A Synthesis of Adenosine 5′-[β-$^{18}O_2$]Triphosphate

By **Gordon Lowe** * and **Brian S. Sproat**, The Dyson Perrins Laboratory, Oxford University, South Parks Road, Oxford OX1 3QY

A synthesis of adenosine 5′-[β-$^{18}O_2$]triphosphate (8) has been achieved by a novel route in an overall yield of 19% based on phosphorus pentachloride. The use of the diphenylmethyl protecting group is a key feature of the synthesis. Some ^{18}O isotope shifts in ^{31}P and ^{13}C n.m.r. spectra are reported.

PHOSPHORYL and adenylyl transferases utilise ATP † as one of their substrates. Phosphoryl transferases catalyse the transfer of the γ-phosphoryl group from ATP to co-substrate breaking the P_γ–OP_β bond, whereas adenylyl transferases catalyse the cleavage of the P_α–OP_β bond by the co-substrate. Enzyme-catalysed phosphoryl transfer reactions have so far been found to occur with inversion of configuration,[1,2] but the distinction between the 'in-line' associative and dissociative mechanisms has been investigated so far only with pyruvate kinase,[3] creatine kinase,[4] and hexokinase.[5] It is evident that if adenosine 5′-[β-$^{18}O_2$]triphosphate could be prepared, this would serve as a substrate for positional isotope exchange experiments [6] for both phosphoryl and adenylyl transferases, and so enable experiments to be performed which would allow a distinction to be made between the associative and dissociative mechanisms for both classes of enzymes. We now report an efficient synthesis of adenosine 5′-[β-$^{18}O_2$]triphosphate.

RESULTS AND DISCUSSION

The synthesis of adenosine 5′-[β-$^{18}O_2$]triphosphate (8) which has been developed (see Scheme), incorporates an entirely new route to ADP, a route necessitated by the ^{18}O labelling required in the final product. A central feature of this synthesis is the use of diphenylmethyl protecting groups, which are easily removed sequentially enabling the triphosphate moiety of ATP to be built up.

It is clear from the scheme that the synthesis would have been considerably shorter had it been possible to prepare diphenylmethyl [$^{18}O_4$]phosphate in a single step in high yield, since reaction of this ester with diphenyl phosphorochloridate would have given the diphosphate triester (4). However, reaction of mono(tri-n-butylammonium) phosphate with either 1 equiv. or a large excess of bromodiphenylmethane in dimethylformamide at 80—85 °C for upwards of 6 h gave only a moderate yield of the desired phosphate monoester (*ca.* 40% yield was estimated by ^{31}P n.m.r. spectroscopy). A similar result was obtained when acetonitrile was used as the solvent. It was apparent that use of bromodiphenylmethane as the alkylating agent would necessitate the use of ion-exchange chromatography to isolate the rather poor yield of phosphate monoester. It was decided at this point to investigate the reaction of phosphoric acid with diphenyldiazomethane (this reagent is easier to prepare and handle than phenyldiazomethane); use of 1 equiv. of the diazo-compound led to the formation of a mixture of the phosphate mono- and di-esters; however, the use of 3 equiv. yielded the crystalline phosphate triester almost quantitatively. The ^{31}P n.m.r. spectrum of tris(diphenylmethyl) [$^{18}O_4$]phosphate (1) admixed with some unlabelled material was recorded (see Figure 1) in order to determine the ^{18}O isotope shift and the ^{18}O isotopic enrichment of the product.

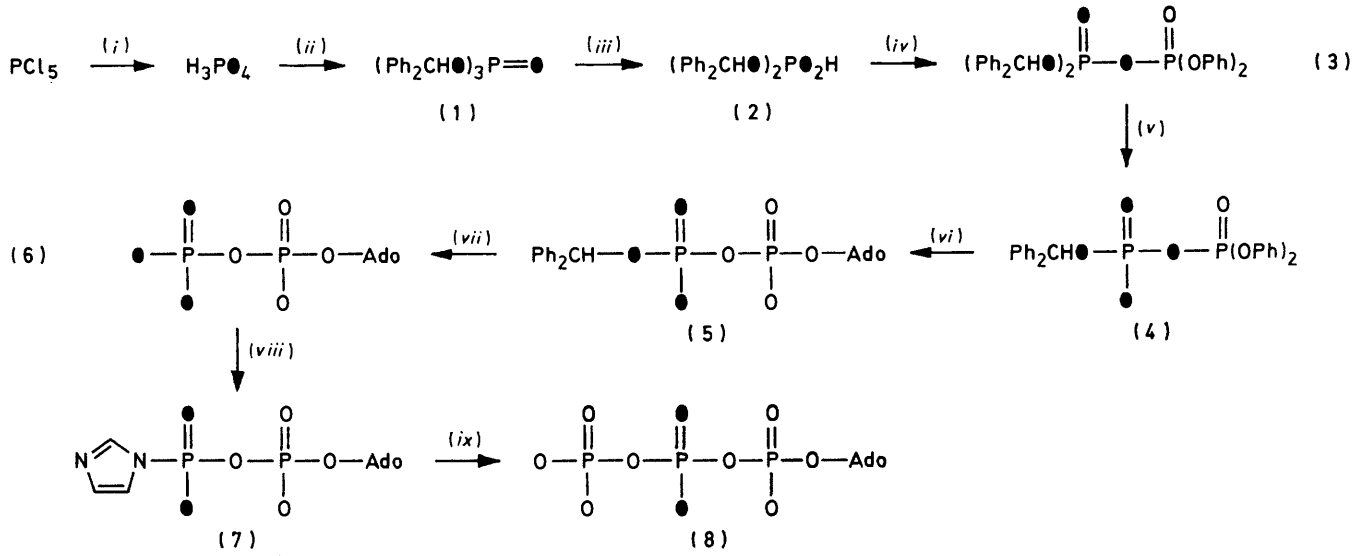
Analogous to the debenzylation of tribenzyl phosphate,[7] the phosphate triester (1) was rapidly converted to the sodium salt of the diester by reaction with sodium iodide in pyridine at 100 °C in virtually quantitative yield; acetone proved to be inferior as the solvent for this particular deprotection. The sodium salt of the diester was converted into bis(diphenylmethyl) [$^{18}O_4$]-phosphoric acid (2), which is totally insoluble in water, simply by acidification of an aqueous solution.

Using a method directly analogous to that used to prepare P^1-dibenzyl P^2-diphenyl diphosphate,[8] reaction of mono(tri-n-butylammonium) bis(diphenylmethyl) [$^{18}O_4$]phosphate with diphenyl phosphorochloridate gave the diphosphate tetraester (3) in high yield. In a preliminary investigation of the reaction of the unlabelled tetraester (3), with mono(tri-n-octylammonium) adenosine 5′-phosphate in pyridine it was found that the product mixture was complex due to the high reactivity of both P^1-diphenyl P^2-bis(diphenylmethyl) diphosphate and of the desired product, P_β-bis(diphenylmethyl) adenosine 5′-diphosphate. Hydrogenolysis of the crude reaction mixture in dimethylformamide gave a 36% yield of P_β-diphenylmethyl adenosine 5′-diphosphate but no ADP. A further modification of the synthesis was sought.

Once again, the diphenylmethyl group proved to be exceptionally useful. In a reaction similar to the debenzylation of tribenzyl phosphate [7] it proved possible to convert the highly reactive diphosphate tetraester (3) into the somewhat less reactive triester, P^1-diphenyl P^2-diphenylmethyl [P^2-$^{18}O_4$]diphosphate (4) using tri-n-butylammonium iodide (a reagent ideally suited for this purpose as it is soluble in dioxan and gives rise to a soluble salt of the diphosphate triester). Reaction was complete and virtually quantitative within 8 h at room temperature as judged by ^{31}P n.m.r. spectroscopy.

Treatment of the diphosphate triester (4) with 1

† Abbreviations; ADP, Adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate; ATP, adenosine 5′-triphosphate; DMF, dimethylformamide; EDTA, ethylenediamine-*NNN′N′*-tetra-acetic acid.



SCHEME ● = ^{18}O; Ado = 5′-adenosyl. Reagents: (*i*) $H_2{}^{18}O$–dioxan; (*ii*) Ph_2CN_2–dioxan; (*iii*) NaI–pyridine, aqueous HCl; (*iv*) $(PhO)_2P(O)Cl$–Bu^n_3N–dioxan; (*v*) $Bu^n_3NH^+I^-$–dioxan; (*vi*) $(n\text{-octyl})_3NH^+AMP^-$–pyridine; (*vii*) H_2, Pd–C, aqueous methanol; (*viii*) *NN*′-carbonyldi-imidazole–DMF; (*ix*) $Bu^n_3NH^+H_2PO_4^-$–DMF

equiv. mono(tri-n-octylammonium) adenosine 5′-phosphate in pyridine gave the desired P^1-(adenosine 5′-), P^2-diphenylmethyl [P^2-$^{18}O_3$]diphosphate (5) in 77% yield based on the diester (2). This reaction is effectively a Michelson synthesis [9] but it is complete within 10 h as compared with the 40 h or so required to obtain a similar yield of an ester of ADP by a normal Michelson synthesis.[9] The P_β-diphenylmethyl [β-$^{18}O_3$]-ADP was readily hydrogenolysed in aqueous methanol only after purification by ion-exchange chromatography. The yield of adenosine 5′-[β-$^{18}O_3$]diphosphate (6) from the diphenylmethyl ester (5) was 93.7% after purification by chromatography. The ^{31}P n.m.r. spectrum of [β-$^{18}O_3$]-ADP admixed with some unlabelled ADP is shown in Figure 2, and it can be estimated that the ^{18}O enrichment is *ca.* 96 atom %.

Finally, the literature procedure of Hecht and Kozarich [10] was used to convert the labelled ADP (6) into adenosine 5′-[β-$^{18}O_2$]triphosphate (8) *via* the intermediate imidazolide (7) which was not isolated. The ^{31}P n.m.r. spectrum of [β-$^{18}O_2$]-ATP (8) admixed with some unlabelled ATP is shown in Figure 3. The overall yield from phosphorus pentachloride to adenosine 5′-[β-$^{18}O_2$]-triphosphate is 19%.

In the course of this synthesis a number of ^{18}O isotope shifts have been measured which for convenience are

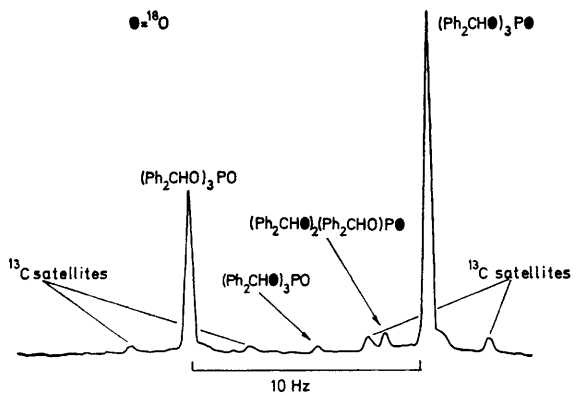


FIGURE 1 The ^{31}P n.m.r. spectrum at 121.493 MHz of tris-(diphenylmethyl) [$^{18}O_4$]phosphate (60 mg, 99.2 μmol) admixed with tris(diphenylmethyl) phosphate (30 mg, 50.3 μmol) and dissolved in $CDCl_3$ (2.2 ml). ^{31}P N.m.r. parameters were: 100 transients; band-width 400 Hz; memory size 4K, but 28K of zeroes added before Fourier-transformation; pulse repetition rate 5.12 s; pulse angle 87.7°; broad-band proton-noise decoupling; line broadening −0.5 Hz; Gaussian broadening 0.3

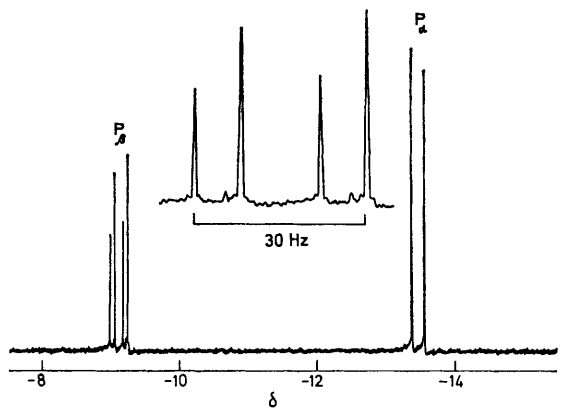


FIGURE 2 The ^{31}P n.m.r. spectrum at 121.493 MHz of adenosine 5′-[β-$^{18}O_3$]diphosphate (34 μmol) admixed with adenosine 5′-diphosphate (25 μmol) and dissolved in aqueous 2-amino-2-methylpropane-1,3-diol hydrochloride buffer (2.2 ml, 100 mM, pH 9.0, 25% D_2O) containing EDTA (10 mM). The expansion is of the P_β resonances. ^{31}P N.m.r. parameters were: 200 transients; band-width 1 200 Hz; memory size 8K, but 24K of zeroes added before Fourier transformation; pulse repetition rate 3.41 s; pulse angle 77°; broad-band proton-noise decoupling; line broadening −0.6 Hz; Gaussian broadening 0.3

brought together in the Table. The previously observed trend, that the isotope shift increases as the bond order increases, is supported.[11] The ^{18}O isotope shift on the

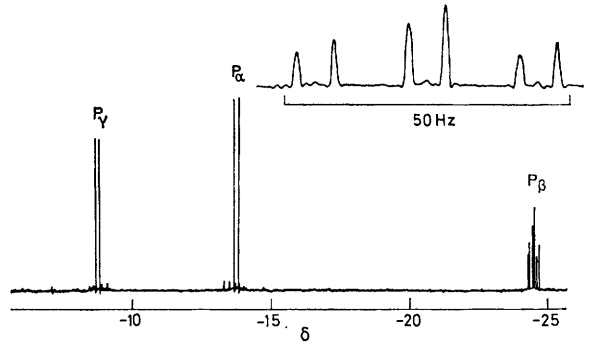


FIGURE 3 The ^{31}P n.m.r. spectrum at 121.493 MHz of adenosine 5′-[β-$^{18}O_2$]triphosphate (25 μmol) admixed with adenosine 5′-triphosphate (20 μmol) and dissolved in aqueous 2-amino-2-methylpropane-1,3-diol hydrochloride buffer (2.2 ml, 100 mM, pH 9.0, 25% D_2O) containing EDTA (10 mM). The expansion is of the Pβ resonances. ^{31}P n.m.r. parameters were: 400 transients; band-width 3 012 Hz; memory size 8K, but 24K of zeroes added before Fourier transformation; pulse repetition rate 1.36 s; pulse angle 77°; broad-band proton-noise decoupling; line broadening −1.4 Hz; Gaussian broadening 0.35

^{13}C resonance in tris(diphenylmethyl) [$^{18}O_4$]phosphate, however, is almost twice as large as in diphenylmethanol (0.020 p.p.m.).[12] The ^{18}O isotope shifts in ^{13}C n.m.r. spectra appear to be more sensitive to factors other than bond order,[12,13] than is found in the ^{31}P n.m.r. spectra of phosphate derivatives.[11,14]

TABLE

^{18}O Isotope shifts (p.p.m.) (per bond) on ^{31}P and ^{13}C resonances

	P–O	P···O	P=O	C–O
$(Ph_2CHO)_3P{=}O$	0.016		0.040	0.036
ADP [β-$^{18}O_3$]		0.023		
ATP [β-$^{18}O_2$]		0.027		

EXPERIMENTAL

^1H N.m.r. spectra were recorded on a Perkin-Elmer R32 spectrometer at 90 MHz. ^{31}P and ^{13}C N.m.r. spectra were recorded at 121.493 and 75.47 MHz respectively on a Bruker WH-300 spectrometer, using quadrature detection and broad-band proton-noise decoupling. Signal averaging was performed by an Aspect 2000 computer interfaced with the spectrometer. Chemical shifts (δ) are reported as positive for resonances downfield from the reference, and are referred to internal tetramethylsilane for ^1H and ^{13}C n.m.r. spectra and to external trimethyl phosphate in D_2O for ^{31}P n.m.r. spectra.

Tris(diphenylmethyl) [$^{18}O_4$]*Phosphate* (1).—$H_2{}^{18}O$ (1 g, 50 mmol, 99 atom %, B.O.C. Prochem Ltd.) in dry dioxan (15 ml) was added dropwise over 20 min, with rigorous exclusion of atmospheric moisture, to a stirred suspension of phosphorus pentachloride (2.082 g, 10 mmol) in dry dioxan (15 ml). The clear solution was kept for 2 days to ensure complete hydrolysis and then solvent was carefully removed *in vacuo* at room temperature leaving a crystalline residue of [$^{18}O_4$]phosphoric acid. This residue was dried thoroughly, and any occluded hydrogen chloride was removed by addition and evaporation of dry dioxan (2 × 20 ml).

A solution of freshly prepared diphenyldiazomethane[15] [5.958 g, 30 mmol; the compound was estimated spectrophotometrically at 525 nm (ε 94) in dioxan solution] in dry dioxan (40 ml) was added dropwise over 0.5 h, with stirring and exclusion of moisture, to a solution of the [$^{18}O_4$]phosphoric acid (10 mmol) in dry dioxan (20 ml). The deep purple colour was rapidly discharged and a vigorous evolution of nitrogen occurred. A sufficient excess of diphenyldiazomethane (*ca.* 300 mg) was then added such that the solution became orange-pink in colour, indicating that all the phosphoric acid had been converted to the triester. Solvent was removed *in vacuo* to leave a pale pink crystalline solid, which was recrystallised from acetone. *Tris(diphenylmethyl)* [$^{18}O_4$]*phosphate* (5.47 g, 90.5%) was obtained as small pale cream rhombic crystals, m.p. 128—129 °C after thorough drying in high vacuum over phosphorus pentaoxide. The microanalytical data were obtained on an unlabelled sample prepared in a preliminary experiment by the above procedure (Found: C, 78.8; H, 5.7; P. 5.2. $C_{39}H_{33}O_4P$ requires C, 78.5; H, 5.6; P, 5.2%); δ_H ($CDCl_3$) 6.25 (3 H, d, $^3J_{PH}$ *ca.* 9 Hz, Ph_2CHOP) and 7.13 (30 H, m, Ph); δ_P ($CDCl_3$) −4.9570 (s; signal split into a quartet with $^3J_{PH}$ 8.5 Hz on removing the ^1H decoupling). A very weak singlet at δ −4.9168 was due to $(Ph_2CH^{18}O)_3P^{16}O$, and a weak singlet at δ −4.9413 was due to $(Ph_2CH^{16}O)(Ph_2CH^{18}O)_2P^{18}O$, giving isotope shifts of 0.0156 ± 0.0002 and 0.0402 ± 0.0002 p.p.m. for a singly bonded ^{18}O and a doubly bonded ^{18}O, respectively. A small quantity of $(Ph_2CH^{16}O)_3P^{16}O$ added as a reference for the isotope shift measurements had δ_P − 4.8700. Also observed coupling from ^{13}C at C-1 of the phenyl groups was $^3J_{CP}$ 5.286 ± 0.025 Hz. The isotopic enrichment per oxygen atom was calculated as 99% from the ^{31}P n.m.r. spectrum. δ_C ($CDCl_3$) 81.12 (d, $^2J_{CP}$ 5.2 Hz, Ph_2CHOP), 126.69 (s, phenyl C^2 and C^6), 127.71 (s, phenyl C^4), 128.19 (s, phenyl C^3 and C^5), 140.26 (d, $^3J_{CP}$ *ca.* 5 Hz, phenyl C^1). ^{13}C N.m.r. spectrum of $(Ph_2CH^{18}O)_3P^{18}O$ plus $(Ph_2CH^{16}O)_3P^{16}O$, recorded with a sweep-width of 800 Hz, and a filter-width of 100 Hz such that only the benzylic carbon was observed, showed the expected two doublets, the isotope shift at ^{13}C due to a singly bonded ^{18}O being 0.0356 ± 0.0003 p.p.m. and $^2J_{CP}$ 5.224 ± 0.025 Hz; $\lambda_{max.}$ (MeOH) 203 nm (ε 66 900) and 259 nm (ε 1 440). The latter band shows vibrational fine structure. *m/e* 605 ($MH^{+\bullet}$) by field-ionisation mass spectrometry (VG Micromass ZAB-IF spectrometer). A small 603 peak was also present, due to molecules containing 3 ^{18}O atoms and 1 ^{16}O atom.

Bis(diphenylmethyl) [$^{18}O_4$]*Phosphoric Acid* (2).—A solution of tris(diphenylmethyl) [$^{18}O_4$]phosphate (5.2 g, 8.6 mmol) and dry sodium iodide (1.289 g, 8.6 mmol) in dry pyridine (70 ml) was heated at 100 °C for 20 min under anhydrous conditions. The initially clear pale yellow solution turned deep red-brown after *ca.* 5 min and after 10 min a flocculent precipitate of sodium bis(diphenylmethyl) [$^{18}O_4$]phosphate started to form. When cool, acetone (100 ml), was added to the almost solid mixture and the precipitate was collected by centrifugation, washed with AnalaR acetone (5 × 150 ml), then re-suspended in acetone (100 ml), filtered off at the pump, and sucked as dry as possible. The material was then dried thoroughly in a vacuum desiccator over phosphorus pentaoxide. Sodium bis(diphenylmethyl) [$^{18}O_4$]-phosphate was obtained as a pale cream powder (3.96 g, 100%); δ_H ([$^2H_4$]MeOH) 6.14 (2 H, d, $^3J_{PH}$ *ca.* 10 Hz, Ph_2CHOP) and 7.16 (20 H, m, Ph); δ_P (MeOH) −3.90 (s; signal split into a triplet with $^3J_{PH}$ 9.9 Hz, on removing the ^1H decoupling).

The sodium salt (3.96 g, 8.6 mmol) was dissolved in deionised water, and a small quantity of insoluble material was removed by filtration. Aqueous hydrogen chloride (4.3 ml, 2M) was added with stirring to the clear filtrate, and a white gelatinous precipitate was formed immediately. The precipitate was collected by filtration after 10 min, washed with water, and then sucked as dry as possible. The damp solid was then dried thoroughly *in vacuo* over phosphorus pentaoxide. *Bis(diphenylmethyl)* [$^{18}O_4$]*phosphoric acid* was obtained as a white powder (2.07 g, 55%). A small sample of the product was recrystallised from acetone to give very fine white needles, m.p. 78—80 °C. The microanalytical data were obtained on the unlabelled sample prepared in a preliminary experiment by the above procedure (Found: C, 72.7; H, 5.5; P, 7.0. $C_{26}H_{23}O_4P$ requires C, 72.6; H, 5.4; P, 7.2%); δ_H ([$^2H_4$]MeOH) 6.24 (2 H, d, $^3J_{PH}$ 8.6 Hz, Ph_2CHOP) and 7.22 (20 H, s, Ph); δ_P ($CHCl_3$) −2.95 (s; signal split into a triplet with $^3J_{PH}$ 8.4 Hz on removing the ^1H decoupling); $\lambda_{max.}$ (MeOH) 203 nm (ε 44 700), and 259 nm (ε 831). The latter band shows vibrational fine structure (5.5-nm spacing); *m/e* 439 ($MH^{+\bullet}$) by field-ionisation mass spectrometry.

P^1-*Diphenyl* P^2-*Bis(diphenylmethyl)* [P^{2}-$^{18}O_4$]*Diphosphate* (3).—A solution of tri-n-butylammonium bis(diphenylmethyl) [$^{18}O_4$]phosphate (2.945 g, 4.72 mmol) in dry dioxan (50 ml) was added dropwise during 2 h to a vigorously stirred solution of diphenyl phosphorochloridate (1.268 g, 4.72 mmol) in dry dioxan (50 ml) in a dry glove-box. The clear solution was then stirred for a further 3 h, by which time the reaction was complete (by ^{31}P n.m.r. spectroscopy). No attempt was made to isolate this reactive intermediate, which was used immediately in the next stage of the synthesis; $\delta_{P'}$ (dioxan) −17.74 (d, $^2J_{PP'}$ 17.3 Hz, P^2) and −28.35 (d, $^2J_{PP'}$ 17.3 Hz, P^1).

P^1-*Diphenyl* P^2-*Diphenylmethyl* [P^{2}-$^{18}O_4$]*Diphosphate* (4).—To the solution of P^1-diphenyl P^2-bis(diphenylmethyl) [P^{2}-$^{18}O_4$]diphosphate (*ca.* 4.72 mmol) in dry dioxan (100 ml) was added with stirring a solution of freshly prepared tri-n-butylammonium iodide (2.958 g, 9.44 mmol) in dry dioxan (30 ml); the iodide was prepared from tri-n-butylamine and concentrated aqueous hydrogen iodide in dioxan, and after evaporation of solvent *in vacuo* the orange gum was rendered anhydrous by addition and evaporation of 3 × 30 ml aliquots of dry dioxan. The orange-brown solution was stirred under anhydrous conditions in a glove-box and the progress of the reaction was monitored by ^{31}P n.m.r. spectroscopy. The mono-deprotection of the diphosphate tetraester was complete and virtually quantitative after 8 h, and the deep red-brown solution was evaporated to dryness *in vacuo* to leave the tri-n-butylammonium salt of P^1-diphenyl P^2-diphenylmethyl [P^{2}-$^{18}O_4$]diphosphate as a red-brown syrup; δ_P (dioxan) −15.62 (d, $^2J_{PP}$ 16.5 Hz, P^2) and −26.37 (d, $^2J_{PP}$ 16.5 Hz, P^1).

P^1-(*Adenosine* 5′-) P^2-*Diphenylmethyl* [P^{2}-$^{18}O_3$]*Diphosphate* (5).—A solution of anhydrous mono(tri-n-octylammonium) adenosine 5′-phosphate [9] (3.308 g, 4.72 mmol) in dry pyridine (45 ml) was added to the residual syrup containing P^1-diphenyl P^2-diphenylmethyl [P^{2}-$^{18}O_4$]diphosphate (*ca.* 4.72 mmol), and the clear red-brown solution was stirred at room temperature under rigorously anhydrous conditions. The ^{31}P n.m.r. spectrum recorded after 10 h showed the reaction to be complete, as the only signals present were a singlet at δ −14.82 due to diphenyl phosphate and the AB pattern of the desired product, with resonances at δ −14.50 (d, $^2J_{PP}$ 18.4 Hz, P^1) and −15.08 (d, $^2J_{PP}$ 18.4 Hz, P^2). Solvent was removed *in vacuo* at room temperature and the residual syrup was dissolved in aqueous triethylammonium hydrogencarbonate (700 ml, 100 mM) pH 7.8 and the solution shaken with carbon tetrachloride (2 × 500 ml) to remove iodine. The opalescent aqueous layer was separated, shaken with ether (500 ml) to remove any tri-n-octylamine, and then chromatographed on a column (38.5 × 5 cm) of DEAE-Sephadex A-25, HCO_3^- form, with a linear gradient of aqueous triethylammonium hydrogencarbonate (0.1—0.5M over 48 h, total volume 12 l) pH 7.8. The product was eluted at a buffer concentration of *ca.* 0.37M. The pooled fractions containing the product were evaporated to dryness under reduced pressure (*ca.* 0.1 mmHg) at room temperature. Residual triethylammonium hydrogencarbonate was then removed by repeated addition and evaporation of dry methanol (5 × 20 ml), leaving the bis(triethylammonium) salt of P^1-(adenosine 5′-) P^2-diphenylmethyl [P^{2}-$^{18}O_3$]diphosphate as a white foam {57 500 A_{259} units, 77% based on bis(diphenylmethyl) [$^{18}O_4$]phosphoric acid}; δ_P (100 mM aqueous 2-amino-2-methylpropane-1,3-diol · hydrochloride containing 10 mM EDTA and 25% D_2O, pH 9.0) −14.27 (d, $^2J_{PP}$ 19.5 Hz, P^1) and −14.88 (d, $_2J_{PP}$ 19.5 Hz, P^2).

Adenosine 5′-[β-$^{18}O_3$]*Diphosphate* (6).—The bis(triethylammonium) P^1-(adenosine 5′-) P^2-diphenylmethyl [P^{2}-$^{18}O_3$]diphosphate (3.64 mmol) was dissolved in methanol–water (120 ml, 2 : 1 v/v) containing triethylamine (506 μl, 3.64 mmol) and shaken with a mixture of 10% palladium–charcoal (1 g) and palladium black (0.1 g) in an atmosphere of hydrogen at room temperature and pressure. Uptake of hydrogen was complete in 1 h, and the catalyst was filtered off on a glass sinter covered with a glass fibre paper. The catalyst was then washed with 50% aqueous ethanol (6 × 100 ml) containing ammonia (0.3M) until $A_{259\ nm}$ of the filtrate had fallen to nearly zero. The combined filtrates were evaporated to dryness *in vacuo* to leave a white foam (diphenylmethane crystallised out during the evaporation and the bulk of it sublimed off) which was chromatographed on a column (50 × 2.5 cm) of DEAE-Sephadex A-25, HCO_3^- form, with a linear gradient of aqueous triethylammonium hydrogencarbonate (0.1—0.6M over 24 h, total volume 4 l) pH 7.8. The product was eluted at a buffer concentration of *ca.* 0.38M and was isolated as its bis(triethylammonium) salt (52 550 A_{259} units, 93.7%).

^{31}P N.m.r. spectrum of [β-$^{18}O_3$]-ADP (34 μmol) admixed with ADP (25 μmol) and dissolved in aqueous 2-amino-2-methylpropane-1,3-diol hydrochloride buffer (2.2 ml, 100 mM, pH 9.0, 25% D_2O) containing EDTA (10 mM) gave δ_P (for ADP) −9.063 (d, $^2J_{PP}$ 22.5 Hz, P_β) and −13.457 (d, $^2J_{PP}$ 22.5 Hz, P_α); δ_P (for [β-$^{18}O_3$]-ADP) −9.131 (d, $^2J_{PP}$ 22.5 Hz, P_β) and −13.457 (d, $^2J_{PP}$ 22.5 Hz, P_α). A trace of [β-$^{18}O_2$]-ADP with $\delta(P_\beta)$ −9.108 was also observed. The isotope shift at P_β of ADP caused by three non-bridging ^{18}O atoms is 0.0679 ± 0.0006 p.p.m. An isotopic enrichment of 96 atom % ^{18}O was calculated from the observed peak intensities for the P_β resonances of [β-$^{18}O_3$]-ADP and [β-$^{18}O_2$]-ADP.

Adenosine 5′-[β-$^{18}O_2$]*Triphosphate* (8).—The title compound was prepared using the procedure developed by Hecht and Kozarich for converting ADP to ATP.[10] Bis(triethylammonium) adenosine 5′-[β-$^{18}O_3$]diphosphate (3.38 mmol) was converted to the bis-pyridinium salt with the pyridinium form of Dowex-50W 50 × 4-200-R (70 ml) in aqueous pyridine (120 ml, 10% pyridine by volume). The resin was removed by filtration after 15 min, washed with

water (4 × 40 ml), and the combined filtrates were evaporated to dryness under reduced pressure to leave a white foam. Tri-n-butylamine (1.253 g, 6.76 mmol) was added to a solution of the pyridinium salt in pyridine–water (50 ml, 4 : 1 v/v) and the clear solution was evaporated to dryness under reduced pressure to leave the bis(tri-n-butylammonium) salt of adenosine 5′-[β-$^{18}O_3$]diphosphate as a colourless glass. This salt was dried by addition and evaporation of dry dimethylformamide (5 × 50 ml). A solution of *NN′*-carbonyldi-imidazole (2.740 g, 16.9 mmol) in dry dimethylformamide (30 ml) was added to a solution of the bis(tri-n-butylammonium) adenosine 5′-[β-$^{18}O_3$]diphosphate (3.38 mmol) in dry dimethylformamide (40 ml) in a dry glove-box, and the solution was stirred at room temperature for 17 h. Dry methanol (1.095 ml, 27.04 mmol) was then added to the pale yellow solution containing the desired P_β-imidazolyl adenosine 5′-[β-$^{18}O_2$]diphosphate (7) to decompose the excess of *NN′*-carbonyldi-imidazole, and after 30 min a solution of anhydrous mono(tri-n-butylammonium) phosphate (4.789 g, 16.9 mmol) in dry dimethylformamide (15 ml) was added with stirring. A small quantity of a white precipitate formed almost immediately, and after 28 h solvent was removed under reduced pressure. The residual syrup was then chromatographed on a column (49 × 5 cm) of DEAE-Sephadex A-25, HCO_3^- form, with a linear gradient of aqueous triethylammonium hydrogencarbonate (0.25—0.7M over 48 h, total volume 12 l) pH 8.0. The product (*ca.* 30 000 A_{259} units) was eluted at a buffer concentration of *ca.* 0.52M and was isolated as its tris(triethylammonium) salt after removal of solvent and buffer as described previously. This salt was converted to the sodium salt by the method of Haley and Yount.[16] Trisodium adenosine 5′-[β-$^{18}O_2$]triphosphate (1.169 g, 53.5%), a free-flowing white powder, was stored at −20 °C. A solution of the product (9.67 mg) in aqueous triethylammonium hydrogencarbonate (25 ml, 250 mM) pH 8 had $A_{259\ nm}$ (1 mm) 0.922, giving a molecular weight of 646.

The ^{31}P n.m.r. spectrum of [β-$^{18}O_2$]-ATP (25 μmol) admixed with ATP (20 μmol) and dissolved in aqueous 2-amino-2-methylpropane-1,3-diol hydrochloride buffer (2.2 ml, 100 mM, pH 9.0, 25% D_2O) containing EDTA (10 mM) gave δ_P (ATP) −8.647 (d, $^2J_{P\beta,P\gamma}$ 20.0 Hz, P_γ), −13.711 (d, $^2J_{P\alpha,P\beta}$ 19.7 Hz, P_α), −24.425 (t, P_β); δ_P ([β-$^{18}O_2$]-ATP) −8.647 (d, $^2J_{P\beta,P\gamma}$ 20.0 Hz, P_γ), −13.711 (d, $^2J_{P\alpha,P\beta}$ 19.7 Hz, P_α), −24.479 (t, P_β). Some [β-^{18}O]-ATP with $\delta(P_\beta)$ −24.452 was also observed. The isotope shift at P_β caused by two non-bridging ^{18}O atoms is 0.0545 ± 0.0015 p.p.m. An isotopic enrichment of 95 atom % ^{18}O was calculated from the observed peak intensities for the P_β resonances of [β-$^{18}O_2$]-ATP and [β-^{18}O]-ATP.

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