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Efficient Synthesis of [2′-^{18}O]Uridine and Its Incorporation into Oligonucleotides: A New Tool for Mechanistic Study of Nucleotidyl Transfer Reactions by Isotope Effect Analysis

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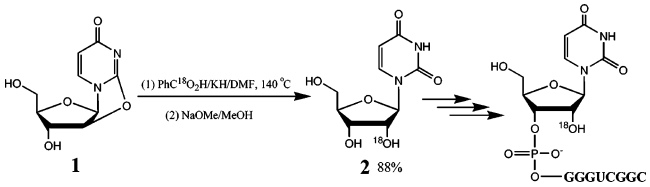

Lack of sufficient quantities of isotopically labeled materials has precluded the use of heavy atom isotope effects to investigate mechanisms of nucleotidyl transfer reactions in nucleic acids. Here we achieve regioselective opening of 2,2′-cyclouridine with [^{18}O$_2$]benzoic acid/potassium hydride, allowing an efficient "one-pot" synthesis of [2′-^{18}O]uridine in 88% yield. Conversion to the corresponding phosphoramidite enables solid-phase synthesis of [2′-^{18}O] RNA substrates for isotope effect studies with nucleotidyl transferases and hydrolases.

A wide range of enzymes, including both protein and RNA catalysts, promotes cleavage of RNA phosphodiester bonds by attack of the adjacent 2′-OH and displacement of the 5′-O leaving group. In solution, this reaction is catalyzed by both acid and base, and both stepwise and concerted reaction mechanisms are observed.[1] Despite significant effort, little direct experimental evidence exists that determines which specific mechanism is followed for enzyme-catalyzed RNA cleavage. The effect of isotopic substitution on chemical reaction kinetics and equilibria provides an especially powerful way to investigate enzymatic transition states and their active site interactions. Isotopic substitution represents the smallest possible chemical perturbation of a catalytic system but can nonetheless have important influences on chemical reactivity.[2] Interpretation of these isotope effects on reactivity can provide essential information for defining chemical mechanism. For nucleotidyl transfer reactions, the nucleophile isotope effect on the attacking 2′-O in particular offers the opportunity to distinguish unambiguously between stepwise and concerted reaction mechanisms.[3]

Technical challenges severely limit the use of isotope effects to study phosphoryl transferase enzymes that operate on nucleic acids. One such limitation involves access to nucleic acid substrates bearing the desired site-specific isotope enrichment. Here we describe an efficient synthesis of [2′-^{18}O]uridine, which enables synthesis of RNA substrates for isotope effect analyses on protein and RNA enzymes that catalyze nucleophilic attack by the 2′-OH.

Few strategies exist for the synthesis of isotope-enriched nucleosides, particularly sugar isotopomers.[4] Previously, Pang et al. obtained [3′-^{18}O]uridine in low yield via reversible hydration (HCl/H$_2$^{18}O) of a 3′-ketouridine derivative followed by reduction and separation from the predominant xylo epimer.[5] Recently, Wnuk et al. reported access to [3′-^{18}O]-1-(β-D-arabinofuranosyl)uracil through a six-step synthesis from 2,3′-cyclouridine involving a Fox thermal rearrangement as the key step.[6] However, this strategy generated arabinofuranosyl derivatives and could not be used to prepare [2′-^{18}O]uridine. Another strategy reported in the literature to prepare ^{18}O-labeled sugar isotopomers makes use of S$_N$2-substituted reaction of triflate with ^{18}O-labeled nucleophiles.[7] Unfortunately, the 2′-β triflate derivative of uridine failed to undergo the analogous reaction to generate [2′-^{18}O]uridine.

Commercially available 2,2′-cyclouridine (**1**) could provide direct access to [2′-^{18}O]uridine (**2**) if an ^{18}O-enriched oxygen nucleophile can be made to attack the ribose C-2′ regioselectively. Initial attempts were directed toward hydrolysis of **1** under alkaline conditions (1 N NaOH, MeOH, rt, overnight). Consistent with previous observations,[6] the reaction gave only 1-(β-D-arabinofuranosyl)uracil, indicating that hydroxide attacks exclusively at the nucleobase C-2 position. Therefore, to gain access to **2** from **1**, we sought to identify an oxygen nucleophile with altered regioselectivity.

Ueda has summarized the reactions of 2,2′-cyclouridine with various nucleophiles.[8] Unfortunately, none of the reported examples (Table 1) can be used to synthesize [2′-^{18}O]uridine (**2**). In general, reactions of 2,2′-cyclouridine with nucleophiles have two possible regiochemical outcomes: (1) attack at the

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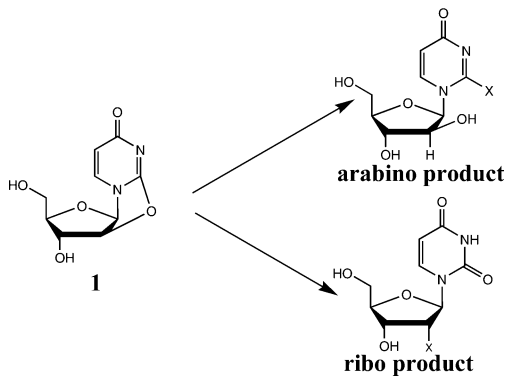
TABLE 1. Reaction of 2,2′-Cyclouridine with Various Nucleophiles

entry	nucleophile	solvent	*T* (°C)	time (h)	product	yield[a] (%)
1[6]	NaOH	MeOH/H_2O	rt	16	arabino	69
2[11]	$Mg(OMe)_2$	MeOH	65	5	ribo	92
3[12]	$B(OMe)_3$	MeOH+$CH(OMe)_3$	150	42	ribo	86
4[13]	NH_3	MeOH	37	48−168	arabino	16
5[14]	NaN_3+BzOH	HMPA	150	<1	ribo	65
6[15]	H_2S+Et_3N	DMF	20	120	arabino	70
7[16]	EtSH+reagent 1[b]	DMF	60	12	ribo	93
8[16]	*t*-BuSH+reagent 1[b]	DMF	100	16	ribo	94
9[17]	AcSH	dioxane	110	6	ribo	65
10[18]	PhSe−SePh+$NaBH_4$	EtOH	78	1	ribo	90
11[19]	HF	dioxane	100−110	18	ribo	40−50
12[20]	HCl	dioxane	75−80	18	ribo	89
13[21]	LiBr	dioxane	60	6	ribo	98
14[22]	NaI+TsOH·H_2O	acetone	50	2.5	ribo	98

[a] Yields are for pure ribo and arabino product. [b] Reagent 1: *N,N,N′,N′*-tetramethylguanidine.

SCHEME 1. The Two Modes of Nucleophilic Attack to 2,2′-Cyclouridine



nucleobase C-2 (nucleobase attack) to give C-2-substituted 1-(β-D-arabinofuranosyl)uracil derivatives, or (2) attack at the pentose C-2′ (ribose attack) to give 2′-substituted uridine derivatives (Scheme 1).[9] Inspection of the literature data reveals an apparent regioselectivity trend: for the same nucleophilic atom, the nucleophile associated with substituents having large electronegativity values (χ_P)[10] tends to favor ribose attack. To illustrate, for sulfur nucleophiles, *t*-BuSH, EtSH, or AcSH (χ_P of C is 2.55) favor ribose attack,[16,17] while H_2S (χ_P of H is 2.20) favors nucleobase attack.[15] For oxygen nucleophiles, NaOMe (χ_P of Na is 0.93) favors nucleobase attack, while $Mg(OMe)_2$ (χ_P of Mg is 1.31) and $B(OMe)_3$ (χ_P of B is 2.03) favor ribose attack.[11,12] With nitrogen nucleophiles, NH_3 (χ_P of H is 2.20) favors nucleobase attack,[13] whereas NaN_3 (χ_P of N is 3.04) favors ribose attack.[14] We used this apparent empirical trend to guide our experiments. To favor nucleophilic attack at ribose, we sought to replace the hydrogen atoms of H_2O with substituents that have larger χ_P values.

Although in dimethylformamide (DMF) $Mg(OMe)_2$ favors ribose attack to form 2′-*O*-methyluridine,[11] we cannot access uridine readily from 2′-*O*-methyluridine because the methyl group is difficult to remove. We reasoned that sodium acetate (NaOAc) might favor ribose attack as an acetyl group withdraws electrons more strongly than a methyl group. Hydrolytic removal of the acetyl group from the resulting product would then allow facile access to uridine. The commercial availability of [$^{18}O_2$]-NaOAc makes this strategy especially attractive. We heated NaOAc with **1** in DMF at 140 °C for 24 h. TLC showed that uridine formed along with the regioisomer 1-(β-D-arabinofuranosyl)uracil in a ratio of 2:3 ribo/arabino. Using potassium acetate (KOAc) rather than NaOAc improved the ribo/arabino ratio to 3:2. Despite this partial success, the poor solubility of NaOAc and KOAc in DMF and the significant amount of byproduct from nucleobase attack (which in larger scale reactions proved difficult to separate from the desired uridine by column chromatography) led us to explore other alternatives.

To enhance solubility in DMF, we tested potassium benzoate (KOBz) as the nucleophile, hoping that the greater electron-withdrawing power[23] of the benzoyl moiety (relative to acetyl moiety) might improve regioselectivity. We heated a mixture of **1** (1.0 equiv) and KOBz (1.0 equiv) in DMF at 140 °C and monitored the reaction by TLC. After 1 week, TLC revealed that little starting material remained, and that two major products and one minor product had formed. The major products corresponded to uridine and 2′-*O*-benzoyluridine. The minor product co-migrated with 1-(β-D-arabinofuranosyl)uracil and presumably forms via benzoate attack on the nucleobase. After treating the crude reaction mixture with NaOMe/MeOH to

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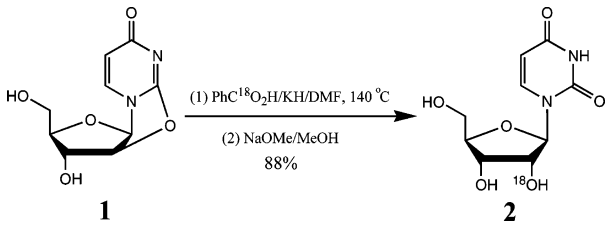
SCHEME 2. Synthesis of [2′-^{18}O]Uridine



convert 2′-*O*-benzoyluridine to uridine, the ^{1}H NMR spectrum indicated that uridine and 1-(β-D-arabinofuranosyl)uracil formed in a 5:1 ratio.

Using 2 equiv of KOBz instead of 1 equiv had little effect on the reaction rate or the ratio of the ribo/arabino products. However, we found that the presence of benzoic acid accelerated the reaction and improved regioselectivity further.[24] A mixture of BzOH (1.0 equiv) and KOBz (1.0 equiv) at 140 °C in DMF consumes **1** completely within 48 h to give the products in a ratio of 20:1 ribo/arabino.[25] The increased reaction rate and regioselectivity upon addition of benzoic acid may reflect acid catalysis, in which protonation of the imino nitrogen alters the regiochemical outcome of the reaction.

We then exploited our observations to synthesize the target isotopomer, **2** (Scheme 2). Acidic hydrolysis of benzonitrile in $H_2{}^{18}O$ gave [$^{18}O_2$]benzoic acid containing 80% isotope enrichment (MS).[6] To avoid loss of ^{18}O, we prepared a mixture of 1:1 [$^{18}O_2$]benzoic acid and potassium [$^{18}O_2$]benzoate by treating [$^{18}O_2$]benzoic acid (2.0 equiv) with potassium hydride (1.0 equiv) in DMF. After addition of **1** (1.0 equiv), the mixture was heated to 140 °C. After 4 days, TLC showed that **1** was almost completely consumed. After removal of DMF under high vacuum, we treated the residue with NaOMe/MeOH to convert 2′-[$^{18}O_2$]benzoyluridine to [2′-^{18}O]uridine. After column chromatography, we obtained **2** in 88% yield (80% isotope enrichment as determined by MS). As expected, the new compound gave essentially the same ^{1}H and ^{13}C NMR spectra as an authentic sample of uridine. In addition, we observed an ^{18}O-induced ^{13}C NMR shift[26] of 1.54 Hz upfield for 2′-C of [2′-^{18}O]uridine, further confirming the position of ^{18}O.[7a]

We transformed [2′-^{18}O]uridine to the corresponding phosphoramidite **3** using standard methods (see Supporting Information).[27] We used **3** to incorporate [2′-^{18}O]uridine into two oligonucleotides, [2′-^{18}O]UG and [2′-^{18}O]UGGGUCGGC, according to standard RNA synthesis protocols. After deprotection and purification by reversed-phase HPLC, MALDI-TOF MS confirmed the masses of the oligonucleotides, giving the expected [M − H]$^-$ peaks at *m*/*z* 590 and 2883, while the wild-type 5′-UG and 5′-UGGGUCGGC gave [M − H]$^-$ peaks at *m*/*z* 588 and 2881, respectively.

In conclusion, regiochemistry of nucleophilic reactions with 2,2′-cyclouridine appears to be influenced by the chemical context of the nucleophilic atom and the presence of acid. Using these hypotheses as a guide, we developed reaction conditions in which an oxygen nucleophile (potassium benzoate in the presence of benzoic acid) favors ribose attack over nucleobase attack by 20-fold. This regioselectivity allows for efficient synthesis of [2′-^{18}O]uridine and its phosphoramidite from 2,2′-cyclouridine and [$^{18}O_2$]benzoic acid. This approach may also be applied for converting the 2,3′-cyclouridine to [3′-^{18}O]uridine. We can now construct RNA substrates containing [2′-^{18}O]-uridine isotopologues, thereby enabling isotope effect analyses of protein and RNA enzymes that catalyze 2′-*O*-transphosphorylation reactions. Through known transformations of uridine,[28] we may also access [2′-^{18}O] isotope-enriched cytidine, adenosine, and guanosine.

Experimental Section

[2′-^{18}O]Uridine (2): To a pressure tube (35 mL) under argon were added anhydrous DMF (20 mL), KH (35%, 457 mg, 4 mmol), and [$^{18}O_2$]benzoic acid (1.01 g, 8 mmol, 2 equiv). After the reaction was stirred for 10 min at rt, 2,2′-cyclouridine (904 mg, 4 mmol, 1 equiv) was added. The mixture was heated to 140 °C for 4 days. The reaction mixture was concentrated to dryness under vacuum. The residue was dissolved in methanol (20 mL), and sodium methoxide in methanol (30%, 1.52 mL, 2 equiv) was added. The mixture was stirred overnight at rt, and acetic acid (690 μL, 3 equiv) was added. The mixture was stirred at rt for an additional 10 min and concentrated to dryness under vacuum. The residue was purified by silica gel chromatography, eluting with 10% methanol in ethyl acetate, to give **2** (859 mg, 88%) as a white solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.30 (br, 1H), 7.88 (d, J = 8.2 Hz, 1H), 5.78 (m, 1H), 5.65 (d, J = 8.2 Hz, 1H), 5.36 (br, 1H), 5.07 (br, 2H), 4.01 (m, 1H), 3.95 (m, 1H), 3.83 (m, 1H), 3.60 (m, 1H), 3.55 (m, 1H); ^{13}C NMR (125.8 Hz, DMSO-d_6) δ 163.5, 151.1, 141.1, 102.1, 88.0, 85.2, 73.9, 70.2, 61.2; HRMS calcd for $C_9H_{12}N_2O_5{}^{18}O$, [MH$^+$] 247.0816, found 247.0818.

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Supporting Information Available: A scheme and experimental details for synthesis of phosphoramidite **3**; ^{1}H and ^{13}C NMR spectra for compounds **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) This reactivity trend should be viewed with caution, as it is based on a limited number of independent examples rather than systematic investigation of factors such as solvent, pH, temperature, bases, and the "chemical context" of the nucleophilic atom.

(25) In the presence of 2 equiv of BzOH (without KOBz), the reaction produced only a trace amount of product as indicated by TLC, presumably due to the weak nucleophilicity of benzoic acid.

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