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

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Chapter 6

The Synthesis of ADP-Ribosylated Nucleic Acids

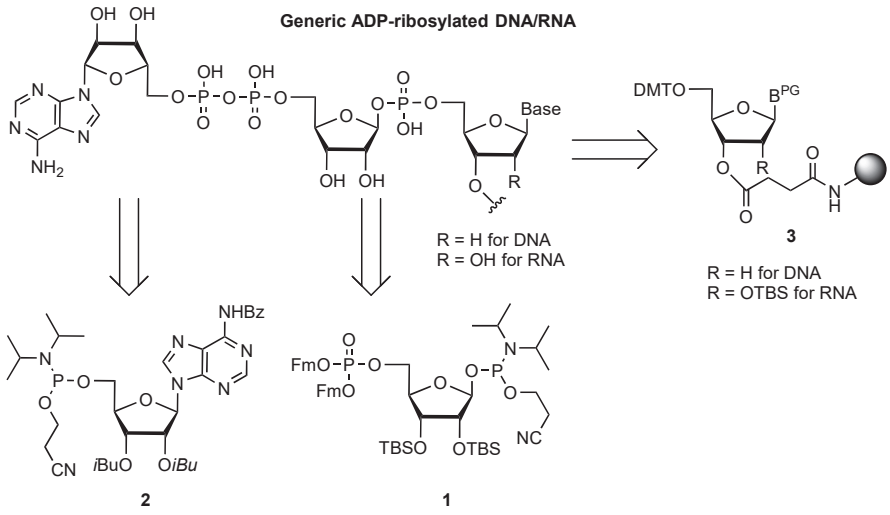
Introduction

Research in the field of ADP-ribosylation is mostly focused on the elucidation of the pathways and mechanisms that regulate ADP-ribosylation of specific amino acids in certain proteins. However, the ADP-ribosylation of biopolymers other than polypeptides, such as nucleic acids, starts to attract attention as well. An early example of ADP-ribosylation of DNA was discovered with proteins originating from the cabbage butterfly[1] and certain shellfish[2] that catalyze the formation of not only N^2-(a-ADP-ribos-1-yl)-2'-deoxyguanosine but also its b-anomer in duplex DNA. Interestingly these studies indicate an involvement of ADP-ribosylation in host-pathogen conflicts.[3] Another nucleobase modification is represented by the sequence specific modification of thymine in single stranded DNA by the bacterial toxin-antitoxin system DarT-DarG.[4] Moreover, recent *in vitro* studies showed that mammalian ARTs are able to ADP-ribosylate DNA[5–9] and RNA[10] oligomers, provided with a 5'- or 3'- terminal phosphate. In the resulting structures, the anomeric configurations of these terminal phosphates linked to the ADP-ribosyl moiety are not yet established. ADP-ribosylation of DNA can occur in both double and single stranded oligomers, seems to be dependent on the localization of the strand break and is probably independent of the nucleotide sequence. DNA oligomers are modified with ADPr by PARP1 and PARP2[6] but PARP3 appears to be most proficient and it is reported that nucleotide fragments, ADP-ribosylated by PARP3 can serve as primers for further modification to give PAR chains.[8] The reversal of the ADPr-modification can be accomplished by several hydrolases, such as MacroD1,[11] MacroD2, PARG, TARG and ARH3[6,10] that are able to remove the ADPr moiety from the terminal phosphate of DNA. The physiological significance of the *mono*-ADP-ribosylation of single-stranded breaks in DNA on the 5'-phosphorylated site is underscored by the fact that the modification is recognized by DNA ligases which ligate to the damaged site and allow DNA restoration by nucleophilic attack of the 3'-OH to the 5'-phosphorylated strand.

Although various *in vitro* studies have shown that terminal phosphorylated DNA/RNA can be ADP-ribosylated and several hydrolases can reverse this modification much is still unknown and cellular *in vitro* and subsequent *in vivo* studies are needed to establish the presence of ADP-ribosylated nucleic acids and to elucidate their functions.[5,9,12] This research will benefit from the presence of well-defined molecular tools, accessible by organic synthesis. For instance, structural elucidation of complexes of ADP-ribosylated DNA/RNA fragments and catalytic domains of both ARTDs and ARHs will provide valuable information as to their respective activity and specificity. This chapter describes the development of a synthetic procedure to ADP-ribosylated DNA and RNA.

Retrosynthetic analysis

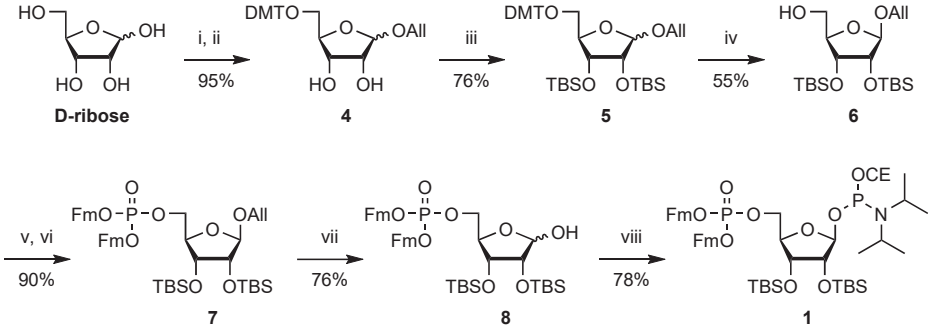
A generic structure of a DNA and RNA nucleotide with the ADP-ribosyl moiety at the 5'–phosphate in the b-configuration is depicted in Scheme 1. To make the building blocks compatible with standardized, automated oligonucleotide synthesis, phosphoramidite **2** was chosen for the adenosine moiety, a building block that was also applied in the automated synthesis of ADPr oligomers.[13] For the ribosyl moiety, key building block **1** was designed as (i) the TBS protecting group is often employed in RNA synthesis, (ii) the anomeric amidite enables coupling to the 5'-OH of the protected DNA/RNA oligomer and (iii) the Fm-protected phosphotriester on the 5-OH allows not only for a mild deprotection of the phosphate triester prior to coupling with amidite **2** but is also orthogonal to all protecting groups of the nucleobases. The standard solid support for oligonucleotide synthesis, controlled pore glass[14] (CPG) equipped with an amino alkyl linker was chosen as suitable. CPG can be functionalized via an ester linkage with the 3'(2') OH of any given (deoxy)ribonucleoside,[15] leading to **3** as solid support provided with the first (deoxy)nucleoside. In case of DNA building blocks, no additional protecting group is required and in case of RNA, the usual 2'-OTBS protected nucleotides can be used.



Scheme 1. Retrosynthetic analysis of generic, ADP-ribosylated nucleotides

Results and discussion

Functionalized CPG resin **3** is commercially available and building block **2** was synthesized by a known procedure[16] (see Chapter 4), leaving the synthesis of key ribosyl building block **1** (Scheme 2). The preparation commenced with Fischer glycosylation of D-ribose with allyl alcohol followed by the regioselective installation of the DMT protecting group on the 5'-OH, giving **4** in 95% yield. Protection of the 2'- and 3'-OH with silyl groups by treatment of **4** with TBS-Cl and imidazole provided **5**. Next, the DMT protecting group was removed by acidolysis with TFA which furnished **6** in 55% yield. It is noteworthy that only the β-anomer could be obtained after flash column chromatography, explaining the moderate yield. Next, a two-step phosphorylation reaction with known bis-Fm-protected phosphoramidite (see Chapter 3) followed by oxidation of the intermediate phosphite yielded triester **7** in a 90% yield. De-allylation of **7** was affected in a two-step procedure whereby the allyl group is isomerized by an iridium catalyst and the obtained enol ether is hydrolyzed by I_2/$NaHCO_3$/H_2O. Finally, **8** is treated with commercially available 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite and DIPEA to give crude **1**. Manual purification of **1** with standard silica gel chromatography, neutralized by adding TEA to the solvent to prevent hydrolysis of the anomeric amidite, proofed cumbersome as the addition of TEA triggered the cleavage of the Fm protecting groups. Therefore, purification of **1** was performed by automated column chromatography using a pH-neutral silica cartridge which furnished key building block **1** as the β-anomer in a total yield of 21% over 8 steps.

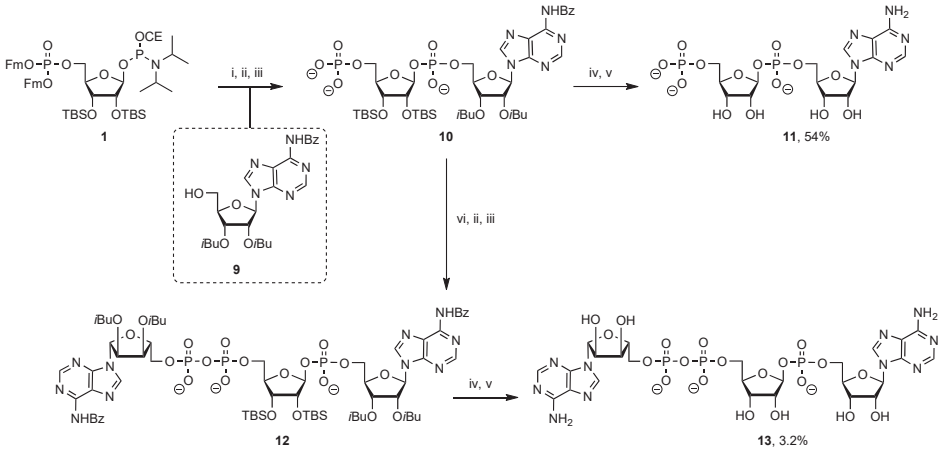


Scheme 2. Synthesis of key building block **1**, the ribosyl moiety suitable for on-resin nucleotide synthesis. Reagents and conditions: i) Ac-Cl, All-OH. ii) DMT-Cl, pyr. iii) TBS-Cl, imidazole, DMF. iv) TFA, TIS, DCM. v) $(FmO)_2PN(iPr)_2$, DCI, MeCN. vi) *t*BuOOH, nonane. vii) $Ir(COD)(Ph_2MeP)_2{\cdot}PF_6$, H_2, THF, then I_2, $NaHCO_3$, H_2O. viii) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIPEA, DCM.

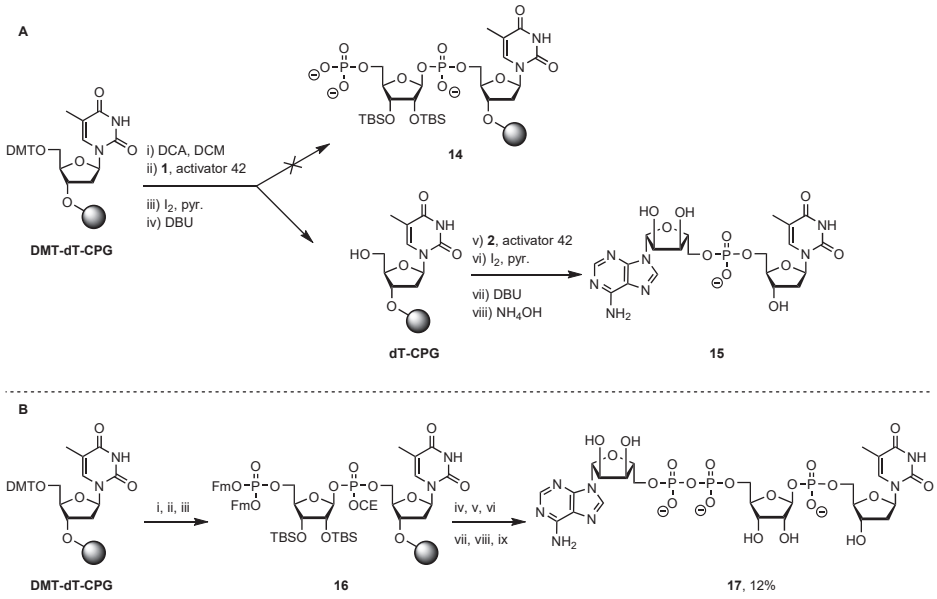
In order to establish whether building block **1** is indeed suitable for the synthesis of DNA/RNA oligomers ADP-ribosylated at their 5' end, a solution-phase exploratory study was performed (Scheme 3). Therefore, adenosine nucleoside **9** (an intermediate *en route* to amidite **2**) was chosen as a model since it mimics the protecting group pattern of the intermediates in the solid-phase synthesis. Amidite **1** was coupled to adenosine **9** using ETT as activator and the reaction progress was monitored by ^{31}P-NMR, showing quantitative conversion of **1** into the expected phosphite intermediate. Subsequent CSO-mediated oxidation led to the formation of a second phosphate triester and the obtained, fully protected crude product was not isolated but TEA was added to the reaction mixture. Monitoring of the reaction progress by LC-MS analysis showed that not only the cyanoethyl group but also one of the two Fm-groups were cleaved off almost instantly. On the other hand, the removal of the second Fm-group required overnight stirring to give trianion **10**. Next, the TBS groups were removed by concentration of the reaction mixture and treatment of the resulting amorphous oil with TEA·3HF in THF. Monitoring by LC-MS analysis showed that it took two days to completely remove the TBS protecting groups. Finally, ammonolysis of the remaining protecting groups (*i*Bu and Bz) with NH_4OH followed by HPLC purification furnished **11** in a 54% yield over 5 steps. Encouraged by this successful result, which indicated that the anomeric phosphodiester survived the applied reaction conditions, the pyrophosphate moiety was appended using the P^{III} – P^{V} procedure as described in earlier chapters. To achieve this, the phosphomonoester of **10** was coupled under influence of ETT with adenosine amidite **2**. Ensuing oxidation of the phosphite intermediate with CSO and the removal of the phosphate protecting groups with TEA furnished intermediate **12**. Analogously to the deprotection procedure for **11**, intermediate **12** was first treated with TEA·3HF and subsequently with NH_4OH to yield ADPr-P-RNA **13**. Although the LC-MS trace of the crude reaction mixture showed the predominant formation of product **13**, HPLC purification proved difficult and homogeneous target **13** was isolated in 3% yield only. Nevertheless, the exploratory study toward **11** and **13** shows that the chemistry for both the anomeric phosphodiester and the pyrophosphate function is viable, indicating that the solid phase synthesis to ribosylated nucleic acid fragments is feasible.

At this stage the solid phase synthesis of ribosylated DNA was investigated and it was decided to adopt the conditions of the standard automated DNA synthesis with regards to the type and concentration of the reagents and reaction times.[17] Commercially available DMT-dT-CPG (Scheme 4) was first treated with a DCA solution in DCM to cleave off the DMT protecting group. Next, anomeric phosphoramidite **1** was coupled to the 5'-OH of dT-CPG under the influence of 5-[3,5-Bis(trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42®). Subsequent oxidation with a I_2 solution in pyridine and water, standard in automated oligonucleotide synthesis,[17] and removal of the cyanoethyl and Fm phosphate protecting groups with DBU should result in immobilized **14**. Surprisingly, after performing a second

coupling cycle with adenosine amidite **2** and subsequent deprotection/cleavage, the LC-MS analysis of the crude product revealed **15** to be the major product, while the expected product was missing. In order to find a productive pathway to **14** several adjustments of the above procedure were made. However, increasing the reaction time of the coupling of building block **1**, repetition of the coupling cycle and use of the activator ETT did not lead to formation of the desired product. Fortunately, oxidation of the phosphite intermediate with CSO rather than I_2, resulted in the effective formation of immobilized **16**. Introduction of the pyrophosphate moiety by DBU mediated cleavage of the phosphate protecting groups, followed by coupling of adenosine amidite **2** with protected intermediate **16** and again, oxidation of the phosphate-phosphite intermediate with CSO proceeded without noteworthy side products as was observed by LC-MS analysis of the crude products. Deprotection and subsequent cleavage from the CPG support led to the silylated precursor of **17**. The remaining silyl ethers were removed by treatment of the crude product with a 4:3:2 v/v% solution of TEA·3HF:TEA:NMP solution. Direct injection of the reaction mixture on a HW40 size exclusion column (after quenching with an NH_4OAc buffer solution) resulted in homogenous **17** in 12% yield.



Scheme 3. Exploratory study for coupling of key building block **1** in solution. Reagents and conditions: i) **9**, ETT, MeCN. ii) CSO, MeCN. iii) TEA. iv) TEA·3HF, THF. v) NH_4OH, H_2O. vi) **2**, ETT, MeCN.



Scheme 4. A: Attempted coupling of building block **1** to DMT-dT-CPG using I_2 as oxidating agent and formation of side product **15**. **B:** Solid-phase synthesis of partially protected 5'-ADP-ribosylated thymidine **17**. Reagents and conditions: i) DCA, DCM. ii) **1**, activator 42®, MeCN. iii) CSO, MeCN. iv) DBU, MeCN. v) **2**, activator 42®, MeCN. vi) CSO, MeCN. vii) DBU, MeCN. viii) NH_4OH, H_2O. ix) TEA·3HF, TEA, NMP.

Conclusion

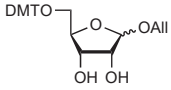
The important *in vitro* discoveries of various ADP-ribosylated nucleic acids raises questions how these DNA and RNA modifications occur *in vivo* and what biological role they play. The synthesis of well-defined fragments of ADP-ribosylated nucleic acid oligomers with and without labels will contribute to finding the answers. This chapter describes the first solution phase synthesis of ADP-ribosylated adenosine 5'-phosphate (**13**) and the first solid phase synthesis of ADP-ribosylated deoxythymidine 5'-phosphate (**17**). It turned out that oxidation of phosphite intermediates with CSO toward the target compounds is of prime importance for the successful synthesis of ADP-ribosylated nucleic acids. The developed procedure to ADP-ribosylated deoxythymidine 5'-phosphate lays down a basis for the future synthesis ADP-ribosylated nucleic acids.

Experimental section

General synthetic procedures

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

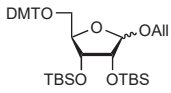
1-*O*-allyl-5-*O*-(4,4'-dimethoxytrityl)- α,β-D-ribofuranoside (4)



D-Ribose (7.51 g, 50 mmol) was suspended in allyl alcohol (125 mL, 0.4 M) and acetyl chloride (2.5 mL, 35 mmol, 0.7 eq.) was added. The reaction was stirred for 2 hours, then quenched with 6 mL pyridine and concentrated *in vacuo*. The residue was co-evaporated with pyridine, dissolved in pyridine (100 mL, 0.5 M) and DMT-Cl (17.8 g, 52.5 mmol, 1.05 eq.) was added. The reaction was stirred overnight after which the volume was reduced by evaporation *in vacuo* till about 20% of the original volume. The residue was taken up in EtOAc (1.000 mL) and carefully quenched with sat. aq. $NaHCO_3$. The suspension was transferred into a separatory funnel and the organic layer was washed with sat. aq. $NaHCO_3$, 1 M $CuSO_4$ and brine respectively. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (3 -> 10% acetone in DCM) yielded the title compound as an α:β mixture (23.48 g, 47.67 mmol, 95%). *α-anomer* **Rf:** 0.33 in 5% acetone in DCM. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.45 – 7.40 (m, 2H, DMT arom.), 7.33 – 7.25 (m, 5H, DMT arom.), 7.23 – 7.14 (m, 2H, DMT arom.), 6.86 – 6.78 (m, 4H, DMT arom.), 5.93 (dddd, *J* = 17.2, 10.3, 6.2, 5.2 Hz, 1H, OCH_2CHCH_2), 5.31 (dq, *J* = 17.2, 1.6 Hz, 1H, OCH_2CHCH_{2a}), 5.22 (dq, *J* = 10.4, 1.3 Hz, 1H, OCH_2CHCH_{2b}), 5.15 (d, *J* = 4.4 Hz, 1H, H-1), 4.33 (ddt, *J* = 12.9, 5.2, 1.5 Hz, 1H, $OCH_{2a}CHCH_2$), 4.29 – 4.22 (m, 1H, H-2), 4.20 – 4.07 (m, 2H, H-4 + $OCH_{2b}CHCH_2$), 4.02 – 3.96 (m, 1H, H-3), 3.77 (s, 6H, 2x CH_3 DMT), 3.31 (dd, *J* = 10.2, 3.8 Hz, 1H, H-5_a), 3.14 (dd, *J* = 10.2, 3.9 Hz, 1H, H-5_b), 3.02 (d, *J* = 9.9 Hz, 1H, 2' OH), 2.69 (d, *J* = 7.9 Hz, 1H, 3' OH). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 158.5, 144.8, 136.0, 135.9 (Cq DMT arom.), 133.9 (OCH_2CHCH_2), 130.1, 130.1, 129.2, 128.2, 127.9, 127.9, 126.9 (CH DMT arom.), 117.9 (OCH_2CHCH_2), 113.2 (CH DMT arom.), 101.2 (C-1), 86.2 (Cq DMT), 84.6 (C-4), 72.1 (C-2), 71.7 (C-3), 69.0 (OCH_2CHCH_2), 63.8 (C-5), 55.3, 55.3 (CH_3 DMT). *β-anomer* **Rf:** 0.13 in 5% acetone in DCM. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.50 – 7.44 (m, 2H, DMT arom.), 7.38 – 7.13 (m, 7H, DMT arom. + residual pyridine overlap), 6.84 – 6.78 (m, 4H, DMT arom.), 5.80 (dddd, *J* = 17.2, 10.3, 6.2, 5.2 Hz, 1H. OCH_2CHCH_2), 5.19 (dq, *J* = 17.2, 1.6 Hz, 1H, OCH_2CHCH_{2a}), 5.13 (dq, *J* = 10.4, 1.4 Hz, 1H, OCH_2CHCH_{2b}), 5.00 (d, *J* = 1.0 Hz, 1H, H-1), 4.28 (dd, *J* = 6.5, 4.8 Hz, 1H, H-3), 4.17 (ddt, *J* = 12.7, 5.1, 1.5 Hz, 1H, $OCH_{2a}CHCH_2$), 4.13 – 4.04 (m, 2H, H-3 + H-4), 3.94 (ddt, *J* = 12.7, 6.2, 1.4 Hz, 1H, $OCH_{2b}CHCH_2$), 3.77 (s, 6H, 2x CH_3 DMT), 3.32 – 3.20 (m, 2H, H-5). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 158.5, 145.0,

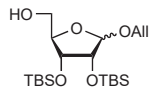
136.2 (Cq DMT arom.), 134.1 (OCH_2CHCH_2), 130.2, 130.2, 129.2, 128.3, 128.0, 126.9, 125.4 (CH arom. DMT), 117.5 (OCH_2CHCH_2), 113.3, 113.2 (CH arom. DMT), 106.4 (C-1), 86.2 (Cq DMT), 82.3 (C-4), 75.4 (C-2), 72.7 (C-3), 68.6 (OCH_2CHCH_2), 65.0 (C-5), 55.3 (CH_3 DMT). **HRMS:** $[C_{29}H_{32}O_7 + Na]^+$ found: 515.2042, calculated: 515.2040

1-*O*-allyl-5-*O*-(4,4'-dimethoxytrityl)-2,3-*O*-bis-*tert*-butyldimethylsilyl-α,β-D-ribofuranoside (5)



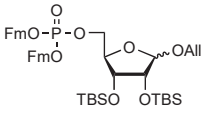
Compound **4** (23.48 g, 47.67 mmol) was co-evaporated with toluene and dissolved in DMF (240 mL, 0.2 M). Imidazole (9.74 g, 143 mmol, 3.0 eq.) and TBS-Cl (50 wt% in toluene, 50 mL, 143 mmol, 3.0 eq.) were added and the reaction was stirred overnight. The reaction was concentrated *in vacuo* and the residue was taken up in sat. aq. $NaHCO_3$ (1.000 mL). The water layer was extracted thrice with Et_2O and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (5 -> 10% Et_2O in pentane) yielded the title compound as an α:β mixture (26.05 g, 36.13 mmol, 76%). **Rf:** 0.63 (α-anomer) and 0.71 (β-anomer) in 10% Et_2O in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.57 – 7.51 (m, 2H, DMT arom. α + β), 7.48 – 7.32 (m, 5H DMT arom. α + β), 7.31 – 7.16 (m, 6H, DMT arom. α + β), 6.87 – 6.76 (m, 5H, DMT arom. α + β), 5.89 (dddd, *J* = 16.8, 10.3, 6.3, 5.1 Hz, 1H, OCH_2CHCH_2 α + β), 5.41 – 5.12 (m, 2H, OCH_2CHCH_2 α + β), 4.93 (s, 1H, H-1 β), 4.32 (ddt, *J* = 12.8, 5.2, 1.5 Hz, 1H, $OCH_{2a}CHCH_2$ α + β), 4.25 – 4.11 (m, 2H, H-3 + H-4 α + β), 4.11 – 4.03 (m, 1H, $OCH_{2b}CHCH_2$ α + β), 3.96 (dd, *J* = 3.6, 1.0 Hz, 1H, H-2 β), 3.83 – 3.73 (m, 7H, 2x CH_3 DMT α + β), 3.32 (dd, *J* = 10.2, 2.3 Hz, 1H, H-5 β), 3.02 (dt, *J* = 10.5, 5.3 Hz, 1H, H-5 β), 0.93 (s, 2H, *t*Bu TBS α), 0.91 (s, 9H, *t*Bu TBS β), 0.80 (s, 2H, *t*Bu TBS α), 0.73 (s, 9H, *t*Bu TBS β), 0.10 (dd, *J* = 8.3, 4.9 Hz, 7H 2x Me TBS α + β), -0.01 (s, 1H, Me TBS α), -0.05 (s, 3H, Me TBS β), -0.13 (s, 1H, Me TBS α), -0.20 (s, 3H, Me TBS β). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 158.5, 145.1, 136.5, 136.4 (Cq DMT arom.), 134.4 (OCH_2CHCH_2 β), 130.3, 130.3, 130.2, 128.5, 128.3, 127.9, 127.8, 126.8, 126.7 (CH DMT arom. α + β), 117.5 (OCH_2CHCH_2 β), 116.1 (OCH_2CHCH_2 α), 113.2, 113.1 (CH DMT arom. α + β), 106.4 (C-1 β), 102.0 (C-1 α), 85.8 (Cq DMT arom. β), 84.2 (C-4 α), 81.5 (C-4 β), 76.4 (C-2 β), 74.0 (C-2 α), 72.3 (C-3 β), 72.2 (C-3 α), 68.6 (OCH_2CHCH_2 β), 68.5 (OCH_2CHCH_2 α), 64.2 (C-5 β), 63.7 (C-5 α), 55.3 (CH_3 DMT α + β), 26.2, 25.9, 25.9 (CH_3 *t*Bu TBS α + β), 18.2, 18.1 (Cq *t*Bu TBS α + β), -4.0, -4.3, -4.5, -5.0 (Me TBS α + β). **HRMS:** $[C_{41}H_{60}O_7Si_2 + Na]^+$ found: 743.3767, calculated: 743.3770.

1-*O*-allyl-2,3-*O*-bis-*tert*-butyldimethylsilyl-β-D-ribofuranoside (6)



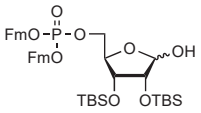
Compound **5** (26.05 g, 36.13 mmol) was dissolved in 5% TFA in DCM (180 mL total volume, 0.2 M). The reaction was stirred for 3 hours before the majority of the TFA was quenched with TEA. The reaction was diluted with DCM, washed with sat. aq. $NaHCO_3$ and brine consecutively. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (5 -> 10% EtOAc in pentane) yielded the title compound (only β-anomer, 8.49 g, 19.9 mmol, 55%). **Rf:** 0.37 in 10% Et_2O in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.02 – 5.80 (m, 1H, OCH_2CHCH_2), 5.38 – 5.16 (m, 2H, OCH_2CHCH_2), 4.81 (s, 1H, H-1), 4.28 (dd, *J* = 7.5, 4.2 Hz, 1H, H-3), 4.20 (ddt, *J* = 13.0, 5.3, 1.5 Hz, 1H, $OCH_{2a}CHCH_2$), 4.09 – 3.98 (m, 2H, H-4 + $OCH_{2b}CHCH_2$), 3.94 (d, *J* = 4.1 Hz, 1H, H-2), 3.83 (dd, *J* = 12.1, 2.5 Hz, 1H, H-5_a), 3.56 (dd, *J* = 12.1, 3.3 Hz, 1H, H-5_b), 0.96 – 0.86 (m, 18H, 2x *t*Bu TBS), 0.16 – 0.02 (m, 12H, 4x Me TBS). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 134.1 (OCH_2CHCH_2), 117.8 (OCH_2CHCH_2), 106.7 (C-1), 82.8 (C-4), 76.9 (C-2), 70.9 (C-3), 69.1 (OCH_2CHCH_2), 61.6 (C-5), 26.0, 25.9 (CH_3 *t*Bu TBS), 18.2, 18.2 (Cq *t*Bu TBS), -4.1, -4.4, -4.5, -4.9 (Me TBS). **HRMS:** $[C_{20}H_{42}O_5Si_2 + Na]^+$ found: 441.2464, found: 441.2463.

1-*O*-allyl-2,3-*O*-bis-*tert*-butyldimethylsilyl-5-(*O*-difluorenylmethyl phosphate)-β-D-ribofuranoside (7)



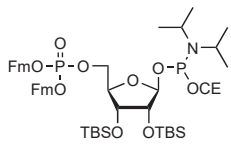
Compound **6** (570 mg, 1.36 mmol) was co-evaporated with toluene and dissolved in a 0.25 M solution of DCI in MeCN (10.9 mL, 2.73 mmol, 2.0 eq.). A 0.2 M stock solution of bis-(9H-fluoren-9-ylmethyl)-*N,N*-diisopropylamidophosphite in MeCN (8.15 mL, 1.63 mmol, 1.2 eq.) was added and the solution was stirred at room temperature. After 15 minutes of stirring, a 5.5 M solution of *t*BuOOH in nonane (2.5 mL, 13.6 mmol, 10 eq.) was added and the reaction was stirred for an additional hour. The reaction was quenched with sat. aq. $NaHCO_3$ and taken up in EtOAc. The resulting solution was washed with sat. aq. $NaHCO_3$ and brine consecutively, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (20% EtOAc in pentane) yielded the title compound as a white foam (1.04 g, 1.22 mmol, 90%). **Rf:** 0.44 in 20% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.71 (dddt, *J* = 9.7, 7.6, 2.0, 0.9 Hz, 4H, Fm arom.), 7.53 (ddq, *J* = 14.5, 7.5, 1.0 Hz, 4H, Fm arom.), 7.41 – 7.29 (m, 4H, Fm arom.), 7.29 – 7.19 (m, 6H, Fm arom.), 5.76 (dddd, *J* = 16.9, 10.3, 6.4, 5.0 Hz, 1H, OCH_2CHCH_2), 5.21 – 5.02 (m, 2H, OCH_2CHCH_2), 4.78 (s, 1H, H-1), 4.35 – 4.23 (m, 4H, 2x CH_2 Fm), 4.23 – 4.05 (m, 6H, H-3 + H-4 + H-5_a + 2x CH Fm + $OCH_{2a}CHCH_2$), 3.98 (dt, *J* = 10.6, 5.2 Hz, 1H, H-5_b), 3.91 (d, *J* = 3.8 Hz, 1H, H-2), 3.83 (ddt, *J* = 13.0, 6.4, 1.3 Hz, 1H, $OCH_{2b}CHCH_2$), 0.89 (s, 9H, *t*Bu TBS), 0.86 (s, 9H, *t*Bu TBS), 0.07 (s, 3H, Me TBS), 0.06 (s, 3H, Me TBS), 0.04 (s, 3H, Me TBS), 0.02 (s, 3H, Me TBS). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 143.3, 143.2, 141.4, 141.4 (Cq Fm), 134.1 (OCH_2C, 127.9, 127.2, 125.4, 125.3, 125.3, 120.1, 120.1, 120.0 (CH arom. Fm), 117.5 (OCH_2CHCH_2), 106.0 (C-1), 80.4, 80.3 (C-4), 76.3 (C-2), 71.8 (C-3), 69.5, 69.5, 69.4, 69.4 (CH_2 Fm), 68.2 (OCH_2CHCH_2), 67.7, 67.7 (C-5), 48.1, 48.0 (CH Fm), 25.9, 25.9 (CH_3 *t*Bu TBS), 18.2, 18.1 (Cq *t*Bu TBS), -4.1, -4.4, -4.5, -4.9 (Me TBS). **^{31}P NMR** (162 MHz, $CDCl_3$) δ -0.8. **HRMS:** $[C_{48}H_{63}O_8Si_2 + Na]^+$ found: 877.3695, found: 877.3691.

2,3-*O*-bis-*tert*-butyldimethylsilyl-5-(*O*-difluorenylmethyl phosphate)-α,β-D-ribofuranoside (8)



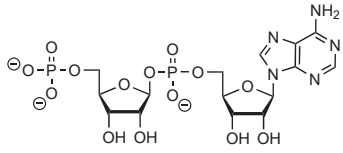
Compound **7** (1.04 g, 1.22 mmol) was dissolved in THF (6 mL, 0.2 M) and the reaction was purged with N_2. $Ir(COD)(Ph_2MeP)_2{\cdot}PF_6$ (15 mg, 0.018 mmol, 0.015 eq.) was added, followed by purging with H_2 for 15 seconds before the reaction was put under N_2 atmosphere again. The reaction was stirred for 1 hour before the reaction was diluted with 3.5 mL THF followed by the addition of 3.5 mL of sat. aq. $NaHCO_3$ and I_2 (464 mg, 1.83 mmol, 1.5 eq.). The reaction was stirred vigorously for an additional hour. The reaction was taken up in EtOAc and the organic layer was washed twice with sat. aq. $Na_2S_2O_3$ followed by brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash column chromatography (20 -> 40% EtOAc in pentane) yielded the title compound as an 2:1 α:β mixture (753 mg, 0.92 mmol,76%). **Rf:** 0.51 in 40% EtOAc in pentane. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.75 – 7.66 (m, 8H, Fm arom. α + β), 7.59 – 7.43 (m, 8H, Fm arom. α + β), 7.42 – 7.18 (m, 16H, Fm arom. α + β), 5.08 (d, J = 1.8 Hz, 1H, H-1 β), 5.01 (dd, J = 11.2, 4.1 Hz, 1H, H-1 α), 4.41 (ddt, J = 32.1, 10.0, 6.3 Hz, 2H, CH_2 Fm β), 4.35 – 4.30 (m, 1H, H-3 β), 4.30 – 4.19 (m, 5H, H-5_a $β_a$ + 2x CH_2 Fm α), 4.19 – 4.05 (m, 7H, H-4 α + CH Fm α + β +CH_2 Fm β), 4.03 – 3.94 (m, 3H, H-3 α + H-4 β + H-5_b β), 3.93 – 3.87 (m, 2H, H-2 α + β), 3.84 (dd, J = 5.9, 4.4 Hz, 2H, H-5 α), 0.94 – 0.82 (m, 36H, *t*Bu TBS α + β), 0.13 – -0.01 (m, 24H, Me TBS α + β). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 143.3, 143.2, 143.1, 143.1, 143.0, 143.0, 141.5, 141.5, 141.4, 141.4, 141.4 (Cq Fm α + β), 128.0, 128.0, 127.9, 127.2, 127.2, 127.2, 125.3, 125.2, 125.1, 125.1, 120.1, 120.1, 120.1, 120.1, 120.0 (CH Fm arom. α + β), 102.7 (C-1 β), 97.7 (C-1 α), 82.7, 82.7 (C-4 α), 80.0, 80.0 (C-4 β), 77.2 (C-2β), 73.5 (C-3 α), 72.5 (C-2 α), 70.6 (C-3 β), 70.0, 70.0, 69.6, 69.5, 69.5, 69.5, 69.4, 69.4 (CH_2 Fm α + β), 67.4, 67.4 (C-5 β), 66.8, 66.7 (C-5 α), 48.1, 48.0, 48.0, 48.0, 47.9, 47.9, 47.9, 47.8 (CH Fm α + β), 25.9, 25.9, 25.8 (CH_3 *t*Bu TBS α +β), 18.3, 18.2, 18.1, 18.0 (Cq *t*Bu TBS α + β), -4.2, -4.4, -4.5, -4.6, -4.6, -4.7, -5.0, -5.0 (Me TBS α + β). **^{31}P NMR** (202 MHz, $CDCl_3$) δ -0.8 (β), -0.9 (α). **HRMS:** $[C_{45}H_{59}O_8Psi_2 + Na]^+$ found: 837.3382, calculated: 837.3378.

1'-*O*-(2',3'-*O*-bis-*tert*-butyldimethylsilyl-5'-(*O*-difluorenylmethyl phosphate)-β-D-ribofuranoside)-2-cyanoethyl-*N*-*N*-diisopropylphosphoramidite (1)



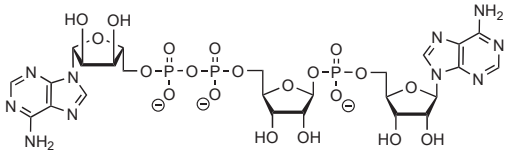
Compound **8** (2.45 g, 3.00 mmol, 1.0 eq.) was co-evaporated thrice with toluene and dissolved in anhydrous DCM (30 mL, 0.1 M). DIPEA (1.57 mL, 9.00 mmol, 3.0 eq.) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.00 mL, 4.50 mmol, 1.5 eq.) were added to the reaction and after 1.5 hours, TLC indicated full conversion of starting material. The reaction was diluted with DCM and washed with brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Automated column chromatography performed on a Biotage® Isolera™ Spektra Four system using neutral Biotage Zip (0 -> 20% Et_2O in pentane) afforded the title compound as a white foam and a mixture of diastereomers (S_P/R_P) (2.38 g, 2.34 mmol, 78%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.76 – 7.65 (m, 4H, Fm arom.), 7.57 – 7.45 (m, 4H, Fm arom.), 7.42 – 7.31 (m, 4H, Fm arom.), 7.30 – 7.20 (m, 4H, Fm arom.), 5.15 (d, J = 9.6 Hz, 1H, H-1), 4.39 – 4.08 (m, 10H, H-2/3 + H-4 + H-5 + CH_2 Fm + + CH_{2a} Fm + 2x CH Fm), 4.04 (tt, J = 10.8, 5.6 Hz, 1H, CH_{2b} Fm), 3.87 (dd, J = 31.7, 3.3 Hz, 1H, H-2/3), 3.79 – 3.61 (m, 2H, ROC$H_2$$CH_2$CN), 3.61 – 3.42 (m, 2H, 2x CH *i*Pr), 2.42 (dddd, J = 33.8, 15.5, 11.0, 6.0 Hz, 2H, ROCH$_2$CH_2CN), 1.18 – 1.05 (m, 12H, 4x CH_3 *i*Pr), 0.93 – 0.85 (m, 18H, *t*Bu TBS), 0.14 – 0.02 (m, 12H, Me TBS). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 143.3, 143.3, 143.2, 143.1, 141.5, 141.4, 141.4 (Cq Fm), 128.0, 127.9, 127.9, 127.2, 127.2, 127.2, 127.2, 125.4, 125.4, 125.3, 125.3, 125.3, 125.2 , 120.1, 120.1, 120.1, 120.0, 120.0, 120.0 (CH Fm arom.), 118.3, 117.5 (CN), 102.5, 102.4, 102.0, 101.8 (C-1), 80.3, 80.3, 79.9, 79.8 (C-4), 77.5, 77.3 (C-2/3), 71.8, 71.3 (C-2/3), 69.5, 69.4, 69.4, 69.3, 69.3 (C-5), 68.5, 68.5, 67.9, 67.8 (CH_2 Fm), 59.0, 58.9, 58.3, 58.1 (ROCH_2CH_2CN), 48.1, 48.1, 48.0, 48.0, 48.0 (CH Fm), 43.6, 43.6, 43.5, 43.5 (CH *i*Pr), 26.0, 25.9, 25.9 (CH_3 *t*Bu TBS), 24.7, 24.6, 24.6, 24.5, 24.4, 24.4 (CH_3 *i*Pr), 20.3, 20.3, 20.2 (ROCH$_2$CH_2CN), 18.1, 18.1, 18.1 (Cq *t*Bu TBS), -4.1, -4.3, -4.5, -4.5, -4.9, -4.9 (Me TBS). **^{31}P NMR** (202 MHz, $CDCl_3$) δ 149.1, 147.3, -0.9, -1.0.

5-*O*-phosphate-β-D-ribofuranoside-(5'-*O*-adenosine)-phosphate (11)



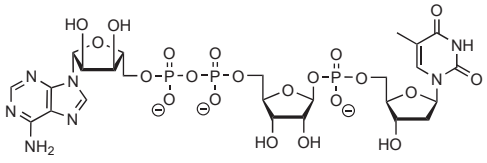
Compound **1** (51 mg, 50 µmol, 1.0 eq.) and adenosine **9** (31 mg, 60 µmol, 1.2 eq.) were co-evaporated thrice with toluene and a solution of ETT (0.25 M in MeCN, 400 µL, 100 µmol, 2.0 eq.) was added. The reaction was stirred for 15 minutes before a CSO solution (0.5 M in MeCN, 500 µL, 250 µmol, 5.0 eq.) was added. The solution was stirred for 30 minutes after which TEA (105 µL, 750 µmol, 15 eq.) was added and the solution was stirred an additional 16 hours. The reaction was concentrated *in vacuo* and co-evaporated with toluene. The resulting oil was dissolved in THF (0.5 mL, 0.1 M) and TEA·3HF (163 µL, 1.0 mmol, 20 eq.) was added. After 2 days, LC-MS analysis revealed full conversion of the starting material (Rt = 4.85 minutes in a 10 -> 90% MeCN gradient flow). To the solution, 3 mL of a 28% NH_4OH solution in water was added and the reaction was stirred overnight. The reaction was concentrated *in vacuo* and the resulting residue was taken up in Milli-Q water. The water layer was washed with EtOAc and the water layer was collected and concentrated *in vacuo*. Purification by prep-HPLC using a HILIC column and repeated lyophilization of the fractions containing product furnished the title compound as a white solid (16.4 mg, 27 µmol, 54%). **^{1}H NMR** (850 MHz, D_2O) δ 8.45 (s, 1H, adenine), 8.23 (s, 1H, adenine), 6.10 (d, J = 6.2 Hz, 1H, H-1'), 5.42 (d, J = 6.4 Hz, 1H, H-1), 4.74 (dd, J = 6.2, 5.1 Hz, 1H, H-2'), 4.50 (dd, J = 5.1, 3.1 Hz, 1H. H-3'), 4.36 (p, J = 2.8 Hz, 1H, H-4'), 4.25 (dd, J = 7.4, 4.5 Hz, 1H), 4.12 (ddd, J = 11.6, 4.7, 3.0 Hz, 1H, H-5'$_a$), 4.11 – 4.06 (m, 3H, H-2 + H-4 + H-5'$_b$), 4.03 (ddd, J = 11.3, 5.7, 3.4 Hz, 1H, H-5$_a$), 3.90 (dt, J = 11.3, 6.4 Hz, 1H. H-5$_b$). **^{13}C NMR** (214 MHz, D_2O) δ 156.0 (Cq adenine), 153.0 (CH adenine), 150.1 (Cq adenine), 140.9 (CH adenine), 119.6 (Cq adenine), 103.7 + 103.6 (C-1), 87.7 (C-1'), 85.3 + 85.2 (C-4'), 82.8 + 82.7 (C-4), 76.1 + 76.0 (C-2), 75.2 (C-2'), 71.5 (C-3'), 71.1 (C-3), 67.0 (C-5), 66.0 (C-5'). **^{31}P NMR** (202 MHz, D_2O) δ 1.1, -1.5.

(5-*O*-adenosine-diphosphate-β-D-ribosyl)-5'-*O*-adenosine phosphate (13)



Compound **1** (51 mg, 50 µmol, 1.0 eq.) and adenosine **9** (31 mg, 60 µmol, 1.2 eq.) were co-evaporated thrice with toluene and a solution of ETT (0.25 M in MeCN, 400 µL, 100 µmol, 2.0 eq.) was added. The reaction was stirred for 15 minutes before a CSO solution (0.5 M in MeCN, 500 µL, 250 µmol, 5.0 eq.) was added. The solution was stirred for 30 minutes after which TEA (105 µL, 750 µmol, 15 eq.) was added and the solution was stirred an additional 16 hours. The reaction was concentrated *in vacuo* and co-evaporated with toluene. The residue was co-evaporated thrice with toluene and compound **2** (71 mg, 100 µmol, 2.0 eq., separately co-evaporated with toluene) was added and a solution of ETT (0.25 M, 800 µL, 200 µmol, 4.0 eq.) was added. The reaction was stirred for 15 minutes before a CSO solution (0.5 M in MeCN, 800 µL, 400 µmol, 8.0 eq.) was added. The solution was stirred for 30 minutes after which DBU was added (112 µL, 750 µmol, 15 eq.) and the reaction was stirred 30 minutes. The reaction was concentrated *in vacuo* and co-evaporated in toluene. The resulting oil was dissolved in THF (0.5 mL, 0.1 M) and TEA·3HF (163 µL, 1.0 mmol, 20 eq.) was added. After 2 days, LC-MS analysis revealed full conversion of the starting material (Rt = 6.12 minutes in a 10 -> 90% MeCN gradient flow). To the solution, 3 mL of a 28% NH_4OH solution in water was added and the reaction was stirred overnight. The reaction was concentrated *in vacuo* and the resulting residue was taken up in Milli-Q water. The water layer was washed with EtOAc and the water layer was collected and concentrated *in vacuo*. Purification by prep-HPLC using a HILIC column and repeated lyophilization of the fractions containing product furnished the title compound as a white solid (1.44 mg, 1.62 µmol, 3.2%). **^{1}H NMR** (850 MHz, D_2O) δ 7.12 – 7.07 (m, 2H, adenine), 6.94 – 6.90 (m, 2H, adenine), 5.90 (d, J = 5.6 Hz, 1H, H-1 adenosine), 5.88 (d, J = 6.0 Hz, 1H, H-1 adenosine), 5.33 (d, J = 6.4 Hz, 1H, H-1 ribosyl), 4.60 – 4.55 (m, 2H, 2x H-2 adenosine), 4.40 – 4.35 (m, 2H, 2x H-3 adenosine), 4.24 – 4.19 (m, 2H, 2x H-4 adenosine), 4.11 – 4.05 (m, 3H, 2x CH_{2a} H-5 adenosine + H-3 ribosyl), 4.03 – 3.91 (m, 6H, 2x CH_{2b} adenosine + H-2 + H-4 + H-5 ribosyl). **^{13}C NMR** (peaks extracted from HSQC spectrum, 214 MHz, D_2O) δ 130.5, 130.5, 115.5, 115.5 (C-H adenine), 102.6 (C-1 ribosyl), 86.7, 86.3 (C-1 adenosine), 83.6 (C-4 adenosine), 81.5 (C-3 ribosyl), 75.0 (C-4 ribosyl), 74.4, 74.2 (C-2 adenosine), 70.1, 70.1, 70.1 (C-3 adenosine + C-2 ribosyl), 68.4, 66.8, 65.0, 65.0, 64.8 (C-5 adenosine + ribosyl). **^{31}P NMR** (202 MHz, D_2O) δ -1.4, -10.4. **HRMS:** $[C_{25}H_{35}N_{10}O_{20}P_3 + H]^+$ found: 889.1313, calculated: 889.1315.

(5-*O*-adenosine-diphosphate-β-D-ribosyl)-5'-*O*-thymidine phosphate (17)



In a fritted syringe, DMT-dT-CPG resin (300 mg, 10 µmol) was flushed with a 5 v/v% DCA solution in DCM until no yellow color appeared by addition of fresh DCA solution, indicating full removal of the DMT group. The resin was washed with anhydrous MeCN and flushed with N_2 to remove traces of water. Next, ribosyl building block **1** (0.1 M in MeCN, 400 µL, 40 µmol, 4.0 eq.) with a solution of activator 42® (0.25 M in MeCN, 600 µL, 150 µmol, 15 eq.). The resin was shaken for 10 minutes and the procedure was repeated to ensure maximum conversion. The phosphite intermediate was oxidized by treatment of the resin with a 0.3 M CSO solution (2x 10 minutes).The Fm protecting group was removed by treatment of the resin with a 10% DBU solution in MeCN (4x 5 minutes). The resin was washed with anhydrous MeCN and flushed with N_2 to remove traces of water. Next, adenosine amidite **2** (0.1 M in MeCN, 400 µL, 40 µmol, 4.0 eq.) was added to the resin, followed

by a solution of activator 42® (0.25 M in MeCN, 600 µL, 150 µmol, 15 eq.). The resin was shaken for 10 minutes and the procedure was repeated to ensure maximum conversion. The intermediate was oxidized by treatment of the resin with a 0.3 M CSO solution (2x 10 minutes). The cyano-ethyl protecting group was removed by treatment of the resin with a 10% DBU solution in MeCN (4x 5 minutes). Finally, the resin was shaken overnight in a 5 mL 28% NH_4OH solution in water. Afterwards, the resin was filtered and the filtrate was concentrated *in vacuo*. The remaining white solid was taken up in Mili-Q water (10 mL) and transferred in a tube followed by centrifugation. The supernatant was carefully collected and transferred into a round bottom flask and concentrated *in vacuo*. The residue was taken up in a 4:3:2 NMP:TEA·3HF:TEA solution (1.5 mL total volume) and the reaction mixture was transferred into a 15 mL tube. The reaction was shaken overnight and quenched by the addition of 1.5 mL of a 0.15 M NH_4OAc buffer. The resulting solution was directly injected on a HW40 column and eluted with NH_4OAc buffer. Fractions containing the product were concentrated and co-evaporated with 1:1 MeCN:Milli-Q water and lyophilized yielding the title compound as a white solid (1.07 mg, 1.24 µmol, 12%). **^{1}H NMR** (850 MHz, D_2O) δ 8.49 (s, 1H, adenine), 8.21 (s, 1H, adenine), 7.64 – 7.59 (m, 1H, thymine), 6.18 (dd, J = 8.3, 6.1 Hz, 1H, H-1' thymidine), 6.08 (d, J = 5.7 Hz, 1H, H-1' adenosine), 5.43 (d, J = 6.5 Hz, 1H, H-1' ribosyl), 4.70 (t, J = 5.4 Hz, 1H, H-2' adenosine), 4.57 (dt, J = 5.1, 2.3 Hz, 1H, H-3' thymidine), 4.49 (dd, J = 5.1, 3.7 Hz, 1H, H-3' adenosine), 4.35 (d, J = 3.4 Hz, 1H, H-4' adenosine), 4.30 (dd, J = 7.2, 4.4 Hz, 1H, H-3' ribosyl), 4.20 (s, 2H, H-5' adenosine), 4.18 – 4.08 (m, 4H, H-2' ribosyl + H-4' ribosyl + H-4' thymidine + CH_{2a} thymidine/ribosyl), 4.06 (dt, J = 11.4, 3.9 Hz, 1H, CH_{2a} thymidine/ribosyl), 4.04 – 3.98 (m, 2H, CH_{2b} thymidine + CH_{2b} ribosyl), 2.25 – 2.16 (m, 2H, H-2' thymidine), 1.83 (s, 3H, CH_3 thymine). **^{13}C NMR** (214 MHz, D_2O): δ 167.4 (C=O thymine), 155.6 (Cq adenine/thymine), 152.5 (CH adenine), 149.9 (Cq adenine/thymine), 141.1 (CH adenine, signal taken from HSQC) 138.1 (CH thymine), 119.5, 112.7, 112.5 (Cq adenine/thymine), 103.6 (C-1' ribosyl), 88 (C-1' adenosine).0, 86.9 (C-4' ribosyl), 86.0 (C-1' thymidine), 84.8 (C-4' adenosine), 82.5 (C-4' thymidine), 76.2 (C-2' ribosyl), 75.4 (C-2' adenosine), 72.5 (C-3' thymidine), 71.2, 71.2 (C-3' ribosyl + C-3' adenosine), 67.8, 66.4 (C-5' ribosyl + C5' thymidine), 66.1 (C-5' adenosine), 39.7 (C-2' thymidine), 12.6 (CH_3 thymine) . **^{31}P NMR** (202 MHz, D_2O, no EDTA was added): δ -1.7, -10.5. **HRMS:** $[C_{25}H_{36}N_7O_{21}P_3 + H]^+$ found: 864.1246, calculated: 864.1250.

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