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Adjusting the Structure of 2′-Modified Nucleosides and Oligonucleotides via C4′-α-F or C4′-α-OMe Substitution: Synthesis and Conformational Analysis

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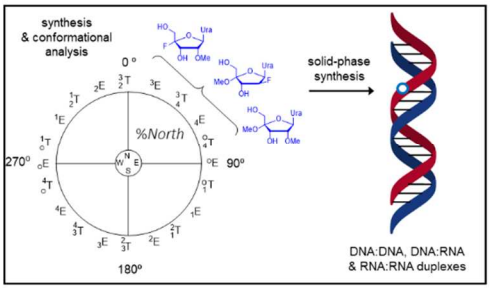


ABSTRACT

We report the first syntheses of three nucleoside analogs, namely 2′,4′-diOMe-rU, 2′-OMe,4′-F-rU, and 2′-F,4′-OMe-araU via stereoselective introduction of fluorine or methoxy functionalities at the C4′ alpha position of a 4′,5′-olefinic intermediate. Conformational analyses of these nucleosides and comparison to other previously reported 2′,4′-disubstituted nucleoside analogs make it possible to evaluate the effect of fluorine and methoxy substitution on sugar pucker, as assessed by NMR, X-ray diffraction and computational methods. We found that that C4′-α-F/OMe substituents reinforce the C3′-endo (*North*) conformation of 2′-OMe-rU. Furthermore, the predominant C2′-endo (*South/East*) conformation of 2′-F-araU switches to C3′-endo upon introduction of these substituents at C4′. The nucleoside analogs were incorporated into DNA and RNA oligonucleotides via standard phosphoramidite chemistry and their effects on thermal stability of homo- and heteroduplexes were assessed via UV thermal melting experiments. We found that 4′ substituents can modulate the binding affinity of the parent 2′-modified oligomers,

inducing a mildly destabilizing or stabilizing effect depending on the duplex type. This study expands the spectrum of oligonucleotide modifications available for rational design of oligonucleotides therapeutics.

INTRODUCTION

Beyond their applications as small-molecule therapeutic agents, nucleoside analogs are extensively used for oligonucleotide modification in gene silencing[1,2-4] and, more recently, gene editing applications.[5-7,8] In this context, the conformation of the nucleotide components is a main factor in defining binding affinity towards the target RNA or ssDNA which determines, to a large extent, the therapeutic and off-target effects. Many different modifications in the carbohydrate moiety have been explored in order to modulate the conformation of the sugar ring.[9] Electronegative substituents at C2′ in the ribo (α) configuration[10] such as methoxy (OMe),[11,12] methoxyethyl (MOE),[13] and fluoro (F)[14,15] tailor the sugar pucker towards the C3′-endo (*North*) conformation, whereas electronegative C2′-β-substituents (e.g., 2′-F) drive the sugar towards the *South/East* conformation. Nucleosides that adopt the C3′-endo conformation (i.e. 2′-F, 2′-OMe, 2′-MOE ribonucleotides) are widely used in RNA targeting applications (e.g., siRNAs, AONs).[16,17-19]

We and others have recently shown that modification at the 4′-position can also have a strong effect on sugar pucker preference.[20-26] We first designed 2′,4′-difluorinated ribonucleosides with the rationale that a C4′-α-fluorine would reinforce the *North* conformation of 2′-F-ribonucleosides. Indeed, as shown by NMR and molecular modeling, 2′,4′-diF-rU adopts exclusively the *North* conformation without requiring a bridge linking the 2′ and 4′ positions as seen in LNA, cEt and their derivatives.[21,27] In the case of 2′-F-araU, introduction of a C4′-α-fluorine caused a switch from *South* to mainly *North* pucker (~8:2% *North:South* ratio), pointing to a dominant F-C4′-O4′ anomeric effect.[20] In a more recent study we showed that 2′-F,4′-OMe-rU also adopts a *Northern* conformation (~ 9:1 N/S),[23,28] and that siRNAs containing several 2′-F,4′-OMe-rU units in the sense or antisense strands triggered RNAi-mediated gene silencing with efficiencies comparable to that of 2′-F-rU.[20,22,23] Furthermore, the C4′-α-OMe moiety conferred increased nuclease resistance due to the close proximity between 4′-OMe substituent and the vicinal 5′- and 3′-phosphate group.[23]

Guided by these results, we report herein the synthesis of several new 2′,4′-modified arabino and ribonucleoside analogs and study their conformation via NMR, X-ray crystallography and computational methods (Figure 1). When appropriate, comparisons are drawn with the conformation of the previously reported 2′,4′-modified analogs (Figure 1). All new 2′,4′-modified arabino and ribonucleoside analogs were converted into their respective phosphoramidite derivatives for incorporation into DNA and RNA strands by solid-phase methods, and the resulting modified strands mixed with complementary DNA or RNA strands to study their binding properties.

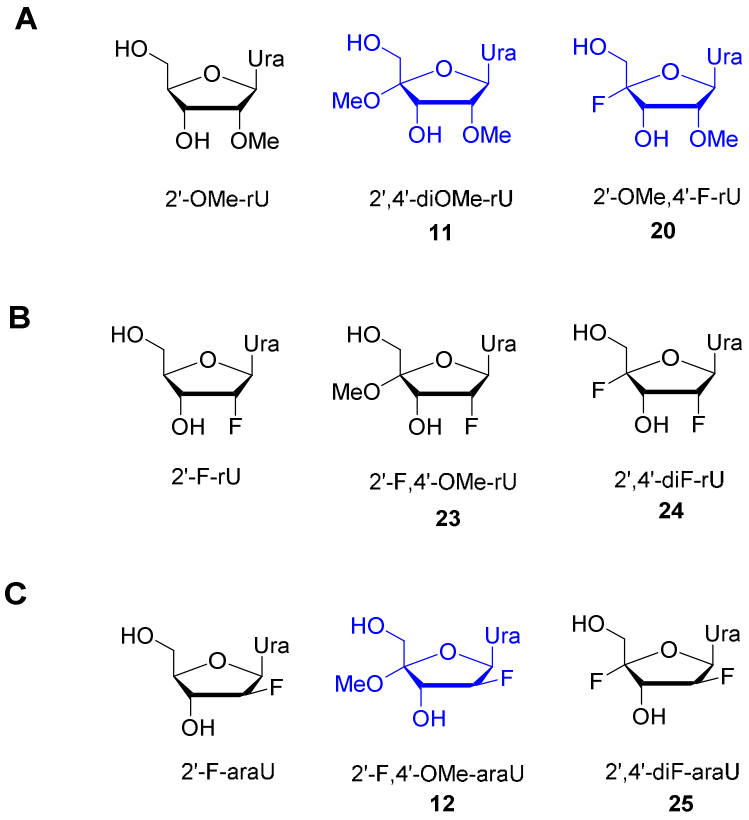
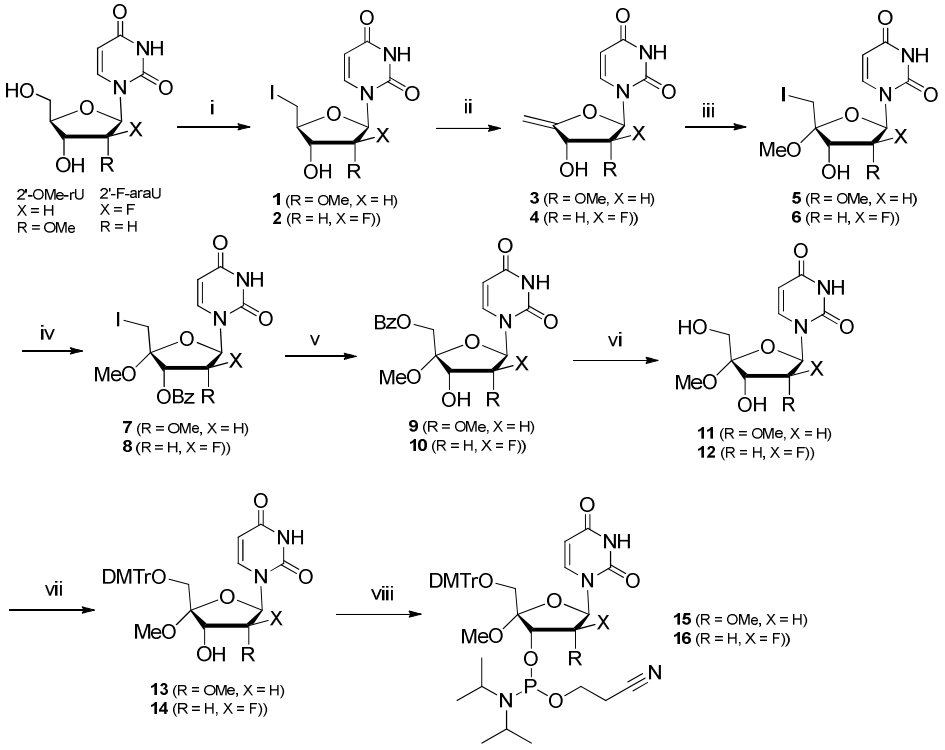


Figure 1. Structures of nucleoside analogues studied in this work. A) 2′-OMe-modified ribonucleosides. B) 2′-F-modified ribonucleosides. C) 2′-F-modified arabinonucleosides. The synthesis of those coloured in blue are reported herein. Ura is uracil nucleobase.

RESULTS AND DISCUSSION

Synthesis. We first focused on installing a OMe group at C4′ of 2′-OMe-rU and 2′-F-araU, following our previously published protocols,[23] producing two new compounds 2′,4′-diOMe-rU (**11**) and 2′-F,4′-OMe-araU (**12**), respectively. Briefly, 2′-OMe-rU and 2′-F-araU were first converted to the 5′-iodine derivative to allow for elimination reaction with DBU in THF to provide the desired alkene derivatives, **3** and **4**, respectively (Scheme 1).

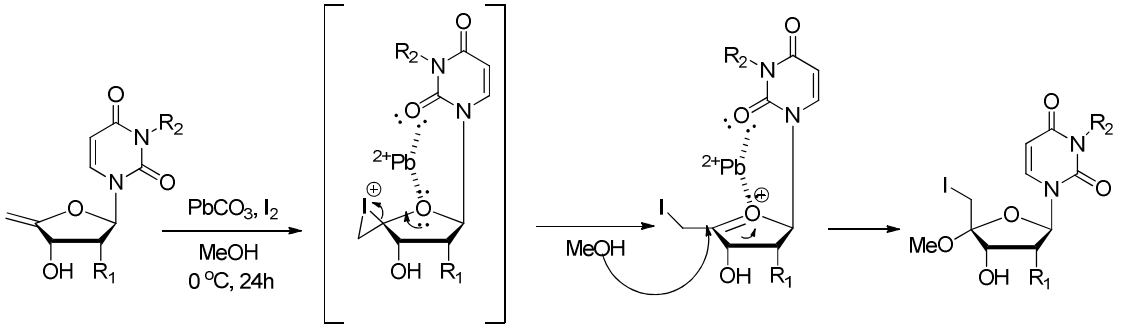
Scheme 1. Synthesis of 2′,4′-diOMe-rU (**11**) and 2′-F,4′-OMe-araU (**12**) and their phosphoramidite derivatives (**15**) and (**16**), respectively.[a]



[a]Reagents and conditions: (i) I_2, PPh_3/ACN, pyridine, rt, 48 h, **1**: 91%, **2**: 93% ; (ii) DBU/THF, rt, overnight, **3**: 86%, **4**: 78% (iii) $PbCO_3$, I_2/MeOH, 0 °C, overnight, **5**: 82%, **6**: 77% (iv) BzCl/pyridine, rt, overnight, **7**: 77%, **8**: 78% (v) *m*CPBA/DCM, H_2O, 40 °C, 6 h, **9**: 62%, **10**: 85% (vi) 2M NH_3/MeOH, rt, overnight, **11**: 81%. **12**: 86% (vii) DMTrCl, pyridine, 45 °C, 2 h, **13**: 57%, **14**: 78%, (viii **15**) 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA/THF, rt, 7 h, **15**: 42%, **16**: 86%.

Subsequent reaction with $PbCO_3$ and I_2 in methanol yielded the corresponding 5′-iodo-C4′-alpha-OMe in 82 and 77% yield for **5** and **6,** respectively, with high stereoselectivity. $PbCO_3$ is essential for the stereoselectivity achieved in this reaction.[23,29] In its absence, a mixture of alpha and beta anomers together with other uncharacterized products were observed in the ^{1}H-NMR of the reaction crude mixture (Figure S3). We propose a mechanism in which $PbCO_3$ coordinates to the ring oxygen effectively blocking the beta face of the nucleoside and forcing the MeOH to attack from the 4′-alpha face exclusively (Scheme 2). When Pb^{2+} is replaced by a smaller cation such as Ag^{2+}, we observed a two-fold decrease in stereoselectivity (Supporting Information, Figures S1 and S2), supporting the mechanism shown in Scheme 2. This observation is corroborated by the finding that protection of the N3 position with the benzyloxymethyl acetal (BOM) protecting group led to the same α-stereoselectivity at C4′ (Scheme 2; Supporting Information, Figure S4).

Scheme 2. Proposed mechanism and role of $PbCO_3$ in the reaction of a 2′-substituted 4′,5′-olefin with $PbCO_3/I_2$ in MeOH. R_1 = OMe, F; R_2 = H or BOM group.

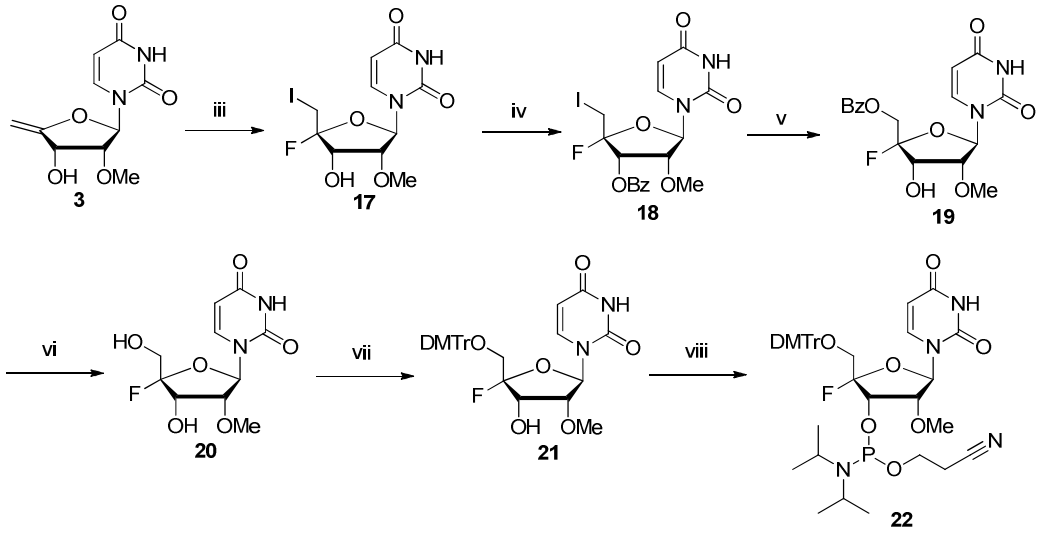


Benzoylation of the 3′-OH with concomitant migration of the Bz group followed by treatment with *m*CPBA in water saturated dichloromethane and ammonia deprotection, afforded the target nucleosides **11** and **12**. Their corresponding phosphoramidites **15** and **16** were obtained using conventional methods by first treatment with DMTrCl in pyridine to obtain **13** and **14** followed by treatment with $ClP(OCEt)N(iPr)_2$ in the presence in DIPEA and THF to afford phosphoramidites **15** and **16** in 42% and 86% yield respectively.

We further exploited the synthetic utility of the 4′,5′-olefin of the 2′-OMe-rU intermediate (**3**), to prepare the 4′-fluorinated analog of 2′-OMe-rU (Scheme 3).[21] The 4′-fluorination was accomplished by slow addition of I_2 to a suspension of AgF and **3** in acetonitrile at 0 °C to obtain

17 in 65% yield. Unprotected nucleoside **20** was obtained in good overall yield after benzoylation of the 3′-OH, oxidative iodine substitution reaction with *m*CPBA in water/DCM, and final ammonia deprotection. Dimethoxytritylation followed by treatment with 2-cyanoethyl *N,N,N,N*-tetraisopropylphosphorodiamidite in the presence of 4,5-dicyanoimidazole in ACN and DCM[30] produced the target phosphoramidite **22** in 64% yield.

Scheme 3. Synthesis of 2′-OMe,4′-F-rU, (**20**) and its phosphoramidite derivative (**22**).



[a]Reagents and conditions: (iii) AgF, I_2/ACN, 0 °C, 30 min, **17**: 65% (iv) BzCl/pyridine, rt, overnight, **18**: 83% (v) *m*CPBA/DCM, H_2O, 40 °C, 6 h, **19**: 83 % (vi) 2M NH_3/MeOH, rt, overnight, **20**: 95 %, (vii) DMTrCl, pyridine, 45 °C, 2 h, **21**: 60%, (viii) 2-cyanoethyl *N,N,N,N*-tetraisopropyldiphosphoramidite, DCI/ACN solution, DCM, rt, 30 min, **22**: 64%.

NMR Conformational Analysis. With our set of nucleosides in hand, we proceeded to analyse their sugar conformations by NMR spectroscopy. The percentage of *North* and *South* conformers of each ribonucleoside in solution was calculated by applying the equation S(%) = 10 × $J_{H1'\text{-}H2'}$,[21,31] where $J_{H1'\text{-}H2'}$ is the $^1H-^1H$ coupling constant between the ribose ring H1′ and H2′ hydrogens (Table 1 and Table S1). Table 1 summarizes, in descending order, the percentage of *North* conformation for all the nucleosides under study in three different solvents. Substitution of the 4′-H for electronegative substituents 4′-F and 4′-OMe resulted in a larger *North* bias in all 2′,4′-substituted nucleosides relative to their corresponding 2′-substituted nucleosides. This effect was more pronounced in the case of 4′-fluoro substitution, in agreement with a fluorine

being a better acceptor of hyperconjugation than a methoxy group,[32] thus providing stronger 4′-anomeric and gauche effects.

Table 1: Percentage of different nucleoside in the *North* conformation in various solvents at 298K.

Entry	Nucleoside	% *North* in Solution[a,b]		
		Solvent		
		CD_3OD	*D_2O*	*CD_3CN*
1	2′,4′-diF-rU[c]	100	100	100
2	2′-OMe,4′-F-rU	86.0	87.0	93.0
3	2′-F, 4′-OMe-rU[d]	86.0	87.0	87.0
4	2′-F-rU[d]	81.0	85.0	80.0
5	2′-F,4′-OMe-araU	80.5	80.0	78.0
6	2′,4′-diF-araU[e]	-	80.0	-
7	2′,4′-diOMe-rU	70.0	72.0	74.0
8	2′-OMe-rU	64.0	60.0	60.0
9	rU[d]	54.0	55.5	54.0
10	2′-F-araU	38.0	40.0	44.0

[a] Obtained from equation: $S(\%) = 10 \times J_{H1'-H2'}$
[b] Sugar pucker populations for 2′-F-araU and 2′-F,4′-OMe-araU were estimated according to Martínez-Montero *et al.*[20]
[c] Previously acquired data from Martínez-Montero *et al.*[21]
[d] Previously acquired data from Malek-Adamian, E. *et al.*[23]
[e] Previously acquired data from Martínez-Montero *et al.*[20]

To assess changes in sugar pucker equilibria as a function of temperature, $J_{H1'-H2'}$ coupling constants were measured in 10 K intervals over the range of 323-193 K in CD_3OD (Supporting Information), and the %*North* at each temperature was calculated (Table S2). Assuming that a two-state *N/S* equilibrium is maintained, all nucleosides under study progressively became more

North as the temperature was reduced, consistent with the notion that the *North* conformation is thermodynamically more favoured. In contrast, 2′,4′-diF-rU adopted exclusively the *North* conformation when heated to 323 K, confirming the conformational rigidity imposed by the two fluorine atoms. In order to quantify electronic interactions that favor the *North* conformation, we then turned to computational methods.

Computational Analysis. Recently, we developed and validated a molecular dynamics-based protocol for analyzing the conformation of nucleosides in solution.[28] We applied this protocol to the abovementioned set of 4′-modified nucleosides to get more insight into the origin of their conformational preferences. For each stable conformation we determined the pseudorotational phase angle P, the puckering amplitude φ_{max} (the maximum degree of pucker) and the appropriate dihedral angles (Supporting Information, Section S5). In addition, relevant hyperconjugation and anomeric effects and possible intramolecular hydrogen bonding were also computed (Supporting Information). Predicted *N/S* ratios determined by either Boltzmann population distribution analysis or energy differences between conformations were in close agreement with the experimentally determined *N/S* ratios (Supporting Information). This further confirmed the accuracy of the method and the generated conformations could now be further analyzed.

Lowest Energy Conformations and Electronic Effects. Anomeric and hyperconjugation effects through the means of natural bond orbital (NBO) analysis were evaluated as described in our recent study.[28] The anomeric effect $nO_{4'} \rightarrow \sigma^*_{C4'OMe/F}$ (Figure 2A) favours the *North* conformation except for 2′-F,4′-OMe-araU; the strength of the anomeric effect followed the order: 2′-OMe,4′-F-rU (P = 55.6°) > 2′,4′-diOMe-rU (P = 60.5°) > 2′-F,4′-OMe-araU (P = 47.2 °).

Several factors seem to influence the strength of the anomeric effect: a) the puckering amplitude of the sugar ring, with a weaker effect observed for lower amplitudes for both *North* and *South* conformations, b) the hyperconjugation accepting ability of the 4′ substituent, and c) the P angle, with deviations from ideal envelope conformations associated with weaker anomeric effects (Supporting Information).

In the case of 2′-F,4′-OMe-araU, a plausible explanation for the *South* conformation exhibiting a slightly larger anomeric effect is that the *North* conformation (P = 47.2°, φ_{max} = 22.1°) deviates

from an ideal envelope conformation (P = 54°, φ_{max} = 25-45°).[33] This leads to a poor overlap between the lone pair on the anomeric oxygen and $\sigma^*_{C4'OMe}$ due to orbital misalignment. However, 2′-F,4′-OMe-araU was shown to be predominantly *North*, and as such another electronic effect apart from the anomeric effect should be the driving force behind the observed conformation. Indeed, the data in Table S40 suggests that the $\sigma_{C3'H3'} - \sigma^*_{C4'OMe}$ hyperconjugation effect **(**Figure 2C) is the most pronounced computed electronic effect (with an energy difference of ~1.9 kcal/mol in favour of *North*), similar in strength to 2′,4′-diOMe-rU (~2.0 kcal/mol) and 2′-OMe,4′-F-rU (~3.1 kcal/mol).

Interestingly, this effect is exhibited in conjunction with a $\sigma_{C3'C4'} - \sigma^*_{C2'H}$ hyperconjugation effect (Figure 2B) (~0.8 kcal/mol in favour of the *South* conformer) that is significantly lower than that observed for 2′-OMe,4′-F-rU (~3.5 kcal/mol) and 2′,4′-diOMe-rU (~2.2 kcal/mol), most likely afforded by orientation of the fluorine in the arabino configuration. As such, the loss of an important hyperconjugation effect favouring the *South* pucker ($\sigma_{C3'C4'} - \sigma^*_{C2'H}$), coupled with a strong hyperconjugation effect favouring the *North* pucker ($\sigma_{C3'H3'} - \sigma^*_{C4'OMe}$) contribute to the observed *North* conformation of 2′-F,4′-OMe-araU.

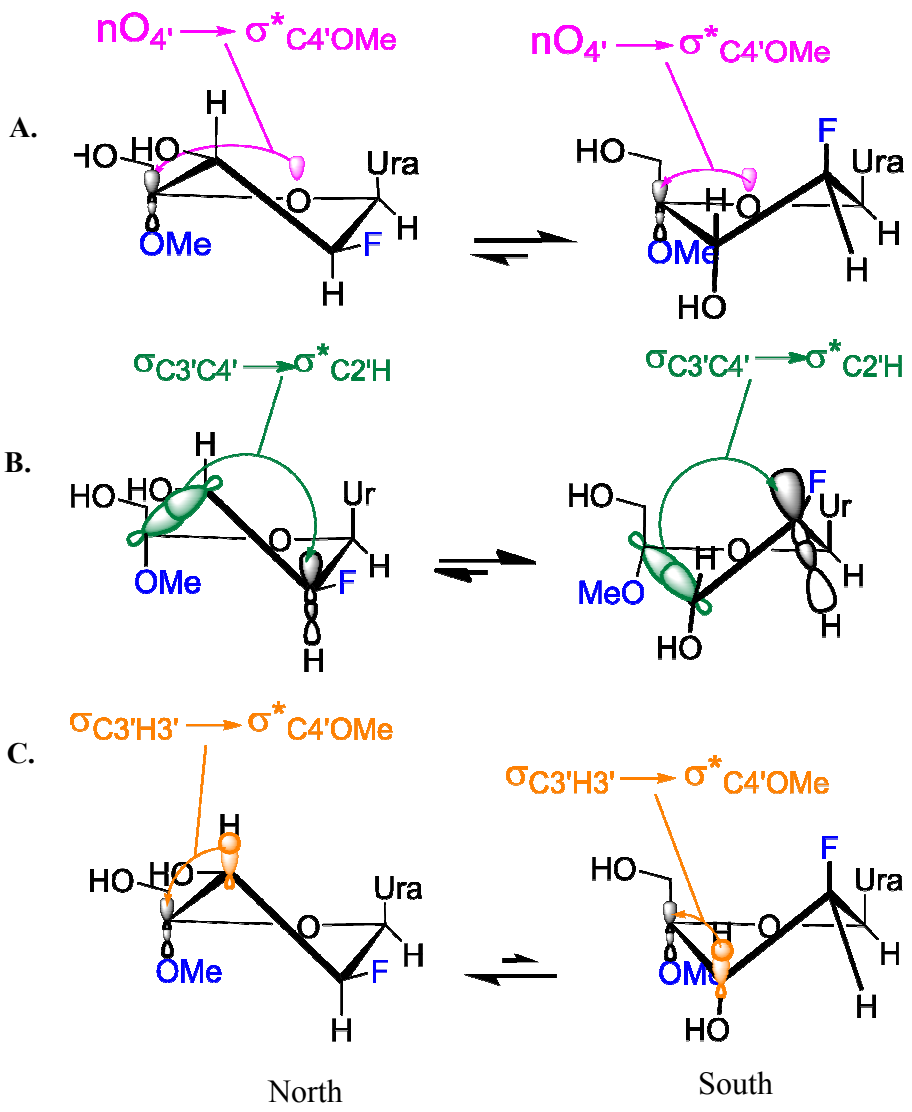


Figure 2. Stereoelectronic effects in 2′-F,4′-OMe-araU : **A)** Depiction of the $nO_{4'} \rightarrow \sigma^*_{C4'OMe}$ anomeric effect. **B)** Depiction of the $\sigma_{C3'C4'} \rightarrow \sigma^*_{C2'H}$ hyperconjugation effect. **C)** Depiction of the $\sigma_{C3'H3'} \rightarrow \sigma^*_{C4'OMe}$ hyperconjugation effect.

A Comparison between Crystal and Predicted Structures. The structures of 2′,4′-diOMe-rU and 2′-OMe,4′-F-rU, first synthesized in this study, were unambiguously confirmed by X-ray crystallography. Additionally, we obtained the crystal structures of previously reported 2′,4′-diF-rU and 2′-F,4′-OMe-rU .[28] These were compared to their predicted conformations by computational analysis and the superposition of these are shown in Figure 3.

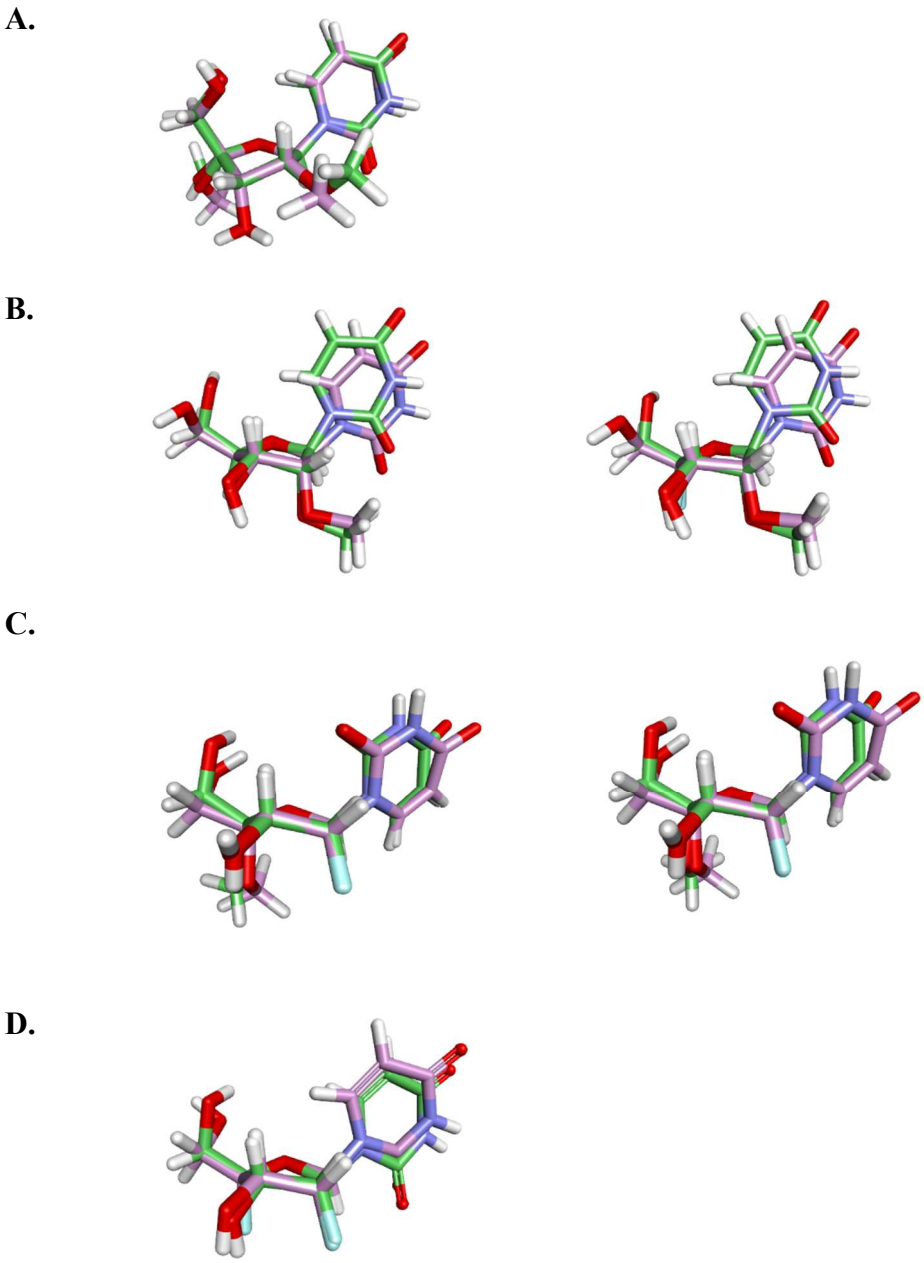


Figure 3. Superposition between the predicted conformation (green) and the crystal structure (pink). **A)** 2′,4′-diOMe-rU, heavy atom RMSD = 0.47Å. **B)** 2′-OMe,4′-F-rU *left)* unit 1, heavy atom RMSD = 0.82Å and *right)* unit 2, heavy atom RMSD = 0.82Å. **C)** 2′-F,4′-OMe-rU *left)* unit 1, heavy atom RMSD = 0.51 Å and *right)* unit 2, heavy atom RMSD = 0.59 Å. **D)** 2′,4′-diF-rU, heavy atom RMSD = 0.59Å.

2′-OMe,4′-F-rU was crystalized in its most stable conformation (P_1 = 22.4°, $\varphi_{1\text{-max}}$ = 34.1°, P_2 = 21.2°, $\varphi_{2\text{-max}}$ = 33.8°). In the case of 2′-F,4′-OMe-rU (4′-exo)., two slightly different conformations appeared in the asymmetric unit crystal (P_1 = 62.3°, $\varphi_{1\text{-max}}$ = 36.6°, and P_2 = 60.5°, $\varphi_{2\text{-max}}$ = 37.1°). However, the orientation of the base and the 3′ and 5′ hydroxyls differ from the

computational data. In contrast, 2′,4′-diOMe-rU and 2′,4′-diF-rU were crystallized in the *South* conformation (P = 163.2°, φ_{max} = 39.5°) and the *East* conformation (P = 74.7°, φ_{max} = 35.4°[28]), respectively. Clearly, these results do not depict how nucleosides behave in solution, and hence crystal packing and intermolecular hydrogen bonding interactions between molecules in different unit cells likely affect the conformation of the nucleoside in the crystal.[34]

Oligonucleotide Synthesis. To systematically compare the effect of the structure of the three novel 2′,4′-modified nucleotides discussed above with the parent 2′-modified nucleotides and other 2′,4′-modifications previously described in our laboratory, we performed single incorporations in a central location of a previously used oligonucleotide sequence.[22] To achieve this, phosphoramidite **15**, **16**, and **22** were incorporated into DNA and RNA oligonucleotides via standard phosphoramidite chemistry (see Experimental Section) with coupling efficiencies similar to 2′-F-araU and 2′-OMe-rU amidites. Oligonucleotides were purified by ion exchange HPLC using 1 M lithium perchlorate buffer. All 2′,4′-modified oligonucleotides were examined by ESI mass spectrometry and confirmed to possess the correct mass (Table S35, Supporting Information)

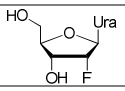
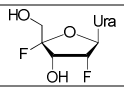
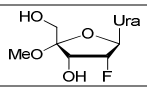
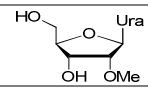
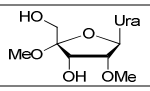
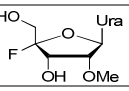
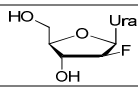
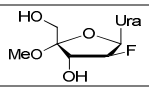
Melting Temperature of Modified Duplexes. Thermal stability was assessed by determining the T_m values of 5′-d(CCA T**X**A TAG C) and 5′-r(CCA U**X**A UAG C) hybridized with complementary DNA or RNA (Table 2). Incorporation of 2′,4′-diOMe-rU in DNA resulted in a destabilization effect when paired with either complementary DNA or RNA (ΔT_m = -1.5 °C). In contrast, its incorporation into RNA had a positive impact on thermal stability whether the target was a complementary DNA or RNA strand (ΔT_m = + 1.5 °C). This suggests that the geometrical constrains imposed by the two bulky OMe groups are well-accommodated in the A-form structure adopted by RNA:RNA duplexes. Similarly, the 2′-OMe,4′-F-rU analog had a destabilizing effect when incorporated into a DNA:DNA duplex. However, the effect was neutral or slightly stabilizing when incorporated in either DNA:RNA hybrids (ΔT_m = neutral; +0.5 °C) or RNA:RNA duplex (ΔT_m = +1.5 °C). Comparison with the isomeric 2′-F,4′-OMe-rU analog previously reported indicates that permutation of the fluorine and methoxy substituents has little effect on the thermal stability of the corresponding modified duplexes (Table 2).[23]

Comparison of 2′,4′-diF-rU with 2′-F,4′-OMe-rU suggest that a 4′-OMe provides a significant improvement over a 4′-F in stability of hybrid duplexes and slight improvement in RNA

duplexes (Table 2).[22] The origins for this behavior remains unclear; however, we hypothesize that the greater inductive effect of F versus OMe (at C4′) diminishes internucleotide 2′C−H···O4′ hydrogen bonds that contribute to the stabilization of A-like duplex structures.[35] This interaction is enhanced in 2′-F-rU by the polarization of the 2′C−H bond by the geminal fluorine at C2′.[22] While this effect should also operate in 2′,4′-diF-rU and 2′-F,4′-OMe-rU, the electronegative substituents at C4′ is expected to affect the charge distribution on the furanose sugar ring (e.g., F at C2′). The consequence of this would be a reduction in both the acceptor capability of O4′ and the polarization of the 2′C−H bond in the order 4′-F > 4′-OMe > 4′-H, consistent with the T_m changes in DNA/RNA and RNA/RNA duplexes by 2′-F-rU (stabilizing), 2′-F,4′-OMe-rU (stabilizing-neutral), and 2′,4′-diF-rU (destabilizing-neutral). Clearly, high-field NMR analysis is needed to gain a better understanding of these subtle structural effects.

Incorporation of 2′-F,4′-OMe-araU into DNA:DNA duplexes is destabilizing (ΔT_m = -2.5 °C), an expected result if the predominant *North* conformation of the nucleoside is maintained in the duplex. By the same token, incorporation of 2′-F,4′-OMe-araU into an RNA duplex is slightly stabilizing, in contrast to 2′-F-araU which adopts mainly a *South/East* conformation and hence causes destabilization of the same duplexes. In the context of DNA:RNA hybrids, 2′-F-araU adopts a *South/East* pucker, allowing the formation of internucleosidic C-H···F-C pseudohydrogen bonds at pyrimidine-purine steps and resulting in the stabilizing effect observed when it is incorporated in the DNA strand of DNA-RNA hybrids.[36,37] This is consistent with the observed stabilization (ΔT_m = +1.5 °C) when a single dT is replaced with 2′-F-araU at a pyrimidine-purine step. Such pseudohydrogen bonding is unlikely to form in a North conformer (2′-F,4′-OMe-araU) hence losing the stabilization typical of 2′-F-araU nucleotides when placed in DNA (ΔT_m = 0.0 °C; Table 2).

Table 2. Comparison of the relative melting temperatures of duplexes containing a single incorporation of modified uridine nucleotide.[a]

Duplex Sequence (5′ to 3′) (3′ to 5′)	ΔT_m/mod. (°C)							
	2′-F ribo modifications			2′-OMe ribo modifications			2′-F arabino modifications	
	2′-F	2′,4′-diF[b]	2′F,4′OMe[b]	2′-OMe	2′,4′-diOMe	2′OMe,4′F	2′-araF	2′araF,4′OMe
								
d(CCAT**X**ATAGC) d(GGTAATATCG)	-0.5	n.d.	-1.0	-1.0	-1.5	-1.5	-0.5	-2.5
d(CCAT**X**ATAGC) r(GGUAAUAUCG)	+2.0	-9.4	+1.5	+1.5	-1.5	+0.5	+1.5	0.0
d(CCAU**X**AUAGC) d(GGTAATATCG)	+1.5	-1.5	+1.5	+1.5	+1.5	0.0	-4.5	0.0
d(CCAU**X**AUAGC) r(GGUAAUAUCG)	+1.5	-0.5	0.0	+1.0	+1.5	+1.5	-1.5	+0.5

[a] T_m values were measured in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1 mM EDTA with a strand concentration of 1.5 μM where ΔT_m = (T_m of modified duplex – T_m of corresponding unmodified duplex). **X** indicates the modified uridine nucleotide: 2′-F-rU, 2′,4′-diF-rU, 2′-F-4′-OMe-rU, 2′-OMe-rU, 2′,4′-diOMe-rU, 2′-OMe-4′-F-rU, 2′-araF, or 2′-F-4′-OMe-araU. [b] Values obtained from previous report.[22,23]

CONCLUSIONS

Three new 2′,4′-modified nucleosides, 2′-F,4′-OMe-araU, 2′-OMe,4′-F-rU, and 2′,4′-diOMe-rU, were synthesized with high stereoselectivity from 4′,5′-olefinic nucleoside precursors using $PbCO_3$ as the catalyst. Computational studies suggest that both electronic and steric effects influence their sugar pucker, favoring in all cases the *North* form. A combination of two hyperconjugation effects ($\sigma_{C3'H3'} - \sigma^*_{C4'OMe}$ and $\sigma_{C3'C4'} - \sigma^*_{C2'H}$) drives the conformation of 2′-F,4′-OMe-araU towards *North*. Evaluation of the duplex stabilizing properties of these new monomers and comparison with the parent 2′-modified nucleotide analogs shows that the 4′ modification clearly impacts duplex stability. The *North* 2′,4′-disubsituted RNA analogues 2′-OMe,4′-F-rU, and 2′,4′-diOMe-rU and the ANA analogue 2′-F,4′-OMe-araU are well tolerated in RNA:RNA duplexes, stabilizing or nearly neutral with respect to effects of duplex thermal stability (+0.5 to +1.5 °C/nt). This outcome suggests that these analogues (and those previously reported by our group) may be very well suited for siRNA and guide RNAs, allowing retention of an A-form structure and negligible effects on thermal stability, while introducing modified nucleotides foreign to serum nucleases and immunostimulatory receptors. The neutral or

minimally destabilizing character of 2′-OMe,4′-F-rU; 2′,4′-diF-rU; 2′-F,4′-OMe-araU in DNA:RNA contexts could be useful for CRISPR/Cas9 technologies where slightly destabilizing chemical modifications in the sgRNA have been shown to reduce off-target effects while keeping gene editing efficiency.[7] This study expands the "chemist's toolbox" of nucleoside (and RNA/DNA) modifications available for a wide range of applications. Further investigations are currently in progress in our laboratory to assess the full potential of the oligonucleotide modifications described herein.

EXPERIMENTAL SECTION

Procedures and Experimental Data. HRMS was obtained by ESI-MS using a Thermo Scientific Exactive Plus Orbitrap mass spectrometer or a Bruker Daltonics Maxis Impact quardupole time-of-flight (QTOF) mass spectrometer.

Synthesis of 2′,4′-diOMe-rU. *5′-iodo-2′-deoxy-2′-methoxyuridine (**1**).* A suspension of 2′-OMe uridine (2.0 g, 7.752 mmol) was prepared in anhydrous acetonitrile (160 mL) and pyridine (8 mL). Iodine (2.56 g, 10.08 mmol) and then triphenylphosphine (2.85 g, 10.85 mmol) were added under inert atmosphere to the reaction mixture. The reaction was left to stir at room temperature for 48 hours, after which the solvents were removed by vacuum and the resulting residue was purified by column chromatography (1-10% MeOH in CH_2Cl_2) to give **1** as a yellow foam (2.59 g, 91%): R_f (10% MeOH/CH_2Cl_2) 0.45; ^{1}H NMR (500 MHz, CD_3OD) δ 7.74 (d, J = 8.1 Hz, 1H), 5.91 (d, J = 3.9 Hz, 1H), 5.74 (d, J = 8.1 Hz, 1H), 4.07 (t, J = 5.8 Hz, 1H), 3.95 (dd, J = 5.6, 4.0 Hz, 1H), 3.84 (td, J = 5.9, 4.6 Hz, 1H), 3.59 (dd, J = 11.0, 4.6 Hz, 1H), 3.50 (s, 3H), 3.45 (dd, J = 11.0, 5.9 Hz, 1H). ^{13}C NMR (126 MHz, CD_3OD) δ 166.2, 152.1, 142.8, 103.2, 89.9, 84.2, 84.1, 74.1, 59.2, 5.8. HRMS (ESI+) m/z calcd for $C_{10}H_{13}IN_2NaO_5$ [M + Na]+ 390.9761, found 390.9760.

*4′,5′-Didehydro-2′,5′-dideoxy-2′-methoxyuridine (**3**).* A solution of **1** (2.58 g, 7.030 mmol) was prepared in anhydrous THF (66 mL) under inert atmosphere. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was added dropwise to the solution (6.31 mL, 42.18 mmol) over a period of 5 minutes. The reaction was left to stir at room temperature overnight. The reaction mixture was then evaporated and purified by column chromatography (1-12% MeOH/CH_2Cl_2) to afford the alkene as a white foam (1.45 g, 86%): R_f (10% MeOH/CH_2Cl_2) 0.42; ^{1}H NMR (500 MHz, CD_3OD) δ 7.48 (d, J = 8.1 Hz, 1H), 6.11 (d, J = 3.9 Hz, 1H), 5.74 (dd, J = 8.1, 1.0 Hz, 1H), 4.67

(dt, *J* = 5.4, 1.5 Hz, 1H), 4.53 (t, *J* = 1.8 Hz, 1H), 4.35 (dd, *J* = 2.2, 1.3 Hz, 1H), 4.00 (dd, *J* = 5.2, 3.9 Hz, 1H), 3.51 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 165.9, 163.0, 151.9, 141.4, 103.4, 89.7, 86.2, 83.0, 69.7, 58.7. HRMS (ESI+) m/z calcd for $C_{10}H_{12}N_2NaO_5$ [M + Na]+ 263.0644, found 263.0636.

*2'-Deoxy-2',4'-dimethoxy-5'-iodo uridine (**5**).* A suspension of alkene **3** (1.45 g, 6.046 mmol) and $PbCO_3$ (3.23 g, 12.09 mmol) was prepared in anhydrous methanol (41 mL). A solution of iodine (3.07 g, 12.09 mmol) in methanol (11 mL) was added dropwise to the rapidly stirring solution at 0 ^{o}C. The reaction was left to stir overnight at room temperature, the solvents were concentrated under vacuum, and the resulting residue was purified by column chromatography (2-5% MeOH/CH_2Cl_2) to afford the product as an orange foam (1.96 g, 82%): R_f (10% MeOH/CH_2Cl_2) 0.73; ^{1}H NMR (500 MHz, CD_3OD) δ 7.66 (d, *J* = 8.1 Hz, 1H), 5.89 (d, *J* = 3.8 Hz, 1H), 5.75 (d, *J* = 8.1 Hz, 1H), 4.51 (d, *J* = 7.2 Hz, 1H), 3.95 (dd, *J* = 7.2, 3.8 Hz, 1H), 3.66 (d, *J* = 11.4 Hz, 1H), 3.51 (d, *J* = 11.4 Hz, 1H), 3.47 (s, 3H), 3.38 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 165.7, 151.6, 142.9, 104.7, 103.3, 90.6, 82.8, 74.2, 59.5, 3.3. HRMS (ESI+) m/z calcd for $C_{11}H_{15}IN_2NaO_6$ [M + Na]+ 420.9872, found 420.9861.

*3'-O-Benzoyl-2'-deoxy-2',4'-dimethoxy-5'-iodo uridine (**7**).* A suspension of the nucleoside **5** (1.45 g, 3.643 mmol) was prepared in pyridine (5 mL). Benzoyl chloride (508 μL, 4.372 mmol) was slowly added dropwise to the mixture, and the reaction was left to stir at room temperature overnight, following which the solvents were concentrated under vacuum. The resulting residue was purified by column chromatography (2-5% MeOH/CH_2Cl_2) to afford the product as an orange solid (1.41 g, 77%): Rf (10% MeOH/CH_2Cl_2) 0.78; ^{1}H NMR (500 MHz, CD_3OD) δ 8.55 (dt, *J* = 4.6, 1.7 Hz, 1H), 8.14 – 8.11 (m, 2H), 7.75 (d, *J* = 8.0 Hz, 1H), 7.67 – 7.62 (m, 1H), 7.53 – 7.49 (m, 2H), 5.98 (d, *J* = 4.0 Hz, 1H), 5.78 (d, *J* = 8.1 Hz, 1H), 5.64 (d, *J* = 7.3 Hz, 1H), 4.41 (dd, *J* = 7.3, 4.0 Hz, 1H), 3.67 (d, *J* = 2.7 Hz, 2H), 3.44 (s, 3H), 3.37 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 166.9, 151.9, 149.6, 143.5, 139.0, 134.7, 134.0, 131.0, 130.7, 129.6, 125.7, 105.6, 103.6, 92.3, 82.0, 75.3, 59.7, 4.2. HRMS (ESI+) m/z calcd for $C_{18}H_{19}IN_2NaO_7$ [M + Na]+ 525.0135, found 525.0132.

*5'-O-Benzoyl-2'-deoxy-2',4'-dimethoxy uridine (**9**)*. A solution of nucleoside **7** (1.41 g, 2.805 mmol) was prepared in CH_2Cl_2 (79 mL) and H_2O (4.5 mL). 3-Chloroperoxybenzoic acid (*m*CPBA) (77% purity, 2.52 g, 11.22 mmol) was added slowly to the stirring solution. The

reaction was heated to 40 °C and allowed to react for 6 hours, after which the solvents are concentrated and the resulting residue was purified by column chromatography (30-70% EtOAc in hexanes) to afford the product as a white foam (683 mg, 62%): R_f (50% EtOAc/hexanes) 0.56; ^{1}H NMR (500 MHz, CD_3CN) δ 7.95 (dd, *J* = 8.5, 1.3 Hz, 2H), 7.74 (d, *J* = 8.2 Hz, 1H), 7.68 (t, *J* = 7.5 Hz, 1H), 7.54 – 7.50 (m, 2H), 5.86 (d, *J* = 2.4 Hz, 1H), 5.66 (d, *J* = 8.1 Hz, 1H), 4.68 (d, *J* = 12.0 Hz, 1H), 4.60 (d, *J* = 7.0 Hz, 1H), 4.47 (d, *J* = 12.0 Hz, 1H), 4.10 – 4.03 (m, 1H), 3.50 (s, 3H), 3.47 (s, 3H). ^{13}C NMR (126 MHz, CD_3CN) δ 169.9, 167.3, 150.4, 143.6, 136.4, 134.5, 132.8, 130.8, 129.6, 105.9, 102.9, 93.2, 82.9, 72.7, 62.3, 60.0, 20.8, 14.4. HRMS (ESI+) m/z calcd for $C_{18}H_{20}N_2NaO_8$ [M + Na]+ 415.1117, found 415.1122.

*2′-Deoxy-2′,4′-dimethoxy uridine (**11**)*. A solution of the nucleoside **9** (683 mg, 1.742 mmol) was prepared in methanolic ammonia (2M, 10 mL) and stirred overnight at room temperature. The resulting mixture was evaporated to remove solvents and then purified by column chromatography (2-20% MeOH/CH_2Cl_2) to afford the product as a white foam (406 mg, 81%): R_f (10% MeOH/CH_2Cl_2) 0.54; ^{1}H NMR (500 MHz, CD_3OD) δ 7.88 (d, *J* = 8.1 Hz, 1H), 6.01 (d, *J* = 3.0 Hz, 1H), 5.70 (d, *J* = 8.1 Hz, 1H), 4.43 (d, *J* = 6.7 Hz, 1H), 3.85 – 3.83 (dd, *J* = 3.5, 6.7 Hz, 1H), 3.84 – 3.82 (d, *J* = 12.0 1H), 3.63 (d, *J* = 11.8 Hz, 1H), 3.51 (s, 3H), 3.39 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 166.0, 151.9, 142.5, 107.7, 103.0, 90.1, 84.1, 71.1, 61.0, 59.7, 50.1. HRMS (ESI+) m/z calcd for $C_{11}H_{16}N_2NaO_7$ [M + Na]+ 311.0855, found 311.0852.

*5′-O-[Bis(4-methoxyphenyl)phenylmethyl]-2′-deoxy-2′,4′-dimethoxy uridine (**13**)*. A solution of nucleoside **11** (300 mg, 1.164 mmol) was prepared in pyridine (15 mL) under argon. 4,4′-dimethoxytrityl chloride (552 mg, 1.628 mmol) was added, and the reaction was allowed to stir for 2 h at 45 °C. Pyridine was subsequently evaporated under vacuum, and the resulting residue purified by column chromatography using de-acidified silica gel by treatment with 10% trimethylamine in CH_2Cl_2 and MeOH/CH_2Cl_2 washing (1−3% MeOH/CH_2Cl_2). This afforded dimethoxytritylated nucleoside **13** as a white foam (391 mg, 57%). R_f (10% MeOH/CH_2Cl_2) 0.64; ^{1}H NMR (500 MHz, CD_3OD) δ 7.89 (d, *J* = 8.1 Hz, 1H), 7.43 – 7.40 (m, 2H), 7.33 – 7.28 (m, 6H), 7.27 – 7.23 (m, 1H), 6.90 – 6.86 (m, 4H), 5.97 (d, *J* = 2.0 Hz, 1H), 5.24 (d, *J* = 8.1 Hz, 1H), 4.66 (d, *J* = 6.8 Hz, 1H), 3.85 (dd, *J* = 6.8, 2.0 Hz, 1H), 3.78 (s, 6H), 3.53 (s, 3H), 3.49 (d, *J* = 10.0 Hz, 1H), 3.34 (d, *J* = 10.0 Hz, 1H), 3.19 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 166.00, 160.36, 151.69, 145.82, 142.51, 136.62, 136.42, 132.91, 131.54, 131.48, 131.45, 129.50, 129.46,

128.96, 128.65, 128.63, 128.13, 114.24, 113.99, 113.94, 107.34, 102.80, 90.66, 88.58, 84.25, 71.91, 62.42, 59.94, 58.44, 55.74, 55.69, 49.94. HRMS (ESI+) m/z calcd for $C_{32}H_{34}N_2NaO_9$ [M + Na]+ 613.2162, found 613.2160.

*5′-O-[Bis(4-methoxyphenyl)phenylmethyl]-2′-deoxy-2′,4′-dimethoxy uridine, 3′-[2-cyanoethyl N,N-bis(1-methylethyl)phosphoramidite] (**15**).* Dimethoxytritylated nucleoside **13** (391 mg, 0.663 mmol), was dried under vacuum overnight and coevaporated with MeCN four times. Dry THF (7 mL) was added under argon. To the solution was added $EtN(iPr)_2$ (603 μL, 3.315 mmol) and then $ClPOCEtN(iPr)_2$ (163 μL, 0.729 mmol). The reaction mixture was stirred for 7 hours at room temperature. After the reaction reached completion, the mixture was extracted using DCM and saturated sodium bicarbonate, the organic phases were collected and dried using $MgSO_4$ to finally be filtered and concentrated for direct loading on a deacidified silica column (eluent: 30-70% EtOAc in hexanes) to afford phosphoramidite **15** as a white foam (220 mg, 42%). R_f (50% EtOAc/hexanes) 0.38. ^{1}H NMR (500 MHz, CD_3CN) δ 9.03 (s, 1H), 7.83 – 7.62 (m, 1H), 7.45 – 7.39 (m, 2H), 7.35 – 7.22 (m, 7H), 6.88 (dd, *J* = 8.2, 4.4 Hz, 4H), 6.00 (dd, *J* = 13.9, 2.7 Hz, 1H), 5.17 (dd, *J* = 8.2, 4.7 Hz, 1H), 4.74 (ddd, *J* = 47.1, 11.2, 6.9 Hz, 1H), 3.93 (td, *J* = 7.7, 7.1, 2.7 Hz, 1H), 3.77 (d, *J* = 2.3 Hz, 6H), 3.66 – 3.58 (m, 2H), 3.46 (d, *J* = 13.7 Hz, 3H), 3.43 – 3.36 (m, 1H), 3.31 – 3.23 (m, 1H), 3.20 (d, *J* = 18.8 Hz, 3H), 2.68 (td, *J* = 6.4, 5.3 Hz, 1H), 2.55 (ddd, *J* = 15.2, 6.6, 5.4 Hz, 1H), 1.27 – 1.09 (m, 11H), 1.07 (d, *J* = 6.7 Hz, 2H). ^{13}C NMR (126 MHz, CD_3CN) δ 163.56, 159.70, 150.86, 145.40, 141.28, 135.96, 132.37, 131.19, 131.10, 129.22, 129.04, 128.76, 128.19, 127.92, 119.36, 113.95, 107.58, 102.81, 102.70, 90.24, 89.93, 87.90, 83.24, 82.97, 72.33, 72.22, 71.54, 71.42, 63.18, 62.55, 59.55, 59.37, 59.33, 55.78, 55.76, 50.69, 50.41, 43.96, 43.86, 24.96, 24.90, 24.80, 24.67, 20.73. ^{31}P NMR (203 MHz, CD_3CN) δ 150.70, 150.26. HRMS (ESI+) m/z calcd for $C_{41}H_{51}N_4NaO_{10}P$ [M + Na]+ 813.3241, found 813.3243.

Synthesis of 2′-F,4′-OMe-araU. *5′-iodo-2′-deoxy-2′-fluoro arabino uridine (**2**).* The same procedure was followed as previously described, and the results matched experimental findings.[4]

*4′,5′-Didehydro-2′,5′-dideoxy-2′-fluoro arabino uridine (**4**).* A solution of **2** (2.4 g, 6.761 mmol) was prepared in anhydrous THF (61 mL) under inert atmosphere. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was added dropwise to the solution (6.1 mL, 40.56 mmol) over a period of 5 minutes. The reaction was left to stir at room temperature overnight. The reaction mixture was then evaporated and purified by column chromatography (1-12% $MeOH/CH_2Cl_2$) to afford the

alkene as a white foam (1.2 g, 78%). R_f (10% MeOH/CH_2Cl_2) 0.59; ^{1}H NMR (500 MHz, CD_3OD) δ 7.47 (dd, *J* = 8.2, 2.1 Hz, 1H), 6.51 (dd, *J* = 19.4, 3.3 Hz, 1H), 5.72 (d, *J* = 8.1 Hz, 1H), 5.11 – 4.95 (m, 1H), 4.69 – 4.68 (m, 1H), 4.67 – 4.65 (m, 1H), 4.47 (dd, *J* = 2.3, 0.8 Hz, 1H). ^{13}C NMR (126 MHz, CD_3OD) δ 166.0, 161.8, 142.6, 102.7, 95.6, 94.1, 88.4, 86.9, 74.5. ^{19}F NMR (471 MHz, CD_3OD) δ -204.41, -204.43, -204.44, -204.45, -204.45, -204.48, -204.52, -204.52, -204.55, -204.56, -204.58. HRMS (ESI+) m/z calcd for $C_9H_9FN_2NaO_4$ [M + Na]+ 251.0444, found 251.0436.

*2',5'-Dideoxy-2'-fluoro-5'-iodo-4'-methoxy arabino uridine (**6**).* A suspension of alkene **4** (2 g, 8.772 mmol) and $PbCO_3$ (4.69 g, 17.54 mmol) was prepared in anhydrous methanol (56 mL). A solution of iodine (4.45 g, 17.54 mmol) in methanol (16 mL) was added dropwise to the rapidly stirring solution at 0 ^{o}C. The reaction was left to stir overnight at room temperature, the solvents were concentrated under vacuum, and the resulting residue was purified by column chromatography (2-10% MeOH/CH_2Cl_2) to afford the product as a yellow foam (2.6 g, 77%): R_f (10% MeOH/CH_2Cl_2) 0.62; ^{1}H NMR (500 MHz, CD_3OD) δ 7.72 (dd, *J* = 8.1, 1.7 Hz, 1H), 6.26 (dd, *J* = 12.0, 5.3 Hz, 1H), 5.74 (d, *J* = 8.1 Hz, 1H), 5.13 (ddd, *J* = 54.4, 5.3, 4.5 Hz, 1H), 4.55 (dd, *J* = 24.6, 4.5 Hz, 1H), 3.80 (dd, *J* = 11.6, 0.9 Hz, 1H), 3.54 (d, *J* = 11.6 Hz, 1H), 3.42 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 165.7, 151.8, 142.8, 103.5, 102.5, 97.7, 96.1, 82.9, 79.7, 1.7. ^{19}F NMR (471 MHz, CD_3OD) δ -201.20, -201.23, -201.26, -201.28, -201.32, -201.35, -201.37, -201.40. HRMS (ESI+) m/z calcd for $C_{10}H_{12}FIN_2NaO_5$ [M + Na]+ 408.9673, found 408.9655.

*3'-O-Benzoyl-2'-deoxy-2'-fluoro-5'-iodo-4'-methoxy arabino uridine (**8**).* A suspension of the nucleoside **6** (3.4 g, 8.854 mmol) was prepared in pyridine (13 mL). Benzoyl chloride (1.2 mL, 10.63 mmol) was slowly added dropwise to the mixture, and the reaction was left to stir at room temperature overnight, following which the solvents were concentrated under vacuum. The resulting residue was purified by column chromatography (2-5% MeOH/CH_2Cl_2) to afford the product as a yellow foam (3.4 g, 78%): Rf (10% MeOH/CH_2Cl_2) 0.78; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.59 (s, 1H), 8.11 – 7.98 (m, 2H), 7.84 (dd, *J* = 8.1, 1.9 Hz, 1H), 7.74 – 7.66 (m, 1H), 7.62 – 7.50 (m, 2H), 6.33 (dd, *J* = 10.8, 5.9 Hz, 1H), 5.99 (dd, *J* = 24.1, 4.8 Hz, 1H), 5.75 (t, *J* = 4.1 Hz, 2H), 3.89 (d, *J* = 11.5 Hz, 1H), 3.71 (d, *J* = 11.4 Hz, 1H), 3.35 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.9, 161.9, 149.2, 141.0, 133.0, 128.8, 127.9, 101.0, 100.2, 93.7, 92.2,

77.6, 48.2, 2.1. ^{19}F NMR (471 MHz, DMSO-d_6) δ -198.48, -198.51, -198.56, -198.59, -198.61, -198.64, -198.67. HRMS (ESI+) m/z calcd for $C_{17}H_{16}FIN_2NaO_6$ [M + Na]+ 512.9935, found 512.9922.

*5′-O-Benzoyl-2′-deoxy-2′-fluoro-4′-methoxy arabino uridine (**10**).* A solution of nucleoside **8** (1 g, 2.041 mmol) was prepared in CH_2Cl_2 (28 mL) and H_2O (2.3 mL). 3-Chloroperoxybenzoic acid (*m*CPBA) (77% purity, 1.8 g, 8.163 mmol) was added slowly to the stirring solution. The reaction was heated to 40 ^oC and allowed to react for 6 hours, after which the solvents are concentrated and the resulting residue was purified by column chromatography (30-80% EtOAc in hexanes) to afford the product as a white foam (840 mg, 85%): R_f (50% EtOAc/hexanes) 0.47; ^{1}H NMR (500 MHz, CD_3CN) δ 9.16 (s, 1H), 8.05 (dt, *J* = 8.4, 1.8 Hz, 2H), 7.69 – 7.64 (m, 1H), 7.55 – 7.50 (m, 2H), 7.40 (dd, *J* = 8.1, 1.7 Hz, 1H), 6.30 (dd, *J* = 11.4, 5.3 Hz, 1H), 5.46 (d, *J* = 8.2 Hz, 1H), 5.17 (dt, *J* = 53.9, 5.1 Hz, 1H), 4.67 (dd, *J* = 12.2, 1.0 Hz, 1H), 4.55 (d, *J* = 8.9 Hz, 1H), 3.78 (s, 1H), 3.47 (s, 3H), 3.45 – 3.31 (m, 1H). ^{13}C NMR (126 MHz, CD_3CN) δ 167.0, 165.6, 151.8, 142.3, 134.7, 130.7, 129.8, 103.8, 102.5, 97.4, 95.8, 82.7, 77.9, 62.0, 50.7. ^{19}F NMR (471 MHz, CD_3CN) δ -202.02, -202.05, -202.07, -202.10, -202.14, -202.16, -202.19, -202.21. HRMS (ESI+) m/z calcd for $C_{17}H_{17}FN_2NaO_7$ [M + Na]+ 403.0917, found 403.0899.

*2′-deoxy-2′-fluoro-4′-methoxy arabino uridine (**12**)*. A solution of the nucleoside **10** (290 mg, 0.7513 mmol) was prepared in methanolic ammonia (2M, 10 mL) and stirred overnight at room temperature. The resulting mixture was evaporated to remove solvents and then purified by column chromatography (2-20% MeOH/CH_2Cl_2) to afford the product as a white foam (180 mg, 86%): R_f (10% MeOH/DCM) 0.31; ^{1}H NMR (500 MHz, CD_3OD) δ 7.84 (dd, *J* = 8.1, 1.2 Hz, 1H), 6.32 (dd, *J* = 8.1, 5.8 Hz, 1H), 5.71 (d, *J* = 8.1 Hz, 1H), 5.20 (dt, *J* = 54.8, 5.8 Hz, 1H), 4.50 (dd, *J* = 24.7, 5.8 Hz, 1H), 3.90 (dd, *J* = 12.0, 1.6 Hz, 1H), 3.68 (d, *J* = 12.0 Hz, 1H), 3.40 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 165.9, 152.0, 142.6, 105.8, 102.4, 97.6, 96.0, 82.3, 75.4, 59.7, 50.3. ^{19}F NMR (471 MHz, CD_3OD) δ -202.82, -202.84, -202.88, -202.89, -202.94, -202.96, -202.99, -203.01. HRMS (ESI+) m/z calcd for $C_{10}H_{13}FN_2NaO_6$ [M + Na]+ 299.0655, found 299.0641.

*5′-O-[Bis(4-methoxyphenyl)phenylmethyl]-2′-deoxy-2′-fluoro-4′-methoxy arabino uridine (**14**).* A solution of nucleoside **12** (240 mg, 0.869 mmol) was prepared in pyridine (12 mL) under argon. 4,4′-dimethoxytrityl chloride (412 mg, 1.217 mmol) was added, and the reaction was allowed to

stir for 2 h at 45 °C. Pyridine was subsequently evaporated under vacuum, and the resulting residue purified by column chromatography using de-acidified silica gel by treatment with 10% trimethylamine in CH_2Cl_2 and MeOH/CH_2Cl_2 washing (1−3% MeOH/CH_2Cl_2). This afforded dimethoxytritylated nucleoside **14** as a white foam (390 mg, 78%). R_f (10% MeOH/CH_2Cl_2) 0.57; ^{1}H NMR (500 MHz, CD_3CN) δ 9.16 (s, 1H), 7.64 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.47 – 7.41 (m, 2H), 7.38 – 7.23 (m, 8H), 6.90 (d, *J* = 8.8 Hz, 4H), 6.27 (dd, *J* = 8.5, 5.7 Hz, 1H), 5.34 (d, *J* = 8.2 Hz, 1H), 5.18 (dt, *J* = 54.4, 5.7 Hz, 1H), 4.62 (dd, *J* = 23.5, 5.6 Hz, 1H), 3.78 (s, 6H), 3.68 (s, 1H), 3.45 (d, *J* = 10.3 Hz, 1H), 3.30 (d, *J* = 10.3 Hz, 1H), 3.17 (s, 3H), 1.97 (s, 1H). ^{13}C NMR (126 MHz, CD_3CN) δ 163.6, 159.8, 151.1, 145.5, 141.6, 136.2, 136.2, 131.1, 129.0, 128.1, 114.2, 104.4, 104.3, 102.5, 97.18, 95.65, 87.91, 81.91, 81.77, 76.65, 76.46, 61.50, 55.95, 50.47, 34.75, 23.06, 15.05. ^{19}F NMR (471 MHz, CD_3CN) δ -202.56, -202.58, -202.61, -202.63, -202.67, -202.69, -202.72, -202.74. HRMS (ESI+) m/z calcd for $C_{31}H_{31}FN_2NaO_8$ [M + Na]+ 601.1962, found 601.1955.

*5'-O-[Bis(4-methoxyphenyl)phenylmethyl]-2'-deoxy-2'-fluoro-4'-methoxy arabino uridine, 3'-[2-cyanoethyl N,N-bis(1-methylethyl)phosphoramidite] (**16**).* Dimethoxytritylated nucleoside **14** (250 mg, 0.432 mmol), was dried under vacuum overnight and coevaporated with MeCN four times to remove water. Dry THF (4.5 mL) was added under argon. To the solution was added $EtN(iPr)_2$ (385 µL, 2.162 mmol) and then $ClPOCEtN(iPr)_2$ (106 µL, 0.476 mmol). The reaction was allowed to stir for 7 hours at room temperature. After the reaction reached completion, the mixture was extracted using DCM and saturated sodium bicarbonate, the organic phases were collected and dried using $MgSO_4$ to finally be filtered and concentrated for direct loading on a deacidified silica column (eluent: 30-70% EtOAc/hexanes) to afford phosphoramidite **16** as a white foam (290 mg, 86%). R_f (3:2 EtOAc/hexanes) 0.66. ^{1}H NMR (500 MHz, CD_3CN) δ 9.01 (s, 1H), 7.76 (td, *J* = 8.0, 1.3 Hz, 1H), 7.47 – 7.38 (m, 2H), 7.37 – 7.22 (m, 7H), 6.92 – 6.85 (m, 4H), 6.38 (ddd, *J* = 20.4, 7.3, 6.1 Hz, 1H), 5.47 – 5.30 (m, 1H), 5.19 (dd, *J* = 23.9, 8.2 Hz, 1H), 5.04 – 4.89 (m, 1H), 4.06 (q, *J* = 7.1 Hz, 1H), 3.78 (d, *J* = 2.6 Hz, 6H), 3.67 – 3.50 (m, 4H), 3.29 (t, *J* = 9.8 Hz, 1H), 3.19 (d, *J* = 8.4 Hz, 3H), 2.63 (t, *J* = 6.0 Hz, 1H), 2.58 – 2.50 (m, 1H), 1.23 – 1.09 (m, 10H), 1.04 (d, *J* = 6.7 Hz, 2H). ^{13}C NMR (126 MHz, CD_3CN) δ 163.4, 159.9, 159.9, 145.4, 141.6, 141.3, 136.0, 131.3, 131.2, 129.2, 129.0, 128.2, 128.2, 114.2, 102.5, 88.20, 61.60, 60.26, 60.12, 59.55, 55.97, 55.94, 50.98, 50.77, 44.35, 44.25, 44.19, 44.09, 24.98, 24.92, 24.87, 24.78, 24.73, 24.68, 20.74, 14.51. ^{19}F NMR (471 MHz, CD_3CN) δ -199.10, -199.11, -199.13, -

199.15, -199.16, -199.18, -199.21, -199.23, -199.25, -199.26, -199.28, -199.30, -199.46, -199.47, -199.49, -199.51, -199.53, -199.54, -199.56, -199.58, -199.59, -199.63, -199.64, -199.66. HRMS (ESI+) m/z calcd for $C_{40}H_{48}FN_4NaO_9P$ [M + Na]+ 801.3041, found 801.3036.

Synthesis of 2′-OMe,4′-F-rU. *2',5'-Dideoxy-2'-methoxy-5'-iodo-4'-fluorouridine (**17**):* A suspension of the nucleoside **3** (100 mg, 0.417 mmol) and AgF (211 mg, 1.667 mmol) was prepared in dry acetonitrile (6 mL). The suspension was brought to 0 ^oC followed by dropwise addition of a solution of I_2 (211 mg, 0.833 mmol) in dry acetonitrile (3.4 mL) over a period of 10 minutes. The reaction mixture was then allowed to stir for 30 minutes and then extracted with ethyl acetate and brine to afford the product as a yellow foam (104 mg, 65%): Rf (10% MeOH/DCM) 0.45; ^1H NMR (500 MHz, CD_3OD) δ 7.60 (d, *J* = 8.0 Hz, 1H), 5.93 (d, *J* = 2.1 Hz, 1H), 5.73 (d, *J* = 8.1 Hz, 1H), 4.62 (dd, *J* = 19.4, 7.2 Hz, 1H), 4.11 – 4.07 (m, 1H), 3.63 (dd, *J* = 11.5, 5.6 Hz, 1H), 3.55 (d, *J* = 11.6 Hz, 1H), 3.52 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 165.9, 151.6, 143.7, 115.2, 103.5, 94.07, 82.55, 72.80, 59.86, 2.49. ^{19}F NMR (471 MHz, CD_3OD) δ -112.56, -112.57, -112.60, -112.61, -112.64, -112.65. HRMS (ESI+) m/z calcd for $C_{10}H_{12}FIN_2NaO_5$ [M + Na]+ 408.9673, found 408.9652.

*3'-O-Benzoyl-2'-deoxy-2'-methoxy-5'-iodo-4'-fluorouridine (**18**)*: A suspension of the nucleoside **17** (400 mg, 1.042 mmol) was prepared in pyridine (1.5 mL). Benzoyl chloride (145 µL, 1.250 mmol) was slowly added dropwise to the mixture, and the reaction was left to stir at room temperature overnight, following which the solvents were concentrated under vacuum. The resulting residue was purified by column chromatography (2-5% MeOH/DCM) to afford the product as a yellow foam (420 mg, 83%): Rf (50% EtOAc/hexanes) 0.43; ^1H NMR (500 MHz, CD_3CN) δ 9.13 (s, 1H), 8.09 (dd, *J* = 8.3, 1.4 Hz, 2H), 7.69 (t, *J* = 7.5 Hz, 1H), 7.49 (t, *J* = 7.8 Hz, 2H), 7.44 (d, *J* = 8.1 Hz, 1H), 5.89 (d, *J* = 2.5 Hz, 1H), 5.71 – 5.68 (m, 1H), 5.67 (d, *J* = 7.3 Hz, 1H), 4.49 (dd, *J* = 7.2, 2.6 Hz, 1H), 3.70 (dd, *J* = 11.8, 8.1 Hz, 1H), 3.57 (dd, *J* = 21.6, 11.8 Hz, 1H), 3.35 (s, 3H), 2.11 (s, 1H). ^{13}C NMR (126 MHz, CD_3CN) δ 167.8, 166.2, 163.7, 151.0, 143.4, 134.8, 134.1, 130.7, 129.5, 103.7, 95.33, 80.78, 73.12, 59.87, 3.42. ^{19}F NMR (471 MHz, CD_3CN) δ -107.29, -107.31, -107.33, -107.33, -107.34, -107.35, -107.37, -107.39. HRMS (ESI+) m/z calcd for $C_{17}H_{16}FIN_2NaO_6$ [M + Na]+ 512.9935, found 512.9929.

*5'-O-Benzoyl-2'-deoxy-2'-methoxy-4'-fluorouridine (**19**)*: A solution of nucleoside **18** (560 mg, 1.143 mmol) was prepared in CH_2Cl_2 (32 mL) and H_2O (1.8 mL). 3-Chloroperoxybenzoic acid

(*m*CPBA) (77% purity, 1.03 g, 4.571 mmol) was added slowly to the stirring solution. The reaction was heated to 40 ^{o}C and allowed to react for 6 hours, after which the solvents are concentrated and the resulting residue was purified by column chromatography (30-70% EtOAc in hexanes) to afford the product as a white foam (360 mg, 83%): R_f (50% EtOAc/hexanes) 0.53; ^{1}H NMR (500 MHz, CD_3CN) δ 9.10 (s, 1H), 8.06 – 8.02 (m, 2H), 7.68 – 7.64 (m, 1H), 7.54 – 7.50 (m, 2H), 7.33 (d, *J* = 8.2 Hz, 1H), 5.92 (d, *J* = 1.4 Hz, 1H), 5.49 (dd, *J* = 8.2, 1.4 Hz, 1H), 4.62 – 4.60 (m, 1H), 4.58 (d, *J* = 7.5 Hz, 1H), 4.54 (d, *J* = 7.5 Hz, 1H), 4.04 (dd, *J* = 6.9, 1.5 Hz, 1H), 3.61 (s, 1H), 3.51 (s, 3H). ^{13}C NMR (126 MHz, CD_3CN) δ 166.3, 163.7, 150.8, 142.3, 134.6, 130.6, 129.7, 103.3, 93.89, 81.07, 71.61, 71.43, 62.70, 62.36, 60.02. ^{19}F NMR (471 MHz, CD_3CN) δ -121.94, -121.96, -121.97, -121.99, -122.00, -122.02. HRMS (ESI+) m/z calcd for $C_{17}H_{17}FN_2NaO_7$ [M + Na]+ 403.0917, found 403.0911.

*2′-deoxy-2′-methoxy-4′-fluorouridine (**20**)*. A solution of the nucleoside **19** (140 mg, 0.3646 mmol) was prepared in methanolic ammonia (2M, 10 mL) and stirred overnight at room temperature. The resulting mixture was evaporated to remove solvents and then purified by column chromatography (2-20% MeOH/CH_2Cl_2) to afford the product as a white solid (85 mg, 95%): R_f (10% MeOH/DCM) 0.38. ^{1}H NMR (500 MHz, CD_3OD) δ 7.84 (d, *J* = 8.1 Hz, 1H), 6.11 (d, *J* = 1.4 Hz, 1H), 5.69 (d, *J* = 8.1 Hz, 1H), 4.48 (dd, *J* = 20.2, 6.6 Hz, 1H), 3.90 (dd, *J* = 6.6, 1.5 Hz, 1H), 3.77 (dd, *J* = 11.9, 3.5 Hz, 1H), 3.73 (dd, *J* = 11.9, 3.8 Hz, 1H), 3.57 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 166.0, 151.7, 142.2, 119.4, 117.6, 91.85, 83.01, 70.14, 60.91, 59.74. ^{19}F NMR (471 MHz, CD_3OD) δ -124.29, -124.30, -124.31, -124.34, -124.34, -124.35. HRMS (ESI+) m/z calcd for $C_{10}H_{13}FN_2NaO_6$ [M + Na]+ 299.0655, found 299.0639.

*5′-O-[Bis(4-methoxyphenyl)phenylmethyl]-2′-deoxy-2′-methoxy-4′-fluoro uridine (**21**).* A solution of nucleoside **20** (80 mg, 0.290 mmol) was prepared in pyridine (4 mL) under argon. 4,4′-dimethoxytrityl chloride (137 mg, 0.406 mmol) was added, and the reaction was allowed to stir for 2 h at 45 °C. Pyridine was subsequently evaporated under vacuum, and the resulting residue purified by column chromatography using de-acidified silica gel by treatment with 10% trimethylamine in CH_2Cl_2 and MeOH/CH_2Cl_2 washing (1−3% MeOH/CH_2Cl_2). This afforded dimethoxytritylated nucleoside **21** as a white foam (100 mg, 60%). R_f (10% MeOH/CH_2Cl_2) 0.62; ^{1}H NMR (500 MHz, CD_3OD) δ 7.87 (d, *J* = 8.1 Hz, 1H), 7.42 – 7.38 (m, 2H), 7.35 – 7.31 (m, 2H), 7.30 – 7.26 (m, 5H), 6.90 – 6.86 (m, 4H), 6.10 (d, *J* = 1.0 Hz, 1H), 5.27 (d, *J* = 8.1 Hz,

1H), 4.73 (dd, *J* = 21.2, 6.5 Hz, 1H), 3.96 (dd, *J* = 6.5, 1.0 Hz, 1H), 3.79 (s, 6H), 3.58 (d, *J* = 1.8 Hz, 3H), 3.51 (dd, *J* = 10.0, 3.2 Hz, 1H), 3.37 – 3.33 (m, 1H). ^{13}C NMR (126 MHz, CD_3OD) δ 166.0, 160.4, 151.6, 145.5, 142.2, 136.4, 136.1, 131.4, 129.4, 129.0, 128.2, 119.0, 117.2, 114.3, 102.9, 92.14, 88.73, 82.93, 70.83, 62.30, 61.93, 59.84, 55.74. ^{19}F NMR (471 MHz, CD_3OD) δ -120.41, -120.45, -121.06, -121.11. HRMS (ESI+) m/z calcd for $C_{31}H_{31}FN_2NaO_8$ [M + Na]+ 601.1962, found 601.1932 .

*5'-O-[Bis(4-methoxyphenyl)phenylmethyl]-2'-deoxy-2'-fluoro-4'-methoxy arabino uridine, 3'-[2-cyanoethyl N,N-bis(1-methylethyl)phosphoramidite] (**22**).* Dimethoxytritylated nucleoside **21** (140 mg, 0.242 mmol), was coevaporated with MeCN four times to remove water and dried under vacuum overnight. Dry DCM (0.540 mL) was added under argon. 2-Cyanoethyl N,N,N,N-tetraisopropyldiphosphoramidite (77 μL, 0.242 mmol) was added to the solution followed by dropwise addition of a solution of 4,5-dicyanoimidazole (DCI) in MeCN (26 mg, 0.243 mL). The reaction mixture was stirred for 30 minutes at room temperature. The reaction progress was monitored by TLC (1:1 EtOAc, Hexanes). After the reaction reached completion, the mixture was extracted using DCM and saturated sodium bicarbonate, the organic phases were collected and dried using Na_2SO_4 to finally be filtered and concentrated for direct loading on a deacidified silica column (eluent: 30-70% EtOAc in hexanes) to afford phosphoramidite **22** as a white foam (120 mg, 64%). R_f (50% EtOAc/hexanes) 0.48. ^{1}H NMR (500 MHz, CD_3CN) δ 9.03 (s, 1H), 7.64 (dd, *J* = 29.1, 8.1 Hz, 1H), 7.46 – 7.38 (m, 2H), 7.36 – 7.23 (m, 7H), 6.92 – 6.83 (m, 4H), 6.06 (d, *J* = 4.6 Hz, 1H), 5.25 (dd, *J* = 8.1, 2.5 Hz, 1H), 4.84 – 4.67 (m, 1H), 4.09 – 4.00 (m, 1H), 3.78 (d, *J* = 3.2 Hz, 6H), 3.74 – 3.68 (m, 1H), 3.62 (dq, *J* = 10.5, 6.7 Hz, 2H), 3.50 (s, 3H), 3.44 – 3.32 (m, 2H), 3.12 (dd, *J* = 7.3, 5.6 Hz, 1H), 2.71 – 2.49 (m, 2H), 1.21 – 1.10 (m, 9H), 1.06 – 0.96 (m, 3H). ^{13}C NMR (126 MHz, CD_3CN) δ 163.7, 159.9, 159.9, 150.9, 145.3, 141.5, 141.3, 135.9, 131.3, 131.2, 131.2, 129.1, 129.0, 128.2, 114.2, 103.1, 103.0, 92.77, 92.57, 88.10, 82.10, 81.54, 70.70, 59.75, 59.71, 59.59, 59.51, 55.96, 55.93, 44.24, 44.13, 44.03, 25.07, 25.01, 24.98, 24.93, 24.80, 24.75, 21.03, 20.97, 15.07. ^{19}F NMR (471 MHz, CD_3CN) δ -117.92, -117.96, -118.65, -118.69. ^{31}P NMR (203 MHz, CD_3CN) δ 151.33, 150.22. HRMS (ESI+) m/z calcd for $C_{40}H_{48}FN_4NaO_9P$ [M + Na]+ 801.3041, found 801.3034. Note: When $ClPOCEtN(iPr)_2$ was used as the phosphitylating reagent for this reaction, it produced significantly lower yield of product..

Computational Methods. All computations were performed according to our previously established protocol.[28]

Solid Phase Synthesis. All oligonucleotides (ONs) were synthesized on an Applied Biosystems (ABI) 3400 DNA synthesizer at 1 μmol scale using Unylinker CPG as solid support, mesh 500A. DNA phosphoramidites and 2′,4′-modified phosphoramidites were prepared as 0.1 M solutions in acetonitrile, RNA phosphoramidites as 0.15 M solutions in acetonitrile. 5-Ethylthiotetrazole was used as activator, 3% trichloroacetic acid in dichloromethane was used to deblock DMTr groups, acetic anhydride in tetrahydrofuran (THF) and 16% N-methylimidazole in THF was used to cap unreacted 5′-OH groups, and 0.1 M I_2 in 1:2:10 pyridine/water/THF was used for oxidation of phosphite triester linkages. DNA monomers were coupled for 200 s (300 s for dG); all other phosphoramidites were coupled for 600 s (900 s for rG). All 2′,4′-modified phosphoramidites were coupled for 1200 s. Deprotection and cleavage from the solid support for DNA oligonucleotides was achieved with concentrated aqueous ammonia for 48 h at room temperature. After decanting to remove the CPG, the deprotection solution was removed under vacuum in a SpeedVac lyophilizer. For RNA-containing oligonucleotides, deprotection and cleavage from the solid support was achieved with 3:1 aqueous ammonia/EtOH for 48 h at room temperature, and desilylation was achieved in neat TREAT-HF (150 μL) with shaking at room temperature for 48 h. Purifications were performed by HPLC, using a Protein Pak DEAE 5PW analytical anionexchange column. A stationary phase of Milli-Q water and a mobile phase of 1 M $LiClO_4$ in water was used for analysis and purification using a gradient of 0−60% over 37 min. Following purification, excess $LiClO_4$ salts were removed using NAP-25 sephadex size-exclusion columns. Oligonucleotides were quantitated by UV (extinction coefficients were determined using the IDT OligoAnalyzer tool (www.idtdna.com/analyzer/Applications/OligoAnalyzer). Extinction coefficients for RNA were used for oligonucleotides containing 2′,4′-modifications.

Thermal Denaturation Studies. Equimolar amounts of complementary sequences were combined, dried, and rediluted in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1 mM EDTA (1 mL). They were then transferred into cuvettes in a Varian UV spectrophotometer. The samples were heated at 90 °C and the cooled to 5 °C. The change in

absorbance at 260 nm was then monitored upon heating from 5 to 90 °C. Melting temperatures were determined using the hyperchromicity method.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR spectra for compounds **1**-**22**, some crystal structures, and additional computational results such as relevant electronic effects, PMFs and puckering distributions for selected nucleosides. Biological activity of nucleosides **11**, **12**, **20**, and **23** against 60 cancer cell lines from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute is included. Additional T_m data and mass analysis of reported oligonucleotides. Available free of charge from the ACS website.

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