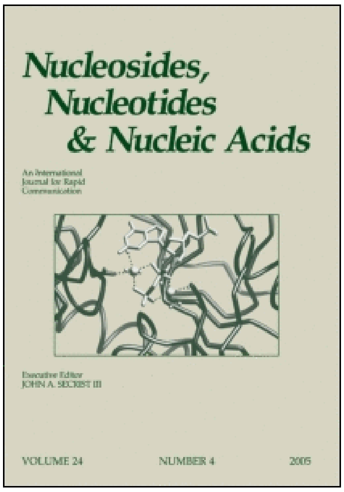

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^{18}O LABELED NUCLEOSIDES. 2. A GENERAL METHOD FOR THE SYNTHESIS OF SPECIFICALLY LABELED PYRIMIDINE RIBONUCLEOSIDES[1]

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Abstract - A facile method for the synthesis of highly enriched ^{18}O labeled pyrimidine ribonucleosides is described using uridine as a model compound. The isotopic label may be selectively incorporated into the base moiety at O^2 or into the ribose portion of the molecule at the 5' position. In addition, both positions may be labeled and this is the first report of a method for labeling of both the base and sugar moieties of pyrimidine ribonucleosides. The site and level of isotope incorporation may be determined mass spectrometrically.

INTRODUCTION

The use of stable isotopically labeled compounds as internal standards for quantitative measurements by gas chromatography-mass spectrometry to solve various chemical, biochemical and clinical problems is an area of rapid growth.[2,3] Although this technique has been successful with compounds of sufficient volatility to permit their gas phase analysis, applications in the nucleic acid field have not been reported until recently. There are two principle reasons for the paucity of reports in this area.

First, the low volatility of nucleosides and nucleotides has precluded the use of gas chromatography for the separation of nucleoside (tide) mixtures from either synthetic reactions or biological materials. However, this difficulty has largely been surmounted by the use of volatile derivatives, most especially the permethyl[4] and trimethylsilyl (TMS) analogs.[5] The second problem is the lack of general procedures for the incorporation of stable isotopes into nucleic acid components.

Although synthetic methods describing the incorporation of oxygen-18[6,7] and deuterium[8,9] into the heterocyclic portion of nucleosides have been reported, procedures for labeling the sugar moiety have been limited,[10,11] with the exception of deuterium incorporation at the 5'-position.[7,12] Also, there have been no general procedures described[13] for the labeling of both the base and carbohydrate portions of nucleosides. Because extensive enzymatic modification of nucleosides can occur in biological systems, isotopic labels in both parts of these molecules would significantly aid the mass spectrometric structure elucidation[14] of nucleoside metabolites isolated from in vivo systems by permitting the identification of specific base and/or sugar residues.

Anhydronucleosides have been shown to be versatile intermediates for the synthesis and interconversion of pyrimidine nucleosides.[15] Unfortunately, most ring opening reactions of the anhydropyrimidine nucleosides result in the formation of pentofuranosides possessing the arabino- or xylo- configurations rather than the desired ribo- isomer. An exception is the ring opening reaction of O^2,5'-anhydrouridine[16-18] which has been shown to give uridine under either acidic[7,19] or basic[18] conditions. Because of the primary importance of nucleic acids in biochemical processes and the current interest in the clinical applications of nucleoside analogs,[20] we would like to report a facile, high yield procedure for the incorporation of oxygen-18 into both the aglycone and sugar portions of uridine starting with O^2,5'-anhydrouridine.

MATERIALS AND METHODS

Materials. Water containing 98.3 atom percent ^{18}O was purchased from KOR Isotopes, Cambridge, Mass. Uridine and 2',3'-O-isopropylidine uridine were purchased from Sigma Chemical Co., St. Louis, MO. Trimethylsilyl (TMS) derivitizing agents were purchased from Pierce Chemical Co., Rockford, IL. Thin layer chromatography (TLC) was performed using SilicAr 7GF, 5x20 cm, 250 μm plates (obtained of Analtech, Inc., Newark, DL) developed in ethyl acetate, n-propanol, water; 4/1/2(v/v/v), upper phase. Preparative TLC was performed using the same conditions with the exception that 20x20 cm, 200 μm plates were used. All other reagents used were of the highest quality available commercially.

Mass Spectra were acquired using a Varian 311A mass spectrometer at 70 eV ionizing energy and an ion source temperature of 290°. Underivatized samples were introduced using a direct probe. The mass range was scanned from 45 to 600 amu at 5 sec/decade and the data was stored and processed with a Varian SS200 data system. An average spectrum of each sample was obtained by averaging scans across the maxima of the integrated total ion chromatogram.

Gas Chromatography of the trimethylsilyl derivatives[5] was performed using a Varian 3700 gas chromatograph equipped with a 6' (3%) OV-17 packed GC column with an initial temperature of 180°C programmed at 40/min and carrier gas flow rate of 30 ml/min. The injector port temperature was 250°C and the glass jet separator was 290°C.

Isotope Incorporation Levels were determined from the M-15 ion[14] region of the spectrum of the TMS derivatives in order to avoid the possibility of pyrolysis. The mass range from 500 to 550 amu was scanned at a rate of 25 sec/decade and an average spectrum was obtained from which isotope levels were measured.

Sample Purity was confirmed using TLC, GC (TMS derivative) and mass spectrometry.

SYNTHESIS

O^2,5'-Anhydro-2',3'-isopropylidene uridine (1).

To a stirred solution of 2',3'-isopropylidene uridine (284 mg, 1.0 mmol) and triphenylphosphine (262 mg, 1.0 mmol) in dry tetrahydrofuran (THF) (15 ml) was added diethylazodicarboxylate (DEAD) (200 mg, 1.15 mmol). The mixture was allowed to stir at room temperature for 12 h and the precipitate was collected by filtration to give 1 (165 mg, 0.62 mmol) in 62% yield. mp 274°-276°C (lit.[17] 275-277°C); R_f=0.5.

2',3'-Isopropylidene-[O^2-^{18}O]uridine (2).

A solution of 2.0 N Na^{18}OH was prepared by the cautious addition of sodium metal (46 mg) to $H_2{}^{18}O$ (1.0 ml) under an argon atmosphere. Compound 1 (100 mg, 0.38 mmol) was dissolved in Na^{18}OH (250 μl) and the solution stirred at room temperature for 4 h. The solution was neutralized to pH 7 by addition of 0.1N HCl and the solvent removed *in vacuo*. The resulting residue was triturated with hot $CHCl_3$ (3 x 10 ml), the solution was filtered and the filtrate was evaporated to dryness under reduced pressure to give 2 (105 mg, 0.37 mmol) in 98% yield. R_f=0.8.

[O^2-^{18}O]Uridine (3).

A sample of 2 (50 mg, 0.17 mmol) was dissolved in trifluoroacetic acid/water (9:1; v/v, 1 ml) and the reaction was stirred for 30 min at room temperature. The solvents were then removed *in vacuo* and the residue triturated with ether (10 ml) to give 3 (37 mg, 0.15 mmol) in 85% yield. R_f=0.5.

O^2,5'-Anhydro-2'3'-isopropylidine-[O^2-^{18}O]uridine (4).

A mixture of 3 (110 mg, 0.38 mmol) and triphenylphosphine (99 mg, 0.38 mmol) was dissolved in THF (10 ml). To this solution was added DEAD (76 mg, 0.44 mmol) and the reaction was stirred at room temperature for 12 h. The crystals which had formed were collected by filtration to afford 4 (71 mg, 0.26 mmol) in 70% yield. The product was homogenous on tlc (R_f=0.8) and mass spectrometry showed a molecular ion ($M^{+\cdot}$) at *m/z* 268.

2',3'-Isopropylidene[5'-^{18}O]uridine (5).

Compound 4 (20 mg, 0.07 mmol) was dissolved in 2.0N NaOH (100 µl) and allowed to stir at room temperature for 4 h. The reaction was neutralized to pH 7 with 0.1N HCl and the solvent removed *in vacuo*. The residue was triturated with hot $CHCl_3$ (3 x 10 ml), filtered, and after evaporation of the solvent gave 5 (19 mg, 0.06 mmol) in 90% yield.

[5'-^{18}O]Uridine (6).

A sample of 5 (10 mg, 0.04 mmol) was dissolved in 9:1 (v/v) trifluoroacetic acid-water (500 µl). The solution was stirred for 30 min at room temperature and then the solvents were removed *in vacuo*. The resulting residue was triturated with ether (10 ml) to give 6 (7 mg, 0.03 mmol) in 85% yield. R_f=0.5.

2'-,3'-Isopropylidene[O^2,5'-$^{18}O_2$]uridine (7).

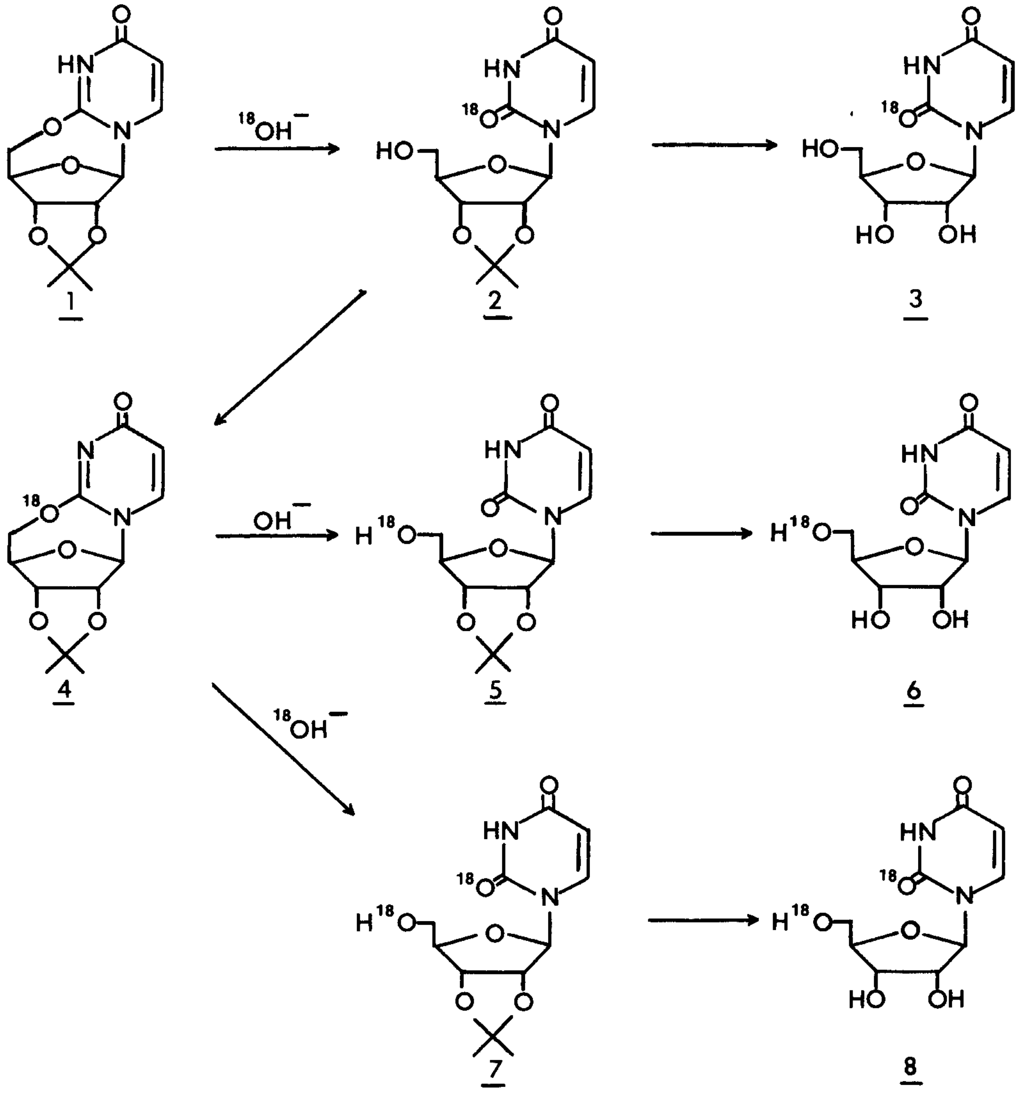
A sample of 4 (50 mg, 0.19 mmol) was dissolved in 2.0N Na^{18}OH and stirred at room temperature for 12 h. The reaction was neutralized to pH 7 with the cautious addition of 0.1N HCl and the solvents were removed *in vacuo*. The residue was triturated with $CHCl_3$ (3 x 10 ml) which, upon removal under reduced pressure, gave 6 (51 mg, 0.18 mmol) in 95% yield. R_f=0.8. MS: $M^{+\cdot}$ observed at *m/z* 288.

[O^2,5'-$^{18}O_2$]Uridine (8).

A sample of 7 (20 mg, 0.07 mmol) was dissolved in 9:1 (v/v) trifluoroacetic acid-water (1 ml). The solution was allowed to stir for 30 min at room temperature and then the solvents removed *in vacuo*. Trituration of the residue with ether (10 ml) gave 8 (16 mg, 0.06 mmol) in 90% yield. R_f=0.5.

RESULTS AND DISCUSSION

The synthetic procedure used to synthesize [O^2-^{18}O]uridine (3), [5'-^{18}O]uridine (6) and [O^2,5'-^{18}O$_2$]uridine (8) is shown in the Reaction Scheme. Basic hydrolysis of the anhydro bridge with Na^{18}OH proceeds by nucleophilic attack specifically at the C2 position of the heterocyclic



REACTION SCHEME

base to afford 2 which is deblocked[21] to give 3. Mass spectrometry of free 3 showed a +2 shift of the molecular ion of 3 relative to uridine, indicating the incorporation of one oxygen-18 atom. The B+2H ion is observed at m/z 115 while the sugar ion at m/z 133 does not shift indicating the label is located in the heterocyclic moiety. The ion at m/z 69[22] also remains unshifted which indicates the ^{18}O atom is located exclusively at the C2 position of the base.

Reformation of the O^2,5'-anhydro bridge by "recycling" 2 provides the key intermediate compound 4. Subsequent reaction conditions will determine the position of the label and provide a means of specifically labeling the 5' position of the ribose moiety or incorporating a second ^{18}O atom into the molecule at the C2 position. Treatment of 4 with unlabeled NaOH results in cleavage of the anhydro linkage with the label, in this case, going to the 5' position. Deblocking of 5 yields [5'-^{18}O]-uridine (6) whose mass spectrum shows a +2 shift in the sugar ion to m/z 135. No shift is observed in the base related ions which indicates the label is specifically in the sugar portion of the molecule. The absence of a shift in the B+30, B+44 and B+60[14] ions eliminates the 2' and 4' oxygens as the labeled positions. Differentiation of the 3' and 5' oxygens in the free compounds is not possible because the M-30 and M-31 ions, arising by specific loss of the 5'-hydroxymethylene group, are weak in the spectrum of pyrimidine ribonucleosides. However, the trimethylsilyl derivatives of all ribonucleosides show an ion at m/z 103 which is indicative of the 5' position and this ion is observed to shift to m/z 105 in the spectrum of the TMS_4 derivative of 6, which unequivocally establishes the 5' position as the site of the ^{18}O label in 6. Alternatively, the anhydro bond may be broken by $Na^{18}OH$ with the resultant incorporation of a second ^{18}O atom which, after removal of the isopropylidene group, affords the di^{18}O labeled 8. Analysis of the mass spectra of 8 and the TMS derivative of 8 indicates that one ^{18}O atom is located in the 2 position of the uracil ring and the other ^{18}O is at the 5' position of the ribose ring. The observed shifts of diagnostic ions used to assign the position(s) of the ^{18}O label are shown in Table 1.

The level of isotope incorporation, sample purity and, as mentioned above, the site of labeling in the sugar moiety were determined by conversion of 3, 6 and 8 to their TMS derivatives.[5] The level of isotope incorporation was obtained by introduction of the sample into the mass spectrometer using a direct insertion probe, heating the probe and slowly scanning (25 sec/decade) the mass range from 500-550 amu. The acquired spectra were then averaged around to peak maximum to eliminate bias. The

TABLE 1. Ions (m/z) Used to Assign Site of Label

Ion^a	Uridine	3	6	8
$M^{+\bullet}$	244	246	246	248
B+2H	113	115	113	115
S	133	133	135	135
m/z 103 (TMS)	103	103	105	105

^{a}S=sugar ion; m/z 103=5'-CH$_2$OTMS

TABLE 2. Relative Intensities of the M-15 Ion (TMS Derivatives) Used to Determine Extent of ^{18}O Incorporation

	m/z 517	518	519	520	521	522	523	% Labeled
Uridine	100	42.2	23.1	5.7	-	-	-	0
3	2.0	1.2	100	42.9	26.2	7.2	2.2	98
6	2.8	1.8	100	43.2	23.5	6.8	2.0	97
8	0.2	-	2.7	1.3	100	42.3	23.5	97

amount of unlabeled, monolabeled and dilabeled 3, 6 and 8 was then determined by the relative intensity of the ions at m/z 517, 519 and 521, respectively, after correcting for the isotopic contribution of heavy isotopes, primarily silicon. The analysis of these results showed the ^{18}O incorporated into the O^2 position of 3 was quantitative with 98% labeled and 2% unlabeled. Similar results were obtained with 6 and 8 and the results are presented in Table 2.

In summary, the method offers a simple means of synthesizing pyrimidine ribosides labeled specifically in the base, the sugar or both moieties with one or two oxygen-18 atoms. The yield of product is moderate but the level of isotope incorporation is essentially quantitative and the reaction should be applicable to any pyrimidine riboside capable of forming an anhydro analog. We are currently extending this synthetic scheme to other pyrimidine nucleosides, including ribo, arabino and deoxyribosides, of clinical and bioligical significance. We believe these specifically labeled compounds will have utility, when combined with mass spectrometry, in studying the biochemistry and metabolism of both naturally occuring and synthetic nucleosides of clinical importance.

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