Synthesis of New Transglycosidically Tethered 5′-Nucleotides Constrained to a Highly Biologically Relevant Profile

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A new motif for restricting 5′-nucleotides to highly biologically relevant conformations has been developed. The 5′,6-oxomethylene transglycosidically tethered versions of uridine 5′-monophosphate and 2′-deoxyuridine 5′-monophosphate (**1** and **2**, respectively) were synthesized in 10−11 steps from their respective natural nucleoside precursors along routes general to the preparation of tethered versions of a wide variety of 5′-nucleotide-based compounds. In both routes, a shelf-stable 6-hydroxymethyl pyrimidine nucleoside 5′-carboxaldehyde is the key intermediate. It exists in a carbohydrate-like fashion in a cyclic hemiacetal form under aprotic conditions. The phosphorylated cyclic hemiacetals **1** and **2** were isolated as binary mixtures of 5′-diastereomers differing principally in the trajectory of the phosphate group with respect to the carbohydrate. By ^{1}H NMR, both **1** and **2** were demonstrated to be stable to hydrolysis at ambient temperature in D_2O solution for at least 2 months. The oxomethylene transglycosidic tether as deployed in **1** and **2** leaves all of the native 5′-nucleotide molecular recognition sites intact while it restricts the framework to a low-energy anti glycosyl conformation and an extended phosphate disposition. This provides a spatial presence that approximates nearly three-quarters of the protein-bound 5′-nucleotide ligands described in the Protein Data Bank. The tether has a low structural and electronic impact, occupies a region of space (over the β-face of the furan ring) seldom penetrated by proteins, and should be accommodated as readily on purine-based 5′-nucleotide frameworks as on pyrimidine-based ones. Because of its unique and attractive features, this new motif for the conformational restriction of 5′-nucleotides is expected to be useful for producing probes of structure/function relationships and in assessing the conformational binding requirements that enzymes and receptor sites have for their natural 5′-nucleotide-based ligands.

Introduction

The conformational restriction of nucleotides with tethers designed to limit primarily the flexibility of the carbohydrate moiety has received an enormous amount of attention recently.[1−10] Most of these carbohydrate-tethered nucleotide units are restricted only in furanose puckering so that when incorporated into DNA or RNA strands, they preorganize those oligomers for an enhanced molecular recognition by other nucleic acids.[2] Tethers that have been attached to the glycon portion of nucleotide units include the β-2′,3′-oxoethylene,[3] the α-3′,4′-propylene,[4] the α-3′,5′-azaethylene,[5] the α-2′,4′-(2-oxapropano),[6] and the α-3′,1′-azamethylene tether.[7] Others more extensively developed are the β-3′,5′-ethylene tether of bicyclo-DNA[8] and its cyclopropane-fused version in tricyclo-DNA[9] and the α-2′,4′-oxomethylene tether of LNA (locked nucleic acids).[10]

Far less attention has been paid to the design and use of tethered nucleotides as biochemical probes for characterizing proteins that have 5′-nucleotide monomers or small molecules containing these units as their ligands. This is surprising, given the plethora of NMPs, NDPs, NTPs, and other biologically important small molecules that contain these units such as the NDP-carbohydrates and enzyme cofactors such as NAD, FAD, and coenzyme A. Cyclopropanated carbocyclic bicyclo[3.1.0]hexane versions of the antiviral agent AZT and its 5′-monophosphate have been prepared as carbohydrate conformationally locked probes of the kinase and HIV-1 reverse transcriptase enzymes key to its therapeutic efficacy,[11] and similarly tethered versions of 2′-deoxyadenosine were used as probes of the enzyme adenosine deaminase.[12] The

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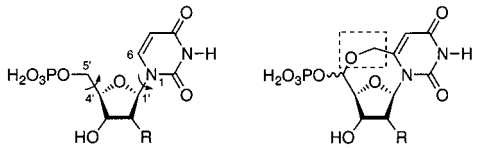
LNA[13] and β-2′,3′-oxomethylene-tethered[14] versions of AZT were also reported recently, but they were devoid of anti-HIV activity. There are very few examples of transglycosidically constrained nucleotide analogues. Among them are the 8,2′-thio-tethered version of ATP and 2,2′-anhydro-UTP synthesized as potential agonists for the $P2X_2$-purinoceptors.[15]

Conformational restriction is a powerful technique that selects a predetermined subset of native rotamers for probing the binding requirements of a biomacromolecule for its natural ligand. In the case of the ubiquitous 5′-nucleotide unit, the rotational disposition of just two bonds, the C4′−C5′ bond and the glycosyl bond (C1′−N1 for pyrimidines and C1′−N9 for purines), largely determines the molecule's spatial presentation, which is fine-tuned by the furanose puckering. A search of the Protein Data Bank[16] using ReLiBase[17] reveals 981 instances of a protein-bound 6-unsubstituted pyrimidine or 8-unsubstituted purine 5′-nucleotide-based ligand. The resolution of these structures notwithstanding, nearly three-quarters of them (711) have a C5′-to-C6 (pyrimidine) or C5′-to-C8 (purine) separation of ≤4.5 Å indicative of an anti glycosyl disposition (pyrimidine C6 or purine C8 over the furanose ring), and most have an extended phosphate disposition. Interestingly, the region of space over the β-face of the furan ring is not utilized by proteins when they bind 5′-nucleotide ligands: only 4 of those 711 PDB entries have a protein heavy atom located within 3.5 Å of *both* the C5′ and the pyrimidine C6 or purine C8. Therefore, a transglycosidic tether in this region of space should not only impose a useful rigidity on the 5′-nucleotide framework by simultaneously restricting the C4′−C5′ and the glycosyl bond rotations, but importantly, it should not encumber protein binding based on steric considerations.

In this context, the transglycosidic tethering of pyrimidine 5′-nucleotides between C5′ and C6 and purine 5′-nucleotides between C5′ and C8 is particularly attractive. 5′-Nucleotide biochemical probes restricted in this manner would be anti glycosyl rotamers with laterally extended phosphate groups, a disposition known to be favored by isolated 5′-nucleotides[18] and very well represented by those found bound to proteins by X-ray methods. Unfortunately, no such highly biomimetic and thus broadly useful set of compounds has yet been reported.[19] We now describe the synthesis and aqueous solution stability of the first two members of a new class of highly biomimetic transglycosidically tethered 5′-nucleotides that should provide valuable probes of the conformational binding requirements that proteins have for 5′-nucleotide ligands.

Results and Discussion

Our initial efforts focused on the uridine nucleotides, an important subclass of endogenous nucleotides.[20] Although the zero-atom-tethered 6,5′-cyclouridines[21] and the one-atom-tethered 6,5′-methanouridines[22] are known, the electron-withdrawing enone portion of the pyrimidin-6-yl moiety could labilize a 5′-phosphate group installed on these strained platforms under certain conditions. A two-atom-saturated tether would insulate the phosphate from such lability and, further, would not introduce much ring strain, but a simple 6,5′-ethylene tether appeared to be too difficult to construct. From our earlier investigations of the 6-formyluridines[23,24] and especially our recent examination of 6-formyl- and 6-(hydroxymethyl)-uridine 5′-carboxaldehydes,[25] we came to expect that the 5′,6-oxomethylene tethered uridine and 2′-deoxyuridine 5′-monophosphates (**1** and **2**, respectively) would be readily accessed and hydrolytically stable.



The synthesis of the 5′,6-oxomethylene-tethered 5′-UMP **1** is shown in Scheme 1. 2′,3′-*O*-(Methoxymethylidene)uridine (**3**),[26] consistently obtained only as a 55:45 mixture of diastereomers,[27] was 5′-*O*-TBDMS-protected to **4** (90%), which was then C6-formylated to **5** in excellent yield via its Miyasaka dianion.[28] Aldehyde **5** was reduced in near quantitative yield to give alcohol **6**, which was then O7-protected with an Fmoc group (90%). The choice of this group was critical because it could be removed without producing a spiro-dihydrouridine like the one we obtained when removing (TBAF) a TBDPS

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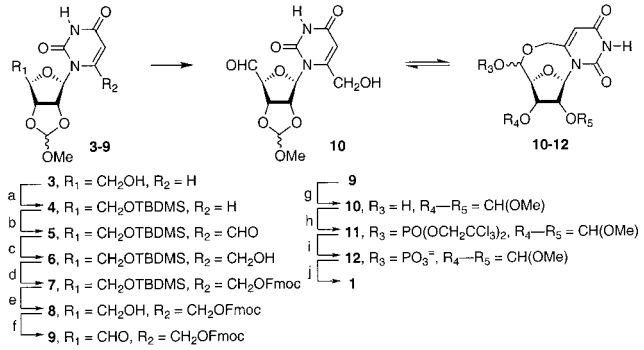
Scheme 1. Synthesis of the Tethered 5′-UMP 1[a]



[a] Conditions: (a) TBDMS−Cl, $C_3H_4N_2$, 45 °C, 16 h (90%). (b) LDA (2.5 equiv), THF, −78 °C, 1.5 h, then HCO_2Me, THF, −78 °C, 30 min (97%). (c) $NaBH_4$, MeOH, 23 °C, 30 min (99%). (d) Fmoc-Cl, C_5H_5N, 23 °C, 16 h (90%). (e) NH_4F (5 equiv), MeOH, 65 °C, 1 h (57%). (f) Dess−Martin periodinane, CH_2Cl_2, 23 °C, 2 h (88%). (g) NH_3, EtOH, 4 °C, 16 h (83%). (h) BuLi (2 equiv), THF, −78 °C, 5 min, then $(Cl_3CCH_2O)_2P(O)Cl$, −78 °C, 15 min (15−20%). (i) Zn(Cu), DMF, 23 °C, 6 h, then Chelex (99%). (j) 10% aq AcOH, 23 °C, 1.5 h, then MeOH, 65 °C, 0.5 h (90%).

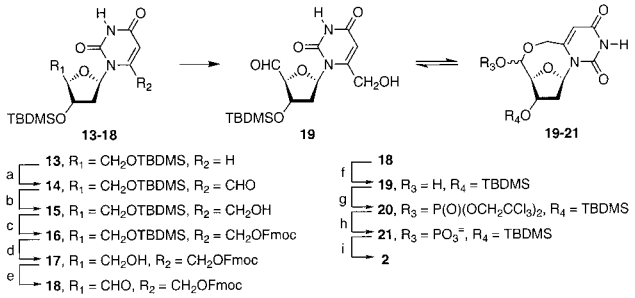
group from a related nucleoside.[29] We examined many options for removing the 5′-*O*-TBDMS group from **7** without affecting the other protecting groups, but only Robins' method (excess NH_4F in refluxing MeOH)[30] worked satisfactorily. Alcohol **8** was isolated in pure condition in a yield (57%) that mainly reflects the difficulty of separating it from the excess NH_4F.

Dess−Martin periodinane oxidation[31] of **8** smoothly afforded aldehyde **9** (88%). Although we were able to remove the Fmoc group of **9** with anhydrous Et_3N/pyridine[32] and follow this immediately by an in situ phosphorylation, this sequence surprisingly captured the "open" hydroxy-aldehyde form of **10**. This product (see Experimental Section) provided useful spectroscopic data for comparison to that of the desired phosphorylated cyclic hemiacetal, constitutional isomer **11**.

The Fmoc group of **9** could be removed using ethanolic NH_3, and the Fmoc-NH_2 byproduct (identified by MS) was removed by rapid filtration through SiO_2 to give pure **10** in 83% yield. As was expected on the basis of its carbohydrate-like structure, **10** exists exclusively in a cyclic hemiacetal form in aprotic solvents. As a 1:1 mixture of epimeric hemiacetals, the mixture of four diastereomers in **10** gave a complex ^{1}H NMR spectrum. Countering this difficulty was the advantage that **10** was readily desiccated to a nonhygroscopic, shelf-stable powder, ideal for preparing and storing this key intermediate in large quantities.

Phosphorylation of the cyclic hemiacetal form of **10** was successful when Shiba's methodology was employed.[33] This approach involves the generation of a hemiacetal anion with BuLi at −78 °C followed by the rapid introduction of a dialkylphosphorochloridate.[34] For our purposes, we selected Scheit's bis-(2,2,2-trichloroethyl) phosphate protection strategy[35,36] because it permitted the removal of the phosphate protecting groups under nonhydrolytic conditions. The low yield of **11** (15−20%) from Shiba phosphorylation of **10** with the appropriate reagent is likely due solely to the poor solubility and, hence, sluggish reactivity of the dianion.[37] Conditions that give a higher yield of phosphorylated product are being sought. The ^{1}H NMR spectrum of the phosphorylated hemiacetal **11** revealed it to be a mixture of four diastereomers. Particularly diagnostic was the downfield-shifted (ca. 6.6 ppm) NMR signal for the anomeric H1′ located in the range (6.6−6.8 ppm) of the diastereomeric 5′,6-oxomethylene-tethered 2′,3′,5′-tri-*O*-acetyluridine we reported recently.[25] The stable protected tethered nucleotide **11** was easily purified by chromatography.

Scheme 2. Synthesis of the Tethered 5′-dUMP 2[a]



[a] Conditions: (a) LDA (5 equiv), THF, −78 °C, 3.5 h, then HCO_2Me, THF, −78 °C, 5 h (45%). (b) $NaBH_4$, MeOH, 23 °C, 30 min (99%). (c) Fmoc-Cl, C_5H_5N, 23 °C, 16 h (62%). (d) catalytic PPTS, EtOH, 23 °C, 16 h (77%). (e) Dess−Martin periodinane, CH_2Cl_2, 23 °C, 2 h (98%). (f) NH_3, EtOH, 4 °C, 12 h (86%). (g) BuLi (2 equiv), THF, −78 °C, 5 min, then $(Cl_3CCH_2O)_2P(O)Cl$, −78 °C, 15 min (15−20%). (h) Zn(Cu), DMF, 23 °C, 6 h, then Chelex (99%). (i) 10% aq AcOH, 23 °C, 16 h (90%).

The synthesis (Scheme 2) that culminated in the 5′,6-oxomethylene-tethered 5′-dUMP (**2**) was similar, but with other noteworthy features. Others have observed no dianion formation when **13** is treated with LDA,[39] but we successfully generated its Miyasaka 3,6-diyl dilithium species with 5 equiv of LDA. Treatment with HCO_2Me gave the desired 6-carboxaldehyde **14** in 45% yield. The structure of **14** was established by ^{1}H and ^{13}C NMR and MS and also by using it to prepare target **2**.

The reduction of **14** to **15** (99%) and the Fmoc protection of **15** to **16** (62%) each proceeded without incident. Because of the greater lability of a TBDMS group at a primary alcohol position, we could regioselectively O5′-

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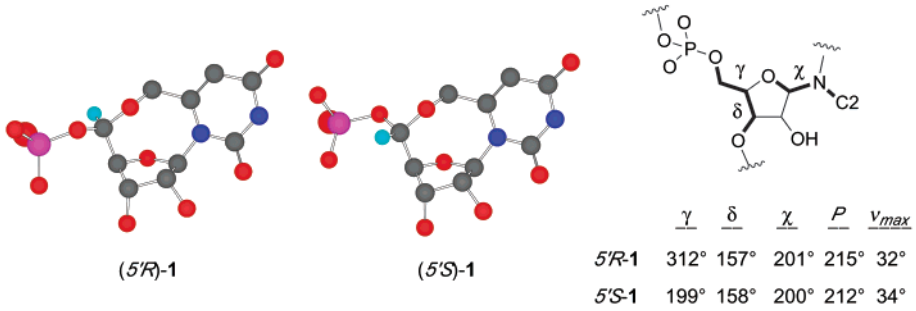

Figure 1. MM conformational analysis of 5′-(*R*)-**1** and 5′-(*S*)-**1**. Definitions: γ = O5′−C5′−C4′−C3, δ = C5′−C4′−C3′−O3′, χ = O4′−C1′−N1−C2, P = arctan[0.3249(ν4 + ν1 − ν3 − ν0)ν2 − 1], ν_{max} = ν2(cos$P$) − 1, ν0 = C4′−O4′−C1′−C2, ν1 = O4′−C1′−C2′−C3, ν2 = C1′−C2′−C3′−C4′, ν3 = C2′−C3′−C4′−O4′, ν4 = C3′−C4′−O4′−C1′. Where ν2 is negative, 180 is added to P.

desilylate **16** in EtOH containing catalytic PPTS (77%). Dess−Martin periodinane oxidation of the resulting **17** (98%) followed by removal of the Fmoc group in aldehyde **18** (86%) then gave **19**. Like **10**, key intermediate **19** exists exclusively in a cyclic hemiacetal form in anhydrous aprotic solvents. It was desiccated to a nonhygroscopic, shelf-stable powder and then recrystallized from absolute EtOH. Shiba phosphorylation of **17** did proceed, but in a low yield, similar to that with **10**. The protected phosphate **20** was deblocked to **21** (99%), and the 3′-*O*-TBDMS group from **21** was then removed by hydrolysis under conditions (10% aqueous AcOH, 23 °C, 16 h) that clearly show a neighboring group participation by the phosphate.[40] The 5′,6-oxomethylene-tethered 5′-dUMP target **2** was obtained as a 5:1 mixture of diastereomers. Importantly, it was found to be just as hydrolytically stable as **1**.

Spatial Presentation. Even before the diastereomers of **1** and **2** are examined in great structural detail by X-ray crystallographic or high-field two-dimensional NMR spectral methods, their spatial presentation can be assessed in a reasonably accurate manner. This information will be valuable to enzymologists or other biochemists who would use these transglycosidically oxomethylene-tethered 5′-nucleotides as probes of protein structure/function relationships. The disposition of the 5′-6-oxomethylene tether in the crystalline intramolecular cyclic hemiacetal form of 1-β-D-ribofuranosyluracil-6-carboxaldehyde as revealed by X-ray crystallography[24] was used as a starting point for MM energy minimizations of 5′-(*R*)- and 5′-(*S*)-**1** performed to reveal the topographical aspects of the lowest steric energy forms. Parameters most useful in comparing these structures to untethered naturally occurring 5′-nucleotide counterparts are shown in Figure 1 for these diastereomers of **1**. Other calculations revealed that oxomethylene-tethered purine and pyrimidine nucleotides are quite similar to each other, as are the ribofuranosyl and 2′-deoxyribofuranosyl variants.

As expected, the native O5′−C5′−C4′−C3′ (γ) torsion angle is the major difference between 5′-(*R*)- and 5′-(*S*)-**1**. Near-classical staggered conformations of rotation about the C5′−C4′ bond were evident. The γ value of 312° for 5′-(*R*)-**1** is that of an −*sc* (−*gauche*) form. For comparison, −*sc* (−*gauche*) rotamers in the Nucleic Acids Database (NDB) have an average γ value of 286°. The γ value of 199° for 5′-(*S*)-**1** is that of an *ap* (trans) form. For comparison, *ap* rotameric nucleotides in the NDB have an average γ value of 178°. With these C5′−C4′ bond rotational restrictions, the oxomethylene-tethered 5′-nucleotides cannot achieve the +*sc* (+*gauche*) forms found in A-DNA (γ = 49−66°), B-DNA (γ = 33−57°), or A-RNA (γ = 0−46°). One-half of the units in Z-DNA (γ = 57−93°) cannot be replicated, but the other half (γ = 159−197°) could be reasonably approximated by a 5′-(*S*) oxomethylene-tethered unit. While their utility in the study of natural DNA and RNA features appears to be limited, the oxomethylene-tethered 5′-nucleotides could very well find some utility in replicating such local DNA/RNA topological features as "hairpin" turns, bulges, and "flipped" residues.

The δ values of 5′-(*R*)- and 5′-(*S*)-**1** close to 160° are common ones for natural 5′-nucleotides, and their glycosyl torsion angles (χ) near 200° are clearly in the anti range, defined to be between 170 and 280°. This is typical of the majority of nucleosides and nucleotides, both pyrimidine and purine. The flexibility of their tether should enable the oxomethylene-tethered 5′-nucleotides to "sweep out" a range of χ values perhaps as large as 30−50°. The pseudorotation (P) values of 212 and 216° place them in the $^4{}_3T$ (C-4′-*endo*/C-3′-*exo* twist) section of the continuum of ring-puckered states.[41] Importantly, though, transglycosidically oxomethylene-tethered 5′-nucleotides should display an equilibrium of ^3E (C-3′-*endo*) and ^2E (C-2′-*endo*) conformations. The puckering amplitude (ν_{max}) values are somewhat smaller than the average value of 36−37° typically found for nucleosides and nucleotides, indicating that **1** and **2** have a slightly more flat furan ring than is common.[18,41]

Conclusions

In summary, we have developed a general synthetic methodology for new highly biomimetic 5′,6-oxomethylene-tethered 5′-nucleotides that can be used to probe the requirements that biomacromolecules have for binding their 5′-nucleotide ligands. Key features of this new motif include a low structural and electronic disturbance caused by the tether, a preservation of all native hydrogen bond donating and accepting recognition sites, and a conformational restriction about the glycosyl linkage that enforces the most commonly found bioactive anti form. The fact that diastereomers are generated by the synthetic attachment of a tether to the normally prochiral C5′ locus is valuable, since it makes it highly likely that, once separated, the 5′-epimers will become a useful pair

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of biochemical probes used in parallel. The synthesis of these oxomethylene-tethered 5′-nucleotides is somewhat long (10−11 steps), but most of the steps give high yields and do not involve overly complicated reaction conditions or manipulations. The analogous 5′,8-oxomethylene-tethered purine 5′-nucleotides should be accessible along similar routes from suitable 8-substituted purine nucleoside precursors,[42] and work in this direction is already under way.

Experimental Section

Materials and General Methods. The Dess−Martin periodinane reagent obtained from Omega, Inc., of Québec, Canada, was kept under argon at <0 °C and allowed to warm to 23 °C just prior to use. 9-Fluorenylmethyl chloroformate was from Avocado, and *tert*-butyldimethylchlorosilane was from Spectrum. LDA (2.0 M) in heptane/THF/C_6H_5Et and 1.6 M BuLi in hexanes were from Aldrich, as were the uridine, 2′-deoxyuridine, bis(2,2,2-trichloroethyl)phosphorochloridate, $NaBH_4$, and NH_4F. Anhydrous DMF, THF, CH_2Cl_2, C_5H_5N, and CH_3OH were also from Aldrich. These were used as received. The CD_3OD was from Cambridge Isotope Laboratories. Chelex 100, 100−200 mesh, Na^+ form analytical-grade chelating resin from BioRad was washed first with 0.1 N NH_4HCO_3 and then with water immediately prior to use. Aldrich 98% $Cu(OAc)_2$ and activated[43] Eastman practical-grade Zn dust were used to prepare the Zn(Cu) couple with a 16% Cu content according to a published procedure.[38] The Zn(Cu) couple was stored at 23 °C under anhydrous Et_2O under argon.

Thin-layer chromatography (TLC) was performed on Analtech silica gel GF Uniplates. Radial preparative-layer chromatography was performed on a Chromatotron instrument (Harrison Research, Inc., Palo Alto, CA) using Adsorbosil-Plus P_{254} from Alltech Associates. This was mixed with $CaSO_4$ binder (VWR) and zinc silicate phosphor (Sigma) before using it to prepare the plates. Column chromatography was performed using Merck silica gel-60 (200−430 ASTM), and ascending preparative-layer chromatography was performed on 2 mm silica gel-GF plates from Analtech. Visualizations employed short-wave (254 nm) UV light. Nucleosides **3**−**11** and **13**−**20** (i.e., those up to the penultimate compounds **12** and **21**) were all obtained as chromatographically homogeneous foam/solids by chromatography on SiO_2. Most of these clung tenaciously to the organic solvents that were used as eluents (e.g., CH_2Cl_2, MeOH, EtOAc), even after drying at 23 °C in vacuo for up to 48 h, by ^{1}H NMR. Some of the elemental combustion analytical data reflect this trace solvent content.

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, using internal tetramethylsilane or external 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS) (δ = 0.0 for ^{1}H), $CHCl_3$ (δ = 77.0 for ^{13}C), or $(CD_3)_2SO$ (δ = 39.5 for ^{13}C) as a reference. The intermediates on the route to the 5′,6-oxomethylene-tethered 5′-UMP target **1** were diastereomeric 2′,3′-O-methoxymethylidenated nucleosides with complex ^{1}H NMR spectra. Therefore, all intermediates on both routes were additionally characterized by low-resolution desorption chemical ionization mass spectrometry (DCI MS). These spectra (data summarized in Tables 1 and 2 in Supporting Information) were recorded using ammonia as the chemical reagent gas. For DCI MS analysis of **1**, **2**, **12**, and **21**, derivatization using BSTFA containing 1% TMSCl (Regis Chemical Co.) and pyridine was employed. Fourier transform high-resolution mass spectra were recorded by matrix-assisted laser desorption/ionization (MALDI HRMS) at The Scripps Research Institute Center for Mass Spectrometry, La Jolla, CA. Elemental microanalyses were obtained from Atlantic Microlab, Inc., Atlanta, GA. Molecular mechanics energy minimizations were performed using CambridgeSoft's Chem 3D Pro version 5.0.

2′,3′-*O*-(Methoxymethylidene)uridine (3). A suspension of uridine (2.685 g, 11 mmol) in 7 mL of $(MeO)_3CH$ was treated with $pTsOH\cdot H_2O$ (80 mg, catalytic) and the mixture stirred at 23 °C for 1.75 h, by which time complete dissolution had occurred. TLC (5% MeOH/CH_2Cl_2) revealed that most of the uridine had been consumed and that **3** was present together with a higher R_f value byproduct (estimated 25%) subsequently identified as an O5′-dimethoxymethylated nucleoside by ^{1}H NMR. The reaction mixture was quenched by the addition of 2 drops of concentrated NH_4OH, and it was then diluted to 50 mL with CH_2Cl_2 and filtered through a pad of Celite in a Büchner funnel. The Celite pad was washed with 5% MeOH/CH_2Cl_2, and the filtrate and washing were combined and rotary evaporated to a colorless foam/gum. Purification by radial chromatography using 7% MeOH/CH_2Cl_2 as the eluent gave 1.87 g (60%) of **3** as a white powder/foam: ^{1}H NMR ($CDCl_3$) δ 9.4 (bs, 1), 7.41 (d, J = 8.2 Hz, 1), 7.36 (d, J = 8.2 Hz, 1), 5.99 (s, 1), 5.93 (s, 1), 5.76 (s, 1), 5.74 (s, 1), 5.70 (d, J = 3.0 Hz, 1), 5.59 (d, J = 3.0 Hz, 1), 5.15−5.02 (m, 4), 4.42 (pseudo-q, 1), 4.26 (pseudo-q, 1), 3.94 (d, J = 12.4 Hz, 1), 3.83 (d, J = 12.4 Hz, 1), 3.40 (s, 3), 3.33 (s, 3). An ^{1}H NMR spectrum of **3** recorded in $(CD_3)_2SO$ solution matched the data given in the first report of this compound.[26] It was consistently obtained as a 55:45 mixture of diastereomers rather than as a single diastereomer.[27]

2′,3′-*O*-(Methoxymethylidene)-5′-*O*-(*tert*-butyldimethylsilyl)uridine (4). The 1.87 g (6.54 mmol) of **3** from above was rendered MeOH-free by repetitive azeotrope with toluene, dissolved in 6.5 mL of anhydrous DMF, and treated with 1.08 g (7.1 mmol) of TBDMS-Cl and 556 mg (8.17 mmol) of imidazole. The reaction mixture was kept at 45 °C for 16 h, diluted with EtOAc, and extracted eight times with water to remove the DMF. The organic phase was dried (Na_2SO_4) and rotary evaporated to a gum, which was purified by filtering a concentrated CH_2Cl_2 solution of it through a pad of SiO_2 in a sintered glass funnel. Elution first with pure CH_2Cl_2 as the eluent removed traces of the silylating reagent, and then 10% MeOH/CH_2Cl_2 was used to remove the product. Rotary evaporation gave, after drying in vacuo at 23 °C, 2.35 g (90%) of **4** as a colorless oil/gum: ^{1}H NMR ($CDCl_3$) δ 8.2 (bs, 1), 7.65 (d, J = 8.2 Hz, 1), 7.58 (d, J = 8.2 Hz, 1), 6.09 (d, J = 3.0 Hz, 1), 5.99 (s, 1), 5.94 (d, J = 3.0 Hz, 1), 5.92 (s, 1), 5.61 (s, 1), 5.58 (s, 1), 4.92−4.64 (m, 4), 4.45 (pseudo-q, 1), 4.31 (pseudo-q, 1), 3.93 (d, J = 12.4 Hz, 1), 3.83 (d, J = 12.4 Hz, 1), 3.43 (s, 3), 3.35 (s, 3), 0.91 (s, 9), 0.09 (s, 6). Anal. Calcd for $C_{17}H_{28}N_2O_7Si$: C, 50.98; H, 7.05; N, 6.99. Found: C, 50.66; H, 6.95; N, 6.98 and C, 50.65; H, 6.88; N, 6.93.

2′,3′-*O*-(Methoxymethylidene)-5′-*O*-(*tert*-butyldimethylsilyl)uridine-6-carboxaldehyde (5). A sample of **4** was readied by eluting an EtOAc solution of it through a short pad of SiO_2, evaporating the eluate to dryness, and then coevaporating the residue several times with toluene and keeping the initially gummy white solid under vacuum at 23 °C overnight. A solution of this now white powder (4.4 g, 11 mmol) in 100 mL of anhydrous THF under argon was cooled to −78 °C and then transferred dropwise via cannula under positive argon pressure to a −78 °C solution of 13.7 mL (27.5 mmol, 2.5 equiv) of 2 M LDA in 86 mL of THF under argon. The dianion mixture was stirred for 1.5 h at −78 °C, and then HCO_2Me (1.7 mL, 1.65 g, 27.5 mmol, 2.5 equiv) was introduced dropwise. Stirring was continued for 30 min at −78 °C, and then AcOH (1.57 mL, 1.65 g, 27.5 mmol, 2.5 equiv) was added followed immediately by 30 mL of 1 M pH 7 phosphate buffer. The mixture was allowed to warm to 23 °C, and then most of the THF was removed by rotary evaporation at 23 °C. EtOAc (400 mL) and water (50 mL) were added, and the layers were separated; the organic phase was washed twice with water (50 mL) and then dried (Na_2SO_4). TLC (5% MeOH/CH_2Cl_2) revealed essentially pure 2,4-DNP-positive **5** (R_f 0.29) together with only a very slight trace of **4** (R_f 0.42). Rotary evaporation gave **5** as a yellow foam, and this was purified by applying a concentrated CH_2Cl_2 solution of it directly to a short pad of SiO_2 (ca. 30 g) in a Büchner funnel and first eluting with pure CH_2Cl_2 and then

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with 10% MeOH/CH_2Cl_2. Rotary evaporation followed by coevaporation with toluene gave, after drying in vacuo at 23 °C overnight, 4.57 g (97%) of pure **5** as a yellow gum. ^{1}H NMR ($CDCl_3$) δ 9.65 (s, 1), 9.63 (s, 1), 8.21 (s, 1), 6.58 (s, 1), 6.47 (s, 1), 6.23 (two s, both 1), 6.00 (d, J = 3.0 Hz, 1), 6.94 (d, J = 3.0 Hz, 1), 5.19 (m, 1), 5.01 (m, 1), 4.89 (m, 1), 4.33 (pseudo-q, 1), 4.11 (pseudo-q, 1), 4.20 (d, J = 12.4 Hz, 1), 3.41 (d, J = 12.4 Hz, 1), 3.42 (s, 3), 3.33 (s, 3), 0.91 (s, 9), 0.09 (s, 6). Anal. Calcd for $C_{18}H_{28}N_2O_8Si$: C, 50.45; H, 6.59; N, 6.54. Found: C, 52.25; H, 7.35; N, 7.40 and C, 52.14; H, 7.27; N, 7.38.

6-Hydroxymethyl-2′,3′-*O*-(methoxymethylidene)-5′-*O*-(*tert*-butyldimethylsilyl)uridine (6). A solution of 3.965 g (9.25 mmol) of **5** in 275 mL of MeOH was treated portionwise with 770 mg (20 mmol, 2.2 equiv) of $NaBH_4$, and the reaction mixture was stirred for 1 h at 23 °C. A 5.35 g sample of NH_4-Cl was added, and after it had dissolved, the reaction mixture was evaporated to near dryness at 35 °C. The residue was treated with 250 mL of EtOAc and 20 mL of water; the layers were separated, and the aqueous phase was washed with an additional 20 mL of EtOAc. The combined organic phases were dried (Na_2SO_4) and rotary evaporated to give 3.94 g (99%) of **6** as a yellow powder/foam: ^{1}H NMR ($CDCl_3$) δ 5.96 (s, 1), 5.92 (d, J = 3.0 Hz, 1), 5.89 (s, 2), 5.82 (s, 1), 5.80 (d, J = 3.0 Hz, 1), 5.38 (m, 2), 5.00 (m, 1), 4.88 (m, 1), 4.52 (s, 2), 4.32 (pseudo-q, 1), 4.10 (pseudo-q, 1), 3.84 (m, 2), 3.39 (s, 3), 3.31 (s, 3), 0.90 (s, 9), 0.08 (s, 6). Anal. Calcd for $C_{18}H_{30}N_2O_8Si$: C, 50.22; H, 7.02; N, 6.51. Found: C, 52.20; H, 7.49; N, 7.32 and C, 52.08; H, 7.61; N, 7.32.

6-[(9-Fluorenylmethyloxycarbonyl)oxymethyl]-2′,3′-*O*-(methoxymethylidene)-5′-*O*-(*tert*-butyldimethylsilyl)uridine (7). A solution of 3.38 g (7.5 mmol) of **6** in 30 mL of anhydrous C_5H_5N under argon was treated with 2.6 g (10 mmol) of 9-fluorenylmethyl chloroformate and the reaction mixture kept at 23 °C overnight. The bulk of the C_5H_5N was removed by rotary evaporation in vacuo at <35 °C, and the residue was twice treated with 50 mL portions of toluene and rotary evaporated. The residue was then dissolved in 300 mL of CH_2Cl_2, and the organic solution was extracted five times with 50 mL portions of water and then dried (Na_2SO_4). The solution was concentrated to about 30 mL by rotary evaporation, and then it was applied to a short pad of SiO_2 that had been preequilibrated with CH_2Cl_2 in a sintered glass funnel. Elution with CH_2Cl_2 removed the excess Fmoc reagent, and then elution with 5% MeOH/CH_2Cl_2 gave 4.37 g (90%) of **7** as a yellow foam/powder: ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 7.7 Hz, 2), 7.60 (d, J = 7.7 Hz, 2), 7.39 (pseudo-t, 2), 7.31 (pseudo-t, 2), 5.98 (s, 1), 5.86 (s, 1), 5.83 (s, 2), 5.77 (s, 1), 5.62 (s, 1), 5.31 (t, J = 5 Hz, 1), 5.05 (m, 2), 4.89 (m, 1), 4.46 (d, J = 5 Hz, 2), 4.32 (pseudo-q, 1), 4.25 (pseudo-q, 1), 3.82 (m, 2), 3.31 (s, 3), 3.29 (s, 3), 0.90 (s, 9), 0.06 (s, 6). Anal. Calcd for $C_{33}H_{40}N_2O_{10}$-Si: C, 60.72; H, 6.18; N, 4.29. Found: C, 59.84; H, 6.71; N, 5.38 and C, 59.93; H, 6.62; N, 5.34.

6-[(9-Fluorenylmethyloxycarbonyl)oxymethyl]-2′,3′-*O*-(methoxymethylidene)uridine (8). A solution of 2.47 g (3.8 mmol) of **7** in 100 mL of MeOH was treated with 2.5 g (67 mmol) of NH_4F and then heated at reflux for 1 h. TLC (5% MeOH/CH_2Cl_2) indicated that **7** had been consumed, so the reaction mixture was allowed to cool to 23 °C and then rotary evaporated onto 35 g of SiO_2 at <23 °C. This was pumped dry in vacuo and then placed on top of 35 g of SiO_2 in a sintered glass funnel. The SiO_2 was eluted first with pure CH_2Cl_2 to remove the trace of an Fmoc-related compound that had formed and then with 5% MeOH/CH_2Cl_2 to give, after drying in vacuo overnight, 1.17 g (57%) of **8** as a pale orange solid/foam: ^{1}H NMR ($CDCl_3$) δ 7.77 (d, J = 7.7 Hz, 2), 7.61 (d, J = 7.7 Hz, 2), 7.42 (pseudo-t, 2), 7.32 (pseudo-t, 2), 5.99 (s, 1), 5.90 (s, 1), 5.84 (s, 1), 5.72 (d, J = 3.0 Hz, 1), 5.67 (d, J = 3.0 Hz, 1), 5.55 (d, J = 5.0 Hz, 1), 5.34 (m, 1), 5.05 (s, 2), 4.48 (m, 2), 4.38 (pseudo-q, 1), 4.27 (t, J = 5.0 Hz, 1), 4.18 (pseudo-q, 1), 3.90 (d, J = 12 Hz, 1), 3.82 (d, J = 12 Hz, 1), 3.30 (s, 3), 3.29 (s, 3). Anal. Calcd for $C_{27}H_{26}N_2O_{10}$: C, 60.22; H, 4.87; N, 5.20. Found: C, 61.16; H, 5.29; N, 5.37 and C, 61.31; H, 5.35; N, 5.35.

6-[(9-Fluorenylmethyloxycarbonyl)oxymethyl]-2′,3′-*O*-(methoxymethylidene)uridine 5′-Carboxaldehyde (9). A 1.44 g (3.4 mmol) sample of the Dess−Martin periodinane was added to 12.5 mL of anhydrous CH_2Cl_2 under argon. After stirring for 30 min, the mixture was first treated with 167 μL (2.1 mmol) of anhydrous pyridine and then treated dropwise with a solution of 1.17 g (2.17 mmol) of **8** in 12.5 mL of anhydrous CH_2Cl_2. Stirring was continued for 2 h at 23 °C, at which time TLC (5% MeOH/CH_2Cl_2) revealed that **9**, identical in R_f value to **8** but 2,4-DNP-positive, had formed. The mixture was diluted to 125 mL with CH_2Cl_2 and treated with 60 mL of saturated aqueous $NaHCO_3$ and 12.5 mL of saturated aqueous $Na_2S_2O_3$. After the mixture was stirred rapidly for 30 min, the layers were separated and the aqueous layer was extracted twice with CH_2Cl_2 (25 mL). The combined organic phases were back-extracted with water and then dried (Na_2-SO_4). Rotary evaporation gave 1.04 g (89%) of **9** as a pale yellow foam/powder: ^{1}H NMR ($CDCl_3$) δ 9.42 (s, 1), 9.40 (s, 1), 7.77 (d, J = 7.7 Hz, 2), 7.61 (d, J = 7.7 Hz, 2), 7.42 (pseudo-t, 2), 7.32 (pseudo-t, 2), 5.91 (s, 1), 5.90 (s, 1), 5.86 (s, 1), 5.84 (s, 1), 5.82 (s, 1), 5.75 (m, 2), 5.35 (m, 1), 5.10 (m, 1), 5.05 (m, 1), 4.48 (m, 2), 4.34 (pseudo-q, 1), 4.26 (m, 2), 3.31 (s, 3), 3.28 (s, 3). Anal. Calcd for $C_{27}H_{24}N_2O_{10}$: C, 60.45; H, 4.51; N, 5.22. Found: C, 59.57; H, 4.88; N, 5.44 and C, 59.50; H, 4.82; N, 5.39.

6-(Hydroxymethyl)-2′,3′-*O*-(methoxymethylidene)uridine 5′-Carboxaldehyde (10). A solution of 420 mg (0.78 mmol) of **9** in 40 mL of absolute EtOH was cooled to 0 °C, and then anhydrous NH_3 was bubbled in until an excess had clearly been delivered. The solution was kept at 4 °C overnight and then was allowed to warm to 23 °C for 1.5 h. Rotary evaporation at 23 °C gave a residue that was treated with 25 mL of toluene and rotary evaporated again to dryness at 23 °C. The residue (357.5 mg after it was placed in vacuo at 23 °C) was triturated three times with 25 mL portions of anhydrous Et_2O to remove the Fmoc-NH_2 byproduct. This solid remaining was placed in vacuo next to P_2O_5 over several days, giving 203 mg (83%) of **10a** as a white nonhygroscopic powder. A 1.0 g sample of **9** treated similarly but purified by radial chromatography (10 then 12.5% MeOH/CH_2Cl_2 as eluents) after Et_2O trituration gave 447 mg (76%) of **10**, which exists as a mixture of diastereomeric cyclic hemiacetals in anhydrous aprotic solvents, but as a mixture of diastereomeric methyl hemiacetals in MeOH: ^{1}H NMR (CD_3OD) δ 5.99 (d, J = 4 Hz), 5.93 (s, 1), 5.89 (d, J = 4 Hz, 1), 5.82 (s, 1), 5.28 (m, 1), 5.00 (m, 1), 4.65 (m, 1), 4.52 (d, J = 12 Hz, 1), 4.22 (d, J = 12 Hz, 1), 3.42 (s, 3), 3.28 (s, 3); ^{13}C NMR (CD_3OD) δ 166, 157, 152, 120, 119, 102, 99, 97, 94, 92, 86, 85, 83, 64, 61, 55, 52, 51, 18, 15; MALDI HRMS calcd for $C_{12}H_{15}N_2O_8$ $(MH)^+$ 315.0823, found 315.0823. Anal. Calcd for $C_{12}H_{14}N_2O_8$: C, 45.86; H, 4.49; N, 8.91. Found: C, 46.46; H, 5.02; N, 8.29 and C, 46.42; H, 5.01; N, 8.35.

6-[Di-(2,2,2-trichloroethyl)phosphoryloxymethyl]-2′,3′-*O*-(methoxymethylidene)uridine 5′-Carboxaldehyde. This untethered constitutional isomer of **11** was obtained when we removed the Fmoc group of **10** under strictly anhydrous, aprotic conditions[32] and then immediately phosphorylated the nucleoside produced. It gave valuable ^{1}H NMR and mass spectral data for comparison to those of the desired tethered isomer **11**. A solution of 268 mg (0.5 mmol) of **10** in 2.5 mL of anhydrous pyridine under argon was treated with 70 μL (51 mg, 0.5 mmol) of CaH_2-dried Et_3N, and the reaction mixture was stirred at 23 °C for 1 h. It was cooled to 4 °C in an ice bath and then treated with 284 mg (0.75 mmol) of bis(2,2,2-trichloroethyl)phosphorochloridate. The reaction mixture was kept at 0 °C for 20 h, and then it was diluted with 25 mL of CH_2Cl_2 and vigorously stirred with 10 mL of water for 2 h. The layers were separated, and the organic phase was extracted four times with water (10 mL) and dried (Na_2SO_4). Rotary evaporation gave a residue that was twice coevaporated with 50 mL of toluene to remove residual pyridine. The residue was pumped free of volatiles at 23 °C for 1 h, and then it was dissolved in ca. 3 mL of CH_2Cl_2. This solution was added to 5 g of SiO_2 in a sintered glass funnel, and this was then eluted first with pure CH_2Cl_2 to remove dibenzofulvene and then with 5% MeOH/CH_2Cl_2 to give a mixture of nucleoside products. Rotary evaporation gave 170 mg of a pale yellow foam that

was pumped dry in vacuo at 23 °C overnight. TLC (5% MeOH/CH_2Cl_2) revealed three components, and separation by radial chromatography (2.5% MeOH/CH_2Cl_2 as the eluent) followed by ascending preparative chromatography (4% MeOH/CH_2Cl_2) gave 36 mg of 6-[di(2,2,2-trichloroethyl)phosphoryloxymethyl]-2′,3′-*O*-(methoxymethylidene)uridine 5′-carboxaldehyde, characterized by ^{1}H NMR and DCI MS. Similar treatment of a 72 mg sample of **10** gave, after radial chromatographic separation, 30 mg of this same nucleoside: ^{1}H NMR ($CDCl_3$) δ 9.43 (s, 1), 9.41 (s, 1), 6.00−5.82 (m, 2), 5.30 (m, 1), 5.15 (m, 1), 4.72 (m, 2), 3.49 (s, 3), 3.39 (s, 3). The DCI mass spectrum (data provided in Table 1 in Supporting Information) revealed a diagnostic pair of peaks for the [B + H^+] and [B + NH_4^+] species at 483 and 500 amu, respectively, consistent with the untethered nature of this undesired constitutional isomer. Base peaks like these were absent, as anticipated, in the DCI mass spectrum of the desired, tethered regioisomer **11** as well as in those of **12** and **1** (derived from **11**) and in those of their counterparts **21** and **2** in the tethered 5′-dUMP synthesis.

5′,6-Oxomethylene-2′,3′-*O*-(methoxymethylidene)uridine 5′-Monophosphate, Di(2,2,2-trichloroethyl) Ester (11). The Shiba procedure[33] was employed: a solution of 72 mg (0.225 mmol) of rigorously dried **10** in 2 mL of anhydrous THF under argon was cooled to −78 °C and treated dropwise with 312 μL of 1.6 M BuLi in hexanes (0.5 mmol, 2.2 equiv). The orange suspension of dianion was stirred at −78 °C for 5 min, and then 128 mg (0.338 mmol, 1.5 equiv) of bis(2,2,2-trichloroethyl)phosphorochloridate was added all at once. The reaction mixture was stirred for 15 min at −78 °C, during which time the suspension became less yellow and cloudy. The cooling bath was removed, and the reaction was quenched by the addition of a mixture of 11 mL of EtOAc and 5.5 mL of saturated aqueous $NaHCO_3$. The mixture was stirred rapidly; then, the layers were separated, and the organic phase was washed with saturated aqueous NaCl and dried (Na_2SO_4). TLC (5% MeOH/CH_2Cl_2) indicated a trace of **10** (R_f 0.22) together with four higher R_f value components, one of them major (**11**, R_f 0.57). Separation by radial chromatography (5% MeOH/CH_2Cl_2 as the eluent) gave 23 mg (15%) of **11** as a pale yellow oil/foam. Similar treatment of a 63 mg sample of **10** gave, again after radial chromatographic separation, 26 mg (20%) of **11**: ^{1}H NMR ($CDCl_3$) δ 9.27 (bs, 1), 9.04 (bs, 1), 6.77 (s, 1), 6.67 (s, 1), 6.52 (s, 1), 6.43 (s, 1), 5.99 (s, 1), 5.90 (s, 1), 5.58 (m, 2), 5.09 (m, 1), 4.87 (m, 1), 4.71 (m, 2), 4.45 (m, 1), 3.44 (s, 3), 3.38 (s, 3), 3.33 (s, 3), 3.30 (s, 3).

5′,6-Oxomethylene-2′,3′-*O*-(methoxymethylidene)uridine 5′-Monophosphate (12). A slight modification of the Imai−Torrence refinement[38] of the Eckstein−Scheit procedure[35] was employed: a solution of the 19 mg (0.03 mmol) of **11** in 0.6 mL of anhydrous DMF was treated with 30 μL of acetylacetone and 39 mg (0.6 mmol) of Zn(Cu) couple that had been freshly prepared with a 16% Cu content. The mixture was stirred vigorously for 6 h at 23 °C during which time the Zn(Cu) couple progressively disappeared. No green color developed, but TLC (5% MeOH/CH_2Cl_2) showed that the deprotection had proceeded smoothly. The solution was then diluted with 18 mL of MeOH and 9 mL of water; Chelex resin (washed first with 0.1 N NH_4HCO_3 and then with water; 15 mL settled volume) was added, and the mixture was stirred for 1 h. The resin was removed by suction filtration through Celite, and the Celite was washed with a small amount of water. The filtrate and washing were combined and rotary evaporated to dryness in vacuo at <40 °C. The residue was coevaporated from absolute EtOH several times, and then it was dried in vacuo overnight to give 13 mg (99%) of **12** as an oil: ^{1}H NMR (D_2O) δ 6.69 (s, 1), 6.59 (s, 1), 6.54 (s, 1), 6.44 (s, 1), 6.20 (s, 1), 6.09 (s, 1), 5.87 (s, 1), 5.82 (s, 1), 5.69 (m, 1), 5.55 (m, 1), 5.13 (m, 1), 5.07 (m, 1), 4.33 (m, 1), 4.27 (m, 1), 4.21 (m, 1), 3.52 (s, 3), 3.50 (s, 3), 3.44 (s, 3), 3.43 (s, 3). MALDI HRMS calcd for $C_{12}H_{14}N_2O_{11}P$ (M − H)$^-$ 393.0341, found 393.0349.

5′,6-Oxomethyleneuridine 5′-Monophosphate (5′,6-Oxomethylene-Tethered 5′-UMP, 1). The 13 mg (0.03 mmol) sample of **12** from above was dissolved in 0.5 mL of D_2O and its ^{1}H NMR spectrum recorded. Then, CD_3CO_2D was added to a 10% concentration, and the ^{1}H NMR spectrum was recorded every 15 min to monitor the hydrolysis of the methoxymethylidene group. Within 1.5 h, the hydrolysis had proceeded to completion, giving a 2′(3′)-O-formylated form of **1**, by NMR. The solution was diluted with water and rotary evaporated, and the residue was twice coevaporated with toluene to remove water and acetic acid. A solution of this residue in 5 mL of MeOH was heated on a steam bath for 30 min to effect the solvolysis of the formate ester. Rotary evaporation afforded 13.4 mg (estimated 90%) of **1** as a white solid contaminated only by NH_4OAc. By ^{1}H NMR, **1** was obtained as a 1:1 mixture of diastereomers (epimeric phosphorylated hemiacetals): ^{1}H NMR (D_2O) δ 6.54 (d, J = 4.6 Hz, 1), 6.28 (d of d, J = 5, 2.7 Hz, 1), 6.02 (pseudo-t, 1), 5.91 (s, 1), 5.86 (s, 1), 5.75 (m, 1), 5.58 (d, J = 7.7 Hz, 1), 5.49 (m, 1), 5.13 (m, 1), 4.68−4.48 (m, 4), 4.40 (m, 1), 4.30 (m, 1). In D_2O solution at 23 °C, the 5′,6-oxomethylene-tethered 5′-UMP **1** remains intact for at least 2 months, by ^{1}H NMR (see Supporting Information). It also remains intact after adsorption onto activated charcoal for 3 days followed by removal with 50% aqueous EtOH containing 4% NH_4OH, by DCI MS. MALDI HRMS calcd for $C_{10}H_{14}N_2O_{11}P$ (M − H)$^-$ 351.0235, found 351.0228.

3′,5′-Di-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine-6-carboxaldehyde (14). Although 3′,5′-di-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine (**13**) has been reported to resist double deprotonation by LDA,[39] we found that a Miyasaka uridin-3,6-diyl dilithium species of this nucleoside could be generated by exposure to 5 equiv of this base: a solution of 3′,5′-di-*O*-(*tert*-butyldimethylsilyl)uridine (7.17 g, 15.7 mmol, prepared in 80% purified yield from uridine) in 150 mL of anhydrous THF at −78 °C under argon was transferred dropwise over 30 min via positive argon pressure through a cannula into a solution of 39 mL of 2 M LDA (78 mmol, 5 equiv) in 150 mL of anhydrous THF at −78 °C under argon. The reaction mixture was stirred at −78 °C for 3.5 h, and then 4.85 mL (78 mmol, 5 equiv) of HCO_2Me was added dropwise. This was slightly exothermic, and some gas evolution was observed. This was likely C═O generated by LDA deprotonation of the excess HCO_2Me. The mixture was stirred at −78 °C for 5 h, and then it was quenched by the dropwise addition of 4.5 mL (78 mmol, 5 equiv) of AcOH. The cooling bath was removed and the mixture allowed to warm to 23 °C under argon overnight. The volatiles were removed by rotary evaporation at less than 30 °C, and the residue was treated with a mixture of EtOAc (300 mL) and water (100 mL). The layers were separated, and the organic layer was dried (Na_2SO_4). TLC (5% MeOH/CH_2Cl_2) showed the presence of nearly equal amounts of the starting material (R_f 0.51) and the 2,4-DNP-positive **14** (R_f 0.38). Rotary evaporation gave 11 g of the mixture, which was separated by careful column chromatography (5% MeOH/CH_2Cl_2), giving 3.42 g (45%) of **14** as a white foam/solid. The identity of **14** as the 6-formyl regioisomer is supported by its NMR spectral characteristics and by its utility in obtaining the final target: ^{1}H NMR ($CDCl_3$) δ 10.0 (s, 1), 9.4 (bs, 1), 6.37 (t, J = 7.3 Hz, 1), 6.06 (s, 1), 4.48 (m, 1), 3.90 (m, 1), 3.83−3.70 (m, 2), 2.37 (m, 1), 2.28 (m, 1), 0.90 (s, 18), 0.08 (s, 12); ^{13}C NMR ($CDCl_3$) δ 184.3, 162.4, 150.0, 148.6, 106.1, 88.2, 86.2, 71.1, 62.0, 41.4, 25.8, 18.3, 17.9, −4.8, −5.7. Anal. Calcd for $C_{22}H_{40}N_2O_6Si_2$: C, 54.51; H, 8.32; N, 5.78. Found: C, 54.97; H, 8.24; N, 6.50 and C, 55.08; H, 8.36; N, 6.39.

6-Hydroxymethyl-3′,5′-di-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine (15). Use of the procedure for the reduction of **5** to **6** allowed reduction of **14**, affording **15** in 99% yield as an off-white foam/solid: ^{1}H NMR ($CDCl_3$) δ 9.11 (bs, 1), 6.26 (t, J = 7.2 Hz, 1), 5.79 (s, 1), 4.51 (s, 2), 3.90 (m, 1), 3.81 (m, 2), 2.64 (m, 1), 2.17 (m, 1), 0.92 (s, 9), 0.89 (s, 9), 0.09 (s, 6), 0.07 (s, 6). Anal. Calcd for $C_{22}H_{42}N_2O_6Si_2$: C, 54.29; H, 8.70; N, 5.76. Found: C, 55.97; H, 8.99; N, 6.32 and C, 55.85; H, 8.89; N, 6.33.

6-[(9-Fluorenylmethyloxycarbonyl)oxymethyl]-3′,5′-di-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine (16). A solution of 1.79 g (4.18 mmol) of **15** in 15 mL of anhydrous pyridine was treated with 1.24 g (4.78 mmol) of 9-fluorenylmethyl chloroformate, and the reaction mixture was allowed to stand

at 23 °C overnight. Toluene was added and the solution rotary evaporated in vacuo at <35 °C. The residue was coevaporated twice more using 90 mL of toluene; then, it was dissolved in 120 mL of EtOAc, and the solution was extracted three times with water (15 mL) and then dried (Na_2SO_4). Rotary evaporation gave a residue that was purified by applying a concentrated solution of it in 50% CH_2Cl_2/hexanes to a short pad of SiO_2 and eluting first with 50% CH_2Cl_2/hexanes to remove residual Fmoc material and then with pure CH_2Cl_2 to afford 1.84 g (62%) of **16** as a pale yellow foam/solid: ^{1}H NMR ($CDCl_3$) δ 8.13 (bs, 1), 7.78 (d, J = 7.5 Hz, 2), 7.60 (d, J = 7.4 Hz, 2), 7.43 (pseudo-t, 2), 7.33 (pseudo-t, 2), 6.18 (t, J = 7.2 Hz, 1), 5.86 (s, 1), 5.17 (d, J = 15 Hz, 1), 5.05 (d, J = 15 Hz, 1), 4.54−4.46 (m, 3), 4.26 (t, J = 7.5 Hz, 1), 3.90 (m, 1), 3.77 (m, 3), 2.74 (m, 1), 2.17 (m, 1), 0.89 (s, 9), 0.88 (s, 9), 0.08 (s, 6), 0.06 (s, 6). Anal. Calcd for $C_{37}H_{52}N_2O_8Si_2$: C, 62.68; H, 7.39; N, 3.95. Found: C, 62.55; H, 7.44; N, 4.13.

6-[(9-Fluorenylmethyloxycarbonyl)oxymethyl]-3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine (17). A solution of 1.35 g (1.9 mmol) of **16** in 7 mL of absolute EtOH was treated with 108 mg (catalytic) of PPTS, and the reaction mixture was allowed to stand at 23 °C overnight. The mixture was kept at 23 °C for 16 h, after which time TLC (5% MeOH/CH_2Cl_2) showed that most of the **16** had reacted. The mixture was rotary evaporated, and the residue was purified first by column chromatography (5% MeOH/CH_2Cl_2) in a sintered glass funnel and then by radial chromatography (4% MeOH/CH_2Cl_2 as the eluent) to afford 875 mg (77%) of **17** as a pale yellow foam/solid: ^{1}H NMR ($CDCl_3$) δ 8.62 (bs, 1), 7.77 (d, J = 7.5 Hz, 2), 7.59 (d, J = 7.4 Hz, 2), 7.43 (pseudo-t, 2), 7.33 (pseudo-t, 2), 5.87 (s and t, J = 7.2 Hz, each 1), 5.05 (s, 2), 4.67 (s, 1), 4.55−4.44 (m, 3), 4.26 (t, J = 7.5 Hz, 1), 3.95−3.65 (m, 4), 3.29 (m, 1), 2.93 (m, 1), 2.08 (m, 1), 0.88 (s, 9), 0.08 (s, 6). Anal. Calcd for $C_{31}H_{38}N_2O_8Si$: C, 62.61; H, 6.44; N, 4.71. Found: C, 61.80; H, 6.87; N, 4.63.

6-[(9-Fluorenylmethyloxycarbonyl)oxymethyl]-3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine 5′-Carboxaldehyde (18). A 975 mg (2.3 mmol) sample of the Dess−Martin periodinane was added to 8.5 mL of anhydrous CH_2Cl_2 under argon. After stirring for 30 min, the mixture was first was treated dropwise with a solution of 875 mg (1.47 mmol) of **17** in 8.5 mL of anhydrous CH_2Cl_2. Stirring was continued for 2 h at 23 °C. The mixture was diluted to 85 mL with CH_2Cl_2 and treated with 40 mL of saturated aqueous $NaHCO_3$ plus 8.5 mL of saturated aqueous $Na_2S_2O_3$. After the mixture was stirred rapidly for 30 min, the layers were separated and the aqueous layer was extracted twice with CH_2Cl_2 (17 mL). The combined organic phases were back-extracted with water and then dried (Na_2SO_4). Rotary evaporation gave 850 mg (98%) of **18** as a pale yellow foam/solid: ^{1}H NMR ($CDCl_3$) δ 9.59 (s, 1), 8.32 (bs, 1), 7.77 (d, J = 7.5 Hz, 2), 7.60 (d, J = 7.4 Hz, 2), 7.43 (pseudo-t, 2), 7.33 (pseudo-t, 2), 5.86 (s and t, J = 7.2 Hz, each 1), 5.05 (s, 2), 4.67 (s, 1), 4.60−4.45 (m, 3), 4.25 (m, 1), 4.00−3.75 (m, 2), 3.29 (m, 1), 3.05 (m, 1), 2.01 (m, 1), 0.90 (s, 9), 0.12 (s, 6). Anal. Calcd for $C_{31}H_{36}N_2O_8Si$: C, 62.82; H, 6.12; N, 4.73. Found: C, 62.26; H, 6.72; N, 4.53.

6-Hydroxymethyl-3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine 5′-Carboxaldehyde (19). A suspension of 850 mg (1.43 mmol) of **18** in 75 mL of absolute EtOH was cooled to 0 °C, and then anhydrous NH_3 was bubbled in until an excess had clearly been delivered. Most of the **18** dissolved within ca. 20 min, and the solution was kept at 4 °C overnight and then allowed to warm to 23 °C over 1.5 h. Rotary evaporation at 23 °C gave a residue that was purified by column chromatography using first CH_2Cl_2 to remove Fmoc byproduct and then 5 and then 10% MeOH/CH_2Cl_2 as eluents to give 454 mg (86%) of **19** as a white powder. This was coevaporated twice with toluene, pumped dry overnight, triturated with a small amount of anhydrous Et_2O, and recrystallized from a small amount of absolute EtOH: mp 216−219 °C dec. Like **10**, nucleoside **19** was found to exist as an equal mixture of diastereomeric cyclic hemiacetals in anhydrous aprotic solvents: ^{1}H NMR [$(CD_3)_2SO$] δ 11.4 (bs, 1), 7.42 (d, J = 5.1 Hz, 1), 7.01 (d, J = 4.8 Hz, 1), 6.58 (t, J = 7.2 Hz, 1), 6.41 (t, J = 7.2 Hz, 1), 5.64 (s, 1), 5.62 (s, 1), 5.48 (d, J = 15 Hz, 1), 5.00 (d, J = 4.5 Hz, 1), 4.95 (t, J = 5 Hz, 1), 4.85 (d, J = 16 Hz, 1), 4.56 (s, 1), 4.55 (d, J = 16 Hz, 1), 4.52 (s, 1), 4.48 (m, 2), 4.32 (m, 2), 4.06 (s, 1), 3.95 (s, 1), 3.92 (d, J = 15 Hz, 1), 3.43 (m, 2), 2.62 (m, 1), 2.22 (m, 1), 0.87 (s, 9), 0.08 (s, 6). Anal. Calcd for $C_{16}H_{26}N_2O_6Si$: C, 51.87; H, 7.07; N, 7.56. Found: C, 49.26; H, 6.71; N, 7.19 and C, 49.23; H, 6.69; N, 7.25.

6-[Di(2,2,2-Trichloroethyl)phosphoryloxymethyl]-2′-deoxy-3′-*O*-(*tert*-butyldimethylsilyl)uridine 5′-Carboxaldehyde (20). A solution 37 mg (0.1 mmol) of rigorously dried **19** in 1.0 mL of anhydrous THF under argon was cooled to −78 °C and treated dropwise with 134 μL of 1.67 M BuLi in hexanes (0.22 mmol, 2.2 equiv). The very thick orange dianion suspension was stirred with difficulty at −78 °C for 5 min, and then 57 mg (0.15 mmol, 1.5 equiv) of bis(2,2,2-trichloroethyl)phosphorochloridate was added all at once. The reaction mixture was stirred for 15 min at −78 °C, during which time the color lightened to yellow and the suspension became thinner. The cooling bath was removed, and the reaction was quenched immediately by the addition of a mixture of 5.5 mL of EtOAc and 2.75 mL of saturated aqueous $NaHCO_3$. The mixture was stirred rapidly; then, the layers were separated, and the organic phase was washed once with saturated aqueous NaCl and dried (Na_2SO_4). Separation by radial chromatography (5% MeOH/CH_2Cl_2 as the eluent) gave 10 mg of starting **19** and 10 mg (20% on the basis of unrecovered **19**) of a 5:1 mixture of **20** diastereomers as a pale yellow oil/foam: ^{1}H NMR ($CDCl_3$) δ 8.6 (bs, 1), 6.86 (t, J = 7.2 Hz, 1), 6.60 (t, J = 7.2 Hz, 1), 5.70 (m, 1), 5.60−5.50 (m, 2), 5.00 (d, J = 15 Hz, 1), 4.75−4.60 (m, 6), 4.40−4.20 (m, 2), 2.60 (m, 1), 2.30 (m, 1), 0.95 (s, 9), 0.89 (s, 9), 0.09 (s, 6), 0.08 (s, 6).

5′,6-Oxomethylene-2′-deoxy-3′-*O*-(*tert*-butyldimethylsilyl)uridine 5′-Monophosphate (21). Use of the procedure for the deprotection of **11** to **12** allowed deprotection of **20**, affording **21** in 99% yield as a pale yellow oil/foam: ^{1}H NMR (D_2O) δ 6.86 (t, J = 7.2 Hz, 1), 6.68 (t, J = 7.2 Hz, 1), 5.88 (s, 1), 5.83 (s, 1), 5.38 (m, 1), 5.12 (d, J = 15.5 Hz, 1), 4.94 (s, 1), 4.93 (d, J = 15.5 Hz, 1), 4.44 (m, 2), 2.69 (m, 1), 2.47 (m, 2), 0.98 (s, 9), 0.96 (s, 9), 0.26 (s, 6), 0.21 (s, 6). MALDI HRMS calcd for $C_{16}H_{26}N_2O_9PSi$ $(M - H)^-$ 449.1151, found 449.1169.

5′,6-Oxomethylene-2′-deoxyuridine 5′-Monophosphate (5′,6-Oxomethylene-Tethered 5′-dUMP, 2). A modification of the procedure for the hydrolysis of **12** to **1** was used. Thus, a solution of **21** in 10% aqueous AcOH was allowed to stand at 23 °C for 16 h and then rotary evaporated to dryness. The residue was twice coevaporated with toluene to remove water and acetic acid to afford 10.3 mg of **2** as an off-white foam/solid contaminated only by NH_4OAc. By ^{1}H NMR, **2** was obtained as a 5:1 mixture of diastereomers (epimeric phosphorylated hemiacetals): ^{1}H NMR (D_2O) δ 6.82 (t, J = 7.2 Hz, 1), 6.69 (t, J = 7.2 Hz, 1), 5.88 (s, 1), 5.84 (s, 1), 5.43 (m, 1), 5.12 (d, J = 15.5 Hz, 1), 4.61 (d, J = 15.5 Hz, 1), 4.44 (s, 2), 2.65 (m, 1), 2.48 (m, 1). In D_2O solution at 23 °C, the 5′,6-oxomethylene-tethered 5′-dUMP **2** remains intact for at least 2 months, by ^{1}H NMR (see Supporting Information). MALDI HRMS calcd for $C_{10}H_{14}N_2O_{10}P$ $(M - H)^-$ 335.0286, found 335.0286.

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Supporting Information Available: Tabular DCI mass spectral data for **1**−**21**; DCI mass spectral plots of **12**, **1**, **21**, and **2**; and periodic ^{1}H NMR spectra of **1** and **2** kept in D_2O solution at 23 °C This material is available free of charge via the Internet at http://pubs.acs.org.

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