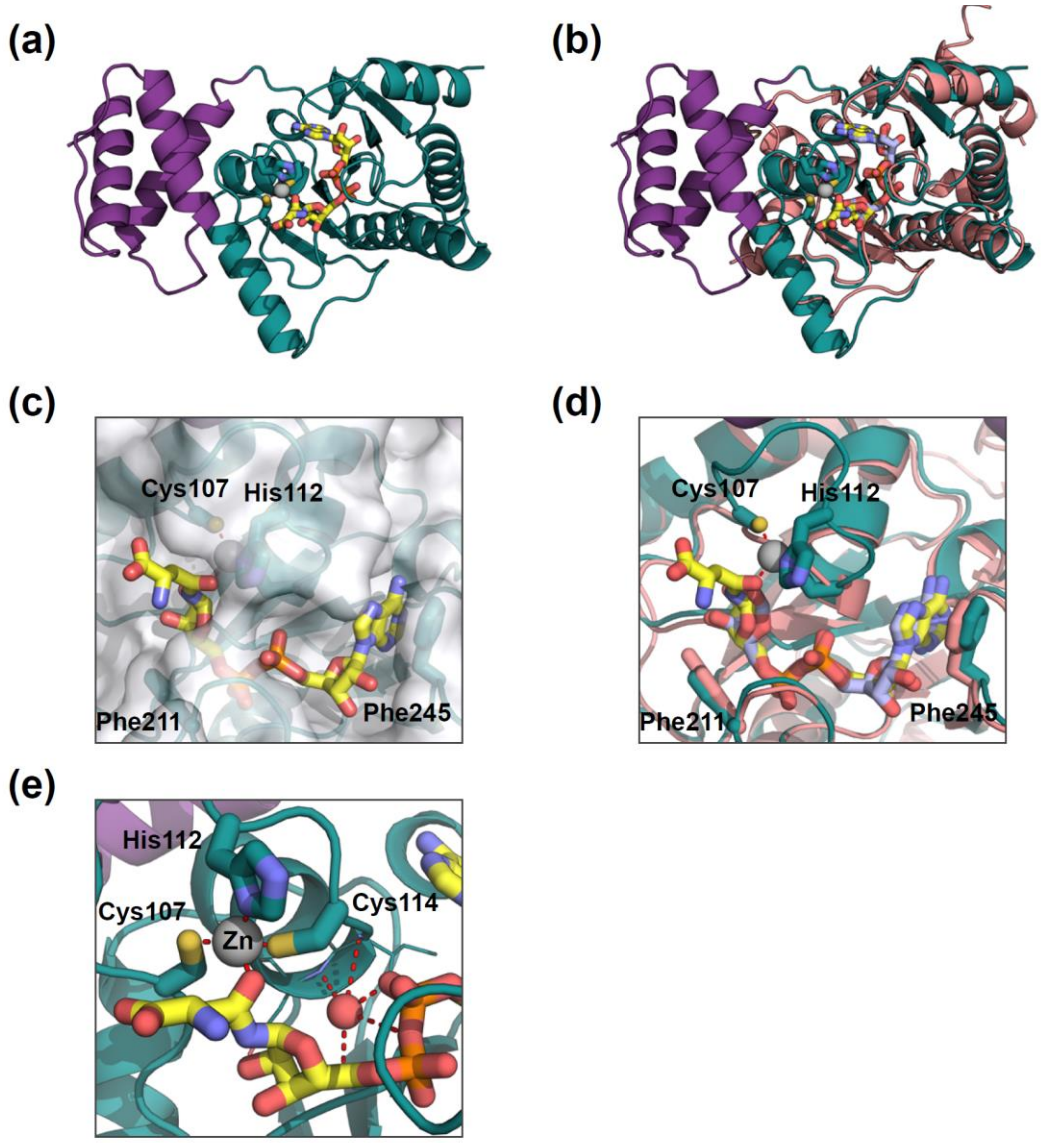


Figure 2. Asn-ADPr as substrate analogue in the crystallisation of *Methanobrevibacter oralis* macrodomain (*Mor*MOD): **(a)** Overall structure of *Mor*MOD in ribbon representation. The classical macrodomain fold is teal, N-terminal extension in purple, the catalytic zinc ion in grey and Asn- ADPr in 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.

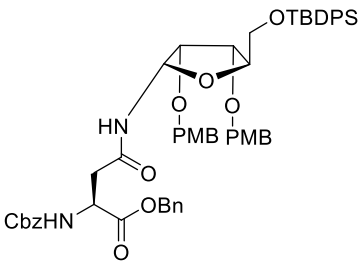
Acknowledgment

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

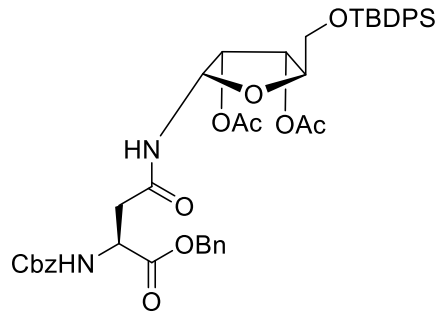
General procedure

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.



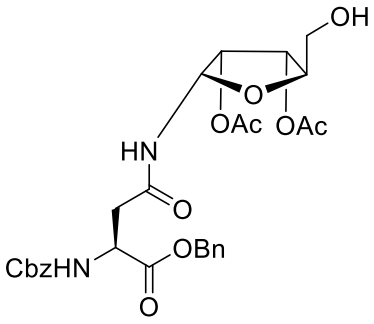
N^{γ}-(2,3-di-*O*-(4-methoxylbenzyl)-5-*O*-*tert*-butyldiphenylsilyl-α-D-ribosyl)-N^{α}-benzyloxycarbonyl asparagine benzyl ester (4)

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)

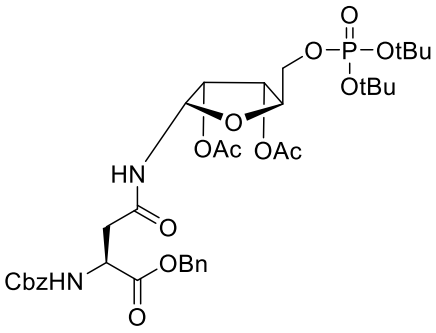
HCl solution (0.74 mL, 0.1M in HFIP) was added into HFIP (15 mL) then the solution was added into **4** (720 mg, 0.74 mmol). The reaction was stirred for 25 minutes at room temperature and quenched with pyridine (0.2 mL). The mixture was co-evaporated under reduced pressure with toluene (3x) and dissolved 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)

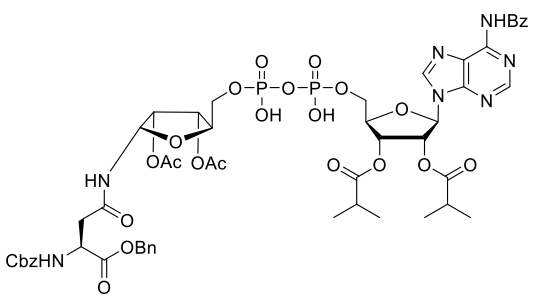
Compounds **5** (318 mg, 0.39mmol) were dissolved in pyridine (4 mL) and HF·Pyridine (0.15 mL, 5.89 mmol) was added. The reaction was stirred for 2.5 hour at 0°C and TLC showed incomplete reversion. Additional HF·Pyridine (0.15 mL, 5.89 mmol) was added and 2 hours later the reaction was quenched by aq. $NaHCO_3$ (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.

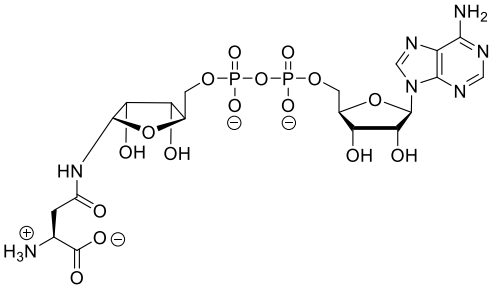


N^{γ}-(N^6-benzoyl-2'',3''-di-*O*-acetyl-2'3'-di-O-isobutyryl-adenosine diphosphate-α-D-ribosyl)-N^{α}-benzyloxycarbonyl asparagine benzyl ester (9)

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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