

Supplementary Information

Constraints on the Emergence of RNA through Non-Templated Primer Extension with Mixtures of Potentially Prebiotic Nucleotides

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1 Materials and Methods

1.1 Characterization of Activated Mononucleotides and Bridged Dinucleotides

- 1.1.1 ribo-cytidine-5'-phosphoro-(2-aminoimidazole) (***rC**)
- 1.1.2 ¹³C, ¹⁵N-ribo-cytidine-5'-phosphoro-(2-aminoimidazole) (**isotope-labeled *rC**)
- 1.1.3 arabino-cytidine-5'-phosphoro-(2-aminoimidazole) (***araC**)
- 1.1.4 threo-cytidine-5'-phosphoro-(2-aminoimidazole) (***tC**)
- 1.1.5 1,3-di-(cytidine-5'-phosphoryl)-2-aminoimidazolium (**rC*rC**)
- 1.1.6 ribo-guanosine-5'-phosphoro-(2-aminoimidazole) (***rG**)
- 1.1.7 arabino-guanosine-5'-phosphoro-(2-aminoimidazole) (***araG**)
- 1.1.8 threo-guanosine-5'-phosphoro-(2-aminoimidazole) (***tG**)
- 1.1.9 1,3-di-(guanosine-5'-phosphoryl)-2-aminoimidazolium (**rG*rG**)

1.2 Oligonucleotide Synthesis

2 Supplementary Scheme and Figures

3 Supplementary Tables

1 Materials and Methods

1.1 Characterization of Activated Mononucleotides and Bridged Dinucleotides

1.1.1 ribo-cytidine-5'-phosphoro-(2-aminoimidazole) (***rC**)

¹H NMR (400 MHz, D₂O) δ 7.75 (d, *J* = 7.6 Hz, 1H), 6.80 (m, 1H), 6.61 (m, 1H), 6.05 (d, *J* = 7.6 Hz, 1H), 5.95 (d, *J* = 4.2 Hz, 1H), 4.25 (t, *J* = 4.5 Hz, 1H), 4.24 – 4.16 (m, 3H), 4.09 (m, 1H).

³¹P NMR (162 MHz, D₂O) δ -11.33.

HRMS (Q-TOF) *m/z*: calculated for [C₁₂H₁₆N₆O₇P]⁻ 387.0824; found: 387.0814.

1.1.2 ¹³C, ¹⁵N-ribo-cytidine-5'-phosphoro-(2-aminoimidazole) (isotope-labeled ***rC**)

¹H NMR (400 MHz, D₂O) δ 7.83 (d, *J* = 8.0 Hz, 1H), 7.37 (d, *J* = 7.9 Hz, 1H), 6.70 – 6.61 (m, 2H), 6.45 (m, 2H), 6.12 (s, 1H), 6.01 (s, 1H), 5.68 (s, 1H), 5.58 (s, 1H), 4.35 – 4.17 (m, 4H), 4.12 (m, 1H), 3.97 – 3.80 (m, 4H), 3.75 (m, 1H).

³¹P NMR (162 MHz, D₂O) δ -11.26 (m).

HRMS (Q-TOF) *m/z*: calculated for [C₃¹³C₉H₁₆N₃¹⁵N₃O₇P]⁻ 399.1037; found: 399.1036.

1.1.3 arabino-cytidine-5'-phosphoro-(2-aminoimidazole) (***araC**)

¹H NMR (400 MHz, D₂O) δ 7.63 (d, *J* = 7.6 Hz, 1H), 6.81 (s, 1H), 6.61 (s, 1H), 6.20 (s, 1H), 6.03 (d, *J* = 7.4 Hz, 1H), 4.40 (m, 1H), 4.23 – 4.01 (m, 4H).

³¹P NMR (162 MHz, D₂O) δ -10.68.

HRMS (Q-TOF) *m/z*: calculated for [C₁₂H₁₆N₆O₇P]⁻ 387.0824; found: 387.0812.

1.1.4 threo-cytidine-5'-phosphoro-(2-aminoimidazole) (***tC**)

¹H NMR (400 MHz, D₂O) δ 7.66 (d, *J* = 7.6 Hz, 1H), 6.58 (td, *J* = 19.3 Hz, 2H), 5.93 (d, *J* = 7.6 Hz, 1H), 5.72 (s, 1H), 4.73 (dd, *J* = 8.3, 3.2 Hz, 1H), 4.49 – 4.37 (m, 2H), 4.06 (s, 1H).

³¹P NMR (162 MHz, D₂O) δ -13.16.

HRMS (Q-TOF) *m/z*: calculated for [C₁₁H₁₄N₆O₆P]⁻ 357.0718; found: 357.0707.

1.1.5 1,3-di-(cytidine-5'-phosphoryl)-2-aminoimidazolium (**rC*rC**)

¹H NMR (400 MHz, D₂O) δ 7.63 (t, *J* = 7.7 Hz, 2H), 6.93 (t, *J* = 2.0 Hz, 2H), 6.01 (d, *J* = 7.6 Hz, 2H), 5.82 (d, *J* = 3.2 Hz, 2H), 4.28 – 4.09 (m, 10H).

³¹P NMR (162 MHz, D₂O) δ -12.68.

HRMS (Q-TOF) *m/z*: calculated for [C₂₁H₂₈N₉O₁₄P₂]⁻ 692.1236; found: 692.1230.

1.1.6 ribo-guanosine-5'-phosphoro-(2-aminoimidazole) (***rG**)

¹H NMR (400 MHz, D₂O) δ 7.89 (s, 1H), 6.66 (t, *J* = 0.5 Hz, 1H), 6.46 (t, *J* = 1.8 Hz, 1H), 5.87 (s, 1H), 4.70 (t, *J* = 5.6 Hz, 1H), 4.38 – 4.33 (m, 1H), 4.28 – 4.22 (m, 1H), 4.11 – 4.01 (m, 2H).

³¹P NMR (162 MHz, D₂O) δ -10.66.

HRMS (Q-TOF) *m/z*: calculated for [C₁₃H₁₆N₈O₇P]⁻ 427.0885; found: 427.0884.

1.1.7 arabino-guanosine-5'-phosphoro-(2-aminoimidazole) (***araG**)

¹H NMR (400 MHz, D₂O) δ 7.79 (s, 1H), 6.67 (dd, *J* = 1.9, 1.3 Hz, 1H), 6.46 (t, *J* = 2.1 Hz, 1H), 6.16 (d, *J* = 5.8 Hz, 1H), 4.48 (t, *J* = 5.9 Hz, 1H), 4.33 (t, *J* = 6.2 Hz, 1H), 4.18 – 3.99 (m, 3H).

³¹P NMR (162 MHz, D₂O) δ -10.59.

HRMS (Q-TOF) *m/z*: calculated for [C₁₃H₁₆N₈O₇P]⁻ 427.0885; found: 427.0878.

1.1.8 threo-guanosine-5'-phosphoro-(2-aminoimidazole) (***tG**)

¹H NMR (400 MHz, D₂O) δ 7.64 (d, *J* = 7.6 Hz, 1H), 6.62 – 6.51 (m, 2H), 5.91 (d, *J* = 7.6 Hz, 1H), 5.69 (s, 1H), 4.71 (dd, *J* = 8.3, 3.2 Hz, 1H), 4.47 – 4.34 (m, 1H), 4.04 (s, 1H).

³¹P NMR (162 MHz, D₂O) δ -12.62.

HRMS (Q-TOF) *m/z*: calculated for [C₁₂H₁₄N₈O₆P]⁻ 397.0779; found: 397.0778.

1.1.9 1,3-di-(guanosine-5'-phosphoryl)-2-aminoimidazolium (**rG*rG**)

¹H NMR (400 MHz, D₂O) δ 7.83 (s, 2H), 6.71 – 6.66 (m, 2H), 5.75 (d, *J* = 5.2 Hz, 2H), 4.65 (t, *J* = 5.3 Hz, 2H), 4.39 (t, 2H), 4.15 – 4.05 (m, 4H), 4.05 – 3.95 (m, 2H).

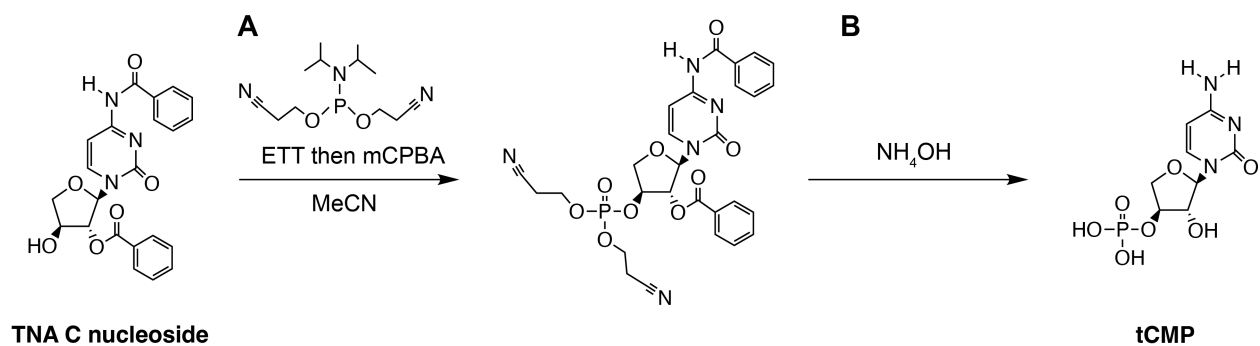
³¹P NMR (162 MHz, D₂O) δ -12.92.

HRMS (Q-TOF) *m/z*: calculated for [C₂₃H₂₈N₁₃O₁₄P₂]⁻ 772.1359; found: 772.1367.

1.2 Oligonucleotide Synthesis

Oligonucleotides were either purchased from IDT or synthesized in-house by solid phase synthesis on the Expedite 8909 DNA/RNA synthesizer. Phosphoramidites and reagents were purchased from ChemGenes (Wilmington, MA) and Glen Research (Sterling, MA). The synthesized oligonucleotides were deprotected and purified by Glen-Pak RNA purification cartridges (Sterling, MA).

2 Supplementary Scheme and Figures



Scheme S1. Synthesis of threose cytidine monophosphate (tCMP) from (A) phosphorylation and (B) deprotection of TNA C nucleoside.

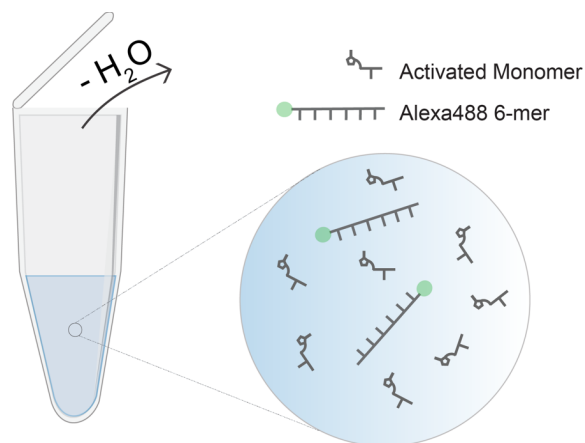
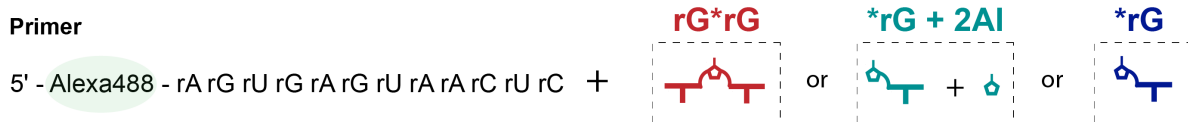
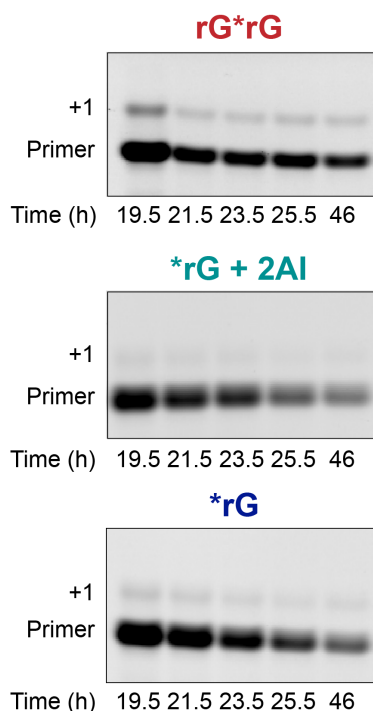
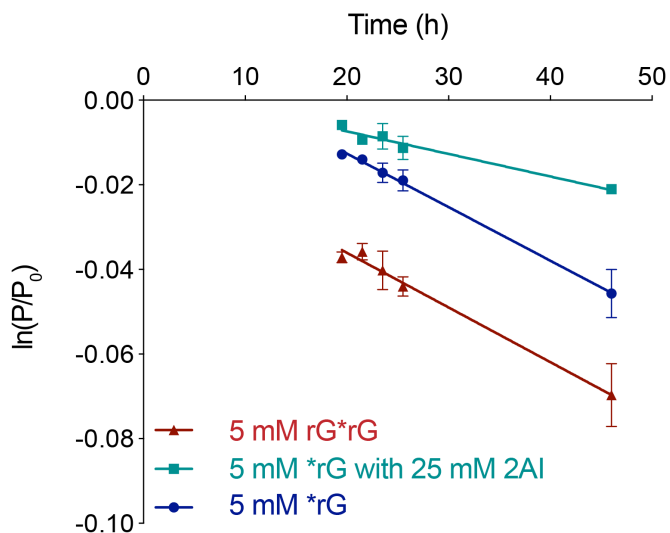


Figure S1. Schematic representation of the spontaneous air-drying experiments. Water in the reaction mixture gradually evaporates under ambient air flow and dry down to a clear paste at the bottom of the Eppendorf tube. During this process, both the primer (XJ-Alexa-6mer, as listed in Table S1) and the activated mononucleotides become concentrated. As a result, the non-templated primer extension speeds up to generate considerable amount of extended products.

A**B****C**

$$k (5 \text{ mM } rG*rG) = 1.3(1) \times 10^{-3} \text{ h}^{-1}$$

$$k (5 \text{ mM } *rG \text{ with } 25 \text{ mM } 2AI) = 5.3(6) \times 10^{-4} \text{ h}^{-1}$$

$$k (5 \text{ mM } *rG) = 1.3(1) \times 10^{-3} \text{ h}^{-1}$$

Figure S2. Mechanism of non-templated primer extension. (A) Schematic representation of a non-templated primer extension reaction using an Alexa488-labeled RNA 12-mer with bridged dinucleotides (rG*rG) or activated mononucleotides (*rG). 2AI was added to reduce the formation of bridged dinucleotides. (B) Gel electrophoresis images of non-templated primer extension reactions using bridged dinucleotides or activated mononucleotides. (C) Kinetic analysis with observed pseudo-first-order reaction rates (k_{obs}). Reaction conditions: 1 μM primer (XJ-Alexa-12mer, Table S1), 200 mM HEPES at pH 8.0, and 50 mM MgCl_2 with 5 mM rG*rG, 5 mM *rG with 25 mM 2AI, or 5 mM *rG. Error bars indicate standard deviations of the mean, $n=2$ replicates.

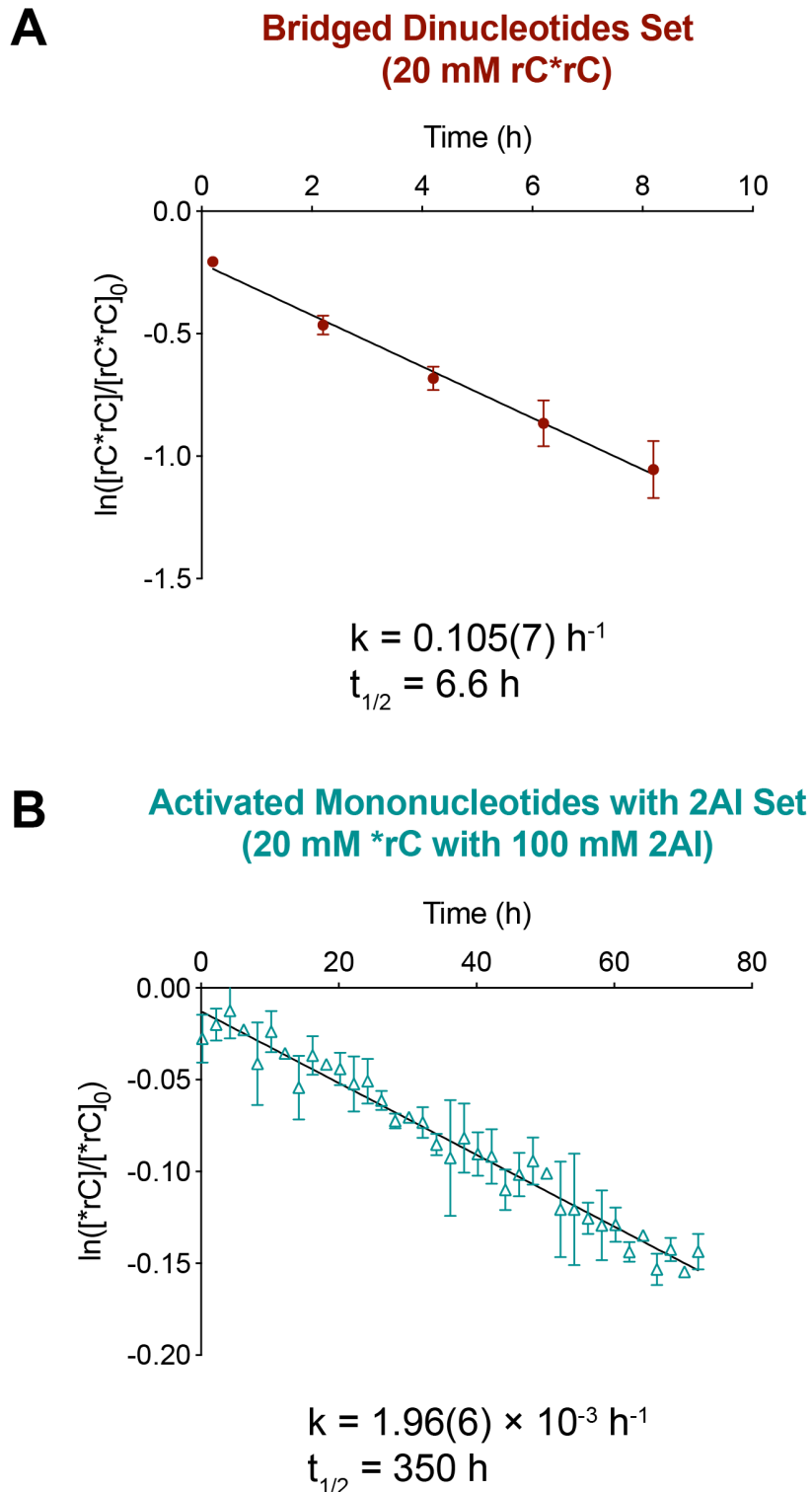


Figure S3. The hydrolysis rates and half-lives of (A) bridged dinucleotides (20 mM rC*rC) and (B) activated mononucleotides with 2AI (20 mM *rC with 100 mM 2AI) derived from the ^{31}P NMR experiments in Figure S5. Reaction conditions: the respective concentration of activated species as indicated above, 50 mM MgCl_2 , 200 mM HEPES at pH 8.0, 10% (v/v) D_2O , incubation at RT. Error bars indicate standard deviations of the mean, $n=2$ replicates.

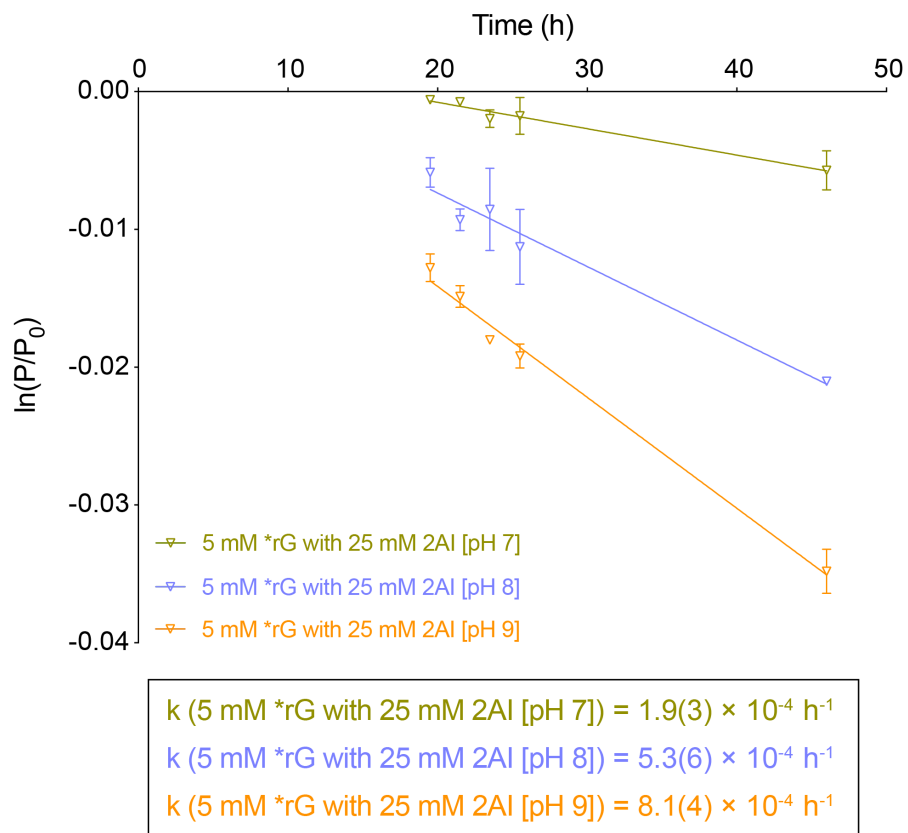


Figure S4. Non-templated primer extension with activated monomers with 2AI at different pHs. Reaction conditions: 5 mM *rG with 5-fold 2AI or 5 mM *rG were added to 1 μ M primer (XJ-Alexa-12mer, Table S1),, 200 mM HEPES pH 7 or 8 or 9 and 50 mM MgCl₂ respectively. Error bars indicate standard deviations of the mean, n=2 replicates.

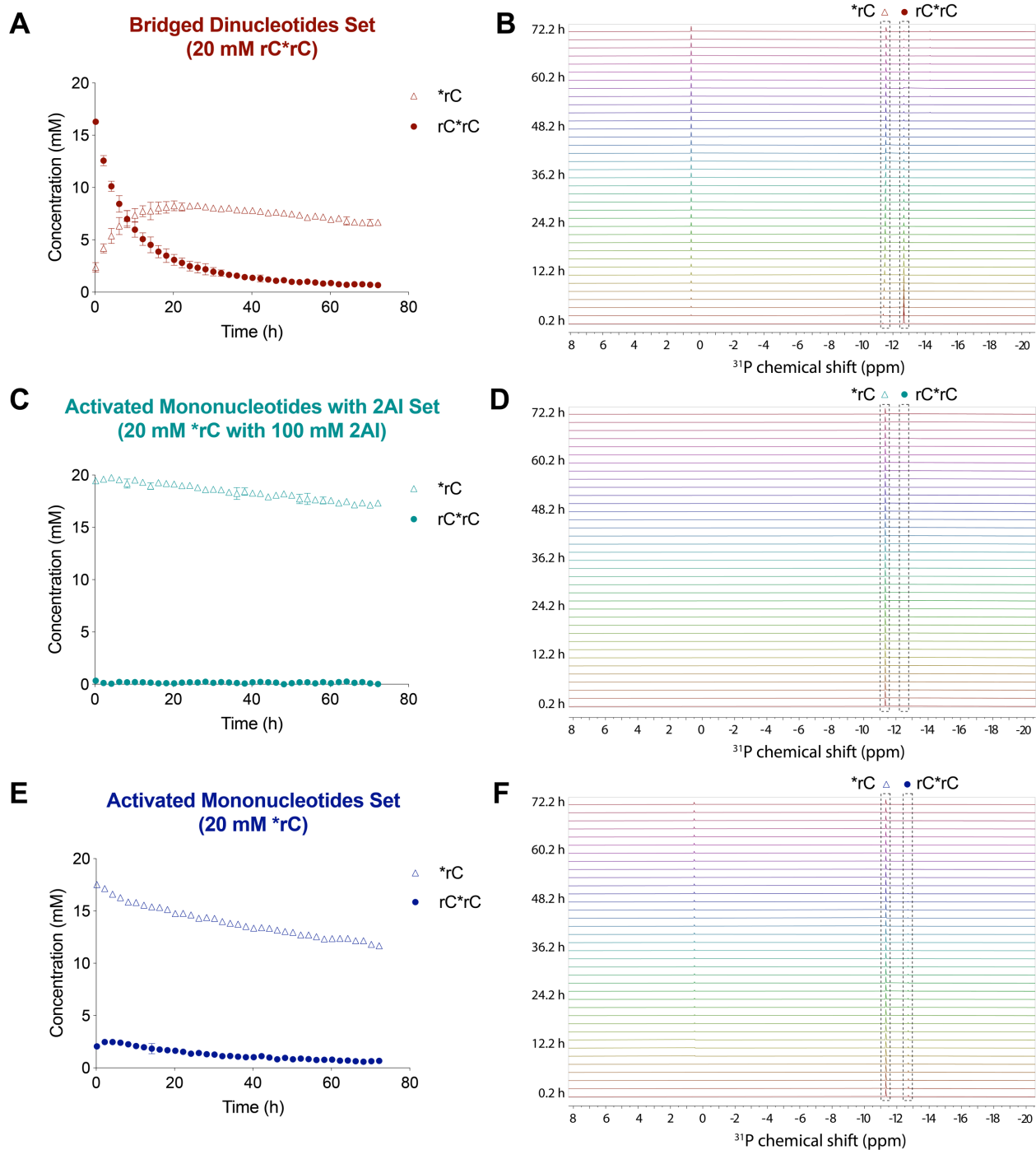


Figure S5. ^{31}P NMR hydrolysis experiments. (A, C, E) Plots of the concentration of active species (*rC and rC*rC) in solution over time. (B, D, F) Stacked ^{31}P NMR spectra with activated species boxed in rectangles. Reaction sets include (A, B) bridged dinucleotides (20 mM rC*rC), (C, D) activated mononucleotides with 2AI (20 mM *rC with 100 mM 2AI), and (E, F) activated mononucleotides sets (20 mM *rC). Reaction conditions: the respective concentrations of activated species as indicated above, 50 mM MgCl_2 , 200 mM HEPES at pH 8.0, 10% (v/v) D_2O , incubation at RT. Error bars indicate standard deviations of the mean, n=2 replicates.

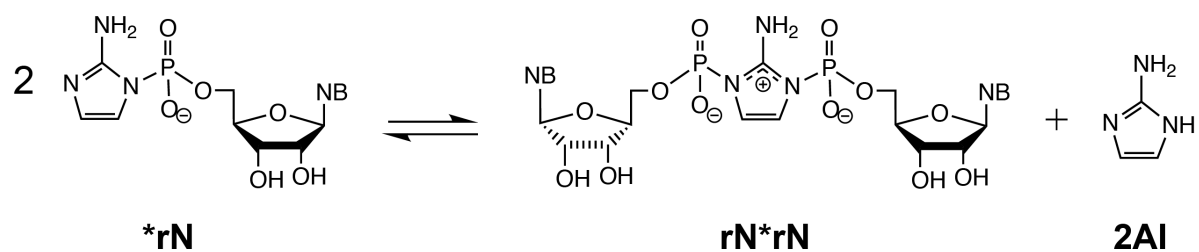
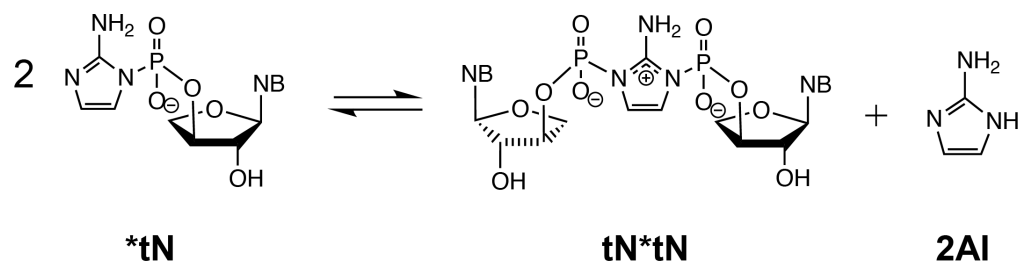
A**B**

Figure S6. Schematic representation of the formation of (A) 5'-5' imidazolium-bridged ribonucleotides (rN*rN) and (B) 3'-3' imidazolium-bridged threonucleotides (tN*tN).

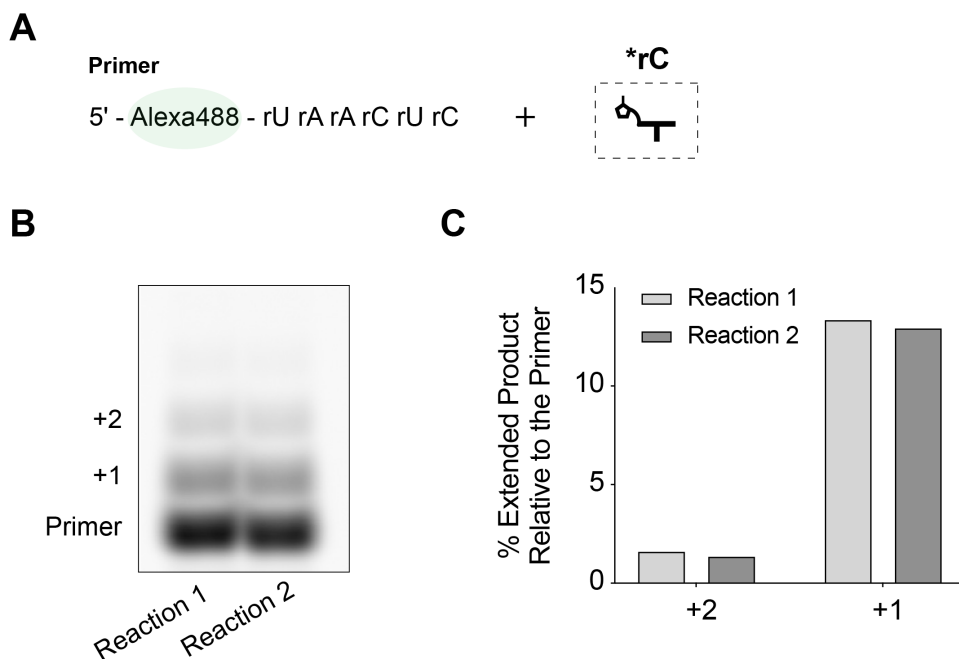


Figure S7. Variability of spontaneous air-drying experiments within the same set (performed at the same time and exposed to the identical ambient air flow rate and moisture level). (A) Schematic representation of non-templated primer extension reaction using an Alexa488-labeled RNA 6-mer with activated mononucleotides (^{*rC}). (B) Gel electrophoresis image of non-templated primer extension reactions within the set. (C) Quantification of extended products relative to the primer (100%). Reaction conditions: 10 μ M primer (XJ-Alexa-6mer, Table S1), 200 mM HEPES pH at 8.0, 50 mM MgCl₂, and 20 mM ^{*rC}. The reaction mixtures were subjected to air-drying to clear pastes under ambient air to accelerate non-templated primer extension (as previously described in Figure S1) and were subsequently quenched at 24h.

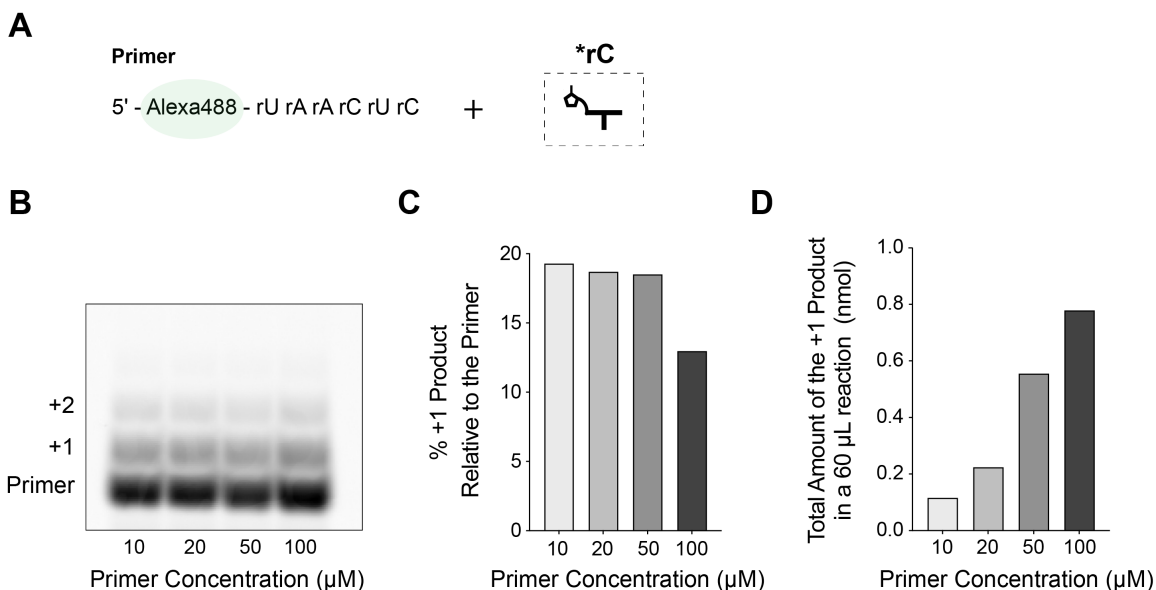


Figure S8. Effect of primer concentrations on spontaneous air-drying experiments. (A) Schematic representation of non-templated primer extension reaction using an Alexa488 labeled RNA 6-mer (Table S1) with activated mononucleotides (\ast rC). (B) Gel electrophoresis image of non-templated primer extension reactions across different primer concentrations. (C) Quantification of +1 product relative to primer (100%). (D) Derived total amount of +1 product at different primer concentrations for a 60 μ L reaction. 100 μ M primer concentration yielded the highest amount of +1 product for characterization and was therefore chosen as the most optimal condition for subsequent spontaneous air-drying and competition experiments. Reaction conditions: primer (10 μ M, 20 μ M, 50 μ M or 100 μ M), 200 mM HEPES pH 8.0, 50 mM MgCl₂ and 20 mM \ast rC. The reaction mixtures were dried down to clear pastes under ambient air to speed up non-templated primer extension (Figure S1) and were subsequently quenched at 24h.

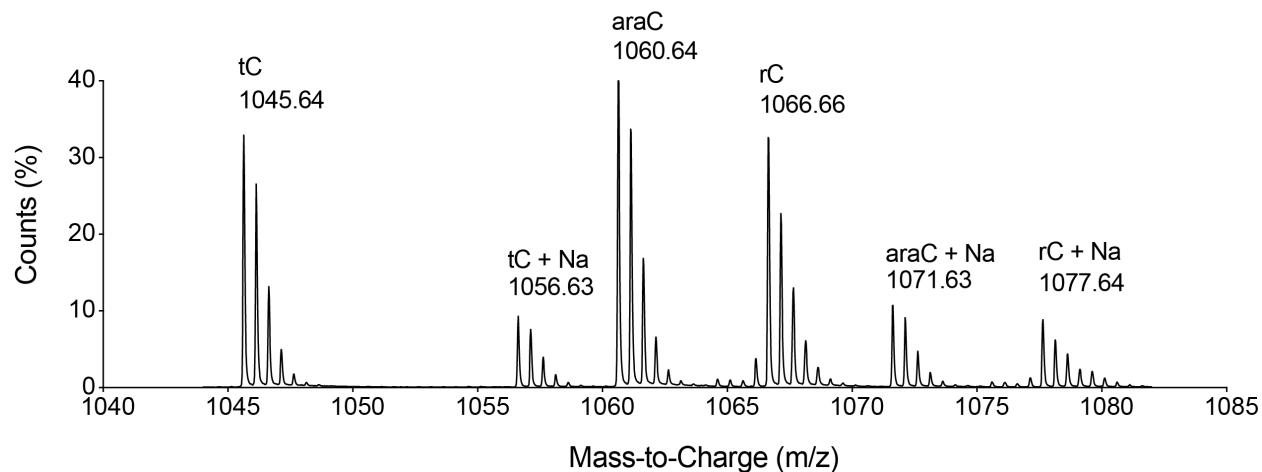


Figure S9. Plot of counts (%) as a function of mass-to-charge (m/z) of the +1 products and their salt adducts of \ast rC: \ast araC: \ast tC = 1:1:1 competition experiment using an 5'-OH RNA 6-mer. Species shown are in the -2 charged state (M-2H)²⁻. Reaction conditions: 100 μ M 6-mer primer (XJ-5, Table S1) and 6.7 mM each of \ast rC, \ast araC and \ast tC. The reactions were allowed to dry spontaneously under ambient air and were subsequently quenched at 24 h. The samples were then desalted on C18 ZipTip pipette tips, followed by injection in HR LC-MS. The generated compound lists using the Agilent MassHunter Qualitative Analysis software were matched with the calculated masses of all possible +1 non-templated primer extension products and their salt adducts (Table S2).

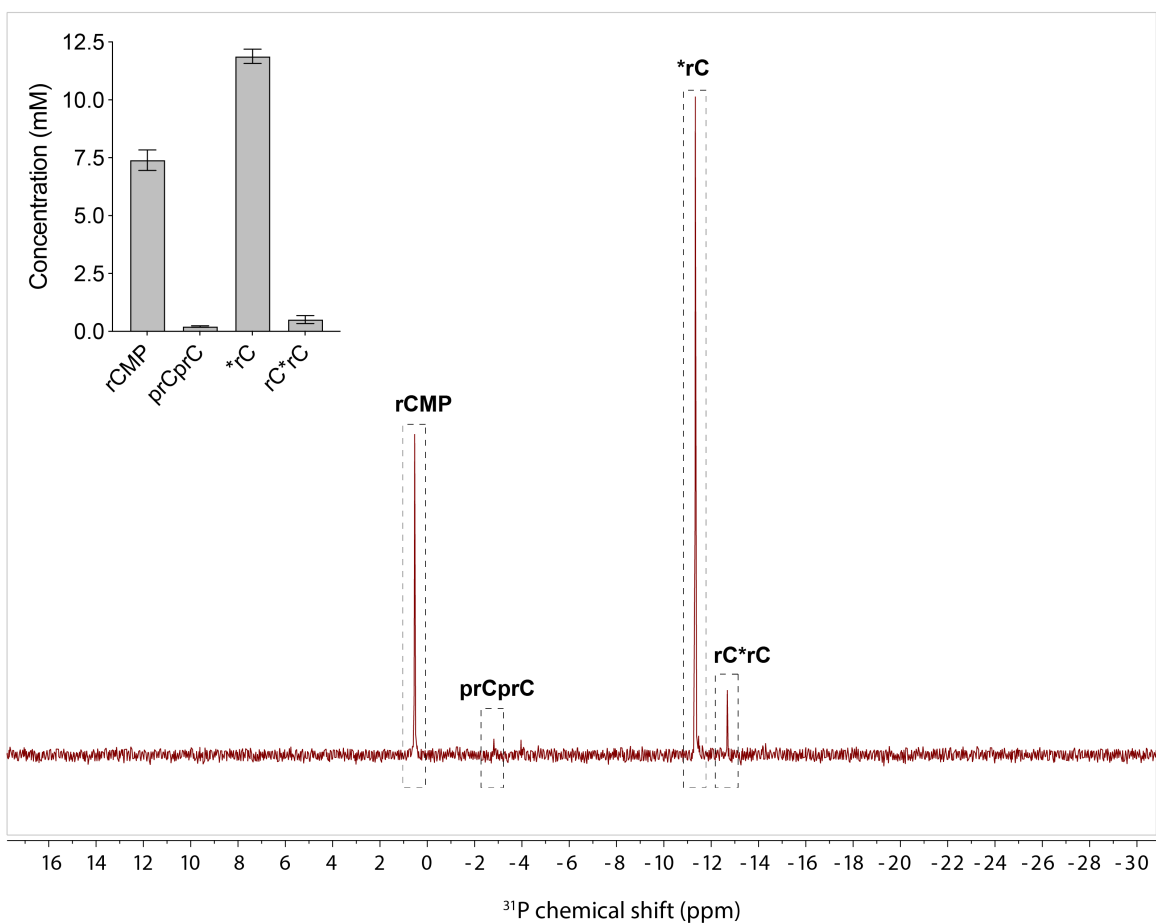


Figure S10. ^{31}P NMR spectrum reveals minimal dimer formation from activated monomers. Inset shows the concentration of different cytidine products: ribonucleoside monophosphate (rCMP), dimer (prCprC), activated mononucleotide (*rC), and bridged dinucleotides (rC*rC). Reaction conditions: 20 mM *rC, 50 mM MgCl_2 , 200 mM HEPES at pH 8.0, 10% (v/v) D_2O , incubation at RT for 72.2 hours. Error bars of the bar plot represent standard deviations of the mean, $n=2$ replicates.

Co-injection of XJ-1: 5' - OH - rU rA rA rC rU rC (the last phosphodiester linkage is 3'-5')
 and XJ-2: 5' - OH - rU rA rA rC rU rC (the last phosphodiester linkage is 2'-5')

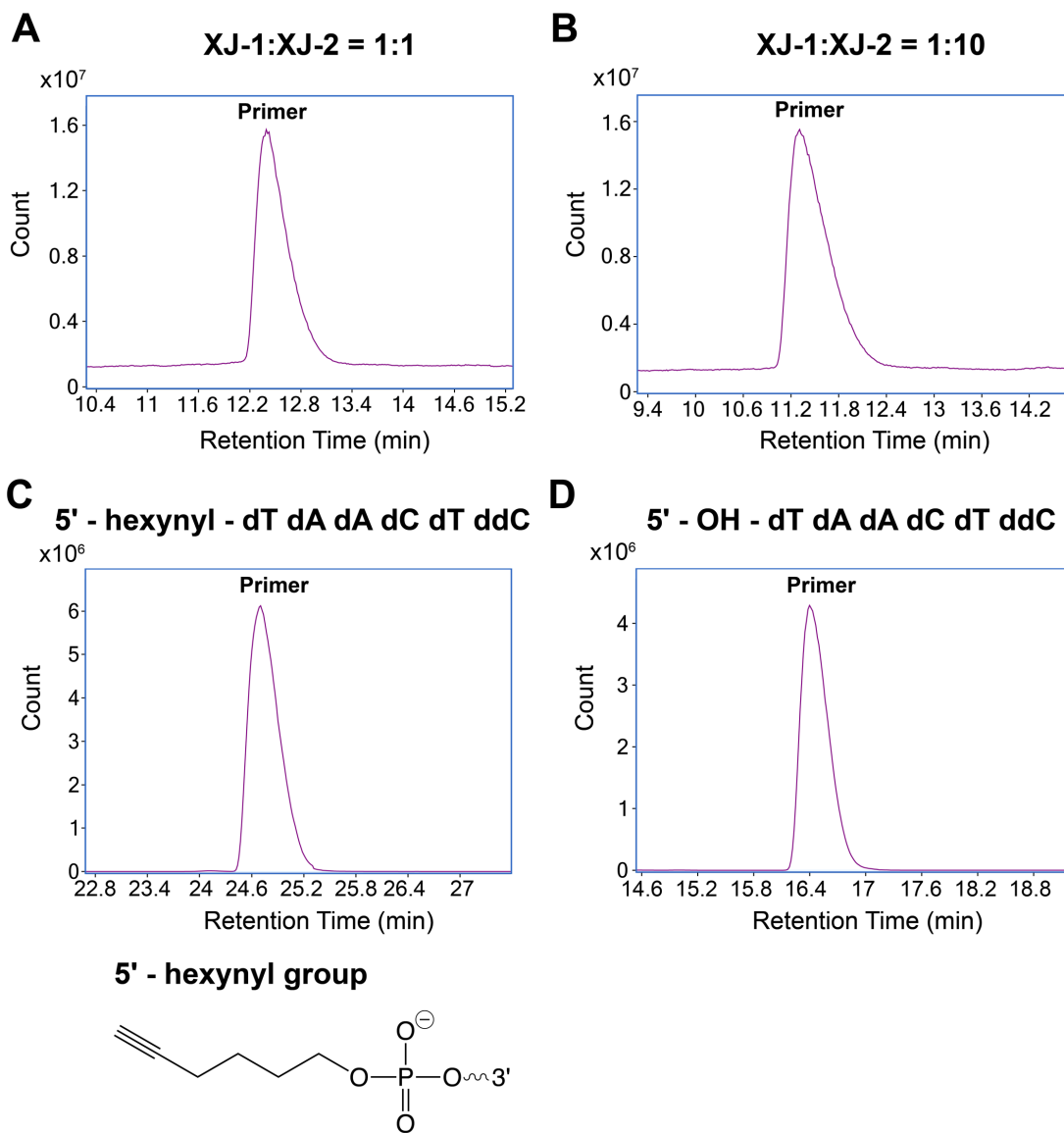


Figure S11. Origin of the double peak pattern in the chromatograms of the competition experiments in Figure 4. (A-B) Total ion chromatograms (TIC) from the co-injection of oligomers ending with 3'-5' (XJ-1, Table S1) and 2'-5' phosphodiester linkages (XJ-2, Table S1) in (A) 1:1 ratio of XJ-1 and XJ-2 and (B) 1:10 ratio of XJ-1 and XJ-2. Note that the XJ-1 and XJ-2 oligomers were synthesized using TOM-protected RNA amidites to exclude the possibility of 2' to 3' migration of the TBDMS group. Standards with 3'-5' and 2'-5' linkage could not be separated on LC-MS. (C) TIC of the non-templated primer extension product of a 5'-hexynyl DNA primer (XJ-9, Table S1) showed that nucleobases did not react. The chemical structure of the 5'-hexynyl group is indicated. (D) TIC of the non-templated primer extension product of a 5'-OH DNA primer (XJ-11, Table S1) showed that 5'-OH did not react with the activated mononucleotides. Reaction conditions of (C)-(D): 20 mM activated mononucleotides, 100 μ M 6-mer primer, 200 mM HEPES at pH 8.0, and 50 mM MgCl₂. The reactions were allowed to dry spontaneously under ambient air and were subsequently quenched at 24 h. The samples were then desalted on C18 ZipTip pipette tips, followed by injection in HR LC-MS.

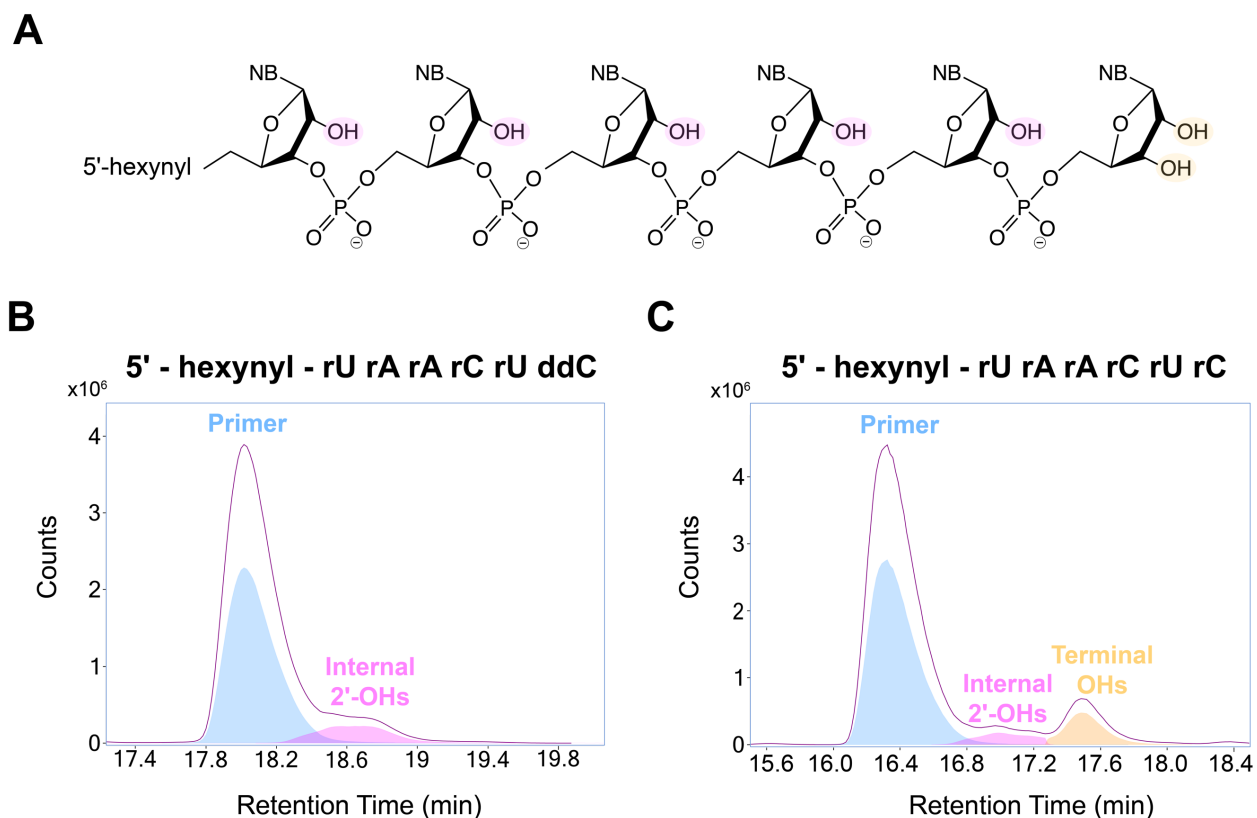


Figure S12. Both internal and terminal hydroxyls can react in non-templated primer extension. (A) Schematic representation of the internal 2'-OHs and terminal OHs in an RNA oligomer (5'-hexynyl-rU rA rA rC rU rC). Overlay of TCC (purple) and ECC (blue: primer; pink: internal 2'-OH reaction; yellow: terminal OH extension) of the non-templated primer extension product with (B) 5'-hexynyl RNA primer ending with dideoxy cytosine (XJ-7, Table S1) and (C) 5'-hexynyl RNA primer (XJ-8, Table S1). Reaction conditions for (B) and (C): 20 mM activated mononucleotides, 100 μ M 6-mer primer, 200 mM HEPES at pH 8.0, and 50 mM $MgCl_2$. The reactions were allowed to dry spontaneously under ambient air and were subsequently quenched at 24 h. The samples were then desalted on C18 ZipTip pipette tips, followed by injection in HR LC-MS.

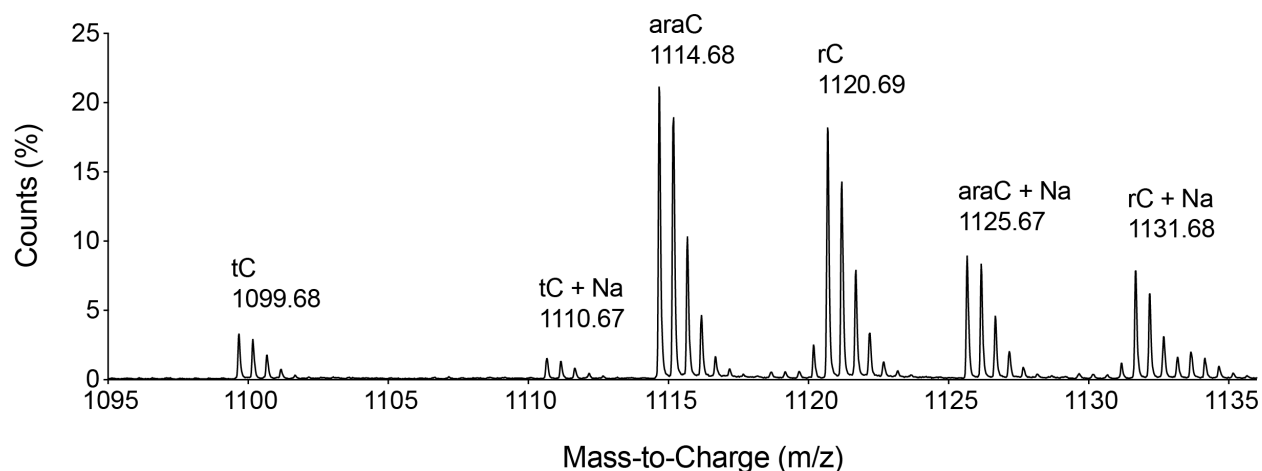


Figure S13. Ion counts (%) as a function of mass-to-charge (m/z) of the +1 products and their salt adducts of *rC:*araC:*tC = 1:1:1 competition experiment (Reaction1, Set 3) with a modified primer. All mass-to-charge ratios here are in the -2 charged state ($M-2H$)²⁻. Reaction conditions: 100 μ M 6-mer primer (XJ-10, Table S1) and 6.7 mM each of *rC, *araC and *tC. The reactions were allowed to dry spontaneously under ambient air and were subsequently quenched at 24 h. The samples were then desalted on C18 ZipTip pipette tips, followed by injection in HR LC-MS. The generated compound lists using the Agilent MassHunter Qualitative Analysis software were matched with the calculated masses of all possible +1 non-templated primer extension products and their salt adducts (Table S3). For further information on the HR LC-MS, please refer to the materials and methods section.

3 Supplementary Tables

Table S1. Sequences of oligonucleotides used in the study.

Name	Source	Sequence (5' → 3')
XJ-FAM-12mer	IDT	5' - FAM - rA rG rU rG rA rG rU rA rA rC rU rC
XJ-Alexa-6mer	IDT	5' - Alexa 488 - rU rA rA rC rU rC
XJ-Alexa-12mer	IDT	5' - Alexa 488 - rA rG rU rG rA rG rU rA rA rC rU rC
XJ-1	In-house	5' - OH - rU rA rA rC rU rC rC (last linkage: 3'- 5')
XJ-2	In-house	5' - OH - rU rA rA rC rU rC rC (last linkage: 2'- 5')
XJ-5	IDT	5' - OH - rU rA rA rC rU rC
XJ-7	IDT	5' - hexynyl - rU rA rA rC rU ddC
XJ-8	IDT	5' - hexynyl - rU rA rA rC rU rC
XJ-9	IDT	5' - hexynyl - dT dA dA dC dT ddC
XJ-10	IDT	5' - hexynyl - dT dA dA dC dT rC
XJ-11	IDT	5' - OH - dT dA dA dC dT ddC
XJ-6mer-rc	IDT	5' - OH - rG rA rG rU rU rA
XJ-15	In-house	5' - FAM - rA rG rU rG rA rG rU rA rA rC rU araC
XJ-16	In-house	5' - rG rA rG rU rU rA rC rU rC rA rC rU

Table S2. Observed and calculated masses of the expected products of competition experiments.

	Observed Mass (Da)	Calculated Mass (Da)
Primer + rC	2135.33	2135.35
Primer + araC	2123.31	2123.32
Primer + tC	2093.30	2093.31
Primer + rC + rC	2452.39	2452.42
Primer + araC + araC	2428.34	2428.36
Primer + tC + tC	N/A	2368.34
Primer + rC + araC	2440.37	2440.39
Primer + rC + tC *	2410.35	2410.38
Primer + araC + tC *	2398.33	2398.35

* Sequence might not be in order.

Table S3. Observed and calculated masses of the expected products of competition experiments with a 5'-hexynyl DNA primer with a 3'-terminal ribonucleotide.

	Observed Mass (Da)	Calculated Mass (Da)
Primer + rC	2243.42	2243.43
Primer + araC	2231.40	2231.41
Primer + tC	2201.38	2201.40

Table S4. Sugar and backbone composition of non-templated products. (A) Nucleotide incorporation (%) of the +1 extended products in the *rC:*araC:*tC = 1:1:1 and 10:1:1 competition experiments. (B) Total and individual extension ratio (internal: terminal) in the non-templated primer extension of 5'-hexynyl RNA primer without and with complementary oligomers. (C) Nucleotide incorporation (%) of the +1 extended products in *rC:*araC:*tC = 1:1:1 and 10:1:1 competition experiments with a modified primer. When calculating the incorporation percentage, the integrated intensity is taken into account from both the +1 extended products and their salt adducts. ^a

A. Incorporation of ribo-, arabino-, and threo-nucleotides in competition experiments ^b

*rC:*araC:*tC = 1:1:1	rC (%)	araC (%)	tC (%)
Reaction 1, Set 1	30.6	47.1	22.3
Reaction 2, Set 1	29.5	48.4	22.2
Reaction 1, Set 2	31.8	41.9	26.3
Reaction 2, Set 2	31.7	41.5	26.8
*rC:*araC:*tC = 10:1:1	rC (%)	araC (%)	tC (%)
Reaction 1, Set 2	86.7	8.7	4.6
Reaction 2, Set 2	84.9	8.1	6.9

B. Presence of complementary oligomers disfavoring extension through internal hydroxyls ^c

Without complementary oligomers	Total Extension Ratio Internal : Terminal	Average Individual Extension Ratio ^d Internal : Terminal
Reaction 1, Set 1	1.0 : 1.6	1.0 : 4.0
Reaction 2, Set 1	1.0 : 1.5	1.0 : 3.8
With complementary oligomers	Total Extension Ratio Internal : Terminal	Average Individual Extension Ratio ^d Internal : Terminal
Reaction 1, Set 1	1.0 : 4.0	1.0 : 10.0
Reaction 2, Set 1	1.0 : 3.6	1.0 : 9.0
Reaction 3, Set 1	1.0 : 3.4	1.0 : 8.4

C. Competition experiments with a 5'-hexynyl DNA primer with a 3'-terminal ribonucleotide

*rC:*araC:*tC = 1:1:1	rC (%)	araC (%)	tC (%)
Reaction 1, Set 1	41.1	43.7	15.3
Reaction 2, Set 1	40.8	43.4	15.8
Reaction 1, Set 2	37.8	44.7	17.5
Reaction 2, Set 2	37.6	42.5	20.0
Reaction 1, Set 3	38.5	47.4	14.1
Reaction 2, Set 3	38.0	48.1	14.0
*rC:*araC:*tC = 10:1:1	rC (%)	araC (%)	tC (%)
Reaction 1, Set 1	88.9	8.3	2.7

Reaction 2, Set 1	89.1	7.9	3.1
Reaction 1, Set 2	89.5	7.8	2.7
Reaction 2, Set 2	89.3	8.0	2.8
Reaction 1, Set 3	89.2	8.8	2.0
Reaction 2, Set 3	88.9	9.1	2.1

^a Within a given set, reactions are independent replicates initiated simultaneously whereas reactions across different sets are independent replicates performed on separate dates. Given that the ambient flow rates and moisture levels can vary daily, reactions performed across different sets exhibited greater variability compared to those within the same set.

^{b,c} $n \geq 2$ replicates.

^d Individual extension ratio was calculated based on the assumption that all internal sites (5 in total) and terminal sites (2 in total) contributed equally to the extension.

^e $n = 6$ replicates.

Table S5. Normalized percentages (%) of the +2 extended products in the *rC:*araC:*tC = 10:1:1 competition experiments. ^a

	Normalized Percentage (%)
Primer + rC + rC	84 (1)
Primer + rC + araC	12 (1)
Primer + rC + tC *	5 (1)

^a $n = 2$ replicates.

* Sequence might not be in order.

Table S6. Observed and calculated masses of the hetero-bridged dinucleotides containing tC in a one-pot mixture of 1:1:1 *rC:*araC:*tC. ^a

	Observed Mass (Da)	Calculated Mass (Da)
rC*tC	674.13	674.13
araC*tC	663.12	663.12

^a Activated mononucleotides (*rC, *araC, and *tC) were mixed in the ratio of 1:1:1. The mixture got spontaneously air-dried. It was then resuspended in LC-MS grade water and immediately submitted for LC-MS analysis.