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2',3'-protected nucleotides as building blocks for enzymatic *de novo* RNA synthesis

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Key words: RNA; modified nucleotides; polymerases; controlled enzymatic synthesis; *de novo* RNA synthesis

Abstract

Besides being a key player in numerous, fundamental biological processes, RNA also represents a versatile platform for the creation of therapeutic agents and efficient vaccines. The production of RNA oligonucleotides, especially those decorated with chemical modifications, cannot meet the exponential demand. Due to the inherent limits of solid-phase synthesis and *in vitro* transcription, alternative, biocatalytic approaches are in dire need to facilitate the production of RNA oligonucleotides. Here, we present a first step towards the controlled enzymatic synthesis of RNA oligonucleotides. We have explored the possibility of a simple protection step of the vicinal *cis*-diol moiety to temporarily block ribonucleotides. We demonstrate that pyrimidine nucleotides protected with acetals, particularly 2',3'-*O*-isopropylidene, are well-tolerated by the template-independent RNA polymerase PUP (polyU polymerase) and highly efficient coupling reactions can be achieved within minutes – an important feature for the development of enzymatic *de novo* synthesis protocols. Even though purines are not equally well-tolerated, these findings clearly demonstrate the possibility of using *cis*-diol-protected ribonucleotides combined with template-independent polymerases for the stepwise construction of RNA oligonucleotides.

Introduction

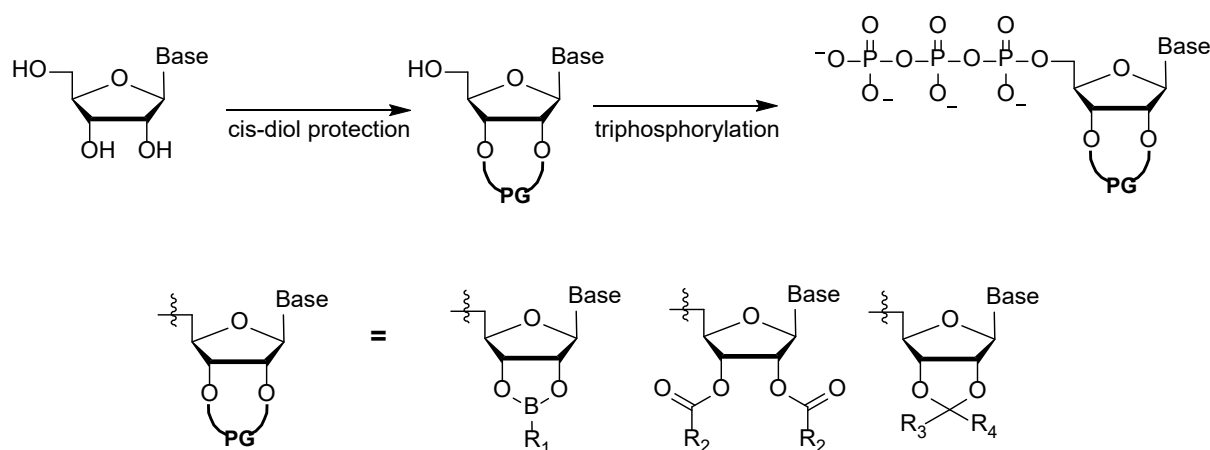
In the early 1990s, RNA oligonucleotides were conceived as promising candidates for the development of therapeutics yet difficult to handle, poorly understood, and challenging to synthesize. The recent FDA-approval of several siRNA drugs and the advent of mRNA vaccines (rewarded by the Nobel Prize in Physiology and Medicine in 2023) have dramatically changed this early vision and thrust RNA in the forefront of drug discovery.^[1] Nonetheless, RNA therapeutics could benefit from a better understanding of the mechanisms underlying existing modalities such as siRNAs, anti-miR, or mRNA vaccines but also from poorly understood RNA molecules such as non-coding RNAs^[2] and other regulatory RNAs^[3] as well as the effect of each of the >100 naturally occurring RNA modifications.^[4] A better understanding of RNA biology however, is tightly tied to our capacity at producing RNA oligonucleotides with or without chemical modifications in both high yields and purity. So far, short RNAs are mainly produced chemically by assembling phosphoramidite building blocks on immobilized nucleosides.^[5] While this method represents the workhorse for the production of therapeutic siRNAs and antisense oligonucleotides,^[6] developing more efficient and sustainable alternatives granting access to longer RNA oligonucleotides remains an important challenge.^[7] Indeed, success of solid-phase assembly of RNA oligonucleotides relies on the careful choice of the 2'-O-protecting groups which directly affects the reactivity of phosphoramidite building blocks.^[8] In addition, chemical synthesis is highly efficient for shorter (i.e. 10-60 nt) sequences, but yields of production rapidly decrease with increasing length of the oligonucleotides.^[9] Chemical synthesis of natural and modified RNA is also impinged by sustainability issues since poor atom economy and the necessity for intricate protecting group strategies generates large amounts of chemical waste.^[10] Alternatively, RNAs can be produced by *in vitro* transcription reactions using nucleoside triphosphates (NTPs) and RNA polymerases.^[11] This highly potent method can be used for the identification of functional nucleic acids via the SELEX (Systematic Evolution of Ligands by EXponential enrichment) protocol,^[12] but also for the industrial-scale production of therapeutic oligonucleotides as showcased by mRNA vaccines during the COVID 19 pandemics.^[13] Notwithstanding these favorable assets, enzymatic synthesis depends on the recognition of modified nucleotides by polymerases and control of the localization of chemically altered nucleotides within the sequence is limited. Hence, alternative methods for the production of modified and unmodified RNA oligonucleotides are in dire need.^[7] Biocatalysis represents an alluring option to improve the sustainability, the purity and yield of oligonucleotide production while simultaneously enabling the control of modification localization. Various biocatalytic strategies have been recently proposed for nucleic acid *de novo* synthesis.^[14] In controlled enzymatic synthesis, temporarily blocked nucleotides are added sequentially at the 3'-termini of immobilized primers by (mainly template-independent) polymerases. Robust protocols have been devised for the *de novo* synthesis of DNA by controlled enzymatic synthesis,^[9b, 15] but this approach has been vastly overlooked for the production of chemically modified oligonucleotides^[16] as well as RNAs.^[17] Challenges in the development of versatile and reliable protocols for efficient controlled enzymatic synthesis of RNA oligonucleotides reside in the identification of suitable sugar and/or nucleobase protecting groups which need to be compatible with RNA polymerases as well as sufficiently robust to withstand enzymatic synthesis but at the same time labile enough to allow for facile and rapid deprotection. Herein, we have explored the possibility of using protecting groups for the *cis*-2',3'-diol of ribonucleosides to generate temporarily blocked nucleotides compatible with controlled enzymatic RNA synthesis. Of the different protecting group strategies that were evaluated, we found that 2',3'-O-isopropylidene-protected pyrimidine triphosphates were incorporated quantitatively within minutes by the template-independent RNA polymerase polyuridine polymerase (PUP). The corresponding

purine nucleotides were more reluctant at acting as substrates for both the PUP and polyadenosine polymerase (PAP) but could still be useful synthons for the production of RNA under controlled enzymatic synthesis conditions. Collectively, we demonstrate a first step towards the step-by-step production of RNA oligonucleotides by incorporating *cis*-2',3'-diol-protected nucleotides into RNA using template-independent polymerases. *Cis*-diol reversible terminators could be directly amenable to the production of unmodified, as well as base- and phosphate-modified RNA oligonucleotides.

Results

2. Design and synthesis of blocked nucleosides and nucleotides

The design of RNA nucleotides equipped with reversible blocking groups presents an additional challenge compared to the corresponding DNA or xenonucleic acids (XNA) nucleoside triphosphates. Indeed, strictly speaking only the 3'-OH moiety of the nucleotide requires a masking group to prevent further incorporation events from occurring but this in turn requires a selective protection of the vicinal *cis*-diol pattern which is notoriously challenging.^[8a, 18] This inherent difficulty mainly stems from the ease of 2',3'-migration during synthesis, lower reactivity of the secondary alcohols at the 2'/3'-positions compared to 5'-OH, and the need for orthogonality with other blocking groups on the nucleosidic scaffold.^[19] In order to reduce synthetic efforts and streamline the production of blocked RNA nucleotides, we opted for a single, *cis*-2',3'-diol-protection step followed by conversion to the corresponding nucleotides (Scheme 1). We also surmised that dual protection of hydroxyl moieties would prevent the formation of 2'-5'-branched RNA from occurring due to the transient blocking of both reactive moieties.

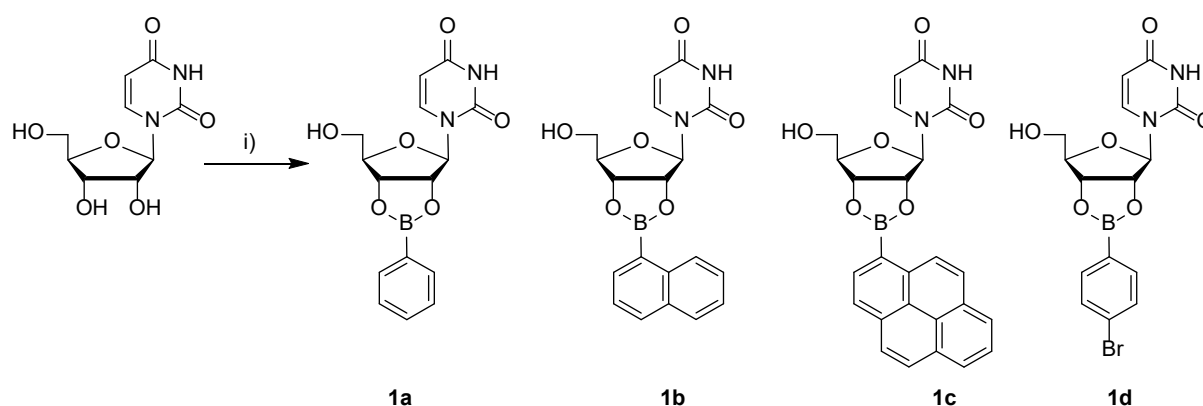


Scheme 1. Description of the rationale involving *cis*-2',3'-diol-protection of ribonucleosides/ribonucleotides and chemical structures of the explored protecting groups.

In addition to opting for a *cis*-2',3'-diol-protection strategy, we decided to explore various typical, well-established blocking groups commonly used in RNA chemistry such as boronic acids,^[20] esters,^[21] and acetals (Scheme 1).^[22]

First, we turned our attention to the formation of uridine nucleosides functionalized with reversible boronic esters (Scheme 1). Boronic acids selectively react with vicinal diols to form five-membered cyclic esters, which made them popular transient protecting groups in synthetic routes to modified ribonucleosides.^[20a, d] Consequently, boronic esters appears as interesting

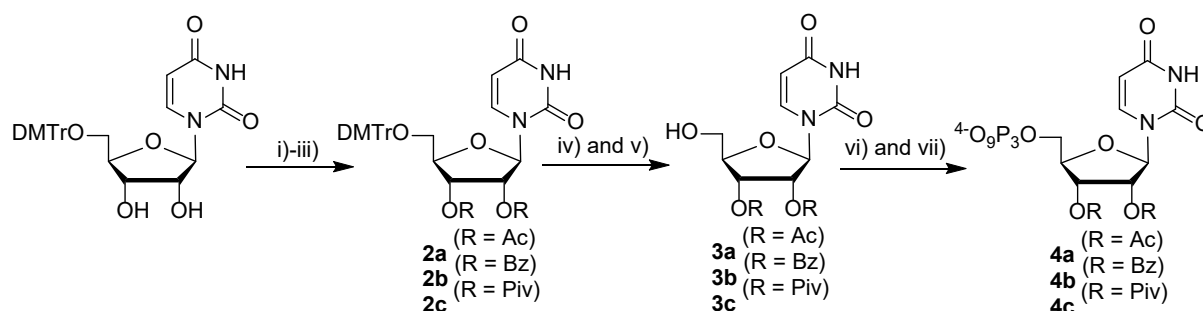
alternatives for enzymatic RNA synthesis due to their compatibility with vicinal diols, relative stability in aqueous conditions, and ease of deprotection. The presence of bulky groups or substituted aromatic residues can stabilize boronic esters against hydrolytic degradation.^[23] As a compromise between enhanced stability and compatibility with polymerase-mediated synthesis, we dwelled on aryl boronates as a choice for potential temporary masking groups. In addition, we considered various substitution patterns on the aromatic moiety of phenyl boronic acid (**1a**) including electron withdrawing elements (**1d**) and extended aromatic systems such as naphthyl (**1b**), and pyrenyl (**1c**) moieties (Scheme 2). The synthesis of the corresponding boronic esters was straightforward and involved treatment of uridine with the corresponding boronic acid in the presence of MgSO₄ to quench the released water molecule. After precipitation in acetonitrile, the corresponding esters were isolated in moderate (~40%) to good (~80%) yields. Boronic esters often display limited stability in aqueous media due to hydrolytic degradation via transesterification or protodeborination.^[20a, 24] Hence, we carried out an ¹H NMR comparative study to investigate whether nucleosides **1a-1d** are stable in water, an important prerequisite for storage and enzymatic reactions with the corresponding nucleotides. To do so, nucleosides **1a-1c** were incubated in a DMSO-*d*₆:D₂O mixture at room temperature for 90 min and ¹H NMR spectra were compared to that of unreacted uridine and the parent boronic esters (Fig. S1-S3, Supporting Information). Under these conditions, all nucleosides were cleanly and completely converted to uridine. Hence, while boronic esters are clearly not suitable as temporary masking groups in enzymatic RNA synthesis, they might represent versatile, transient protecting groups for the preparation of RNA nucleoside analogues given the ease of preparation and the mild deprotection conditions.



Scheme 2. Synthesis of uridine nucleosides with boronic ester protecting groups. Reagents and conditions: i) a) RB(OH)₂, pyridine, reflux, 4-5 h, MgSO₄; b) Precipitation in CH₃CN; 39% (**1a**), 43% (**1b**), 87% (**1c**), 74% (**1d**).

We next considered the possibility of using esters as temporary blocking groups for RNA nucleotides. We have previously reported the compatibility of locked nucleic acid (LNA) nucleotides equipped with various 3'-O-ester moieties.^[16b, c] From these studies, we concluded that 1) esters such as allyl or nitrobenzoyl were not sufficiently robust to be considered as blocking groups of nucleotides; 2) very bulky groups such as mesityl were not tolerated by DNA polymerases; and 3) esters of intermediate size and robustness such as benzoyl and pivaloyl were compatible with enzymatic synthesis despite some hydrolysis caused by the esterase activity of DNA polymerases.^[16a, 25] Based on these considerations, we opted to equip uridine with benzoyl (**2b**) and pivaloyl (**2c**) esters as temporary blocking groups. In addition, since esterase activity has been observed for DNA but not for RNA polymerases, we also considered acetyl (**2a**) as a blocking group. We therefore first prepared the suitably protected nucleosides **2a-2c** by treating directly commercially available 5'-O-DMTr-uridine with either acyl chlorides or acetic anhydride under optimized conditions to minimize N³-acylation

(Scheme 3). The resulting nucleosides were then treated under mild acidic conditions to remove the trityl protecting group and the resulting nucleosides **3a-3c** were converted to triphosphates using either the method based on chlorophosphorinone^[26] (for **4a** and **4b**) or POCl₃^[27] (for **4c**) as phosphorylation reagents. Interestingly, *N*3-benzoyl-bis-2',3'-*O*-benzoyl-uridine, which arises as a side-product of the esterification reaction, was highly refractory to the phosphorylation reactions and no conversion to triphosphate could be observed.

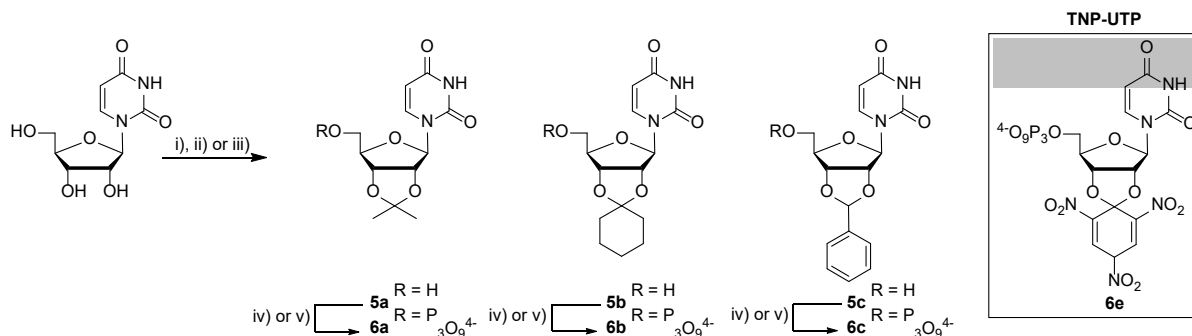


Scheme 3. Synthesis of ester-blocked nucleosides (**3a-3c**) and nucleotides (**4a-4c**). Reagents and conditions: i) Ac₂O, pyridine, rt, 12 h, 85% (**2a**), ii) BzCl, DMAP, DCM, Et₃N, rt, 5.5 h, 82% (**2b**), iii) PivCl, pyridine, rt to 50°C, 7 days, 40% (**2c**); iv) TFA, DCM, rt, 30 min, 80% (**3a**) and 56% (**3c**), v) DCA (2%) in DCM, rt, 25 min, quant. (**3b**); vi) 1) 2-chloro-1,3,2-benzodioxaphosphorin-4-one (1.2 eq.), Pyridine/dioxane (2:1), 0°C, 4.5 h; 2) (nBu₃NH)₂H₂P₂O₇ (1.2 eq.), nBu₃N (1.2 eq.), DMF, 0°C, 2.5 h; 3) I₂ (1.2 eq.), pyridine/H₂O (95:5), rt, 20 min; 1% (**4a**) and 22% (**4b**), vii) 1) POCl₃ (1.1 eq.), PO(OMe)₃, 0°C, 4 h, 2) (nBu₃NH)₂H₂P₂O₇ (1.1 eq.), nBu₃N (1.1 eq.), DMF, 0°C, 1 h; 3) TEAB 1 M, rt, 30 min, 4% (**4c**).

Lastly, besides esters, acetals such as isopropylidene are common protecting groups for the vicinal *cis*-diol pattern of RNA nucleosides and nucleotides. These groups can easily be installed on RNA nucleosides by reacting the corresponding ketones and diols under acidic conditions or by transketalization.^[28] In addition to facile preparation, we surmised that acetals protecting groups might resist against a potential esterase activity of RNA polymerases. Consequently, we set out to synthesize uridine nucleosides and nucleotides equipped with 2',3'-*O*-isopropylidene (**5a** and **6a**), 2',3'-*O*-cyclohexylidene (**5b** and **6b**), and 2',3'-*O*-benzylidene (**5c** and **6c**) moieties (Scheme 4). We also included a 2-(methoxycarbonyl)ethylidene (or Moc-ethylidene) acetal protecting group (**10** and **6d**) since this moiety can be cleaved under basic rather than (often strongly) acidic conditions typical for ketal removal (Scheme 5).^[29] We also included commercially available 2',3'-*O*-trinitrophenyl uridine 5'-triphosphate (TNP-UTP) in this study. While TNP-nucleotides are usually employed as probes to target nucleotide-binding proteins such as enzymes, receptors and structural proteins with an affinity in the micromolar range,^[30] they have never been considered as substrates for template-independent RNA polymerases and strictly speaking are also acetal-blocked nucleotides.

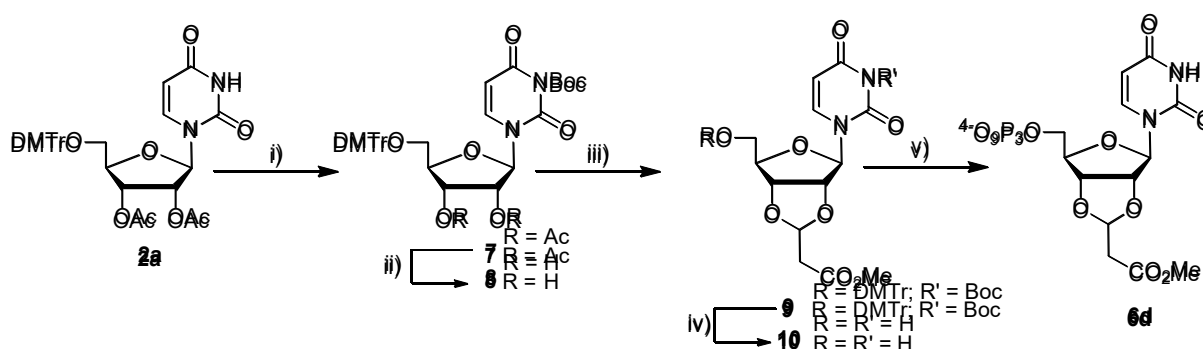
Synthetic routes to acetal-blocked nucleotides **6a-6c** follows standard protocols. Briefly, uridine was converted to the corresponding 2',3'-*O*-isopropylidene-protected nucleoside by treatment with 2,2-dimethoxypropane under acidic conditions in good yields (72%). Nucleotide **6a** was obtained by phosphorylation with a P(III) reagent (6% yield) or POCl₃ (16%). Nucleoside **5b** was obtained in good yields (72%) by reacting uridine directly with cyclohexanone under acidic conditions and triphosphorylation led to the isolation of nucleotide **6b** in low yields (5% regardless of the method). Similarly, when uridine was treated with benzaldehyde under acidic conditions, acetal **5c** could be obtained in acceptable (37%) yields. An increase in reaction

time did not improve the yield of this conversion since degradation of product was observed (data not shown). Finally, nucleotide **6c** was obtained by triphosphorylation of precursor **5c** in low yields (6%) as an inseparable mixture of stereoisomers.



Scheme 4. Synthesis of acetal-protected nucleosides (**5a-5c**) and nucleotides (**6a-6c**). Reagents and conditions: i) 2,2-dimethoxypropane, APTS, acetone, 0°C to reflux, 2h, 72% (**5a**); ii) cyclohexanone, APTS, 50°C, 4h, 82% (**5b**); iii) benzaldehyde, APTS, molecular sieves, 70°C, 12h, 37% (**5c**); iv) 1) 2-chloro-1,3,2-benzodioxaphosphorin-4-one (1.2 eq.), Pyridine/dioxane (2:1), 0°C, 4h30; 2) (nBu₃NH)₂H₂P₂O₇ (1.2 eq.), nBu₃N (1.2 eq.), DMF, 0°C, 2h30; 3) I₂ (1.2 eq.), pyridine/H₂O (95:5), rt, 20min; 6% (**6a**) and 5% (**6b**), v) 1) POCl₃ (1.1 eq.), PO(OMe)₃, 0°C, 4h, 2) (nBu₃NH)₂H₂P₂O₇ (1.1 eq.), nBu₃N (1.1 eq.), DMF, 0°C, 1h; 3) TEAB 1M, rt, 30 min, 16% (**6a**), 5% (**6b**), and 6% (**6c**).

Synthesis of Moc-ethylidene-protected UTP **6d** required first protection of the *N3*-position of the nucleobase **2a**,^[31] to avoid undesired alkylation.^[29] To do so, we converted nucleoside **2a** (Scheme 3) to the corresponding *N3*-Boc-protected analog **7** under standard conditions (Scheme 5). Removal of the *O*-acetyl protecting group under basic conditions^[32] followed by treatment with methyl propynoate in the presence of catalytic amounts of DMAP yielded nucleoside **9** as an inseparable mixture of stereoisomers.^[29] Deprotection of the DMTr and Boc protecting groups produced **10** in good yields. Deprotection of **9** with 5% DCA in DCM for 45 min in the absence of Et₃SiH only led to removal of the DMTr group in 50% yield (data not shown). Finally, nucleotide **6d** was obtained by application of the Ludwig-Eckstein protocol in moderate yields (17%).



Scheme 5. Synthesis of acetal-protected nucleoside **10** and nucleotide **6d**. Reagents and conditions: i) Boc₂O, Et₃N, DMAP, Pyridine, rt, 1h, 88% (**7**); ii) NH₃ in MeOH (4M), 5°C to rt, 3h, 86% (**8**); iii) Methyl propynoate, DMAP, CH₃CN, rt, 30 min, 78% (**9**); iv) 5% TFA in DCM, Et₃SiH, 4-5h, rt, 76% (**10**); v) 1) 2-chloro-1,3,2-benzodioxaphosphorin-4-one (1.2 eq.),

Pyridine/dioxane (2:1), 0°C, 4h30; 2) (nBu₃NH)₂H₂P₂O₇ (1.2 eq.), nBu₃N (1.2 eq.), DMF, 0°C, 2h30; 3) I₂ (1.2 eq.), pyridine/H₂O (95:5), rt, 20min; 17% (**6d**).

3. Biochemical characterization of 2',3'-O-blocked UTPs

With ester- and acetal protected uridine triphosphates **4a-4c** and **6a-6d**, respectively at hand, we next sought to evaluate their capacity at acting as substrates in the context of controlled RNA synthesis. To this effect, our group^[16c] and the laboratory of Church^[17] have independently identified the template-independent PUP and PAP polymerases as suitable candidates. Indeed, these polymerases are capable of acting like the TdT does with 3'-O-blocked DNA nucleotides^[15a-c, 33] and appear to be quite tolerant to modified nucleotides.^[34] Moreover, we surmised that PUP could be employed for the introduction of blocked pyrimidine nucleotides while PAP would be required for the incorporation of similarly modified purines. To verify this hypothesis, we first set out to compare the substrate tolerance of both RNA polymerases with canonical nucleotides. To do so, we performed primer extension (PEX) reactions using a 5'-FAM-labelled, 18 nucleotide long RNA primer (5'-FAM-CAG UCG GAU CGC AGU CAG-3') and each individual rNTP along with each of the template-independent RNA polymerases (Fig. S4A). Gel analysis (PAGE 20%) reveals that PUP equally well-tolerates UTP and ATP as substrates since robust tailing activities could be observed albeit the reaction with ATP led to larger product dispersities and lower size averages, consistent with previous reports.^[35] On the other hand, the PUP was rather reluctant at accepting CTP and GTP as substrates, nonetheless several incorporation events could be observed with full consumption of the primer which would be sufficient for controlled enzymatic synthesis applications. Unsurprisingly, the PAP readily incorporates rA and rC nucleotides into RNA primers.^[36] Overall, the substrate preference for PUP decreases in the order rU~rA > rC > rG and rA > rC > rU > rG for PAP. We also investigated the effect of UTP concentration on the tailing reaction efficiency of the PUP (Fig. S4B). As observed for the TdT,^[37] the tailing efficiency of the PUP-catalyzed reaction strongly increases with UTP concentration when enzyme and initiator (primer) are both kept at a constant concentration.

We next turned to evaluate the substrate tolerance of the blocked nucleotides with template-independent RNA polymerases. First, we evaluated the possibility of using ester-protected UTPs **4a-4c** as substrates for the PUP polymerase to mediate single incorporation events. Analysis of the products stemming from the reaction of **4a** with both PUP and PAP displayed a product distribution reminiscent of that obtained with unmodified UTP (Fig. S5A). This result is consistent with the rather low hydrolytic stability observed for a related 3'-O-Ac-LNA-T nucleoside.^[16b] Surprisingly, equipping UTP with benzoyl (Fig. S6) and pivaloyl (Fig. S5B) masking groups, which had been identified as suitable for single incorporation of LNA-T nucleotides,^[16b] led to low conversion yields (< 40%) and production of n+2 and n+3 side-products or completely abrogated substrate acceptance by the polymerase, respectively.

We next assayed the acetonide-modified nucleotides **6a-6e** under similar PEX reactions with PUP and PAP. We first carried out PEX reactions by supplementing the mixtures with different divalent metal cofactors (Mn²⁺, Co²⁺, Mg²⁺, and Zn²⁺) and nucleotide **6a**. Gel analysis (**Figure 1**) revealed that full conversion of the primer to the corresponding n+1 product could be achieved with little formation of n+2 and degradation products when **6b** was incubated with PUP and Mn²⁺ for 5 h at 37°C (Figure 1). Degradation might occur via an exonuclease activity of the PUP which has been suggested previously^[38] or due to pyrophosphorolysis triggered by the poor substrate tolerance of the polymerase for **6b**.^[39] This analysis also revealed that the

reaction mixtures supplemented with manganese led to the highest conversion yields. We also confirmed the necessity of adding Mn^{2+} by performing PEX reactions without adding any metal cofactor (Fig. S7A). Moreover, lowering the RNA primer concentration led to a large product distribution suggesting an increased rate of deprotection of the modified UTP (Fig. S7B).

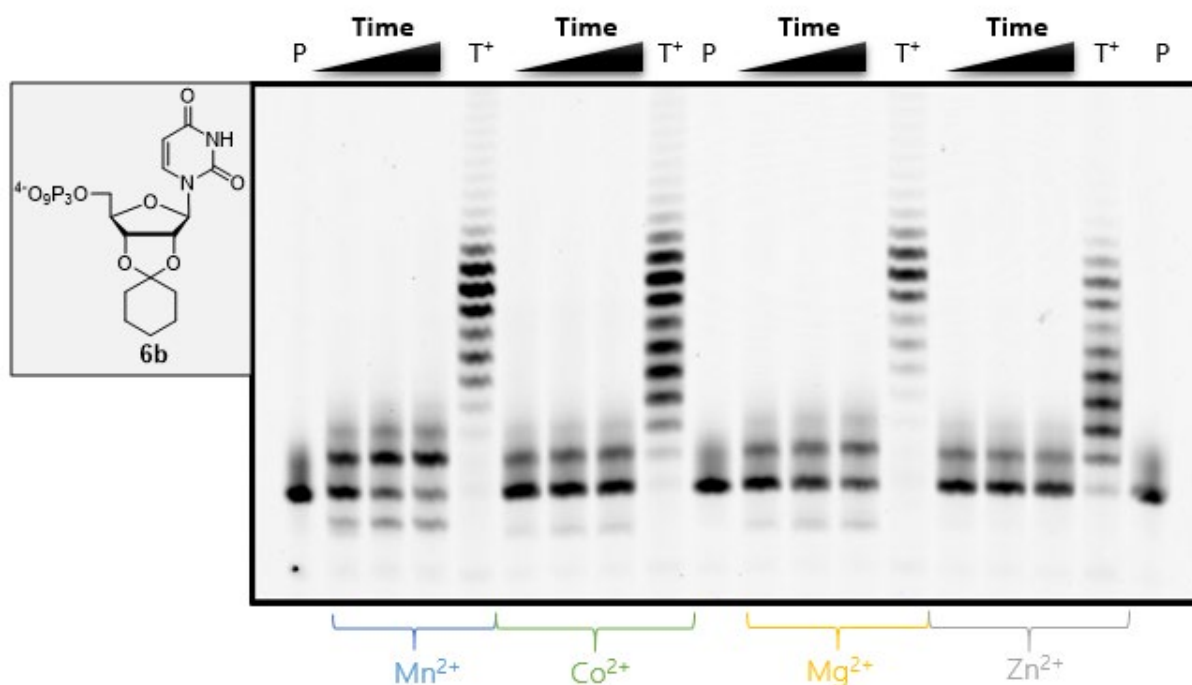


Figure 1. Gel (PAGE 20%) analysis of PEX reaction of 2',3'-O-cyclohexylidene-UTP **6b**. All reactions were incubated with 1 mM of **6b**, 20 pmol of RNA primer, 10 U of PUP, 1 mM of metal (Mn^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} from left to right), 20 U RNase Murine Inhibitor varying reaction time (i.e. 1 h, 3 h, 5 h) at 37°C. Control reactions were carried out in the presence of unmodified UTP for 1h (T+) or primer only (P).

We next evaluated the substrate tolerance of nucleotides **6a** and **6c** equipped with 2',3'-O-isopropylidene and 2',3'-O-benzylidene blocking groups, respectively under the best conditions identified for **6b** with the PUP and PAP polymerases (Fig. 2). Reactions carried out with nucleotide **6c** and the PUP mainly led to the formation of the desired n+1 product with only little n+2 and n+3 by-products. However, conversion of the primer was only in the ~50-70% range. On the other hand, PEX reactions with **6a** and catalysed by the PUP led to more complex product distributions even though the n+1 product also appeared as the main product. Expectedly, the PAP did not readily accept either of these nucleotides as substrates and produced the extended n+1 primer only in moderate yields (~40%).

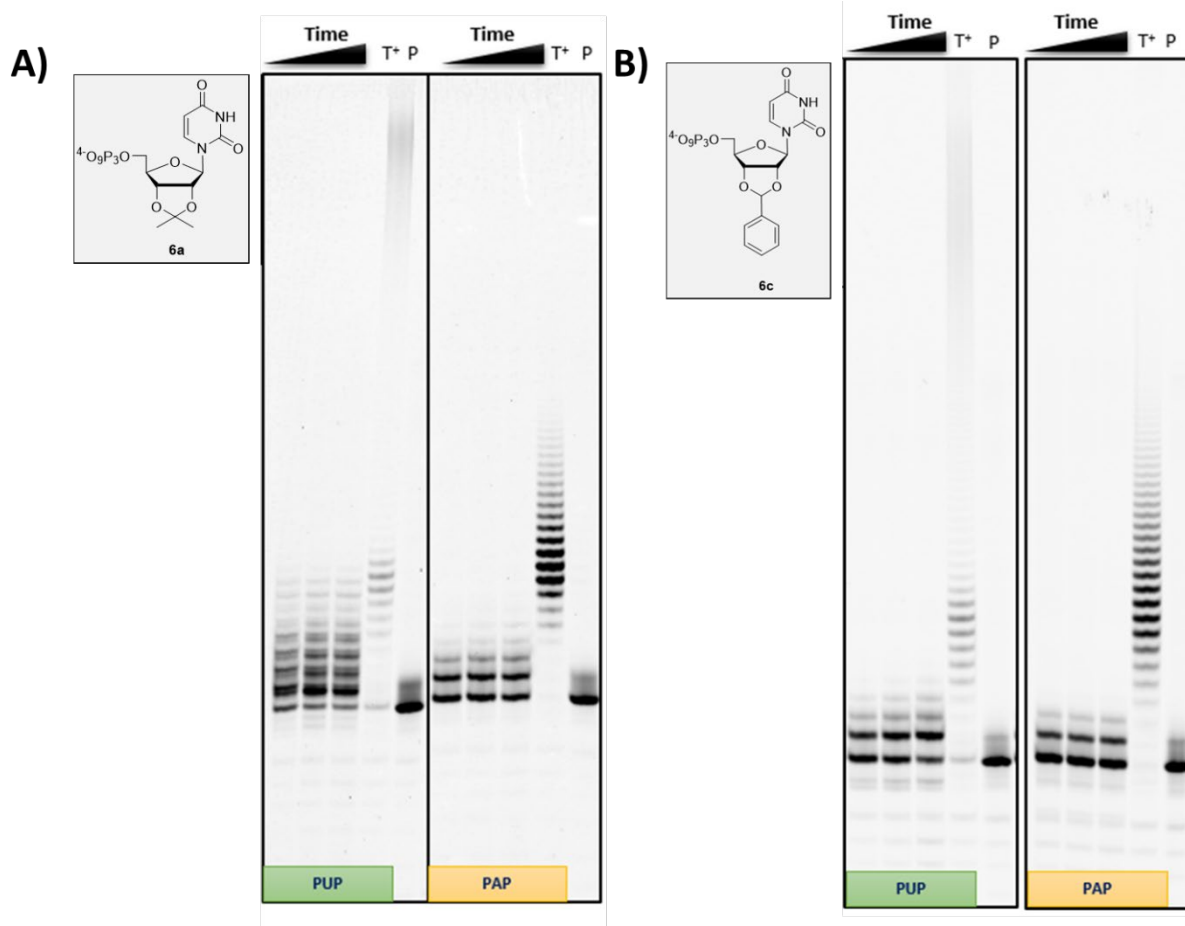


Figure 2. Gel (PAGE 20%) analysis of PEX reaction of 2',3'-O-isopropylidene-UTP **6a** and 2',3'-O-benzylidene-UTP **6c**. All reactions were incubated with 1 mM of N*TP, 20 pmol of RNA primer, 10 U of PUP or PAP, 1 mM of Mn²⁺, 20 U RNase Murine Inhibitor varying reaction time (i.e. 1 h, 3 h, 5 h) at 37°C. Control reactions were carried out in the presence of unmodified UTP for 1 h (T+) or primer only (P).

Encouraged by these initial results, we next sought to optimize the experimental conditions to exclusively produce primers extended by a single modified nucleotide and minimize the rate of hydrolysis of the blocking groups. To do so, we first considered the addition of the crowding agents DMSO and PEG to the reaction mixtures.^[40] When PEX reactions with **6a-6c** were supplemented with either 10% DMSO (Fig. S8A), 20% PEG, or a mixture of DMSO and PEG (Fig. S8B), we observed a marked decrease in n+1 product formation for all conditions. After excluding crowding agents from the optimization parameters, we next evaluated the effect of nucleotide concentration on the outcome of the PEX reactions. We first varied the concentration of nucleotide **6c** in PEX reactions and observed that lower concentrations (i.e. 500 μ M) led to near quantitative conversion of primer to the n+1 product (Fig S9). Even though this finding was surprising since the efficiency of tailing reactions with natural and modified nucleotides usually increases with nucleotide concentration,^[16b, 41] we lowered the range of concentration to 50-500 μ M. Gratifyingly, conditions could be found where the primer was cleanly converted to the expected n+1 product for all three nucleotides **6a-6c** (Fig. 3). Interestingly, analysis of the PEX reactions conducted with **6a** revealed quantitative conversion to the extended primer within 60 min and at concentrations as low as 50 μ M (Fig. 3A). On the other hand, PEX reactions with nucleotide analogs **6b** and **6c** required longer reaction times and slightly higher concentrations to achieve similar conversion efficiencies.

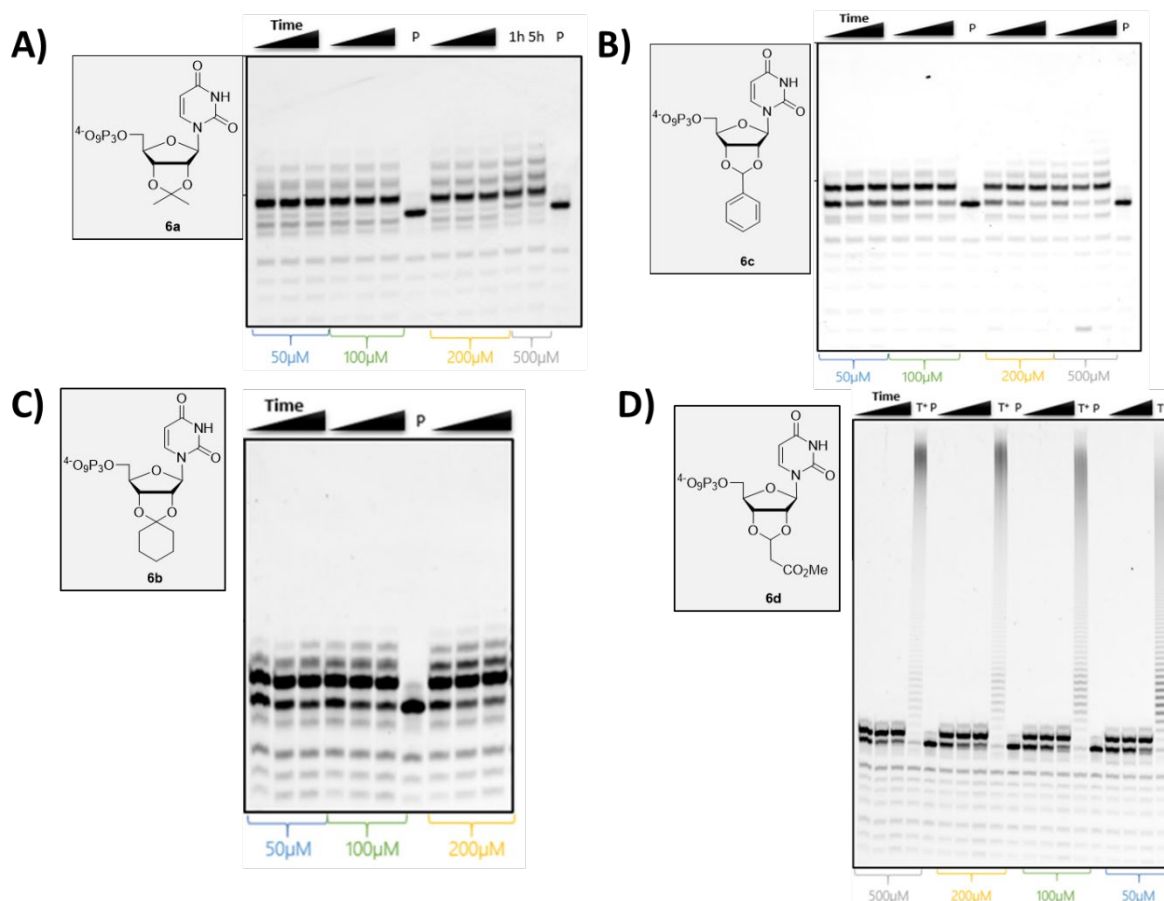


Figure 3. Gel (PAGE 20%) analysis of PEX reaction of N*TP-UTP **6a**, **6b**, **6c** and **6d** with concentration from 50 to 500 μM . All reactions were incubated with 10 pmol of RNA primer, 10 U PUP, 1 mM of Mn^{2+} , 20 U RNase Murine Inhibitor varying reaction time A), B) and C) (i.e. 1 h, 3 h, 5 h) or D) (i.e. 30 min 1 h, 3 h) at 37°C. Primer only (P).

We next applied similar reaction conditions to Moc-ethylidene-protected UTP **6d** (Fig. 3D). Also for this nucleotide analogue, the highest primer conversion yields ($\sim 90\%$) were achieved when the concentration of the incoming triphosphate was kept low (50 μM) albeit with longer reaction times (5 h). We also applied these low triphosphate conditions to ester-blocked nucleotides **4a** and **4c**, which were hydrolysed or not accepted by the polymerase, but to no avail since the PEX reactions either led to the formation of larger distribution of side-products or completely abrogated substrate acceptance by the polymerase, respectively (Fig. S10). A similar outcome was observed when 2',3'-O-trinitrophenyl-UTP **6e** was used in conjunction with the PUP suggesting a polymerase-mediated hydrolysis of the blocking group (Fig. S11).

Having identified a protecting group that is compatible with PUP-mediated RNA synthesis, we next investigated the possibility of reducing the reaction time. Indeed, for efficient *de novo* RNA synthesis, suitable nucleotides need to be accepted by the polymerase, the reactions should be quantitative, and coupling times should be as short as possible. Hence, we carried out PEX reactions with nucleotide with reaction times in the 5 to 60 min range (Fig. 4). This analysis revealed that 1) when nucleotide concentration was kept at 50 μM , the reaction was already complete within 15 min (Fig. 4A) and 2) concentrations as low as 20 μM were still compatible with high yielding nucleotide incorporation, albeit with slightly longer reaction times (30 to 45 min; Fig. 4B).

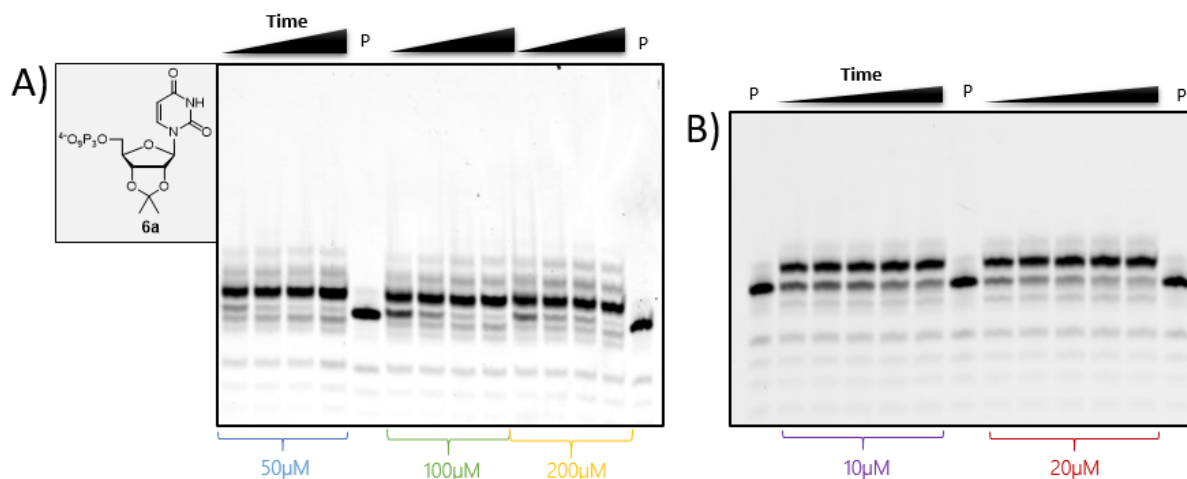
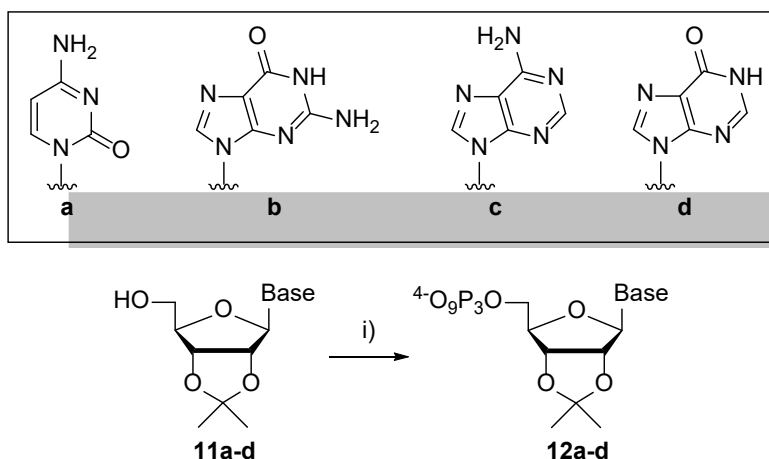


Figure 4. Gel (PAGE 20%) analysis of PEX reaction of 2',3'-O-isopropylidene UTP **6a** at lower concentration and for shorter reaction time. A) Concentration of **6a** from 50 to 200 μM at various reaction time (i.e. 15, 30, 45, 60 min), B) Concentration of **6a** at 10 or 20 μM at various reaction time (i.e. 5, 15, 30, 45, 60 min). All reactions were incubated with 10 pmol of RNA primer, 10 U PUP, 1 mM of Mn^{2+} , 20 U RNase Murine Inhibitor at 37°C. Primer only (P).

Overall, we have identified conditions that permit the production of n+1 elongated ssRNA primers with excellent yields using four different blocking groups, namely 2',3'-O-isopropylidene, 2',3'-O-cyclohexylidene, 2',3'-O-benzylidene, and 2',3'-O-moc-ethylidene. Of these, UTP analogue **6a** displayed the best compatibility with PUP-mediated synthesis since complete conversion of the primer could be achieved in less than 15 min of reaction, at low NTP concentration, and without the occurrence of side-products. Prompted by these encouraging results, we set out to synthesize the full set of 2',3'-O-isopropylidene-blocked NTPs and evaluate the possibility of using these analogues in PUP and/or PAP-mediated RNA synthesis.

4. Synthesis and biochemical characterization of 2',3'-O-isopropylidene ATP, CTP, GTP, ITP

Since cytidine, guanosine, adenosine and inosine nucleosides equipped with a 2',3'-O-isopropylidene group were all commercially available (**11a-d**), we directly used these as substrates in the triphosphorylation reaction following Ludwig-Eckstein procedure to obtain the corresponding nucleotides **12a-d** in low to moderate yields (Scheme 6).



Scheme 6. Synthesis of 2',3'-O-isopropylidene CTP, GTP, ATP, ITP **12a-d**. 1) 2-chloro-1,3,2-benzodioxaphosphorin-4-one (1.2 eq.), Pyridine/dioxane (2:1), 0°C, 4.5 h; 2) (nBu₃NH)₂H₂P₂O₇ (1.2 eq.), nBu₃N (1.2 eq.), DMF, 0°C, 2.5 h; 3) I₂ (1.2 eq.), pyridine/H₂O (95:5), rt, 20 min: 1.5% (**12a**), 19% (**12b**), 1.5% (**12c**), 3% (**12d**).

With the complete set of 2',3'-O-isopropylidene-blocked nucleotides **12a-d** at hand, we evaluated their substrate acceptance by the PUP and PAP polymerases. Cytidine nucleotide **12a** displayed a similar behavior to that of UTP **6a** (Fig. 5), since n+1 product formation could be achieved in near quantitative yields within ~2 min of reaction with a relatively low NTP concentration (40 μM) and at room temperature (instead of 37°C as for **6a** so as to suppress the formation of undesired n+2 product as much as possible). Nonetheless, the formation of very low amounts (< 5%) of n+2 products was observed, suggesting a slightly reduced hydrolytic stability of the acetonide moiety when installed on CTP than UTP. Increasing both the reaction time and the nucleotide concentration were deleterious and led to the formation of complex product distributions (Fig. S12 and S13).

Surprisingly, GTP **12b** was not readily accepted as a substrate by the PAP, irrespective of the concentration involved in the PEX reaction since only low (< 10%) conversion yields to the expected n+1 product could be achieved (Fig. S14). Since the PUP displayed a certain capacity at incorporating unmodified rGTP into RNA (Fig. S4A), we also investigated whether this enzyme could be coerced to produce the desired n+1 product. When GTP **12b** was supplied in low concentration (50 μM) to the PEX reaction mixtures, the primer was converted to the corresponding n+1 product in moderate yields (~50%) and increasing the reaction time did not seem to improve the conversion rate (Fig. S15). At higher concentrations, slower running products appear on the gel analysis of the reaction products suggesting a partial deblocking of the isopropylidene moiety. We also investigated whether other metal cofactors could positively influence the outcome of the PEX reactions with GTP **12b**, but to no avail (Fig. S16). When ATP **12c** was evaluated as a substrate for the PAP polymerase, important product dispersities could be observed even at low nucleotide concentrations (Fig. S17). A similar outcome was observed when the PAP was replaced by the PUP polymerase (Fig. S18) and ATP **12c** with inosine analogue **12d** (Fig. S19). The inherent instability of the 2',3'-O-isopropylidene blocking group on purines is in stark contrast with that observed for pyrimidines where very little hydrolysis occurred. Intrigued by these observations, we sought to install a more robust blocking group on a purine nucleotide and evaluate whether hydrolysis could be prevented without impeding efficient incorporation. To do so, we prepared 2',3'-O-cyclohexylidene-blocked ATP analogue **13** by application of the protocol outlined in Scheme 4 for uridine (see Experimental Section). With **13** at hand, we carried out PEX reactions with both the PAP and PUP polymerases (Fig. S20 and S21). As expected, no hydrolysis of the

more robust cyclohexylidene moiety was observed with both enzymes but n+1 product formation was very modest. Despite screening different experimental conditions, n+1 product formation by the PAP did not exceed 10-20%, suggesting that bulkier blocking groups on purines are not well tolerated by these template-independent RNA polymerases.

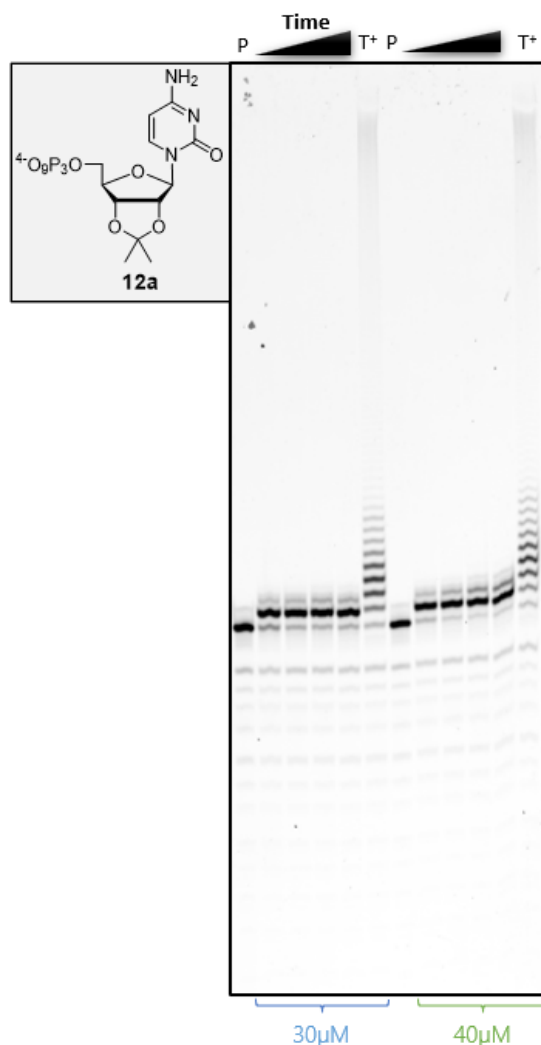


Figure 5. Gel (PAGE 20%) analysis of PEX reaction of 2',3'-O-isopropylidene CTP **12a**. A) Concentration of **12a** at 30 or 40 μM at various reaction time (i.e. 1 min 45, 5, 15, 30 min). All reactions were incubated with 10 pmol of RNA primer, 10 U PUP, 1 mM of Mn^{2+} , 20 U RNase Murine Inhibitor at 23°C. Primer only (P).

5. Chemical deprotection of elongated products

After biochemical verification of the compatibility of all blocked nucleotides with the PUP and PAP polymerases for the elongation of ssRNA primers, we carried out large-scale PEX reactions with nucleotides that were best tolerated. The resulting products were then analysed by MALDI-TOF after purification with an RNA Cleanup Kit. This analysis clearly demonstrated the chemical integrity of the n+1 products obtained by PEX reactions with nucleotides **6a-6c** and further proved the stability of the acetonide protecting groups under elongation reaction conditions (Fig. S22). By application of this protocol we also investigated the nature of the products stemming from reactions carried out with CTP **13a**. This analysis confirms that n+2 product is formed and the blocking group partially hydrolysed (Fig. S23). After confirmation by

MALDI-TOF of the formation of the targeted ssRNA n+1 products, we tested different conditions aiming at removing the acetal protecting groups. Deprotection of O,O-isopropylidene and -benzylidene protecting groups is usually accomplished under acidic conditions.^[42] Unlike DNA, RNA is much more resistant to acidic hydrolysis and the ribophosphodiester linkages are believed to be most stable at pH 4-5.^[43] Hence, we screened several acidic deprotection conditions compatible with acetal removal by incubating the unreacted RNA primer and analysing the integrity of the oligonucleotide by gel electrophoresis (Fig. S24). Even though most conditions led to degradation of the oligonucleotide, some conditions appeared to be compatible with RNA. When we applied these conditions to the n+1 product obtained by PEX reaction with nucleotide **6a**, only incubation with 2% DCA in H₂O led to partial deprotection of the isopropylidene moiety (Fig. 6). We also investigated other, less common, deprotection methods such as incubation with BCl₃ 12% in MeOH or ZrCl₄ (10mol%) but these did not permit removal of the 2',3'-isopropylidene protecting group and mainly led to degradation of the oligonucleotide (Fig. S25A). A similar outcome was observed when n+1 products obtained with nucleotides **6c** and **6d** were treated with an acidic Dowex resin or neat pyrrolidine for 1 h at 50°C (Fig. S25B).

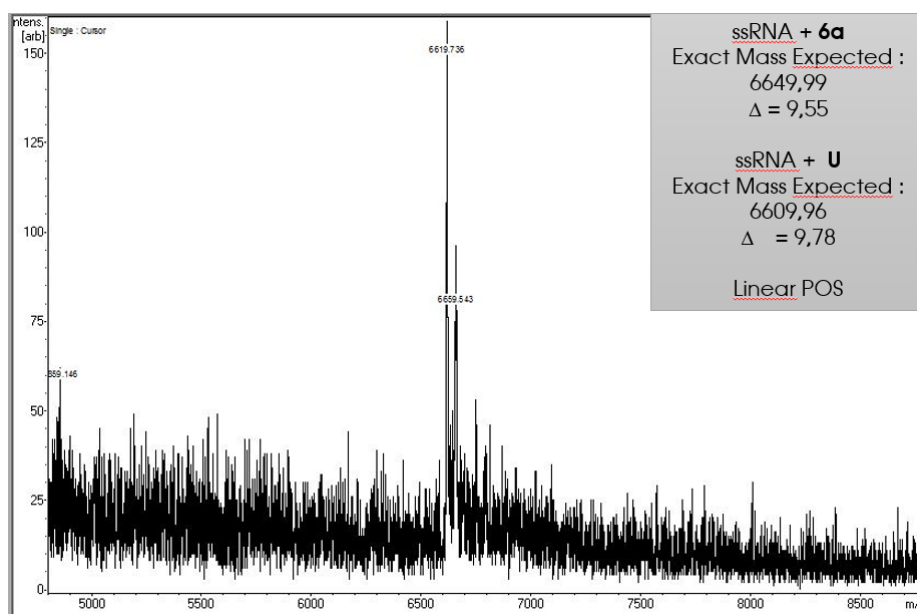


Figure 6. MALDI-TOF analysis of the deprotection of RNA primer elongated with a single **6a** nucleotide. The deprotection conditions involved incubation for 2.5 h in the presence of 2% DCA in H₂O at rt.

Discussion

Production of nucleic acids by *de novo* enzymatic synthesis is challenging for DNA and even more arduous for sugar modified substrates such as RNA or XNAs. The major difficulties reside in the identification of suitable and matching nucleotide/polymerase couples. Indeed, the protecting groups present on the nucleotide needs to be sufficiently stable to prevent hydrolysis from occurring during both enzymatic synthesis and upon storage but concomitantly must be labile enough to facilitate mild and rapid deprotection after incorporation. Moreover, polymerases need to obey the strict requirement of tolerating sugar-modified residues and incorporate single nucleotides with high efficiency and low coupling times. Preferentially, polymerases should display template-independent activity. Here, we have explored the possibility of using *cis*-diol protecting groups in conjunction with the template-independent RNA

polymerases PUP and PAP. While 2',3'-*O*-acetals are not yet the ideal blocking groups, they present a number of fundamentally important characteristics for efficient enzymatic *de novo* RNA synthesis. Indeed, pyrimidine nucleotides equipped with 2',3'-*O*-isopropylidene moieties are excellent substrates for the commercially available PUP polymerase and allow for the high yielding production of extended primers within *minutes* and without formation of any side-products. The protected nucleotides are also stable upon storage in aqueous or buffered solutions. In addition, deprotection can be achieved by application of acidic conditions under which RNA is noticeably stable. These conditions are also compatible with certain base-modifications (e.g. *N*-methyl-pseudouridine) or phosphate alterations (e.g. phosphorothioates) which are key constituents of mRNA vaccines and therapeutic siRNAs, respectively.^[1b] In addition, the simultaneous blocking of both hydroxyl moieties prevents the formation of undesired 2'-5'-branched RNA.

We found that the hydrolytic stability of the 2',3'-*O*-isopropylidene moiety installed on nucleotides and during enzymatic reactions follows the order U > C > A, G, I. Hence, modulation of the substitution pattern of the isopropylidene scaffold (e.g. by introducing electron-withdrawing moieties) or alternate deprotection conditions are expected to favour completion of the deprotection reactions for pyrimidines, uridine particularly. On the other hand, purine nucleotides equipped with 2',3'-*O*-isopropylidene masking groups are not well tolerated by any of the template-independent polymerases and appear to be partially hydrolysed during enzymatic synthesis. This low substrate tolerance might originate from a deviation from the C3'-*endo* sugar pucker^[44] and might be remediated by using engineered polymerases.^[17] Alternatively, a different protecting group strategy can be used for purine (e.g. 3'-*O*-allyl^[17]) and pyrimidine (e.g. 2',3'-*O*-isopropylidene) nucleotides to ensure efficient enzymatic incorporation of all ribonucleotides.

Conclusions

Herein, we have explored the possibility of using *cis*-diol protected nucleotides for PUP/PAP-mediated *de novo* RNA synthesis. Amongst the protecting groups that were screened, 2',3'-*O*-isopropylidene offers a good compromise between robustness, bulkiness, polymerase recognition, and deprotection conditions at least for pyrimidine nucleotides. We have indeed demonstrated that equipping UTP and CTP with such a temporary masking group, permits the high yielding and efficient production of the RNA primers extended by a single nucleotide. Even though pyrimidines presenting such a modification pattern are not ideal candidates, these results bode well for the identification of potent blocking groups for enzymatic *de novo* RNA synthesis. Alternative *cis*-diols, including cyclic phosphate and silyl ethers, combined with engineered polymerases are currently investigated in our laboratory to favour single incorporation events of blocked nucleotides, particularly of purines, and identify mild and efficient deprotection conditions.

Experimental Section

General protocols for triphosphorylation of blocked nucleosides

Method A (Ludwig-Eckstein)

The suitably protected nucleoside (1 eq.) was dissolved in a pyridine:dioxane 2:1 mixture (1.5 mL). The reaction was cooled down to 0°C. Salicyl chlorophosphite (1.5 eq.) was then added

portion wise under Argon atmosphere. The reaction was stirred for 4 h at 0°C. After this time, tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (1.5 eq.) was diluted in DMF (1 mL), this freshly prepared solution was then added to the mixture. Tributylamine (1.5 eq.) was added dropwise. The reaction was stirred for a further 1.5 h. Iodine (1.5 eq.) was dissolved in a pyridine/H₂O 95:5 mixture (1 mL), this solution was added dropwise to the mixture. After an additional 30 min, the reaction was quenched by addition of Na₂S₂O₃ sat. solution until disappearance of yellow coloration. The reaction was evaporated to dryness. The crude was dissolved in a minimum amount of H₂O. The crude solution was poured dropwise to a 2% NaClO₄ in acetone solution for precipitation. Eppendorfs were centrifuged for 10-15 min. The acetone was poured out slowly and the white precipitate was dried under high-vacuum.

A) The crude was then purified by HPLC on anion exchange column DNAPac™ PA-100 BioLCTM (Thermo Scientific), 22 x 250 mm, Flow: 10 mL/min; gradient 0% B for 5 min then 0 to 100% B in 25 min and 100% B for 5 min (A: 10 mM TEAB; B: 1 M TEAB) at rt.

B) The collected fraction was then lyophilized and purified again by HPLC using C18 column Kinetex (Phenomenex) 250 x 10.0 mm, 5 μM, 100Å, Flow: 2 mL/min; Gradient: 0 to 50% of B in 30 min (A: 20 mM TEAA, B: MeCN) at rt. Fractions of interest were lyophilized giving the desired pure 5'-triphosphate as a white foam.

Method B (Yoshikawa protocol)

The suitably nucleoside (1 eq.) was solubilised in trimethylphosphite (c = 0.25 M) at 0°C. Freshly distilled phosphorous oxychloride was added dropwise (1.1 eq.). The reaction was stirred for 4 h at 0°C under argon atmosphere. Bis-tributylammonium pyrophosphate (1.1 eq.) was dissolved in dry DMF. This previous solution and TBA (1.1 eq.) were added dropwise to the reaction mixture at 0°C. The reaction was stirred for 1 h at 0°C. The reaction was quenched by addition of TEAB 1 M solution and let stirred for 30 min at room temperature. The reaction was evaporated to dryness, co-evaporated with water. The crude was dissolved in a minimum amount of H₂O. The crude solution was poured dropwise to a 2% NaClO₄ in acetone solution for precipitation. Eppendorfs were centrifuged for 10-15 min. The acetone was poured out slowly and the white precipitate was dried under high-vacuum. This solid was then purified by HPLC on anion exchange column DNAPac™ PA-100 BioLCTM (Thermo Scientific), 22 x 250 mm, Flow: 10 mL/min; gradient 0% B for 5min then 0 to 100% B in 25 min and 100% B for 5 min (A: 10 mM TEAB; B: 1 M TEAB) at rt.

General protocol of PUP/PAP-mediated extension reactions: RNA primer (20 pmol) is incubated with the modified nucleoside triphosphates (at a given concentration) with a metal cofactor and the PUP or PAP polymerase (10 U) in 1X reaction buffer (supplied with the polymerase; 10 μL final volume) at 37 °C for indicated reaction times. The reaction mixtures were then purified by Nucleospin columns and quenched by the addition of an equal volume of loading buffer (formamide (70%), ethylenediaminetetraacetic acid (EDTA, 50 mM), bromophenol (0.1%), xylene cyanol (0.1%)). The reaction products were then resolved by electrophoresis (PAGE 20%) and visualized by phosphorimager analysis.

Synthetic procedures and nucleotide characterization

2',3'-bis-*O*-acetyluridine-5'-triphosphate (4a)

Triphosphorylation method B was followed, starting from 85 mg (0.259 mmol) of **3a**. After HPLC purification, the nucleotide **4a** was obtained in a very low yield (1.5 mg, 0.003 mmol, 1 %). The quantity was estimated by UV measurement taking the ϵ value of UTP as a reference (9.8 L mmol⁻¹ cm⁻¹). Data are in accordance with those reported in the literature.^[21b]

¹H NMR (500 MHz, D₂O): δ 7.91 (d, J = 8.12 Hz, 1H), 6.16 (d, J = 4.94 Hz, 1H), 5.98 (d, J = 8.10 Hz, 1H), 5.51-5.46 (m, 2H), 4.55-4.51 (m, 1H), 4.33-4.28 (m, 1H), 4.26-4.20 (m, 1H), 2.17 (s, 3H), 2.11 (s, 3H).

³¹P NMR (202 MHz, D₂O): δ -10.93 (d, J = 19.3 Hz, 1P), -11.66 (d, J = 19.9 Hz, 1P), -23.26 (t, J = 20.2 Hz, 1P)

HRMS (ESI) m/z [M-H]⁻ calcd for C₁₃H₁₉N₂O₁₇P₃ 566.9824; Found 566.9828.

2',3'-bis-*O*-benzoyluridine-5'-triphosphate (4b)

Triphosphorylation method A was followed starting from 62 mg (0.137 mmol) of **3b**. Product **4b** was purified by 1) anion exchange followed by 2) C18, affording 21 mg (0.030 mmol, 22 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 8.09 (d, J = 8.12 Hz, 1H), 7.98 (d, J = 8.44 Hz, 2H), 7.79 (d, J = 8.44 Hz, 2H), 7.67 (t, J = 7.46 Hz, 1H), 7.59 (t, J = 7.48 Hz, 1H), 7.47 (t, J = 7.84 Hz, 2H), 7.35 (t, J = 7.86 Hz, 2H), 6.44 (d, J = 5.96 Hz, 1H), 6.07 (d, J = 8.12 Hz, 1H), 5.92 (dd, J = 5.66, 3.66 Hz, 1H), 5.84 (t, J = 5.86 Hz, 1H), 4.81-4.84 (m, 1H), 4.37-4.52 (m, 2H).

³¹P NMR (202 MHz, D₂O): δ -10.91 (d, J = 19.7 Hz, 1P), -11.66 (d, J = 19.7 Hz, 1P), -23.27 (t, J = 17.8 Hz, 1P).

¹³C NMR (125 MHz, D₂O): δ 166.9, 166.6, 166.0, 151.4, 141.9, 134.4, 134.3, 129.6, 129.5, 128.8, 128.7, 128.2, 127.7, 103.1, 87.0, 81.6, 74.1, 71.9, 65.2.

HRMS (ESI): m/z [M-H]⁻ calcd for C₂₃H₂₃N₂O₁₇P₃ 691.0137; Found 691.0137.

2',3'-bis-*O*-pivaloyluridine-5'-triphosphate (4c)

Triphosphorylation method A was followed starting from 50 mg (0.121 mmol) of **3c**. Product **4b** was purified by 1) anion exchange only affording 3.2 mg (0.005 mmol, 4 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 7.89 (d, J = 8.15 Hz, 1H), 6.12 (d, J = 6.63 Hz, 1H), 5.95 (d, J = 8.12 Hz, 1H), 5.45 (dd, J = 5.20, 2.74 Hz, 1H), 5.38 (t, J = 6.02 Hz, 1H), 4.53-4.49 (m, 1H), 4.27-4.22 (m, 1H), 4.21-4.15 (m, 1H), 1.20 (s, 9H), 1.09 (s, 9H)

³¹P NMR (202 MHz, D₂O): δ -9.88 (d, J = 20.1 Hz, 1P), -11.5 (d, J = 18.4 Hz, 1P), -22.2 (t, J = 18.4 Hz, 1P).

¹³C NMR (125 MHz, D₂O): δ 180.2, 179.9, 179.8, 166.0, 151.7, 141.3, 103.2, 86.1, 81.9, 81.8, 73.6, 71.6, 38.6, 26.3, 26.2, 22.5.

HRMS (ESI): m/z [M-H]⁻ calcd for C₁₉H₃₁N₂O₁₇P₃ 651.0763; Found 651.0768.

2',3'-*O*-isopropylideneuridine-5'-triphosphate (6a)

Triphosphorylation method A was followed starting from 150 mg of **5a** (0.528 mmol). Product **6a** was purified by 1) anion exchange and then 2) C18 affording 44.0 mg (0.084 mmol, 16 %) of the desired nucleotide.

Triphosphorylation method B was followed starting from 74 mg of **5a** (0.297 mmol). Product **6a** was purified by 1) anion exchange only affording 10.0 mg (0.019 mmol, 6 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 7.69 (d, *J* = 7.96 Hz, 1H), 5.85 (d, *J* = 3.31 Hz, 1H), 5.77 (d, *J* = 7.94 Hz, 1H), 4.95 (dd, *J* = 6.25, 2.62 Hz, 1H), 4.89 (dd, *J* = 6.22, 3.33 Hz, 1H), 4.46-4.42 (m, 1H), 4.11-4.07 (m, 2H), 1.45 (s, 3H), 1.28 (s, 3H).

³¹P NMR (202 MHz, D₂O): δ -11.01 (d, *J* = 20.1 Hz, 1P), -11.98 (d, *J* = 20.1 Hz, 1P), -23.43 (t, *J* = 20.1 Hz, 1P)

¹³C NMR (125 MHz, D₂O): δ 179.4, 166.3, 151.4, 142.3, 114.4, 101.9, 92.6, 85.0, 84.9, 84.3, 80.8, 65.8, 65.7, 26.1, 24.3.

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₂H₁₉N₂O₁₅P₃ 522.9926; Found 522.9930.

2',3'-O-cyclohexylideneuridine-5'-triphosphate (6b)

Triphosphorylation method A was followed starting from 95 mg of **5b** (0.151 mmol). Product **6b** was purified by 1) anion exchange only affording 8.2 mg (0.015 mmol, 5 %) of the desired nucleotide.

Triphosphorylation method B was followed starting from 75 mg of **5b** (0.231 mmol). Product **6b** was purified by 1) anion exchange only affording 6.6 mg (0.012 mmol, 5 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 7.87 (d, *J* = 8.11 Hz, 1H), 5.98-5.94 (m, 2H), 5.10-5.05 (m, 2H), 4.65-4.61 (m, 1H), 4.28-4.18 (m, 2H), 1.77-1.61 (m, 6H), 1.61-1.51 (m, 2H), 1.51-1.37 (m, 2H).

³¹P NMR (202 MHz, D₂O): δ -7.87 (d, *J* = 18.6 Hz, 1P), -11.48 (d, *J* = 18.8 Hz, 1P), -22.0 (t, *J* = 19.1 Hz, 1H).

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₅H₂₃N₂O₁₅P₃ 563.0239; Found 563.0243.

2',3'-O-benzylideneuridine-5'-triphosphate (6c)

Triphosphorylation method A was followed starting from 93.5 mg of **5c** (0.281 mmol). Product **6c** was purified by 1) anion exchange only affording 10.5 mg (0.018 mmol, 6 %) of the desired nucleotide as a mix of two diastereoisomers.

¹H NMR (500 MHz, D₂O): δ 7.93 (d, *J* = 8.13 Hz, 0.4H), 7.87 (d, *J* = 8.11 Hz, 0.6H), 7.69-7.65 (m, 0.8H), 7.63-7.60 (m, 1.2H), 7.57-7.50 (m, 3H), 6.26 (s, 0.6H), 6.14 (s, 0.4H), 6.13-6.10 (m, 1H), 5.98-5.94 (m, 1H), 5.26-5.18 (m, 2H), 4.68-4.64 (m, 0.6H), 4.38-4.27 (m, 2H).

³¹P NMR (202 MHz, D₂O): δ -10.91 (d, *J* = 19.5 Hz, 1P), -11.64 (d, *J* = 19.9 Hz, 0.6P), -11.97 (d, *J* = 20.0 Hz, 0.4P), -23.29 (t, *J* = 19.7 Hz, 1P)

¹³C NMR (125 MHz, D₂O): δ 166.4, 142.8, 134.6, 130.5, 128.9, 128.8, 127.1, 127.0, 107.0, 103.6, 102.2, 101.8, 92.8, 91.5, 85.0, 84.1, 83.6, 82.6, 80.0, 58.6.

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₆H₁₉N₂O₁₅P₃ 570.9926; Found 570.9929.

2',3'-O-Moc-ethylideneuridine-5'-triphosphate (6d)

Triphosphorylation method A was followed starting from 84 mg (0.256 mmol) of **11**. Product **6d** was purified by 1) anion exchange and 2) C18 affording 25.4 mg (0.045 mmol, 17 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 7.77 (d, *J* = 8.10 Hz, 0.7H), 7.71 (d, *J* = 8.10 Hz, 0.3H), 5.86-5.79 (m, 2H), 5.59 (t, *J* = 4.85 Hz, 0.3H), 5.41 (t, *J* = 4.54 Hz, 0.7H), 4.98-4.91 (m, 2H), 4.57-4.54 (m, 0.7H), 4.43-4.39 (m, 0.3H), 4.20-4.09 (m, 2H), 3.65 (s, 2.1H), 3.63 (s, 0.9H), 2.89 (d, *J* = 4.57 Hz, 1.4H), 2.81 (d, *J* = 4.91 Hz, 0.6H).

³¹P NMR (202 MHz, D₂O): δ -10.97 (d, *J* = 19.6 Hz, 1P), -11.77 (dn *J* = 19.8 Hz, 0.3P), -12.04 (d, *J* = 20.0 Hz, 0.7P), -23.41 (t, *J* = 19.8 Hz, 1P).

¹³C NMR (125 MHz, D₂O): δ 179.4, 171.6, 166.3, 151.4, 142.8, 142.4, 103.9, 102.1, 101.8, 101.2, 92.6, 91.5, 84.6, 83.8, 82.3, 80.0, 65.84, 65.79, 52.5, 38.6, 38.4, 30.2.

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₃H₁₉N₂O₁₇P₃ 566.9824; Found 566.9820.

2',3'-O-isopropylidene-5'-triphosphate cytidine (12a)

Triphosphorylation method A was followed starting from 70 mg (0.247 mmol) of 2',3'-isopropylidene cytidine **11a**. Product **12a** was purified by 1) anion exchange and 2) C18 affording 1.9 mg (0.0037 mmol, 1.5 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 8.52 (s, 1H), 8.30 (s, 1H), 6.30 (d, *J* = 3.43 Hz, 1H), 5.43 (dd, *J* = 6.07, 3.45 Hz, 1H), 5.29 (dd, *J* = 6.09, 1.99 Hz, 1H), 4.75-4.70 (m, 1H), 4.32-4.20 (m, 2H), 1.71 (s, 3H), 1.50 (s, 3H).

³¹P NMR (500 MHz, D₂O): δ -10.89 (d, *J* = 19.6 Hz, 1P), -11.76 (d, *J* = 19.6 Hz, 1P), -23.25 (t, *J* = 19.8 Hz, 1P).

¹³C NMR (125 MHz, D₂O): δ 142.4, 114.2, 95.7, 93.2, 85.0, 80.8, 65.8, 26.2, 24.3.

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₂H₂₀N₃O₁₄P₃ 522.0085; Found 522.0082

2',3'-O-isopropylidene-5'-triphosphate guanosine (12b)

Triphosphorylation method A was followed starting from 70 mg (0.216 mmol) of 2',3'-isopropylidene guanosine **11b**. Product **12b** was purified by 1) anion exchange and 2) C18 affording 23 mg (0.041 mmol, 19 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 8.05 (s, 1H), 6.04 (d, *J* = 3.15 Hz, 1H), 5.33 (dd, *J* = 6.06, 3.20 Hz, 1H), 5.21 (dd, *J* = 6.08, 2.17 Hz, 1H), 4.56-4.60 (m, 1H), 4.15-4.22 (m, 2H), 1.62 (s, 3H), 1.42 (s, 3H).

³¹P NMR (500 MHz, D₂O): δ -10.97 (d, *J* = 19.4 Hz, 1P), -11.74 (d, *J* = 19.3 Hz, 1P), -23.33 (t, *J* = 19.2 Hz, 1P).

¹³C NMR (125 MHz, D₂O): δ 178.8, 158.6, 153.8, 151.2, 137.7, 115.8, 114.8, 90.0, 84.7, 84.6, 83.7, 81.3, 65.7, 26.1, 24.4.

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₃H₂₀N₅O₁₄P₃ 562.0147; Found 562.0141.

2',3'-O-isopropylidene-5'-triphosphate adenosine (12c)

Triphosphorylation method A was followed starting from 80 mg (0.260 mmol) of 2',3'-isopropylidene adenosine **11c**. Product **12c** was purified by 1) anion exchange and 2) C18 affording 1.9 mg (0.0035 mmol, 1,3 %) of the desired nucleotide. Data are in accordance with those reported in the literature.^[45]

¹H NMR (500 MHz, D₂O): δ 8.82 (s, 1H), 8.30 (s, 1H), 6.29 (d, *J* = 3.44 Hz, 1H), 5.43 (dd, *J* = 6.08, 3.44 Hz, 1H), 5.29 (dd, *J* = 6.08, 2.00 Hz, 1H), 4.75-4.70 (m, 1H), 4.31-4.19 (m, 2H), 1.71 (s, 3H), 1.50 (s, 3H).

³¹P NMR (500 MHz, D₂O): δ -10.89 (d, *J* = 19.6 Hz, 1P), -12.06 (d, *J* = 19.6 Hz, 1P), -23.30 (t, *J* = 19.5 Hz, 1P).

¹³C NMR (125 MHz, D₂O): δ 179.0, 154.2, 151.0, 148.6, 140.6, 118.4, 114.9, 90.4, 84.8, 84.7, 84.0, 81.4, 65.9, 26.2, 24.4.

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₃H₂₀N₅O₁₃P₃ 546.0198; Found 546.0200.

2',3'-O-isopropylidene-5'-triphosphate inosine (12d)

Triphosphorylation method A was followed starting from 100 mg (0.32 mmol) of 2',3'-isopropylidene inosine **11d**. Product **12d** was purified by 1) anion exchange and 2) C18 affording 6 mg (0.011 mmol, 3 %) of the desired nucleotide.

¹H NMR (500 MHz, D₂O): δ 8.41 (s, 1H), 8.22 (s, 1H), 6.30 (d, *J* = 3.15 Hz, 1H), 5.44 (dd, *J* = 6.02, 3.23 Hz, 1H), 5.28 (dd, *J* = 6.10, 1.86 Hz, 1H), 4.72-4.68 (m, 1H), 4.27-4.17 (m, 2H), 1.67 (s, 3H), 1.46 (s, 3H).

³¹P NMR (500 MHz, D₂O): δ -10.93 (d, *J* = 19.5 Hz, 1P), -11.81 (d, *J* = 19.8 Hz, 1P), -23.30 (t, *J* = 19.6 Hz, 1P).

¹³C NMR (125 MHz, D₂O): δ 179.1, 158.6, 148.5, 146.0, 140.0, 114.8, 90.8, 85.0, 84.1, 81.44, 65.8, 26.1, 24.3.

HRMS (ESI): *m/z* [M-H]⁻ calcd for C₁₃H₁₉N₄O₁₄P₃ 547.0038; Found 547.0041.

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Conflict of interest

The authors declare no conflict of interest.

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