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Nucleosides with self-complementary hydrogen-bonding motifs: Synthesis and base-pairing studies of two nucleosides containing the imidazo[4,5-*d*]pyridazine ring system

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Abstract—Synthesis and base-pairing studies of two 2′-deoxyribonucleosides, containing a common heterocyclic base, 7(4)-amino-5(6)*H*-imidazo[4,5-*d*]pyridazin-4(7)one (**1** and **2**), have been reported. The synthesis was accomplished by base-promoted deoxyribosylation of ethyl 5(4)-cyanoimidazole-4(5)-carboxylate (**6**), followed by ring-closure with hydrazine hydrate. The ^{1}H NMR-based base-pair studies were conducted using DMF-d_7 as a solvent by measuring changes in chemical shifts of the amino, hydrazide, imidazole H-2, and the sugar H-1′ protons of the nucleosides with variations in concentrations and temperatures. Large downfield chemical shifts were observed for the NH, NH_2, and to a lesser extent for the H-1′ protons when the temperature was lowered from 25 to 0 °C, and then further down to −50 °C in 10 degree intervals. The observed experimental data are consistent with the results of molecular modeling studies. Nucleoside **2** exhibited low level antiviral activity against HIV-1 in CEM-SS cells with an IC_{50} of 89.2 μM. No cellular toxicity was observed at the highest concentration of the compound tested.

1. Introduction

There has been a surge of interest recently in unnatural nucleic acid bases with novel base-pairing and hydrophobic characteristics as probes for fidelity and function of DNA polymerases[1–5] as well as for potential expansion of the genetic code.[6–25] Much attention is focused on the design of unnatural Watson–Crick base-pair partners that resemble a purine in one strand and a pyrimidine in the other strand of a double-helix, a classic example being the often-quoted *iso*G:*iso*C pair reported several years ago by Benner and coworkers.[26–28] By contrast, little effort is devoted to the design of molecules which can effectively form self-complementary base-pairs, substituting for both a purine and a pyrimidine, and thus the same nucleobase occupying both strands of the double-helix. A notable exception is a recent report by Matsuda and coworkers on the formation of self-complementary base-pairs by the designed imidazopyridopyrimidine nucleosides.[29] However, the latter are 5:6:6-fused tricyclic ring systems that have little, if any, resemblance to the natural 5:6-fused purines. In this context, a self-pairing monocyclic or bicyclic heterocyclic base that closely mimics a natural pyrimidine or a purine would be of considerable interest. We report herein the design, synthesis, and preliminary base-pairing studies of two isomeric nucleoside analogues, **1** and **2**, possessing a common, self-pairing nucleobase with a 5:6-fused heterocyclic ring system, and has the structural resemblance of a purine. Also reported are the results of in vitro anti-HIV screening studies that showed that while **2** is a weak inhibitor of the viral replication, **1** is completely inactive.

2. Results and discussion

The target nucleosides are **1** and **2**, which contain the common heterocyclic base 7(4)-amino-5(6)*H*-imidazo[4,5-*d*]pyridazin-4(7)one. The nucleobase has four

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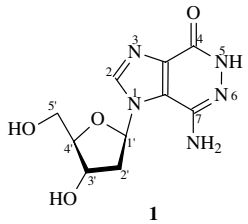

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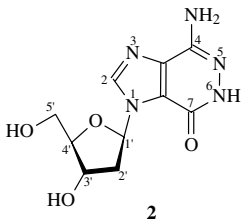

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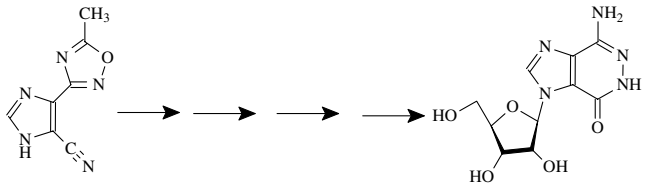
possible sites for base-pairing, including the two H-bond donor sites (NH_2 and NH), and two H-bond acceptor sites (=N and =O).

The two nucleosides are each capable of base-pairing with the self or with each other. Our molecular modeling studies (see Fig. 1) reveal that only three of the four possible sites in each can effectively participate in the Watson–Crick base-pairing at any one moment, be it with the self or with each other. Furthermore, base-pairing with the self, for example, **1**:**1** (Fig. 1A) or **2**:**2** (Fig. 1B), would transpose the two respective backbone sugars into a rare motif befitting a parallel stranded double-helix.[30] The intermolecular base-pairing between **1** and **2** (Fig. 1C), on the other hand, results in an anti-parallel strand array, analogous to that of a natural B-DNA duplex.[30] The side-views of the complexes between **1**:**1** (Fig. 1A) and **1**:**2** (Fig. 1C) show near-perfect, flat, coplanar structures containing the two base-paired nucleobases, while the homo complex **2**:**2** (Fig. 1B) reveals a slight bend in the center. The two sugar residues on each end of the complexes protrude themselves in either a parallel (Fig. 1A and B) or anti-parallel (Fig. 1C) orientation, depending upon whether the base-pairing is between **1**:**1**, **2**:**2** or **1**:**2**, respectively.

Several years ago, Townsend and coworkers reported an elegant synthesis of nucleoside **3**, the ribose analogue of **2**, in several steps commencing from the imidazole derivative **4**.[31] A drawback of the synthesis, however, is the poor yield (17%) in the final base-catalyzed transformation of the oxadiazole ring of **4** into the target imidazo[4,5-*d*]pyridazine ring system of **3**. We have devised an alternative, more efficient synthesis to access not only its deoxy analogue **2**, but also the latter's regioisomer **1**, both of which are required for our studies. We used classical chemical methods of glycosylation of a suitably substituted imidazole derivative to synthesize both **1** and **2**.

The synthesis of **1** and **2** starts from the commercially available 4,5-dicyanoimidazole (**5**) (Scheme 1), which was converted to the corresponding cyano-ester (**6**) by ethanolysis with concentrated sulfuric acid.[32] Deoxyribosylation of **6** with the α-chlorosugar **7**,[33,34] employing



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the sodium salt glycosylation procedure,[35] was quantitative, and gave the desired isomeric nucleosides **8** (39%) and **9** (61%), which were separated by silica gel flash chromatography. The identities of the two isomers were established by carrying through the synthesis to the target nucleosides **1** and **2**, which could be easily distinguished by ^{1}H NMR NOE studies (see below). The toluoyl protecting groups of the sugar moieties of **8** and **9** were removed by treatment with *tert*-butylamine in methanol. This reaction step also resulted in a complete exchange of the ethyl ester group for methyl, forming **10** (93%) or **11** (95%), respectively. The latter, upon reaction with hydrazine hydrate in methanol at ice-cold temperature, provided the intermediate **12** or **13**, which, without isolation and purification, was treated with sodium ethoxide in ethanol at reflux to obtain the target nucleoside **1** (76%) or **2** (81%), respectively. While the ^{1}H and ^{13}C NMR, and mass spectral data, as well as elemental microanalyses, were consistent with their assigned structures, yet they did not provide a clear distinction between the two isomeric nucleosides **1** and **2**. The two structures were unequivocally assigned using Nuclear Overhauser Effect Spectroscopy (NOESY). The proximity of the exocyclic NH_2 protons with the anomeric H-1′ proton was detected in **1** but not in **2**, thus providing a conclusive evidence for their structural identities.

Base-pair studies of **1**, **2**, and **1** + **2** were conducted using a 400 MHz ^{1}H NMR spectrometer with DMF-d_7 as a solvent. ^{1}H NMR spectroscopy is ideally suited for monitoring base-pair hydrogen-bonding between nucleosides[36–38] as well as for differentiation between base-pairing and stacking interactions.[37,39] In most cases, upfield chemical shifts of ring protons are observed upon vertical stacking of bases, whereas base-pairing leads to downfield shifts of the participating protons.[39] The change in chemical shifts of the exocyclic amino and the endocyclic imino protons would provide the evidence for base-pair interactions, but it is necessary to ascertain that the observed shifts are not due to interactions of nucleosides with the solvent or due to self-association stacking interactions between bases. Therefore, the chemical shifts of both **1** and **2** were individually monitored in dilute solutions at various concentrations ranging from 0.01 to 0.08 M and at two different temperatures, 25 and 0 °C. The results (see Tables I–IV in Supplementary material) show that there is little, if any, concentration-dependent stacking self-association in these dilute solutions as monitored by the chemical shifts of the NH, NH_2, imidazole H-2, or anomeric H-1′ signals, all of which were shifted slightly downfield rather than upfield. While there was only a small gradual change (0.3–7.7 Hz) in concentration-dependent base-pair interactions at both 25 and 0 °C in going from 0.01 to 0.08 M, a vast temperature-dependent downfield shift was observed for each of the NH and NH_2 signals (and to a lesser extent, for the H-2 signal) in going from 25 to 0 °C. For example, while the observed maximum differences in chemical shifts for **1** at 25 and 0 °C in going from 0.01 to 0.08 M are 3.5 Hz (NH) and 7.7 Hz (NH), respectively, a large

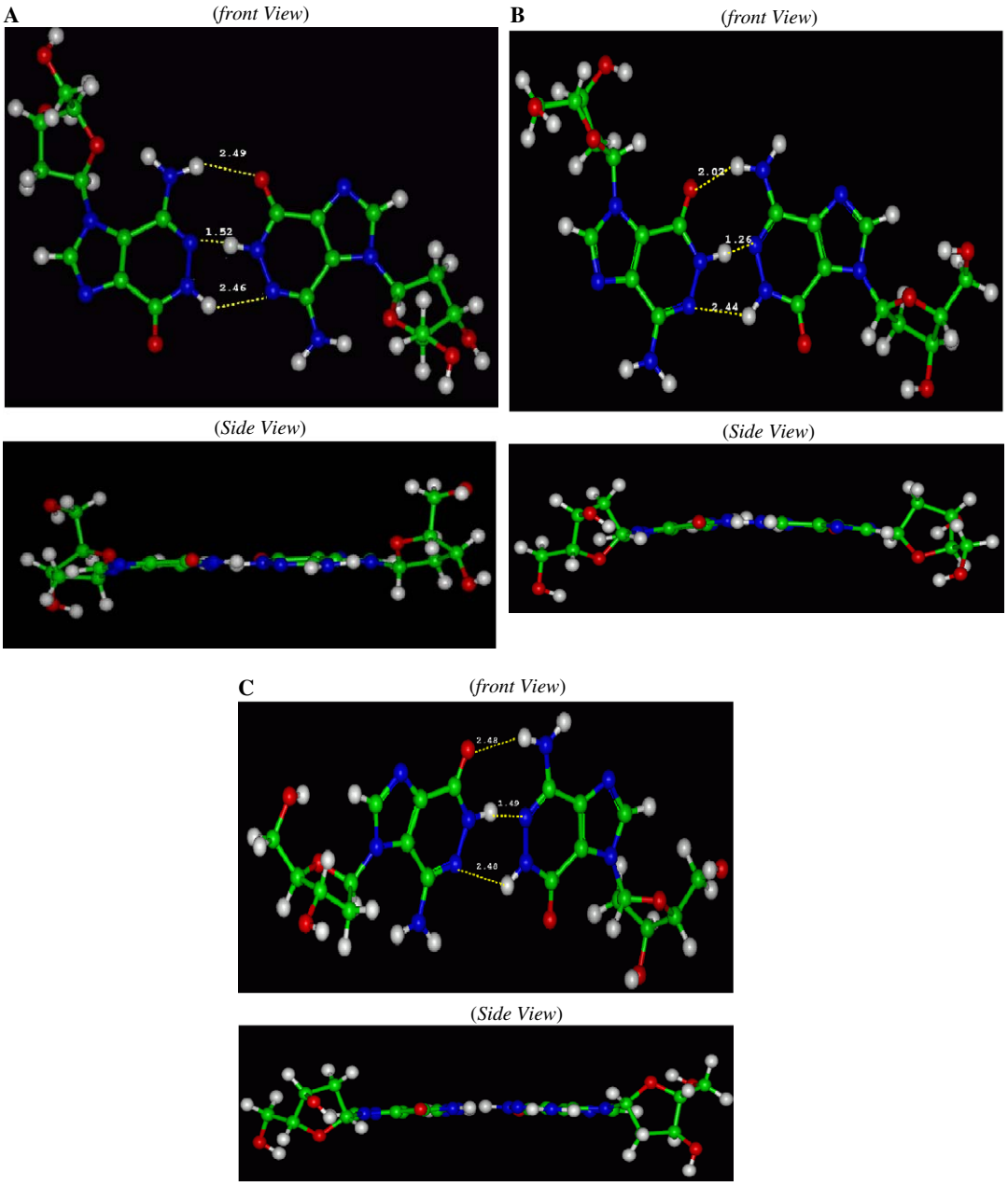
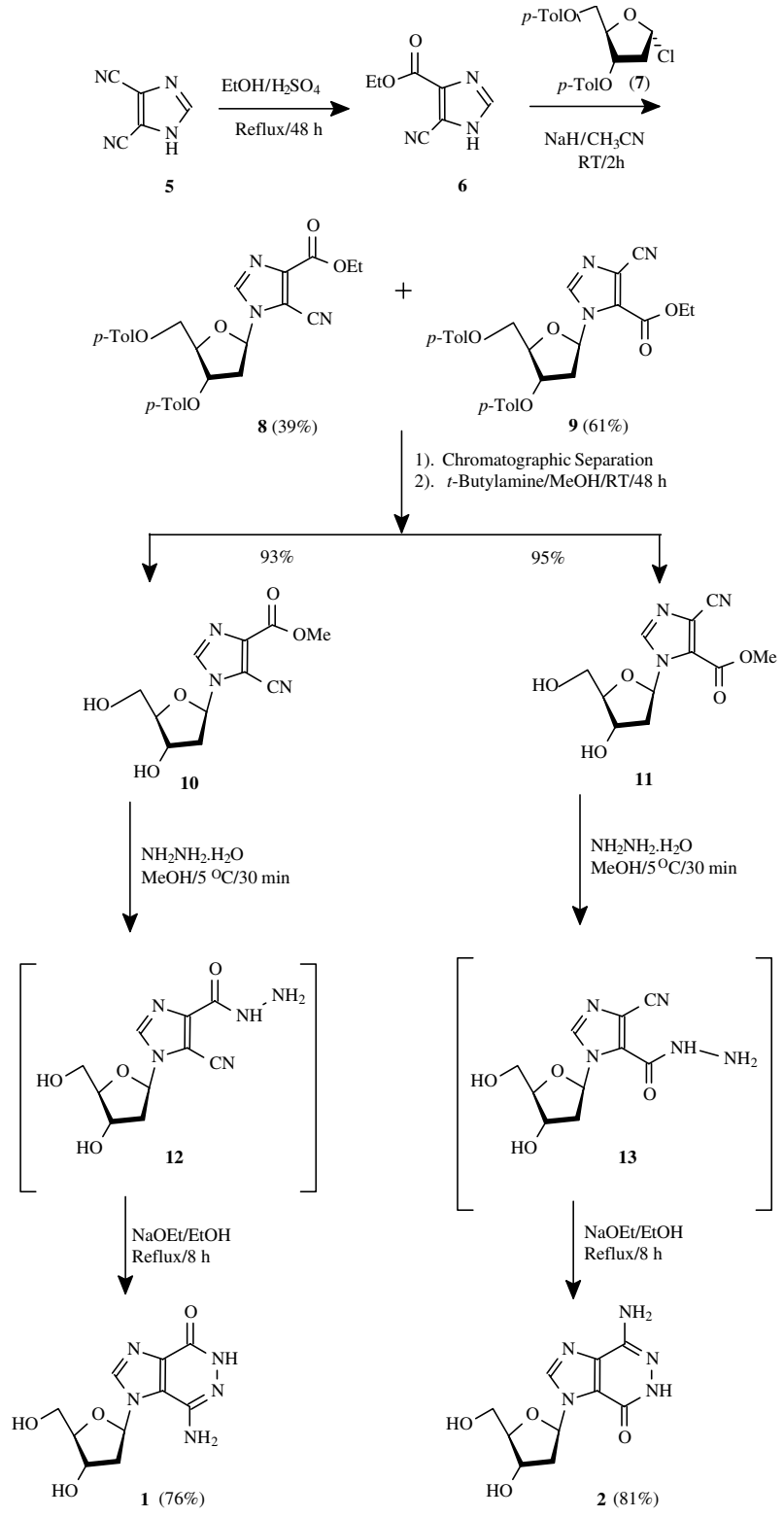


Figure 1. Self-complementary base-pair arrays of nucleosides **1** and **2**: (A) between **1** and **1**, (B) between **2** and **2**, and (C) between **1** and **2**.

difference in chemical shifts was observed for each of the NH, NH_2, and H-2 signals of **1** in going from 25 to 0 °C. These values range from 48 to 52 Hz (NH), 36 to 39 Hz (NH_2), and 15 to 17 Hz (H-2). Interestingly, there was a slight upfield shift (−1.2 to −1.4 Hz) of H-1′ signals when the temperature was lowered from 25 to 0 °C. This is not too surprising in view of the fact that the lower temperature would result in a restricted rotation of the NH_2 protons at position-7 of **1**, causing steric constraints in the vicinity of H-1′. In corroboration of this notion is the absence of such upfield shifts in the H-1′ signal of **2** where the NH_2 group is directed away from H-1′.

The concentration-dependent differences in chemical shifts for nucleoside **2** in 0.01–0.08 M range are also small, although somewhat higher than those for **1**, with the largest values of 9.6 Hz (NH) and 12.7 Hz (NH) at 25 and 0 °C, respectively. As the observed shifts are all downfield, they can once again be attributed to intermolecular base-pairing rather than vertical stacking interactions. As with **1**, a dramatic difference in chemical shifts was observed for each of the NH, NH_2, and H-2 signals in going from 25 to 0 °C. These values range from 46 to 49 Hz (NH), 62 to 65 Hz (NH_2), and 12 to 13 Hz (H-2). The concentration-dependent ^{1}H NMR studies were then conducted using equimolar mixtures of **1** and **2** (see Tables V and VI in the Supplementary material). A graph of chemical shifts in Hz versus concentration in M for the NH and NH_2 signals of **1** and **2** in a mixture of **1** + **2** at both 25 and 0 °C are shown in Figures 2A and B. As



Scheme 1.

is evident from the graphs, there is a considerable change in concentration-dependent chemical shifts of the NH protons (see Fig. 2A) in the region 0.001–0.08 M (ΔHz for **1** = 28.8 and 26.5 Hz; ΔHz for **2** = 28.8 and 36.2 Hz at 25 and 0 °C, respectively). While the two NH signals of **1** and **2** are coalesced at 25 °C at all concentrations, they appear as two distinct lines at 0 °C. However, as was the case with the individual components of the mixture, there is a sharp downfield jump in chemical shifts of NH at each concentration in going from 25 to 0 °C (ΔHz for **1** = 44–48 Hz; ΔHz for **2** = 52–59.6 Hz). The

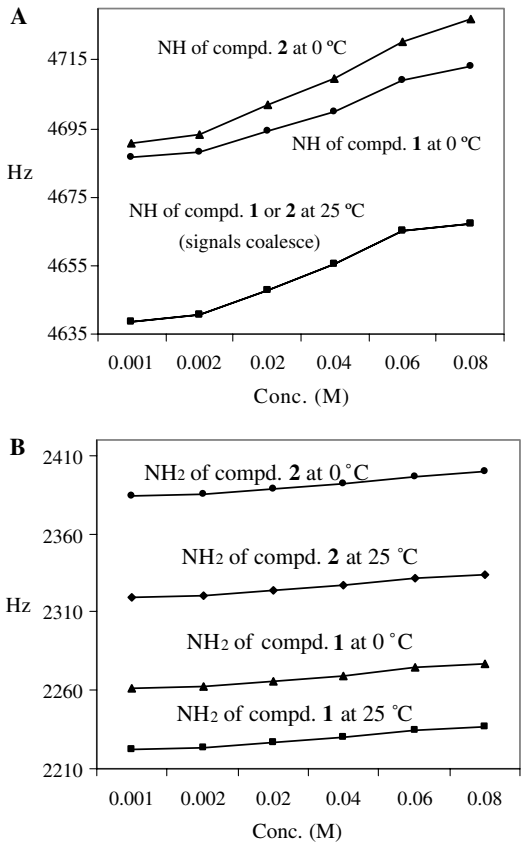


Figure 2. Concentration-dependent ^{1}H NMR studies of a mixture of compounds **1** and **2** at 25 and 0 °C: (A) NH signals of **1** and **2**, and (B) NH_2 signals of **1** and **2** in DMF-d_7.

concentration-dependent chemical shifts of the NH_2 protons (see Fig. 2B), by contrast, are considerably less pronounced than those of NH protons in the region 0.002–0.06 M (ΔHz for **1** = 11.8 and 12.4 Hz; ΔHz for **2** = 11.3 and 11.8 Hz at 25 and 0 °C, respectively). Furthermore, the NH_2 signals of **1** and **2** have two distinctly separate absorptions even at 25 °C, the signal of **2** appearing farther downfield than that of **1**. The most conspicuous feature of the NH_2 absorptions is the sharp downfield jump in chemical shifts at each concentration in going from 25 to 0 °C (ΔHz for **1** = 39–41 Hz; ΔHz for **2** = 64–66 Hz). The concentration-dependent chemical shifts of the H-2 protons of **1** and **2** in a mixture of **1** + **2** are rather small in the range 0.001–0.08 M (ΔHz = 9–10 Hz at both 25 and 0 °C). The changes in chemical shifts of the same signals at each concentration in going from 25 to 0 °C, while noticeable (ΔHz for **1** = 16–17 Hz; ΔHz for **2** = 12–14 Hz), are still considerably smaller than those for NH and NH_2 protons. Likewise, the concentration-dependent chemical shifts of the H-1′ signals of **1** and **2** in the range 0.001–0.08 M at both 25 and 0 °C are also small (ΔHz ≈ 4 Hz). Once again, as was the case with studies of individual nucleosides described earlier, there was a slight upfield shift of the H-1′ signals of **1** (ΔHz = −0.9 to −2.5 Hz) and a slight downfield shift of the same signal in **2** (ΔHz = 1.9–3.2 Hz) in going from 25 to 0 °C.

Variable temperature ^{1}H NMR studies were then conducted using 0.08 M concentration for each of **1**, **2**, and **1** + **2** at 10 degree intervals in the range from 0 to −50 °C (see Figs. 3–5) by monitoring the change in chemical shifts of the same NH, NH_2, imidazole H-2, and anomeric H-1′ signals as before. The results (see Tables VII–IX in Supplementary material) reveal dramatic downfield shifts for the NH, NH_2, and H-2 protons, ranging by as much as 114 and 90 Hz, respectively, for the NH and NH_2 of **1**, 103 and 157 Hz, respectively, for the NH and NH_2 of **2**, 103 and 89 Hz, respectively, for the NH and NH_2 of **1** in **1** + **2**, and 115 and 156 Hz, respectively, for the NH and NH_2 of **2** in **1** + **2**. Obviously, there is a sudden and sharp downfield jump in chemical shifts of both NH and NH_2 protons in going from 25 to 0 °C, which then steadily falls further downfield as the temperature is decreased. Also, while the NH signals of **1** and **2** are coalesced at 25 °C, they begin to separate when the temperature is gradually lowered. Apparently, there is a fast exchange of the two NH protons among the four ring N atoms present in the six-membered ring of **1** and **2** at 25 °C. The two NH_2 signals, by contrast, are separated even at 25 °C, and the temperature-dependent change in chemical shifts of NH_2 is more pronounced in **2** than in **1**, possibly because of the steric constraints imposed by the sugar H-1′ of **1** in base-pairing, as described earlier.

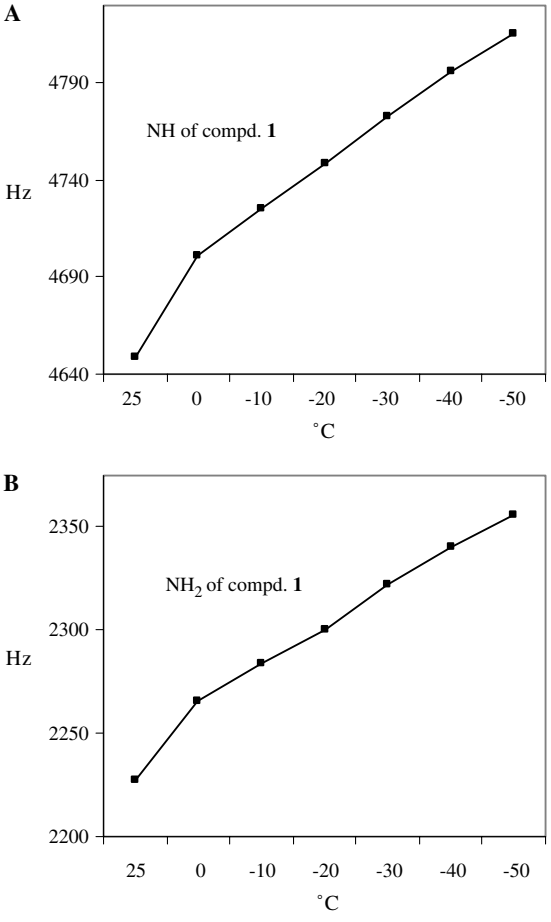


Figure 3. Temperature-dependent ^{1}H NMR chemical shifts of (A) NH and (B) NH_2 protons of nucleoside **1** in DMF-d_7.

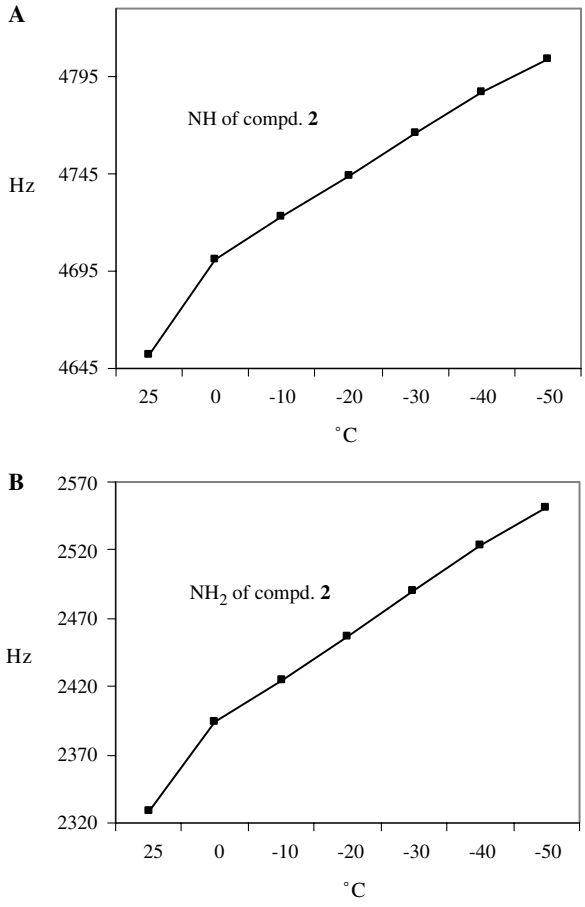


Figure 4. Temperature-dependent ^{1}H NMR chemical shifts of (A) NH and (B) NH_2 protons of nucleoside **2** in DMF-d_7.

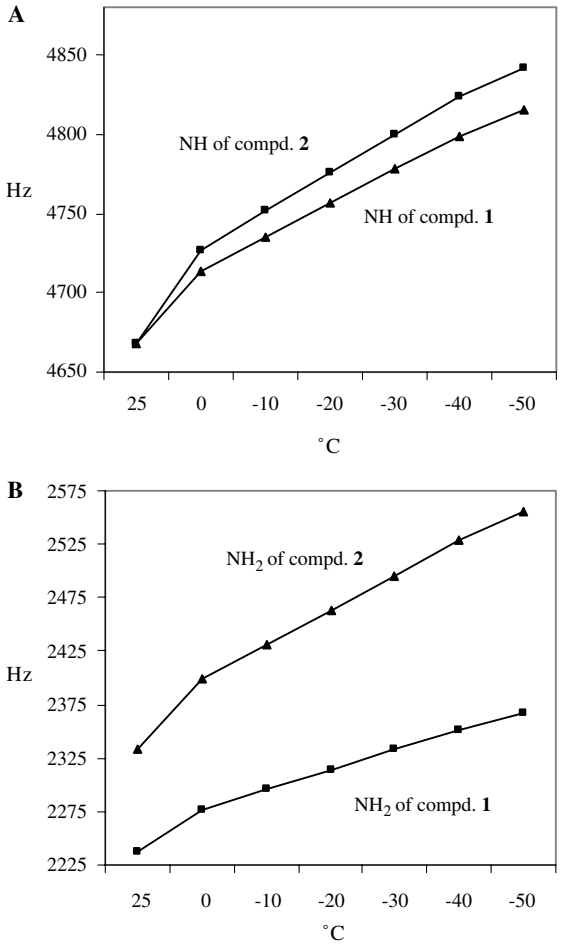


Figure 5. Temperature-dependent ^{1}H NMR chemical shifts of (A) NH and (B) NH_2 protons of nucleosides **1** and **2** in an equimolar mixture of **1** + **2** in DMF-d_7.

The temperature-dependent downfield chemical shifts of the H-2 signal of **1** and **2** in the range from 0 to −50 °C in the above experiments are also significant as revealed by the values of 40 Hz for **1**, 29 Hz for **2**, 39 Hz for **1** in **1** + **2**, and 30 Hz for **2** in **1** + **2**. Once again, a conspicuous observation concerned the H-1′ signal of **1** both in an isolated run and in a **1** + **2** mixture, which showed an upfield rather than a downfield shift.

Finally, as mentioned under Section 1, nucleoside **2** exhibited a weak antiviral activity against HIV-1 in CEM-SS cells with a 50% inhibitory concentration (IC_{50}) of 89.2 μM and a 50% cytotoxic concentration (TC_{50}) of >200 μM, the highest concentration tested.

3. Conclusion

We have designed and successfully synthesized two isomeric nucleosides **1** and **2**, containing a common heterocyclic base, 7(4)-amino-5(6)*H*-imidazo[4,5-*d*]pyridazine-4(7)one, in three steps commencing from ethyl 5(4)imidazole-4(5)carboxylate (**6**). Our molecular modeling studies suggest that **1** and **2** can form stable base-pair complexes with the self (homo) or with each other (hetero), in a parallel or anti-parallel orientation, respectively. The ^{1}H NMR-based base-pair studies are in agreement with the results of molecular modeling. Compound **2** is a weak inhibitor of HIV-1 in vitro, while **1** is inactive.

4. Experimental

4.1. Base-pair studies using H NMR spectroscopy

All samples were directly prepared in 5 mL NMR tubes using 0.5 mL DMF-d_7 in concentrations ranging 0.001–0.08 M. Variable temperature (25 to −50 °C) ^{1}H NMR experiments were conducted with a 400 MHz NMR spectrometer (JEOL JNM-ECX) following the manufacturer's procedures and instructions. Briefly, the procedure involves generation of a constant flow of nitrogen gas by heating liquid nitrogen in the metal Dewar. The gas is supplied to the surroundings of the sample via the probe heater. The desired temperature can be adjusted by heating the nitrogen gas with the probe heater and controlling its power. Tetramethylsilane (TMS) was used as an internal reference standard for NMR measurements.

4.2. Ethyl 5-cyano-1-[(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl)-β-D-erythropento-furanosyl]imidazole-4-carboxylate (8) and ethyl 4-cyano-1-[(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl)-β-D-erythropento-furanosyl]imidazole-5-carboxylate (9)

To a solution of **6**[32] (3.30 g, 0.02 mol) in anhydrous acetonitrile (175 mL), sodium hydride (60% in oil, 2 g, 50 mmol) was added and the mixture was stirred at rt under nitrogen atmosphere for 30 min. 2-Deoxy-3,5-di-*O*-*p*-toluoyl-α-D-erythropento-furanosyl chloride (**7**)[33,34] (7.76 g, 0.02 mol) was added portionwise over a period of 1 h. After the addition was complete, the reaction mixture was allowed to stir at rt for 2 h. It was then filtered and evaporated under reduced pressure. The residue was purified by flash chromatography on a silica gel column using a mixture of 4:1 hexanes/ethyl acetate as an eluant for isolation of **8** and **9**. The spectral and analytical data for **8** and **9** are as follows:

4.3. Compound 8

Yield 3.71 g, 39%; mp 69 °C; R_f 0.11 (2:1 hexane/ethyl acetate); IR 2235, 1716, 1612, 1268, 1178, 1096, 1019, 752 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz), δ 7.94 (s, 1H, H-2), 7.92 (d, J = 3.6 Hz, 2H, ArH), 7.82 (d, J = 7.82 Hz, 2H, ArH), 7.24 (dd, J = 8.1 Hz, 4H, ArH), 6.29 (dd, J = 6 + 3 Hz, 1H, 1′H), 5.69–5.67 (m, 1H, 3′H), 4.76–4.63 (m, 3H, 4′, 5′ and 5″-H), 4.43 (q, J = 6.9 Hz, 2H, CH_2), 2.97–2.91 (m, H, 3″H), 2.72–2.62 (m, 2H, 2′,2″H), 2.43 (s, 3H, Ar-CH_3), 2.39 (s, 3H, Ar-CH_3), 1.40 (t, J = 6.9 Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.4, 165.2, 159.4, 144.2, 143.8, 137.2, 129.2, 129.0, 128.8, 128.7, 125.8, 125.4, 108.9, 106.4, 86.2, 83.4, 73.9, 63.0, 61.3, 39.3, 21.2, 21.1. Mass (FAB) m/z 518.20 (MH^+). Anal. Calcd for $C_{28}H_{27}N_3O_7$: C, 64.98; H, 5.26; N, 8.12. Found: C, 64.82; H, 5.28; N, 8.08.

4.4. Compound 9

Yield 5.92 g, 61%; mp 128 °C; R_f 0.41 (2:1 hexane/ethyl acetate); IR 2241, 1720, 1611, 1265, 1203, 1176, 1086, 1018, 756 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz), δ 8.11 (s, 1H, H-2), 7.93 (d, J = 8.1 Hz, 2H, ArH), 7.80 (d, J = 7.8 Hz, 2H, ArH), 7.23 (dd, J = 7.8 Hz, 4H, ArH), 6.72 (t, J = 6.6 Hz, 1H, 1′H), 5.60–5.58 (m, 1H, 3′H), 4.71–4.67 (m, 3H, 4′, 5′ and 5″H), 4.42 (q, J = 7.2 Hz, 2H, CH_2), 3.08–3.05 (m, 2H, 2′,2″H), 2.42 (s, 3H, Ar-CH_3), 2.39 (s, 3H, Ar-CH_3), 1.43 (t, J = 7.5 Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.5, 165.3, 157.3, 144.0, 143.9, 138.2, 129.2, 129.0, 128.8, 128.7, 126.7, 125.7, 125.6, 120.6, 112.81, 87.92, 83.3, 73.8, 63.1, 61.8, 40.4, 21.2, 21.1. Mass (FAB) m/z 518.20 (MH^+). Anal. Calcd for $C_{28}H_{27}N_3O_7$: C, 64.98; H, 5.26; N, 8.12. Found: C, 64.95; H, 5.39; N, 8.11.

4.5. Methyl 5-cyano-1-[(2′-deoxy)-β-D-erythropento-furanosyl]imidazole-4-carboxylate (10)

Compound **8** (3.23 g, 0.0063 mol) was placed in dry methanol (35 mL) and *tert*-butylamine (2.28 g, 0.0312 mol) was added, and the reaction mixture was stirred at room temperature for 48 h, when a TLC (chloroform/methanol, 10:1) showed the completion of reaction. Deprotected methyl ester **10** was isolated by flash silica gel chromatography, using 15:1 chloroform/methanol solvent system. Yield 1.56 g, 93%; mp 145 °C; R_f 0.29 (chloroform/methanol, 10:1); IR 3354, 3110, 2238, 1725, 1551, 1439, 1240 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz), δ 8.41 (s, 1H, H-2), 6.16 (t, J = 5.96 Hz, 1H, 1′H), 5.41–5.40 (d, J = 4.6 Hz, 1H, OH), 4.94 (t, J = 5.64 Hz, 1H, OH), 4.33–4.31 (m, 1H, CH), 3.86–3.84 (d, 1H, CH), 3.84 (s, 3H, CH_3), 3.56–3.45 (m, 1H, 2′-H), 2.61–2.36 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 160.9, 141.0, 140.3, 110.5, 107.6, 89.1, 87.1, 70.5, 60.4, 52.6, 40.5. Mass (FAB) m/z 268.20 (MH^+). Anal. Calcd for $C_{11}H_{13}N_3O_5$: C, 49.44; H, 4.90; N, 15.72. Found: C, 49.42; H, 4.96; N, 15.46.

4.6. Methyl 4-cyano-1-[(2′-deoxy)-β-D-erythropento-furanosyl]imidazole-5-carboxylate (11)

Compound **9** (5.17 g, 0.01 mol) was placed in anhydrous methanol (50 mL) and *tert*-butylamine (3.65 g, 0.05 mol) was added, and the reaction mixture was stirred at room temperature for 48 h, when a TLC (chloroform/methanol, 10:1) showed the completion of reaction. Deprotected methyl ester **11** was isolated by silica gel flash column chromatography, using 20:1 $CHCl_3$–MeOH solvent system. Yield 2.46 g, 93%; mp 131 °C; R_f 0.38 (chloroform/methanol, 10:1); IR 3361, 3109, 2241, 1723, 1541, 1437, 1207 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz), δ 8.55 (s, 1H, H-2), 6.50 (t, J = 5.52 Hz, 1H, 1′H), 5.32–5.31 (d, J = 4.56 Hz, 1H, OH), 5.12 (t, J = 5.04 Hz, 1H, OH), 4.30–4.25 (m, 1H, CH), 3.88 (s, 3H, CH_3), 3.86–3.84 (d, 1H, CH), 3.68–3.55 (m, 1H, 2′-H), 2.44–2.27 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 158.2, 141.2, 140.9, 114.7, 88.5, 88.1, 60.7, 53.0, 40.4. Mass (FAB) m/z 268.20 (MH^+). Anal. Calcd for $C_{11}H_{13}N_3O_5$: C, 49.44; H, 4.90; N, 15.72. Found: C, 49.07; H, 4.92; N, 15.34.

4.7. 7-Amino-1-[(2′-deoxy)-β-D-erythropento-furanosyl]-1H-imidazol[4,5-*d*]pyridazin-4(5H)-one (1) and 4-amino-1-[(2′-deoxy)-β-D-erythropento-furanosyl]-3H-imidazol[4,5-*d*]pyridazin-7(6H)-one (2)

To a solution of **10** or **11** (0.534 g, 0.002 mol) in anhydrous methanol (15 mL), hydrazine hydrate (2 mL) was added. This reaction mixture was stirred at ice-cold temperature for 30 min. Then methanol and unreacted hydrazine hydrate were removed under vacuum. The residue was refluxed in ethanolic solution of sodium ethoxide (2 mL, 0.35 mmol). After 8 h reflux, the reaction mixture was brought to room temperature and the separated solid was filtered and washed with ethanol.

4.8. Compound 1

Recrystallized from ethanol. Yield 0.43 g, 76%; mp > 300 °C; R_f 0.20 (chloroform/methanol/ammonium hydroxide, 2:1:0.25); IR 3432, 3339, 3216, 3106, 1657, 1628, 1594, 1566, 1461, 1221 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.61 (s, 1H, CONH), 8.48 (s, 1H, H-2), 6.43 (t, J = 5.96 Hz, 1H, 1′-H), 5.49 (s, 2H, NH_2), 5.35 (s, 1H, OH), 5.00 (s, 1H, OH), 4.33–4.35

(m, 1H, 4′-H), 3.90–3.89 (m, 1H, 3′-H), 3.56–3.44 (m, 1H, 5′,5″H), 2.65–2.38 (m, 2H, 2′,2″H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 157.5, 142.0, 140.3, 138.3, 126.7, 88.6, 85.8, 70.2, 61.2, 40.8. Mass (FAB) *m*/*z* 268.20 (MH^+). Anal. Calcd for $C_{10}H_{13}N_5O_4$: C, 44.94; H, 4.90; N, 26.21. Found: C, 44.70; H, 4.94; N, 26.11.

4.9. Compound 2

Recrystallized from methanol. Yield 0.46 g, 81%; mp > 300 °C; R_f 0.44 (chloroform/methanol/ammonium hydroxide, 2:1:0.25); IR, 3415, 3308, 3332, 3158, 1662, 1636, 1584, 1469, 1200 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 11.55 (s, 1H, CONH), 8.57 (s, 1H, H-2), 6.78–6.75 (t, J = 6.4 Hz, 1H, 1′H), 5.78 (s, 2H, NH_2), 5.29 (s, 1H, OH), 5.02 (s, 1H, OH), 4.31–4.32 (s, 1H, 4′H), 3.85–3.84 (m, 1H, 3′H), 3.60–3.51 (m, 1H, 5′,5″H), 2.46–2.31 (m, 2H, 2′,2″H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 154.7, 145.5, 142.2, 135.9, 125.1, 88.6, 85.9, 70.7, 61.7, 41.7. Mass (FAB) *m*/*z* 268.20 (MH^+). Anal. Calcd for $C_{10}H_{13}N_5O_4$: C, 44.94; H, 4.90; N, 26.21, Found, C, 44.79; H, 4.93; N, 26.21.

4.10. Molecular modeling studies

Molecular modeling studies were performed on a PS/2 workstation, employing the Biosym/InsightII software (Accelrys, San Diego), coupled with Discover algorithm for energy minimization. Nucleosides **1** and **2** were separately built and energy-minimized before association into a complex and further energy minimization. The complex was minimized to convergence using the steepest descent and conjugate gradient forcefield protocols of the software program.

4.11. Antiviral screening

Antiviral assays were performed at Southern Research Institute (SRI), Frederick, Maryland.

4.11.1. Cell preparation. CEM-SS cells were passaged in T-75 flasks prior to use in the antiviral assay. On the day preceding the assay, the cells were split 1:2 to assure they were in an exponential growth phase at the time of infection. Total cell and viability quantification was performed using a hemacytometer and Trypan blue exclusion. Cell viability was greater than 95% for the cells to be utilized in the assay. The cells were resuspended at 5×10^4 cells/mL in tissue culture medium and added to the drug-containing microtiter plates in a volume of 50 μL.

4.11.2. Virus preparation. The lymphocytropic virus strain HIV-1_{RF} was obtained from the NIH AIDS Research and Reference Reagent Program, and was grown in CEM-SS cells for the production of stock virus pools. For each assay, a pre-titered aliquot of virus was removed from the freezer (−80 °C) and allowed to thaw slowly to room temperature in a biological safety cabinet. The virus was resuspended and diluted into tissue culture medium such that the amount of virus added to each well in a volume of 50 μL was the amount determined to give between 85% and 95% cell killing at 6 days post-infection. $TCID_{50}$ calculations by endpoint titration in CEM-SS cells indicated that the multiplicity of infection of these assays was approximately 0.01.

4.11.3. Assessment of IC_{50} and TC_{50} values. Samples were evaluated for antiviral efficacy with triplicate measurements using 6 concentrations at half-log dilutions in order to determine the IC_{50} values and with duplicate measurements to determine cellular cytotoxicity (TC_{50}). At assay termination, the assay plates were stained with the soluble tetrazolium-based dye MTS (CellTiter 96 Reagent, Promega) to determine cell viability and quantify compound toxicity. At termination of the assay, 20–25 μL MTS reagent was added per well, and the microtiter plates were then incubated for 4–6 h at 37 °C, 5% CO_2 to assess cell viability. The plate was read spectrophotometrically at 490/650 nm with a Molecular Devices Vmax plate reader. Using an in-house computer program, % CPE reduction, % cell viability, and IC_{50} and TC_{50} values were calculated. AZT was evaluated in parallel as a positive control in the anti-HIV assay. The computed values for **2** are: IC_{50} = 89.2 μM and TC_{50} > 200 μM, while those for AZT are: IC_{50} = 15.6 nM and TC_{50} > 500 nM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.05.043. Description of the general experimental procedure and Tables I–IX listing concentration and temperature-dependent ^{1}H NMR chemical shifts of target nucleosides **1** and **2**, either alone or as a mixture (seven pages), can be found in the supplementary data associated with the online version of this journal.

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