Supporting Information

Nonenzymatic template-directed synthesis of mixed-sequence 3′-NP-DNA up to 25 nucleotides long inside model protocells

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Table of Contents

Materials and Methods S3

General information S3

Synthesis of activated nucleotides S3

Template-directed synthesis of 3′-NP-DNA S5

LC-MS analysis of primer extension reactions S6

Supporting Figures and Tables S7

SI Figure S1 : ^{31}P NMR degradation profile of 3′-NH_2-2AIpddT in presence or absence of $MgCl_2$. S8

SI Figure S2: Nonenzymatic primer extension on RNA homotemplates 3′-NNNN (N= G, A, T, or C). S9

SI Figure S3: ^{31}P NMR and LR-MS characterization of 3′-NH_2-2AIpddC showing the presence of the imidazolium bridged dinucleotide species. S10

SI Figure S4: Nonenzymatic primer extension on RNA heterotemplates 3′-NCCC (N= G, A, or C). S11

SI Figure S5: Timecourse analysis of fidelity assessment experiment (adapted from Sanger sequencing) for 2-letter RNA template sequences 3'-CUCUC-5' and 3'-GAGAG-5' S12

SI Figure S6: Timecourse analysis of fidelity assessment experiment (adapted from Sanger sequencing) for sequence 3'-ACUGCC AA-5' S13

SI Figure S7: Nonenzymatic primer extension on 4-letter mixed-sequence RNA templates 3'-ACUGCC AA-5'. S14

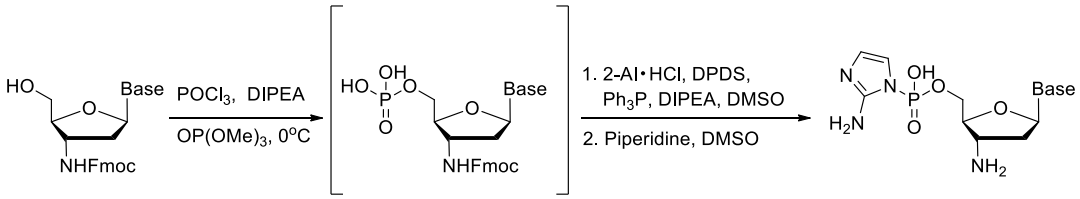
SI Table S1: Summary of oligonucleotide sequences used in this study S15

References S16

Materials and Methods

General information. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. 2-aminoimidazole HCl was purchased from Combi-Blocks, Inc. (San Diego, CA). Starting materials 3′-NH_2-2′,3′-dideoxyadenosine, 3′-NH_2-2′,3′-dideoxyguanosine, and 3′-NH_2-2′,3′-dideoxythymidine were purchased from Alfa Aesar (Reston, VA). 3′-NH_2-2′,3′-dideoxycytidine was purchased from Carbosynth (Compton, Berkshire, UK). Fmoc-protected compounds **1.A**, **1.G**, **1.C**, and **1.T** were synthesized according to previously published procedures (*1*, *2*). All riboside-5′-phosphoro-(2-aminoimidazolide) monomers were prepared according to previously published procedures (*3*). Reverse phase flash chromatography was performed using prepacked RediSep Rf Gold C18Aq 50 g columns from Teledyne Isco (Lincoln, NE). ^{1}H and ^{31}P NMR spectra were acquired at 400 MHz and 162 MHz, respectively, on a Varian Oxford AS-400 NMR spectrometer. Chemical shifts are reported in parts per million. ^{1}H NMR was referenced using the solvent resonance as the internal standard (HDO, 4.79 ppm at 25 °C). ^{31}P NMR was referenced using the internal standard $PO(OEt)_3$ or $PO(OMe)_3$ with chemical shifts of -3 ppm and 0 ppm, respectively. Data were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet), integration, and coupling constants. LRMS and HRMS were carried out on a Bruker Esquire 6000 ion trap and an Agilent 6520 QTOF LC-MS, respectively. All oligonucleotide sequences were synthesized in-house on an Expedite 8900 nucleic acid synthesizer (Millipore; Billerica, MA). All fluorescence single-wavelength readings were conducted using a Molecular Devices SpectraMax i3 (Sunnyvale, CA).

Synthesis of activated nucleotides. Cytosine 5′-phosphoro-2-aminoimidazolide, adenosine 5′-phosphoro-2- aminoimidazolide, uridine 5′-phosphoro-2-aminoimidazolide and guanosine 5′-phosphoro-2- aminoimidazolide were synthesized according to a previously described procedure.(*3*)



1.A (Base = adenine)
1.G (Base = guanine)
1.C (Base = cytosine)
1.T (Base = thymine)

2.A (Base = adenine)
2.G (Base = guanine)
2.C (Base = cytosine)
2.T (Base = thymine)

3′-NH_2-2-AIpddA (Base = adenine)
3′-NH_2-2-AIpddG (Base = guanine)
3′-NH_2-2-AIpddC (Base = cytosine)
3′-NH_2-2-AIpddT (Base = thymine)

Synthesis of 3′-NH_2-2′,3′-dideoxyriboside-5′-phosphoro-2-aminoimidazolides

General procedure 1: Preparation of 3′-NH_2-2′, 3′-dideoxyriboside-5′-monophosphates.

To a pre-chilled mixture of 3′-NHFmoc-2′, 3′-dideoxyriboside (**1.A – 1. T**, 1 eq.) in $OP(OMe)_3$ (0.1 M with respect to the nucleoside) was added $POCl_3$ (4 equiv) under vigorous stirring. The resultant mixture was allowed to stir at 0 °C. After complete solubilization of the nucleoside, DIPEA (0.5 eq.) was added drop-wise to the stirring reaction. Three additional portions of DIPEA were added (0.5 eq. each) at 20 min intervals. Once the starting material disappeared by low resolution – mass spectrometry (LR-MS) analysis, the reaction was quenched using 1 M TEAB (5-volumes, pH 7.5). Any precipitate observed after the quench was resolubilized using a minimal amount of MeCN. The product was partially purified by reverse phase flash chromatography using gradient elution between (A) aqueous 2 mM TEAB (pH 7.5) and (B) acetonitrile. The product was eluted between 0% and 70% B over 13 CVs with a flow rate of 40 mL/min. Fractions containing product were pooled and most of the solvent was removed in *vacuo*, followed by freeze-drying under high-vacuum at room temperature. The 3′-NHFmoc-2′, 3′-dideoxyriboside-5′-monophosphate was used without further purification.

General procedure 2: Preparation of 3′-NH_2-2′, 3′-dideoxyriboside-5′-phosphoro-(2-aminoimidazolide) monomers.

To a solution of 3′-NHFmoc-2′, 3′-dideoxyriboside-5′-monophosphate (**2.A – 2.T**, 1 eq.), 2-aminoimidazolium chloride (5 eq.), DIPEA (5 eq.) in DMSO (30 mM with respect to nucleoside) was added triphenylphosphine (10 eq.) and DPDS (9 eq.) under stirring at room temperature. After 30 min, more triphenylphosphine (10 eq.) and DPDS (10 eq.) was added to the reaction if starting material remained by ^{31}P NMR analysis. Once the reaction reached completion, the Fmoc protecting group was removed by treatment with DIPEA (3 eq.) and piperidine (20 eq.) for 10 min at 20 °C. The reaction mixture was added to a pre-chilled solution of acetone/diethyl ether (1 : 1 v/v, 500 mL) to which 2 mL of saturated $NaClO_4$ solution in acetone had been added. After the precipitate had settled out, the majority of the supernatant was removed using pipette-suction and the remaining suspension was centrifuged at 3,000 rpm for 3 min. The pellets were washed with acetone/diethyl ether (1 : 1 v/v, 3 x 40 mL). The product was purified by reverse phase flash chromatography using gradient elution between (A) aqueous 2 mM TEAB buffer (pH 7.5) and (B) acetonitrile. The product was eluted between 0% and 15% B over 13 CVs with a flow rate of 40 mL/min. Fractions containing products were pooled, the pH raised to 9.5-10, aliquoted in 1.5 mL centrifuge tubes (approximately 1-2 μmol per tube) and lyophilized at -15 °C.

3′-NH_2-2′, 3′-dideoxyadenosine-5′-phosphoro-(2-aminoimidazole) (3′-NH_2-2AIpddA)

The 3′-NH_2-2′, 3′-dideoxyadenosine-5′-phosphoro-(2-aminoimidazole) was prepared on a 0.5 mmol scale following general procedures 1 and 2. ^{1}H NMR (400 MHz, D_2O) δ: 8.29 (s, 1H), 8.25 (s, 1H), 6.51 (m, 1H), 6.42-6.40 (m, 3H), 4.15-4.00 (m, 3H), 3.86 (dd, 1H, J = 8 & 14 Hz), 2.89 (m, 1H), 2.53 (m, 1H). Peaks corresponding to residual TEAB were observed at 3.22 ppm and 1.30 ppm. ^{31}P (162 MHz, D_2O): -11.04. λ_{max} = 259 nm (H_2O). LRMS (ESI-ion trap): calc for $[C_{13}H_{18}N_9O_4P - (H^+)]^-$ 394.1147, found 394.1. LRMS (ESI- ion trap): calc for $[C_{13}H_{18}N_9O_4P + (H^+)]^+$ 396.1292, found 396.3. HRMS (ESI-QTOF): calc for $[C_{13}H_{18}N_9O_4P - (H^+)]^-$ 394.1147, found 394.1183

3′-NH_2-2′, 3′-dideoxyguanosine-5′-phosphoro-(2-aminoimidazole) (3′-NH_2-2AIpddG)

The 3′-NH_2-2′, 3′- dideoxyguanosine -5′-phosphoro-(2-aminoimidazole) was prepared on a 0.5 mmol scale following general procedure 1 and 2. ^{1}H NMR (400 MHz, D_2O) δ: 7.91 (s, 1H), 6.58 (m, 1H), 6.43 (m, 1H), 6.21 (dd, 1H, J = 4 & 7 Hz), 4.13-3.99 (m, 3H), 3.82 (dd, 1H, J = 7 & 14 Hz), 2.81 (m, 1H), 2.42 (m, 1H). Peaks corresponding to residual TEAB were observed at 3.21 ppm and 1.29 ppm. ^{31}P (162 MHz, D_2O): - 11.06. λ_{max} = 252 nm (H_2O). LRMS (ESI-ion trap): calc for $[C_{13}H_{18}N_9O_5P - (H^+)]^-$ 410.1096, found 410.1. LRMS (ESI- ion trap): calc for $[C_{13}H_{18}N_9O_5P + (Na^+)]^+$ 434.1061, found 434.1. HRMS (ESI-QTOF): calc for $[C_{13}H_{18}N_9O_5P - (H^+)]^-$ 410.1096, found 410.1112

3′-NH_2-2′, 3′-dideoxycytidine-5′-phosphoro-(2-aminoimidazole) (3′-NH_2-2AIpddC)

The 3′-NH_2-2′, 3′-dideoxycytidine-5′-phosphoro-(2-aminoimidazole) was prepared on a 0.5 mmol scale following general procedures 1 and 2. ^{1}H NMR (400 MHz, D_2O) δ: 7.78 (d, 1H, J = 8 Hz), 6.78 (m, 1H), 6.62 (m, 1H), 6.19 (t, 1H, J = 6 Hz), 6.02 (d, 1H, J = 8 Hz), 4.19 (m, 1H), 4.07 (m, 1H), 3.96 (m, 1H), 3.54 (dd, 1H, J = 8 & 14 Hz), 2.33 (dd, 2H, J = 6 & 7 Hz). Peaks corresponding to residual TEAB were observed at 3.21 and 1.29 ppm. ^{31}P (162 MHz, D_2O): - 10.86. λ_{max} = 271 nm (H_2O). LRMS (ESI-ion trap): calc for $[C_{12}H_{18}N_7O_5P - (H^+)]^-$ 370.1034, found 370.1. LRMS (ESI- ion trap): calc for $[C_{12}H_{18}N_7O_5P + (H^+)]^+$ 372.1180, found 372.2. HRMS (ESI-QTOF): calc for $[C_{12}H_{18}N_7O_5P - (H^+)]^-$ 370.1034, found 370.1062.

3′-NH_2-3′-deoxythymidine-5′-phosphoro-(2-aminoimidazole) (3′-NH_2-2AIpddT)

The 3′-NH_2-2′, 3′-dideoxythymidine-5′-phosphoro-(2-aminoimidazole) was prepared on a 0.5 mmol scale following general procedures 1 and 2. ^{1}H NMR (400 MHz, D_2O) δ: 7.58 (s, 1H), 6.78 (m, 1H), 6.64 (m, 1H), 6.23 (dd, 1H, J = 5 & 7 Hz), 4.19 (m, 1H), 4.07 (m, 1H), 3.97 (m, 1H), 3.65 (dd, 1H, J = 7 & 14 Hz), 2.43 (m, 1H), 2.33 (m, 1H), 1.91 (s, 3H). Peaks corresponding to residual TEAB were observed at 3.21

ppm and 1.29 ppm. ^{31}P (162 MHz, D_2O): - 11.53. λ_{max} = 267 nm (H_2O). LRMS (ESI-ion trap): calc for $[C_{13}H_{19}N_6O_6P - (H^+)]^-$ 385.1025, found 385.1. LRMS (ESI- ion trap): calc for $[C_{13}H_{19}N_6O_6P + (H^+)]^+$ 387.1176, found 387.2. HRMS (ESI-QTOF): calc for $[C_{13}H_{19}N_6O_6P - (H^+)]^-$ 385.1025, found 385.1072.

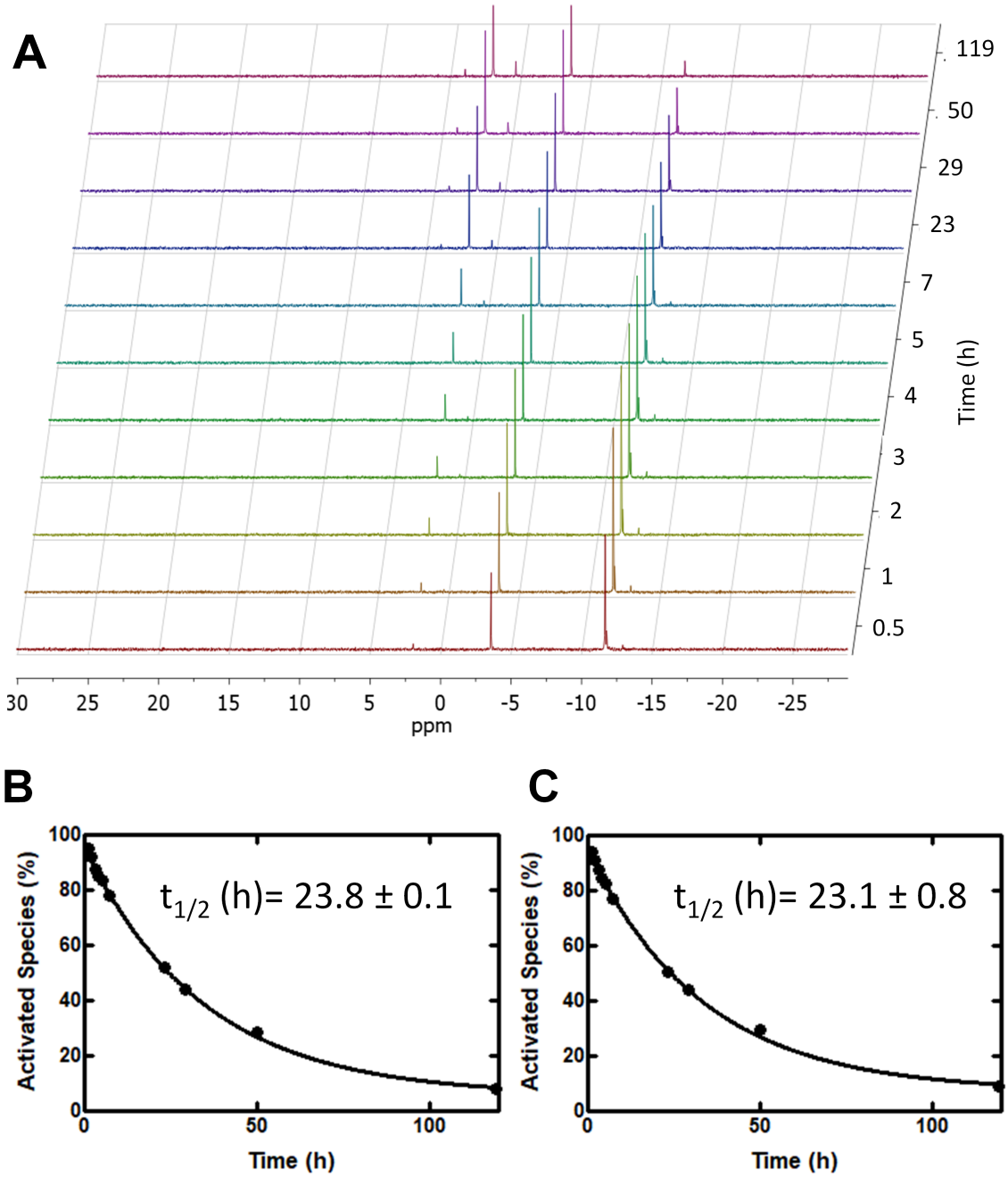
Primer extension reactions. Unless otherwise indicated, primer extension reactions on RNA templates to generate a 3′-NP-DNA complementary strand were performed according to previously reported procedures with slight modifications.(*3*) Monomer stocks were typically resuspended in water at a concentration of 100 mM. Reaction quenching buffers contained 15 mM EDTA, 20 μM complementary RNA and 90 % (v/v) formamide. Quenched samples were heated to 100 °C (120 °C for primers extended by 12 or more nucleotides) for 2 min and cooled quickly to room temperature (typically 25 °C). Primer extension products were resolved by 20 % (19:1) denaturing PAGE with 7 M urea. Full length products were inferred from the stepwise addition of mononucleotides. All primer extension reactions within vesicles were prepared and processed according to previously reported procedures (*4*). Briefly, vesicle samples were prepared with 0.1 M of total lipid, pre-annealed primer (13 μM) / template (15 μM) in 200 mM Na^+-bicine, pH 8.5. Samples were tumbled for 12 h, extruded to 100 nm and tumbled for at least 3 h. Vesicles were separated from unencapsulated primer/template on a Sepharose 4B size exclusion column. Primer extension reactions were initiated by first adding pre-mixed $MgCl_2$ and Na^+-citrate (1:4 ratio) and vortexing vigorously, followed by adding activated monomers to a final concentration of 10 mM, and vortexing once more vigorously. After tumbling for the indicated times, an aliquot of vesicles was purified a second time by size exclusion chromatography and the vesicle fractions collected and pooled. Triton X-100 was added to a concentration of 1% v/v and the RNA was precipitated with cold ethanol (at least 4 volumes, typically 3-4 mL) at -20°C for 12 h, then centrifuged at 4 °C. The RNA pellets were washed twice with 80% cold ethanol (-20°C), dissolved in 8 M Urea 1 x TBE loading buffer and products were resolved by 20 % (19:1) denaturing PAGE with 7 M urea.

Activated mononucleotide degradation profiling by ^{31}P NMR. 3′-NH_2-2AIpddT (10 mM) in a 500 μL aqueous solution containing 200 mM HEPES buffer (pH 8.0), 3 mM or 50 mM $MgCl_2$, 10 mM NaCl, 20 % (v/v) D_2O and 0.06 % (v/v) $PO(OEt)_3$ as a reference was studied by ^{31}P NMR spectroscopy. Spectra were periodically collected at 25 °C in triplicate with times (h) equal to 0.5, 1, 2, 3, 4, 5, 7, 23, 29, 51.2, and 119.

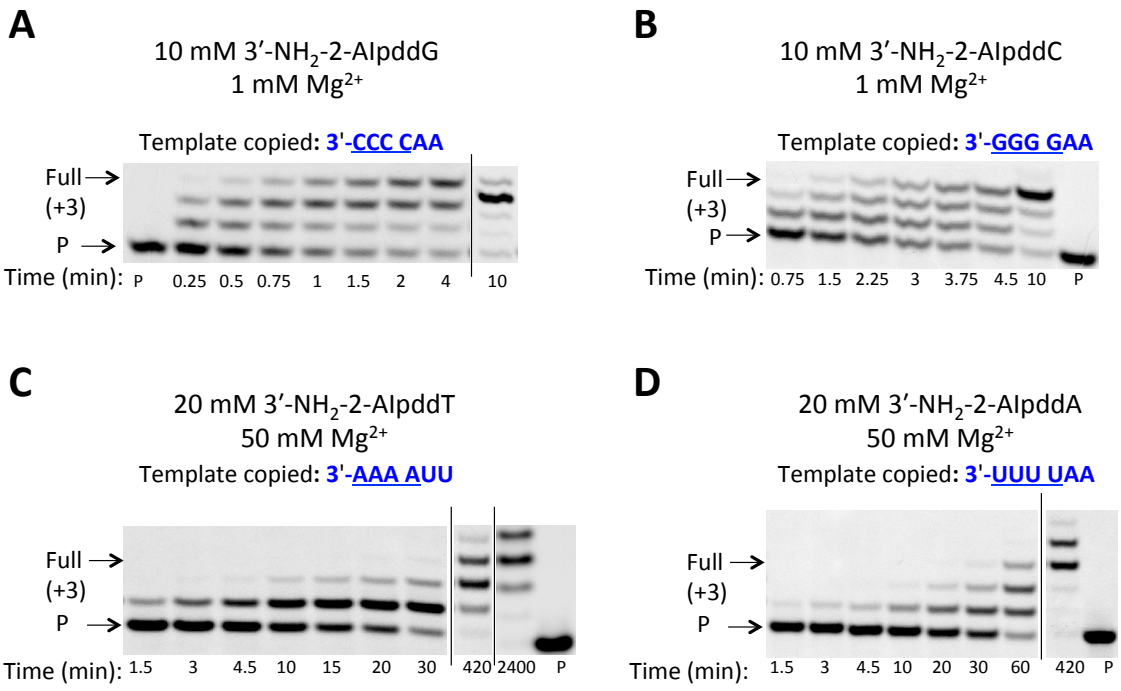
LC-MS analysis of 3′-NP-DNA synthesis on RNA templates. Primer extension reactions (60 μL) containing 10 μM primer, 13 μM template, 50 mM $MgCl_2$, 200 mM Na^+-bicine (pH 8.5), and 10 mM activated monomers were allowed to react for 4 h. Reactions were stopped by first adding EDTA and NH_4OAc to the reaction to reach a concentration of 42 mM and 0.83 M, respectively. Reaction mixtures were precipitated using 4 volumes of ethanol at -80 °C for at least 2h, and centrifuged at 12,000 g at 4 °C for 30 min. Samples were washed with aqueous ethanol (80% v/v, 2 x 400 μL). Samples (approximately 100 pmol) were resolved and analyzed using an

Agilent 1200 HPLC coupled to an Agilent 6230 TOF. Liquid chromatography was conducted using an IP-RP-HPLC on a 100 mm × 1 mm (length × i.d.) Xbridge C18 column with 3.5 μm particle size (Waters, Milford, MA) using gradient elution between (A) aqueous 200 mM 1,1,1,3,3,3-hexafluoro-2- propanol with 1 mM triethylamine, pH 7.0, and (B) methanol. Samples were eluted using a gradient of 2.5-15 % B over 16 min, followed by 15-60 % B over 20 min at a flow rate of 100 μL/min at 50 °C. Samples were analyzed in negative mode from 239 m/z to 3200 m/z. Acquired mass spectra were background subtracted and deconvoluted using Agilent's MassHunter software.

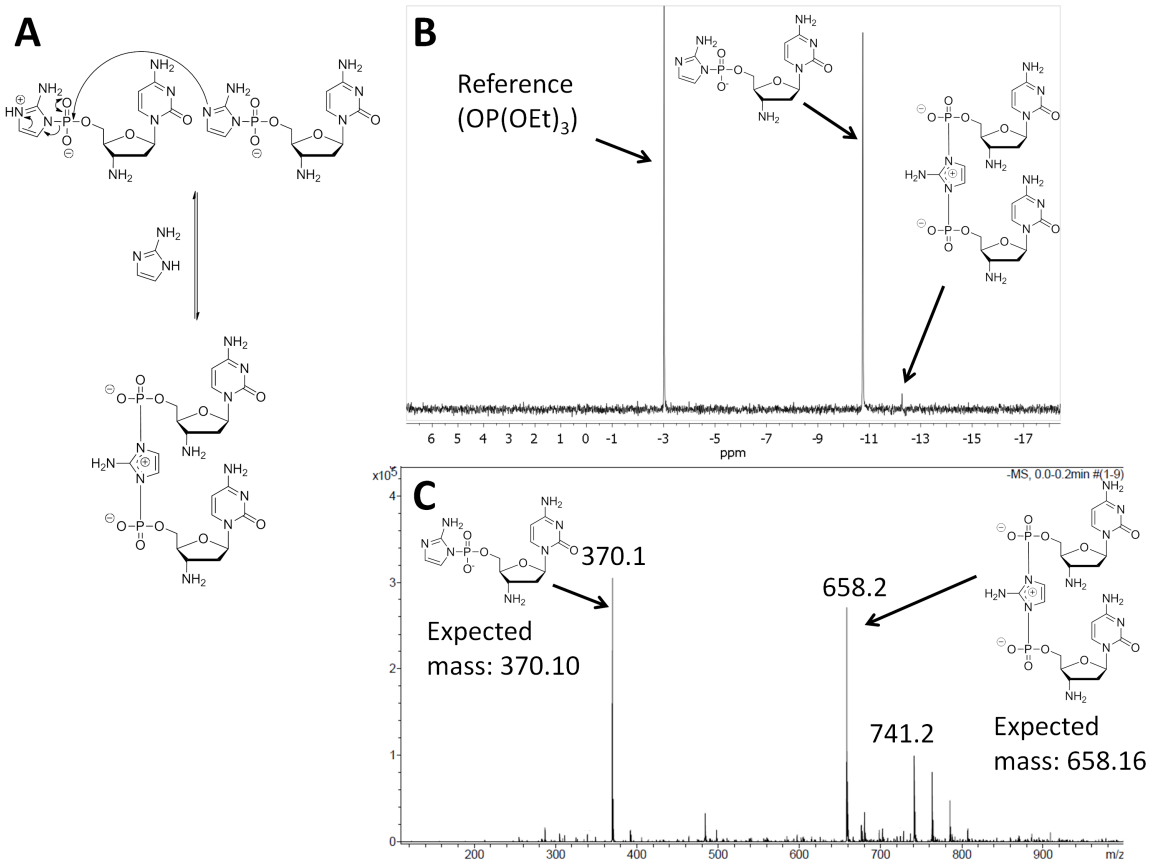
Supporting Figures and Tables



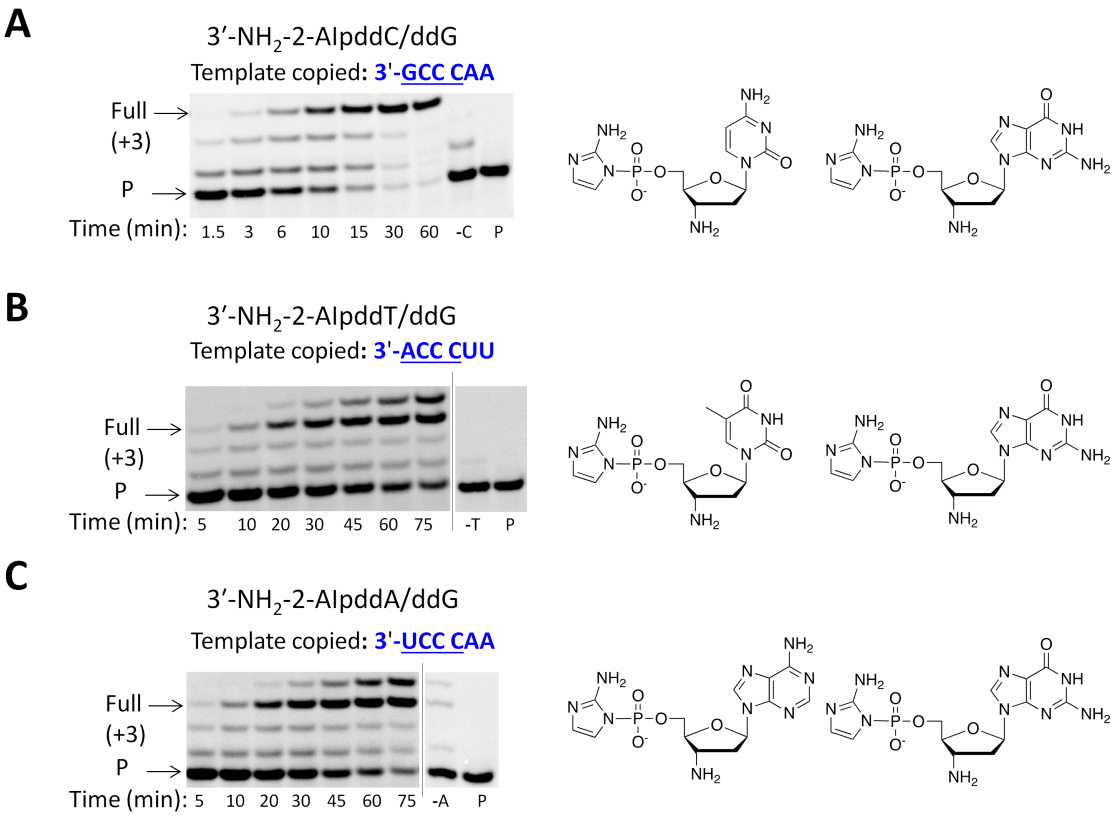
SI Figure S1: A) representative degradation profile of 3′-NH2-ImpddT (10.0 mM) in a 500 µL aqueous solution containing 200 mM HEPES buffer (pH 8.0), 10% D_2O, and $PO(OEt)_3$ (as a reference set at -3.0 ppm), 50 mM $MgCl_2$ (or no $MgCl_2$) studied by ^{31}P NMR spectroscopy at 162 MHz on a Varian NMR spectrometer. Time-points were collected according to times reported on the y-axis. Activated species (%) vs. time (h) (B) in the presence of $MgCl_2$ (50 mM) or (C) in the absence of $MgCl_2$ addition. Half lives are reported in h. Data points are reported as the mean +/- s.d. from triplicate experiments.



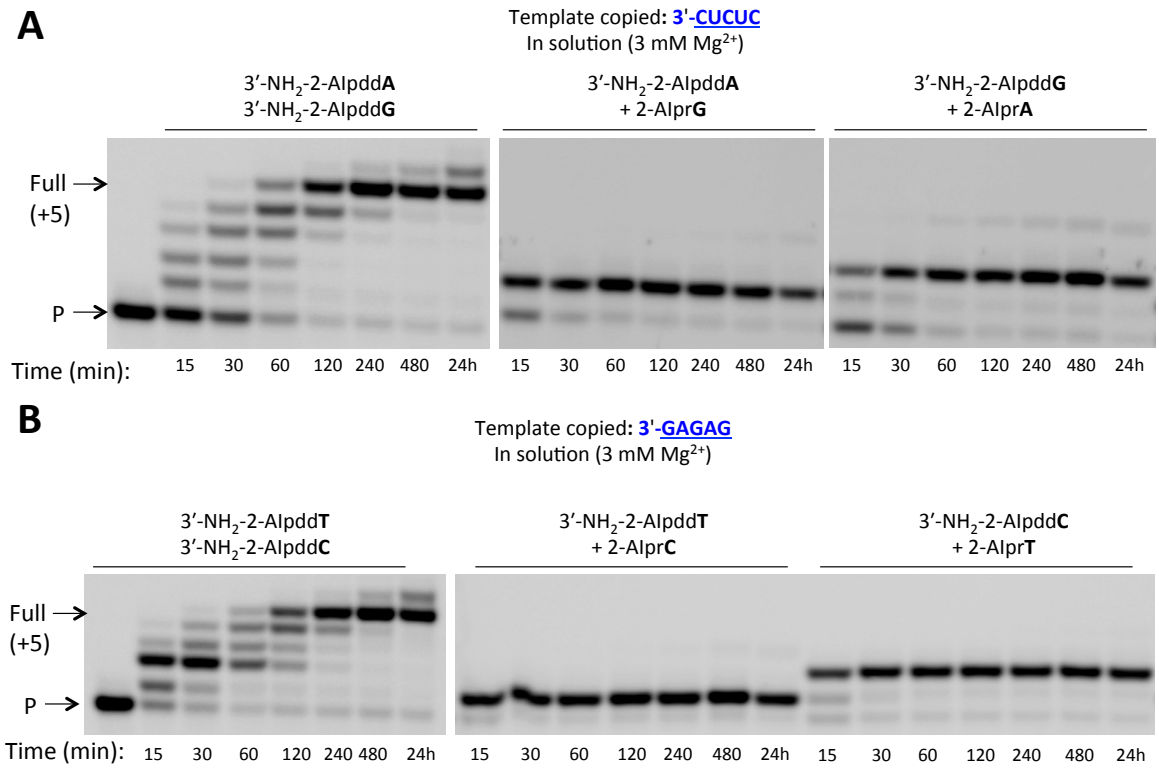
SI Figure S2: Nonenzymatic primer extension on RNA homotemplates. All reactions were conducted at pH 8.5, 25 °C, and 200 mM Na^+-bicine with monomer and Mg^{2+} concentrations reported in each panel. PAGE analysis of the products of the primer extension reaction in solution for sequence (A) 3′-CCCC with 3′-NH_2-2AIpddG, (B) 3′-GGGG with 3′-NH_2-2AIpddC, (C) 3′-AAAA with 3′-NH_2-2AIpddT (D) 3′-UUUU with 3′-NH_2-2AIpddA. P stands for primer alone. Note that more Mg^{2+} (50 mM) was required for primer extension reaction for sequence (C) 3′-AAAA with 3′-NH_2-2AIpddT (D) 3′-UUUU with 3′-NH_2-2AIpddA given the slower reaction rates.



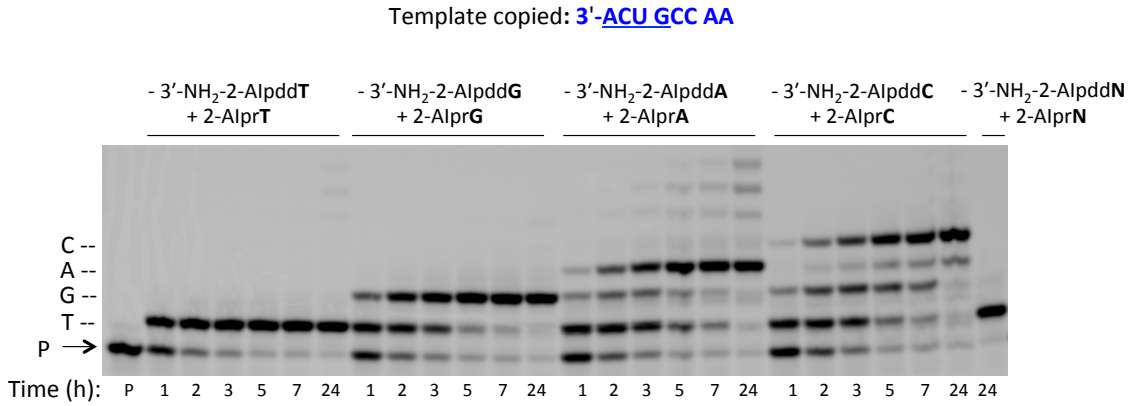
SI Figure S3: Supporting evidence for the formation of the imidazolium-bridged dinucleotide species. A) The proposed reaction of a 3′-NH_2-2AIpddC anion and 3′-NH_2-2AIpddC zwitterion produces the imidazolium-bridged dicytidine species and free 2-AI. B) ^{31}P (162 MHz) NMR spectra of 5 mM 3′-NH_2-2AIpddC incubated in 200 mM Na^+-bicine, pH 8.5, 50 mM $MgCl_2$ at 25°C for approximately 5 minutes, showing the presence of 3′-NH_2-2AIpddC and the imidazolium-bridged dicytidine species at -10.74 ppm and -12.27 ppm, respectively. C) LRMS (ESI-ion trap, negative mode) spectrum of 3′-NH_2-2AIpddC resuspended in water to a final concentration of approximately 0.2 mM. The sample was injected directly. Signals corresponding to the 3′-NH_2-2AIpddC (m/z = 370.1 & 741.2 for the M-H^+ and 2M-H^+, respectively) and the imidazolium-bridged dicytidine species (m/z = 658.2) are observed, as previously reported (*5*, *6*).



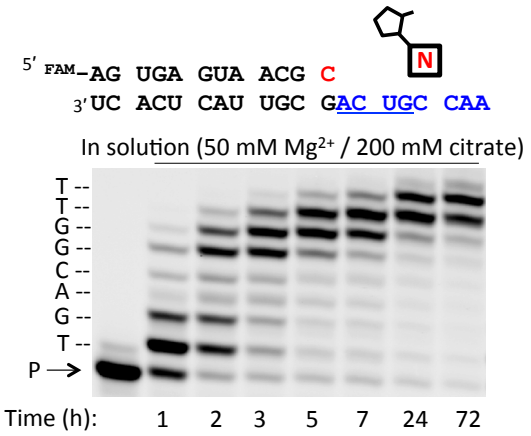
SI Figure S4: Chemical transcription of RNA heterotemplates 3′-NCCC (N= G, A, or C) by primer extension to generate a complementary 3′-NP-DNA product. All reactions were conducted at pH 8.5, 25 °C, and 200 mM Na^+-bicine with 5 mM of each activated mononucleotide and 3 mM Mg^{2+}. PAGE analysis of the products of the primer extension reaction in solution for sequence (A) 3′-GCCC with 3′-NH_2-2AIpddC and 3′-NH_2-2AIpddG, (B) 3′-ACCC with 3′-NH_2-2AIpddT and 3′-NH_2-2AIpddG, (C) 3′-UCCC with 3′-NH_2-2AIpddA and 3′-NH_2-2AIpddG. Poor extension was observed with 3′-NH_2-2AIpddG alone (60 min for (A), 75 min for (B) and (C)) suggesting a primer extension process with good fidelity (lanes are labeled as "-N" where N is for the activated mononucleotide other than 3′-NH_2-2AIpddG. P, primer. Chemical structures of activated mononucleotides are shown for clarity.



SI Figure S5: Timecourse analysis of fidelity assessment experiment (adapted from Sanger sequencing) for 2-letter mixed-sequences. Left panels: PAGE analysis of 3′-NP-DNA primer extension products on RNA templates (A) 3′-CUCUC-5′ and (B) 3′-GAGAG-5′. All reactions were conducted at pH 8.5, 25 °C, and 200 mM Na^+-bicine with 10 mM of (A) A + G or (B) C + T activated mononucleotides and 3 mM Mg^{2+}. Right panels: the indicated 3′-NH_2-2AIpddN was replaced by the corresponding 2AIprN, which generated chain-terminated products following ribonucleotide incorporation. P, primer. High fidelity reactions were observed, using this methodology, for all cases except in the right panel (A), where misincorporation of 3′-NH_2-2AIpddG allowed for synthesis of < 5% of longer extension products.



SI Figure S6: Timecourse of fidelity assessment experiment. PAGE analysis of the products of the chemical transcription of mixed-sequence RNA template 3′-ACUG by primer extension to generate a complementary 3′-NP-DNA product. All reactions were conducted at pH 8.5, 25 °C, and 200 mM Na^+-bicine with 10 mM of each activated mononucleotide and 3 mM Mg^{2+}. As noted above each timecourse, the indicated 3′-NH_2-2AIpddN was replaced by the corresponding 2AIprN, which generated chain-terminated products because low Mg^{2+} is insufficient to allow for primer extension of a primer terminated in a ribonucleotide. P, primer.



SI Figure S7: Nonenzymatic primer extension on a mixed sequence RNA template using 2AI activated 3′-amino nucleotides. The template-directed synthesis reaction is the same as in Figure 4 of the main text, but at 50 mM Mg^{2+} and 200 mM Na^{+}-citrate, in order to compare to results reported in Fig. 7A of the manuscript. The primer extension reaction was carried out using 10 mM activated NP monomers (3′-NH_2-2AIpddA, 3′-NH_2-2AIpddG, 3′-NH_2-2AIpddC, 3′-NH_2-2AIpddT), 50 mM $MgCl_2$, 200 mM sodium citrate, 200 mM Na^{+}-bicine pH 8.5, 25 °C. P, primer.

SI Table S1: Summary of oligonucleotides sequences used in this study†

Name	Sequence (5′ →3′)	Figure associated with oligomer
P1	56-FAM-rArGrU rGrArG rUrArA rCrG-ddCNH$_2$	Primer: Fig 4, 5, 7, S2, S3, S4, S5, S6
P2	rArGrU rGrArG rUrArA rCrG-ddCNH$_2$	Primer: Fig 3, 4B
T1	r(GAG AG GCG UUA CUC ACU AAAAAA)	Template: Fig 3A, S4
T2	r(CUCUC GCG UUA CUC ACU AAAAAA)	Template: Fig 3B, S4
T3	r(AAC CGU CAG CGU UAC UCA CUU UUU UU)	Template: Fig 4, 7A, 7B S2, S3, S4, S5, S6
T4	r(UUNNNN GCG UUA CUC ACU)	Template: Fig 5, 7C
P3	56-FAM-rCrGrCrUrCrGrA-ddCNH$_2$	Primer: Fig 6A, 6C, 7D
P4	56-FAM-rArGrG rUrCrA rCrC-ddGNH$_2$	Primer: Fig 6B, 7D
T5	r(GCC ACC UCA G UCA GUC GAG CG)	Template: Fig 6A, 7D
T6	r(GCG CCU CUU CAG CCC GCC CGG UGA CCU)	Template: Fig 6B, 7D
T7	r(GCC ACC UCA G UCA GCC ACC UCA G UCA GUC GAG CG AAAAAAAAAAAAAAAAA)	Template: Fig 6C, 7D

† 56-FAM, fluorescein.

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