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Synthesis and Properties of 5-Cyano-Substituted Nucleoside Analog with a Donor–Donor–Acceptor Hydrogen-Bonding Pattern

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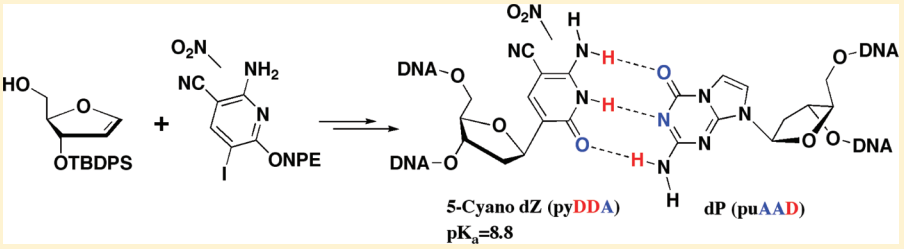
Supporting Information



ABSTRACT: 6-Aminopyridin-2-ones form Watson–Crick pairs with complementary purine analogues to add a third nucleobase pair to DNA and RNA, if an electron-withdrawing group at position 5 slows oxidation and epimerization. In previous work with a nucleoside analogue trivially named d**Z**, the electron withdrawing unit was a nitro group. Here, we describe an analogue of d**Z** (cyano-d**Z**) having a cyano group instead of a nitro group, including its synthesis, pK_a, rates of acid-catalyzed epimerization, and enzymatic incorporation.

One goal of synthetic biology[1,2] is to create molecular systems that support genetics and Darwinian evolution but with molecular structures different from natural DNA and RNA.[3−9] In this regard, among the most successful synthetic additions to natural DNA are two nonstandard nucleosides, 6-amino-5-nitro-3-(1′-β-D-2′-deoxyribofuranosyl)-2(1*H*)-pyridone (trivially called d**Z**) and 2-amino-8-(1′-β-D-2′-deoxyribofuranosyl)imidazo[1,2-*a*]-1,3,5-triazin-4(8*H*)-one (trivially called d**P**).[10] The pair between their two nucleobases is joined by a hydrogen bond donor–donor–acceptor pattern on the small pyrimidine analogue (from the major to the minor groove, pyDDA) and a hydrogen bond acceptor-acceptor–donor pattern on the purine analogue (puAAD). The success of this nonstandard pair has been explained by a theory that focuses on "unshared pairs" of electrons presented by nucleobases to the minor groove; this electron density is believed to be a recognition element sought by DNA polymerases.[11] As both d**Z** and d**P** have this electron density, the d**Z**:d**P** pair was expected to be accepted relatively easily by DNA polymerases, even when its elements are incorporated consecutively into a DNA template and (consequently) a polymerase-generated product (Figure 1).[12]

Creating a small pyrimidine analogue that presented the hydrogen bond donor–donor–acceptor pattern proved to be challenging, however. The parent pyridine lacking a nitro group, in our hands (but see the growing literature on this species[13]) proved to be too sensitive to oxidation and too basic to serve as a working component of a genetic material. To mitigate these undesirable properties, a nitrogen was introduced to create a pyrazine aromatic ring that supported the same hydrogen bonding donor–donor–acceptor pattern. This, however, proved to be susceptible to epimerization.[14]

The nitropyridine structure was then examined under the hypothesis that a strongly electron-withdrawing group would stabilize the pyridine ring against oxidation, raise the pK_a of the pyridine nitrogen, and stabilize the deoxyribonucleoside analogue against epimerization.[15] This level of theory proved able to support a design. The nitropyridine deoxyribonucleoside d**Z** epimerizes only at low pH, with the rate of the epimerization negligible at neutral pH. The material was also quite stable against oxidation, surviving in its protected form in standard DNA synthesis. Further, the d**Z**:d**P** pair in duplex DNA was synthesized by many DNA polymerases without difficulty, and even duplexes containing multiple consecutive d**Z**:d**P** pairs.

However, the pK_a of the d**Z** heterocycle (measured in the nucleoside) is 7.8. While this is not problematic for many applications, in its deprotonated form, d**Z** is a good mismatch for dG. This mismatching is evidently responsible for low rates of loss of **Z**:**P** pairs in six letter GACTZP PCR, where those pairs must be copied again and again with high fidelity.[16]

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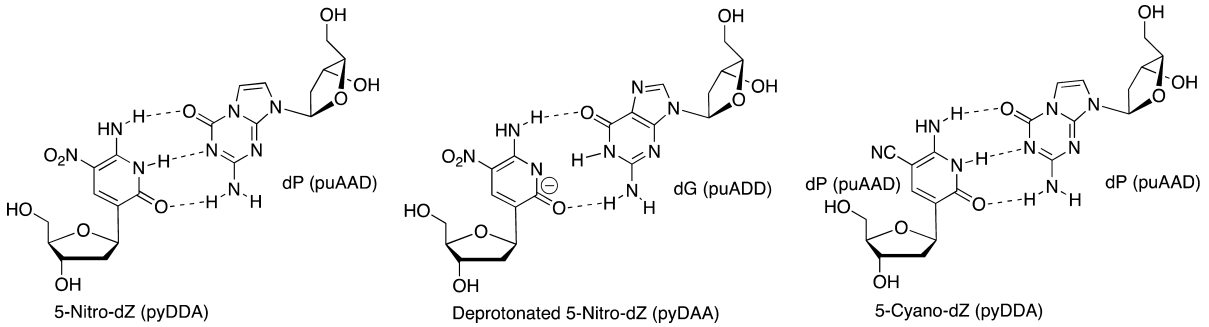
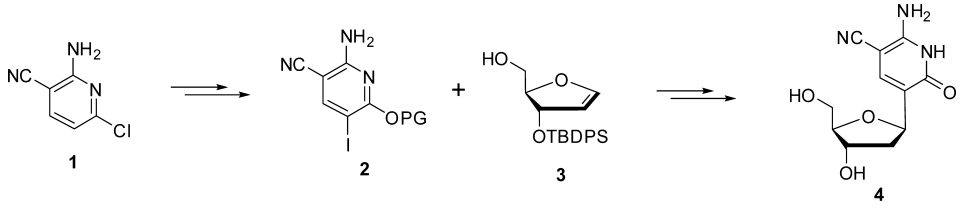
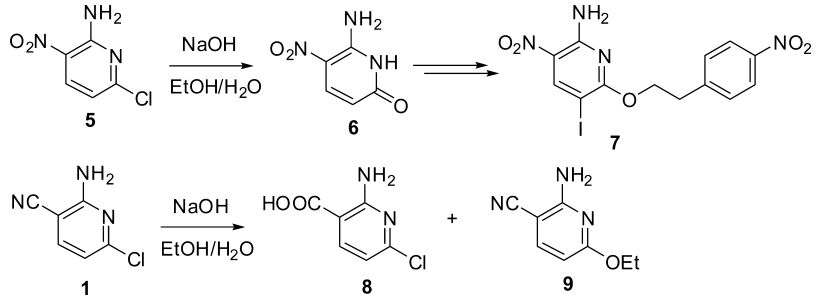


Figure 1. Two different implementations of the hydrogen-bonding donor−donor−acceptor (pyDDA) pattern forming a pair with d**P**, the first on a nitrogen-substituted aminopyridone (d**Z**), the second with a cyano-substituted aminopyridone (cyano-d**Z**). The deprotonated nitropyridine mispairs with dG.

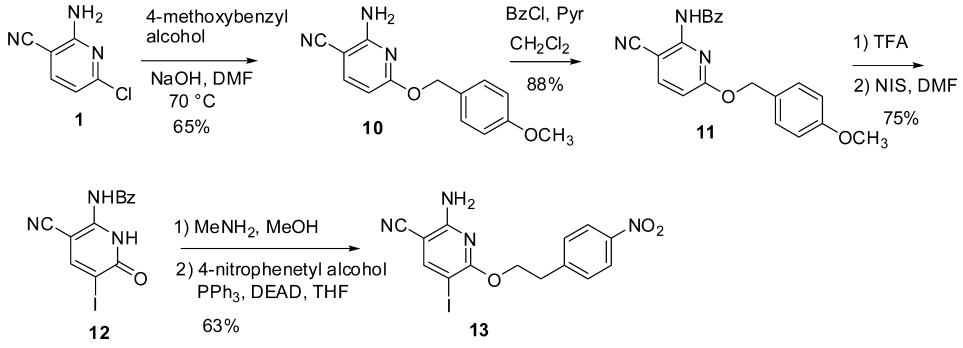
Scheme 1. Synthetic Strategy for 5-Cyano-Substituted pyDDA Nucleoside



Scheme 2. Hydrolysis of Nitro- and Cyano-Substituted Heterocycles by Sodium Hydroxide



Scheme 3. Synthesis of Cyano-Substituted Heterocycle 13 Suitable for Heck Coupling



Seeking to fix this problem, we asked whether other electron-withdrawing groups might also be attached to a pyridine that implements the donor−donor−acceptor hydrogen-bonding pattern, in particular, a cyano group. By its Hammett parameters, the cyano group is slightly less electron withdrawing than the nitro group.[17] We expected, therefore, that the cyano analogue of d**Z** might have a slightly higher pK_a, perhaps at the cost of a marginally lower stability against epimerization. Further, because a cyano group is slightly smaller than a nitro group, we speculated that it would be less likely to be viewed as "foreign" by various enzymes that act on DNA; the replacement does not change much the electron density that it presents to the minor groove.

We report here the synthesis of the deoxyribose derivative of 2-amino-3-cyanopyridin-6-one (cyano-d**Z**) and its triphosphate (cyano-d**ZTP**), together with studies of its properties, including a comparative study of its rate of epimerization. We also report experiments challenging a panel of DNA polymerases to incorporate 2-amino-3-cyanopyridin-6-one nucleotides (cyano-d**ZTP**) opposites d**P**.

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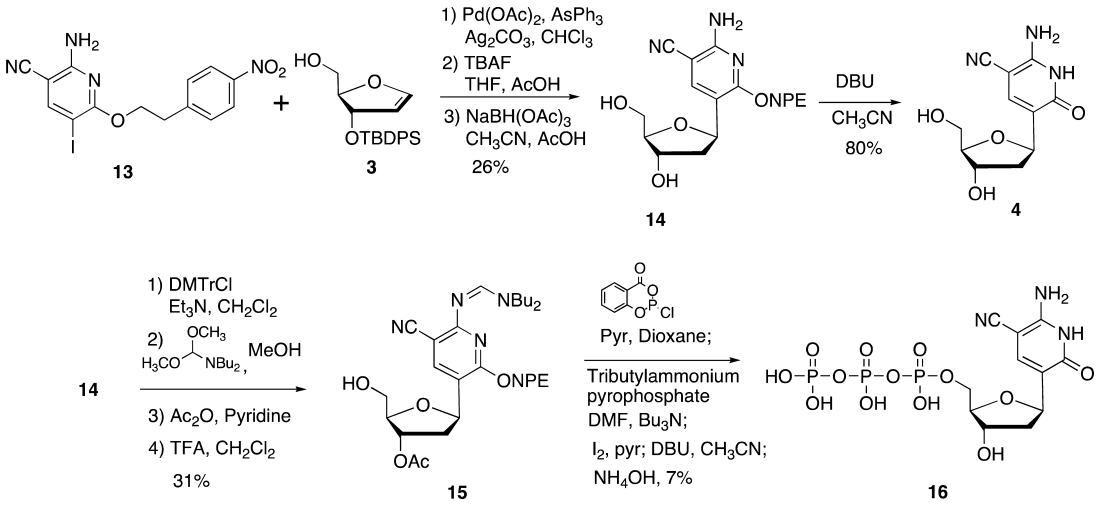
Scheme 4. Synthesis of 5-Cyano-Substituted pyDDA Nucleoside 4 and Its Triphosphate 16



The strategy for synthesizing 5-cyano-substituted pyDDA nucleoside exploited the palladium-catalyzed Heck coupling of the suitable heterocycle with known glycal **3** (Scheme 1).[18]

Our initial attempt to synthesize the cyano heterocycle for Heck coupling, which followed literature procedures developed for the nitro compound[15] was unsuccessful (Scheme 2). For the nitro compound, treatment of 2-amino-6-chloro-3-nitropyridine (**5**) with sodium hydroxide in ethanol/water mixture gave the desired 6-amino-5-nitro-2(1*H*)-pyridinone (**6**) exclusively.[10,15] However, when commercial 2-amino-6-chloro-3-cyanopyridine (**1**) was treated under the same conditions, 2-amino-6-chloro-3-pyridinecarboxylic acid (**8**) was obtained as a major product with a small amount of 2-amino-6-chloro-3-ethoxypyridine (**9**). When this reaction was performed in the absence of ethanol, only hydrolysis of the cyano group was observed, giving 2-amino-6-chloro-3-pyridinecarboxylic acid (**8**) as the only product. This implies that the hydrolysis of the cyano group is more facile than the hydrolysis of chloropyridine. Also, nucleophilic substitution by alkoxide anion is more facile than the hydrolysis of chloropyridine for these 5-cyano group substituted heterocycles.

The observed reactivity of 2-amino-6-chloro-3-cyanopyridine suggested that an acid labile alkoxide might be used to protect the pyridinone oxygen. This suggestion was adopted as a working hypothesis, even though it required several more synthetic steps to implement (Scheme 3). Thus, treatment of 2-amino-6-chloro-3-cyanopyridine (**1**) with 4-methoxybenzyl alcohol in the presence of sodium hydroxide provided **10**. After protection of the amine of **10** as its benzoyl amide, removal of 4-methoxybenzyl by trifluoroacetic acid followed by iodination with *N*-iodosuccinimide in DMF gave **12**. In contrast, direct removal of 4-methoxybenzyl by trifluoroacetic acid gave lower yield of the product and needed more laborious purification process. After removal of the benzoyl group, the oxygen of the pyridinone unit in **12** was converted to a *p*-nitrophenylethyl ether under Mitsunobu reaction condition giving **13**. It should be noted that the removal of the benzoyl group in **12** before the Mitsunobu reaction was essential, as the reaction failed to create the *p*-nitrophenylethyl ether if the benzoyl group remains.

With iodide **13** in hand, the synthesis of the nucleoside and its triphosphate was straightforward (Scheme 4). Heck coupling of the iodinated heterocycle (**13**) to the glycal (**3**) using palladium acetate with triphenylarsine in anhydrous chloroform proceeded as planned, as did subsequent deprotection with TBAF followed by reduction with $NaBH(OAc)_3$. These steps provided the NPE-protected nucleoside **14**. The NPE group was then removed with DBU in anhydrous acetonitrile to give 5-cyano-substituted nucleoside **4** with pyDDA hydrogen-bonding pattern. For the synthesis of the corresponding triphosphate, the 5′-hydroxyl, 2-amino, and 3′-hydroxyl group were sequentially protected with dimethoxytrityl chloride, *N*,*N*-dibutylformamide dimethyl acetal, and acetic anhydride, and then the 5′-DMT group was removed in acid to give **15**. The triphosphate **16** (cyano-d**ZTP**) was synthesized using the standard Ludwig–Eckstein procedure.[19]

The pK_a of the 2′-deoxyribonucleoside analogue **4** was estimated from a series of UV spectra collected at 323 nm (λ_{max} for deprotonated **4**) and 338 nm (λ_{max} for protonated **4**) as the pH of the aqueous solution was adjusted by adding dilute aqueous solutions of HCl and NaOH. The plot of ratio of absorbance at 323 nm/338 nm, shown in Figure S1 (Supporting Information), gave a pK_a value for **4** = 8.8 ± 0.1. This value is approximately one pK_a unit higher than that for the 5-nitro-substituted nucleoside[15] and one pK_a unit lower than those for the natural nucleosides.

The 2′-deoxyribonucleoside **4** (Figures S2 and Figure S3, Supporting Information) epimerizes at pH 7.0 much more slowly than at pH 2.1 but faster in both cases than the nitro analogue. Thus, at room temperature at pH 7.0 ("biological" conditions), 83% of the 5-cyano pyDDA nucleoside remained without epimerization after incubation for 8 days. However, the heating of the solution accelerated the epimerization rate; after 1 day at 55 °C at pH 7.0, about half of the nucleoside epimerized.

As perhaps expected for the specific acid catalysis mechanism shown for the epimerization of pyrazine pyDDA nucleoside analogues and observations with nitro-d**Z** by Hutter et al.,[15] the epimerization of cyano-d**Z** was much faster at lower pH than higher pH. Consistent with the Hammett sigma values for cyano and nitro (0.66 and 0.78, respectively, for σ_{para}), the pK_a of the cyano analogue was higher (by a full unit) than the pK_a of the nitro analogue. Consistent with the hypotheses behind our design, the pseudo-first-order rate constant for the epimerization of the cyano nucleoside at pH 2.1 at room temperature was only ~12 fold faster than for the epimerization

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of the nitro nucleoside. This gives a "two point" Hammett ρ of 9.

Six DNA polymerases (Taq, Vent (exo$^-$), Bsu, Klenow (exo$^-$), 9° Nm, and Therminator) and two reverse transcriptases (SuperScript II RT (SS), and HIV RT) were screened for their ability to incorporate one cyano-d**Z** nucleotide opposite a d**P** in the template (Figure 2).

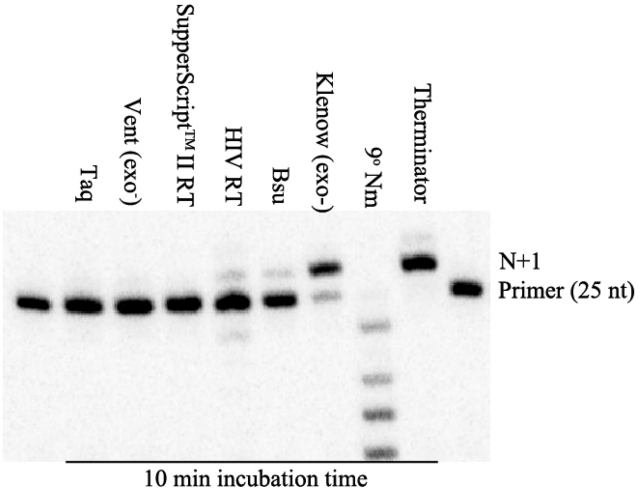

Figure 2. Standing-start primer extension experiments testing the ability of various polymerases to incorporate cyano-d**Z**TP (**16**) opposite template d**P**.

Surprisingly, however, several polymerases (Taq, Vent, and Superscript reverse transcriptase) showed no detectable addition with cyano-d**Z**; these did accept nitro-d**Z**. Two of the polymerases (Bsu DNA polymerases and HIV reverse transcriptase) gave very small amounts of extension. In contrast, Klenow (exo-) and Therminator showed efficient incorporation of cyano-d**Z**. Klenow (exo-) DNA polymerase displayed high fidelity with cyano-d**ZTP**, even after long incubation times (30 min). In contrast, Therminator DNA polymerase yielded detectable mismatched N+2 bands upon longer incubation (Figure S4, Supporting Information). The other four enzymes accept cyano-d**ZTP** (Figure 2). These results can be compared with published results showing that nitro-d**Z** is easily accepted by all of these polymerases.[12,16]

While the different physical organic properties (pK_a and epimerization) were expected based on semiquantitative theory, the results with DNA polymerases were not. Many studies have shown that most DNA polymerases and reverse transcriptases accept d**Z** opposite d**P**, many with high efficiency. In contrast, only a few polymerases accepted the cyano-d**Z** triphosphate opposite d**P**, and only one Family A polymerase examined (Klenow) and only one Family B polymerase examined (Therminator) performed adequately to be useful. This difference arises even though both analogues implement the py(DDA) hydrogen bonding pattern, and both present electron density to the minor groove at the same position.

As a hypothesis to explain the last observation, we suggest that the nitro group forms a hydrogen bond to one hydrogen on the exocyclic amino group of d**Z**. In contrast, the cyano has a nonbonding interaction with that hydrogen. We speculate that nonbonding interaction forces the amino group out of the plane of the aromatic system. This feature would likely be recognized as "foreign" by some polymerases.

This notwithstanding, this is (to our knowledge) the first example of the failure of the "minor groove electron density" model for polymerase interactions with substrates as a rule to support the design of nucleoside analogues. Thus, it should interest the many laboratories seeking to develop a synthetic biology based on alternative nucleic acids.

EXPERIMENTAL SECTION

2-Amino-6-chloro-3-pyridinecarboxylic Acid (8). A mixture of 2-amino-6-chloro-3-cyanopyridine (**1**, 1.0 g, 6.51 mmol) and NaOH (1.50 g) in H_2O (10 mL) and ethanol (20 mL) was refluxed for 1 h. The mixture was acidified with concentrated HCl, and the resulting solid was filtered, washed with water, and dried to give a white solid (0.60 g, 53%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.6 (br s, 1H), 7.86 (d, 1H, J = 8.7 Hz), 7.06 (s, 2H), 6.37 (d, 1H, J = 8.7 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 165.99, 161.70, 150.13, 142.51, 112.78, 106.67; MS (ESI) m/z calcd for $C_6H_6ClN_2O_2$ $(M + H)^+$ 173/175, found 173/175.

3-Cyano-6-(4-methoxybenzyloxy)-2-pyridinamine (10). A mixture of 2-amino-6-chloro-3-cyanopyridine (**1**, 6.0 g, 39.1 mmol), 4-methoxybenzyl alcohol (14.4 mL), and NaOH (9.0 g) in DMF (150 mL) was heated to 70 °C overnight. After being cooled to rt, the mixture was poured into H_2O (500 mL) and extracted with ethyl acetate. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (silica, ethyl acetate/hexanes = 1:2) to give a white solid (6.48 g, 65%): mp 120−121 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.59 (d, 1H, J = 8.4 Hz), 7.39 (d, 2H, J = 8.7 Hz), 7.08 (br s, 2H), 6.92 (d, 2H, J = 9.0 Hz), 6.06 (d, 1H, J = 8.4 Hz), 5.26 (s, 2H), 3.74 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.9, 161.8, 159.8, 143.4, 130.6, 129.3, 118.2, 114.5, 101.3, 79.8, 67.5, 55.8; HRMS (ESI) m/z calcd for $C_{14}H_{13}N_3O_2Na$ $(M + Na)^+$ 278.0900, found 278.0909, $C_{28}H_{26}N_6O_4Na$ $(2M + Na)^+$ 533.1908, found 533.1904.

***N*-[3-Cyano-6-(4-methoxybenzyloxy)-2-pyridinyl]benzamide (11).** To a mixture of **10** (6.48 g, 25.4 mmol) and pyridine (6.1 mL, 75 mmol) in dichloromethane (150 mL) was added benzoyl chloride (4.4 mL, 38 mmol). The mixture was stirred at rt overnight, poured into brine (400 mL), and extracted with dichloromethane. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (silica, ethyl acetate/hexanes = 1:2) to give a white solid (8.0 g, 88%): mp 117−118 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (br s, 1H), 8.05 (d, 1H, J = 8.4 Hz), 7.90−7.95 (m, 3H), 7.50−7.65 (m, 3H), 7.38 (d, 2H, J = 9.0 Hz), 6.91 (d, 2H, J = 9.0 Hz), 5.38 (s, 2H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.7, 163.3, 159.9, 152.5, 145.6, 133.8, 133.1, 129.7, 129.3, 128.1, 127.5, 115.5, 114.2, 106.1, 92.1, 68.8, 55.5; HRMS (ESI) m/z calcd for $C_{21}H_{17}N_3O_3Na$ $(M + Na)^+$ 382.1162, found 382.1176, $C_{21}H_{17}N_3O_3K$ $(M + K)^+$ 398.0901, found 398.0906, $C_{21}H_{16}N_3O_3Na_2$ $(M - H + 2Na)^+$ 404.0982, found 404.0980.

***N*-[3-Cyano-5-iodo-2(1*H*)-pyridonyl]benzamide (12).** A mixture of **11** (1.9 g, 5.3 mmol) and trifluoroacetic acid (8 mL) was stirred at rt for 10 min and then diluted with diethyl ether (50 mL). The mixture was filtered, and the solid was suspended in a mixture of methanol and diethyl ether. The precipitate was filtered and dried. The solid was suspended in DMF (20 mL) and treated with *N*-iodosuccinimide (1.9 g). The mixture was stirred at rt for 1 h and poured into water (100 mL). The mixture was filtered, and the solid was suspended in a mixture of methanol and diethyl ether. The precipitate was filtered and dried to give a light yellow solid (1.45 g, 75%): mp >220 °C dec; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.68 (br s, 1H), 8.54 (s, 1H), 7.93 (d, 2H, J = 7.2 Hz), 7.5−7.7 (m, 3H), 6.75 (br s, 1H); HRMS (ESI) m/z calcd for $C_{13}H_9IN_3O_2$ $(M + H)^+$ 365.9739, found 365.9736.

3-Cyano-5-iodo-6-[2-(4-nitrophenyl)ethoxy]-2-pyridinamine (13). A mixture of **12** (1.45 g, 4.0 mmol) and methylamine (40% in water, 10 mL) in methanol (30 mL) was stirred at rt overnight. The mixture was concentrated under reduced pressure, and the residue was suspended in a mixture of methanol and diethyl ether. The precipitate was filtered and dried. The solid was transferred to a flask and charged

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with triphenylphosphine (1.85 g, 7.0 mmol), 4-nitrophenethyl alcohol (1.18 g, 7.0 mmol), and anhydrous THF (20 mL). The mixture was treated with diethyl azodicarboxylate (1.23 mL, 7.0 mmol) at 0 °C and stirred at rt for 1 h. It was poured into brine (50 mL) and extracted with ethyl acetate. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (silica, CH_2Cl_2/hexanes = 2:1) to give a white solid (1.04 g, 63%): mp 160−161 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (d, 2H, J = 9.0 Hz), 7.87 (s, 1H), 7.46 (d, 2H, J = 9.0 Hz), 5.30 (br s, 2H), 4.50 (t, 2H, J = 6.6 Hz), 3.18 (t, 2H, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.5, 158.7, 151.7, 145.9, 130.3, 123.97, 115.1, 86.8, 66.9, 63.3, 35.4; HRMS (ESI) m/z calcd for $C_{14}H_{12}IN_4O_3$ $(M + H)^+$ 410.9949, found 410.9940, $C_{14}H_{11}IN_4O_3Na$ $(M + Na)^+$ 432.9738, found 432.9768.

2-Amino-3-cyano-5-(2′-deoxy-β-D-ribofuranosyl)-6-[2-(4-nitrophenyl)ethoxy]-2-pyridine (14). Palladium acetate (110 mg, 0.49 mmol) and triphenylarsine (299 mg, 0.98 mmol) were dissolved in chloroform (15 mL), and the mixture was stirred at rt for 30 min. The mixture was then added to a solution of **13** (1.00 g, 2.44 mmol), glycal **3** (0.95 g, 2.68 mmol), and silver carbonate (1.35 g, 4.88 mmol) in chloroform (15 mL). The resulting mixture was refluxed overnight. After being cooled to rt, the mixture was filtered through Celite and washed with ethyl acetate. The combined filtrate was concentrated in vacuo. The residue was purified by flash chromatography (silica, ethyl acetate/hexanes = 1:1) to give a white solid (∼1.0 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 (d, 2H, J = 8.7 Hz), 7.7−7.8 (m, 4H), 7.4−7.6 (m, 8H), 6.80 (s, 1H), 5.41 (dd, 1H, J = 4.2, 1.5 Hz), 5.37 (br s, 2H), 4.71 (m, 1H), 4.45 (t, 2H, J = 6.6 Hz), 4.29 (s, 1H), 3.8−3.9 (m, 2H), 3.13 (t, 2H, J = 6.6 Hz). This material, without further characterization, was dissolved in THF (40 mL), and acetic acid (0.2 mL) and TBAF (1 M in THF, 2 mL) were added. The mixture was stirred at rt for 30 min, poured into brine (120 mL), and extracted with ethyl acetate. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (silica, ethyl acetate/hexanes = 2:1) to give a white solid (443 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (d, 2H, J = 8.7 Hz), 7.46 (d, 2H, J = 8.7 Hz), 7.44 (s, 1H), 6.03 (br s, 2H), 5.10 (dd, 1H, J = 11.4, 6.3 Hz), 4.45−4.55 (m, 2H), 3.9−4.1 (m, 3H), 3.17 (t, 2H, J = 6.3 Hz), 2.88 (dd, 1H, J = 18.3, 11.4 Hz), 2.59 (dd, 1H, J = 18.0, 6.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 213.0, 164.3, 158.3, 147.1, 146.2, 142.9, 130.3, 123.9, 116.6, 109.0, 82.9, 82.2, 66.6, 61.2, 40.7, 35. 4. This material was dissolved in acetic acid (12 mL) and acetonitrile (12 mL), treated with sodium triacetoxyborohydride (600 mg, 2.83 mmol), and stirred at rt for 2 h. The mixture was poured into brine (100 mL) and extracted with ethyl acetate. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (silica, ethyl acetate) to give a white solid (257 mg, 26% for three steps): mp 55−57 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 8.14 (d, 2H, J = 9.0 Hz), 7.55 (d, 2H, J = 9.0 Hz), 7.54 (d, 1H, J = 0.3 Hz), 4.97 (dd, 1H, J = 10.8, 5.4 Hz), 4.56 (t, 2H, J = 6.5 Hz), 4.36 (m, 1H), 3.90 (dd, 1H, J = 6.0, 3.3 Hz), 3.71 (d, 2H, J = 3.3 Hz), 3.17 (t, 2H, J = 6.3 Hz), 2.18 (ddd, 1H, J = 13.2, 12.1, 6.3 Hz), 1.97 (ddd, 1H, J = 13.2, 5.4, 1.5 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 164.0, 159.0, 147.0, 146.8, 141.2, 130.2, 123.2, 117.1, 111.1, 88.1, 80.2, 78.2, 72.9, 66.1, 61.9, 39.6, 35.0; HRMS (ESI) m/z calcd for $C_{19}H_{21}N_4O_6$ $(M + H)^+$ 401.1456, found 401.1461, $C_{19}H_{20}N_4O_6Na$ $(M + Na)^+$ 423.1275, found 423.1287, $C_{19}H_{19}N_4O_6Na_2$ $(M\text{-}H + 2Na)^+$ 445.1095, found 445.1109, $C_{38}H_{40}N_8O_{12}Na$ $(2M + 2Na)^+$ 823.2658, found 823.2654.

6-Amino-5-cyano-3-(2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (4). A mixture of **14** (30 mg, 0.075 mmol) and DBU (57 mg, 0.375 mmol) in acetonitrile (3 mL) was stirred at rt for 2 days. The mixture was concentrated, and the residue was purified by flash chromatography (silica, CH_2Cl_2/MeOH = 4:1) to give a white solid (15 mg, 80%): mp 130−131 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.66 (s, 1H), 4.97 (dd, 1H, J = 11.1, 5.1 Hz), 4.37 (d, 1H, J = 6.0 Hz), 3.90 (d, 1H, J = 2.4 Hz), 3.72 (m, 2H), 2.20 (ddd, 1H, J = 13.2, 12.1, 5.7 Hz), 1.95 (dd, 1H, J = 12.6, 5.1 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 162.4, 154.2, 147.2, 117.7, 102.1, 88.1, 83.1, 78.5, 73.3, 61.8, 39.8; UV λ_{max} (triethylammonium acetate, pH 7.0) 338 nm (ε 15800); HRMS (ESI) m/z calcd for $C_{11}H_{12}N_3O_4$ $(M - H)^-$ 250.0833, found 250.0834, $C_{11}H_{11}N_3NaO_4$ $(M - 2H+Na)^-$ 272.0653, found 272.0663.

(3-Cyano-5-(2′-deoxy-3′-*O*-acetyl-β-D-ribofuranosyl)-*N*-[(dibutylamino)methylene]-6-[2-(4-nitrophenyl)ethoxy]-2-pyridinamine (15). To a mixture of **14** (200 mg, 0.50 mmol) and triethylamine (0.2 mL) in dichloromethane (5 mL) was added 4,4′-dimethoxytrityl chloride (185 mg, 0.55 mmol). The mixture was stirred at rt overnight. The mixture was poured into brine (20 mL) and extracted with dichloromethane. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (silica, ethyl acetate/hexanes = 2:1) to give a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.15 (d, 2H, J = 8.7 Hz), 7.45 (d, 2H, J = 8.7 Hz), 7.2−7.4 (m, 10H), 6.75−6.85 (m, 4H), 5.65 (br s, 2H), 4.99 (dd, 1H, J = 10.8, 5.4 Hz), 4.61 (m, 1H), 4.48 (t, 2H, J = 6.6 Hz), 4.00 (m, 1H), 3.78 (s, 6H), 3.39 (ddd, 2H, J = 23.1, 10.2, 3.3 Hz), 3.15 (t, 2H, J = 6.3 Hz),2.4−2.5 (m, 1H), 2.0−2.15 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 158.8, 158.3, 147.0, 146.3, 144.3, 141.8, 135.5, 135.5, 130.3, 128.4, 128.1, 127.3, 123.8, 117.1, 113.4, 110.3, 86.9, 82.3, 78.9, 73.6, 66.4, 63.3, 55. 4, 40.2, 35.4. This material and *N*,*N*-dibutylformamide dimethyl acetal (0.3 mL) were dissolved in methanol (3 mL) and stirred at room temperature overnight. The mixture was evaporated and purified by flash chromatography (neutral silica, ethyl acetate/hexanes = 1:1). The major fraction was collected and evaporated. This material, without further characterization, was dissolved in dichloromethane (10 mL) with acetic anhydride (0.3 mL), DMAP (5 mg), and pyridine (0.6 mL). The mixture was stirred at rt overnight. The mixture was poured into brine (20 mL) and extracted with dichloromethane. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (neutral silica, ethyl acetate/hexanes = 1:2). The major fraction was collected and evaporated. This material, without further characterization, was dissolved in dichloromethane (5 mL), treated with trifluoroacetic acid (0.1 mL), and stirred at rt for 10 min. The mixture was neutralized by aqueous sodium bicarbonate and extracted with methylene chloride. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (neutral silica, ethyl acetate/hexanes = 1:1) to give a sticky syrup (90 mg, 31% for four steps): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (s, 1H), 8.17 (d, 2H, J = 8.7 Hz), 7.84 (d, 1H, J = 0.6 Hz), 7.48 (d, 2H, J = 8.7 Hz), 5.30 (dd, 1H, J = 10.8, 4.8 Hz), 5.17 (dd, 1H, J = 7.8, 1.8 Hz), 4.55 (t, 2H, J = 6.6 Hz), 4.03 (m, 1H), 3.84 (m, 2H), 3.45−3.55 (m, 2H), 2.51 (ddd, 1H, J = 13.8, 4.8, 0.9 Hz), 2.27 (t, 1H, J = 6.0 Hz), 2.08 (s, 3H), 1.75−1.9 (m, 1H), 3.15−3.35 (m, 4H), 0.9−1.7 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.1, 162.9, 162.5, 159.6, 155.5, 147.1, 146.4, 140.1, 130.3, 123.9, 122.2, 117.2, 87.4, 85.4, 76.4, 66.1, 63.5, 52.4, 47.4, 46.23, 42.1, 40.0, 35.54, 31.2, 30.9, 29.6, 29.3, 21.2, 20.5, 20.4, 19.8, 14.0, 13.8; HRMS (ESI) m/z calcd for $C_{30}H_{40}N_5O_7$ $(M + H)^+$ 582.2922, found 582.2913, $C_{30}H_{39}N_5NaO_7$ $(M + Na)^+$ 604.2742, found 604.2744.

6-Amino-5-cyano-3-(2′-deoxy-5′-*O*-triphosphate-β-D-ribofuranosyl)-2(1*H*)-pyridone (16). To a solution of **15** (87 mg, 0.15 mmol) in pyridine (0.8 mL) and dioxane (2.4 mL) was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (48 mg, 0.24 mmol) in dioxane (1.0 mL) at room temperature. After 15 min, a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 2.4 mL, 0.48 mmol) and tributylamine (0.27 mL, 1.1 mmol) was added. After 20 min, a solution of iodine (61 mg, 0.24 mmol) and water (0.095 mL) in pyridine (4.76 mL) was added. After 30 min, the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, until color disappears). The pyridine and dioxane were removed in vacuo. The residue was dissolved in acetonitrile (3 mL) and DBU (0.2 mL). The mixture was stirred at room temperature overnight. The volatiles were removed in vacuo and dissolved in ammonium hydroxide (10 mL). The mixture was stirred at room temperature overnight. Ammonia was removed by rotary evaporation, and the residue was diluted with water (20 mL). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 × 250 mm, eluent A = water, eluent B = 1 M aq NH_4HCO_3, gradient to 50% B in 30 min, flow rate =10 mL/min, t_R = 25 min) gave the triphosphate as a white solid after lyophilization

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(11 μmol, 7.3%): ^{1}H NMR (300 MHz, D_2O) δ 7.60 (s, 1H), 4.90 (dd, 1H, J = 11.7, 5.1 Hz), 4.44 (d, 1H, J = 5.7 Hz), 3.9−4.1 (m, 3H), 2.1−2.3 (m, 1H), 1.88 (dd, 1H, J = 13.5, 5.1 Hz); ^{31}P NMR (121 MHz, D_2O) δ −7.9 (m, 1P), −10.3 (m, 1P), −21.2 (m, 1P); HRMS (ESI) m/z calcd for $C_{11}H_{15}N_3O_{13}P_3$ $(M - H)^-$ 489.9823, found 489.9846, $C_{11}H_{14}N_3O_{13}P_3Na$ $(M - 2H + Na)^-$ 511.9643, found 511.9624.

Measuring the pK_a of 4. An aqueous solution (30 mL) of **4** (ca. 0.5 mg) was acidified to pH 4 by the addition of dilute aqueous HCl (10 mM). The pH of the solution was then varied by additions of various amounts of dilute aqueous NaOH (10 mM or 100 mM). UV scans (220−500 nm) were taken at the various pHs. The pK_a was determined by plotting the pH versus the quotient of absorption at two different wavelengths (323 nm/338 nm).

Measuring the Rate of Epimerization of 4. Compound **4** was dissolved in a dilute HCl aqueous solution (pH 2.1), a solution of 40 mM NaOAc buffer at pH 4.0, and a solution of 25 mM Et_3N−HOAc buffer (pH 7.0). The concentration of **4** was 0.4 mM. The solutions were incubated at room temperature or 55 °C. At time intervals, aliquots (10 μL) were removed, neutralized with aqueous triethylammonium bicarbonate buffer (50 mM; pH 8; 0.1 mL) and analyzed by analytical rp-HPLC (Sunfire C18 5 μm, 3.0 × 150 mm, eluent A = 50 mM TEAB, eluent B = 35% CH_3CN and 65% 50 mM TEAB, gradient from 100% A to 70% A, 30% B in 30 min, flow rate 0.5 mL/min).

Standing Start Primer Extension Reactions Using Cyano-dZTP (16). Primer (25mer): 5′-GCGAATTAACCCTCACTAAAG-TACG-3′. **P**-Template (51mer): 5′-GCGTAA-ACGACTCACTATAGACGACGTATTTAGGAGGGTAATCGC-3′.

To prepare the template-primer complex, γ-^{32}P-labeled primer (4 μL of 1 μM solution, 4 pmol), unlabeled primer (4 μL of 10 μM solution, 40 pmol), and **P**-template (60 μL of 1 μM solution, 60 pmol) were annealed by incubation at 95 °C for 5 min followed by slow cooling to room temperature. Incubation mixtures were created by mixing template-primer solution (1 μL), cyano-d**ZTP** (**16**, 1 μL of 1 mM solution, final concentration 100 μM), buffer (supplied by the manufacturer and diluted as instructioned), and water (to give, after enzyme addition, a total volume of 10 μL). Extension was initiated by adding enzyme (DNA polymerase or reverse transcriptase, 1 unit each). The mixture was then incubated at recommended temperatures for the specified times. The reaction was quenched by adding 10 mM EDTA in formamide loading buffer (20 μL), and the products were resolved by 14% PAGE.

ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton-X-100, pH 8.8) was used for *Taq*, Vent (exo$^-$), 9° Nm, and Therminator. NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$,1 mM dithiothreitol) was used for Bsu and Klenow (exo$^-$). First-strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 200 mM DTT) was used for SuperScript II RT. HIV RT buffer (50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$) was used for reverse transcriptase. Temperatures used were 42 °C for SuperScript II Reverse Transcriptase, 37 °C for Bsu and Klenow (exo$^-$) DNA polymerases, and 72 °C all others.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

pK_a and epimerization of compound **4**, standing-start primer extension experiments of **16**, and ^{1}H, ^{13}C, and ^{31}P NMR spectra for compounds **10**−**14**, **4**, **15**, and **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The authors and their institution have intellectual property covering certain of these compounds and processes using them.

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