

added, and the resultant suspension was stirred at 75 °C under argon. After 9 h, the reaction mixture was poured into water, extracted with diethyl ether, and processed in the usual way to yield the benzoate mixture as a pale yellow oil (0.087 g, 89%): chromatographic purification PTLC (solvent A); IR (neat), ν_{\max} 2970, 1725, 1450, 1270, 710 cm^{-1} ; ^1H NMR (100 MHz) (CDCl_3) δ 1.38 (s, 3, $\text{C}(\text{CH}_3)_2$), 1.5 (s, 3, $\text{C}(\text{CH}_3)_2$), 1.70–2.4 (m, 4), 2.50–2.75 (m, 1), 2.77–3.10 (m, 1), 3.40 (s, 3, OCH_3), 3.58–4.38 (m, 4, H-4,5,6,6'), 4.88 (s, 1, H-1), 5.55 (m, 1, H-8), 7.50, 8.05 (2m, 5, O_2CPh); ^{13}C NMR (80 MHz) (CDCl_3) δ 25.32 ($\text{C}(\text{CH}_3)_2$), 26.85 ($\text{C}(\text{CH}_3)_2$), 36.07, 39.22 (C-7,9), 42.81 (C-3), 50.56 (C-2), 54.66 ($\text{OC}-\text{H}_3$), 66.00 (C-6), 78.07 (C-8), 79.10 (C-4), 89.41 (C-5), 128.49, 129.66, 130.61 (O_2CPh), 166.17 (O_2CPh); MS, m/e 347 ($\text{M}^+ - \text{CH}_3$), 331 ($\text{M}^+ - \text{OCH}_3$).

The benzoate **18b** (0.010 g, 0.03 mmol) was dissolved in dry methanol (10 mL), a trace of sodium methoxide was added and the solution was

stirred at room temperature for 18 h. The reaction solution was evaporated and the resulting salts extracted with methylene chloride. The extract was evaporated to yield a clear syrup that contained as the major product a 25:1 mixture of **18a** and **17a**. This syrup displayed the following characteristics: TLC R_f 0.11 (solvent A); GLC retention time 5.0 min (**17a**), 5.25 min (**18a**), ultra bond, 70 °C/4 min, 8 °C/min up to 250 °C; ^1H NMR (80 MHz) (CDCl_3) δ 1.35 (s, 3, $\text{C}(\text{CH}_3)_2$), 1.45 (s, 3, $\text{C}(\text{CH}_3)_2$), 1.50–2.10 (m, 5), 2.20–2.95 (m, 3), 3.35 (s, 3, OCH_3), 3.50–4.50 (m, 4, H-4,5,6,6'), 4.79 (s, 1, H-1); ^{13}C NMR (80 MHz) (CDCl_3) δ 25.20 ($\text{C}(\text{CH}_3)_2$), 26.81 ($\text{C}(\text{CH}_3)_2$), 39.02, 42.10 (C-7,9), 42.68 (C-3), 49.93 (C-2), 54.54 (OCH_3), 66.02 (C-6), 74.22 (C-8), 79.12 (C-4), 89.51 (C-5), 109.71 (C-1), 111.24 ($\text{C}(\text{CH}_3)_2$).

Acknowledgment. We are grateful to the Natural Sciences and Engineering Research Council of Canada for support of this work.

Oxygen Chiral Phosphate in Uridyl(3'→5')adenosine by Oxidation of a Phosphite Intermediate: Synthesis and Absolute Configuration†

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Abstract: Coupling of 2',5'-silylated uridine and 2',3'-silylated N^6 -benzoyladenine with dichloromethoxyphosphine furnished dinucleoside monophosphite triesters. Oxidation of these intermediates with [^{17}O , ^{18}O]H₂O and iodine afforded the diastereoisomeric phosphate triesters of fully protected [^{17}O , ^{18}O]UpA having the oxygen labels in the P=O group. The diastereoisomeric mixture of major and minor components was separated by column chromatography. Stereospecific cleavage of the phosphate protecting group by thiophenolate followed by desilylation with $(\text{Bu})_4\text{NF}$ yielded the oxygen chiral isotopomers of [^{17}O , ^{18}O]UpA. The incorporation of ^{17}O was demonstrated by ^{17}O NMR spectroscopy and of ^{18}O by ^{31}P NMR spectroscopy. Hydrolysis of the major isotopomer with nuclease P1 in [^{17}O , ^{18}O]H₂O with inversion of configuration at phosphorus yielded [^{16}O , ^{17}O , ^{18}O]AMP. This was converted into the isotopomers of the respective cyclic 3',5'-phosphate with inversion of configuration. Methylation of the latter followed by ^{31}P NMR spectroscopy established the absolute isotopic configuration of the [^{16}O , ^{17}O , ^{18}O]AMP as S_p . The absolute configurations of the diastereoisomeric triesters from the phosphite oxidation are therefore assigned as S_p for the major isomer and R_p for the minor isomer. Consequently, the resulting deprotected isotopomers of [^{17}O , ^{18}O]UpA have the opposite R_p and S_p configurations, respectively. Methylation of (R_p)-[^{17}O , ^{18}O]UpA gave two diastereoisomeric triesters whose absolute configurations were established by inspection of the ^{18}O isotope shifts on phosphorus in the ^{31}P NMR spectrum. An unknown absolute isotopic configuration of [^{18}O]UpA can now be determined by methylation and subsequent ^{31}P NMR spectroscopy.

The elucidation of the stereochemical course of action of many nucleotide and polynucleotide processing enzymes has been accomplished to a large extent by the use of phosphorothioate analogues.^{2–5} More recently, considerable advances have been made in the study of this type of reaction by the use of some or all of the three stable isotopes of oxygen, ^{16}O , ^{17}O , and ^{18}O , for the synthesis of stereospecifically labeled phosphate esters.^{6–8} Syntheses of oxygen chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoesters and their configurational assignments have been described by Lowe et al.⁶ and by Knowles and associates,⁷ and these compounds have since been used for a wide variety of mechanistic studies in the realm of phosphoryl transfer reactions. Interest in this field has also recently centered around the introduction of oxygen isotopes by the stereospecific replacement of sulfur by ^{17}O or ^{18}O .⁹

The analysis of the chirality of oxygen chiral nucleoside monophosphates has been facilitated by the development of a method¹⁰ that depends on the different effects of ^{17}O and ^{18}O in ^{31}P NMR spectroscopy.^{11–13} More specifically, a 5'-[^{16}O , ^{17}O , ^{18}O]nucleotide^{10a} (which can be obtained by stereospecific

enzymic cleavage of an oxygen chiral dinucleoside monophosphate in oxygen isotope enriched water¹⁴) is converted into the respective

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† Dedicated to Prof. F. Cramer on the occasion of his 60th birthday.

isotopomers of the 3',5'-cyclonucleotide by a chemical procedure shown to proceed with inversion of configuration at phosphorus. The isotopomeric differences in the resulting cyclic diesters are then localized by methylation to two diastereoisomeric triesters of known absolute configuration at phosphorus. Examination of the ^{31}P NMR spectra of these compounds permits identification of the labels and consequently allows the absolute isotopic configuration of the 5'-[^{16}O , ^{17}O , ^{18}O]nucleotide precursor to be deduced.

Up until now the only preparation of a stereospecifically labeled phosphate group in an internucleotide linkage has been accomplished for a deoxydinucleoside phosphate by the stereospecific replacement of sulfur in a separated deoxydinucleoside phosphorothioate diastereoisomer.¹⁴ We now wish to report a novel, facile, and likely universal method for the stereochemically controlled oxygen labeling of ribo- and deoxyribodinucleoside monophosphates. Since dinucleoside monophosphite triesters can be oxidized by iodine-water to the corresponding phosphate triesters,¹⁵ the use of oxygen isotope enriched water should lead to diastereoisomers with a chiral phosphorus having the oxygen label in the phosphoryl group of the triester. We demonstrate here the use of such a method for the synthesis of the stereochemically pure isotopomers of [^{17}O , ^{18}O]uridylyl(3'→5')adenosine ([^{17}O , ^{18}O]UpA) and assign their absolute isotopic configurations at phosphorus.

Results and Discussion

For the synthesis of an oxygen labeled UpA an appropriate 2',5'-protected uridine and 2',3'-protected adenosine had to be prepared. In both cases the *tert*-butyldimethylsilyl (TBDMS) protecting group was chosen. This type of protection has been introduced into oligonucleotide chemistry by Ogilvie et al.¹⁶ The TBDMS group is quite stable against hydrolytic cleavage and oxidation, and moreover, it can be introduced into nucleosides under mild conditions. Cleavage is accomplished readily with tetrabutylammonium fluoride because of the high affinity of fluoride ions for silicon. 2',5'-*O*-Bis(*tert*-butyldimethylsilyl)uridine (**1a**) was prepared according to a known method¹⁷ but by using an excess of pyridine. For the preparation of the 2',3'-protected *N*⁶-benzoyladenine the nucleoside was treated with 5 equiv of TBDMS chloride, yielding first the trisilylated derivative **2a**. The acid-sensitive 5'-silyl group was then split off by 80% acetic acid.¹⁶ However, partial removal of the 2'-silyl protecting group was also unfortunately observed. A cleaner removal of the silyl groups was effected by the use of zinc bromide, a reagent normally used for the removal of trityl groups.¹⁸ Compound **2a** was dissolved in nitromethane together with the standard zinc bromide solution. TLC showed that the deprotection reaction was complete after 12 h at room temperature and that no depurination had occurred. Compound **2b** was isolated in 81% yield.¹⁹ Structural assignment of the silylated compounds was made by ^{13}C NMR spectroscopy (Table I), and ^{29}Si NMR spectroscopy essentially demonstrated

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Table I. ^{13}C Chemical Shifts in ^1H Decoupled NMR Spectra of Protected [^{17}O , ^{18}O]UpA Diastereoisomers and Their Parent Nucleosides^a

	C-2	C-4	C-5	C-6	C-8	C-1'	C-4'	C-2'	C-3'	C-5'	C=O	OCH ₃	(CH ₃) ₃	CSi	(CH ₃) ₃ Si
1a	150.26	162.78	102.40	140.21		88.70	84.96	76.62	70.46	62.67			25.84, 25.58	18.30, 17.89	-4.92 → -5.69
2a	153.10	150.00	123.80	152.20	142.50	89.00	86.00	76.10	72.40	62.90	164.80		25.90-26.30	18.00-18.70	-4.30 → -5.30
2b	152.50	150.80	124.70	150.90	143.50	91.40	89.70	74.30	74.00	63.00	164.30		25.60-26.30	17.70-18.30	-4.70 → -5.70
4a: U	150.06	162.70	102.74	139.93		87.05	82.45 (d, 8.1)	74.98	71.51	63.10		54.84 (d, 6.2)	25.94, 25.84	18.33-18.04	-4.32 → -5.59
4a: A	152.63	150.24	123.89	151.47	142.08	89.85	83.98	75.09	74.80	66.23 (d, 4.8)	164.97		25.74, 25.51	17.93	
4b: U	149.82	162.52	102.65	139.67		87.52	83.05 (d, 8.0)	74.95	72.04	62.81		54.69 (d, 6.0)	25.91, 25.83	18.34, 18.04	-4.38 → -5.54
4b: A	152.73	150.15	123.80	151.46	142.41	89.73	83.59 (d, 3.2)	75.05	74.44	66.97 (d, 5.6)	164.67		25.70, 25.56	18.02, 17.93	

^a δ values are reported relative to tetramethylsilane; coupling constants (*J*) are in hertz.

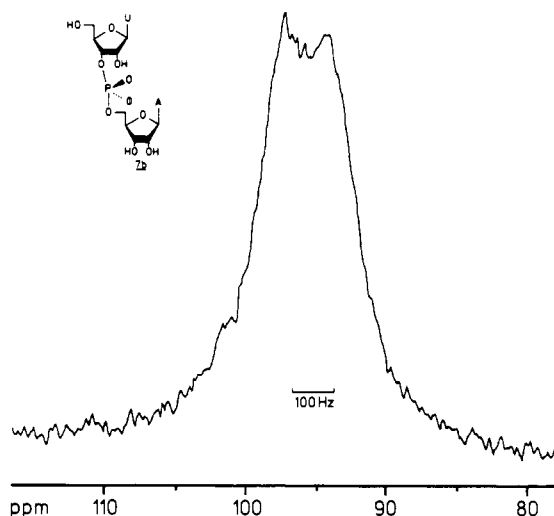


Figure 1. Phosphorus-coupled ^{17}O NMR spectrum of (R_p) - ^{17}O UpA at 33.9 MHz and 95 °C in D_2O -triethylammonium bicarbonate (pD 8.5).

5'-monophosphate, and digestion by ribonuclease A and spleen phosphodiesterase proved that the dinucleoside monophosphates were connected by a 3'→5' linkage. No 2'→5' isomer was detected by HPLC on a RP-18 column. The 3'→5' isomers **7a** and **7b** were eluted after 24 min, whereas for (2'→5')UpA a retention time of 16 min was found. The 33.8-MHz ^{17}O NMR spectra of the oxygen-labeled UpAs were measured at 95 °C in D_2O at a pD maintained at 8.5 by triethylammonium bicarbonate. Because of the reduced molecular weight in comparison to the blocked compounds, the ^{17}O NMR spectra showed better resolution. Compound **7b** gave a broad doublet with a $^1J_{\text{PO}}$ of approximately 100 Hz (Figure 1). This doublet was located at ca. 96 ppm, being in the range of that of cyclic ^{17}O , ^{18}O dAMP.²⁸

The presence of ^{16}O and ^{18}O isotopes in ^{17}O , ^{18}O UpA was confirmed by ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum of compound **7b** was recorded in aqueous EDTA solution, pH 8.0. The spectrum is shown in Figure 2. Two sharp peaks are seen, centered on -0.37 ppm (relative to H_3PO_4) and separated by an isotope shift of 2.42 Hz. Since an ^{18}O isotope shift is always to high field,^{11,12} the resonance to low field is assigned to ^{16}O UpA and the larger resonance to high field to ^{18}O UpA. ^{17}O UpA will not be observed under normal conditions in the ^{31}P NMR spectrum.^{11b,13}

From the isotopic enrichment of the labeled water used for the phosphite oxidation, the ratio of the peak area of the ^{18}O UpA resonance to that of the ^{16}O UpA resonance should be 1:3.79. Examination of the spectrum shows that this is, however, clearly not the case and the respective peaks have relative areas of 1:1.84. This means that during or prior to the oxidation step the phosphite precursor was oxidized to some extent by extraneous ^{16}O water. However, the new isotopic content of the final ^{17}O , ^{18}O UpA can be easily calculated from this spectrum and the original values and was found to be 28.9% ^{16}O , 18.1% ^{17}O , and 53.0% ^{18}O . Thus the final product contained some 12.5% more ^{16}O isotope than would have been expected on the basis of the isotope content of the water used for oxidation alone. It should of course be possible to avoid this contamination in the future.

With the successful synthesis of the isotopomers of ^{17}O , ^{18}O UpA it now remained to determine their absolute configurations, so that they might prove useful for future physical studies or mechanistic investigations of enzymes. It has recently been demonstrated by Potter et al.¹⁴ that it is possible to determine the absolute isotopic configuration of an ^{18}O -labeled deoxydinucleoside phosphate, (S_p) - ^{18}O TpT, by digestion of this compound in ^{17}O -labeled water using an enzyme whose stereochemical course of reaction is known.

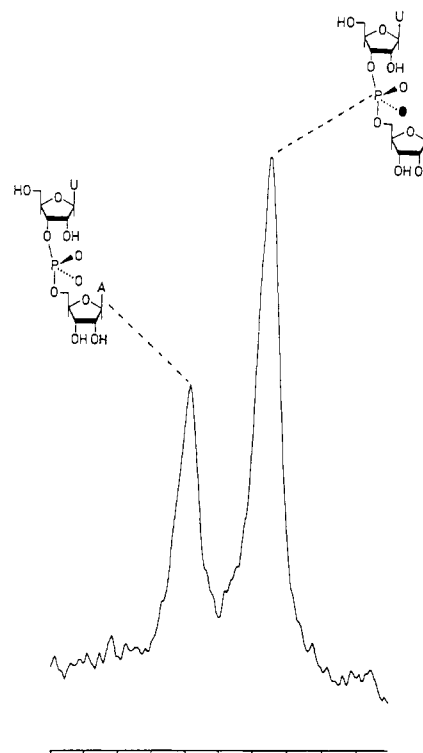
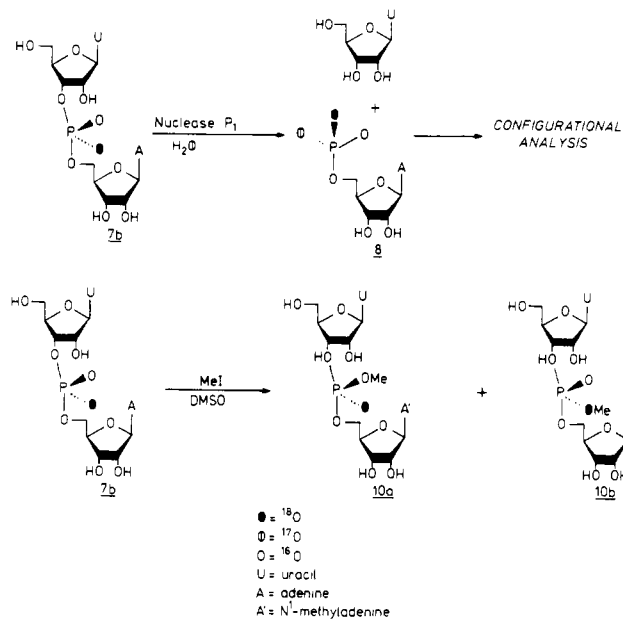


Figure 2. 81.01-MHz ^{31}P NMR spectrum of (R_p) - ^{17}O , ^{18}O UpA (**7b**). Spectrum of (R_p) - ^{17}O , ^{18}O UpA (approximately 10 mM) containing 28.9% intrinsic ^{16}O UpA. Signals observable are from ^{16}O UpA, δ -0.35, and ^{18}O UpA, δ -0.38, (isotope shift 2.42 ± 0.06 Hz) measured from 85% H_3PO_4 as the external standard. The spectrum was recorded in 100 mM EDTA, pH 8.0, and 25% D_2O ; NMR parameters were as follows: sweep width, 1000 Hz; pulse width, 17 μs ; acquisition time, 16.38 s; data collection in 16K, Fourier transform in 32K; number of transients, 1856; line broadening, 0.06 Hz; scale, 1 Hz = 1 division. U = uridine; A = adenosine.

Scheme I



Although the ^{17}O , ^{18}O UpA of unknown configuration that we prepared was only some 53% labeled with ^{18}O , it was nevertheless calculated that in principle a similar strategy could be used here. If a digestion were to be carried out in 52.8%-enriched H_2^{17}O , an intensity ratio of the crucial central resonances in the final NMR spectra of the stereochemical analysis would be expected to be 0.67:1.00 for the isotopomers of the axial diastereoisomer

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Table II. Configurational Analysis of (R_p)-[^{17}O , ^{18}O]UpA (7b)^a

	axial diastereoisomer			equatorial diastereoisomer		
	obsd	calcd for [^{17}O , ^{18}O]UpA		obsd	calcd for [^{17}O , ^{18}O]UpA	
		R_p	S_p		R_p	S_p
MeO-P=O	0.97	0.64	0.64	0.93	0.64	0.64
Me●-P=O	0.79	0.67	1.00	1.00	1.00	0.67
MeO-P=●	1.00	1.00	0.67	0.71	0.67	1.00
Me●-P=●	0.48	0.31	0.31	0.32	0.31	0.31

^a The observed relative peak intensities of the ^{31}P NMR resonances (from Figure 3) of the isotopomers of the diastereoisomeric triesters obtained via the cyclization and methylation of [^{16}O , ^{17}O , ^{18}O]AMP derived from the nuclease P1 catalyzed hydrolysis of [^{17}O , ^{18}O]UpA 7b are compared with the calculated values for the hydrolysis of both isotopomers with inversion of configuration at phosphorus. The values were calculated on the basis of the following assumptions: (R_p)-[^{17}O , ^{18}O]UpA starting material contained 28.9% ^{16}O , 18.1% ^{17}O , and 53.0% ^{18}O , and the hydrolysis reaction with nuclease P1 was performed in water of the following isotopic composition: ^{16}O , 9.4%; ^{17}O , 52.8%; ^{18}O , 37.8%.

of methyl cAMP, whereas if 99%-enriched H_2 ^{18}O were to be used, a ratio of only 1.00:0.82 would be observed.

The stereochemical course of the enzyme nuclease P1 has been determined by Potter et al.¹⁴ (Scheme I). This enzyme is highly active on ribodinucleotides and we therefore decided to cleave the [^{17}O , ^{18}O]UpA with this enzyme in ^{17}O -labeled water. The major isotopomer of [^{17}O , ^{18}O]UpA, 7b, was chosen for the configurational analysis. Cleavage of this compound in ^{17}O , ^{18}O -labeled water produced, in addition to achiral isotopomers, [^{16}O , ^{17}O , ^{18}O]AMP of both configurations, the major product being generated by attack of H_2 ^{17}O on the [^{18}O]UpA isotopomer and the minor product being generated from the attack of H_2 ^{18}O on the smaller amount of the [^{17}O]UpA isotopomer present. The excess isotopic chirality in the [^{16}O , ^{17}O , ^{18}O]AMP was analyzed by cyclization to the isotopomers of [^{16}O , ^{17}O , ^{18}O]cAMP followed by methylation according to the procedure of Jarvest et al.^{10a} The ^{31}P NMR spectra of the resulting isotopomers of N^1 -methyl-[^{16}O , ^{17}O , ^{18}O]cAMP methyl ester are shown in Figure 3 and their relative line intensities in Table II.

These spectra are interpreted on the following basis:^{10a} When ^{18}O is bonded to phosphorus it causes an upfield shift in the NMR signal^{11a,12a} that is greater for doubly as compared to singly bonded ^{18}O .^{11b,12b} Additionally, when ^{17}O is bonded to phosphorus, the quadrupolar moment of the ^{17}O nucleus causes a broadening of the ^{31}P NMR signal to such an extent that this resonance is not observed.^{11b,13} Random loss of any one of the three labels from an [^{16}O , ^{17}O , ^{18}O]AMP molecule with equal probability during cyclization gives rise to only one species that does not contain ^{17}O (and is therefore not quenched in the ^{31}P NMR spectrum), namely, that derived from the loss of ^{17}O during the cyclization process. Inspection of the ^{31}P NMR intensity patterns of the signals due to the ^{16}O , ^{18}O isotopomers of the methyl esters of N^1 -methyl-[^{16}O , ^{17}O , ^{18}O]cAMP permits the localization of the residual ^{18}O atom in either a bridging or doubly bonded position in the species that has lost ^{17}O on cyclization, since only this resonance will have no quenched component. As one knows that the cyclization reaction proceeds with inversion of configuration,^{10a} the configuration of the original [^{16}O , ^{17}O , ^{18}O]AMP can be deduced and hence the isotopic configuration of the original [^{17}O , ^{18}O]UpA since the cleavage by nuclease P1 also proceeds with inversion of configuration.¹⁴

Examination of the intensity pattern of the ^{31}P NMR signals in Figure 3 and Table II shows the highest peak in the equatorial series of triesters to be derived from that isotopomer possessing ^{18}O in a bridging bond and in the axial series from that isotopomer possessing ^{18}O in a double bond.

The intensity patterns as set out in Table II correlate well with those predicted and provide an unambiguous stereochemical answer. Exact conformity of peak heights should not be expected

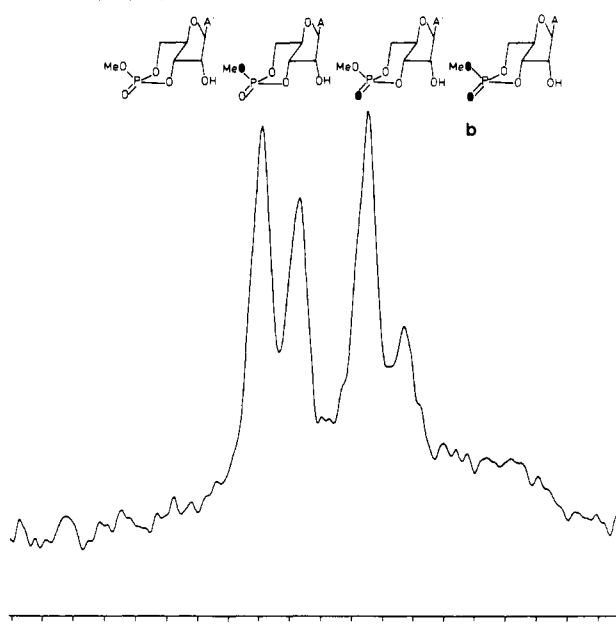
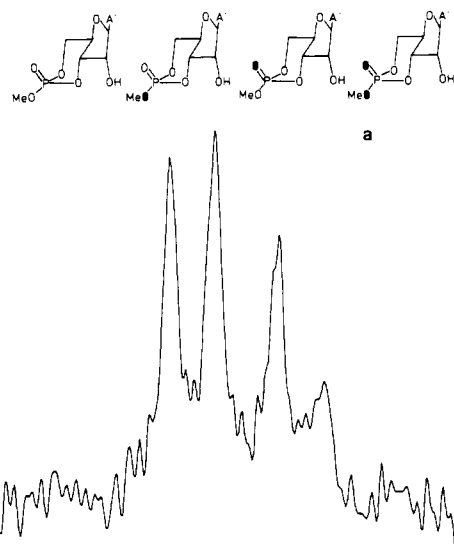


Figure 3. 81.01-MHz ^{31}P NMR spectrum of the isotopomers of N^1 -methyl-cAMP derived from [^{16}O , ^{17}O , ^{18}O]AMP obtained via the nuclease P1 digestion of (R_p)-[^{17}O , ^{18}O]UpA. The upper spectrum (a) shows the isotopomers of the equatorial diastereoisomer; the lower spectrum (b) shows the isotopomers of the axial diastereoisomer. Spectra were obtained from a 15 mM solution in $\text{Me}_2\text{SO}-d_6$ - Me_2SO (50:50 v/v) containing 8-hydroxyquinoline. NMR parameters were as follows: sweep width, 1000 Hz; pulse width, 14 μs ; acquisition time, 8.19 s; data collection in 8K, Fourier transform in 32K; number of transients, 15 000; line broadening, 0.06 Hz; scale, 1 Hz = 1 division. A' = N^1 -methyladenosine.

since the isotopomer signals in the final spectra (Figure 3) are not completely resolved. The heights of the peaks due to the ^{16}O isotopomers of the cAMP methyl esters are also slightly larger than expected, and this represents possibly a small further contamination by ^{16}O during the analysis procedure or the error involved in calculating the isotopic content of [^{17}O , ^{18}O]UpA from the ^{31}P NMR spectra. These data permit the precursor [^{16}O , ^{17}O , ^{18}O]AMP to be designated as (S_p)-[^{16}O , ^{17}O , ^{18}O]AMP (8). Since this molecule was formed by an inversion of configuration at phosphorus, the original [^{17}O , ^{18}O]UpA, 7b, must have the R_p configuration. Thus, to the major product of phosphite oxidation 4b (slower running on TLC and giving a ^{31}P NMR signal at high field relative to the other diastereoisomer) is assigned the S_p configuration, and to the minor product 4a (faster running on

TLC and with the ^{31}P NMR signal at low field relative to the other diastereoisomer) is assigned the R_p configuration.²⁹

It is of interest to establish whether use of a simple methylation and NMR analysis procedure for a stereochemically pure ribo- or deoxyribonucleoside phosphate isotopomer can be used as a reliable guide for determining the absolute isotopic configuration at phosphorus of such a diester, thus making unnecessary the more rigorous and difficult stereochemical analysis as presented here and requiring perhaps 10–20 times less material. Knowing the isotopic configuration at phosphorus for (R_p) - $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]\text{UpA}$ permits us now to assign the resonances in the ^{31}P NMR spectrum of UpA after methylation. The only previous such correlation has been made by Potter et al. for TpT methyl ester,¹⁴ where it was demonstrated that in the ^{31}P NMR spectrum of this compound in Me_2SO solution the resonance at low field could be assigned to (S_p) -TpT methyl ester and that at high field to (R_p) -TpT methyl ester. Similar information for members of the ribonucleoside series would of course be useful as a comparison.

UpA was methylated to two diastereoisomeric methyl esters of uridylyl(3'→5')adenosine according to Potter et al.¹⁴ Examination of the ^{31}P NMR spectra of these diastereoisomers in Me_2SO at this stage showed only a broad resonance centered upon +0.33 ppm relative to external H_3PO_4 . These conditions had previously sufficed for a 7.5-Hz separation of the signals of the diastereoisomers of TpT methyl ester in the deoxy series.¹⁴ Addition of methanol to the solution to a ratio of 50:50 v/v, however, produced a separation of the ribodinucleoside phosphate resonances of ca. 8 Hz, and two sharp signals could be clearly seen (Figure 4a). This is in contrast to what has been observed for TpT methyl ester, where addition of methanol resulted in a considerable decrease in the separation of the ^{31}P NMR signals (B. V. L. Potter, unpublished observation).

Methylation of (R_p) - $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]\text{UpA}$ in an identical fashion gave rise to the ^{31}P NMR spectrum shown in Figure 4b. Four resonances are to be seen, assignable to two unlabeled methyl esters and two methyl esters containing ^{18}O . [The methyl esters derived from (R_p) - $[\text{}^{17}\text{O}]\text{UpA}$ are not observed^{11b,13}]. Two sets of resonances are observed, each pair representing an isotope shift. The low-field pair exhibits clearly a larger isotope shift (3.26 Hz) than the high-field pair (1.28 Hz), thus demonstrating that the former can be assigned to a methyl ester that contains ^{18}O in a double bond and the latter to a methyl ester containing ^{18}O in a single bond in the labeled species in this case. Thus, the signals to low field can be assigned to the ester 10a (S_p configuration) and those to high field to the ester 10b (R_p configuration). Consequently, the absolute configurations of the methyl esters of unlabeled UpA 9a/b can be assigned by a simple comparison of the NMR spectra in Figure 4. These assignments are the same as those previously established for the methyl esters of TpT.

Thus, we present here the configurational assignments of two ribodinucleoside phosphate methyl esters, namely, the fully protected methyl esters 4a and 4b and also the diastereoisomeric methyl triesters of uridylyl(3'→5')- N^1 -methyladenosine 9a and 9b.

These findings, therefore, present the interesting possibility that methylation of either a ribo- or deoxyribodinucleoside phosphate might generally permit the assignment of the resulting S_p diastereoisomer to the ^{31}P NMR resonance at low field and the R_p diastereoisomer to that at high field. It is clear, however, that further examples in both series must be confirmed before such an NMR effect can be turned around and used as a reliable indication of absolute configuration at phosphorus in an acyclic phosphate methyl ester or as a method for the determination of the absolute isotopic configuration of any ^{18}O -labeled dinucleoside phosphate isotopomer.

In summary, we present in this paper a straightforward method for the stereospecific synthesis of isotopomers of both configu-

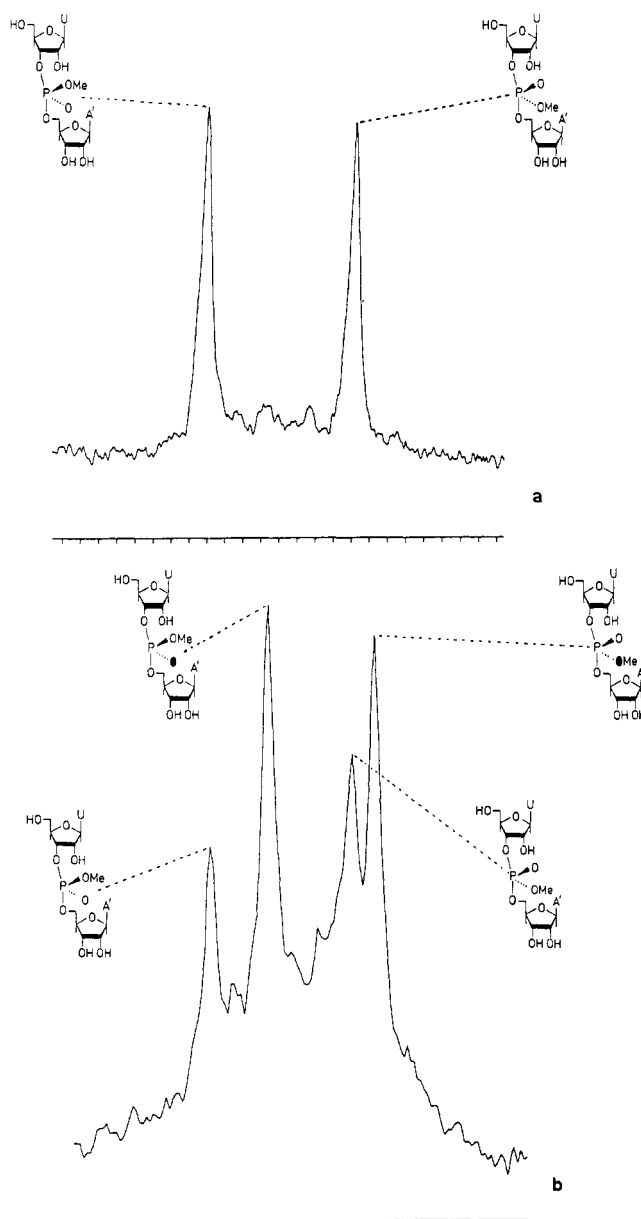


Figure 4. 81.01-MHz ^{31}P NMR spectra of (R_p, S_p) -uridylyl(3'→5')- N^1 -methyladenosine methyl ester (9a/b) and (R_p, S_p) - $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ -uridylyl(3'→5')- N^1 -methyladenosine methyl ester (10a/b). The upper spectrum (a) is of the mixture of diastereoisomers of uridylyl(3'→5')- N^1 -methyladenosine methyl ester (9a/b) [δ -3.11 (S_p) and -3.21 (R_p) measured from external trimethyl phosphate]. Assignments were made after the interpretation of spectrum 4 (b). The lower spectrum (b) represents the mixture of diastereoisomers obtained by the methylation of (R_p) - $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]\text{UpA}$ (7b) [only resonances due to $[\text{}^{16}\text{O}]$ - and $[\text{}^{18}\text{O}]$ -uridylyl(3'→5')- N^1 -methyladenosine methyl ester are observed]. Isotope shifts are as follows: for downfield resonances, 3.26 ± 0.18 Hz; for upfield resonances, 1.28 ± 0.18 Hz. Spectra were taken from an 18 mM solution in $\text{Me}_2\text{SO}-d_6$ -MeOH (50:50 v/v) containing 8-hydroxyquinoline; NMR parameters were as follows: sweep width, 1501 Hz; pulse width, 10 μs ; acquisition time, 5.46 s; data collection in 16K; Fourier transform in 32K; number of transients, 2058; line broadening 0.18 Hz; scale, 1 Hz = 1 division. U = uridine; A' = N^1 -methyladenosine.

rations of ^{17}O - or ^{18}O -labeled deoxydinucleoside or ribodinucleoside phosphates. The absolute configurations of two ribodinucleoside phosphate methyl esters have been assigned, and the absolute isotopic configuration of $[\text{}^{18}\text{O}]\text{UpA}$ can now be determined by a simple methylation procedure.

Experimental Section

Melting points were determined on a Berli apparatus (Wagner & Munz, West Germany) and are not corrected. UV spectra were measured on an Uvikon 810 spectrophotometer (Kontron, Switzerland).

(29) Although the deprotection reaction occurred with retention of configuration at phosphorus, the R,S sequence rules demand that the resulting isotopically labeled product be assigned the opposite absolute configuration to that of its precursor.

NMR spectra were recorded on Bruker WM 250 and WP 200SY spectrometers. δ values are relative to Me_4Si for ^1H , ^{13}C , and ^{29}Si nuclei, relative to external phosphoric acid or trimethyl phosphate for the ^{31}P nucleus, and relative to natural-abundance ^{17}O for the ^{17}O nucleus. Chemical shifts are positive when downfield from the appropriate standard. TLC was performed on silica gel SIL G-25 UV₂₅₄ plates and polyethylenimine-impregnated cellulose CEL 300 PEI/UV₂₅₄ plates (Macherey-Nagel, West Germany) with solvent systems (A) CHCl_3 -MeOH (99:1), (B) CHCl_3 -MeOH (95:5), (C) CHCl_3 -MeOH (9:1), and (D) 1 M LiCl. Silica gel 60, 70–230-mesh ASTM, and 230–400-mesh ASTM (Merck, West Germany) and DEAE-Sephadex A-25 (Pharmacia, Sweden) were used for column chromatography with eluents as indicated. The columns were connected to a Uvicord UV detector and an UltroRac fraction collector (LKB Instruments, Sweden). HPLC was performed on a prepacked LiChrosorb RP-18 column (250 \times 4 mm; Merck, West Germany) connected to an Altex Model 110A pump and an Altex dual-wavelength detector, Model 152 (Altex, Berkeley, CA). As the eluant 0.1 M aqueous ammonium acetate containing 5% acetonitrile was used.

Tetrahydrofuran was dried over sodium wire. Acetonitrile was distilled from P_2O_5 before use. *sym*-Collidine, pyridine, 2,6-lutidine, and diisopropylethylamine were distilled from KOH and stored over 4-Å molecular sieves. Dimethylformamide was dried with barium oxide and distilled under reduced pressure.

Elemental analyses were performed by Mikroanalytisches Labor Beller (Göttingen, West Germany).

^{17}O , ^{18}O H_2O was obtained from the Kernforschungszentrum Karlsruhe (^{16}O , 16.5%; ^{17}O , 21.27%; ^{18}O , 62.23%) and from the Monsanto Research Corp. (Miamisburg, OH; ^{16}O , 9.4%; ^{17}O , 52.8%; ^{18}O , 37.8%). Nucleosides were purchased from Pharma Waldhof (Düsseldorf, West Germany). Ribonuclease A (EC 3.1.27.5) and snake venom phosphodiesterase (EC 3.1.4.1) were purchased from Boehringer Mannheim (Mannheim, West Germany). UpA, spleen phosphodiesterase (EC 3.1.4.18), and nuclease P1 (EC 3.1.30) were obtained from Sigma Chemical Co. (St. Louis, MO).

(R_p , S_p)-2',5'-Bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]-3'-*O*-[(dimethylamino)methoxyphosphino]uridine (**1b**). 2',5'-Bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]uridine¹⁶ (**1a**) (236 mg, 0.5 mmol), which had been dried over P_2O_5 , and *N,N*-diisopropylethylamine (0.4 mL, 2.3 mmol) were dissolved in acid-free chloroform (1.5 mL). The reaction vessel was preflushed with dry nitrogen and chloro(dimethylamino)methoxyphosphine²⁰ (0.13 mL, 1 mmol) was added dropwise with a syringe during a period of 1 min. The solution was stirred for 15 min under nitrogen and then diluted with ethyl acetate. After extraction with a saturated solution of sodium chloride (4 times) the organic layer was dried with sodium sulfate and evaporated in vacuo to yield a pale yellow crude product, **1b**: ^{31}P NMR (CDCl_3) δ 149.84 (s), 149.30 (s) relative to H_3PO_4 .

*N*⁶-Benzoyl-2',3',5'-tris-*O*-[(1,1-dimethylethyl)dimethylsilyl]adenosine (**2a**). *N*⁶-Benzoyladenose¹⁹ (0.78 g, 2.0 mmol), dried over P_2O_5 , *tert*-butyldimethylchlorosilane (1.5 g, 10.0 mmol), and imidazole (1.36 g, 20.0 mmol) were dissolved in a mixture of anhydrous pyridine and *N,N*-dimethylformamide (20 mL, 1:1 v/v) and stirred for 6 h at 60 °C and then 12 h at 25 °C. The reaction mixture was evaporated repeatedly in vacuo, accompanied by additions of benzene to a final volume of 10 mL. The oily residue was dissolved in chloroform and extracted 3 times with water. After drying over anhydrous sodium sulfate the organic layer was evaporated. The resulting oil was applied to a silica gel column (40 \times 3.0 cm, 230–400-mesh ASTM). Elution with chloroform-methanol (99:1 v/v) furnished 0.99 g (69%) of a colorless foam as the main product: TLC (A) R_f 0.26; UV (MeOH) λ_{max} 280, 230 nm (ϵ 20 500, 13 100); ^{29}Si NMR (CDCl_3) δ 22.3, 21.9, 21.0. Anal. Calcd for $\text{C}_{35}\text{H}_{59}\text{N}_5\text{O}_5\text{Si}_3$: C, 58.86; H, 8.33; N, 9.81. Found: C, 58.90; H, 8.47; N, 9.68.

*N*⁶-Benzoyl-2',3'-bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]adenosine (**2b**). Compound **2a** (500 mg, 0.7 mmol) was dissolved in nitromethane (7 mL), a solution of zinc bromide in nitromethane (7 mL, 70 g of anhydrous ZnBr_2 in 500 mL of nitromethane and 5 mL of water) was added, and the mixture was stirred overnight at room temperature. The reaction was then quenched by the addition of 1 M aqueous ammonium acetate (200 mL) and chloroform (50 mL). The organic layer was separated, washed with water, dried over anhydrous sodium sulfate, and evaporated in vacuo. The oily residue was dissolved in chloroform and ether was added until an opalescence appeared. After standing overnight at 3 °C the product crystallized, yielding 342 mg (81%) of colorless needles with mp 189 °C (lit.¹⁹ mp 188–192 °C): ^{29}Si NMR (CDCl_3) δ 22.85, 20.86.

Condensation of Compound 1b with 2b and Oxidation in the Presence of ^{17}O , ^{18}O H_2O . Compound **1b** (290 mg, 0.5 mmol), thoroughly dried, was dissolved in 8 mL of a mixture of dry acetonitrile and tetrahydrofuran (4:1 v/v), and freshly sublimed tetrazole (53 mg, 0.75 mmol) was

added. Then a solution of compound **2b** (151 mg, 0.25 mmol) in the same solvent mixture was added dropwise at room temperature and the reaction mixture was stirred for 24 h. Oxidation of the resulting phosphite **3a/b** was accomplished by addition of iodine (125 mg, 0.5 mmol) in tetrahydrofuran-2,6-lutidine- ^{17}O , ^{18}O H_2O (^{17}O , 21.27%; 4 mL, 2:1:1 v/v/v). The resulting mixture was stirred for another 15 min and then evaporated to dryness. The oily residue was dissolved in chloroform and extracted with an aqueous solution of sodium bisulfite (4 mL of saturated sodium bisulfite solution in 20 mL of water). The organic layer was dried, concentrated, and applied to a silica gel column (20 \times 2.5 cm, 230–400-mesh ASTM). Stepwise elution with chloroform-methanol mixtures of increasing methanol content (100:0, 99.5:0.5, 99:1 v/v) yielded a faster migrating zone of **4a** (100 mg, 35%) and a slower migrating zone of **4b** (67 mg, 23%).

Condensation of 2',5'-Bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]uridine (1a**) with Compound 2b and Dichloromethoxyphosphine and Oxidation by ^{17}O , ^{18}O H_2O .** Dichloromethoxyphosphine (64 μL , 0.68 mmol) was dissolved at -78 °C in a mixture of 0.75 mL of dry tetrahydrofuran containing *sym*-collidine (228 μL , 2.46 mmol). A solution of compound **1a** (293 mg, 0.62 mmol), thoroughly dried, in 0.7 mL of dry tetrahydrofuran was added dropwise over a period of 5 min with vigorous stirring. After 30 min the nucleoside **2b** was added, and the reaction was allowed to proceed for 1 h at -78 °C and for another hour at room temperature. A solution of iodine (170 mg, 0.68 mmol) and ^{17}O , ^{18}O H_2O (^{17}O , 21.27%; 0.25 mL) in tetrahydrofuran (2.0 mL) with *sym*-collidine (0.5 mL) was then added. The oxidation was complete within 15 min at ambient temperature and the mixture was then concentrated in vacuo and the residue was dissolved in chloroform (20 mL). The subsequent steps were identical with those of the method described above, yielding the faster migrating zone of **4a** (88 mg, 15%) and the slower migrating zone of **4b** (239 mg, 46%).

(R_p)- ^{17}O , ^{18}O -2',5'-Bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]-*O*-(*P*-methyluridylyl)(3'→5')-*N*⁶-benzoyl-2',3'-bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]adenosine (**4a**). The content of the faster migrating zone gave colorless needles of **4a** from ether-*n*-hexane: mp 181 °C; TLC (B) R_f 0.70; UV (MeOH) λ_{max} 271 nm (ϵ 27 500); ^{29}Si NMR (CDCl_3) δ 24.08, 23.72, 22.66, 22.23; ^1H NMR (CDCl_3) δ 6.15 (d, $J_{1,2'} = 6$ Hz; 1'-H), 6.04 (d, $J_{1,2'} = 4$ Hz, 1'-H); ^{31}P NMR (CDCl_3) δ 0.71 (relative to H_3PO_4). Anal. Calcd for $\text{C}_{51}\text{H}_{86}\text{N}_{10}\text{O}_{13}\text{PSi}_4$: C, 53.33; H, 7.55; N, 8.54. Found: C, 53.46; H, 7.33; N, 8.68.

(S_p)- ^{17}O , ^{18}O -2',5'-Bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]-*O*-(*P*-methyluridylyl)(3'→5')-*N*⁶-benzoyl-2',3'-bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]adenosine (**4b**). The material from the slower migrating zone furnished colorless needles of **4b** from ether-*n*-hexane: mp 181 °C; TLC (B) R_f 0.63; UV (MeOH) λ_{max} 271 nm (ϵ 26 300); ^{29}Si NMR (CDCl_3) δ 24.06, 23.86, 22.77, 22.14; ^1H NMR (CDCl_3) δ 6.07 (d, $J_{1,2'} = 6$ Hz; 1'-H), 6.02 (d, $J_{1,2'} = 5$ Hz; 1'-H); ^{31}P NMR (CDCl_3) δ 0.50 (relative to H_3PO_4). Anal. Calcd for $\text{C}_{51}\text{H}_{86}\text{N}_{10}\text{O}_{13}\text{PSi}_4$: C, 53.33; H, 7.55; N, 8.54. Found: C, 53.56; H, 7.48; N, 8.51.

(S_p)- ^{17}O , ^{18}O -2',5'-Bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]uridylyl-(3'→5')-*N*⁶-benzoyl-2',3'-bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]adenosine Triethylammonium Salt (**5a**). Compound **4a**, R_p isomer (186 mg, 0.16 mmol), in a solution of thiophenol-triethylamine-*p*-dioxan (6 mL, 1:1:2 v/v/v) was stirred for 45 min at 25 °C, and the reaction mixture was evaporated in vacuo. The oily residue was dissolved in chloroform, applied to a silica gel column (20 \times 2.5 cm), and chromatographed with chloroform-methanol (9:1) as the eluant. The main fraction was pooled and evaporated, yielding a colorless foam of **5a** (162 mg, 81%); TLC (C) R_f 0.43; UV (MeOH) λ_{max} 271 nm (ϵ 25 300).

(R_p)- ^{17}O , ^{18}O -2',5'-Bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]uridylyl-(3'→5')-*N*⁶-benzoyl-2',3'-bis-*O*-[(1,1-dimethylethyl)dimethylsilyl]adenosine Triethylammonium Salt (**5b**). Starting with the S_p isomer **4b** (153 mg, 0.13 mmol) and applying the reaction conditions described above for **4a**, we obtained compound **5b** as a colorless foam (127 mg, 78%); TLC (C) R_f 0.43; UV (MeOH) λ_{max} 271 nm (ϵ 25 100). Anal. Calcd for $\text{C}_{56}\text{H}_{98}\text{N}_{10}\text{O}_{13}\text{PSi}_4$: C, 54.56; H, 7.85; N, 9.09. Found: C, 54.63; H, 7.96; N, 9.13.

(S_p)- ^{17}O , ^{18}O Uridyl(3'→5')adenosine Ammonium Salt (**7a**). Compound **5a** (103 mg, 0.08 mmol) was suspended in a mixture of dioxan (5 mL) and concentrated ammonia (5 mL) and stirred for 24 h until all the material had dissolved. The solution was then evaporated in vacuo, the residue was dissolved in the standard TBAF reagent (5 mL; 0.7 M tetrabutylammonium fluoride in tetrahydrofuran-pyridine-water, 8:1:1 v/v/v), and the resulting mixture was stirred for 2 h at room temperature. After that the solution was evaporated to dryness, and the residue was dissolved in 0.02 M aqueous ammonium bicarbonate and applied onto a DEAE-Sephadex column (30 \times 2.0 cm). Compound **7a** was eluted by a linear gradient of 0.02–0.2 M aqueous ammonium bicarbonate at about 0.12 M and lyophilized, yielding a colorless fluffy material (23 mg, 68%); HPLC [LiChrosorb RP-18, 0.1 M ammonium

acetate–acetonitrile (95:5)] retention time = 24 min at 1.5 mL/min. The peak coincided with that of unlabeled (3'→5')UpA: UV (H₂O) λ_{max} 259 nm.

(*R*_p)-[¹⁷O,¹⁸O]Uridyl(3'→5')adenosine Ammonium Salt (**7b**). Using the same reaction conditions as described for compound **7a** and starting with **5b** (115 mg, 0.09 mmol), we obtained colorless fluffy **7b** (40 mg, 75%). UV and HPLC data were identical with those of the isomer **7a** and (3'→5')UpA. Enzymatic cleavage at 37 °C with spleen phosphodiesterase (0.05 M ammonium acetate, pH 6.5) furnished uridine 3'-monophosphate and adenosine (TLC, PEI–cellulose, solvent D); the same reaction products were obtained by hydrolysis with ribonuclease A (0.01 M Tris–HCl, pH 7.8 37 °C). Snake venom phosphodiesterase digested **7b** to adenosine 5'-monophosphate and uridine (0.01 M Tris–HCl, pH 9.0, 37 °C). Identical cleavage products as described for **7b** were also found for **7a** in all cases.

Configurational Analysis of (*R*_p)-[¹⁷O,¹⁸O]Uridyl(3'→5')adenosine (7b**).** (a) **Hydrolysis of (*R*_p)-[¹⁷O,¹⁸O]UpA by Nuclease P1 in H₂¹⁷O.** [¹⁷O,¹⁸O]UpA **7b** (42.8 μmol), derived by deprotection of **4b** (slower migrating zone), was dissolved in H₂¹⁷O (¹⁷O, 52.8%; 100 μL) and left at room temperature for 30 min. The H₂¹⁷O was removed in vacuo under thoroughly anhydrous conditions, and the solid residue was left on a vacuum line for 15 h. The residue was then dissolved in H₂¹⁷O (350 μL) and nuclease P1 was added as a lyophilized powder (300 μg, ca. 120 units). The solution was incubated at 37 °C and the cleavage reaction monitored by HPLC. The reaction was seen to be essentially complete after 9 min, but the mixture was left for a total time of 30 min at 37 °C and then applied to a DEAE-Sephadex A-25 column (30 × 25 cm) and the reaction products were eluted with a gradient of 1 L each of 50 and 250 mM triethylammonium bicarbonate. Fractions of approximately 20 mL were collected. Uridine (37 μmol) was eluted in fractions 10–17 and [¹⁶O,¹⁷O,¹⁸O]AMP **8** (35.6 μmol) in fractions 72–91. The latter fractions were pooled and evaporated to dryness in vacuo, excess triethylammonium bicarbonate being removed by the evaporation of several volumes of methanol.

(b) **Configurational Analysis of (*S*_p)-[¹⁶O,¹⁷O,¹⁸O]AMP (**8**).** [¹⁶O,¹⁷O,¹⁸O]AMP triethylammonium salt (35.6 μmol) was converted to the tri-*n*-octylammonium salt via the pyridinium salt and cyclized with diphenylphosphochloridate and potassium *tert*-butoxide to the isotopomers of [¹⁶O,¹⁷O,¹⁸O]cAMP by the method of Jarvest et al.^{10a} Purification by DEAE-Sephadex chromatography as described above gave 7.7 μmol (22%) of pure [¹⁶O,¹⁷O,¹⁸O]cAMP, which was eluted in fractions 15–22. After evaporation of solvent in vacuo and conversion to the potassium-18-crown-6 salt, this product was methylated by using methyl iodide in

Me₂SO according to the method of Jarvest et al.^{10a} The ³¹P NMR spectra obtained are shown in Figure 3: ³¹P NMR (Me₂SO-*d*₆–Me₂SO, 50:50 v/v) δ –2.809, –2.827, –2.851, –2.869 (4 s) equatorial series; –4.032, –4.046, –4.074, –4.087, (4 s) axial series (referenced to H₃PO₄).

(*R*_p,*S*_p)-Uridyl(3'→5')-*N*¹-methyladenosine Methyl Ester (**9a/b**). UpA ammonium salt (7 mg, 11.9 μmol) was dissolved in water (5 mL) and the solution stirred with Dowex 50 XW (1 mL, potassium form) for 30 min. The solution was filtered from the resin, and filtrate and washings were evaporated to a small volume. 18-Crown-6 was added (15 mg, 57 μmol) and the resulting solution evaporated thoroughly to dryness in vacuo. The residue was dissolved in dry DMF (5 mL), which was evaporated under anhydrous conditions. This was repeated 3 times. The residue was finally dissolved in Me₂SO-*d*₆ (200 μL) and methyl iodide (100 μL) was added. After the mixture had been stirred for 12 h the methyl iodide was removed in vacuo to give (*R*_p,*S*_p)-uridylyl(3'→5')-*N*¹-methyladenosine methyl ester as a solution in Me₂SO-*d*₆. The methylation was effectively quantitative. To this solution were added a few crystals of 8-hydroxyquinoline, followed by methanol (200 μL). The resulting solution was filtered and the ³¹P NMR spectrum recorded (Me₂SO-*d*₆–MeOH, 50:50 v/v) δ –3.11 (s) for the *S*_p diastereoisomer and –3.21 (s) for the *R*_p diastereoisomer (with trimethyl phosphate as the external standard) (Figure 4a).

(*R*_p,*S*_p)-[¹⁷O,¹⁸O]Uridyl(3'→5')-*N*¹-methyladenosine Methyl Ester (**10a/b**). [¹⁷O,¹⁸O]Uridyl(3'→5')-*N*¹-methyladenosine methyl ester was synthesized from (*R*_p)-[¹⁷O,¹⁸O]UpA (9.2 μmol) exactly as described for the unlabeled compound. ³¹P NMR (Me₂SO-*d*₆–MeOH, 50:50 v/v) δ –3.139, –3.180 (2 s) and –3.237, –3.253 (2 s) (with trimethyl phosphate as the standard) (Figure 4b).

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Note Added in Proof. (a) In the dinucleoside monophosphates and their protected derivatives, the nomenclature [¹⁷O,¹⁸O] is intended to signify the presence of both isotopes at one position at phosphorus. (b) The phosphorus decoupled ¹⁷O NMR spectra of **7a** and **7b** gave both singlets at 93.04 ppm (water, pH 6.1) [Gerlt, J. A., private communication].

A Mechanistic Basis for the Stereoselectivity of Enzymatic Transfer of Hydrogen from Nicotinamide Cofactors

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Abstract: A mechanistic theory explains the stereochemical preferences of dehydrogenases dependent on nicotinamide cofactors that interconvert alcohols and ketones. This theory is based on the principles of stereoelectronic control and assumes that the Gibbs free energies of reactants and products are more nearly matched when bound in the active site of an optimal enzyme than in solution. This theory makes experimentally testable predictions both of the stereochemical preferences of dehydrogenases and of the nature of the free energy profile of enzyme-catalyzed reactions. Consistent with these predictions, we report that the enzyme lactaldehyde reductase (EC 1.1.1.55) from pig kidney catalyzes the transfer of the *pro-R* (A) hydrogen from NADH, and we provide estimates for the "internal" equilibrium constant between the ternary complexes enzyme–alcohol–NAD⁺ and enzyme–ketone–NADH.

Thirty years ago, in a classical demonstration that enzymes could distinguish between enantiotopic groups on substrate molecules, Westheimer and Vennesland showed that dehydrogenases catalyze the stereoselective transfer of hydrogens from the 4-position of reduced nicotinamide cofactors.¹ Subsequent studies showed that dehydrogenases appeared to be dis-

tributed "randomly" between two stereochemical classes, those transferring the *pro-R* hydrogen and those transferring the *pro-S* hydrogen.² These observations present the most puzzling problem

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