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# Synthesis and enzymatic incorporation of a novel, bicyclic pyrimidine nucleoside: a thymidine mimic

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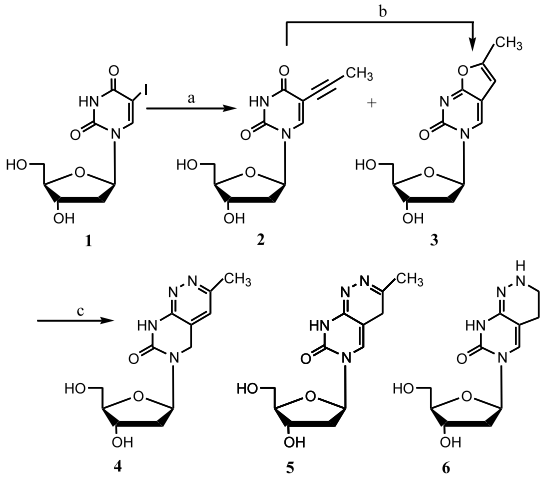
**Abstract**—Nucleophilic ring-opening and rearrangement reaction of a furanopyrimidine nucleoside with anhydrous hydrazine provided a novel, 6,6-bicyclic pyrimidopyridazin-7-one nucleoside (dH, **4**), whose structure was confirmed by X-ray crystallography. This novel nucleoside was converted to its 5′-triphosphate (dHTP) for studies with DNA polymerases and incorporated into a template by using standard phosphoramidite chemistry. In the template, dH directed the incorporation of dATP and to a lesser extent dGTP into the transcript and dHTP was efficiently incorporated at the 3′-end of a primer opposite dA using both exonuclease free Klenow fragment (KF *exo*-) and *Taq* DNA polymerases and extended with natural dNTPs. 

There has been a tremendous interest in the design and synthesis of natural DNA base modified nucleoside mimics for a variety of explorations, such as re-engineering DNA/proteins, gene-therapy, antivirals, duplex stability, base-stacking and mutagenesis.[1–5] As a part of our research program on novel nucleoside analogs, we have been focusing our efforts on the design, synthesis and substrate activity of base modified nucleosides which have the property of base pairing with one or more of the natural bases in DNA duplexes.[6–8] In the latter case, the base would behave in a degenerate fashion and the nucleoside-5′-triphosphate should act as a substrate and show the same recognition features in polymerase catalyzed chain extension. Such analogs have use in labeling and in increasing the molecular diversity in oligonucleotides. We have previously reported on such an analog, a bicyclic pyrimidine mimic capable of base pairing with a specific base/bases in DNA.[6] The extra steric bulk imparted by the second ring added to the pyrimidine ring in bicyclic analogs would certainly influence the duplex stability either by its distorted geometry or by the hydrophobicity/base-stacking. Herein, we report on the synthesis and enzymatic incorporation of an unusual bicyclic analog, which acts essentially as thymidine.

Bicyclic furanopyrimidine nucleoside analogs are known to undergo nucleophilic ring-opening/rearrangement reactions with amines resulting in pyrrolopyrimidine analogs.[9,10] In an effort to derive novel, bicyclic pyrimidine mimics, it was of interest to see the reaction




**Scheme 1.** Synthesis of nucleoside analog dH (**4**). *Reagents and conditions*: (a) propyne, $(PPh_3)_4Pd(0)$, CuI, DMF; (b) CuI, $Et_3N$, MeOH, reflux; (c) $NH_2NH_2$.

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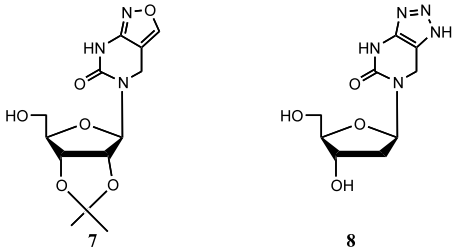
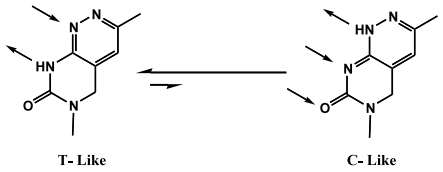
product outcome of the bicyclic furanopyrimidines with hydrazine. To this end (Scheme 1), reaction of 5-iodo-dU with propyne by the Sonogashira cross coupling reaction was exploited to generate an equal mixture of the propynylated derivative **2** and the cyclised furo[2,3-*d*]pyrimidinone **3**, the former undergoing conversion to **3** by treatment with copper(I) iodide. Treatment of the furano derivative **3** with neat anhydrous hydrazine resulted in a product, to which the structure could not be established unambiguously by the initial spectroscopic data. The $^1$H NMR spectrum of the product suggested it to be **5**, and showed it to be a single compound. The UV spectrum showed a $\lambda_{max}$ at 292 nm (compared with only 278 nm for the reduced derivative **6**[11]).

However, our earlier attempts to synthesize the ring system **5** via a different route involving reaction of a $C^4$-triazolo, $C^5$-ethanal pyrimidine derivative with hydrazine failed to give the expected ring product (R. Bazzanini, unpublished data). Similar dihydropyrimidine nucleosides (**7** and **8**) have been noted in the literature, however, these structures were not unambiguously determined. Structure **7** was initially suggested but was ruled out by NMR studies in favor of 7*H*-pyrimido[4,5-*c*]oxazol-6-one analogous to **5**.[12a,b] Similarly, compound **8**, formed by reduction of the 7-azapurine was suggested, but not characterized.[13] To clarify the structure of this hydrazine-derived adduct, crystals from methanol were used to unambiguously assign the structure by X-ray cyrstallography (Fig. 1).[14] The crystal structure pointed to a more remarkable structure, **4**, 6-(β-D-2-deoxyribofuranosyl)-5*H*,8*H*-dihydro-3-methylpyrimido[4,5-*c*][1,2]pyridazin-7-one (dH).



**7** **8**

In order to investigate the influence of the pyridazine ring system and tautomeric state of **4** on the H-bonding capability with natural bases, **4** was converted to its 5′-DMT-3′-phosphoramidite and to its 5′-triphosphate. The nucleoside dH was found to be stable to the reagents used during oligonucleotide synthesis. Thus, oligonucleotides incorporating the nucleoside dH were prepared using standard DNA synthesis procedures, to examine its templating properties with both *Taq* polymerase and exonuclease free Klenow fragment (KF *exo*-). In the template, dH (**4**) directed the incorporation of dATP and to a lesser extent dGTP into the transcript (Fig. 2) with both polymerases. dH therefore behaves as a pyrimidine analog. Extension of the primer beyond the analog with natural dNTPs gave full-length product (data not shown).




The 5′-triphosphate (dHTP) was also efficiently incorporated at the 3′-end of a primer opposite dA, and also to a lesser extent dG (Fig. 3). Further extension of the

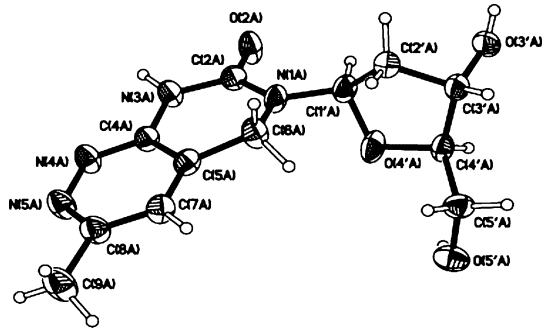



**Figure 1.** Thermal ellipsoid plot (50% probability) of the X-ray crystal structure of the nucleoside dH (**4**). There are two molecules in the asymmetric unit, but they are of similar conformation, and only one is presented for clarity. Atom labeling uses conventional pyrimidine nomenclature.

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5'-    TAATACGACTCACTATAGGGAGA
3'-    ATTATGCTGAGTGATATCCCTCTHGTCA
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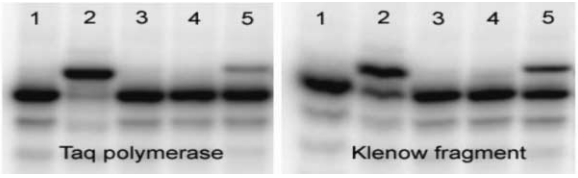


**Figure 2.** Primer extension reactions using template containing dH. The primer (23nt) is elongated by one nucleotide opposite dH when insertion is successful. The data were obtained at 60°C for Taq polymerase, and 37°C for KF *exo*- using 0.5 units polymerase in a total volume of 40 μl, containing 50 pmol primer annealed to 100 pmol template and 40 μM dNTP. Lane 1, primer. 2, primer+dATP. 3, +dTTP. 4,+dCTP. 5,+dGTP.

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5'-    TAATACGACTCACTATAGGGAGA
3'-    ATTATGCTGAGTGATATCCCTCTXTCAG
                    X = A, T, C, G
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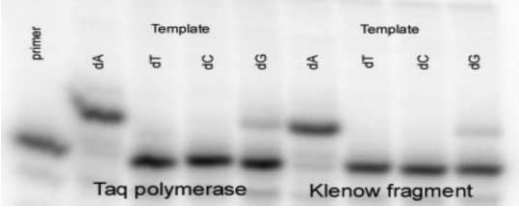


**Figure 3.** Primer extension using dHTP. Extension reactions using primer (23nt) annealed to four different templates were treated with 40 μM dHTP and 0.5 units polymerase, *Taq* or KF (*exo*-), using conditions as in Figure 2.

primer with native dNTPs gave full-length product (data not shown). When dH was present in the template or as its 5′-triphosphate, primer extension was more efficient with *Taq* polymerase than with Klenow fragment. There was no incorporation of natural pyrimidines opposite dH.

As **4** contains a pyridazine ring system, the fact that it behaves as a T/C analog (albeit biased towards T) is remarkable. Such an analog would be expected to behave only as a thymidine analog. The behavior of **4** is more like dihydropyrimidine derivatives. To the best of our knowledge the substrate properties of 2′-deoxy-5,6-dihydocytidine-5′-triphosphate with DNA polymerases have not been studied. However, the ribonucleoside derivative of dihydrocytosine has been shown to behave as a C/T analogue when its 5′-triphosphate is used as a substrate for the RNA polymerase from *M. luteus*.[15]

The novel analog that we have described may be readily modified to give substituents at either C-3 or C-4, which would allow for labeling of the nucleoside. The presence of the aromatic planar ring substituting for the hydrophobic methyl group of thymidine may also be of use for probing base interactions.

In summary, we have described a novel class of nucleoside analogs based on the 5*H*,8*H*-dihydropyrimido[4,5-*c*][1,2]pyridazin-7-one ring system. We have shown the 3-methyl analog (dH) to be a good template base, and as its 5′-triphosphate (dHTP) a substrate for both Taq and Klenow fragment (*exo*-) polymerases. The nucleotide, **4**, behaves as a T≫C mimic exclusively and there was no evidence of tautomerism observed by NMR. Further work is under way to examine its effect on the duplex melting temperatures, and the synthesis of other related analogs and their biological activities.

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12. (a) Loakes, D.; Brown, D. M. *Nucleosides Nucleotides* **1994**, *13*, 679–706; (b) *Spectral data for compound* ***4***: $^1$H NMR (DMSO-$d_6$): $\delta$ 1.80 (m, 1H, 2′), 2.14 (m, 1H, 2′), 2.49 (s, 3H, Me), 3.49 (m, 2H, 5′), 3.62 (q, 1H, 4′), 4.16 (m, 1H, 3′), 4.40 (s, 2H, H-5), 4.79 (t, 1H, OH-5′), 5.13 (d, 1H, OH-3′), 6.24 (t, 1H, 1′), 7.34 (s, 1H, H-4), 10.29 (bs, 1H, NH-8). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 21.1 (Me), 35.1 (2′), 47.8 (C-5), 61.8 (5′), 70.6 (3′), 83.1 (1′), 86.0 (4′), 120.8 (C-4a), 124.8 (C-4), 151.8 (C-7), 153.2 (C-3), 155.2 (C-8a). UV: $\lambda_{max}$ (pH 7) 293 (3290), 242 (7330); (pH 1) 307 (1800), 250 (6790); (pH 12) 278 (10, 370). MS: $m/z$ calcd for $C_{12}H_{16}N_4O_4$: 280.12; observed: M+ $Na^+$ 303.
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14. *Selected crystal data for* ***4***: CCDC 192848. Formula: $C_{12}H_{17}N_4O_4{\cdot}0.5H_2O$. Unit cell parameters: $a=8.0283(4)$, $b=20.8112(6)$, $c=8.1985(4)$, $\alpha=90.00$, $\beta=91.421(2)$, $\gamma=90.00°$, space group $P2_1$.
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