Synthesis and Some Biochemical Properties of a Novel 5,6,7,8-Tetrahydropyrimido[4,5-c]pyridazine Nucleoside

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Dedicated to Professor Jack D. Dunitz on the occasion of his 80th birthday

A novel nucleoside analogue is described based on the pyridazine ring system. The nucleoside was successfully incorporated into DNA *via* both its phosphoramidite and 5'-triphosphate derivatives. Enzymatically, the analogue behaves essentially as thymidine: it is a good substrate for the DNA polymerases Taq and exonuclease-free *Klenow* fragment, leading to full-length products when present in either the primer or template strands. In hybridisation studies, the nucleoside displays ambiguous base-pairing properties, including universal base properties.

Introduction. – Over many years, there has been a continuing interest in nucleoside analogues in which the base residue is modified to provide structures with a variety of altered properties. Our interest was originally sparked by the remarkable *in vitro* mutagenic effects of NH₂OH on bacteriophage T4 and S13 [1][2], demonstrating that the reagent introduced a single class of base transitions *viz*. C \rightarrow T. Structural work in many laboratories showed that at least one explanation depended on the transamination reaction to give N⁴-hydroxycytosine derivatives **1**, (*cf.* [3] and refs. cit. therein). Later, the corresponding reaction with NH₂NH₂ was shown to give N⁴aminocytosine **2** [4]. Both bases as their deoxyribosyl derivatives were mutagenic in prokaryotes [5][6]. The general explanation for their mutagenicity resided in the very large tautomeric shift (10⁴-10⁵) engendered by the more electronegative element attached to the exocyclic N⁴, leading to K_T values close to unity [7]. In particular N⁴aminocytosine had values of 30 (in favor of the amino form) in H₂O and 0.1 in the less polar CHCl₃ [7].

Most applications of this degeneracy in N^4 -hydroxycytosine and related systems involve, in general, duplex base pairing, for example, in diminishing chain multiplicity in probes and primers. Moreover, they have remarkable utility in generating populations of mutant polynucleotides with such bases in templates or as 5'triphosphate substrates in chain extensions and PCR reactions [8–10]. It became apparent that the N^4 -substituent (*e.g.*, in N^4 -methoxyC) lowered the base-pairing interaction energy with A and G due to its preferred *syn*-conformation with respect to the H-bonding face, but fixing the *anti*-conformation by introducing a second ring gives the deoxynucleoside dP **3**. The additional ring in dP had a marked duplex stabilising

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effect [11], with little effect on its K_T value, recently established as 11 in favor of the imino tautomer [12]. The triphosphate dPTP was an excellent polymerase substrate (as a C/T analogue), showing that the additional structural complexity was accepted in the polymerase active site [8][10].

In view of the very favorable tautomeric ratio of **2**, we have attempted to incorporate the exocyclic residue into a ring structure with the intention of suppressing its instability [13][14]. The reduced bicyclic system **4** (R=H, PhCH₂) proved to be chemically unstable, though their 5'-triphosphates were polymerase substrates, being incorporated opposite A and G [14]. Attempts to prepare **5** by hydrazine cyclisation of the 4-triazolo-5-(2-ethanal) nucleoside derived from deoxyuridine failed to give a recognisable product [15]. The observation that furano-pyrimidine nucleosides react with NH₃ to give the corresponding pyrrolo-pyrimidine [16][17] suggested an alternative approach to our problem, which led, however, to an unexpected outcome that we discuss here.

Results and Discussion. – When 5-iododeoxyuridine is cross-coupled with propyne in the presence of tetrakis(triphenylphosphine)palladium(0), the propynylated product **6** and the cyclised furano-pyrimidine **7** were formed in approximately equal amounts (*Scheme 1*). Unreacted **6** was converted to the latter by CuI treatment in a second step. The ring-opening rearrangement reaction of furano-pyrimidine nucleoside derivatives to the corresponding pyrrolo-pyrimidines has been reported [16][17]. Thus, reaction of the furano-pyrimidine derivative **7** with NH₃ yielded the expected pyrrolo-pyrimidine **8**.

Whilst the reaction of furano-pyrimidine derivatives with NH_3 is known, the reaction with other nucleophiles has not been investigated. We reasoned that reaction with NH_2OH and NH_2NH_2 might give rise to 9 and the analogue of 5. These compounds would be analogous to the bicyclic pyrimidines 3 and 4 we have previously studied [8][13][14].

The furano derivative 7, when heated with anhydrous NH_2NH_2 , gave a single product in high yield. The possibility that the compound was 10 was rendered unlikely

Scheme 1. Sonagashira Cross-Coupling Synthesis of Furano-pyrimidine and Conversion to Pyrrolo-pyrimidine Derivative



a) Propyne, [Pd⁰(PPh₃)], CuI, DMF. b) CuI, Et₃N, MeOH.



by the absence of an amino signal in its NMR spectrum, and the UV spectrum showed a λ_{max} of 292 nm compared with 278 nm for 4 (R=H). The failure to synthesis 5 by the alternative route mentioned above was further evidence that the product was not of this general structure. However, crystals obtained from MeOH were submitted to X-ray crystallography and showed that the product had the unexpected structure 11 (dH).

The nucleoside dH crystallized with two molecules in the asymmetric unit (*Fig. 1*). The two independent molecules form a H-bonded pair involving O(7)- and N(8)-atoms of each (N–O distances of 2.85 and 2.81 Å). The base moieties of both molecules are similar structurally, but O(5') of one deoxyribose is observed to be disordered.

Bond lengths observed in the bicyclic base clearly support the assignments indicated in **11** by their general similarity to comparable molecular fragments described in the literature [18], although structural data on closely analogous ring systems are not available. The pyridazine ring is completely planar, but the presence of an sp³-C-atom in the pyrimidine ring causes C(5) and N(6) to be displaced respectively 0.15 and 0.19 Å either side of the mean plane. The bond angle N(6)-C(5)-C(10) is,



Fig. 1. The molecular structure of dH (11) determined by single-crystal X-ray analysis, showing displacement ellipsoids (50% probability). Molecule B is disordered in the region of C(5') (see text).

respectively, $112.72(19)^{\circ}$ and $112.03(18)^{\circ}$ in the two molecules. Evidently, the slight lack of planarity does not present significant obstacles for duplex formation, and, indeed, base stacking is a feature of the extended crystal structure. An off-set, antiparallel arrangement achieves substantial overlap between the more-planar side of the heterocycle, and avoiding unfavourable contacts from H-atoms on C(5) and the exocyclic Me group at the other.

The putative initial ring-closure product related to **5** had clearly tautomerised to aromatise the pyridazine ring, which, in retrospect, is an unsurprising outcome. There is little work described relating to analogous tetrahydropyrimidin-2(1H)-ones for comparison. Thus, compound **12**, formed by reduction of the 7-azapurine, was not supported by structural information [19]. Also, the structure **13**, formed by a NH₂OH ring closure, suggested by its UV spectrum, was ruled out in favor of **14** on the basis of NOE-NMR evidence [20].



Reaction of 7 with NH_2OH gave a more-polar product, and, based on the mass spectrum and NMR data, it became clear that the product was not the desired bicyclic one but the acyclic derivative **15** (*Scheme 2*). Attack by NH_2OH first gives the expected

 N^4 -OH derivative, however, the *N*-OH group was not sufficiently nucleophilic to react with intermediate ketone. This preferentially further reacts with NH₂OH to yield **15**. Reaction of **7** with NaOH similarly opens the furan ring to give the 2-oxopropyl derivative of deoxyuridine **16**.





Hybridisation Studies. From the nucleoside dH (11) its 5'-triphosphate 17 and 5'-(dimethoxytrityl)-3'-phosphoramidite 18 were prepared in the usual manner, and incorporated into oligonucleotides (dH was shown to be stable to the reagents used for DNA synthesis). Starting from the phosphoramidite, oligonucleotide duplexes were prepared for which a number of melting temperatures were measured (*Table*) to determine base-pairing behaviour. Duplexes 1 and 2 (*Table*) are GC and TA controls, respectively. Duplexes 3-6 have the analogue dH base paired opposite each of the natural DNA bases near the centre of the duplex, whilst 7-10 has dH towards the 5'end. In both sets of duplexes, there is a clear preference for base pairing, but whilst, in the centre of the duplex dH pairs preferentially with dA, when near the 5'-terminus, it pairs preferentially with dT. Thus, in one context, it behaves expectedly as a thymidine mimic, in the other as an adenosine mimic. In both cases, however, the duplex stability is the same as that of the TA control.

To assess the effect of a second dH residue, the most stable duplex (10) was substituted first in the centre (duplexes 11-12) then consecutively (duplexes 13-16). For the central substitutions, only two duplexes were prepared (dH opposite dT and dA) as these were found to form the most stable base pairs in the other contexts (duplexes 3-6). Substituting a second residue in the centre resulted in a 12° decrease in T_m , but this was now consistent between the two duplexes. The consecutive substitutions showed only a slight decrease in T_m , and, in this case, the additional substitution behaved more like a universal base analogue. Similarly, when dH was substituted at the 5'-end of the duplex (17-20) the behaviour was more like that of a universal base.

Clearly, there are sequence effects that determine the optimal base-pairing behaviour of dH, so no absolute conclusions may be drawn from this data. In one context, dH behaves as a good T analogue (duplexes 3-6) in another as an A analogue

Duplex		$T_{\rm m}/^\circ$	Duplex		$T_{\rm m}/^{\circ}$
	GTAAAACGA C GGCGCAGT			GT H AAACGA H GGCCAGT	
1	CATTTTGCT G CCGGTCA	73	11	CA T TTTGCT T CCGGTCA	59
			12	CA T TTTGCT A CCGGTCA	58.5
	GTAAAACGA T GGCCAGT				
2	CATTTTGCT A CCGGTCA	71		GT HH AACGACGGCCAGT	
			13	CA TT TTGCTGCCGGTCA	70
	GTAAAACGA H GGCCAGT		14	CA TA TTGCTGCCGGTCA	69
3	CATTTTGCT G CCGGTCA	65.5	15	CA TC TTGCTGCCGGTCA	69
4	CATTTTGCT A CCGGTCA	71	16	CA TG TTGCTGCCGGTCA	69
5	CATTTTGCT C CCGGTCA	61			
6	CATTTTGCT \mathbf{T} CCGGTCA	63		H TAAAACGACGGCCAGT	
			17	C ATTTTGCTGCCGGTCA	63
	GT H AAACGACGGCCAGT		18	G ATTTTGCTGCCGGTCA	63
7	CA C TTTGCTGCCGGTCA	63.5	19	T ATTTTGCTGCCGGTCA	63
8	CA G TTTGCTGCCGGTCA	64	20	A ATTTTGCTGCCGGTCA	63
9	CA A TTTGCTGCCGGTCA	66			
10	CA T TTTGCTGCCGGTCA	71			

Table 1. Thermal Melting (T_m) of Duplexes Containing the Modified Nucleoside dH (11). T_m Measurements were carried out in 6xSSC (0.9M NaCl, 0.09M sodium citrate, pH 7), at an oligomer strand concentration of *ca*. 3 μ M.

(7-10), whilst, in a majority of cases, it behaves as a universal base. A possible explanation for the universal behaviour could be because it contains an aromatic hydrophobic ring, which is common to universal bases [21], and this enhances stacking interactions. Also, like other universal base analogues, the $T_{\rm m}$ is not significantly altered by a second consecutive substitution.

Enzyme Specificity of dH. Although it is likely that the mobile H-atom residues at N³ of the pyrimidine ring of **11**, it was of interest to investigate the biochemical properties of the nucleoside for degenerate base-pairing behaviour. Two sets of primer extension reactions were performed in which dH was either in a template, or its 5'-triphosphate was used to extend the primer. In the first case with dH in the template, primer-extension reactions were performed by addition of a single natural dNTP to determine how dH was copied by the polymerase. Reactions were performed with both exonuclease-free *Klenow* fragment (KF exo⁻) and Taq DNA polymerases. After reaction with a single dNTP, the reaction products were fully extended by the addition of the remaining three dNTPs. As can be seen (*Fig. 2*), both KF exo⁻ and Taq were able to incorporate dATP and, to a much lesser extent, dGTP, indicating that as a template base the pyrimido-pyridazine behaves as a pyrimidine, and preferentially as thymine. With both polymerases, chain extension beyond dH occurred efficiently.

In the second experiment, four templates containing one of the four natural template bases were used to determine how dHTP would be incorporated. Again the analogue behaved as a thymidine mimic, and although it could be incorporated opposite dG, it was much less efficient than as a template base (*Fig. 3*). The reactions were subsequently run with a mixture of all four dNTPs, and, in each case, full-length product was observed, even after dHTP had been incorporated into the growing chain (data not shown). Thus, dH is extended without chain termination, and its triphosphate does not act as an inhibitor to chain extension in these reactions.





Fig. 2. Primer-extension reactions with template dH, and 0.5 units exonuclease-free Klenow fragment (KF exo⁻) or Taq DNA polymerases and 40 μM dNTPs. Data were obtained at 37° for KF exo⁻ and 60° for Taq polymerase. Lanes marked 2', 3', etc. correspond to the subsequent addition of the remaining 3 dNTPs corresponding to lanes 2, 3, respectively.



Fig. 3. Primer-extension reactions with 40 μM dHTP with primer annealed to four different templates (A, T, C and G) and 0.5 units KF exo⁻ or Taq polymerase. Reaction conditions are as for Fig. 2.

There are three environments in which a modified nucleoside needs to be accepted by a polymerase. The easiest of these is when the modification is in the template, and there have been many examples of such chain-extension reactions. The second-mostdifficult situation is the incorporation of a modified nucleoside triphosphate onto the 3'- end of a primer. Simple primer-extension reactions have been used for such reactions. The most difficult task expected of a polymerase, though, is chain extension off a primer with the modification at the 3'-end. Many nucleoside analogues have been incorporated into a growing DNA chain, but once incorporated they behave as chain terminators. In the case of dH, it was able, in primer-extension reactions with either Taq or KF exo⁻, to meet all of these criteria.

One final set of reactions was performed to assess the effectiveness of dH as an enzyme substrate. The two primers used formed a self-complementary duplex with eight base pairs and a four-base overhang (Fig. 4). The primers had at the 5'-end either thymidine or dH, and were used to assess the effectiveness of a ligation followed by digestion reaction, as the ligation product generated the restriction site for Cla I. Each primer was kinased with ³²P-ATP and T4 polynucleotide kinase, and, after heat denaturation of the kinase, allowed to anneal, first to room temperature then at 4° overnight. The primers were then treated with T4 DNA ligase at 16° for 16 h, then heatdenatured at 65° for 20 min. Initial attempts to remove salts and ATP with spin columns resulted in significant loss of DNA too, so the ligation product was treated directly with Cla I restriction enzyme at 37° for 4 h. The products were electrophoretically separated on a 15% acrylamide denaturing gel (data not shown). The results showed that the 5'thymidine primer gave long ligation products, with bands corresponding to the ligation of 12 primers clear before the product band became a smear. For the 5'-dH primer, ligation products could also be observed, though bands corresponding to ligation of up to 8 primers were visible. Whilst the 5'-thymidine ligation product showed a single new short product similar in length to the original primer after digestion with Cla I, the 5'dH ligation product showed no sign of digestion.

T^{*}CGATACGCGTA

ATGCGCATAGCT^{*}



Fig. 4. Partially self-complementary primer used for ligation reactions. Ligation generates a Cla I restriction site denoted by arrows. T* represents site at which dH is substituted.

From these experiments, it was shown that oligonucleotides bearing dH at the 5'end were substrates for both kinase and ligase enzymes, but that DNA containing dH at the scission site for Cla I restriction enzyme was not recognised and, therefore, protected from digestion. Thus, whilst enzymatically it appears that dH behaves as a thymidine mimic, in certain situations, it is not recognised as thymidine, probably due to the bulky aromatic ring.

Conclusions. – We have described the synthesis of a new class of nucleoside analogue, the pyrimido-pyridazine ring system (dH). In hybridisation studies, the

analogue showed no clear preference for behaviour as one of the natural DNA bases, but in enzymatic studies the analogue behaves preferentially as a thymidine mimic. As a template base and as the triphosphate, it behaves mainly as a T analogue, and very slightly as C with DNA polymerases. An explanation for the C-like behaviour would be an equilibrium with the N(8) imino tautomer. dH was a good substrate for DNA polymerases as a template base, as its 5'-triphosphate, and when present at the 3'-terminus. It was also a substrate for kinase and ligase enzymes, but when incorporated into DNA as a thymidine mimic it afforded protection of the DNA to the restriction enzyme Cla I.

Experimental Part

General. TLC was carried out on pre-coated F_{254} silica plates and column chromatography (CC) with Merck silica gel 60. UV Spectra were recorded on a Perkin-Elmer Lambda 20 Spectrophotometer in 10% MeOH in H₂O unless otherwise stated. ¹H- ¹³C- and ³¹P-NMR spectra were obtained on a Varian Mercury VX300 spectrometer, and were obtained in (D₆)DMSO unless otherwise stated. LC/MS chromatograms/spectra were acquired with Micromass LCT spectrometer, Waters 2695 separation module, and Waters 996 PDA UV detector by electrospray ionisation (ES) in negative-ion mode (unless noted otherwise) and orthogonal acceleration time-of-flight (oa-TOF) detection techniques. Waters XTerra MS C18 3.5 cm column (2.1 × 50 mm) was used, unless noted otherwise. Prep. HPLC was performed with Waters Prep LC 4000 system with Prep LC controller, dual-wavelength UV absorbance detector, and Omega Chart recorder in 0.1M triethylammonium bicarbonate (TEAB; pH 7.0) and MeCN. Melting temps. were recorded on a Perkin Elmer Lambda 40 spectrophotometer fitted with a Peltier cell in 6 × SSC (0.9M NaCl, 0.09M sodium citrate, pH 7), at an oligomer strand concentration of ca. 3µM. Absorbance vs. temp, for each duplex was obtained at a heating and cooling rate of 0.5°/min. and melting temperatures (T_m) were determined as the maxima of the differential curves, with an error of $\pm 0.5^\circ$.

 $3-(\beta-D-2-Deoxyribofuranosyl)-6-methylfuro[2,3-d]pyrimidin-2-one (7).$ To a 100-cm³ glass pressure reaction vessel under Ar was added 5-iodo-2-deoxyuridine (7.1 g, 20 mmol), tetrakis(triphenylphosphine)palladium(0) (2.32 g, 2 mmol), and CuI (0.76 g, 4 mmol). Anh. DMF (50 cm³) and Et₃N (4.2 cm³, 30 mmol) were then injected into the reaction vessel, and the mixture was cooled to 0° . At this point, propyne was bubbled into the soln. for 10 min. Upon sealing the pressure vessel, the temp. of the reactants was increased to $55-60^\circ$ via an oil bath. The mixture was allowed to stir under these conditions for 18 h. On cooling, no starting material was detected by TLC. MeOH (25 cm³), Chelex 100 resin (5 g, 200-400 mesh, Na form) and AG 1-X8 resin (5 g, 20-50 mesh, bicarbonate form) were added to the mixture and stirred gently for 1 h. Upon filtration, the soln. was evaporated to an oil under high vacuum. The residue was purified by CC (silica gel; CHCl₃/MeOH 100% to 10:1) yielding $1-(\beta-D-2-deoxyribofuranosyl)5-(prop-1-ynyl)uracil, (6; R_f 0.4) as a tan foam in 45% yield, and 7$ $(R_{\rm f} 0.2)$ as a yellow solid in 50% yield. Compound 6 was converted to 7 by refluxing in MeOH/Et₃N 7:3 containing 5% CuI for 2 h. After CC a 61% yield was obtained. UV: λ_{max} 292, 239. ¹H-NMR: 2.06 (m, 1 H-C(2')); 2.33 (s, Me); 3.38 (m, 1 H-C(2')); 3.65 (m, CH₂(5')); 3.91 (m, H-C(4')); 4.24 (m, H-C(3')); 5.09 (t, 5'-OH); 5.26 (d, 3'-OH); 6.18 (t, H-C(1')); 6.41 (s, H-C(5)); 8.66 (s, H-C(4)). ¹³C-NMR $((D_6)DMSO): 29.8 (Me); 40.3 (C(2')); 60.8 (C(5')); 69.9 (C(3')); 85.3 (C(1')); 88.1 (C(4')); 88.8 (C(5)); 98.3 (C(5));$ (C(4a)); 144.8 (C(4)); 147.8 (C(6)); 150.1 (C(2)); 162.1 (C(7a)). HR-MS: 289.07980 ([M + Na]⁺, $C_{12}H_{14}N_2NaO_5^+$; deviation - 0.94 ppm).

3-(β-D-2-Deoxyribofuranosyl)-3,7-dihydro-6-methyl-2H-pyrrolo[2,3-d]pyrimidin-2-one (8). To 7 (200 mg, 0.75 mmol) was added 10 cm³ of 0.880M NH₄OH soln., and the mixture was stirred at r.t. overnight. After concentration, the product was purified by silica-gel chromatography to give 180 mg (90%) of 8. UV: λ_{max} 335. ¹H-NMR: 1.99 (m, 1 H–(2')); 2.21 (s, Me); 2.32 (m, 1 H–C(2')); 3.64 (m, CH₂(5')); 3.87 (m, H–(4')); 4.23 (m, H–(3')); 5.07 (t, 5'–OH); 5.23 (d, 3'–OH); 5.90 (s, H–C(7)); 6.26 (t, H–C(1')); 8.47 (s, H–C(6)); 11.06 (br. s, NH). ¹³C-NMR ((D₆)DMSO): 13.22 (Me); 41.24 (C(2')); 60.97 (C(5')); 69.88 (C(3')); 86.54 (C(1')); 87.62 (C(4')); 96.82 (C(7)); 108.89 (C(5)); 134.01 (C(6)); 137.68 (C(8)); 153.72 (C(2)); 159.21 (C(4)). HR-MS: 288.09500 ([M + Na]⁺, C₁₂H₁₅N₃NaO⁺₄; deviation – 3.67 ppm).

 $6-(\beta-D-2-Deoxyribofuranosyl)-5,6,7,8-tetrahydro-3-methylpyrimido[4,5-c]pyridazin-7-one (11).$ To 500 mg (1.35 mmol) of **7** was added 6 cm³ of anh. NH₂NH₂. The mixture was allowed to stir at r.t. for 2 h. The excess NH₂NH₂ was then removed by evaporation under high vacuum. The residue was purified by CC (silica gel;

CHCl₃/MeOH 10:1) to yield an off-white crystalline product in 85% yield. X-Ray-quality crystals were obtained from MeOH. UV: λ_{max} 293 (3290), 242 (7330); λ_{min} 268, 223; pH 1: λ_{max} 307 (1800), 250 (6790); λ_{min} 283, 236; pH 12: λ_{max} 278 (10370); λ_{min} 240; ε 260 (μ M) 3.0, ε 280 (μ M) 2.6. ¹H-NMR: 1.80 (m, 1 H–C(2')); 2.14 (m, 1 H–C(2')); 2.49 (s, Me); 3.49 (m, CH₂(5')); 3.62 (m, H–C(4')); 4.16 (m, H–C(3')); 4.40 (s, CH₂(5)); 4.79 (t, 5'–OH); 5.13 (d, 3'–OH); 6.24 (t, H–C(1')); 7.34 (s, H–C(4)); 10.29 (br. s, H–N(8)). ¹³C-NMR: 21.1 (Me); 35.1 (C(2')); 4.78 (C(5)); 61.8 (C(5')); 70.6 (C(3')); 83.1 (C(1')); 86.0 (C(4')); 120.8 (C(4a)); 124.8 (C(4)); 151.8 (C(7)); 153.2 (C(3)); 155.2 (C(8a)). HR-MS: 303.10790 ([M + Na]⁺, C₁₂H₁₆N₄NaO⁺₄; deviation 3.12 ppm).

2'-Deoxy-N⁴-hydroxy-5-(2-hydroxyiminopropyl)cytidine (**15**). Compound **7** (0.85 g, 3.1 mmol) was dissolved in 5 cm³ of MeOH, and a 50% soln. of NH₂OH in H₂O (20 cm³) was added. The mixture was stirred at r.t. for overnight, and the solvent was concentrated. The compound was purified by silica-gel chromatography with CHCl₃/MeOH mixture. To completely remove NH₂OH from the desired compound, either HPLC or a further column was required. Yield: 0.65 g (66%). UV: λ_{max} 232, 275. ¹H-NMR: 1.72 (*s*, Me); 2.00 (*m*, CH₂(2')); 3.72 (*m*, CH₂(5')); 3.75 (*m*, H–C(4')); 4.05 (*s*, CH₂); 4.20 (*m*, H–C(3')); 4.88 (*t*, 5'–OH); 5.20 (*m*, 3'–OH); 6.16 (*t*, H–C(1')); 6.89 (*s*, H–C(6)); 10.36 (*s*, NH). ¹³C-NMR: 13.27 (Me); 32.80 (C(2')); 38.62 (CH₂); 61.77 (C(5')); 70.79 (C(3')); 83.27 (C(1')); 86.86 (C(4')); 106.81 (C(5)); 127.81 (C(6)); 128.05, 143.12, 149.12 (C(2) or C=NOH), 153.00 (C(2) or C=NOH). ES-TOF-MS: 313.37 (C₁₂H₁₇N₄O₆; calc. 313.29). HR-MS: 337.11220 ([*M*+Na]⁺, C₁₂H₁₈N₄NaO₆⁺; deviation – 0.70 ppm).

2'-Deoxy-5-(2-oxopropyl)uridine (**16**). Compound **7** (1 g, 3.7 mmol) was dissolved in 25 cm³ of THF, and 5 cm³ of 2N NaOH soln. was added. The mixture was stirred at r.t. for 4 h. After concentration, the compound was purified by silica-gel chromatography with 0–20% MeOH/CHCl₃. Yield: 0.23 g (22%), UV: λ_{max} 266. ¹H-NMR: 2.08 (*m*, 1 H–C(2')); 2.12 (*s*, Me); 3.40 (*m*, 1 H–C(2')); 3.54 (*m*, CH₂(5')); 3.76 (*m*, H–C(4')); 4.07 (*s*, CH₂COMe); 4.22 (*m*, H–C(3')); 5.01 (*t*, 5'–OH); 5.27 (*d*, 3'–OH); 6.16 (*t*, H–C(1')); 7.71(*s*, H–C(6)); 11.37 (br. *s*, NH). ¹³C-NMR: 29.22 (Me); 38.66 (C(2')); 40.53 (CH₂COMe); 61.31 (C(5')); 70.31 (C(3')); 84.04 (C(1')); 87.31 (C(4')); 108.34 (C(5)); 138.24 (C(6)); 150.24 (C(2)); 162.98 (C(4)); 204.90 (COMe). ES-TOF-MS: 283.33 (C₁₂H₁₅N₂O₆; calc. 283.26). HR-MS: 307.09110 ([*M*+Na]⁺, C₁₂H₁₈N₂NaO₆⁺; deviation 1.51 ppm).

 $6-(\beta-D-2-Deoxyribofuranosyl)-5,6,7,8-tetrahydro-3-methylpyrimido[4,5-c]pyridazin-7-one 5'-Triphosphate$ (dHTP). To 140 mg (0.5 mmol) of dried 11 in a 50-cm³ round-bottom flask under Ar was added 5 cm³ of trimethyl phosphate. The homogenous soln. was cooled via an ice-bath, and redistilled phosphorus oxychloride (70 µl, 0.75 mmol) was added. The mixture was stirred with cooling for 1 h. TLC Analysis indicated the reaction to be ca. 70% complete. Therefore, 25 µl (0.27 mmol, 0.51 equiv.) more phosphorus oxychloride was added, and the mixture was allowed to stir for an additional hour. Both 1M tributylammonium pyrophosphate in anh. DMF (2.5 cm³, 2.5 mmol) and Bu₃N (0.6 cm³, 2.5 mmol) were then simultaneously added slowly to the cooled soln. After addition, the mixture was stirred for 30 min, then warmed to r.t., and stirred for 1 h. Cooled 1M TEAB buffer (pH 7.0) was added to the mixture until the soln. became neutral as observed by pH paper. The buffer was then added to a final volume of 40 cm³, and the mixture was stirred overnight at r.t. The soln. was evaporated under high vacuum to a viscous soln., and the crude mixture was then applied to a 50×300 mm *DeltaPak* (15 μ C18 HPLC column), which was eluted with a linear gradient over 25 min with 0.1M TEAB (pH 7.0) to 0.1M TEAB in 25% MeCN at a flow rate of 130 ml per min. A peak containing the product was collected at 10.5 min. After evaporation, the crude triphosphate was finally purified on a 21 × 250 mm Synchropak Ax100 HPLC column with a linear gradient for 30 min at 15 ml per min of 0.1m TEAB in 40% MeCN to 1m TEAB in 40% MeCN. ³¹P-NMR (D₂O): -9.26 (d); -10.20 (d); -22.43 (t). HPLC (DeltaPak C18, 3.9 × 30 cm, 0.1M TEAB (pH 7.0) to 0.1M TEAB in 25% MeCN in 30 min at 1 ml per min) 12.7 min. UV: λ_{max} 237, 292.

6-[5-O-Dimethoxytrityl-3-O-(2-cyanoethyl N,N-Diisopropylphosphoramidite)-β-D-2-deoxyribofuranosyl]-5,6,7,8-tetrahydro-3-methylpyrimido[4,5-c]pyridazin-7-one. Compound **11** (1.16 g, 4.1 mmol) was co-evaporated with anhydrous pyridine and redissolved in 30 cm³ of anh. pyridine. Dimethoxytrityl chloride (4.15 g, 12.24 mmol) was added to the stirred soln. at r.t. under Ar. After 3.5 h, the mixture was evaporated under reduced pressure, and residue was dissolved in CHCl₃. The org. layer was washed with sat. NaHCO₃ soln., dried (Na₂SO₄) and evaporated under reduced pressure. The residue obtained was purified by CC (silica gel; CHCl₃ \rightarrow CHCl₃/MeOH 95:5) to afford the 6-(5-Dimethoxytrityl-β-D-2-deoxyribofuranosyl)-5,6,7,8-tetrahydro-3-methylpyrimido[4,5-c]pyridazin-7-one (2.35 g, 99%). ES-TOF-MS (cone 100v, MeCN/0.1M TEAB): 581.24 ([MH - 1]).

5'-DMT-Protected **11** (583 mg, 1.0 mmol) was co-evaporated with anh. pyridine, followed by toluene, and dissolved in anh. CH_2Cl_2 (5 cm³). To the stirred soln. under a slow stream of Ar, at r.t., $Et(i-Pr)_2N$ (0.7 cm³, 4.0 mmol) was added, followed by dropwise addition of 2-cyanoethoxy *N*,*N*-diisopropylaminochlorophosphine (0.28 cm³, 1.25 mmol). After 30 min, TLC in 10% MeOH/CHCl₃ indicated completion of the reaction. The mixture was diluted with CH_2Cl_2 , washed with 10% Na_2CO_3 , and the org. layer after drying (Na_2SO_4) was

evaporated under reduced pressure. The residue obtained was purified by CC (silica gel; 4×13 cm) eluted with CH₂Cl₂/AcOEt/Et₃N 2.9:7:0.1 to give 570 mg (73%) of the phosphoramidite. ³¹P-NMR (CDCl₃): 149.66.

X-Ray Analysis. Compound **11** crystallized as colourless stout needles on cooling and subsequent slow evaporation of a MeOH solution. A crystal fragment measuring 0.28 mm × 0.23 mm × 0.14 mm was coated in perfluoropolyether *RS3000* (*Riedel-de Haën*) and mounted on a glass fibre by surface tension followed by rapid cooling in a N₂ stream. Diffraction intensities were measured at 180 K on a *Nonius Kappa CCD* detector with MoK_a radiation ($\lambda = 0.71069$ Å) from a fine focus sealed tube with graphite monochromator. 11028 Reflections $> 2\sigma(I)$ in the range $3.60^{\circ} < \theta < 27.47^{\circ}$ were reduced to 5863 independents by averaging symmetry equivalents, and the structure was routinely solved with SHELXS-90 [22]. Refinement (against F^2) with SHELXL-97 converged at R = 6.74% (R_w 12.95%) for all 5863 reflections (388 parameters, 3 restraints). Cell constants: a = 8.0283(4), b = 20.8112(6), c = 8.1985(4) Å, $b = 91.421(2)^{\circ}$; space group *P*21; formula C₁₂H₁₇N₄O_{4.5}; Z = 4. Crystal data and atomic coordinates have been deposited at the *Cambridge Crystallographic Data Centre*, Deposition No. CCDC-192848.

Oligonucleotide Synthesis. Oligonucleotides were prepared on ABI 380B or 394 DNA synthesisers at 0.2µmol scale. The coupling time for dH phosphoramidite was 15 min. Oligonucleotides were purified either by HPLC (*Dionex Nucleopac*) buffer A 25% formamide, 1 mM NaClO₄, 0.02 mM *Tris*, pH 6.8; buffer B 25% formamide 400 mM NaClO₄, 0.02 mM *Tris*, pH 6.8, or 20% PAGE electrophoresis followed by dialysis. Where necessary, oligonucleotides were further desalted with NAP-10 columns (*Pharmacia*) according to the manufacturers protocol. Modified oligonucleotides were analysed by MALDI-TOF-MS (*Perceptive Biosystems Voyager-DE*).

Primer-Extension Assays.

3'-ATTATGCTGAGTGATATCCCTCT**H**GTCA 5'-TAATACGACTCACTATAGGGAGA

To a soln. of 44 μ (1000 pmol) of the primer 3'-AGA GGG ATA TCA CTC AGC ATA AT containing 5 μ l $10 \times PNK$ buffer (*NEB*) was added 4 μ l of ³²P-ATP and 1 μ l of T4 polynucleotide kinase, and the mixture was heated at 37° for 30 min. 1 μ l of 5 mm ATP (total volume 50 μ l) was added and the reaction left at 37° for a further 30 min and then heated at 95° for 5 min.

50 pmol of the labelled primer was annealed to 100 pmol of the template in H₂O containing 2.5 μ 10 × *Klenow* (*NEB*) buffer. 1 μ of 800 μ m dNTP (final concentration 40 μ m) was added, followed by 0.5U *Klenow* (exo⁻), and reactions incubated at 37° for 30 min (total volume 20 μ l). 5 μ l of each mixture was quenched in 5 μ l of stop soln. To the remainder of the mixture, the remaining three dNTPs were added, and the mixtures incubated for a further 30 min. Samples were taken as above. Prior to separation by *PAGE* (20%) samples were heat-denatured (95°) for 5 min and gels run at 30 W for 4.5 h. Gels were dried and exposed to a phosphorimager screen (*Molecular Dynamics*) and scanned on 425S phosphorimager (*Molecular Dynamics*).

Similar reactions were carried out with *SuperTaq* (*HT Biotech*). After annealing the reactions in *SuperTaq* buffer, 0.5U of *SuperTaq* was added, and the mixtures were incubated at 60° for 15 min. 1 μ l of 1 mM dNTP was added, and the mixtures were incubated for a further 30 min at 60°. Samples were taken as for *Klenow* reactions. The reactions were then chased with the remaining 3 dNTPs.

Primer-Extension Reactions with dH triphosphate.

3'-ATTATGCTGAGTGATATCCCTCT**A**TCAG 3'-ATTATGCTGAGTGATATCCCTCT**T**TCAG 3'-ATTATGCTGAGTGATATCCCTCT**C**TCAG 3'-ATTATGCTGAGTGATATCCCTCT**G**TCAG 5'-TAATACGACTCACTATAGGGAGA

50 pmol of the labelled primer were annealed to 100 pmoles of the template in H₂O containing 2.5 μ l 10 × *Klenow* (*NEB*) buffer. 1 μ l of 1 mM dHTP (final concentration 40 μ M) was added, followed by 0.5U *Klenow* (exo⁻), and mixtures were incubated at 37° for 30 min (total volume 25 μ l). 5 μ l of each mixture was quenched in 5 μ l of stop soln. To the remainder of the mixture, the four dNTPs were added, and the mixtures were incubated for a further 30 min. Samples were taken and electrophoretically separated as above.

Similar reactions were carried out with SuperTaq (HT Biotech). After annealing the mixtures in SuperTaq buffer, 0.5U SuperTaq was added, and the mixtures were incubated at 60° for 15 min. 1 µl of 1 mM dHTP was

added, and the mixtures were incubated for a further 30 min at 60° . Samples were taken as for *Klenow* reactions. The reactions were then chased with the 4 dNTPs.

Ligation with T4 DNA Ligase. The oligonucleotides XCGATACGCGTA, where X is either thymidine or dH, were kinased with ³²P-ATP in T4 DNA ligase buffer. To 40-µl mixtures containing 4 µl of $10 \times$ ligase buffer (final concentration 50 mm *Tris* · HCl, 10 mm MgCl₂, 10 mm DTT, 1 mm ATP, 25 µg/ml BSA), 50 pmol of primer and 1 µl of ³²P-ATP were added units T4 polynucleotide kinase (NEB), and the mixtures were incubated at 37° for 1 h. The mixtures were heated at 95° for 5 min and then allowed to cool to r.t.

To 10 μ l of each of the kinased primer reactions were added 2 μ l of 5 mM ATP and 4U T4 DNA ligase (NEB) in ligase buffer to a final volume of 25 μ l, and the mixtures were incubated at 16° for 16 h. The mixtures were then heated at 60° for 20 min then allowed to cool to r.t., and stored at 4° overnight.

Restriction Digest with Cla I. The ligation products from above were treated with 20U Cla I restriction enzyme (*NEB*), and the mixture incubated at 37° for 4 h. The mixtures were then heated at 60° for 20 min, then allowed to cool to r.t. The products from the ligation and digestion reactions were heated at 95° for 5 min before electrophoretically separated on a 15% acrylamide denaturing gel.

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