Organic & Biomolecular Chemistry

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2018, **16**, 5800

Enzymatic synthesis of base-modified RNA by T7 RNA polymerase. A systematic study and comparison of 5-substituted pyrimidine and 7-substituted 7-deazapurine nucleoside triphosphates as substrates†

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We synthesized a small library of eighteen 5-substituted pyrimidine or 7-substituted 7-deazapurine nucleoside triphosphates bearing methyl, ethynyl, phenyl, benzofuryl or dibenzofuryl groups through cross-coupling reactions of nucleosides followed by triphosphorylation or through direct cross-coupling reactions of halogenated nucleoside triphosphates. We systematically studied the influence of the modification on the efficiency of T7 RNA polymerase catalyzed synthesis of modified RNA and found that modified ATP, UTP and CTP analogues bearing smaller modifications were good substrates and building blocks for the RNA synthesis even in difficult sequences incorporating multiple modified nucleotides. Bulky dibenzofuryl derivatives of ATP and GTP were not substrates for the RNA polymerase. In the case of modified GTP analogues, a modified procedure using a special promoter and GMP as initiator needed to be used to obtain efficient RNA synthesis. The T7 RNA polymerase synthesis of modified RNA can be very efficiently used for synthesis of modified RNA but the method has constraints in the sequence of the first three nucleotides of the transcript, which must contain a non-modified G in the +1 position.

Received 25th June 2018,
Accepted 11th July 2018

DOI: 10.1039/c8ob01498a

rsc.li/obc

Introduction

Studies toward detailed understanding of the structure and many diverse biological functions of nucleic acids require the use of chemically modified DNA or RNA probes.[1] In addition to the classical chemical synthesis of oligonucleotides on solid support,[2,3] enzymatic methods for the synthesis of nucleic acids play an important role.[4–7] The polymerase catalyzed synthesis of DNA using modified 2′-deoxyribonucleoside triphosphates (dNTPs) is very well established[4–6] and there are many commercially available DNA polymerases capable of incorporation of modified nucleotides as well as many diverse methodologies for the construction of single- or double-stranded DNA bearing one, several or many modifications.[6] On the other hand, both chemical and enzymatic syntheses of modified RNA are more problematic and less well established,[7–9] despite the extensive current efforts in the use of modified RNA probes for imaging, chemical biology and therapeutic applications.[6,9,10–16] The enzymatic production of modified RNAs can be based either on RNA polymerase catalyzed synthesis using modified ribonucleoside triphosphates (NTPs) or on enzymatic posttranscriptional modifications of RNA (*e.g.* alkylation by methyltransferases, *etc.*).[6] Posttranscriptional chemical modifications through bioorthogonal chemistry is another alternative approach to modified RNA.[17,18]

The enzymatic synthesis of modified RNA relies almost exclusively on the use of bacteriophage T7 RNA polymerase to avoid the need for complex transcription factors. The T7 RNA polymerase requires specific promoters[19–21] and the presence of guanosines in the +1 and/or +2 positions to ensure efficient transcription initiation.[22] The known examples of polymerase synthesis of modified RNAs include the incorporation of useful functional groups, *i.e.* biotin for affinity probes,[23,24] alkyne or azido groups for click reactions,[25,26] 5-vinylU for further chemical modifications,[27] 5-iodoU for posttranscriptional cross-coupling modification,[28] amino acid-like side chains for selection of aptamers,[29–31] diazirine for cross-linking[32] or fluorophores.[33–37] In the vast majority of cases, the modification was attached at position 5 of uridine tripho-

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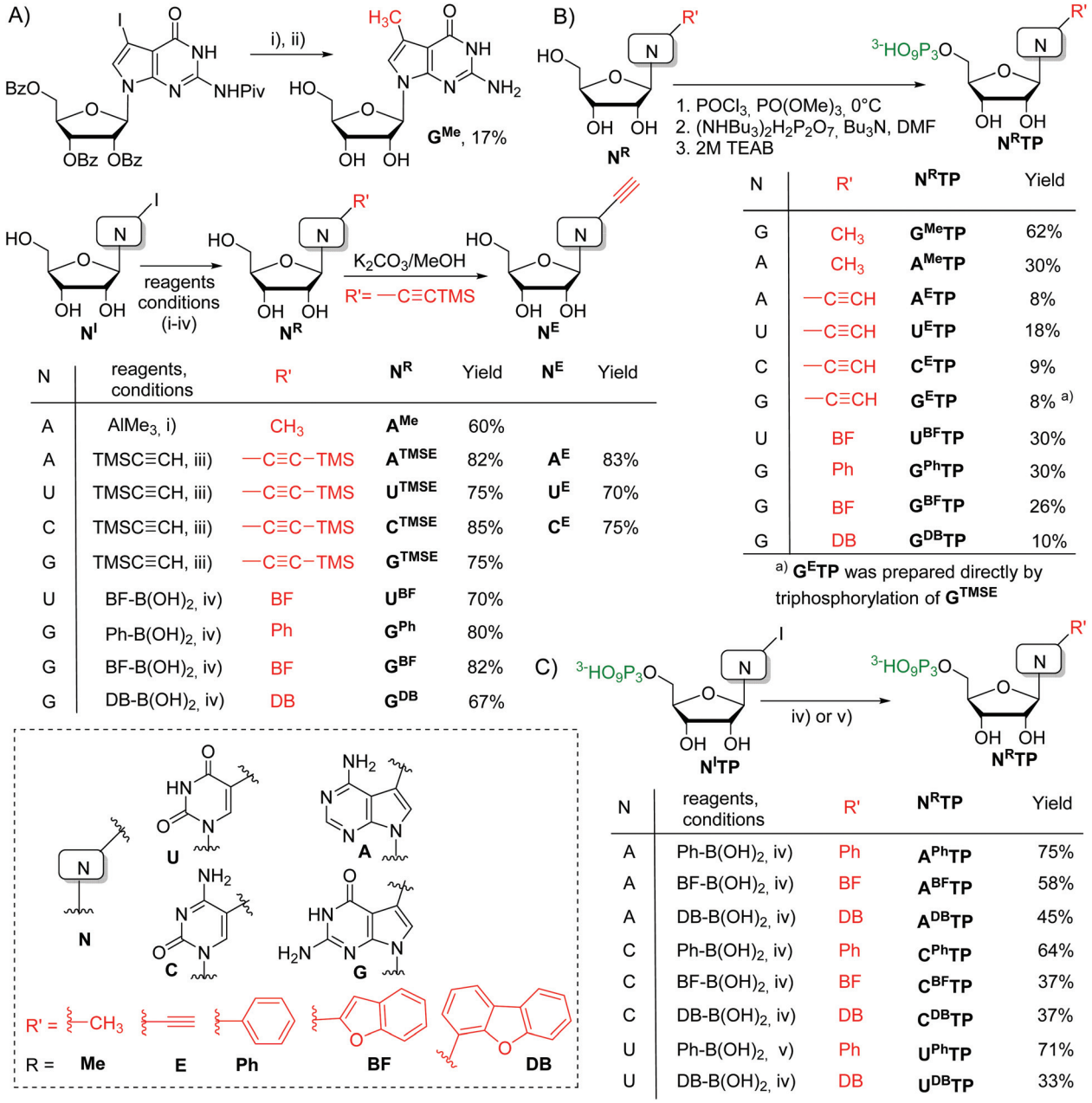
†Electronic supplementary information (ESI) available: Full experimental part, characterization of all compounds, additional gels and copies of spectra. See DOI: 10.1039/c8ob01498a

sphate (UTP) because of easy synthetic access and because modification at this position does not much perturb the structures of RNA duplexes. Only a few scattered examples have been published on the incorporation of modified cytidines.[38–41] Modified adenine NTPs were never studied systematically and just a few examples of successful incorporation of 7-aryl-[42] and 7-ethynyl-7-deazaadenine[43] or -8-aza-7-deazaadenine[44] nucleotides were reported. In addition, fluorescent analogues of NTPs (including GTP)[45] and even formylpyrrole[46–48] or thioisocarbostyryl[49,50] nucleobase surrogates, extending the genetic alphabet, were successfully incorporated by T7 RNA polymerase. Apart from base-modified NTPs, also several examples of 2′-sugar-modified NTPs were reported[51] to be substrates for T7 RNA polymerase. Despite those many excellent works dealing with incorporation of specific modified nucleotides (mostly 5-substituted uracils or non-natural bases), there has been no systematic study and comparison of substrate activity of NTPs bearing different modifications at pyrimidine or 7-deazapurine bases. Since the enzymatic synthesis of base-modified RNA is an important



N	reagents, conditions	R'	N^R	Yield	N^E	Yield
A	$AlMe_3$, i)	CH_3	A^{Me}	60%		
A	TMSC≡CH, iii)	—C≡C—TMS	A^{TMSE}	82%	A^E	83%
U	TMSC≡CH, iii)	—C≡C—TMS	U^{TMSE}	75%	U^E	70%
C	TMSC≡CH, iii)	—C≡C—TMS	C^{TMSE}	85%	C^E	75%
G	TMSC≡CH, iii)	—C≡C—TMS	G^{TMSE}	75%		
U	BF-$B(OH)_2$, iv)	BF	U^{BF}	70%		
G	Ph-$B(OH)_2$, iv)	Ph	G^{Ph}	80%		
G	BF-$B(OH)_2$, iv)	BF	G^{BF}	82%		
G	DB-$B(OH)_2$, iv)	DB	G^{DB}	67%		

N	R'	N^RTP	Yield
G	CH_3	$G^{Me}TP$	62%
A	CH_3	$A^{Me}TP$	30%
A	—C≡CH	A^ETP	8%
U	—C≡CH	U^ETP	18%
C	—C≡CH	C^ETP	9%
G	—C≡CH	G^ETP	8% [a]
U	BF	$U^{BF}TP$	30%
G	Ph	$G^{Ph}TP$	30%
G	BF	$G^{BF}TP$	26%
G	DB	$G^{DB}TP$	10%

[a] G^ETP was prepared directly by triphosphorylation of G^{TMSE}

N	reagents, conditions	R'	N^RTP	Yield
A	Ph-$B(OH)_2$, iv)	Ph	$A^{Ph}TP$	75%
A	BF-$B(OH)_2$, iv)	BF	$A^{BF}TP$	58%
A	DB-$B(OH)_2$, iv)	DB	$A^{DB}TP$	45%
C	Ph-$B(OH)_2$, iv)	Ph	$C^{Ph}TP$	64%
C	BF-$B(OH)_2$, iv)	BF	$C^{BF}TP$	37%
C	DB-$B(OH)_2$, iv)	DB	$C^{DB}TP$	37%
U	Ph-$B(OH)_2$, v)	Ph	$U^{Ph}TP$	71%
U	DB-$B(OH)_2$, iv)	DB	$U^{DB}TP$	33%

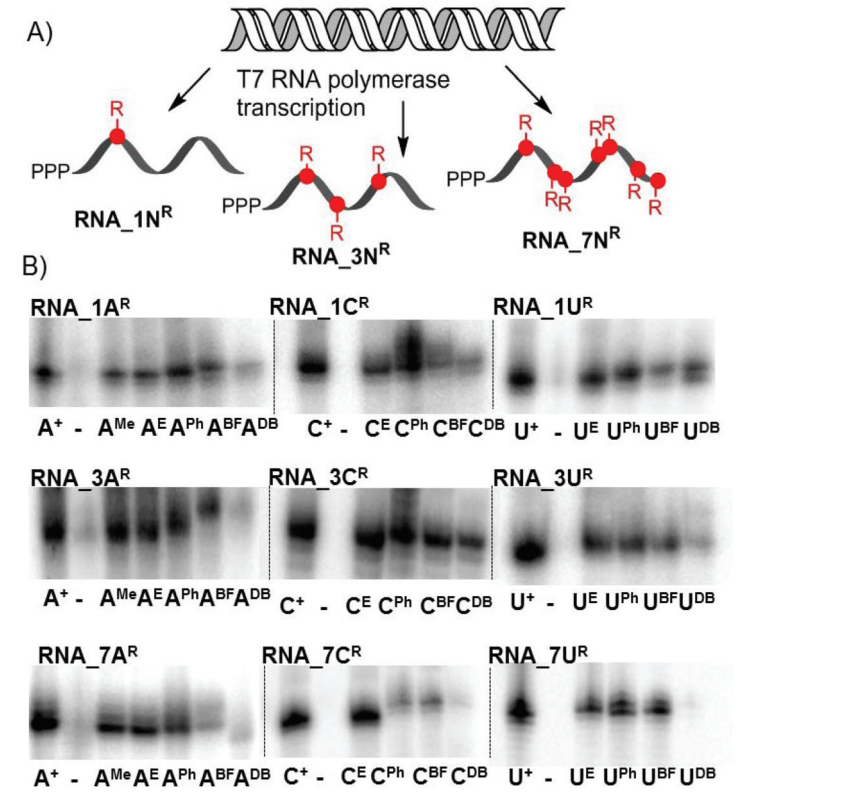
Scheme 1 (A) Synthesis of modified nucleosides, (B) triphosphorylation of nucleosides and (C) cross-coupling reactions on NTPs. Reagents and conditions: (i) HMDS, 40 °C, $AlMe_3$, $Pd(PPh_3)_4$, THF, 80 °C, 2 h; (ii) NaOMe/MeOH, 80 °C, 16 h; (iii) TMS–acetylene, $Pd(PPh_3)_4$, CuI, DMF, r.t., 16 h; (iv) R–$B(OH)_2$, $Pd(OAc)_2$, TPPTS, H_2O/ACN (2 : 1), Cs_2CO_3 100 °C, 2 h for nucleosides or 1 h for NTPs; and (v) R–$B(OH)_2$, Na_2PdCl_4, TPPTS, H_2O, 60 °C, 1 h.

and underexplored area, such a systematic study may facilitate further use of this approach. Therefore we synthesized a small library of NTPs (derived from all four bases) bearing modifications of different bulkiness and systematically studied them as substrates for T7 RNA polymerase.

Results and discussion

Chemistry

In order to study the influence of substituents on incorporation of modified nucleotides by T7 RNA polymerase, we designed a small library of substituted NTPs (**N^{R}TP**). The 7-deazapurine NTPs were substituted at position 7 whereas the pyrimidine NTPs were substituted at position 5. The substituents were chosen with increased bulkiness: methyl (for deazapurines only), ethynyl, phenyl, 2-benzofuryl and dibenzo[*b*,*d*]furan-4-yl. The synthesis (Scheme 1) was based either on preparation of the corresponding modified nucleosides followed by triphosphorylation,[52] or on the direct Suzuki–Miyaura cross-coupling modifications of iodinated NTPs.[53] The 7-methylation of 7-iodoadenosine (unprotected **A^I**) and -guanosine (N^2-pivaloyl-2′,3′,5′-tribenzoyl protected **G^I**) was performed through Pd-catalyzed reactions with trimethylaluminum (Scheme 1A). The Sonogashira cross-coupling reactions of iodinated nucleosides with TMS–acetylene followed by



C) Sequences of the modified RNA transcripts:

RNA_1N^R: +9
RNA_1A^R: pppGGG CCC CU**A^R** UUG UCU CUC UCU UCU CUG CUG UUU CC
RNA_1C^R: pppGGG AGG GA**C^R** UGU GAG UGG AGA UUG UAG GAU UGA GG
RNA_1U^R: pppGGG CCA GC**U^R** CAA GCC ACG CAC CCC ACG GCA CAC AC

RNA_3N^R: +9 +14 +24
RNA_3A^R: pppGGG CCC GU**A^R** UGU U**A^R**C UUG CUC UU**A^R** UCG UCU CUC GC
RNA_3C^R: pppGGG AGG AU**C^R** AGU A**C^R**A GAG GUA UG**C^R** UGG GAU AGG GA
RNA_3U^R: pppGGG CCA GC**U^R** CAC A**U^R**C AAA GAC AC**U^R** ACG ACA CAC AC

RNA_7N^R: +9 +14,15 +24,25 +29 +35
RNA_7A^R: pppGGG CUU GC**A^R** CGU G**A^{R}A^R** UCG CUC UU**A^R** **A^R**UG G**A^R**U CGC G**A^R**
RNA_7C^R: pppGGG AUU GA**C^R** AGU G**C^{R}C^R** UAG AUA UU**C^R** **C^R**GG G**C^R**U AGG G**C^R**
RNA_7U^R: pppGGG CCA GC**U^R** CAA G**U^R** **U^R** ACG CAC CC**U^R** **U^R**CG G**U^R**A CAA A**U^R**

Fig. 1 (A) Scheme of T7 RNA polymerase synthesis of modified RNA; (B) examples of PAGE analysis of the transcripts; and (C) sequences of the modified RNA transcripts, **RNA_XN^R**.

desilylation was used to introduce the ethynyl group and the Suzuki reaction for attachment of the aryl groups. The triphosphorylation of nucleosides was performed according to the literature procedure[52] using $POCl_3$ in $PO(OMe)_3$, followed by reaction with pyrophosphate and triethylammonium bicarbonate (TEAB) to give the desired **N^{R}TP**s in moderate to good yields (Scheme 1B). In the case of synthesis of **G^{E}TP**, the triphosphorylation was performed with silylated nucleoside **G^{TMSE}** and the concomitant desilylation occurred during the workup. Finally, most of the aryl derivatives of ATP, UTP and CTP were prepared by the direct aqueous Suzuki coupling of iodinated **N^{I}TP**s with the corresponding arylboronic acid using a Pd catalyst and triphenylphosphine-3,3′,3″-trisulfonate (TPPTS) ligand.[53] The desired **N^{R}TP**s were obtained in good yields of 33–75% (Scheme 1C).

Biochemistry

All the synthesized **N^{R}TP**s were then systematically tested as substrates for T7 RNA polymerase. Since all the literature examples of T7 RNA polymerase syntheses of modified RNAs use specific promoters containing guanosines in the +1 and/or +2 positions,[19–22] for the testing of incorporation of modified **A^{R}TP**s, **U^{R}TP**s and **C^{R}TP**s, we also designed DNA templates containing the promoter and three guanosines in the +1–+3 position.[54] The 5′-terminal nucleotide in the antisense strand of DNA templates was always a 2′-MeO ribonucleotide to minimize nontemplated nucleotide addition.[55] For each modified base, we designed three different templates encoding the incorporation of either one, three or seven modified nucleotides (in the latter case there were also two adjacent incorporations of modified nucleotides) (Fig. 1, Table S1 in the ESI†). The transcription has been performed in analogy to literature procedures[25–39,54] using [α-^{32}P]-GTP or [α-^{32}P]-ATP and the RNA products were analyzed by PAGE and quantified using QuantityOne software (Fig. 2, means from 2–3 independent experiments). The transcription conditions needed to be carefully optimized to avoid misincorporations, especially when ATP analogues (**A^{R}TP**) were used. Addition of detergent (Triton X-100) and fine-tuning of Mg^{2+} ion concentration led to minimization of misincorporated transcript content in negative control experiments (see Fig. S1 in the ESI†). The RNA products were also characterized by MALDI-TOF, which in most cases confirmed the correct full-length modified RNA (see Table S2 and Fig. S6–S44 in the ESI†). In some cases, we also observed a one-nucleotide longer RNA product containing an additional n + 1 nucleotide incorporated in a non-templated manner.

Fig. 1 and 2 show that the single incorporations of a modified nucleotide, as well as incorporations of three modified nucleotides at separate positions, worked with excellent (for smaller substituents) to moderate (for bulky BF or DB substituents) conversions to give the desired modified RNAs (**RNA_1N^R** or **RNA_3N^R**). Modified nucleotides bearing smaller substituents (Me, E or Ph) were typically comparable or just slightly worse substrates than the natural nucleotides in these experiments. On the other hand, in the more challenging experiments, aiming at incorporation of seven modified nucleotides with two pairs of modified bases in adjacent positions, the polymerase synthesis of **RNA_7N^R** was somewhat less efficient than the synthesis of natural non-modified RNA. The transcriptions with bulky DB-substituted **N^{DB}TP**s did not give any significant amounts of the full-length transcripts.

The study of processing of modified **G^{R}TP**s was more difficult because of the requirements of the T7 RNA polymer-

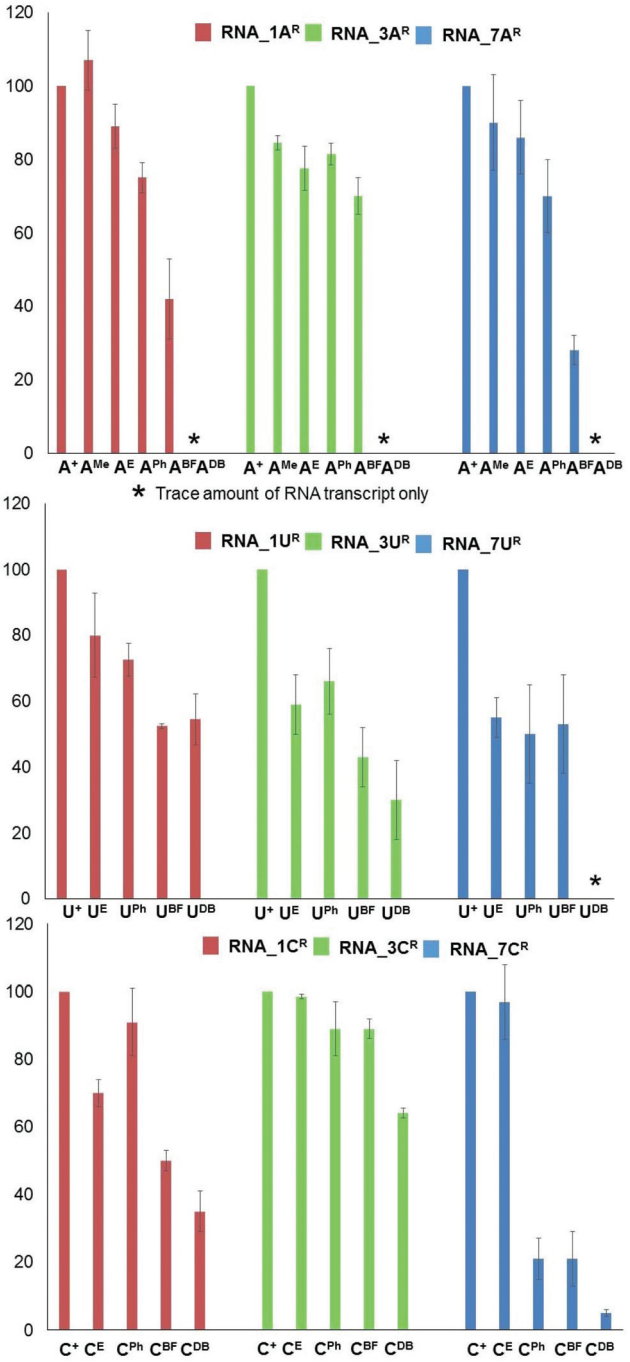


Fig. 2 Quantifications of the relative conversions (compared to transcription with all four natural NTPs: N$^+$) of T7 RNA polymerase transcription experiments.

ase for the presence of guanosines in the +1, +2 and/or +3 positions of the transcript. At first we designed sequences with three adenosines at +1–+3 positions and tried the transcriptions under the same conditions as above. Fig. 3A shows that the transcriptions did not work even for the positive control (using all natural NTPs). Then we designed a sequence commencing with three guanosines and the transcription worked only when natural NTPs were used. This indicates that the modified **G^{R}TP**s are not suitable for transcription initiation. Next, we designed a sequence with only one G at the +1 position (followed by CA). The result was encouraging showing that the transcription worked reasonably well at least for **G^{Me}TP** but still was negligible for all other bulkier **G^{R}TP**s. Apparently, the polymerase does not tolerate a bulky modification at the +1 position. Therefore, we used the sequence GCA in the first positions of the transcript and used guanosine monophosphate (GMP) for initiation of the transcription[56] (Fig. 3B). Now, the transcriptions leading to the difficult **RNA_pg7G^{R}** transcripts proceeded with acceptable efficiency for the smaller modifications (35–50% compared to natural RNA). Again, the most bulky dibenzofuryl modified **G^{DB}TP** gave only trace amounts of the transcript.

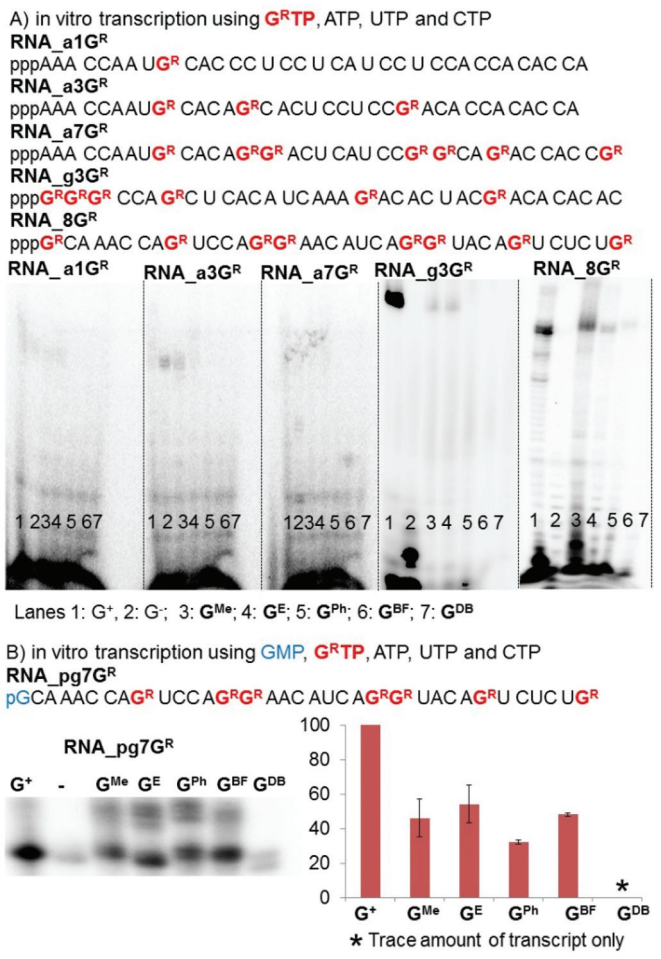


Fig. 3 (A) Scheme of attempted T7 polymerase synthesis of modified RNA using modified **G^{R}TP**s; and (B) synthesis of modified RNA using modified **G^{R}TP**s in the presence of GMP.

Conclusions

We synthesized a complete set of 18 modified pyrimidine or 7-deazapurine NTPs bearing substituents of increasing bulkiness (methyl, ethynyl, phenyl, benzofuryl and dibenzofuryl). For alkyl or ethynyl substituted nucleotides, the best approach was the synthesis of the corresponding nucleoside followed by triphosphorylation. For most of the aryl substituted **N^{R}TP**s, the direct Suzuki–Miyaura cross-coupling of halogenated **N^{I}TP**s with the corresponding arylboronic acid gave the desired modified nucleotides in a single step.

Systematic screening of the incorporation of the modified ribonucleotides into RNA using **N^{R}TP**s as substrates in the presence of T7 RNA polymerase was performed using several DNA templates encoding for different RNA transcripts. It should be noted that the procedures had to be optimized to minimize misincorporations. The assays revealed that the modified pyrimidine nucleotides **U^{R}TP**s and **C^{R}TP**s bearing smaller or mid-sized substituents (up to benzofuryl) worked as good to very good substrates for the T7 RNA polymerase and the nucleotides were incorporated with moderate to good efficiency even into difficult RNA sequences containing 7 modifications with two pairs of modified bases at adjacent positions. The most bulky dibenzofuryl modification still worked reasonably well on pyrimidine NTPs in easier sequences but not in the difficult sequences. Transcriptions with modified 7-deazaadenine **A^{R}TP**s also worked well for smaller or mid-sized modifications (but not for bulky dibenzofuryl). However, special attention had to be paid to optimize the procedure to avoid misincorporations.

Much more problematic and difficult was the polymerase synthesis of RNA containing modified 7-deazaguanosines. The synthesis of RNA commencing with a guanosine-free AAA sequence did not work and the sequence starting with GGG worked only for non-modified GTP. Using a modified promoter encoding for RNA starting with the GCA sequence we at least observed some transcription product when using **G^{Me}TP** (but not the bulkier **G^{R}TP**s) indicating that the polymerase does not tolerate bulky modifications at the +1 position. The problem was solved using GMP for initiation of transcription. In the presence of GMP, the synthesis of **G^{R}**-modified RNA worked well for all **G^{R}TP**s (except for the most bulky **G^{DB}TP**). The lack of transcription with modified **G^{R}TP**s in the absence of GMP could be explained by the fact that the K_m for the +1 substrate is much higher than that for any other elongating position and therefore the sterically hindered nucleotide that can be tolerated in other positions cannot be tolerated in the +1 position.

Most of the previous studies were performed only with 5-modified UTPs[25–37] and with just a few examples of 5-modified CTPs.[38–41] Our current work for the first time compares the same substituents directly linked (without any flexible

tether) to different NTPs. This not only confirms the previous findings[25–39] that the pyrimidine NTPs bearing even bulkier groups at position 5 are good substrates for T7 RNA polymerase, but also shows that the corresponding 7-substituted 7-deazapurine NTPs (with smaller or mid-sized groups up to benzofuryl) can be reasonably efficiently incorporated into RNA. The substituents here were not selected for a specific function but to study the influence of the size of the group on the enzymatic incorporation. Nevertheless, at least the UTP, CTP and 7-deazaATP derivatives bearing smaller hydrophobic substituents certainly have a potential for applications in selection of aptamers or other functional RNAs.[14–16] The limitations for practical use are the first three nucleotides of the transcript (the transcript must start with at least one non-modified guanosine) and problematic use of modified **G^{R}TP**s. The difficult sequences not accessible by T7 polymerase synthesis (*e.g.* any sequence starting with non-G nucleotides) still remain a challenge for future development, which in principle could be solved by engineered RNA polymerases not dependent on specific promoters.[57] Studies in this direction are underway in our laboratory.

Experimental

Synthesis and characterization data of the following known compounds have been reported previously: **A^{E}**, **A^{I}TP** and **A^{Ph}TP**,[39] **C^{E}** and **U^{E}**,[58] **G^{Ph}**, **G^{BF}** and **G^{TMSE}**,[59] **C^{I}TP**,[60] **U^{I}TP**,[61] **U^{Ph}TP**[62] and **U^{BF}TP**.[37] Detailed synthetic procedures and full characterization data for all new compounds are given in the ESI.† Only the most important general procedures are given below.

General procedure A: Suzuki cross-coupling on modified nucleosides

A water/ACN mixture (2 : 1, 15 mL) was added through a septum to an argon purged vial containing an iodinated nucleoside (0.2 mmol), corresponding boronic acid (2.4 mmol) and Cs_2CO_3 (399 mg, 1.2 mmol), followed by the addition of the degassed solution of $Pd(OAc)_2$ (7 mg, 0.02 mmol) and TPPTS ligand (69 mg, 0.12 mmol) in water/ACN (2 : 1, 10 mL). After argon/vacuum exchange, the reaction mixture was left stirring at 100 °C overnight. The mixture was cooled to room temperature, coevaporated with silica gel and purified by high performance reverse phase flash chromatography (0 → 100% MeOH in water) using a C18 RediSep column on a CombiFlash Teledyne ISCO system.

General procedure B: Phosphorylation of modified nucleosides

A modified nucleoside (1 equiv.) was dried at 60 °C overnight under vacuum. It was then suspended in $PO(OMe)_3$, stirred at room temperature for 15 minutes, followed by cooling to 0 °C and addition of $POCl_3$ (1.2 equiv.). The reaction mixture was left stirring at 0 °C (1.5–24 h) and then an ice cold solution of $(NHBu_3)_2H_2P_2O_7$ (5 equiv.) and tributyl amine (5 equiv.) in anhydrous DMF (1–5 mL) was added. The reaction mixture was stirred at 0 °C for another hour. Then an aqueous solution of TEAB (2 M, 2 mL, 4 mmol) was added and the mixture was evaporated under reduced pressure. The residue was coevaporated several times with water. The product was purified with chromatography on a DEAE Sephadex column (0→1.2 M aq. TEAB) and then with HPLC (C-18 column, 0.1 M TEAB in water to 0.1 M TEAB in 50% aq. MeOH); it was coevaporated several times with water and, where possible, converted to the sodium salt form (Dowex 50 in an Na^+ cycle).

General procedure C: Suzuki coupling on iodinated triphosphates

A degassed solution of $Pd(OAc)_2$ (0.5 mg, 0.002 mmol) and TPPTS (6 mg, 0.01 mmol) in water/ACN (2 mL, 2 : 1) was added to the water/ACN (5 mL, 2 : 1) solution of **N^{I}TP** (30 mg, 0.04 mmol), corresponding boronic acid (0.11 mmol) and Cs_2CO_3 (70 mg, 0.2 mmol). The reaction mixture was left stirring for 30 min at 100 °C. After cooling to room temperature, the mixture was concentrated under reduced pressure and the product was purified with HPLC (C-18 column, 0.1 M TEAB in water to 0.1 M TEAB in 50% aq. MeOH), coevaporated several times with water and, where possible, converted to a sodium salt form (Dowex 50 in Na^+ cycle).

In vitro transcription with modified NTPs

A solution of template oligonucleotides (100 μM each) in annealing buffer [Tris (10 mM), NaCl (50 mM), EDTA (1 mM), pH 7.8] was heated to 95 °C for 5 minutes and slowly cooled to 25 °C over a period of 45 minutes. The resulting DNA (50 μM) was used as a template for transcription reactions. *In vitro* transcription reactions were performed in the total volume of 20 μL in 40 mM Tris buffer (pH 7.9) containing modified NTP (2 mM), three natural NTPs (2 mM), DTT (10 mM), $MgCl_2$ (25 mM), Ribolock RNase inhibitor (1 U μL^{-1}), Triton X-100 (0.1%), dsDNA template (0.625 μM), T7 RNA polymerase (2 U μL^{-1}, Thermo Scientific) and [α-^{32}P]-GTP (111 TBq mmol^{-1}, 370 MBq mL^{-1}, 0.4 μL) or [α-^{32}P]-ATP (111 TBq mmol^{-1}, 370 MBq mL^{-1}, 0.4 μL) if transcription was done with modified GTPs. In the negative control experiment, water was used instead of the solution of modified NTP, and in the positive control the natural NTP (2 mM) was used instead. The transcription reactions were performed at 37 °C for 2–4 h. The samples (2 μL) were mixed with RNA loading dye (2 μL, Thermo Scientific), heated to 75 °C for 10 minutes and cooled on ice. The samples were then analyzed using gel electrophoresis on 12.5% denaturing polyacrylamide gel containing 1 × TBE buffer (pH 8) and urea (7 M) at 42 mA for 45 minutes. The gels were dried (85 °C, 75 minutes), autoradiographed and visualized using a phosphoimager (Typhoon 9410, Amersham Biosciences). Transcription efficiencies were determined densitometrically from two or three independent experiments using QuantityOne software.

In vitro transcription with modified GTPs in the presence of GMP

A solution of template oligonucleotides (100 μM) in annealing buffer [Tris (10 mM), NaCl (50 mM), EDTA (1 mM), pH 7.8] was heated to 95 °C for 5 minutes and slowly cooled to 25 °C over a period of 45 minutes. The resulting DNA (50 μM) was used as a template for transcription reactions. *In vitro* transcription reactions were performed in the total volume of 20 mL in 40 mM Tris buffer (pH 7.9) containing modified GTP (2 mM), GMP (30 mM), CTP (2 mM), UTP (2 mM), ATP (2 mM), DTT (10 mM), $MgCl_2$ (25 mM), Ribolock RNase inhibitor (1 U μL^{-1}), Triton X-100 (0.1%), dsDNA template (0.625 μM), T7 RNA polymerase (3 U μL^{-1}, Thermo Scientific) and [α-^{32}P]-ATP (111 TBq $mmol^{-1}$, 370 MBq mL^{-1}, 0.4 μL). In the negative control experiment, water was used instead of the solution of modified GTP, and in the positive control the natural GTP (2 mM) was used instead. The transcription reactions were performed at 37 °C for 4 h. The samples (2 μL) were mixed with RNA loading dye (2 μL, Thermo Scientific), heated to 75 °C for 10 minutes and cooled on ice. The samples were then analyzed by gel electrophoresis on 12.5% denaturing polyacrylamide gel containing 1 × TBE buffer (pH 8) and urea (7 M) at 42 mA for 45 minutes. The gels were dried (85 °C, 75 minutes), autoradiographed and visualized by a phosphoimager (Typhoon 9410, Amersham Biosciences). Transcription efficiency was determined densitometrically from two independent experiments using QuantityOne software.

MALDI-TOF analysis of modified RNAs

A solution of template oligonucleotides (100 μM each) in annealing buffer [Tris (10 mM), NaCl (50 mM), EDTA (1 mM), pH 7.8] was heated to 95 °C for 5 minutes and slowly cooled to 25 °C over a period of 45 minutes. The resulting DNA (50 μM) was used as a template for transcription reactions. *In vitro* transcription reactions were performed in a total volume of 50 μL in 40 mM Tris buffer (pH 7.9) containing modified NTP (2 mM), three natural NTPs (2 mM), DTT (10 mM), $MgCl_2$ (25 mM), Ribolock RNase inhibitor (1 U μL^{-1}), Triton X-100 (0.1%), dsDNA template (1.25 μM), and T7 RNA polymerase (3 U μL^{-1}, Thermo Scientific). The transcription reactions were performed at 37 °C for 2–4 h. Then, DNAse I (2U, Thermo Scientific) was added and the samples were incubated for further 15 minutes. The samples were then purified on NucAway spin columns (Ambion, elution done in DEPC-H_2O) as per the supplier's protocol. Purified samples were analyzed by MALDI-TOF mass spectrometry. The MALDI-TOF spectra were measured on a MALDI-TOF/TOF mass spectrometer with a 1 kHz smartbeam II laser. The measurements were done in the reflection mode by a droplet technique, with the mass range up to 30 kDa. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in a 9/1/1 ratio. The matrix (1 μL) was applied on the target (ground steel) and dried at room temperature. The sample (1 μL) and matrix (1 μL) were mixed and added on top of the dried matrix preparation spot and dried at room temperature.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Funding by the European Regional Development Fund; OP RDE (No. CZ.02.1.01/0.0/0.0/16_019/0000729 to M. H.), the Czech Science Foundation (206-12-G151 to N. M.) and the Czech Academy of Sciences (Praemium Academiae award to MH) is gratefully acknowledged.

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