2’/3’ Regioselectivity of Enzyme-Free Copying of RNA Detected by NMR

Sebastian Motsch, Daniel Pfeffer, and Clemens Richert*\[a\]

cbic_202000014_sm_miscellaneous_information.pdf
Author Contributions

S.M. Conceptualization: Supporting; Data curation: Lead; Methodology: Lead; Writing - Original Draft: Equal; Writing - Review & Editing: Equal
D.P. Conceptualization: Supporting; Data curation: Supporting; Methodology: Supporting; Validation: Equal; Writing - Original Draft: Supporting; Writing - Review & Editing: Supporting.
Table of Contents

1. Materials and Methods ............................................................................................................ S2
2. Synthesis and NMR Spectra of 4-Dimethylaminopyridine-Activated CMP .................. S3
3. Synthesis of RNA Hairpins .................................................................................................. S7
4. Assignment of $^1$H-NMR Spectra for Hairpins ............................................................... S12
5. Confirmation of Peak Assignments via Spiking with Authentic Samples ..................... S16
6. Protocols for Primer Extension Assays ................................................................................. S17
7. $^1$H NMR Spectra of Primer Extension Assays ................................................................. S18
8. Additional Kinetics of Primer Extension Assays with Hairpin 1 ...................................... S24
9. References for Supporting Information .............................................................................. S26
1. Materials and Methods

Reagents. General chemicals and solvents were obtained from commercial sources and further used without purification. Cytidine 5'-monophosphate (CMP, disodium salt) was purchased from Sigma Aldrich (Deisenhofen, Germany) and D₂O (99.9% deuterated) was from Euriso-Top (Saint-Aubin, France). Pre-activated nucleotides 2-Alm-CMP,[S¹] 2-MeIm-CMP,[S²] and OAt-CMP[S³] were synthesized based on protocols given in the literature (vide infra). The 1-ethyl-2-methylimidazole, employed as alternative organocatalyst, was prepared in-house following a published procedure.[S⁴]

NMR Spectroscopy. The NMR spectra were acquired on a Bruker Avance III spectrometer, proton resonance frequency 700 MHz, and a phosphorous resonance frequency of 284 MHz or a Bruker Avance III HD-NanoBay spectrometer, with a proton resonance frequency of 400 MHz and a phosphorous resonance frequency of 162 MHz, using 3 x 100 mm NMR Tubes from Hilgenberg (Malsfeld, Germany). Before ¹H-NMR spectra were recorded, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt was added as internal standard.

MALDI-TOF MS. MALDI-TOF mass spectra were measured on a Bruker Microflex mass spectrometer (Bruker Daltonics, Bremen, Germany). The spectra were acquired in linear negative mode using a 2,4,6-trihydroxyacetophenone solution (0.2 M in ethanol) and a diammonium citrate solution (0.1 M in H₂O) at a ratio of 2:1 (v:v) as matrix and co-matrix.

Chromatography. Purification of preactivated nucleotides was performed via Sep-Pak Vac C18 3cc RP-cartridges (Waters, Milford, MA, USA), previously rinsed with acetonitrile and aqueous triethylammonium acetate buffer solution (0.1 M, pH 7.5). The RNA hairpins were purified by Sep-Pak Vac QMA 3cc IEX-cartridge (Waters, Milford, MA, USA), pre-washed with an aqueous solution of ammonium carbonate (5 mM, pH 8.0).

Solid-Phase RNA Synthesis. RNA solid-phase synthesis was performed on an ABI Expedite 8909 oligonucleotide synthesizer (PerSeptive Biosystems, Framingham, USA) using a slight modification of the default protocol of the manufacturer. The 2'-TBDMS-protected phosphoramidites, as well as immobilized ribonucleosides on controlled-pore glass (cpg), were purchased from Sigma Aldrich (Taufkirchen, Germany). The 3'-TBDMS-protected phosphoramidites and the phosphoramidite of the hexaethyleneglycol (HEG) linker 17-O-(4,4'-dimethoxytrityl)-hexaethyleneglycol-1-{(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite were from ChemGenes (Wilmington, MA, USA).
2. Synthesis and NMR Spectra of 4-Dimethylaminopyridine-Activated CMP

Cytidine 5'-monophosphate (CMP) in its free acid form (1.62 mg, 5.0 μmol) and DMAP (3.7 mg, 30 μmol, 6.0 equiv) were dissolved in D$_2$O (200 μL) and the pH was adjusted to 8.0, using an NaOH stock solution (10 M, D$_2$O). Then, EDC hydrochloride (30 mg, 160 μmol, 32 equiv) was added, resulting in an 800 mM final concentration of the carbodiimide, and the reaction was allowed to proceed for 2 h at 22 °C. Then, the reaction mixture was added to a pre-chilled solution of NaClO$_4$ in acetone/Et$_2$O (0.1 M, 5 mL, 1:1, v/v) at 0 °C. After vigorous shaking, the suspension was centrifuged for 5 min, and the supernatant was discarded. The pellet was washed with acetone (3 mL) and dried under reduced pressure in a desiccator. The crude product was dissolved in aqueous TEAA buffer (1 mL, 50 mM) and loaded onto a C18 SepPac cartridge (500 mg). After washing with aqueous TEAA buffer (4.5 mL, 50 mM) and H$_2$O (4.5 mL), the product was eluted with a solution of MeCN in H$_2$O (4.5 mL, 10% MeCN). Product-containing fractions were pooled and lyophilized. The activated ribonucleotide was obtained as a colorless solid (1.06 mg, 2.48 μmol, 50%). $^1$H-NMR (400 MHz, D$_2$O): $\delta$ [ppm] = 8.15 (dd, $^3J = 7.0$ Hz, 2 H, ArH), 7.45 (d, $^3J = 7.6$ Hz, 1 H, H6), 6.78 (d, $^3J = 7.0$ Hz, 2 H, Ar-H), 5.97 (d, $^3J = 7.5$ Hz, 1 H, H5), 5.71 (d, $^3J = 3.6$ Hz, 1 H, H1'), 4.31 (m, 1 H, H2'), 4.23 (dd, $^3J = 5.0$ Hz, 1 H, H3'), 4.17-4.08 (unresolved multiplets, 3 H, H3', H5', H5''), 3.20 (s, 6 H, 2×CH$_3$); $^{31}$P-NMR (162 MHz, D$_2$O): $\delta$ [ppm] = -5.75; HRMS (ESI-TOF): m/z calcd. for C$_{16}$H$_{23}$N$_5$O$_7$P [M]$^+$ 428.133, found 428.133.
Figure S1. $^1$H-NMR spectrum of 4-dimethylaminopyridine-activated CMP (700 MHz, D$_2$O).

Figure S2. $^{31}$P-NMR spectrum of 4-dimethylaminopyridine-activated CMP (162 MHz, D$_2$O).
Protocols employed for the preparation of known compounds

Cytidine-5′-phosphoro-(2-aminoimidazole) (2-AIm-CMP)

The procedure is based on a protocol previously reported in literature. To a suspension of CMP free acid (9.9 mg, 0.03 mmol), 2-aminoimidazole (65 mg, 0.30 mmol), triphenylphosphine (71 mg, 0.27 mmol) and NEt₃ (60 μL, 0.43 mmol) in dry DMSO (1 mL) under argon atmosphere, 2,2′-dipyridyl disulfide (66 mg, 0.30 mmol) was added. The resulting yellow solution was stirred for 30 min at room temperature. Afterwards, the solution was added dropwise to a pre-chilled solution of NaClO₄ in acetone/Et₂O/NEt₃ (0.1 M, 20:10:1 mL) at 0 °C. After vigorous shaking, the suspension was centrifuged and the supernatant was decanted. The pellet obtained was washed with 10 mL acetone and dried under reduced pressure in a desiccator. From the crude product obtained (47 mg) an aliquot of 6.5 mg was taken, diluted in TEAA buffer (1 mL, 50 mM) and loaded onto a C18 SepPac cartridge (500 mg). The cartridge was rinsed with TEAA buffer (6 mL, 50 mM) and the product was eluted with H₂O (6 mL). Product-containing fractions were lyophilized, and the activated ribonucleotide was obtained as a colorless solid (triethylammonium salt, 1.29 mg, 2.64 μmol, 64%).

1H-NMR (400 MHz, D₂O): δ [ppm] = 7.68 (d, ³J = 7.5 Hz, 1 H, H6), 6.75 (m, 1 H, HIm), 6.63 (m, 1 H, HIm), 6.00 (d, ³J = 7.6 Hz, 1 H, H5), 5.86 (d, ³J = 4.0 Hz, 1 H, H1′), 4.20 (dd, ³J = 4.2 Hz, 1 H, H2′), 4.18 – 4.11 (m, 3 H, H3′, H4′, H5′), 4.06 (m, 1 H, H5″); 31P-NMR (162 MHz, D₂O): δ [ppm] = -8.42; ESI-MS: m/z calcd. for C₁₂H₁₆N₆O₇P [M-H] 387.08, found 387.06.

Cytidine-5′-phosphoro-(2-methylimidazole) (2-MeIm-CMP)

The CMP free acid (78 mg, 0.25 mmol), 2-methylimidazole (328 mg, 4.0 mmol), triphenylphosphine (210 mg, 0.80 mmol), and 2,2′-dipyridyl disulfide (326 mg, 0.80 mmol) were placed in a round-bottom flask and dried under high vacuum for 30 min. The flask was flushed with argon and dry DMF (2.5 mL) was added. Then, triethylamine (80 μL, 0.625 mmol) was added, and the resulting yellow solution was stirred for 4.5 h at 22 °C. The reaction mixture was added to a saturated solution of NaClO₄ in acetone/Et₂O (100 mM, 30 mL, 1:1 v/v) at 0 °C. The suspension was shaken and then kept in an ice bath for 10 min. Afterwards, the suspension was centrifuged and the supernatant removed. The slightly yellow solid thus obtained was washed with Et₂O (10 mL) and dried in a desiccator. An aliquot of the crude (2.0 mg from 120 mg) was applied to a C18 SepPac cartridge (500 mg), followed by washing with TEAA buffer (6 mL, 50 mM) and eluting with H₂O (6 mL). Product-containing fractions were pooled and freeze-dried. The triethylammonium salt of the imidazolide was obtained as a colorless solid (0.91 mg, 1.86 μmol, 45%).
1H-NMR (400 MHz, D2O): δ [ppm] = 7.62 (d, J = 7.6 Hz, 1 H, H6), 7.21 (m, 1 H, HIm), 6.89 (m, 1 H, HIm), 5.94 (d, J = 7.6 Hz, 1 H, H5), 5.85 (d, J = 3.6 Hz, 1 H, H1’), 4.20 (dd, J = 3.6 Hz, 1 H, H1’), 4.21 – 4.10 (m, 3 H, H3’), (H4’, H5’), 3.08 (m, 1 H, H5’), 2.46 (s, 3 H, HIm); 31P-NMR (162 MHz, D2O): δ [ppm] = -8.09; ESI-MS: m/z calcd. for C13H17N5O7P [M-H]- 386.09, found 386.06.

7-Azabenzotriazol-1-yl-cytidine-5’-phosphate (OAt-CMP)

The protocol is based on a literature protocol.[S3] To a solution of HATU (115 mg, 0.3 mmol), HOAt (27 mg, 0.2 mmol) and triethylamine (21 µL, 0.15 mmol) in dry DMF (0.5 mL), a solution of CMP (tetrabutylammonium salt, 56.5 mg, 0.1 mmol, 0.1 M) in dry DMF (1 mL) was added dropwise. The reaction mixture was stirred 2 h at room temperature. Afterwards, the reaction mixture was added dropwise to a pre-cooled, saturated solution of NaClO4 in acetone/Et2O (0.05 M, 20/20 mL) at 0 °C. After vigorous shaking, a centrifugation run was performed, and the supernatant was decanted off. The pellet obtained was washed three times with acetone (5 mL) and dried under reduced pressure in a desiccator. The crude product (46 mg) was obtained as a colorless solid. A sample of the crude (2.3 mg) was purified via RP C-18 chromatography. For this, the product was dissolved in TEAA buffer (1 mL, 50 mM), loaded onto the cartridge (500 mg) and washed with TEAA buffer (4.5 mL, 50 mM) and H2O (4.5 mL). The product was eluted using an aqueous acetonitrile solution (4.5 mL, 5% acetonitrile). Product-containing fractions were pooled and lyophilized. The triethylammonium salt of the active ester was isolated as a colorless solid (2.05 mg, 3.9 µmol, 78%).

1H-NMR (400 MHz, D2O): δ [ppm] = 8.73 (d, J = 4.6 Hz, 1 H, OAt-H6), 8.46 (d, J = 8.4 Hz, 1 H, OAt-H4), 7.63 (d, J = 7.7 Hz, 1 H, H6), 7.56 (dd, J = 8.6 Hz, J = 4.5 Hz, 1 H, OAt-H5), 5.84 (d, J = 4.2 Hz, 1 H, H1’), 5.72 (d, J = 7.6 Hz, 1 H, H5), 4.55 (m, 1 H, H5’), 4.35 (m, 1 H, H5’), 4.31 (dd, J = 5.2 Hz, 1 H, H2’), 4.26 (m, 1 H, H4’), 4.20 (dd, J = 4.7 Hz, 1 H, H3’); 31P-NMR (162 MHz, D2O): δ [ppm] = -0.90; ESI-MS: m/z calcd. for C14H15N7O8P [M-H]- 440.07, found 440.06.
3.  Synthesis of RNA Hairpins

**Synthesis of RNA Hairpins.** This protocol is for hairpin 5'-AGCAG-HEG-CUG-3' (1) and is representative. Automated solid-phase synthesis was performed, starting from cpg loaded with the first guanosine nucleoside (40 mg, 1 μmol). The synthesis was carried out twice and both batches were combined afterwards. The solid support was treated with AMA solution (750 μL, aqueous ammonia/aqueous methylamine 1:1, v/v) for 20 min at 55 °C. The supernatant was removed, and the residue was washed twice with H2O (2 × 1 mL). The aqueous solutions were combined and freed from ammonia and methylamine using nitrogen stream, targeted to the surface of the solution for 3 h. Then, the solution was lyophilized and the resulting solid was dissolved in triethylamine trihydrofluoride (1 mL). The reaction mixture was shaken for 5 h at 22 °C. Methoxytrimethylsilane (8 mL) was added and the mixture was shaken vigorously for 10 min. The formed suspension was centrifuged for 5 min and the supernatant was decanted. Then, the obtained pellet was dissolved in aqueous (NH4)2CO3 solution (1 mL, 5 mM) and loaded onto a QMA-SepPac cartridge (500 mg). Hairpins 1, 2c, and 3,2c were eluted with a step-gradient of (NH4)2CO3 in H2O (0-350 mM carbonate, 25 mM-steps, 6 mL each). In the case of hairpins 3, 4c, and 4,2c, a concentration gradient of 0-500 mM carbonate was used. Fractions containing the desired product were pooled and freeze-dried.

**Purification of Hairpins**

5'-AGCAG-HEG-CUG-3' (1). QMA-SepPac cartridge (500 mg, gradient of 0-350 mM (NH4)2CO3 in H2O, 25 mM-steps, 6 mL each): product eluted at 300-325 mM (NH4)2CO3.

5'-AGCAG-HEG-CUGC-3' (2c). QMA-SepPac cartridge (500 mg, gradient of 0-350 mM (NH4)2CO3 in H2O, 25 mM-steps, 6 mL each): product eluted at 300-325 mM (NH4)2CO3.

5'-AGCAG-HEG-CUG2C-3' (2,2c). QMA-SepPac cartridge (500 mg, gradient of 0-350 mM (NH4)2CO3 in H2O, 25 mM-steps, 6 mL each): product eluted at 300-325 mM (NH4)2CO3.

5'-CGCGGCAG-HEG-CUG-3' (3). QMA-SepPac cartridge (500 mg, gradient of 0-500 mM (NH4)2CO3 in H2O, 25 mM-steps, 6 mL each): product eluted at 475-500 mM (NH4)2CO3.

5'-CGCGGCAG-HEG-CUGC-3' (4c). QMA-SepPac cartridge (500 mg, gradient of 0-500 mM (NH4)2CO3 in H2O, 25 mM-steps, 6 mL each): product eluted at 475-500 mM (NH4)2CO3.
5′-CGCGGCAG-HEG-CUG\textsuperscript{2}C-3′ (4\textsuperscript{2}c). QMA-SepPac cartridge (500 mg, gradient of 0-500 mM (NH\textsubscript{4})\textsubscript{2}CO\textsubscript{3} in H\textsubscript{2}O, 25 mM-steps, 6 mL each): product eluted at 475-500 mM (NH\textsubscript{4})\textsubscript{2}CO\textsubscript{3}.

Table S1. Data for RNA hairpins synthesized.

| Hairpin | Sequence                  | Yield [%] | m/z calc. | Molecular formula          | m/z found |
|---------|---------------------------|-----------|-----------|-----------------------------|-----------|
| 1       | 5′-AGCAG-HEG-CUG-3′       | 51        | 2891      | C\textsubscript{89}H\textsubscript{120}N\textsubscript{33}O\textsubscript{62}P\textsubscript{8} | 2891      |
| 2c      | 5′-AGCAG-HEG-CUGC-3′      | 8         | 3197      | C\textsubscript{99}H\textsubscript{132}N\textsubscript{36}O\textsubscript{69}P\textsubscript{9} | 3198      |
| 2\textsuperscript{2}c | 5′-AGCAG-HEG-CUG\textsuperscript{2}C-3′ | 16       | 3197      | C\textsubscript{99}H\textsubscript{132}N\textsubscript{36}O\textsubscript{69}P\textsubscript{9} | 3197      |
| 3       | 5′-GCACGCAG-HEG-CUG-3′    | 17        | 3863      | C\textsubscript{117}H\textsubscript{156}N\textsubscript{44}O\textsubscript{84}P\textsubscript{11} | 3863      |
| 4c      | 5′-GCACGCAG-HEG-CUGC-3′   | 2         | 4168      | C\textsubscript{126}H\textsubscript{168}N\textsubscript{47}O\textsubscript{91}P\textsubscript{12} | 4169      |
| 4\textsuperscript{2}c | 5′-GCACGCAG-HEG-CUG\textsuperscript{2}C-3′ | 7        | 4168      | C\textsubscript{126}H\textsubscript{168}N\textsubscript{47}O\textsubscript{91}P\textsubscript{12} | 4167      |

Mass Spectra of Hairpins

![Mass spectrum of RNA hairpin 1](image-url)

Figure S3. MALDI-TOF mass spectrum of RNA hairpin 1.
**Figure S4.** MALDI-TOF mass spectrum of RNA hairpin 2c.

**Figure S5.** MALDI-TOF mass spectrum of RNA hairpin 2°c.
Figure S6. MALDI-TOF mass spectrum of RNA hairpin 3.

Figure S7. MALDI-TOF mass spectrum of RNA hairpin 4c.
**Figure S8.** MALDI-TOF mass spectrum of RNA hairpin 4\textsuperscript{c}. 
4. Assignment of $^1$H-NMR Spectra for Hairpins

Figure S9. Peak assignment for hairpin 5'-AGCAG-HEG-CUGC-3' (2c) in $^1$H-NMR (700 MHz). A) Aromatic region, B) region with anomeric and pyrimidine protons; C) Excerpt of NOESY spectrum showing nucleobase to ribose connectivities. Spectra were recorded in D$_2$O containing 60 mM HEPES, 9 mM MgCl$_2$ and 18 mM 1-EtIm at pH 7.5, uncorrected for deuterium effect.
Figure S10. Peak assignment for hairpin 5'-AGCAG-HEG-CUG^2_C-3' (2^2c) in 1H-NMR (700 MHz). A) Aromatic region, B) region with anomeric and pyrimidine protons; C) Excerpt of NOESY spectrum showing nucleobase to ribose connectivities. Spectra were recorded in D_2O containing 60 mM HEPES, 9 mM MgCl_2 and 18 mM 1-EtIm at pH 7.5, uncorrected for deuterium effect.
Figure S11. Peak assignment for hairpin 5'-CGCGGCAG-HEG-CUGC-3' (4c) in $^1$H-NMR (700 MHz). A) Aromatic region, B) region with anomic and pyrimidine protons; C) Excerpt of NOESY spectrum showing nucleobase to ribose connectivities. Spectra were recorded in D$_2$O containing 25 mM HEPES, 6 mM MgCl$_2$ at pH 7.8, uncorrected for deuterium effect.
Figure S12. Peak assignment for 5'-CGCGGCAG-HEG-CUG\textsuperscript{2'}C-3' (4\textsuperscript{2'}c) in \textsuperscript{1}H-NMR (700 MHz). A) Aromatic region, B) region with anomeric and pyrimidine protons; C) Excerpt of NOESY spectrum showing nucleobase to ribose connectivities. Spectra were recorded in D\textsubscript{2}O containing 25 mM HEPES, 6 mM. MgCl\textsubscript{2} at pH 7.8, uncorrected for deuterium effect.
5. Confirmation of Peak Assignments via Spiking with Authentic Samples

Figure S13. Confirmation of peak assignment in $^1$H-NMR (700 MHz) spectra from primer extension assay with hairpin 1 (in situ-activation with 1-EtIm as organocatalyst) by spiking with 2c (15 nmol) and 2$^2$c (15 nmol). Assay conditions: 2 mM 1, 10 mM CMP, 150 mM 1-EtIm, 800 mM EDC, 80 mM MgCl$_2$, 500 mM HEPES, pH 7.5, D$_2$O, 0 °C, 7 d.

Figure S14. Confirmation of peak assignment in $^1$H-NMR spectra (700 MHz) from primer extension assay with hairpin 3 (in situ-activation with 1-EtIm as organocatalyst) by spiking first with 4c (22 nmol) and then with 4$^2$c (22 nmol). The spiking was carried out successively, with NMR spectra recorded after each addition. Black arrows indicate the peaks that increase in intensity upon addition of authentic material. Assay conditions: 2 mM 3, 10 mM CMP, 150 mM 1-EtIm, 800 mM EDC, 80 mM MgCl$_2$, 500 mM HEPES, pH 7.5, D$_2$O, 0 °C, 5 d.
6. Protocols for Primer Extension Assays

Primer extension with in situ activation of ribonucleotides. This protocol is for the extension of hairpin 1 with CMP with in situ activation and is representative. A solution (10 µL) containing 1-ethylimidazole (1.5 µmol, 0.15 M), HEPES (5.0 µmol, 0.5 M) and MgCl₂ (8.0 µmol, 0.08 M) at pH 7.5 in D₂O was added to the hairpin 1 (40 nmol, lyophilized from stock solution). The solution was heated to 75 °C and cooled to 25 °C at a rate of 0.1 °C/s. Separately, EDC hydrochloride (30.7 mg, 160 µmol) was dissolved in D₂O (100 µL) containing CMP (free acid, 6.5 mg, 20 µmol, 20 mM), 1-ethylimidazole (1.4 µL, 15 µmol, 0.15 M), HEPES (11.9 mg, 50 µmol, 0.5 M), and MgCl₂ (0.73 mg, 8.0 µmol, 0.08 M) at pH 7.5. The solution of hairpin 1 (10 µL) was then added to the EDC-containing solution (10 µL), followed by vortexing, and the resulting solution was kept at 0 °C. The progress of the reaction was monitored by MALDI-TOF analysis. For this, analytical samples (0.5 µL) were drawn and diluted with aqueous NH₄OAc solution (20 µL, 1 M, pH 7.5). The solution was treated with Dowex 50WX8 cation exchange resin (1 mg, NH₄⁺ form) and incubated for 15 min. After centrifugation, 1 µL of the supernatant was diluted with H₂O (20 µL) and the resulting solution was analyzed by MALDI-TOF-MS. To determine the regioselectivity, D₂O (150 µL), containing TPS (0.1 equiv) as internal standard was added to the reaction mixture, and NMR spectra (700 MHz, room temperature) were recorded immediately.

Primer extension with preactivated ribonucleotides. This protocol is for the extension of hairpin 1 with 2-AIm-CMP and is representative. The RNA hairpin 1 (40 nmol) was dissolved in D₂O (10 µL) containing HEPES (2.0 µmol, 0.2 M) and MgCl₂ (0.5 µmol, 0.05 M) at pH 7.75, uncorrected for deuterium effect. The hairpin was annealed by heating to 75 °C and cooling to 25 °C at a rate of 0.1 °C/s. Subsequently, a solution containing 2-AIm-CMP (0.4 µmol, 40 mM), HEPES (2.0 µmol, 0.2 M) and MgCl₂ (0.5 µmol, 0.05 M) in D₂O (10 µL) at pH 7.75 was added to the hairpin solution. The reaction mixture was incubated at 25 °C. MALDI-TOF-MS analysis and ¹H-NMR measurement were performed at stated intervals as described in the protocol for primer extension with in situ activation of ribonucleotides.
7. ¹H NMR Spectra of Primer Extension Assays

¹H NMR spectra of primer extension assays with hairpin 1

Figure S15. ¹H-NMR spectrum (700 MHz, D₂O) from primer extension assay with hairpin 1 and in situ-activation with 1-EtIm acquired 7 d after the start of the assay at 0 °C.

Figure S16. ¹H-NMR spectrum (700 MHz, D₂O) from primer extension assay with hairpin 1 and in situ-activation with EtMeIm acquired 13 d after the start of the assay at 0 °C.
Figure S17. $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 1 with 2-AIm-CMP acquired 6 d after the start of the assay at 25 °C.

Figure S18. $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 1 with 2-MeIm-CMP acquired 11 d after the start of the assay at 25 °C.
Figure S19. $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 1 with OAt-CMP acquired 4 d after the start of the assay at 25 °C.
$^1$H NMR spectra of primer extension assays with hairpin 3

**Figure S20.** $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 3 and in situ-activation with 1-EtIm acquired 5 d after the start of the assay at 0 °C.

**Figure S21.** $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 3 with 2-AIm-CMP acquired 5 d after the start of the assay at 25 °C.
Figure S22. $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 3 with 2-MeIm-CMP acquired 7 d after the start of the assay at 25 °C.

Figure S23. $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 3 with OAt-CMP acquired 4 d after the start of the assay at 25 °C.
Figure S24. $^1$H-NMR spectrum (700 MHz, D$_2$O) from primer extension assay with hairpin 3 with DMAP-CMP acquired 4 d after the start of the assay at 25 °C.
8. Additional Kinetics of Primer Extension Assays with Hairpin 1

**Assay with EtMeIm-CMP (in situ activation)**

![Graph showing kinetics](image)

**Figure S25.** Kinetics of primer extension with hairpin 1 (1 mM) at 25 mM CMP with in situ activation, detected by MALDI-TOF MS. Conditions: 250 mM EtMeIm, 800 mM EDC hydrochloride, 80 mM MgCl₂, 500 mM HEPES, D₂O, pH 7.5, 0 °C. No fitting with a simple, monoexponential function is shown because in situ activation requires a minimum of two steps, resulting in more complex kinetics.

**Monoexponential Fits to Kinetics with Pre-activated Monomers**

**Assay with 2-AIm-CMP**

![Graph showing kinetics](image)

**Figure S26.** Kinetics of primer extension with hairpin 1 (1.5 mM), 25 mM 2-AIm-CMP, as detected by MALDI-TOF MS. Symbols are experimental data points and the line is a monoexponential fit. Conditions: 50 mM MgCl₂, 200 mM HEPES, D₂O, pH 7.5, 25 °C.
Assay with 2-Melm-CMP

Figure S27. Kinetics of primer extension with hairpin 1 (1 mM) at 20 mM 2-Melm-CMP, as detected by MALDI-TOF MS. Symbols are experimental data points and the line is a monoexponential fit. Conditions: 50 mM MgCl₂, 200 mM HEPES, D₂O, pH 7.75, 25 °C.

Assay with OAt-CMP

Figure S28. Kinetics of an assay of primer extension with hairpin 1 (2 mM) at 40 mM OAt-CMP, as detected by MALDI-TOF MS. Symbols are experimental data points and the line is a monoexponential fit. Conditions: 50 mM MgCl₂, 200 mM HEPES, D₂O, pH 8.7, 25 °C.
9. References for Supporting Information

[S1] L. Li, P. T. Tam, D. K. O'Flaherty, V. S. Lelyveld, E. C. Izgu, A. Pal, J. W. Szostak, Enhanced nonenzymatic RNA copying with 2-aminoimidazole activated nucleotides. *J. Am. Chem. Soc.* 2017, 139, 1810-1813.

[S2] a) R. Lohrmann, L. E. Orgel, Preferential formation of (2'-5')-linked internucleotide bonds in non-enzymatic reactions. *Tetrahedron* 1978, 34, 853-855.  b) M. Kurz, K. Göbel, C. Hartel, M. W. Göbel, Acridine-labeled primers as tools for the study of nonenzymatic RNA oligomerization. *Helv. Chim. Acta* 1998, 81, 1156–1180.

[S3] S. R. Vogel, C. Deck, C. Richert, Accelerating chemical replication steps of RNA involving activated ribonucleotides and downstream-binding element. *Chem. Commun.* 2005, 4922-4924.

[S4] B. Pilarski, A new method for N-alkylation of imidazoles and benzimidazoles. *Liebigs Ann. Chem.* 1983, 1078-1080.