Synthesis of well-defined ADP-Ribosylated biomolecules

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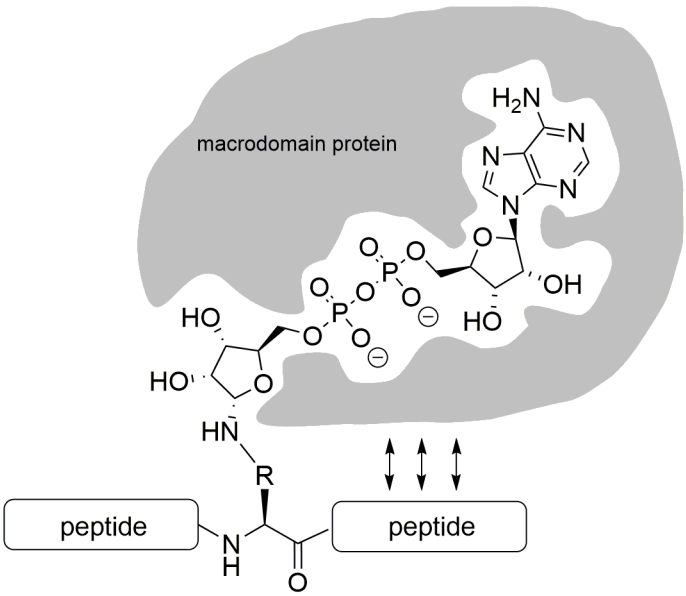
Chapter 3

Synthesis and Macrodomain Binding of *Mono*-ADP-Ribosylated Peptides



mono-ADPribosylated peptide

Abstract: *Mono-ADP-ribosylation is a dynamic post-translational modification (PTM) with important roles in signalling. This chapter describes the synthesis of ADP-ribosylated peptides from histone H2B, RhoA and HNP-1 proteins. The synthesis involves an innovative procedure that make use of pre-phosphorylated amino acid building blocks. Binding assays reveal that the macrodomains of human MacroD2 and TARG1 exhibit distinct specificities for the different ADP-ribosylated peptides, showing that the sequence surrounding ADP-ribosylated residues affects the substrate selectivity of macrodomains.*

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Introduction

Post-translational modifications (PTMs) of proteins play an essential role in cellular signalling and in the regulation of protein-ligand interactions. PTMs occur through the dynamic and usually reversible modification of one or more target amino acids. Adenosine diphosphate ribosylation (ADP-ribosylation) is a phylogenetically ancient PTM and is associated with biological processes that include DNA damage, apoptosis, inflammation and gene regulation.[1,2] The modification involves the enzymatic transfer of an ADP-ribose (ADPr) moiety from β-NAD^+ to the side chain of amino acid residues such as arginine, glutamic acid, aspartic acid, serine, cysteine or asparagine.[1-3] *Mono*-ADP-ribosylation (MARylation) is also a starting point for *poly*-ADP-ribosylation (PARylation), in which multiple ADP-ribose units are appended via the 2'-OH of the covalently linked ADP-ribose to form ADPr-oligomers or polymers (linear and branched).[4-6] There is a large number of ADP-ribosyl transferase enzymes (ARTDs and ARTCs)[7] known to *mono*-ADP-ribosylate acceptor proteins, of which bacterial toxins are the best studied ARTs.[8,9] Recently, new *mono*-ART enzymes have been characterised, several of which have been associated to have a function in human diseases.[10,11] In addition to ARTDs and ARTCs, multiple members of the sirtuin family (SirT3, SirT4 and SirT6) can ADP-ribosylate proteins, expanding the presence of this PTM and its effectors in almost all intracellular organelles.[12,13] However, the mechanism(-s) and function(-s) of *mono*-ADP-ribosylation are considerably less well understood compared to *poly*-ADP-ribosylation, for which multiple effector proteins have been characterized.[14,15]

ADP-ribosylation is a highly reversible modification and multiple ADP-ribosylhydrolases (ARHs) that specifically hydrolyze *poly*-ADPr (ARH3, PARG, ARH1) have been characterized. Until recently, only one enzyme was known to hydrolyze mono-ADP-ribose on arginine residues (ARH1), leaving elusive the hydrolases capable of removing the modification from other residues, such as glutamates. The gap in our knowledge has now been filled with the identification of three macrodomain-containing proteins that display *mono*-ARH activity (MacroD1, MacroD2 and TARG1).[16-18] The identification of the human MacroD1, MacroD2, and TARG1 proteins as mono-ARH enzymes showed for the first time that macrodomain-containing proteins are thus far the only globular protein domains capable of 'reading' and 'erasing' MARylation. However, aside from *in vitro* assays and cell-based experiments with ADP-ribosylated ARTD1/PARP1 and MARylated ARTD10/PARP10 or ARTD1/$PARP1^{E988Q}$ mutant proteins, no other substrates are currently known and the selectivity of macrodomain-containing enzymes for their MARylated protein substrates is unclear. Reduced activity or deficiency of one of these enzymes are linked to embryonic lethality[19] or neuronal degradation (glutamyl ribose 5-phosphate storage disease),[20-22] indicating that reversing cellular *mono*-ADP-ribosylation is biologically important.

To facilitate the analysis of MARylation, MARylated peptides (ADPr peptides) were synthesized and their binding to different macrodomains was investigated. The pioneering studies by van der Heden-van Noort *et al.* on the synthesis of ADPr peptides revealed that the "on-resin" formation of phosphomonoester, the required precursor towards the pyrophosphate, was an inefficient step.[23] Therefore, it was decided to develop pre-phosphorylated amino acid building blocks to circumvent on-resin phosphitylation. First, the synthesis of a histone H2B peptide with ADP-ribosylated Glu2 was attempted.[24-26] Although the synthesis of a phosphoribosylated

glutamic acid building block was accomplished, solid-phase synthesis of a N-terminal tetrapeptide of H2B could not be completed as will be described later. Therefore, glutamatic acid was replaced by glutamine in the H2B N-terminus peptide (**24, 25**, Scheme 5) and MARylated at glutamine, which is resistant to acyl migration and hydrolysis.[23] The MARylation sites from RhoA (Asn41)[27-29] (**26**, Scheme 5) and human neutrophil defensin 1 (HNP-1) (Arg14)[30-32] (**27**, Scheme 5) were also targeted. Citrulline (Cit) was selected as an isostere for arginine (Arg14) since native arginine was expected to be troublesome due to the very high basicity of its side chain and with that a high degree of lability.

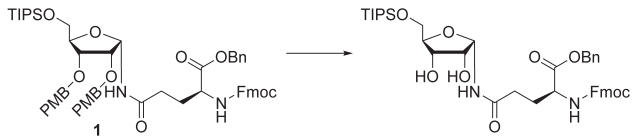
This chapter describes the synthesis of the phosphoribosylated amino acids their incorporation in the peptide substrates and finally the introduction of the ADPr linkages. Furthermore, the obtained MARylated synthetic peptides (**24**-**27**) were carefully analyzed and screened for their binding properties with different macrodomain proteins *in vitro*.

Results and discussion

For the synthesis of *mono*-ADPribosylated peptides, the ribosylated amino acids described in chapter 2 had to undergo a few protecting group manipulations to be compatible with the base-labile strategy chosen for the solid phase peptide synthesis (SPPS).[33] First, the 4-methoxybenzyl ethers (PMB) had to be removed for which a number of different methods are described in literature. Generally, strong oxidizing agents, such as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) or ceric ammonium nitrate (CAN), are used in combination with a biphasic reaction medium to remove PMB groups.[34,35] Thus, DDQ in DCM/H_2O was applied to compound **1** to remove both PMB groups (entry 1, Table 1). This attempt, however, consistently resulted in the formation of 4-methoxybenzylidene as the major product and only a minimal amount of the completely deprotected compound. Next, acidic deprotection methods were investigated as described in the literature where the use of a 10% (v/v) TFA solution in DCM removes multiple PMB groups in a variety of substrates and in a fast and high yielding fashion.[36] To avoid degradation of the starting materials investigated here, a 5% TFA solution was used. These conditions efficiently removed both PMB groups but concomitant anomerization was also observed (entry 2, Table 1). The lowest TFA concentration that still gave complete PMB cleavage was determined to be 3%. However, even under these mild conditions the extent of anomerization remained significant (entry 3, Table 1). Next, attention was turned to a new acidolysis method that was recently published by the group of Stetsenko and that is based on the use of hexafluoroisopropanol (HFIP) and HCl.[37] Applying the reported conditions to compound **1** resulted in the formation of a complex mixture of products (entry 4, Table 1). Therefore, it was investigated if adding small amounts of HCl (0.1 molar equivalents) to the reaction mixture would prevent degradation of the starting material and product. Surprisingly, complete removal of both PMB groups was already achieved within a few minutes by the addition of only 0.1 molar equivalents of HCl and the glycosidic linkage remained intact (entry 5, Table 1).[33,38] Closer investigation of the reaction, using LC-MS and NMR spectroscopy, showed the formation of PMB derived polymers indicating a Friedel-Crafts like mechanism in which released PMB cations react with other PMB molecules to form these polymers.

This results in the release of additional protons needed to drive the reaction to completion. Because HFIP is an expensive and highly toxic solvent, attempts were made to reduce the amounts of HFIP by using DCM or trifluoroethanol (TFE) as a co-solvent. This resulted, however, in slower reactions and anomerization of the glycosidic linkage (entries 6-9, Table 1). The use of HFIP as the solvent and a catalytic amount of HCl proved to be essential for preserving the configuration of the anomeric center.

Table 1. Screening of reaction conditions for PMB cleavage of compound **1**.



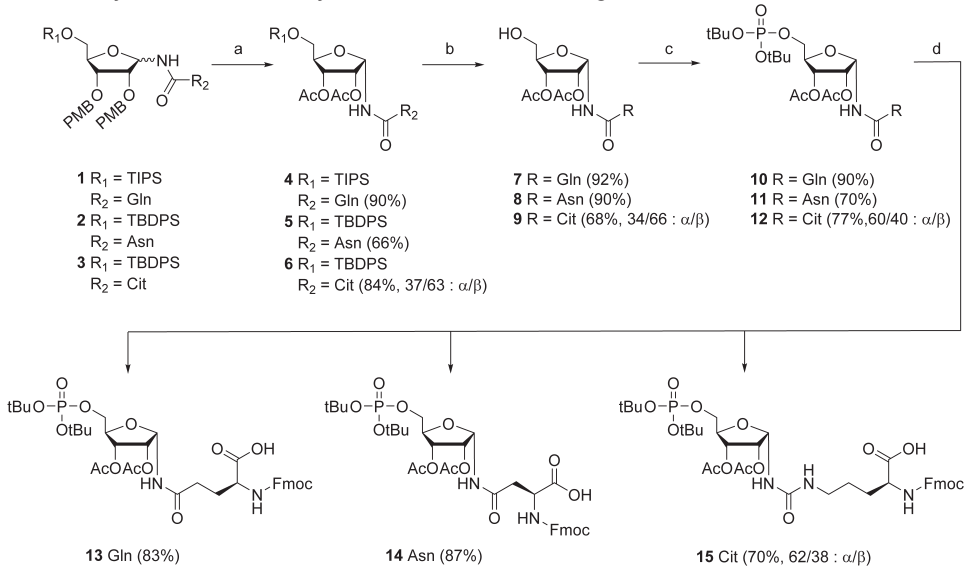
Entry	*Catalyst*	*Solvent*	*alpha/beta*[a]
1	DDQ	DCM/H_2O (9/1)	PMBenzylidene
2	TFA (5% v/v)	DCM	71/29
3	TFA (3% v/v)	DCM	80/20
4	HCl (1 equiv)	HFIP	complex mixture
5	**HCl (0.1 equiv)**	**HFIP**	**91/9**[b]
6	HCl (0.1 equiv)	HFIP/DCM (1/2)	80/20
7	HCl (0.1 equiv)	HFIP/DCM (1/3)	76/24
8	HCl (0.1 equiv)	HFIP/DCM (1/1)	75/25
9	HCl (0.1 equiv)	HFIP/TFE (1/1)	79/21

a) anomeric ratios were determined by ^{1}H-NMR spectroscopy and LC-MS; b) the anomeric ratio of starting material **1** is 91/9 (α/β).

The application of this protocol to compound **2** (Asn) prevented anomerization completely, but for glycosylurea **3** (Cit) considerable anomerization was observed (46/54 = α/β). Subsequent acetylation of the produced diol gave compounds **5** and **6** in good yields. Next, purification by silica gel chromatography allowed separation of the individual anomers. At this stage in the synthesis route, the silyl protecting group at the 5'-OH was removed and a tert-butyl protected phosphate introduced.[39] To this end, the TIPS group in compound **4** was cleaved with triethylamine trihydrofluoride to give compound **7** (Gln) in 92% yield and as pure α anomer. The TBDPS group in compounds **5** and **6** was removed using hydrogen fluoride pyridine and alcohol **8** (Asn) was obtained in 90% yield and as pure α anomer. In contrast, citrulline derivative **9** was obtained as an anomeric mixture (34/66 = α/β), while the anomerically pure compound **6** (α) was used as starting compound for the removal of the TBDPS group. Next, the phosphotriester was installed by phosphitylation of anomerically pure

compounds **7**, **8** and **9** with di-tert-butyl *N*,*N*-diisopropylphosphoramidite and ensuing oxidation.[40] This phosphorylation procedure yielded the di-tert-butyl protected phosphate compounds **10**, **11** and **12** and minimized the unwanted formation of H-phosphonate by-products. Again, ribosylated Cit (**12**) was obtained as a mixture of anomers that were separated by silica gel chromatography. Finally, the benzyl ester protecting group in **10**, **11** and **12** was removed by Pd/C catalyzed hydrogenolysis for 1 hour to afford **13**, **14** and **15** as suitable building blocks for solid phase peptide synthesis (SPPS). Asn derivative **11** had to be treated carefully as aspartimide formation has been observed once during hydrogenolysis. Besides, as anomerization also occurred during the hydrogenation of **12**, (Cit) building block **15** was used as anomeric mixture (α/β : 62/38) in the forthcoming SPPS. The native ADP-ribosylated arginine is known to be configurationally unstable and upon enzymatic synthesis undergoes spontaneous conversion into a mixture of anomers under physiological conditions.[41-43] Therefore it may be appropriate to use phosphoribosylated citrulline **15** as anomeric mixture in the synthesis of ADPribosylated HNP-1.

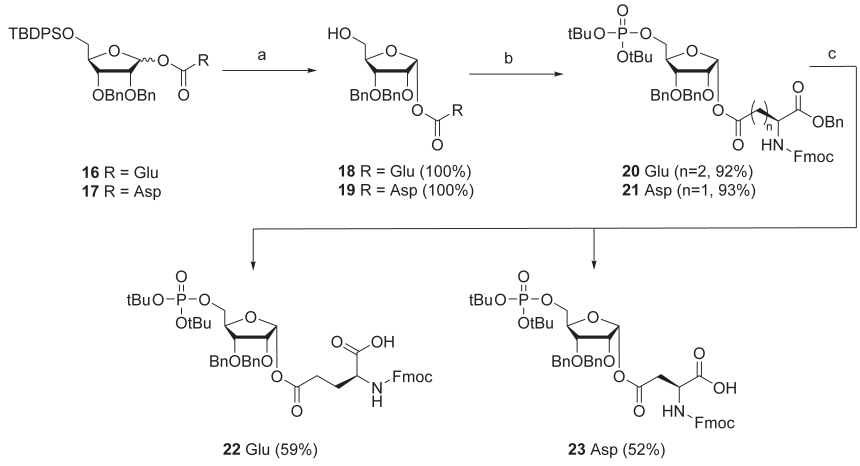
Scheme 1. Synthesis of *N*-ribosylated amino acid building blocks **13**, **14** and **15**.



Reagents and conditions: a) i. HCl, HFIP; ii. Ac_2O, pyridine; b) TBDPS: HF.pyridine, pyridine; TIPS: $Et_3N.3HF$, THF; c)) i. $(tBuO)_2PN(iPr)_2$, 1-Me-Im.HCl (0.3M), 1-Me-Im (0.2M), DMF; ii. tBuOOH; d) H_2, Pd/C, tBuOH/Dioxane/H_2O (4/4/1; v/v/v).

Next, the ribosylated glutamate and aspartate building blocks **16** and **17** were also phosphorylated at the 5'OH position (Scheme 2). The TBDPS group was removed in a quantative manner using HF.pyridine to give compounds **18** and **19**. Phosphorylation of these compounds, employing the previously described method, provided compounds **20** and **21** in excellent yields (92-93%). Hydrogenolysis in the final step of the synthesis route afforded the Fmoc building blocks **22** (Glu) and **23** (Asp) with a yield of 59% and 52%, respectively.

Scheme 2. Synthesis of *O*-ribosylated amino acid building blocks **22** and **23**.

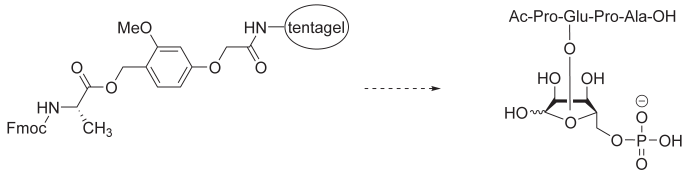


Reagents and conditions: a) HF.pyridine, pyridine; b)) i. $(tBuO)_2PN(iPr)_2$, 1-Me-Im.HCl (0.3M), 1-Me-Im (0.2M), DMF; ii. tBuOOH; c) H_2, Pd/C, tBuOH/Dioxane/H_2O (4/4/1; v/v/v).

3

First, the synthesis of an ADPr peptide derived from histone H2B with an ADP-ribosylation site at residue Glu 2 was attempted. Phosphoribosylated glutamic acid **22** was incorporated in an N-terminal tetrapeptide fragment of histone H2B using standard SPPS and tentagel resin equipped with a highly acid-labile linker (Scheme 3). Repeated treatment with a TFA/DCM (2/98) solution resulted in cleavage of the tetrapeptide from the resin and concomitant removal of the tert-butyl protecting groups. This was followed by hydrogenation with Pd/C to remove the benzyl protecting groups which resulted in a complex mixture of products, as judged by LC-MS. The formation of multiple products was attributed to glycosidic bond cleavage as well as migration of the glutamyl moiety to the liberated 2′-OH and 3′-OH of the ribose followed by the reduction of the anomeric center to a primary alcohol amongst other possible side-reactions. Therefore, attempts to synthesize ADPribosylated glutamate and aspartate peptides was abandoned.

Scheme 3. Synthesis towards ADPribosylated glutamate tetrapeptide.

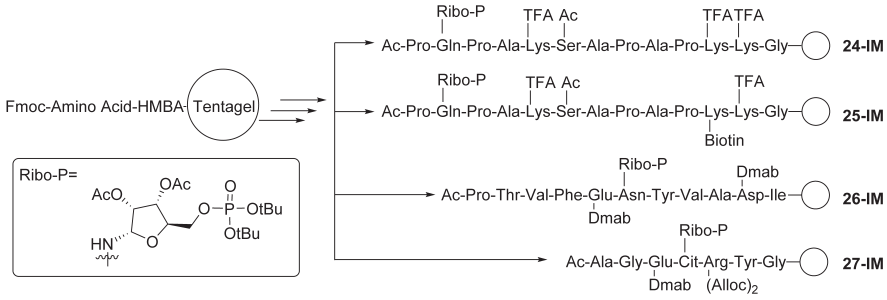


Reagents and conditions: a) i. DBU in DMF; ii. amino acid, HCTU, NMP, DiPEA; b) TFA/DCM; c) H_2, Pd/C

Tentagel resin equipped with the base labile HMBA linker was selected as most suitable for the SPPS and incorporation of the *N*-ribosylated building blocks in target peptides.[23,44] Moreover, the use of the more standard rinkamide linker would require strong acidic conditions which are not compatible with the acid labile glycosidic

linkage and pyrophosphate bond. The first amino acid was manually coupled with the HMBA linker using DIC and DMAP after which the loading was determined with UV-VIS spectroscopy (~ 0.22 mmol/g). Next, the resin (50 µmol) was loaded in an automated peptide synthesizer and standard SPPS protocols were applied to synthesize the intermediate peptides **24-IM** – **27-IM** (Scheme 4). Amino acids with reactive side chains were equipped with base labile protecting groups (PG). However, Ser was used with trityl (Trt) protection and replaced by acetyl protection before coupling of compound **13** in the synthesis towards intermediate peptides **24** and **25**. After coupling of the *N*-ribosylated building blocks and remaining amino acids, the N-terminus was acetylated to prevent any possible side reactions.

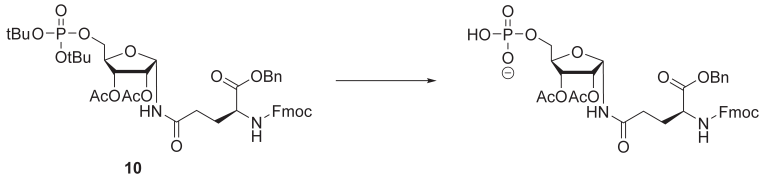
Scheme 4. Synthesis of intermediate phosphoribosyl peptides **24-IM**, **25-IM**, **26-IM** and **27-IM**.



Reagents and conditions: i. 20% piperidine in DMF; ii. amino acid, HCTU, NMP, DiPEA (standard SPPS conditions).

At this stage in the synthesis, the tert-butyl (tBu) protecting groups had to be removed. Initially TFA in DCM was used for this deprotection, as described before in the synthesis of *mono*-ADPribosylated peptides.[39] However, a considerable degree of anomerization was observed when applying these conditions to compound **25-IM.** This was only discovered after obtaining purified ADPr peptide **25** using ^{1}H-NMR spectroscopy (65/35 = α/β). Thus, the tBu cleavage had to be optimized and compound **10** was used as a test substrate. The effectiveness of the previously described HCl/HFIP system was explored for the removal of the two tBu groups. These were effectively removed in 45 minutes with 2% of anomerization using only a half molar equivalent HCl (entry 1, Table 2). Further increasing the amounts of HCl was tolerated as long as the reaction was quenched in time to control the degree of anomerization (entries 2-4, Table 2). However, anomerization could not be prevented when a large excess of HCl (100 equiv) was used (entry 5, Table 2).

Table 2. Cleavage of tBu groups in compound **10** using HCl in HFIP.



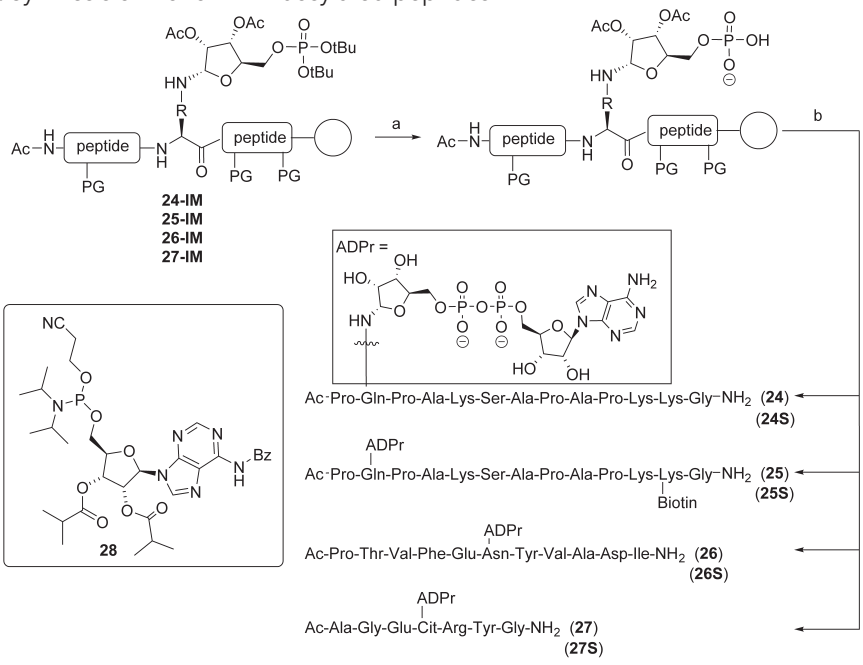
entry	*HCl (eq.)*	*reaction time (min)*	*alpha/beta* [a]
1	0.5	45	98/2
2	1	35	94/6
3	1	24 hours	94/6
4	10	2	94/6
5	100	1	65/35

a) anomeric ratios were determined by ^{1}H-NMR spectroscopy and LC-MS.

One equivalent of HCl in HFIP appeared most suitable. Accordingly, immobilized intermediates **24-IM**, **26-IM** and **27-IM** were shaken for one hour in the presence of one equivalent of HCl in HFIP followed by a pyridine wash (Scheme 5). "On-resin" analysis with ^{31}P-NMR spectroscopy showed almost complete removal of the tBu groups with trace amounts of mono-tBu protected phosphate. Successive treatment with HCl/HFIP for 30 minutes ensured the complete removal. Next, the immobilized 5′-phosphoribosylpeptides were reacted with adenosine phosphoramidite **28** using ethylthiotetrazole (ETT) as activator.[45,46] Oxidation of the intermediate P(III)-P(V) species with camphorsulfonic acid (CSO) followed by cyanoethyl cleavage using 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) afforded protected ADPr peptides **24** – **27**. Next, the Alloc (Arg) and Dmab[47] (Glu and Asp) groups in protected **26** and **27** were removed using $Pd(PPh_3)_4$ and hydrazine, respectively. Finally, ADPr peptides were cleaved from the resin using saturated ammonia in TFE[44], which also removed most remaining protecting groups. The NH_3/TFE mixture proved superior to methanolic ammonia[23] in terms of yield and provides peptide carboxamide as the only product. Addition of concentrated aqueous ammonia to the mixture ensured benzoyl removal from the adenosine-NH_2 to yield crude ADPr peptides **24** – **27**. A combination of RP-HPLC and boronate affinity chromatography[48] was used to purify the products. Repeated lyophilization afforded pure **24** and **25** in 4% (3.3 mg) and 5% (5.6 mg) overall yield, respectively. ADPr peptides **26** and **27** were purified by anion exchange chromatography to yield the products in 1.1% (1.0 mg) and 0.2% (0.3 mg) overall yield. It was hypothesized that the lower yields for peptides **26** and **27** could originate from the hydrazine treatment. The use of Tesser's base as a replacement for hydrazine was investigated resulting in a significant higher yield (6%) but the glycosidic linkage was also affected leading to a complex mixture of products. The purified compounds were carefully analyzed with ^{1}H-NMR spectroscopy to determine the anomeric ratio. The α-anomeric configuration in peptides **24** and **26** was retained while for peptide **25** a ratio of 65/35 (α/β) was observed due to the TFA treatment which demonstrates the effectiveness of the HCl/HFIP procedure. Peptide **27**, Cit, was also obtained as anomeric mixture (60/40; α/β) because Fmoc building block **15** was used as anomeric mixture and anomerization cannot be prevented in any case as described earlier. Finally, four peptides identical to the ADPr peptides but without the ADPr modification were synthesized using standard SPPS to give compounds **24S** – **27S**. These peptides will be used as negative controls in the binding assays.

Having the target peptides in hand, the affinity toward human MacroD2 and TARG1 (Terminal ADP-Ribose protein Glycohydrolase/C6orf130 protein) proteins was established. MacroD2 and TARG1, both MARylation readers and erasers, diverge in

Scheme 5. Synthesis of *mono*-ADPribosylated peptides **24 – 27**.



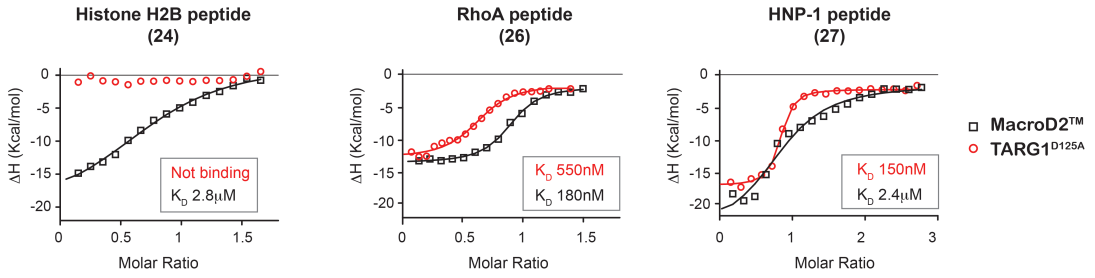
Reagents and conditions: a) HCl, HFIP (5% TFA in DCM for **25-IM**); b) i. compound **28**, ETT, MeCN; ii. CSO, MeCN; iii. DBU, DMF; iv. TFE, NH_3; v. NH_4

sequence. To ensure that the results on the binding are not contaminated by possible catalytic events, point mutants of MacroD2 and TARG1 were used (MacroD2$^{G100E/I189R/Y190N}$ [referred to as MacroD2^{TM}] and TARG1^{D125A}). The catalytic ability of these mutants is impaired, while ADPr and MARylated ARTD10 binding is retained.[49,50]

Where possible, the thermodynamic parameters of the MacroD2 and TARG1 proteins for compounds **24**, **26** and **27** (Figure 1) and for the negative controls **24S**, **26S** and **27S** (Figure 2) were derived using isothermal titration calorimetry. MacroD2^{TM} binds peptide **24** with a K_D of 2.8 ± 0.8 μM, while TARG1^{D125A} shows no binding (Figure 1). This was confirmed using biotinylated ADPr H2B peptide **25** in a streptavidin pull down assay (Figure 3). In contrast, peptide **26** binds both macrodomains with similar affinity (180 ± 80 nM for MacroD2^{TM} and 550 ± 90 nM for TARG1^{D125A}) (Figure 1). Strikingly, ADPr HNP-1 peptide **27** binds much better to TARG1^{D125A} compared to MacroD2^{TM}. The TARG1^{D125A}-derived K_D value for peptide **27** is 150 ± 20 nM, whereas the affinity for MacroD2^{TM} is about 16x lower at 2.4 ± 0.4 μM and ADPr binds TARG1^{D125A} with a ~20x lower affinity at 2.6 μM (Figure 1).[49] These results reveal distinct selectivities of MacroD2^{TM} and TARG1^{D125A} toward ADPr peptides. Indeed, MacroD2^{TM} binds all three tested peptides, irrespective of peptide sequence, length and nature of modified amino acid. This indicates that MacroD2^{TM} tolerates diverse sequence contexts beyond the ADP-ribosylated amino acid. Thus, MacroD2 either exhibits promiscuous binding toward ADPr substrates, or other mechanisms engender the binding specificity of MacroD2 *in vivo*. In contrast, the amino acid context surrounding the ADPr amino acid strongly influences the binding of TARG1^{D125A} towards ADPr peptides, suggesting a more specific target range for

TARG1 that will have to be tested *in vivo*.

A



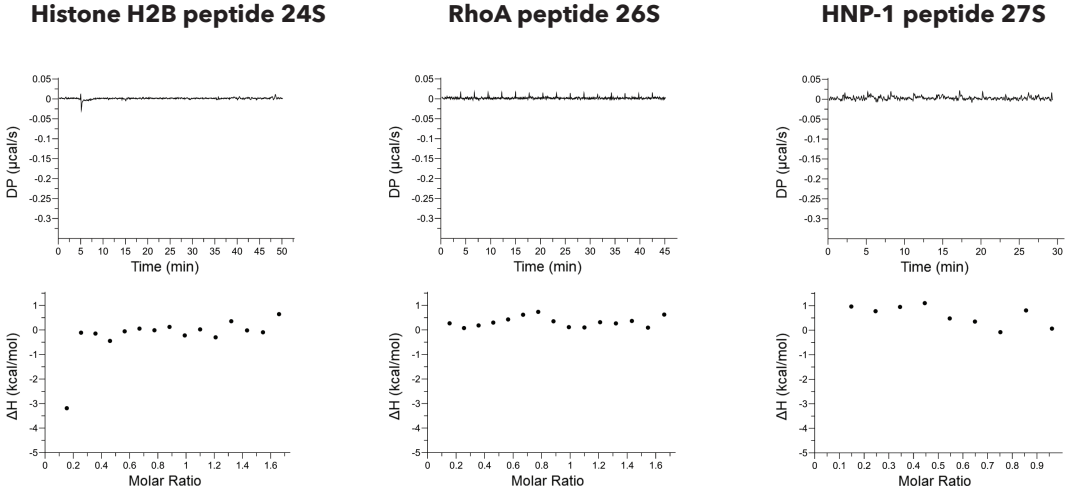
B

Protein	Peptide	K_D (μM)	ΔH (kcal/mol)	ΔG (kcal/mol)	-T ΔS (kcal/mol)	N
MacroD2™	Histone H2B peptide (24)	2.8 (0.8)	-22.7 (0.7)	-7.6 (0.1)	15 (0.7)	0.8 (0.07)
TARG1D125A	Histone H2B peptide (24)			Not binding		
MacroD2™	RhoA peptide (26)	0.18 (0.03)	-13.9 (0.9)	-9.2 (0.08)	4.7 (0.8)	1 (0.06)
TARG1D125A	RhoA peptide (26)	0.55 (0.09)	-10.6 (0.3)	-8.6 (0.1)	2.1 (0.4)	0.7 (0.01)
MacroD2™	HNP-1 peptide (27)	2.4 (0.4)	-27.5 (1.8)	-7.7 (0.1)	19.8 (1.9)	0.8 (0.03)
TARG1D125A	HNP-1 peptide (27)	0.15 (0.02)	-14.5 (0.8)	-9.3 (0.1)	5.1 (0.9)	0.8 (0.02)

Protein	Ligand	K_D (μM)	Reference
MacroD2™	ADP ribose	0.55	G. Jankevicius et al., *Nat. Struc. Mol. Biol.* 2013 [8a]
TARG1D125A	ADP ribose	2.6	R. Sharifi et al., *EMBO J.* 2013 [10b]

Figure 1. Binding affinity of the MacroD2 and TARG1 macrodomain for ADP-ribosylated histone H2B (**24**), RhoA (**26**) and HNP1 **(27)** peptides. (A) Representative isothermal titration calorimetry (ITC) profiles for the titration of **24**, **26** and **27** into a solution containing either MacroD2™ or TARG1D125A. (B) ITC data derived from the interaction measurements between the macrodomains and the ADP-ribosylated peptides. Data are mean (n=3) ± SEM (values in brackets).

A



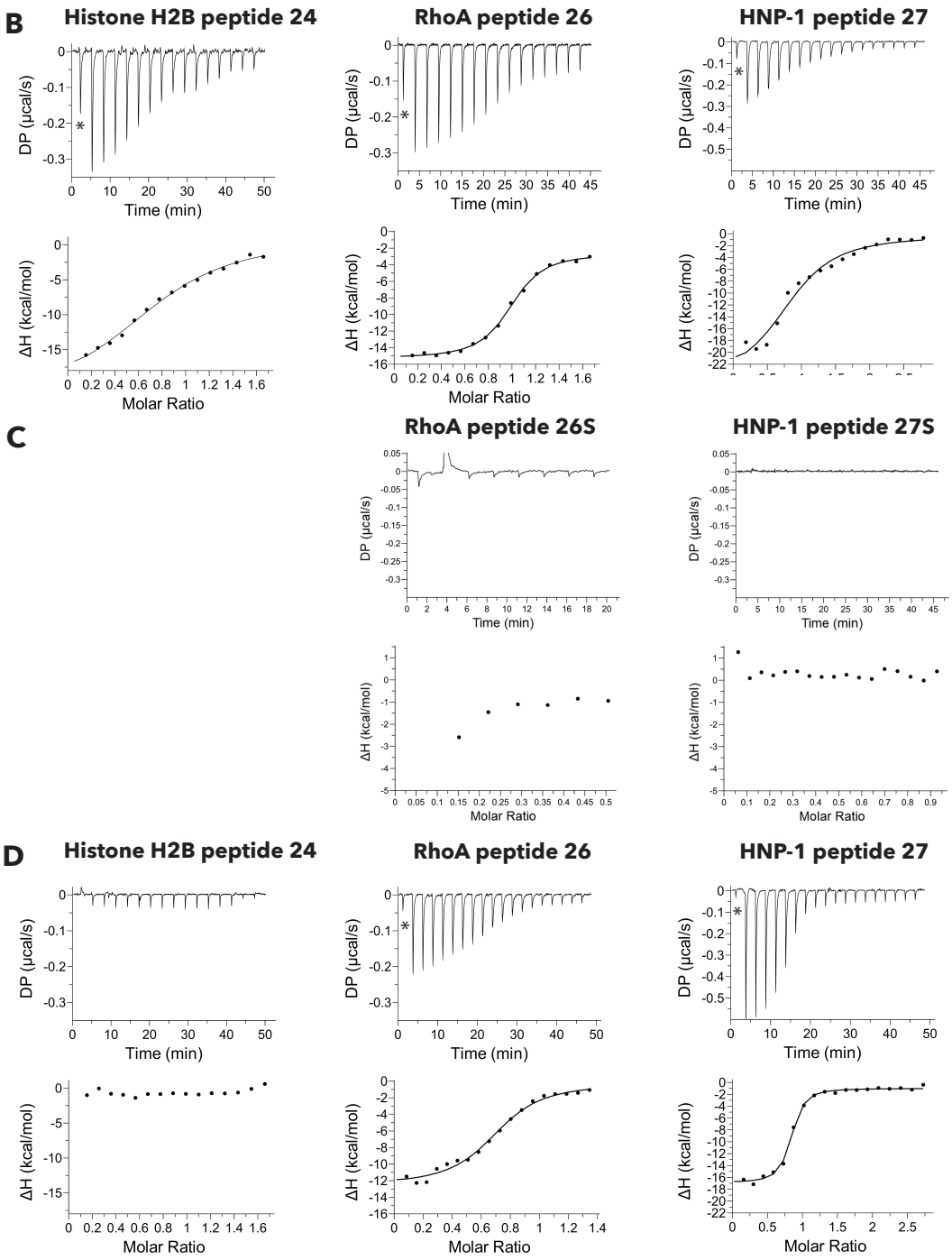


Figure 2. Titration of unmodified (non-ADP-ribosylated) peptides **24S**, **26S** and **27S** and ADP-ribosylated peptides **24**, **26** and **27** with purified MacroD2$^{G100E-I189R-Y190N}$ (A and B) or purified TARG1^{D125A} (C and D) macrodomains. The non-ADP-ribosylated peptide **24S** was not tested for binding with TARG1^{D125A} , since its ADP-ribosylated counterpart does not bind.

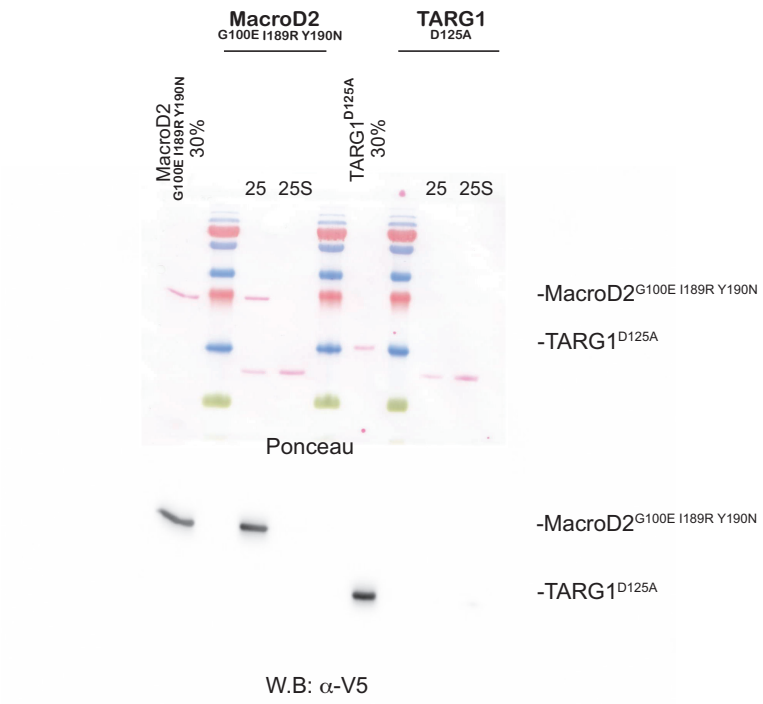


Figure-3. Western blot analysis of streptavidin pull-down assays for biotinylated peptide **25** and its non-ADP-ribosylated counterpart **25S** with recombinant proteins MacroD2 and TARG1.

Conclusion

In summary, the results presented in this chapter provide the first biochemical evidence that macrodomain modules bind chemically-synthesized *mono*-ADP-ribosylated peptides. Moreover, it is demonstrated that the local sequence context surrounding the MARylation site in the histone H2B, RhoA and HNP-1 peptides affects the affinity of a particular macrodomain for their peptide substrates. Realizing that the binding affinity of ADPr peptides containing isosteric replacements instead of native amino acids (Glu and Arg) may be different from the native counterparts. However, the difference is likely to be small since the ADPr moiety recognized by all ADPr binding macrodomains is identical in all cases. Even if the presence of an amine instead of carbonyl was to change the affinity by 0.2-2 kcal/mol (due to the difference in the possible number of hydrogen bonds, for example), then this would be unlikely to create a situation whereby all tested macrodomains have the same affinity and/or same selectivity for distinct MARylated synthetic peptides.

The *mono*-ADP-ribosylated peptides were obtained via a new synthetic methodology based on phosphoribosylated amino acids that is capable to provide synthetic ADP-ribosylated peptides of unprecedented complexity using conventional solid-phase peptide synthesis. The ability to synthesize different designer ADPr-peptides will support future investigations into the emerging role of MARylation as a post-translational modification and to systematically identify the key features that drive the recognition of MARylated substrates by macrodomain modules, providing new insights in the dynamic regulation and biology of cellular *mono*-ADP-ribosylation.

Experimental Section

General procedures for synthesis

All chemicals were used as received unless stated otherwise. All solvents used under anhydrous conditions were stored over 4Å molecular sieves except for methanol which was stored over 3Å molecular sieves. Unless stated otherwise, solvents were removed by rotary evaporation under reduced pressure at 40 °C. TLC analysis, silica gel chromatography, NMR, LCMS, and HRMS techniques were used as described in chapter 2. The reported peptides were synthesized using an automated peptide synthesizer (ABI-433A, Applied Biosystems, Perkin-Elmer) and applying a standard coupling protocol (Fmoc cleavage using 20% piperidine in DMF, coupling of the appropriate amino acid applying a five-fold excess, activation by 5 eq. HCTU in NMP (0.25M) and 12.5 eq. DiPEA in NMP (1.25 M) for 1 hour) unless stated otherwise. RP-HPLC purification was conducted on an automated HPLC system supplied with a semi-preparative C_{18} column (250 x 10.00 mm, 5 µ, flow: 4 mL/min). Boronate affinity chromatography was performed on a TSKgel Boronate-5PW 10 µm stainless steel column.

General procedures protein–ligand interaction studies

Plasmids.

Bacterial expression constructs of pETM-CN vector-based His6-TEV-V5 tagged MacroD2^{G100EI189RY190N} and TARG1D^{125A} were described previously[8a,10b]

Protein expression and purification.

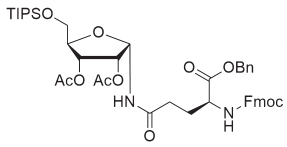
Macrodomains were expressed in *E. coli* Rosetta (DE3) pLysS cells at 18 °C for 18 h after 200 µM IPTG induction. Cell pellets were snap frozen with liquid nitrogen and stored at −80 °C. The thawed pellet was resuspended in lysis buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 20 mM imidazole and protease inhibitor cocktail (Roche)). Lysates were sonicated for 3 × 45 s at medium setting (Branson) until the lysate was not viscous and centrifuged for 45 min at ~45,000*g* at 4 °C. The supernatant was incubated for 1h with Ni-NTA resin (Macherey Nagel), washed five times with 45 mL wash buffer and eluted with wash buffer containing 500 mM imidazole. The histidine tag was cleaved with TEV protease overnight at 4 °C. The proteins were dialyzed overnight in storage buffer (25 mM Tris-Cl, pH 7.5, 0.3 M NaCl, 1 mM DTT, 10% gycerol), concentrated and subjected to gel-filtration chromatography with a Superdex S75 16/60 column (GE Healthcare) using storage buffer as eluent. Peak fractions confirmed by SDS-PAGE and Coomassie staining were pooled and concentrated with 10,000 MWCO concentrators (Amicon). Concentrations were determined by absorbance measurements at 280 nm wavelength by using calculated molar extinction coefficients.

ITC

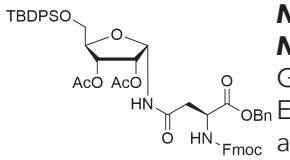
For ITC binding assays both ITC200 and Peaq-ITC instruments (Malvern,GE Healthcare) were used. Before the ITC experiment, the cells where dialyzed in 25 mM Tris, 100 mM NaCl buffer (pH 7.5) and 5 mM 2-Mercaptoethanol overnight at 4 °C. The dialyzed proteins were then centrifugated at 20,000 g for 20 minutes at 4 °C. Protein concentrations were determined as described above. The binding reactions were carried out at 25 °C in 25 mM Tris, 100 mM NaCl buffer (pH 7.5), using 10–30 µM

macrodomains and 100–250 µM of *mono*-ADP-ribosylated and not ADP-ribosylated peptides. The ligand in the injection syringe was at 10 to 15 times higher concentration than the macrodomains. MicroITC PEAQ-ITC Analysis Software (Malvern) was used for data analysis.

General procedure A; removal PMB
Compounds **1** - **3** were dissolved in HFIP (50 mM) and HCl (0.1 eq; 100 mM HCl in HFIP) was added. The reaction was stirred for 15 minutes at room temperature, turned deep red/purple and was quenched with pyridine. The mixture was co-evaporated under reduced pressure with toluene (3x) and dissolved in pyridine (0.1M). Ac_2O (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.

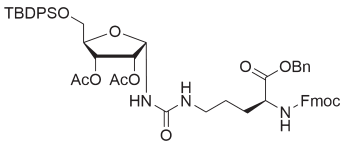


***N*δ-(5-tri-isopropylsilyl-2,3-di-*O*-acetyl-α-D-ribosyl)-*N*α-fluorenylmethoxycarbonyl glutamine benzyl ester (4)**
General procedure A was applied to compound **1** (Pentane/EtOAc, 60/40) to obtain the α-anomer as a white foam (150 mg, 0.18 mmol, yield 90%). ^{1}H-NMR (400 MHz, MeOD-$CDCl_3$) δ: 7.78 (d, *J* = 7.5 Hz, 2H, Fmoc arom.), 7.68 – 7.60 (m, 2H, Fmoc arom.), 7.39 (t, *J* = 7.3 Hz, 2H, arom.), 7.36 – 7.24 (m, 7H, arom.), 6.00 (d, *J* = 5.2 Hz, 1H, H1'), 5.49 – 5.44 (m, 1H, H3'), 5.40 (t, *J* = 5.2 Hz, 1H, H2'), 5.17 (s, 2H, CH_2 Bn), 4.41 (AB, *J* = 10.5, 7.0 Hz, 1H, CH_2 Fmoc), 4.36 – 4.25 (m, 2H, CH_2 Fmoc, CH α-Gln), 4.20 (t, *J* = 7.0 Hz, 1H, CH Fmoc), 4.17 – 4.12 (m, 1H, H4'), 3.84 (d, *J* = 2.5 Hz, 2H, H5'), 2.37 (t, *J* = 7.4 Hz, 2H, CH_2 γ-Gln), 2.25 – 2.13 (m, 1H, CH_2 β-Gln), 2.08 (d, *J* = 4.6 Hz, 6H, CH_3 Ac), 2.08 – 1.94 (m, 1H, CH_2 β-Gln), 1.10 – 1.04 (m, 21H, TIPS). ^{13}C-NMR (100 MHz, MeOD-$CDCl_3$) δ: 174.24, 172.89 (CO α-Gln, CO δ-Gln), 171.22, 170.87 (CO Ac), 158.06 (CO Fmoc), 144.71, 144.53, 142.08, 136.35 (Cq. arom.), 129.21 - 127.78, 125.88, 125.78, 120.58 (arom.), 82.50 (C4'), 79.96 (C1'), 72.69 (C3'), 71.43 (C2'), 67.80 (CH_2 Bn), 67.75 (CH_2 Fmoc), 64.19 (C5'), 54.63 (CH α-Gln), 47.88 (CH Fmoc), 32.98 (CH_2 γ-Gln), 27.99 (CH_2 β-Gln), 20.75, 20.55 (CH_3 Ac), 18.31, 18.29, 12.65 (CH, CH_3,TIPS). IR: 2941, 2864, 1748, 1717, 1699, 1420, 1238, 1067. HRMS $[C_{45}H_{58}N_2O_{11}Si + Na]^+$: 853.3704 found, 853.3702 calculated.



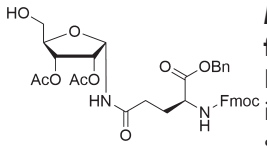
***N*γ-(2,3-di-*O*-acetyl-5-tert-butyldiphenylsilyl-α-D-ribosyl)-*N*α-fluorenylmethoxycarbonyl asparagine benzyl ester (5)**
General procedure A was applied to compound **2** (Pentane/EtOAc, 70/30 – 65/35 – 60/40) to obtain the title compound as a white foam (328 mg, 0.36 mmol, 49%) along with a mixture of anomers (108 mg, 0.12 mmol, 17%). ^{1}H-NMR (400 MHz, MeOD-$CDCl_3$) δ 7.70 (d, J = 7.5 Hz, 2H, arom.), 7.64 (t, J = 7.7 Hz, 4H, arom.), 7.59 – 7.52 (m, 2H, arom.), 7.43 – 7.17 (m, 15H, arom.), 6.05 (d, J = 5.0 Hz, 1H, H1'), 5.52 (t, J = 4.7 Hz, 1H, H3'), 5.45 (t, J = 5.1 Hz, 1H, H2'), 5.14 (s, 2H, CH_2 Bn), 4.62 (t, J = 5.4 Hz, 1H, CH α-Asn), 4.38 – 4.29 (m, 1H, CH_2 Fmoc), 4.29 – 4.20 (m, 1H, CH_2 Fmoc), 4.13 (t, J = 7.1 Hz, 1H, CH Fmoc), 4.10 – 4.04 (m, 1H, H4'), 3.75 – 3.69 (m, 1H, H5'), 3.69 – 3.62 (m, 1H, H5'), 2.92 (AB, J = 15.9, 6.3 Hz, 1H, CH_2 β-Asn), 2.85 (AB, J = 15.8, 4.8 Hz, 1H, CH_2 β-Asn), 2.06 (s, 3H, CH_3 Ac), 2.04 (s, 3H, CH_3 Ac), 1.03 (s, 9H, CH_3 TBDPS). ^{13}C-NMR

(101 MHz, MeOD-$CDCl_3$) δ 171.99, 171.46, 170.99, 170.72 (CO α-Asn, CO γ-Asn, CO Ac), 157.57 (CO Fmoc), 144.43, 144.35, 141.89 (Cq. arom.), 136.23, 136.18 (arom.), 136.05, 133.41, 133.25 (Cq. arom.), 130.50, 130.45, 129.07, 128.85, 128.66, 128.42, 128.40, 128.31, 127.68, 125.72, 125.66, 120.48 (arom.), 81.89 (C4'), 79.80 (C1'), 72.45 (C3'), 71.38 (C2'), 68.00, 67.82 (CH_2 Bn, CH_2 Fmoc), 64.14 (C5'), 51.58 (CH α-Asn), 47.65 (CH Fmoc), 38.11 (CH_2 β-Asn), 27.14 (CH_3 TBDPS), 20.74, 20.56 (CH_3 Ac), 19.64 (Cq. TBDPS). HRMS [$C_{51}H_{54}N_2O_{11}Si$ + Na]$^+$: 921.3388 found, 921.3389 calculated.



N^{ω}-(2,3-di-*O*-acetyl-5-tert-butyldiphenylsilyl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl citrulline benzyl ester (6)

General procedure A was applied to compound **3** (Pentane/EtOAc, 70/30 – 65/35 – 60/40) to obtain the title compound as a white foam (602 mg, 0.64 mmol, 27%) separated from the β-anomer (1.00 g, 1.07 mmol, 44%) along with a mixture of anomers (303 mg, 0.32 mmol, 13%). *α-anomer*: ^{1}H-NMR (400 MHz, MeOD-$CDCl_3$) δ 7.78 – 7.55 (m, 8H. arom.), 7.45 – 7.17 (m, 15H, arom.), 5.98 (d, J = 5.2 Hz, 1H, H1'), 5.60 – 5.52 (m, 1H, H3'), 5.41 (t, J = 5.2 Hz, 1H, H2'), 5.19 – 5.07 (m, 2H, CH_2 Bn), 4.36 (AB, J = 10.5, 7.0 Hz, 1H, CH_2 Fmoc), 4.29 (AB, J = 10.5, 7.1 Hz, 1H, CH_2 Fmoc), 4.23 (dd, J = 9.1, 4.9 Hz, 1H, CH α-Cit), 4.16 (t, J = 6.9 Hz, 1H, CH Fmoc), 4.10 – 4.01 (m, 1H, H4'), 3.76 – 3.63 (m, 2H, H5'), 3.20 – 3.04 (m, 2H, CH_2 δ-Cit), 2.09 (s, 3H, CH_3 Ac), 2.07 (s, 3H, CH_3 Ac), 1.92 – 1.80 (m, 1H, CH_2 β-Cit), 1.76 – 1.63 (m, 1H, CH_2 β-Cit), 1.63 – 1.43 (m, 2H, CH_2 γ-Cit), 1.04 (s, 9H, CH_3 TBDPS). ^{13}C-NMR (101 MHz, MeOD-$CDCl_3$) δ 173.47 (CO α-Cit), 171.25, 170.82 (CO Ac), 159.03 (CO Fmoc), 158.20 (CO ω-Cit), 144.83, 144.64, 142.17 (Cq. arom.), 136.44, 136.37 (arom.), 133.70, 133.53 (Cq. arom.), 130.67, 130.63, 129.26, 129.03, 128.89, 128.59, 128.57, 128.47, 127.86, 125.94, 125.87, 120.62 (arom.), 81.51 (C4'), 81.24 (C1'), 72.87 (C3'), 71.53 (C2'), 67.72 (CH_2 Bn, CH_2 Fmoc), 64.45 (C5'), 55.10 (CH α-Cit), 47.98 (CH Fmoc), 40.01 (CH_2 δ-Cit), 29.58 (CH_2 β-Cit), 27.32 (CH_2 γ-Cit), 27.22 (CH_3 TBDPS), 20.66, 20.52 (CH_3 Ac), 19.79 (Cq. TBDPS). *β-anomer*: ^{1}H-NMR (400 MHz, MeOD-$CDCl_3$) δ 7.79 – 7.56 (m, 8H, arom.), 7.44 – 7.18 (m, 15H, arom.), 5.57 (d, J = 7.2 Hz, 1H, H1'), 5.47 (dd, J = 5.5, 3.1 Hz, 1H, H3'), 5.18 – 5.13 (m, 2H, CH_2 Bn), 5.09 (t, J = 6.4 Hz, 1H, H2'), 4.36 (AB, J = 10.4, 7.1 Hz, 1H, CH_2 Fmoc), 4.29 (AB, J = 10.5, 7.1 Hz, 1H, CH_2 Fmoc), 4.21 (dd, J = 9.0, 4.9 Hz, 1H, CH α-Cit), 4.16 (t, J = 6.9 Hz, 1H, CH Fmoc), 4.01 (q, J = 3.3 Hz, 1H, H4'), 3.80 – 3.65 (m, 2H, H5'), 3.18 – 3.03 (m, 2H, CH_2 δ-Cit), 2.07 (s, 3H, CH_3 Ac), 2.04 (s, 3H, CH_3 Ac), 1.90 – 1.76 (m, 1H, CH_2 β-Cit), 1.74 – 1.60 (m, 1H, CH_2 β-Cit), 1.59 – 1.42 (m, 2H, CH_2 γ-Cit), 1.05 (s, 9H, CH_3 TBDPS). ^{13}C-NMR (101 MHz, MeOD-$CDCl_3$) δ 173.45 (CO α-Cit), 171.35, 171.18 (CO Ac), 159.28 (CO Fmoc), 158.19 (CO ω-Cit), 144.83, 144.65, 142.18, 136.55 (Cq. arom.), 136.39 (arom.), 133.84, 133.57 (Cq. arom.), 130.74, 130.64, 129.26, 129.04, 128.89, 128.63, 128.59, 128.47, 127.86, 125.94, 125.88, 120.61 (arom.), 83.39 (C1'), 82.00 (C4'), 73.97 (C2'), 72.11 (C3'), 67.71 (CH_2 PMB, CH_2 Fmoc), 64.64 (C5'), 55.07 (CH α-Cit), 48.00 (CH Fmoc), 40.06 (CH_2 δ-Cit), 29.55 (CH_2 β-Cit), 27.32 (CH_2 γ-Cit), 27.27 (CH_3 TBDPS), 20.75, 20.56 (CH_3 Ac), 19.84 (Cq. TBDPS). HRMS [$C_{53}H_{59}N_3O_{11}Si$ + H]$^+$: 942.4001 found, 942.3992 calculated.

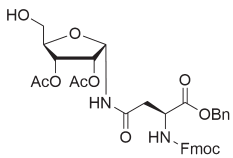


N^{δ}-(2,3-di-*O*-acetyl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl glutamine benzyl ester (7)

Ribosylated glutamine **4** (1.02 g, 1.23 mmol) was dissolved in THF (12 mL) and cooled to 0 °C. $Et_3N.3HF$ (5.3 mL) was slowly added and the reaction was stirred for 16 hours at room temperature. The reaction was carefully quenched by the addition of aq. $NaHCO_3$ (sat.) and extracted with EtoAc. The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography (Pentane/EtOAc, 50/50 – 30/70 – 20/80) to afford the title compound as a white foam (654 mg, 1.1 mmol, 92%). ^{1}H-NMR (400 MHz, MeOD-$CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H, arom.), 7.62 (dd, J = 7.3, 3.7 Hz, 2H, arom.), 7.37 (t, J = 7.5 Hz, 2H, arom.), 7.34 – 7.21 (m, 7H, arom.), 5.95 (d, J = 4.2 Hz, 1H, H1'), 5.38 – 5.28 (m, 2H, H2', H3'), 5.15 (s, 2H, CH_2 Bn), 4.38 (AB, J = 10.5, 6.9 Hz, 1H, CH_2 Fmoc), 4.30 – 4.21 (m, 2H, CH_2 Fmoc, CH Fmoc), 4.16 (t, J = 6.9 Hz, 1H, CH α-Gln), 4.12 – 4.06 (m, 1H, H4'), 3.69 (AB, J = 12.2, 2.5 Hz, 1H, H5'), 3.57 (AB, J = 12.3, 3.3 Hz, 1H, H5'), 2.36 (t, J = 7.4 Hz, 2H, γ-Gln), 2.17 (dq, J = 13.1, 7.4 Hz, 2H, β-Gln), 2.07 (s, 3H, CH_3 Ac), 2.04 (s, 3H, CH_3 Ac), 2.01 – 1.91 (m, 1H, β-Gln). ^{13}C-NMR (100 MHz, MeOD-$CDCl_3$) δ 174.79, 173.14 (CO α-Gln, CO δ-Gln), 171.36, 171.10 (CO Ac), 158.39 (CO Fmoc), 145.03, 144.85, 142.34, 136.81 (Cq. arom.), 129.39, 129.15, 129.01, 128.62, 128.00, 127.97, 126.10, 125.99, 120.77 (arom.), 82.17 (C4'), 80.23 (C1'), 72.34, 71.78 (C2', C3'), 67.93, 67.89 (CH_2 Fmoc, CH_2 Bn), 62.23 (C5'), 54.90 (CH Fmoc), 48.14 (CH α-Gln), 33.00 (CH_2 γ-Gln), 28.07 (CH_2 β-Gln), 20.60, 20.52 (CH_3 Ac). HRMS $[C_{36}H_{38}N_2O_{11}Si + H]^+$: 675.2554 found, 675.2548 calculated.

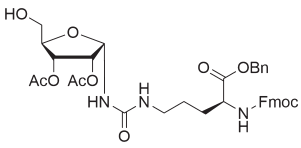
General procedure B; removal TBDPS

Compounds **5** and **6** were dissolved in pyridine (0.1 M) and HF.Pyridine (1.0 mL/mmol substrate) was added. The reaction was stirred for 1 hour at room temperature and carefully quenched by the addition of aq. $NaHCO_3$ (sat.). The reaction mixture was extracted with DCM, the organic layer dried over $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography.



N^{γ}-(2,3-di-*O*-acetyl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl asparagine benzyl ester (8)

General procedure B was applied to compound **5** (Pentane/EtOAc, 50/50 – 27/75 – 0/100) to obtain the title compound as a white foam (135 mg, 0.20 mmol, 90%). ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.73 (d, J = 7.6 Hz, 2H, arom.), 7.58 (d, J = 7.4 Hz, 2H, arom.), 7.35 (t, J = 7.5 Hz, 2H, arom.), 7.31 – 7.20 (m, 7H, arom.), 5.93 (d, J = 4.3 Hz, 1H, H1'), 5.36 – 5.31 (m, 2H, H2', H3'), 5.18 – 5.09 (m, 2H, CH_2 Bn), 4.62 (t, J = 5.8 Hz, 1H, CH α-Asn), 4.35 (AB, J = 10.3, 7.2 Hz, 1H, CH_2 Fmoc), 4.25 (AB, J = 10.3, 7.4 Hz, 1H, CH_2 Fmoc), 4.14 (t, J = 6.9 Hz, 1H, CH Fmoc), 4.10 – 4.04 (m, 2H, H4'), 3.69 (AB, J = 12.3, 2.7 Hz, 1H, H5'), 3.56 (AB, J = 12.4, 3.2 Hz, 1H, H5'), 2.87 (d, J = 5.6 Hz, 2H, CH_2 β-Asn), 2.07 (s, 3H, CH_3 Ac), 2.05 (s, 3H, CH_3 Ac). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 172.15, 171.99, 171.15, 170.89 (CO α-Asn, CO γ-Asn, CO Ac), 157.78 (CO Fmoc), 144.57, 142.07 (Cq. arom.), 136.30, 129.19, 128.97, 128.77, 128.43, 127.80, 125.85, 125.79, 120.60 (arom.), 81.90 (C4'), 79.95 (C1'), 72.10, 71.57 (C2', C3'), 68.05, 67.92 (CH_2 Bn, CH_2 Fmoc), 62.03 (C5'), 51.72 (CH α-Asn), 47.83 (CH Fmoc), 38.15 (CH_2 β-Asn), 20.62, 20.54 (CH_3 Ac). HRMS $[C_{35}H_{36}N_2O_{11}Si + H]^+$: 661.2396 found, 661.2392 calculated.

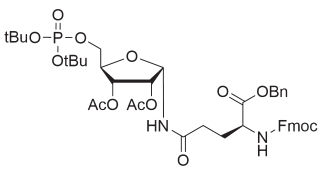


N^{ω}-(2,3-di-*O*-acetyl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl citrulline benzyl ester (9)

General procedure B was applied to compound **6** (Pentane/EtOAc, 50/50 – 40/60 – 0/100) to obtain the title compound (125 mg, 0.18 mmol, 24%) and the β-anomer (231 mg, 0.33 mmol, 44%) separately. *α-anomer:* ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.74 (d, J = 7.5 Hz, 2H, arom.), 7.66 – 7.57 (m, 2H, arom.), 7.44 – 7.21 (m, 9H, arom.), 5.85 (d, J = 4.6 Hz, 1H, H1'), 5.36 (t, J = 5.2 Hz, 1H, H3'), 5.30 (t, J = 4.8 Hz, 1H, H2'), 5.18 – 5.08 (m, 2H, CH_2 Bn), 4.36 (AB, J = 10.4, 7.0 Hz, 1H, CH_2 Fmoc), 4.32 – 4.25 (m, 1H, CH_2 Fmoc), 4.23 (dd, J = 8.7, 4.7 Hz, 1H, CH α-Cit), 4.16 (t, J = 6.8 Hz, 1H, CH Fmoc), 4.06 – 4.00 (m, 1H, H4'), 3.68 (AB, J = 12.3, 2.7 Hz, 1H, H5'), 3.56 (AB, J = 12.3, 3.0 Hz, 1H, H5'), 3.13 (t, J = 6.5 Hz, 2H, CH_2 δ-Cit), 2.09 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac), 1.91 – 1.79 (m, 1H, CH_2 β-Cit), 1.76 – 1.62 (m, 1H, CH_2 β-Cit), 1.61 – 1.44 (m, 2H, CH_2 γ-Cit). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 173.50 (CO α-Cit), 171.26, 170.86 (CO Ac), 159.23 (CO Fmoc), 158.24 (CO ω-Cit), 144.90, 144.70, 142.22, 136.64 (Cq. arom.), 129.30, 129.07, 128.93, 128.51, 127.89, 125.97, 125.90, 120.66 (arom.), 81.47 (C4'), 81.25 (C1'), 72.36 (C3'), 71.78 (C2'), 67.74 (CH_2 Bn, CH_2 Fmoc), 62.25 (C5'), 55.12 (CH α-Cit), 48.04 (CH Fmoc), 40.02 (CH_2 δ-Cit), 29.61 (CH_2 β-Cit), 27.31 (CH_2 γ-Cit), 20.57, 20.49 (CH_3 Ac). β-anomer: ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.73 (d, J = 7.4 Hz, 2H, arom.), 7.66 – 7.53 (m, 2H, arom.), 7.38 – 7.15 (m, 9H, arom.), 5.56 (d, J = 6.6 Hz, 1H, H1'), 5.34 – 5.26 (m, 1H, H3'), 5.18 – 5.05 (m, 3H, H2', CH_2 Bn), 4.40 – 4.30 (m, 1H, CH_2 Fmoc), 4.30 – 4.24 (m, 1H, CH_2 Fmoc), 4.21 (dd, J = 8.4, 4.6 Hz, 1H, CH α-Cit), 4.13 (t, J = 6.6 Hz, 1H, CH Fmoc), 4.03 – 3.93 (m, 1H, H4'), 3.71 – 3.60 (m, 2H, H5'), 3.17 – 3.05 (m, 2H, CH_2 δ-Cit), 2.05 (s, 3H, CH_3 Ac), 2.00 (s, 3H, CH_3 Ac), 1.90 – 1.78 (m, 1H, CH_2 β-Cit), 1.74 – 1.61 (m, 1H, CH_2 β-Cit), 1.58 – 1.45 (m, 2H, CH_2 γ-Cit). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 173.57 (CO α-Cit), 171.48, 171.26 (CO Ac), 159.46 (CO Fmoc), 158.29 (CO ω-Cit), 144.99, 144.78, 142.27, 142.25, 136.77 (Cq. arom.), 129.33, 129.07, 128.95, 128.56, 127.95, 126.04, 125.98, 120.71 (arom.), 84.24 (C1'), 82.67 (C4'), 74.46 (C2'), 72.44 (C3'), 67.77, 67.72 (CH_2 Bn, CH_2 Fmoc), 62.53 (C5'), 55.22 (CH α-Cit), 48.09 (CH Fmoc), 40.19 (CH_2 δ-Cit), 29.60 (CH_2 β-Cit), 27.39 (CH_2 γ-Cit), 20.64, 20.49 (CH_3 Ac). HRMS $[C_{37}H_{41}N_3O_{11}Si + H]^+$: 704.2817 found, 704.2814 calculated.

General procedure C; phosphorylation

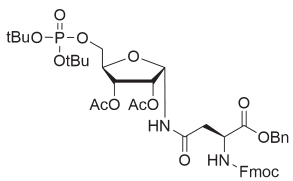
Compounds **7-9** were co-evaporated with dioxane (3x) and subsequently a mixture of 1-methyl-imidazole.HCl (3 eq.) and 1-methyl-imidazole (2 eq.) in dry DMF (10 mL) was added under an atmosphere of argon. Di-*tert*-butyl-*N,N*-diisopropylphosphoramidite (1.5 eq.) was added and the reaction was stirred at room temperature for 15 minutes. Then tBuOOH (5.5 M in nonane) (10 eq.) was added and the reaction mixture was stirred for 1 hour. The reaction was quenched upon addtion of aq. $NaHCO_3$ (sat.) and extracted with Et_2O. The organic layer was dried ($MgSO_4$), concentrated *in vacuo* and purified by silica gel chromatography.



N^{δ}-(2,3-di-*O*-acetyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl glutamine benzyl ester (10)

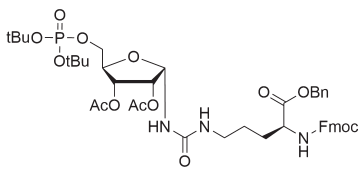
General procedure C was applied to compound **7** (Toluene/EtOAc, 50/50 – 40/60 – 30/70) to obtain the title

compound (781 mg, 0.90 mmol, 90%). ^{1}H-NMR (400 MHz, MeOD-$CDCl_3$) δ 7.75 (d, J = 7.5 Hz, 2H, arom.), 7.65 – 7.57 (m, 2H, arom.), 7.36 (t, J = 7.4 Hz, 2H, arom.), 7.33 – 7.20 (m, 7H, arom.), 5.99 (d, J = 2.5 Hz, 1H, H1'), 5.37 – 5.28 (m, 2H, H2' H3'), 5.15 (s, 2H, CH_2 Bn), 4.38 (AB, J = 10.4, 7.0 Hz, 1H, CH_2 Fmoc), 4.31 – 4.20 (m, 2H, CH_2 Fmoc, H4', CH α-Gln), 4.16 (t, J = 6.9 Hz, 1H, CH Fmoc), 4.10 – 4.00 (m, 2H, H5'), 2.35 (t, J = 7.3 Hz, 2H, CH_2 γ-Gln), 2.23 – 2.12 (m, 1H, CH_2 β-Gln), 2.08 (s, 3H, CH_3 Ac), 2.05 (s, 3H, CH_3 Ac), 2.02 – 1.93 (m, 1H, CH_2 β-Gln), 1.47 (s, 18H, CH_3 tBu). ^{13}C-NMR (101 MHz, MeOD-$CDCl_3$) δ 174.61, 173.18 (CO α-Gln, CO δ-Gln), 171.23, 171.03 (CO Ac), 158.44 (CO Fmoc), 145.10, 144.91, 142.41, 136.90 (Cq. arom.), 129.43, 129.18, 129.06, 128.67, 128.05, 126.16, 126.05, 120.81 (arom.), 84.81, 84.77, 84.73, 84.70 (Cq. tBu), 80.37 (C1'), 80.07, 79.99 (C4'), 72.30, 71.52 (C2', C3'), 67.97, 67.90 (CH_2 Fmoc, CH_2 Bn), 67.27, 67.21 (C5'), 54.98 (CH Fmoc), 48.19 (CH α-Gln), 33.05 (CH_2 γ-Gln), 30.15, 30.11 (CH_3 tBu), 28.09 (CH_2 β-Gln), 20.55, 20.48 (CH_3 Ac). ^{31}P-NMR (162 MHz, MeOD-$CDCl_3$) δ -9.48. HRMS [$C_{44}H_{55}N_2O_{14}P$ + H]$^+$: 867.3470 found, 867.3464 calculated.



N^{γ}-(2,3-di-*O*-acetyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-*N*^{α}-fluorenylmethoxycarbonyl asparagine benzyl ester (11)

General procedure C was applied to compound **8** (Pentane/EtOAc, 60/40 – 50/50 – 40/60) to obtain the title compound (113 mg, 0.13 mmol, 70%). ^{1}H-NMR (400 MHz, MeOD-$CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H, arom.), 7.61 (d, J = 7.4 Hz, 2H, arom.), 7.37 (t, J = 7.5 Hz, 2H, arom.), 7.33 – 7.20 (m, 7H, arom.), 6.00 (d, J = 4.1 Hz, 1H, H1'), 5.39 – 5.27 (m, 2H, H2', H3'), 5.14 (s, 2H, CH_2 Bn), 4.63 (t, J = 6.0 Hz, 1H, CH α-Asn), 4.34 (AB, J = 10.1, 7.4 Hz, 1H, CH_2 Fmoc), 4.29 – 4.19 (m, 2H, CH_2 Fmoc, H4'), 4.15 (t, J = 7.0 Hz, 1H, CH Fmoc), 4.10 – 4.01 (m, 2H, H5'), 2.92 – 2.78 (m, 2H, CH_2 β-Asn), 2.09 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac), 1.47 (s, 18H, CH_3 tBu). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 171.30, 170.81, 170.10, 169.92 (CO α-Asn, CO γ-Asn, CO Ac), 156.95 (CO Fmoc), 143.84, 143.76, 141.22, 135.58 (Cq. arom.), 128.30, 128.04, 127.87, 127.55, 126.94, 125.01, 124.96, 119.71 (arom.), 83.67, 83.64, 83.61, 83.58 (Cq. tBu), 79.23 (C1'), 78.91, 78.84 (C4'), 71.18, 70.39 (C2', C3'), 67.09, 67.02 (CH_2 Bn, CH_2 Fmoc), 66.07, 66.03 (C5'), 50.90 (CH α-Asn), 46.99 (CH Fmoc), 37.27 (CH_2 β-Asn), 29.13, 29.10 (CH_3 tBu), 19.56, 19.50 (CH_3 Ac). ^{31}P-NMR (162 MHz, MeOD) δ -9.21. HRMS [$C_{43}H_{53}N_2O_{14}P$ + H]$^+$: 853.3315 found, 853.3307 calculated.



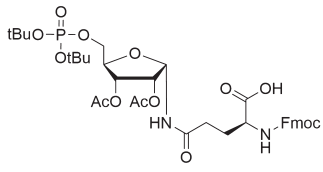
N^{ω}-(2,3-di-*O*-acetyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-*N*^{α}-fluorenylmethoxycarbonyl citrulline benzyl ester (12)

General procedure C was applied to compound **9** (α-anomer) (Pentane/EtOAc, 40/60 – 30/70 – 20/80) to obtain the title compound (104 mg, 0.12 mmol, 45%) and the β-anomer (75 mg, 0.08 mmol, 32%) separately. General procedure C was applied to compound **9** (β-anomer) (Pentane/EtOAc, 40/60 – 30/70 – 20/80) to obtain the title compound (116 mg, 0.13 mmol, 15%) and the β-anomer (559 mg, 0.62 mmol, 73%) separately. *α-anomer:* ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.75 (d, J = 7.5 Hz, 2H, arom.), 7.65 – 7.58 (m, 2H, arom.), 7.36 (t, J = 7.4 Hz, 2H, arom.), 7.33 – 7.20 (m, 7H, arom.), 5.92 (d, J = 4.9 Hz, 1H, H1'), 5.35 (t, J = 5.2 Hz, 1H, H3'), 5.29 (t, J = 5.1 Hz, 1H,

H2'), 5.19 – 5.09 (m, 2H, CH_2 Bn), 4.36 (AB, J = 10.5, 7.0 Hz, 1H, CH_2 Fmoc), 4.29 (AB, J = 10.3, 7.3 Hz, 1H, CH_2 Fmoc), 4.22 (dd, J = 9.0, 4.7 Hz, 1H, CH α-Cit), 4.20 – 4.12 (m, 2H, H4', CH Fmoc), 4.09 – 3.98 (m, 2H, H5'), 3.13 (t, J = 6.6 Hz, 2H, CH_2 δ-Cit), 2.09 (s, 3H, CH_3 Ac), 2.08 (s, 3H, CH_3 Ac), 1.91 – 1.79 (m, 1H, CH_2 β-Cit), 1.76 – 1.61 (m, 1H, CH_2 β-Cit), 1.60 – 1.50 (m, 2H, CH_2 γ-Cit), 1.48 (s, 18H, CH_3 tBu). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 173.44 (CO α-Cit), 171.03, 170.66 (CO Ac), 158.89 (CO Fmoc), 158.19 (CO ω-Cit), 144.87, 144.67, 142.19, 142.17, 136.59 (Cq. arom.), 129.28, 129.04, 128.89, 128.49, 128.47, 127.87, 127.85, 125.96, 125.89, 120.64 (arom.), 81.29 (C1'), 79.39, 79.32 (C4'), 72.38 (C3'), 71.20 (C2'), 67.74, 67.71 (CH_2 Bn, CH_2 Fmoc), 67.13, 67.08 (C5'), 55.11 (CH α-Cit), 48.02 (CH Fmoc), 40.02 (CH_2 δ-Cit), 30.12, 30.09 (CH_3 tBu), 29.59 (CH_2 β-Cit), 27.30 (CH_2 γ-Cit), 20.54, 20.46 (CH_3 Ac). ^{31}P-NMR (162 MHz, MeOD-$CDCl_3$) δ -10.67. *β-anomer:* ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H, arom.), 7.66 – 7.59 (m, 2H, arom.), 7.40 – 7.21 (m, 9H, arom.), 5.59 (d, J = 6.6 Hz, 1H, H1'), 5.33 (dd, J = 5.4, 3.6 Hz, 1H, H3'), 5.19 – 5.08 (m, 2H, CH_2 Bn), 5.05 (t, J = 6.0 Hz, 1H, H2'), 4.35 (AB, J = 10.4, 7.1 Hz, 1H, CH_2 Fmoc), 4.28 (AB, J = 10.4, 7.3 Hz, 1H, CH_2 Fmoc), 4.21 (dd, J = 9.0, 4.8 Hz, 1H, CH α-Cit), 4.16 (t, J = 6.9 Hz, 1H, CH Fmoc), 4.13 – 4.08 (m, 1H, H4'), 4.08 – 3.99 (m, 2H, H5'), 3.13 (t, J = 6.6 Hz, 2H, CH_2 δ-Cit), 2.07 (s, 3H, CH_3 Ac), 2.03 (s, 3H, CH_3 Ac), 1.91 – 1.79 (m, 1H, CH_2 β-Cit), 1.74 – 1.61 (m, 1H, CH_2 β-Cit), 1.60 – 1.51 (m, 2H, CH_2 γ-Cit), 1.48 (d, J = 9.5 Hz, 18H, CH_3 tBu). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 173.56 (CO α-Cit), 171.30, 171.10 (CO Ac), 159.40 (CO Fmoc), 158.36 (CO ω-Cit), 145.09, 144.88, 142.35, 142.33, 136.92 (Cq. arom.), 129.40, 129.12, 128.99, 128.62, 128.02, 128.01, 126.13, 126.07, 120.77 (arom.), 84.21 (C1'), 80.35, 80.28 (C4'), 74.24 (C2'), 71.91 (C3'), 67.82, 67.72 (CH_2 Bn, CH_2 Fmoc), 67.53, 67.48 (C5'), 55.28 (CH α-Cit), 48.19 (CH Fmoc), 40.21 (CH_2 δ-Cit), 30.15, 30.12 (CH_3 tBu), 29.65 (CH_2 β-Cit), 27.52 (CH_2 γ-Cit), 20.56, 20.43 (CH_3 Ac). ^{31}P-NMR (162 MHz, MeOD-$CDCl_3$) δ -10.96. HRMS $[C_{45}H_{58}N_3O_{14}P + H]^+$: 896.3736 found, 896.3729 calculated.

General procedure D; hydrogenation

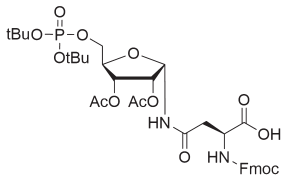
Compounds **10-12** were dissolved in tBuOH/Dioxane/H_2O (50 mM; 4/4/1 v/v/v), Pd/C (150 mg/mmol substrate) was added and the reaction mixture was placed under a H_2 atmosphere. The reaction was stirred at room temperature for 1 hour, filtered over celite, concentrated *in vacuo* and purified by silica gel chromatography.



N^{δ}-(2,3-di-*O*-acetyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl glutamine (13)

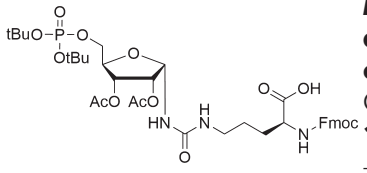
General procedure D was applied to compound **10** (DCM/MeOH/AcOH, 99/0/1 – 94/5/1 – 89/10/1) to yield the title compound as a white foam (521 mg, 0.67 mmol, 83%). ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.74 (d, J = 7.6 Hz, 2H, Fmoc), 7.62 (dd, J = 11.0, 7.7 Hz, 2H, Fmoc), 7.36 (t, J = 7.4 Hz, 2H, Fmoc), 7.28 (t, J = 7.4 Hz, 2H, Fmoc), 6.01 (d, J = 3.8 Hz, 1H, H1`), 5.38 – 5.28 (m, 2H, H2', H3'), 4.34 (AB, J = 10.2, 7.3 Hz, 1H, CH_2 Fmoc), 4.32 – 4.27 (m, 1H, CH_2 Fmoc), 4.27 – 4.23 (m, 1H, H4'), 4.23 – 4.15 (m, 2H, CH α-Gln, CH Fmoc), 4.10 – 4.05 (m, 1H, H5'), 4.05 – 3.99 (m, 1H, H5'), 2.38 (t, J = 7.5 Hz, 2H, CH_2 γ-Gln), 2.23 – 2.12 (m, 1H, CH_2 β-Gln), 2.09 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac), 2.03 – 1.95 (m, 1H, CH_2 β-Gln), 1.47 (s, 18H, CH_3 tBu). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 175.05, 174.64 (CO α-Gln, CO δ-Gln), 171.05, 170.83 (CO

Ac), 158.05 (CO Fmoc), 144.86, 144.69, 142.15 (Cq. arom.), 128.49, 127.87, 125.97, 125.90, 120.65 (arom.), 84.60, 84.57, 84.54, 84.51 (Cq. tBu), 80.09 (C1'), 79.89, 79.82 (C4'), 72.13, 71.24 (C2', C3'), 67.74 (CH_2 Fmoc), 67.02, 66.97 (C5'), 54.58 (CH Fmoc), 47.98 (CH α-Gln), 33.15 (CH_2 γ-Gln), 30.10, 30.07 (CH_3 tBu), 28.63 (CH_2 β-Gln), 20.58, 20.48 (CH_3 Ac). ^{31}P-NMR (162 MHz, MeOD-$CDCl_3$) δ -9.50. HRMS [$C_{37}H_{49}N_2O_{14}P$ + H]$^+$: 777.2996 found, 777.2994 calculated.



N^{γ}-(2,3-di-*O*-acetyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl asparagine (14)

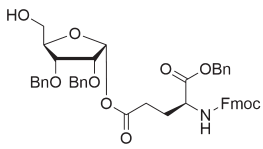
General procedure D was applied to compound **11** (DCM/EtOH/AcOH, 99/0/1 – 96/3/1 – 94/5/1) to yield the title compound as a white foam (390 mg, 0.48 mmol, 87%). ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H, Fmoc), 7.63 (d, J = 7.4 Hz, 2H, Fmoc), 7.37 (t, J = 7.5 Hz, 2H, Fmoc), 7.29 (t, J = 7.4 Hz, 2H, Fmoc), 6.00 (d, J = 2.3 Hz, 1H, H1'), 5.34 (d, J = 3.1 Hz, 2H, H2', H3'), 4.55 (t, J = 6.0 Hz, 1H, CH α-Asn), 4.35 (AB, J = 10.3, 7.3 Hz, 1H, CH_2 Fmoc), 4.32 – 4.26 (m, 1H, CH_2 Fmoc), 4.25 – 4.18 (m, 2H, CH Fmoc, H4'), 4.10 – 3.98 (m, 2H, H5'), 2.87 (AB, J = 15.8, 5.2 Hz, 1H, CH_2 β-Asn), 2.81 (AB, J = 15.7, 6.9 Hz, 1H, CH_2 β-Asn), 2.10 (s, 3H, CH_3 Ac), 2.07 (s, 3H, CH_3 Ac), 1.47 (s, 18H, CH_3 tBu). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 174.26 (CO α-Asn), 172.12, 171.20, 171.00 (CO γ-Asn, CO Ac), 157.99 (CO Fmoc), 144.90, 144.85, 142.26 (Cq. arom.), 128.58, 127.96, 126.06, 126.03, 120.72 (arom.), 84.74, 84.71, 84.68, 84.64 (Cq. tBu), 80.25 (C1'), 80.03, 79.96 (C4'), 72.23, 71.38 (C2', C3'), 68.01 (CH_2 Fmoc), 67.10, 67.06 (C5'), 51.81 (CH α-Asn), 48.06 (CH Fmoc), 38.61 (CH_2 β-Asn), 30.13, 30.10 (CH_3 tBu), 20.57, 20.49 (CH_3 Ac). ^{31}P-NMR (162 MHz, MeOH) δ -9.24. HRMS [$C_{36}H_{47}N_2O_{14}P$ + H]$^+$: 763.2839 found, 763.2838 calculated.



N^{ω}-(2,3-di-*O*-acetyl-5-*O*-(di-tert-butyl)-phosphoryl-α/β-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl citrulline (15)

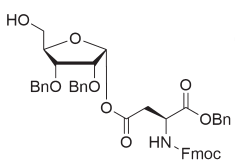
General procedure D was applied to compound **12** (α-anomer) (DCM/EtOH/AcOH, 99/0/1 – 94/5/1 – 92/7/1) to yield the title compound as a mixture of anomers (62 mg, 77 μmol, 70%, α/β : 62/38). General procedure D was also applied to compound **12** (β-anomer) (DCM/EtOH/AcOH, 99/0/1 – 94/5/1 – 92/7/1) to yield the title compound as a mixture of anomers (335 mg, 0.42 mmol, 68%, α/β : 24/76). ^{1}H-NMR (500 MHz, MeOD-$CDCl_3$) δ 7.77 (d, J = 8.2 Hz, 4H, arom. Fmoc), 7.65 (t, J = 8.0 Hz, 4H, arom. Fmoc), 7.37 (t, J = 7.4 Hz, 4H, arom. Fmoc), 7.30 (t, J = 7.4 Hz, 4H, arom. Fmoc), 5.92 (d, J = 5.1 Hz, 1H, H1'-α), 5.58 (d, J = 6.5 Hz, 1H, H1'-β), 5.36 (t, J = 5.2 Hz, 1H, H3'-α), 5.32 (dd, J = 5.5, 3.6 Hz, 1H, H3'-β), 5.29 (t, J = 5.3 Hz, 1H, H2'-α), 5.04 (t, J = 6.1 Hz, 1H, H2'-β), 4.35 (d, J = 6.9 Hz, 4H, CH_2 Fmoc), 4.23- 4.15 (m, 5H, CH Fmoc, CH α-Cit, H4'-α), 4.13 – 4.09 (m, 1H, H4'-β), 4.08 – 4.00 (m, 4H, H5'-β, H5'-α), 3.20 – 3.13 (m, 4H, CH_2 δ-Cit), 2.11 (s, 3H, CH_3 Ac), 2.09 (s, 6H, CH_3 Ac), 2.05 (s, 3H, CH_3 Ac), 1.94 – 1.82 (m, 2H, CH_2 β-Cit), 1.76 – 1.64 (m, 2H, CH_2 β-Cit), 1.64 – 1.52 (m, 4H, CH_2 γ-Cit), 1.50 – 1.47 (m, 36H, CH_3 tBu). ^{13}C-NMR (126 MHz, MeOD-$CDCl_3$) δ 175.51 (CO α-Cit), 171.38, 171.23, 171.17, 170.85 (CO Ac), 159.42, 159.12 (CO Fmoc), 158.35 (CO ω-Cit), 145.04, 144.87, 142.33 (Cq. arom. Fmoc), 128.58, 127.98, 126.06, 120.72 (arom. Fmoc), 84.17 (C1'-β), 81.45 (C1'-α), 80.35, 80.28 (C4'-β), 79.59,

79.52 (C4′-α), 74.23 (C2′-β), 72.51 (C3′-α), 71.93 (C3′-β), 71.33 (C2′-α), 67.79 (CH_2 Fmoc), 67.49, 67.44 (C5′-β), 67.31, 67.26 (C5′-α), 54.91 (CH α-Cit), 48.20 (CH Fmoc), 40.28, 40.19 (CH_2 δ-Cit), 30.15, 30.12 (CH_3 tBu), 29.94, 29.89 (CH_2 β-Cit), 27.49, 27.43 (CH_2 γ-Cit), 20.58, 20.53, 20.44 (CH_3 Ac). ^{31}P-NMR (162 MHz, MeOD-$CDCl_3$) δ -9.20, -9.48. HRMS [$C_{38}H_{52}N_3O_{14}P$ + H]$^+$: 806.3258 found, 806.3260 calculated.



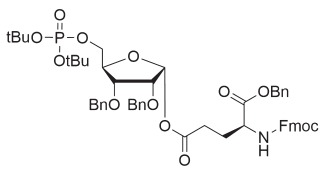
O^{δ}-(2,3-di-*O*-benzyl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl glutamic acid benzyl ester (18)

General procedure B was applied to compound **16** (Pentane/EtOAc, 60/40 – 50/50) to obtain the title compound (1.20 g, 1.78 mmol, 100%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.72 (d, J = 7.5 Hz, 2H, arom.), 7.56 (d, J = 7.3 Hz, 2H, arom.), 7.40 – 7.18 (m, 19H, arom.), 6.28 (s, 1H, H1′), 5.73 (d, J = 8.2 Hz, 1H, NH), 5.16 – 5.03 (m, 2H, CH_2 Bn), 4.67 (d, J = 12.3 Hz, 1H, CH_2 Bn), 4.59 – 4.48 (m, 3H, CH_2 Bn), 4.46 – 4.38 (m, 1H, CH α-Glu), 4.38 – 4.31 (m, 2H, CH_2 Fmoc), 4.28 (s, 1H, H4′), 4.16 (t, J = 6.5 Hz, 1H, CH Fmoc), 3.94 (s, 2H, H2′, H3′), 3.59 (AB, J = 12.1, 2.9 Hz, 1H, H5′), 3.40 (AB, J = 12.2, 3.1 Hz, 1H, H5′), 2.53 – 2.40 (m, 2H, CH_2 γ-Glu), 2.26 – 2.16 (m, 1H, CH_2 β-Glu), 2.08 – 2.01 (m, 1H, CH_2 β-Glu). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 172.16 (CO α-Glu), 171.74 (CO δ-Glu), 156.10 (CO Fmoc), 149.45 (arom.), 143.86, 143.78, 141.24, 137.93 (Cq. arom.), 137.44 (arom.), 136.23 (Cq. arom.), 135.26, 128.56, 128.45, 128.39, 128.17, 127.98, 127.96, 127.81, 127.73, 127.68, 127.08, 125.11, 123.84, 119.93 (arom.), 95.16 (C1′), 85.46 (C4′), 78.27, 74.99 (C2′, C3′), 72.98, 72.50 (CH_2 Bn, CH_2 Fmoc), 67.19, 66.95 (CH_2 Bn), 62.28 (C5′), 53.52 (CH α-Glu), 47.14 (CH Fmoc), 30.77 (CH_2 γ-Glu), 26.83 (CH_2 β-Glu). HRMS [$C_{46}H_{45}N_2O_{10}$ + H]$^+$: 772.3121 found, 772.3116 calculated.



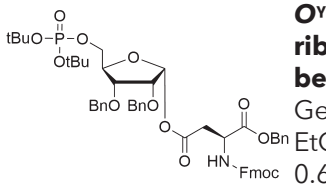
O^{γ}-(2,3-di-*O*-benzyl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl aspartic acid benzyl ester (19)

General procedure B was applied to compound **17** (Pentane/EtOAc, 70/30 – 60/40) to obtain the title compound (457 mg, 0.69 mmol, 100%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.71 (t, J = 8.0 Hz, 2H, arom.), 7.55 (t, J = 6.5 Hz, 2H, arom.), 7.40 – 7.16 (m, 19H, arom.), 6.75 (d, J = 9.0 Hz, 1H, NH), 6.26 (d, J = 3.8 Hz, 1H, H1′), 5.06 (d, J = 12.5 Hz, 1H, CH_2 Bn), 4.99 (d, J = 12.5 Hz, 1H, CH_2 Bn), 4.73 (dt, J = 8.6, 4.3 Hz, 1H, CH α-Asp), 4.64 (d, J = 12.8 Hz, 1H, CH_2 Bn), 4.57 – 4.49 (m, 3H, CH_2 Bn), 4.34 (qd, J = 10.7, 7.4 Hz, 2H, CH_2 Fmoc), 4.27 – 4.20 (m, 1H, H4′), 4.13 (t, J = 6.9 Hz, 1H, CH Fmoc), 3.91 (dd, J = 6.2, 1.9 Hz, 1H, H3′), 3.90 – 3.85 (m, 1H, H2′), 3.67 – 3.59 (m, 1H, H5′), 3.44 – 3.35 (m, 1H, H5′), 3.18 (AB, J = 17.2, 4.8 Hz, 1H, CH_2 β-Asp), 2.91 (AB, J = 17.1, 4.0 Hz, 2H, CH_2 β-Asp). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 170.24 (CO α-Asp), 169.76 (CO γ-Asp), 156.44(CO Fmoc), 144.04, 143.81, 141.34, 141.29, 137.75, 137.33, 135.48 (Cq. arom.), 128.63, 128.57, 128.49, 128.30, 128.18, 128.04, 127.93, 127.73, 127.70, 127.11, 127.08, 125.18, 125.16, 120.01 (arom.), 95.08 (C1′), 86.25 (C4′), 78.46 (C2′), 74.77 (C3′), 73.00, 72.64 (CH_2 Bn), 67.39, 67.02 (CH_2 Bn, CH_2 Fmoc), 62.54 (C5′), 50.64 (CH α-Asp), 47.25 (CH Fmoc), 37.10 (CH_2 β-Asp). HRMS [$C_{45}H_{43}NO_{10}$ + H]$^+$: 758.2965 found, 758.2960 calculated.



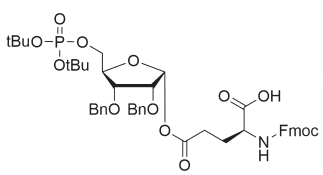
O^{δ}-(2,3-di-*O*-benzyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl glutamic acid benzyl ester (20)

General procedure C was applied to compound **18** (Pentane/EtOAc, 60/40 – 50/50) to obtain the title compound (382 mg, 0.40 mmol, 92%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.72 (d, J = 7.4 Hz, 2H, arom.), 7.57 (d, J = 7.0 Hz, 2H, arom.), 7.42 – 7.16 (m, 19H, arom.), 6.32 (d, J = 2.5 Hz, 1H, H1'), 5.73 (d, J = 8.1 Hz, 1H, NH), 5.12 (s, 2H, CH_2 Bn), 4.70 (d, J = 12.2 Hz, 1H, CH_2 Bn), 4.64 – 4.51 (m, 3H, CH_2 Bn), 4.48 – 4.39 (m, 1H, CH α-Glu), 4.36 (d, J = 7.0 Hz, 3H, CH_2 Fmoc, H4'), 4.17 (t, J = 7.1 Hz, 1H, CH Fmoc), 4.05 – 3.95 (m, 2H, H3', H2'), 3.94 – 3.86 (m, 1H, H5'), 3.86 – 3.78 (m, 1H, H5'), 2.55 – 2.40 (m, 2H, CH_2 γ-Glu), 2.30 – 2.15 (m, 1H, CH_2 β-Glu), 2.10 – 2.01 (m, 1H, CH_2 β-Glu), 1.40 (s, 9H, CH_3 tBu), 1.39 (s, 9H, CH_3 tBu). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 171.90 (CO α-Glu), 171.63 (CO δ-Glu), 155.98 (CO Fmoc), 143.79, 143.71, 141.17, 137.75, 137.30, 135.21 (Cq. arom.), 128.47, 128.40, 128.33, 128.27, 128.09, 127.95, 127.89, 127.74, 127.66, 127.59, 127.00, 125.04, 119.85 (arom.), 94.89 (C1'), 83.53, 83.46 (C4'), 82.60, 82.59, 82.54, 82.53 (Cq. tBu), 78.04, 74.84 (C2', C3'), 72.90, 72.47 (CH_2 Bn), 67.07, 66.87 (CH_2 Bn, CH_2 Fmoc), 65.93, 65.88 (C5'), 53.47 (CH α-Glu), 47.07 (CH Fmoc), 30.72 (CH_2 γ-Glu), 29.72, 29.69, 29.69, 29.66 (CH_3 tBu), 26.69 (CH_2 β-Glu). ^{31}P-NMR (162 MHz, $CDCl_3$) δ -9.55. HRMS $[C_{54}H_{62}N_2O_{13}P + H]^+$: 964.4041 found, 964.4032 calculated.



O^{γ}-(2,3-di-*O*-benzyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl aspartic acid benzyl ester (21)

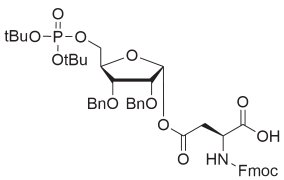
General procedure C was applied to compound **19** (Pentane/EtOAc, 70/30 – 60/40) to obtain the title compound (611 mg, 0.64 mmol, 93%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.71 (t, J = 7.6 Hz, 2H, arom.), 7.59 – 7.47 (m, 2H, arom.), 7.42 – 7.14 (m, 19H, arom.), 6.73 (d, J = 8.8 Hz, 1H, NH), 6.28 (d, J = 3.7 Hz, 1H, H1'), 5.06 (d, J = 12.5 Hz, 1H, CH_2 Bn), 4.99 (d, J = 12.5 Hz, 1H, CH_2 Bn), 4.73 (dt, J = 9.0, 4.5 Hz, 1H, CH α-Asp), 4.64 (d, J = 12.7 Hz, 1H, CH_2 Bn), 4.55 (d, J = 12.8 Hz, 1H, CH_2 Bn), 4.51 (s, 2H, CH_2 Bn), 4.40 – 4.32 (m, 2H, CH_2 Fmoc), 4.31 (s, 1H, H4'), 4.14 (t, J = 6.8 Hz, 1H, CH Fmoc), 3.99 – 3.95 (m, 1H, H3'), 3.95 – 3.91 (m, 1H, H2'), 3.91 – 3.84 (m, 1H, H5'), 3.84 – 3.72 (m, 1H, H5'), 3.18 (AB, J = 17.1, 4.8 Hz, 1H, β-Asp), 2.97 – 2.90 (m, 1H, β-Asp), 1.39 (d, J = 6.1 Hz, 18H, CH_3 tBu). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 170.13 (CO α-Asp), 169.56 (CO γ-Asp), 156.32 (CO Fmoc), 143.94, 143.74, 141.26, 141.21, 137.57, 137.22, 135.42 (Cq. arom.), 128.56, 128.49, 128.43, 128.21, 128.12, 128.08, 127.96, 127.85, 127.65, 127.62, 127.04, 127.01, 125.09, 125.06, 119.93 (arom.), 94.89 (C1'), 84.27, 84.20 (C4'), 82.76, 82.70 (Cq. tBu), 78.09 (C2'), 74.64 (C3'), 72.92, 72.57 (CH_2 Bn), 67.28, 66.91 (CH_2 Bn, CH_2 Fmoc), 65.95, 65.90 (C5'), 50.58 (CH α-Asp), 47.17 (CH Fmoc), 37.00 (CH_2 β-Asp), 29.81, 29.78, 29.75 (CH_3 tBu). ^{31}P-NMR (162 MHz, $CDCl_3$) δ -9.58. HRMS $[C_{53}H_{60}NO_{13}P + H]^+$: 950.3886 found, 950.3875 calculated.



O^{δ}-(2,3-di-*O*-benzyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl glutamic acid (22)

General procedure D was applied to compound **20** (DCM/

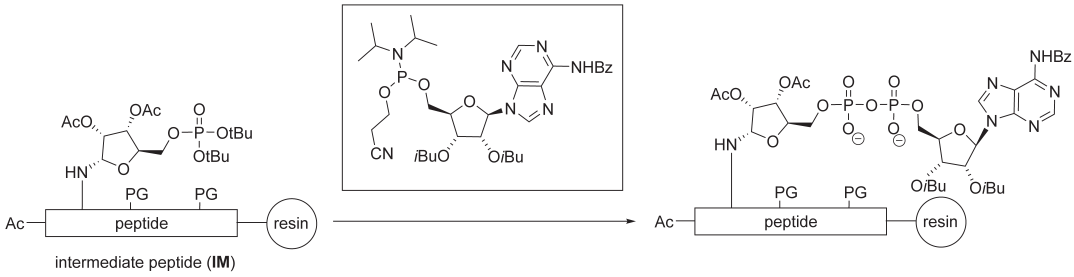
EtOH/AcOH, 99/0/1 – 94/5/1) to obtain the title compound (479 mg, 0.55 mmol, 59%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.73 (d, J = 7.5 Hz, 2H, arom.), 7.58 (d, J = 6.9 Hz, 2H, arom.), 7.42 – 7.21 (m, 14H, arom.), 6.31 (s, 1H, H1.), 5.64 (d, J = 8.0 Hz, 1H, NH), 5.52 (bs, 1H, OH), 4.70 (d, J = 12.3 Hz, 1H, CH_2 Bn), 4.62 – 4.54 (m, 3H, CH_2 Bn), 4.45 – 4.38 (m, 1H, CH α-Glu), 4.39 – 4.33 (m, 3H, CH_2 Fmoc, H4'), 4.19 (t, J = 7.0 Hz, 1H, CH Fmoc), 3.98 – 3.96 (m, 2H, H2', H3'), 3.93 – 3.87 (m, 1H, H5'), 3.84 – 3.77 (m, 1H, H5'), 2.62 – 2.45 (m, 2H, CH_2 γ-Glu), 2.29 – 2.19 (m, 1H, CH_2 β-Glu), 2.06 – 1.97 (m, 1H, CH_2 β-Glu), 1.40 (s, 9H, CH_3 tBu), 1.39 (s, 9H, CH_3 tBu). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 173.92 (CO α-Glu), 172.13 (CO δ-Glu), 156.23 (CO Fmoc), 143.98, 143.89, 141.38, 137.60, 137.25 (Cq. arom.), 128.63, 128.60, 128.37, 128.19, 128.10, 127.99, 127.80, 127.21, 125.27, 120.04 (arom.), 94.96 (C1'), 83.53, 83.46 (C4'), 83.38, 83.34, 83.32, 83.29 (Cq. tBu), 77.90, 74.90 (C2', C3'), 73.16, 72.73 (CH_2 Bn), 67.14 (CH_2 Fmoc), 66.25, 66.20 (C5'), 53.36 (CH α-Glu), 47.26 (CH Fmoc), 30.87 (CH_2 γ-Glu), 29.90, 29.88, 29.87, 29.85 (CH_3 tBu), 27.42 (CH_2 β-Glu). ^{31}P-NMR (162 MHz, $CDCl_3$) δ -10.11.



O^{γ}-(2,3-di-*O*-benzyl-5-*O*-(di-tert-butyl)-phosphoryl-α-D-ribosyl)-N^{α}-fluorenylmethoxycarbonyl aspartic acid (23)

General procedure D was applied to compound **21** (DCM/MeOH/AcOH, 100/0/0.2 – 95/5/0.2) to obtain the title compound (81 mg, 94 µmol, 52%). ^{1}H-NMR (500 MHz, MeOD) δ 7.77 (d, J = 2.7 Hz, 1H, arom.), 7.75 (d, J = 2.7 Hz, 1H, arom.), 7.64 – 7.59 (m, 4H, arom.), 7.40 – 7.22 (m, 14H, arom.), 6.26 (d, J = 4.2 Hz, 1H, H1'), 4.69 – 4.51 (m, 5H, CH α-Asp, CH_2 Bn), 4.32 (d, J = 7.0 Hz, 3H, CH_2 Fmoc, H4'), 4.15 (t, J = 6.9 Hz, 1H, CH Fmoc), 4.00 (dd, J = 6.2, 4.3 Hz, 1H, H2'), 3.95 (dd, J = 6.3, 2.1 Hz, 1H, H3'), 3.90 (ABX, J = 11.1, 5.2, 3.6 Hz, 1H, H5'), 3.82 (ABX, J = 11.2, 5.3, 3.5 Hz, 1H, H5'), 2.93 (d, J = 5.9 Hz, 2H, CH_2 β-Asp), 1.38 (d, J = 1.4 Hz, 18H, CH_3 tBu). ^{13}C-NMR (126 MHz, MeOD) δ 173.81 (CO α-Asp), 171.22 (CO γ-Asp), 158.29 (CO Fmoc), 145.25, 145.11, 142.54, 142.52, 139.21, 138.95 (Cq. arom.), 129.59, 129.49, 129.35, 129.15, 129.06, 128.91, 128.75, 128.17, 128.15, 126.26, 120.92 (arom.), 96.39 (C1'), 85.03, 84.96 (C4'), 84.80, 84.73 (Cq. tBu), 78.98 (C2'), 76.12 (C3'), 73.88, 73.55 (CH_2 Bn), 67.94, 67.69 (C5'), 67.64 (CH_2 Fmoc), 51.80 (CH α-Asp), 48.37 (CH Fmoc), 37.65 (CH_2 β-Asp), 30.13, 30.10 (CH_3 tBu). ^{31}P-NMR (202 MHz, MeOD) δ -9.43.

General procedure E: on-resin tBu cleavage and pyrophosphate formation



intermediate peptide (**IM**)

HFIP (2 mL) and HCl (12.3 M, 1 eq.) were added and the resin was shaken for 1 hour at room temperature. The resin was filtered and washed with pyridine and DCM respectively. The resin was analyzed with ^{31}P-NMR spectroscopy (swelling in DMF and inserting aceton capillary) to determine the tBu cleavage. ^{31}P-NMR indicated almost

complete tBu cleavage and a minimal amount of mono-tBu phosphate. Therefore, the resin was treated a second time with HFIP (2 mL) and HCl (1 eq.) for 30 minutes. The resin was washed with pyridine and DCM respectively and placed under an argon atmosphere. The resin was extensively washed with ACN to remove traces of water. Next, ETT (6 eq., 0.25M in ACN) and adenosine-amidite[51] (= *N*^{6}-benzoyl-9-[5-*O*-(*N*,*N*-di-isopropylamino-*O*-cyanoethyl)phosphoramidite-2,3-di-*O*-isobutyryl-β-**D**-ribofuranosyl]-adenine) (3 eq.) in ACN (0.3 M) were added. The reaction was shaken for 30 minutes. The resin was filtered, washed with ACN and CSO (2 mL, 0.5 M in ACN) was added. The resin was shaken for 10 minutes, filtered, washed with ACN and DBU (2 mL, 0.5 M in DMF) was added. The mixture was shaken for 10 minutes, filtered and washed with DMF, ACN and DCM respectively.

Ac-Pro-Gln(N^6-[5'-*O*-adenosine diphosphate-α-D-ribosyl])-Pro-Ala-Lys-Ser-Ala-Pro-Ala-Pro-Lys-Lys-Gly-NH$_2$ (24)

The intermediate peptide was synthesized on an automated peptide synthesizer using standard SPPS protocols starting from Tentagel® S HMBA resin (225 mg, 50 µmol) which was preloaded with Fmoc-Gly-OH using DIC, DMAP in DCM and the loading was determined by UV-VIS spectroscopy (0.22 mmol/g based on Fmoc loading). The following amino acids were used: Fmoc-Lys(TFA)-OH, Fmoc-Pro-OH, Fmoc-Ser(Trt)-OH and Fmoc-Ala-OH. The resin was transferred to a fritted syringe before coupling of phosphoribosylated glutamine **13**. The resin was repeatedly treated with 5% TFA in DCM until no yellow colour appeared upon addition of the fresh TFA solution indicating complete Trt removal. After washing with DCM and NMP, Ac_2O (10% $^v/_v$) and DMAP (cat.) in NMP (2 mL) was added. The resin was shaken for 20 minutes after which the resin was washed with NMP and DCM respectively. Next, compound **13** (116 mg, 150 µmol), HCTU (150 µmol), DIPEA (300 µmol) in NMP (1.5 mL) were added and the resin was shaken for 16 hours. The resin was washed with NMP and DCM prior to coupling of the last amino acid, Fmoc-Pro-OH, using the standard cycle. Final Fmoc cleavage, capping and washing (NMP, DCM) yielded the intermediate peptide **24-IM**. Next, general procedure E was applied to the resin to obtain the crude protected ADPr-peptide. The resin was treated with a saturated solution of NH_3 in trifluoroethanol (5 mL) for 4 hours to cleave the peptide from the resin and remove the protecting groups (PG). Finally, conc. aq. NH_4OH (5 mL) was added and the resin was shaken for 16 hours to remove the Bz from the exocyclic amine of adenosine. The resin was filtered, washed with H_2O and the filtrate concentrated under reduced pressure. The crude was dissolved in a minimal amount of MeOH and a few drops of AcOH, precipitated in ice-cold Et_2O and centrifuging (5.0 min at 4000 RPM) followed by decantation of the supernatant yielded the crude title compound. RP-HPLC using a gradient of 5 – 20 % B (eluens: A. 10 mM NH_4OAc in H_2O, B. MeCN) yielded the title compound. Semi-pure fractions were applied to a boronate affinity column to yield the title compound. The combined fractions were lyophilized to yield the title compound as a white solid (3.3 mg, 1.8 µmol, 4% based on initial loading). ^{1}H NMR (500 MHz, D_2O) δ 8.55 (s, 1H, H2), 8.31 (s, 1H, H8), 6.17 (d, J = 6.1 Hz, 1H, H1'-adenosine), 5.67 (d, J = 4.5 Hz, 1H, H1'-ribose). ^{31}P-NMR (162 MHz, D_2O): δ -10.34, -10.47, -10.63, -10.76. (EDTA was added to sharpen signals). LC-MS (5 - 20 % MeCN [1% TFA] in 12.5 min), Rt = 6.38. HRMS [$C_{74}H_{121}N_{23}O_{29}P_2$ + 2H]$^{2+}$: 929.9165 found, 929.9161 calculated, [$C_{74}H_{121}N_{23}O_{29}P_2$ + 3H]$^{3+}$: 620.2800 found, 620.2798 calculated.

Ac-Pro-Gln-Pro-Ala-Lys-Ser-Ala-Pro-Ala-Pro-Lys-Lys-Gly-NH_2 (24S)
The peptide was synthesized on an automated peptide synthesizer using standard SPPS protocols starting from Tentagel® S RAM resin (100 µmol). RP-HPLC using a gradient of 5 – 15 % B (eluens: A. 0.5 % TFA in H_2O, B. MeCN) and lyophilisation of the appropriate fractions yielded the title compound (33.5 mg, 25 µmol, 25 % based on initial loading). LC-MS (0 - 20 % MeCN [1% TFA] in 12.5 min), Rt = 6.63; (10 - 50 % MeCN [1% TFA] in 12.5 min), Rt = 1.45. HRMS [$C_{59}H_{100}N_{18}O_{16}$ + H]$^+$: 1317.7656 found, 1317.7637 calculated, [$C_{59}H_{100}N_{18}O_{16}$ + 2H]$^{2+}$: 659.3851 found, 659.3855 calculated, [$C_{59}H_{100}N_{18}O_{16}$ + 3H]$^{3+}$: 439.9260 found, 439.9261 calculated

Ac-Pro-Gln(N^{δ}-[5′-*O*-adenosine diphosphate-α-D-ribosyl])-Pro-Ala-Lys-Ser-Ala-Pro-Ala-Pro-Lys-Lys(biotin)-Gly-NH_2 (25)
First Fmoc-Lys(Mtt)-OH was coupled with Fmoc-Gly-Tentagel® S HMBA resin (225 mg, 50 µmol) using standard SPPS protocols. The resin was repeatedly rinsed with TFA (5 %$^v/_v$) in DCM until no yellow colour appeared upon addition of fresh TFA solutionand the resin was neutralized with a solution of DIPEA (10 %$^v/_v$) in NMP. Next, biotin (5 eq.), PyBOP (5 eq.), DIPEA (10 eq.) in NMP (1.4 mL) were added and the resin shaken for 1 hour. The resin was washed and loaded in an automated peptide synthesizer to couple the following amino acids: Fmoc-Lys(TFA)-OH, Fmoc-Pro-OH, Fmoc-Ser(Trt)-OH and Fmoc-Ala-OH using standard SPPS protocol. The resin was transferred to a fritted syringe before coupling of compound **13**. The resin was repeatedly treated with 5% TFA in DCM until no yellow colour appeared upon addition of the fresh TFA solution indicating complete Trt removal. After washing with DCM and NMP, Ac_2O (10% $^v/_v$) and DMAP (cat.) in NMP (2 mL) was added. The resin was shaken for 20 minutes after which the resin was washed with NMP and DCM respectively. Next, compound **13** (2 eq.), PyBOP (2 eq.), DIPEA (4 eq.) in NMP (1.3 mL) were added and the resin was shaken for 16 hours. The resin was washed with NMP and DCM prior to coupling of the last amino acid, Fmoc-Pro-OH, using the standard cycle. Final Fmoc cleavage, capping and washing (NMP, DCM) yielded the intermediate peptide **25-IM**. Next, modified general procedure E (5% TFA in DCM for 40 minutes) was applied to the resin to obtain the crude protected ADPr-peptide. The resin was treated with a saturated solution of NH_3 in trifluoroethanol (5 mL) for 4 hours to cleave the peptide from the resin and remove the protecting groups (PG). Finally, conc. aq. NH_4OH (5 mL) was added and the resin was shaken for 16 hours to remove the Bz from the exocyclic amine of adenosine. The resin was filtered, washed with H_2O and the filtrate concentrated under reduced pressure. The crude was dissolved in a minimal amount of MeOH and a few drops of AcOH, precipitated in ice-cold Et_2O and centrifuging (5.0 min at 4000 RPM) followed by decantation of the supernatant yielded the crude title compound. RP-HPLC using a gradient of 10 – 20 % B (eluens: A. 10 mM NH_4OAc in H_2O, B. MeCN) yielded the title compound. Semi-pure fractions were applied to a boronate affinity column to yield the title compound. The combined fractions were lyophilized to yield the title compound as a white solid (5.6 mg, 2.7 µmol, 70/30;α/β, 5% based on initial loading). ^{1}H NMR (500 MHz, D_2O) δ 8.53 (s, 1H, H2), 8.28 (s, 1H, H8), 6.15 (d, J = 6.0 Hz, 1H, H1′-adenosine), 5.65 (d, J = 4.3 Hz, 0.7H, H1′-α ribose), 5.45 (d, J = 6.3 Hz, 0.3H, H1′-β ribose). ^{31}P-NMR (202 MHz, D_2O): δ -10.32, -10.35, -10.42, -10.60, -10.64, -10.68, -10.70, -10.75. (EDTA was added to sharpen signals). LC-MS (5 - 20 % MeCN [1% TFA] in 12.5 min), Rt = 8.31, (0 - 25 % MeCN [10 mM NH_4OAc]

in 15 min), Rt = 7.56. HRMS [$C_{84}H_{135}N_{25}O_{31}P_2S$ + 2H]$^{2+}$: 1042.9559 found, 1042.9549 calculated, [$C_{74}H_{121}N_{23}O_{29}P_2$ + 3H]$^{3+}$: 695.6398 found, 695.6390 calculated.

Ac-Pro-Gln-Pro-Ala-Lys-Ser-Ala-Pro-Ala-Pro-Lys-Lys(biotin)-Gly-NH_2 (25S)
The peptide was synthesized on an automated peptide synthesizer using standard SPPS protocols starting from Tentagel® S RAM resin (50 µmol) and biotin was introduced as described before. RP-HPLC using a gradient of 5 – 25 % B (eluens: A. 0.5 % TFA in H_2O, B. MeCN) and lyophilisation of the appropriate fractions yielded the title compound (6.92 mg, 4.5 µmol, 9 % based on initial loading). LC-MS (0 - 20 % MeCN [1% TFA] in 12.5 min), Rt = 8.83; (10 - 50 % MeCN [1% TFA] in 12.5 min), Rt = 3.90; (10 - 90 % MeCN [1% TFA] in 12.5 min), Rt = 3.38. HRMS [$C_{69}H_{114}N_{20}O_{18}S$ + 2H]$^{2+}$: 772.4248 found, 772.4243 calculated, [$C_{69}H_{114}N_{20}O_{18}S$ + 3H]$^{3+}$: 515.2853 found, 515.2853 calculated.

Ac-Pro-Thr-Val-Phe-Glu-Asn(N$^{\gamma}$-[5'-*O*-adenosine diphosphate-α-D-ribosyl])-Tyr-Val-Ala-Asp-Ile-NH_2 (26)
The intermediate peptide was synthesized on an automated peptide synthesizer using standard SPPS protocols starting from Tentagel® S HMBA resin (225 mg, 50 µmol) which was preloaded with Fmoc-Ile-OH using DIC, DMAP in DCM and the loading was determined by UV-VIS spectroscopy (0.22 mmol/g based on Fmoc loading). The following amino acids were used: Fmoc-Asp(Dmab)-OH, Fmoc-Glu(Dmab)-OH, Fmoc-Pro-OH, Fmoc-Tyr-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Thr-OH, Fmoc-Phe-OH and compound **14**. The resin was transferred to a fritted syringe and was capped with Ac_2O (50 eq.) and DIPEA (50 eq.) in NMP (2 mL) for 1 hour to give intermediate peptide **26-IM**. Next, general procedure E was applied to the resin to obtain the crude protected ADPr-peptide. The resin was shaken 3 x 5 minutes with 2 %$^{v}/_{v}$ hydrazine in DMF (2 mL) to remove the Dmab groups and washed with NMP and DCM. The resin was treated with a saturated solution of NH_3 in trifluoroethanol (5 mL) for 4 hours to cleave the peptide from the resin and remove the protecting groups (PG). Finally, conc. aq. NH_4OH (5 mL) was added and the resin was shaken for 16 hours to remove the Bz from the exocyclic amine of adenosine. The resin was filtered, washed with H_2O and the filtrate concentrated under reduced pressure. The crude was dissolved in a minimal amount of MeOH and a few drops of AcOH, precipitated in ice-cold Et_2O and centrifuging (5.0 min at 4000 RPM) followed by decantation of the supernatant yielded the crude title compound. Strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded the title compound as a white solid (0.98 mg, 0.53 µmol, 1.1 % based on initial loading). *[The same synthesis (100 µmol) and final deprotection using Tesser's base (dioxane, methanol and aqueous NaOH) followed by strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization gave a complex mixture of products (11.8 mg, 6.4 µmol, 6 % based on initial loading).]* ^{1}H NMR (500 MHz, D_2O) δ 8.48 (s, 1H, H2), 8.21 (s, 1H, H8), 7.29 – 7.19 (m, 3H, arom. Phe), 7.15 (d, J = 7.3 Hz, 2H, arom. Phe), 7.02 (d, J = 8.5 Hz, 2H, arom. Tyr), 6.75 (d, J = 8.4 Hz, 2H, arom. Tyr), 6.08 (d, J = 5.8 Hz, 1H, H1'-adenosine), 5.64 (d, J = 4.4 Hz, 1H, H1'-ribose). ^{31}P-NMR (202 MHz, D_2O): δ -10.38, -10.49, -10.64, -10.74. (EDTA was added to sharpen signals). LC-MS (5 - 50 % MeCN [1% TFA] in 12.5 min), Rt = 7.90. HRMS [$C_{76}H_{110}N_{18}O_{32}P_2$ + H]$^{+}$: 1849.7084 found, 1849.7082 calculated, [$C_{76}H_{110}N_{18}O_{32}P_2$ +

2H]$^{2+}$: 925.3572 found, 925.3577 calculated, [$C_{76}H_{110}N_{18}O_{32}P_2$ + 2Na]$^{2+}$: 936.8469 found, 936.3487 calculated.

H_2N-Pro-Thr-Val-Phe-Glu-Asn-Tyr-Val-Ala-Asp-Ile-NH_2 (26S)
The peptide was synthesized on an automated peptide synthesizer using standard SPPS protocols starting from Tentagel® S RAM resin (50 µmol). RP-HPLC using a gradient of 5 –% B (eluens: A. 0.5 % TFA in H_2O, B. MeCN) and lyophilisation of the appropriate fractions yielded the title compound (8.6 mg, 6.8 µmol, 14% based on initial loading). LC-MS (0 - 20 % MeCN [1% TFA] in 12.5 min), Rt = 10.37; (10 - 50 % MeCN [1% TFA] in 12.5 min), Rt = 6.80; (10 - 90 % MeCN [1% TFA] in 12.5 min), Rt = 4.91. HRMS [$C_{59}H_{87}N_{13}O_{18}$ + H]$^+$: 1266.6379 found, 1266.6365 calculated, [$C_{59}H_{87}N_{13}O_{18}$ + 2H]$^{2+}$: 633.8223 found, 633.8219 calculated.

Ac-Ala-Gly-Glu-Cit(N$^{\omega}$-[5′-*O*-adenosine diphosphate-α-D-ribosyl])-Arg-Tyr-Gly-NH_2 (27)
The peptide was synthesized starting from Fmoc-Gly-Tentagel® S HMBA resin (550 mg, 100 µmol) using standard coupling conditions and using DBU (2 %$^v/_v$) in DMF for Fmoc cleavage. First, Fmoc-Tyr-OH was coupled which was followed by acetylation for 1 hour (Ac_2O, DIPEA, DMAP and NMP). Fmoc-Arg(Alloc)$_2$-OH was coupled for 1 hour and subsequently compound **15** (3 eq.) by shaking the resin for 16 hours. Then, the following amino acids were used: Fmoc-Glu(Dmab)-OH, Fmoc-Gly-OH and Fmoc-Ala-OH. Finally acetylation using Ac_2O (0.5 mL) and DIPEA (0.8 mL) in NMP (4 mL) for 2 x 10 minutes yielded the intermediate peptide **27-IM**. Next, general procedure E was applied to the resin to obtain the crude protected ADPr-peptide. The Alloc was removed by shaking the resin 3 x 15 mintues with dimethylbarbituric acid (46 mg) and $Pd(PPh_3)_4$ (20 mg) in DCM (5 mL). Next, the Dmab was removed by shaking the resin 3 x 5 minutes with 2 %$^v/_v$ hydrazine in DMF (2 mL) and was washed with NMP and DCM. The resin was treated with a saturated solution of NH_3 in trifluoroethanol (5 mL) for 4 hours to cleave the peptide from the resin and remove the protecting groups (PG). Finally, conc. aq. NH_4OH (5 mL) was added and the resin was shaken for 16 hours to remove the Bz from the exocyclic amine of adenosine. The resin was filtered, washed with H_2O and the filtrate concentrated under reduced pressure. The crude was dissolved in a minimal amount of MeOH and a few drops of AcOH, precipitated in ice-cold Et_2O and centrifuging (5.0 min at 4000 RPM) followed by decantation of the supernatant yielded the crude title compound. Strong anion exchange chromatography on Source 15Q (10mm x 10cm) using gradient elution with NH_4OAc (20 mM to 1.0 M) and repeated lyophilization afforded the title compound as a white solid (0.31 mg, 0.2 µmol, 60/40;α/β, 0.2 % based on initial loading). ^{1}H-NMR (500 MHz, D_2O) δ 8.48 (s, 1H, H2), 8.46 (s, 1H, H2), 8.22 (s, 1H, H8), 8.21 (s, 1H, H8), 7.06 (d, J = 8.6 Hz, 2H, arom. Tyr), 7.03 (d, J = 8.6 Hz, 2H, arom. Tyr), 6.76 (d, J = 8.6 Hz, 3H, arom. Tyr), 6.74 (d, J = 8.5 Hz, 2H, arom. Tyr), 6.12 – 6.07 (m, 2H, H1′α-, H1′β-adenosine), 5.54 (d, J = 4.4 Hz, 1H, H1′α-ribose), 5.36 (d, J = 6.3 Hz, 1H, H1′β-ribose). ^{31}P-NMR (202 MHz, D_2O): δ -10.17, -10.28, -10.32, -10.43, -10.60, -10.62, -10.70, -10.72. (EDTA was added to sharpen signals). LC-MS (10 - 50 % MeCN [1% TFA] in 12.5 min), Rt = 4.47. HRMS [$C_{50}H_{76}N_{18}O_{25}P_2$ + H]$^+$: 1391.4777 found, 1391.4777 calc, [$C_{50}H_{76}N_{18}O_{25}P_2$ + 2H]$^{2+}$: 696.2428 found, 696.2425 calculated.

Ac-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-NH_2 (27S)
The peptide was synthesized on an automated peptide synthesizer using standard SPPS protocols starting from Tentagel® S RAM resin (50 µmol). RP-HPLC using a gradient of 5 –% B (eluens: A. 0.5 % TFA in H_2O, B. MeCN) and lyophilisation of the appropriate fractions yielded the title compound (3.6 mg, 4.2 µmol, 9 % based on initial loading). LC-MS (10 - 50 % MeCN [1% TFA] in 12.5 min), Rt = 3.10; (10 - 90 % MeCN [1% TFA] in 12.5 min), Rt = 1.49. HRMS $[C_{35}H_{56}N_{14}O_{11} + H]^+$: 849.4321 found, 849.4326 calculated, $[C_{35}H_{56}N_{14}O_{11} + 2H]^{2+}$: 425.2194 found, 425.2199 calculated.

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