Synthesis of Adenosine 5′[(*R*)α-^{17}O]Triphosphate

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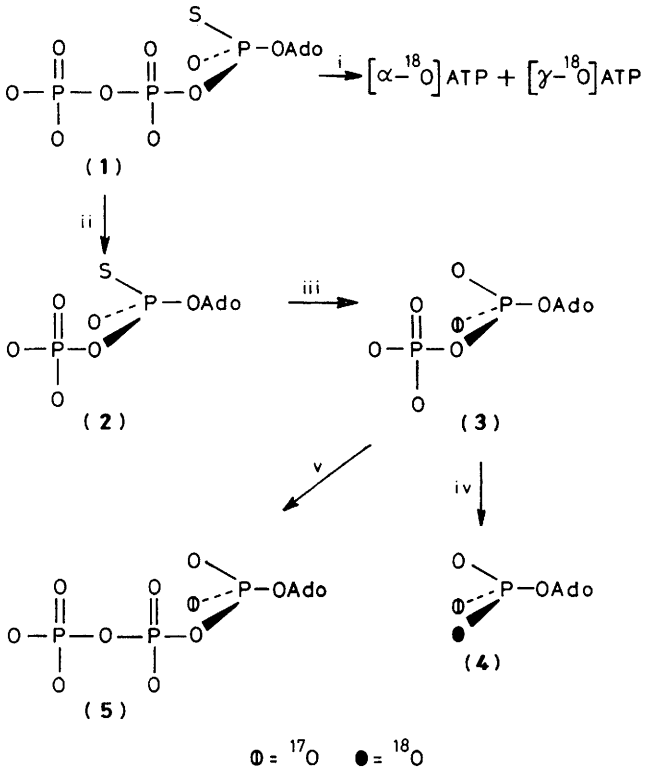
Adenosine 5′[(*S*)α-thio]triphosphate is converted predominantly into adenosine 5′[γ-^{18}O]triphosphate by bromine in [^{18}O]water, but adenosine 5′[(*S*)α-thio]diphosphate gives adenosine 5′[(*R*)α-^{17}O]diphosphate on treatment with bromine in [^{17}O]water which can be converted enzymically into adenosine 5′[(*R*)α-^{17}O]-triphosphate.

A method has been developed for the analysis of the chirality of [^{16}O, ^{17}O, ^{18}O] phosphate esters,[1] which makes possible the determination of the stereochemical course of not only phosphokinases,[2] phosphomutases,[3] phosphatases,[4] and phospho-

diesterases,[5] but also the nucleotidyl transferases, a group of enzymes which harness the chemical potential of ATP for the biosynthesis of all the major biopolymers. In order to undertake a stereochemical investigation of this class of enzymes, however, it is necessary to make ATP (or another nucleoside 5′-triphosphate) chiral at P_α, ideally by isotopic substitution. We report here a synthesis of adenosine 5′[(*R*)α-^{17}O]triphosphate.

Adenosine 5′[(*S*)α-thio]triphosphate (**1**) can be prepared enzymically from adenosine 5′-phosphorothioate,[6] and its chirality at P_α has been rigorously established.[7] Although there appears to be no example in the literature of the conversion of an *O,O*-dialkyl phosphorothioate into a dialkyl phosphate, *S,O,O*-trialkyl phosphorothioates have been converted into dialkyl phosphates with bromine–water,[8] and into trialkyl phosphates with bromine in an alcohol; in the latter case the reaction was shown to occur with inversion of configuration at phosphorus.[9]

Preliminary experiments showed that *O,O*-diethyl phosphorothioate was rapidly (<4 min) and quantitatively converted into diethyl phosphate by bromine–water at ambient temperature. Since bromine–water is known to convert adenine nucleotides into 8-bromo-adenine nucleotides in buffer (pH 4.0 over several hours) at ambient temperature,[10] it was necessary to establish that the reaction time was sufficiently short to avoid brominating the adenine ring. Adenosine 5′-phosphorothioate[11] (35 μmol) in water (70 μl) containing bromine (88 μmol) was kept for 4 min at ambient temperature and then the excess of bromine destroyed with sodium hydrogen sulphite. Adenosine 5′-phosphate was obtained quantitatively as shown by ^{1}H and ^{31}P n.m.r. spectroscopy and adenylate deaminase assay.



Scheme 1. *Reagents:* i, Br_2, [^{18}O]water; ii, hexokinase or myosin, Mg^{2+}; iii, Br_2, [^{17}O]water; iv, snake venom phosphodiesterase, [^{18}O]water; v, phosphoenolpyruvate, pyruvate kinase, Mg^{2+}, K^+.

Adenosine 5′[(*S*)α-thio]triphosphate (**1**) was rapidly (<4 min) converted into ATP by bromine–water at ambient temperature, but when the reaction was run in [^{18}O]water, the ^{18}O incorporated into ATP was distributed between P_α and P_γ in the ratio of 1 : 4 as shown by ^{31}P n.m.r. spectroscopy.[12] This was presumably due to the activated intermediate reacting directly with [^{18}O]water to give [α-^{18}O]ATP and intramolecularly with the γ-phosphate residue to give adenosine 5′-trimetaphosphate[13] which would hydrolyse to [γ-^{18}O]ATP (Scheme 1).

In order to circumvent this problem, adenosine 5′[(*S*)α-thio]triphosphate (**1**) was hydrolysed to adenosine 5′[(*S*)α-thio]diphosphate (**2**) with hexokinase[14] (or myosin[15]). Adenosine 5′[(*S*)α-thio]diphosphate (**2**) (300 μmol) in [^{17}O]water (420 μl; 4 atom % ^{18}O, 43 atom % ^{17}O, and 53 atom % ^{18}O) containing bromine (1.5 mmol) was kept for 4 min at ambient temperature and then the excess of bromine destroyed by the addition of anhydrous sodium hydrogen sulphite. The [^{17}O]-water can be recovered by lyophilization on a vacuum line and the adenosine 5′[α-^{17}O]diphosphate (**3**) isolated by ion-exchange chromatography on DEAE-Sephadex A25, with elution with triethylammonium hydrogen carbonate buffer, pH 7.6. A portion of the adenosine 5′[α-^{17}O]diphosphate (**3**) was hydrolysed in [^{18}O]water by snake venom phosphodiesterase to adenosine 5′[^{16}O, ^{17}O, ^{18}O]phosphate (**4**) which was shown to have the (*S*)-configuration at phosphorus by our established analytical procedure.[1] The ^{31}P n.m.r. spectrum of the adenosine 5′[^{16}O, ^{17}O, ^{18}O]phosphate after cyclization and methylation is shown in Figure 1. Since the hydrolysis of adenosine 5′-triphosphate by snake venom phosphodiesterase has been shown to proceed with retention of configuration at phosphorus,[5a] the adenosine 5′[α-^{17}O]diphosphate must be the (R_p)-diastereoisomer (**3**), and hence the displacement of sulphur from adenosine 5′[(*S*)α-thio]diphosphate with bromine–water proceeds with inversion of configuration. Comparison of the observed relative intensities of the ^{31}P resonances from Figure 1 with those calculated for substitution with inversion of configuration, however, indicates that a small amount of racemization has occurred; the best fit of the data indicated that the reaction proceeds with 93% inversion and 7% retention of configuration (Table 1).

Table 1. Comparison of the observed relative peak intensities of the ^{31}P resonances taken from Figure 1 with the calculated values expected for conversion of adenosine 5′[(*S*)α-thio]diphosphate into adenosine 5′[α-^{17}O]diphosphate with 100% and 93% inversion of configuration at P_α. The isotopically labelled diastereoisomeric triesters were derived by cyclization followed by methylation of the adenosine 5′[^{16}O, ^{17}O, ^{18}O]phosphate obtained by hydrolysing the adenosine 5′[α-^{17}O]diphosphate with snake venom phosphodiesterase in [^{18}O]water (80 atom % ^{18}O).

	Equatorial triester			Axial triester		
		Calculated for			Calculated for	
	Observed	100% Inversion	93% Inversion 7% Retention	Observed	100% Inversion	93% Inversion 7% Retention
MeO–P=O	0.41	0.40	0.41	0.41	0.40	0.41
Me^{18}O–P=O	1.00	1.00	1.00	0.72	0.67	0.71
MeO–P=^{18}O	0.71	0.67	0.71	1.00	1.00	1.00
Me^{18}O–P=^{18}O	0.51	0.44	0.45	0.48	0.44	0.45

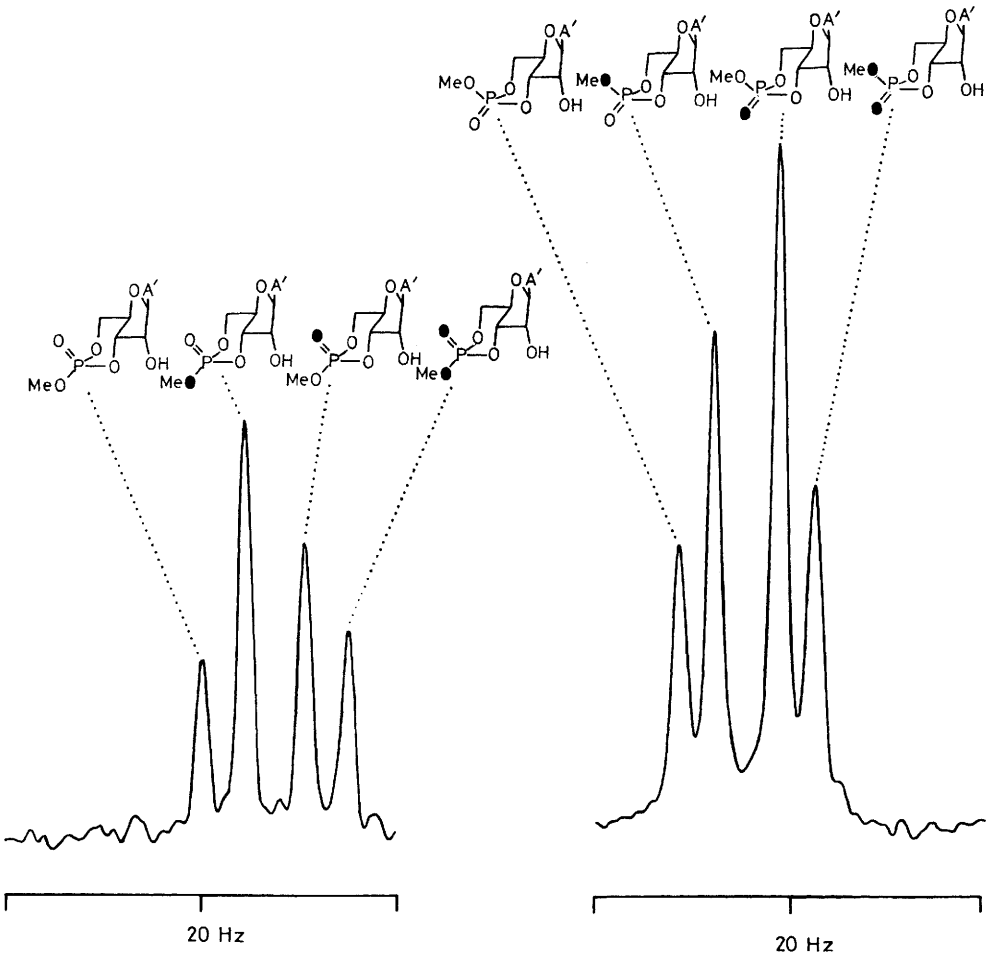


Figure 1. ^{31}P N.m.r. spectrum (121.5 MHz) of the equatorial and axial triesters derived by the cyclization and methylation of the adenosine 5′[^{16}O, ^{17}O, ^{18}O]phosphate obtained by snake venom phosphodiesterase-catalysed hydrolysis of adenosine 5′[α-^{17}O]-diphosphate in [^{18}O]water. The ratio of the [$^{16}O_{ax}$, $^{18}O_{eq}$]-to [$^{18}O_{ax}$, $^{16}O_{eq}$]-triesters shows that the adenosine 5′[^{16}O, ^{17}O, ^{18}O]phosphate has the (S_p)-configuration and hence the adenosine 5′[α-^{17}O]diphosphate has the (R_p)-configuration as shown in Scheme 1. ● = ^{18}O. A′ = N^1-methyladenine. The solvent and ^{31}P n.m.r. parameters are as reported previously.[1]

Adenosine 5′[(*R*)α-^{17}O]diphosphate (**3**) was then converted into adenosine 5′[(*R*)α-^{17}O]triphosphate (**5**) with pyruvate kinase and phosphoenolpyruvate. The ^{31}P n.m.r. spectrum of the adenosine 5′[(*R*)α-^{17}O]triphosphate (**5**), isolated by ion exchange chromatography on DEAE-Sephadex A25 (with triethylammonium hydrogen carbonate buffer, pH 7.6) is compared with that of ATP in Figure 2. If the ^{17}O site was fully enriched in adenosine 5′[(*R*)α-^{17}O]triphosphate (**5**) only the P_β and P_γ resonances would be observed in the ^{31}P n.m.r. spectrum since ^{17}O directly bonded to ^{31}P causes nuclear electric quadrupolar relaxation of the ^{31}P signal.[16] In ATP (Figure 2b) the P_α resonance is more intense than those of P_β and P_γ in the proton-decoupled spectrum owing to the nuclear Overhauser effect; in the spectrum of adenosine 5′[(*R*)α-^{17}O]-triphosphate (Figure 2a) the P_α resonance is seen to be less intense than those of P_β and P_γ.

From the relative intensities it can be calculated that the ^{17}O enrichment at P_α is about 40 atom %. The expanded P_α resonance in the spectrum of adenosine 5′[(*R*)α-^{17}O]triphosphate (Figure 2a inset) is seen to be two doublets due to ATP (minor doublet) and adenosine 5′[(*R*)α-^{18}O]triphosphate (major doublet) whereas the expanded P_β resonance is a single triplet as expected; the separation of the P_α doublets is caused by the upfield ^{18}O isotope shift.[12] From the ^{31}P n.m.r. spectrum the isotopic composition of the labelled site in adenosine 5′[(*R*)α-^{17}O]triphosphate can be calculated to be 5 atom % ^{16}O, 40 atom % ^{17}O, and 55 atom % ^{18}O, in good agreement with the isotopic composition of the [^{17}O]water used in the synthesis (*vide supra*). This material is now being used to investigate the stereochemical course of a wide range of nucleotidyl transferases.

Added in proof: The substitution of S by ^{18}O in nucleoside phosphorothioates has also been achieved with *N*-bromosuccinimide in dioxan/[^{18}O]water, but the claim that the reaction is stereospecific is not justified by the spectroscopic evidence.[17] When we treated thymidine 3′,5′(*R*)-cyclic phosphorothioate with *N*-bromosuccinimide in dioxan/[^{18}O]-water under the conditions used by Connolly *et al.*[17] (we are grateful to Professor Eckstein for informing us of this work prior to publication), the thymidine 3′,5′-cyclic[^{18}O]phosphate obtained was shown by ^{31}P n.m.r. spectroscopy (after methylation) to consist of 77% of the S_p and 23% of the R_p stereoisomers. In subsequent correspondence Professor Eckstein informed us that he has confirmed that the reaction of *N*-bromosuccinimide in dioxan/[^{18}O]water is not stereospecific and has now found that replacement of S by ^{18}O in both cyclic and acyclic phosphorothioates occurs with *ca.* 80% inversion and 20% retention of configuration at phosphorus (personal communication).

Cyanogen bromide in [^{18}O]water has also been used to prepare the R_p and S_p stereoisomers of adenosine 5′[α-^{18}O]-diphosphate from the S_p and R_p diastereoisomers of adeno-

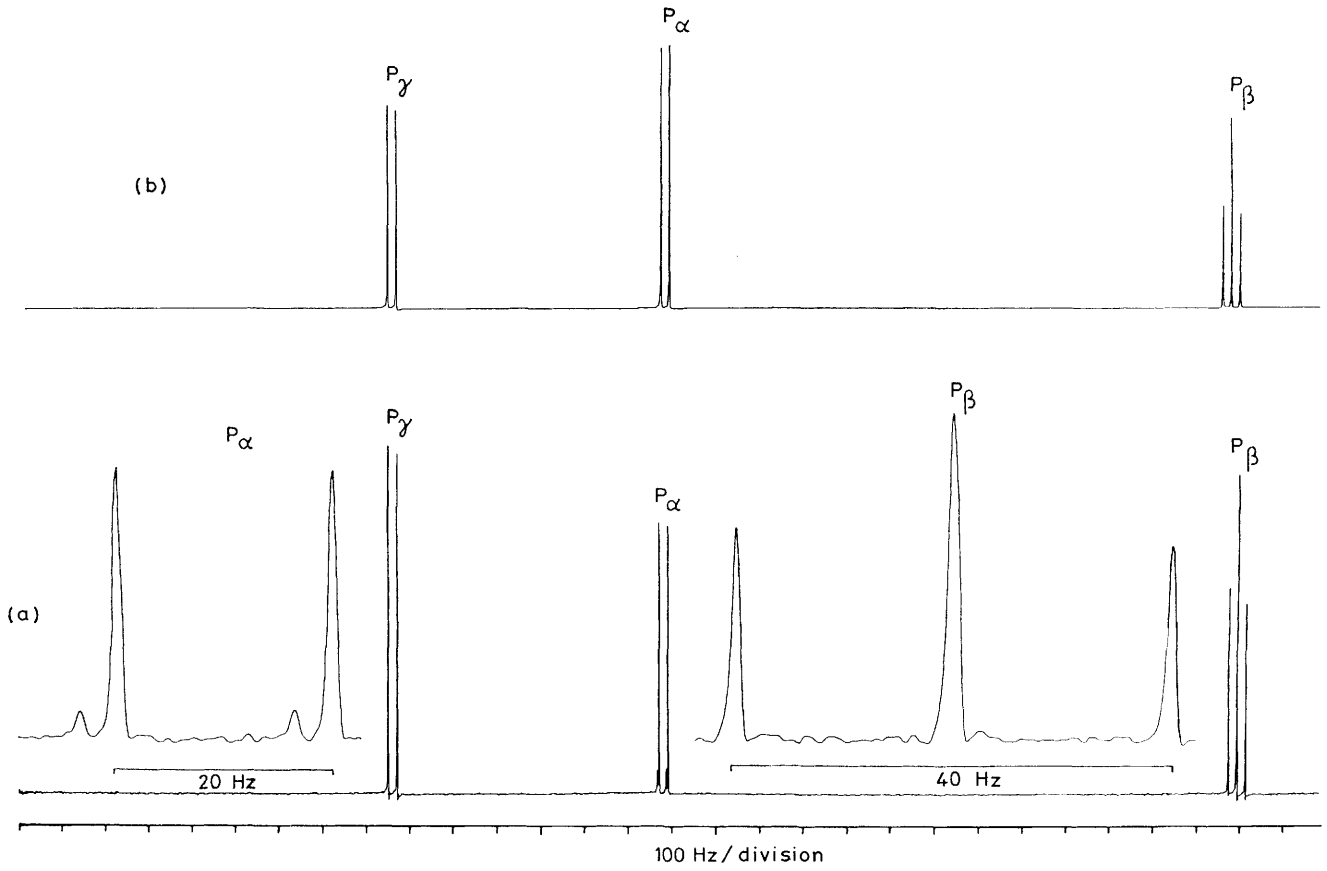


Figure 2. ^{31}P N.m.r. spectrum (121.5 MHz) of (a) adenosine 5′[(*R*)α-^{17}O]triphosphate with expansion of the P_α and P_β resonances, and (b) ATP, in 50% D_2O containing ethylenediaminetetra-acetic acid (10 mM) at pH 9.0. ^{31}P N.m.r. parameters are: offset 1150 Hz, sweep width 3012 Hz, acquisition time 1.36 s, pulse width (angle) 15 μs (75°), gaussian multiplication (line broadening-1.2 Hz, gaussian broadening 0.4) in 8K, and Fourier transform in 32K.

sine 5′[1-thio,2-cyanoethyl]diphosphate but the ^{31}P n.m.r. spectra reported for determining the stereochemistry at P_α of the adenosine 5′[α-^{18}O]diphosphates again do not permit an accurate assessment to be made of the stereoselectivity of the reaction.[18] Moreover the synthetic route is significantly longer than the procedure reported here or by Connolly *et al.*[17]

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