SITE-SPECIFIC LABELLING OF CAGED ATP WITH DEUTERIUM OR ^{18}OXYGEN

John E.T. Corrie* and Gordon P. Reid

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

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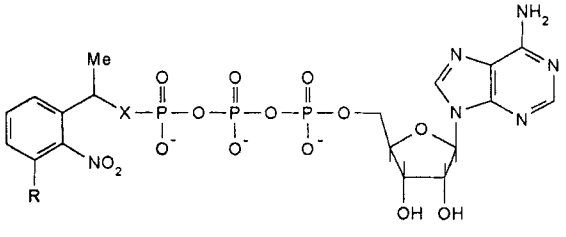
SUMMARY

[3-D]2-Nitroacetophenone and [alcohol-^{18}O]-1-(2-nitrophenyl)ethyl alcohol were prepared and used to synthesise labelled P^3-1-(2-nitrophenyl)ethyl esters of ATP ("caged ATP") with isotope present either as deuterium on the 3-position of the nitrosubstituted ring or as ^{18}oxygen in the bridging position between the terminal phosphate and the nitrophenylethyl group. The availability of the deuterated compounds enabled complete assignment of their ^{1}H NMR spectra.

In connexion with studies of the mechanism of photochemical cleavage of caged ATP (the P^3-1-(2-nitrophenyl)ethyl ester of adenosine triphosphate **1**), [1,2] we required versions **2** and **3** of the compound specifically labelled with deuterium or ^{18}oxygen respectively. This paper describes the synthesis of the required labelled precursors, their elaboration into the compounds **2** and **3** and relevant related chemistry.



1 R = H, X = ^{16}O
2 R = D, X = ^{16}O
3 R = H, X = ^{18}O

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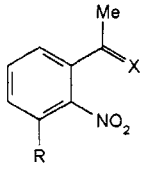
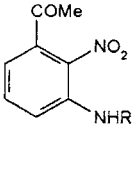
For the deuterated compound **2**, we planned to synthesise [3-D]2-nitroacetophenone **5**, which could be converted via its hydrazone to the diazo compound **7** and hence to the compound **2** as previously described for the unlabelled species [1]. We proposed to incorporate the deuterium label via reductive deamination of a suitably substituted aromatic amine.

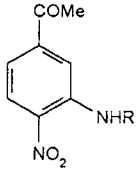


4 R = H, X = O **6** R = H, X = N_2
5 R = D, X = O **7** R = D, X = N_2

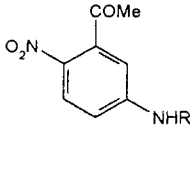
Nitration of 3-acetamidoacetophenone has been the subject of 3 separate reports [3,4,5] each of which used different reaction conditions and reported differing proportions of isomeric nitration products. The method described by Leonard and Boyd [5] with fuming nitric acid alone gave as the principal product the 2-nitro isomer **8a**, easily isolated by crystallisation. In our hands the reported [4,5] hydrolysis of the acetamide under strongly acidic conditions gave an intractable tar, but with dilute aqueous methanolic HCl the required amine **8b** was readily obtained. Since the other isomeric acetamides **9a** and **10a** could in principle be used to prepare ring-deuterated nitroacetophenones, they were also isolated from the nitration mixture and hydrolysed to their parent amines **9b** and **10b**. Spectral data for all these compounds are recorded in the Experimental section.



8a R = Ac
8b R = H



9a R = Ac
9b R = H



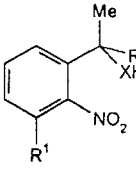
10a R = Ac
10b R = H

The amine **8b** was diazotised and reduced with D_3PO_2 [6,7] to give the deuterated compound **5** in 44% yield, with 95% deuterium incorporation determined by ^1H NMR spectroscopy There was no evidence of deuterium other than at the 3-position. The availability of the deuterated 2-nitroacetophenone facilitated interpretation of the ^1H NMR spectrum, which in the unlabelled isomer **4** shows two doublets of doublets at δ 8.10 (J_{ortho} 8.2, J_{meta} 1.0) and 7.45 (J_{ortho} 7.6, J_{meta} 1.3) and two doublets of triplets at δ 7.74 and 7.62. In the 3-deuterated compound **5**, the signal at δ 8.10 was

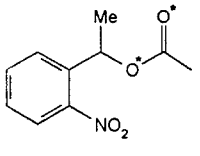
almost obliterated and therefore corresponds to H-3, while the signal at δ 7.62 was collapsed to a broadened *ortho*-coupled doublet, and therefore corresponds to H-4. As expected, the signal at δ 7.74 (H-5) was collapsed to an *ortho*-coupled triplet and the signal at δ 7.45 (H-6) was unaffected. Since a proton *ortho* to a carbonyl group would normally be expected to be deshielded, the appearance of the signal from H-6 as that at the highest field of the aromatic protons implies that in 2-nitroacetophenone the carbonyl group is twisted out of the plane of the aromatic ring, while the nitro group remains in the plane.

The deuterated ketone **5** was converted as described above to the labelled caged ATP **2**, and again the presence of the isotope enabled an assignment of the ^{1}H NMR spectrum of protons on the nitro-substituted ring. Because of the presence of two diastereoisomers, which arise from the racemic centre in the 1-(2-nitrophenyl)ethyl group, the expected multiplicity of the 4 signals is made more complex since corresponding protons in each diastereoisomer have slightly different chemical shifts [cf. ref. 8 for differences elsewhere in the two isomers]. However the assignments are easily made as described above. Thus the signals centred at δ 7.85, 7.77, 7.61 and 7.34 (D_2O solution) correspond respectively to H-3, H-6, H-5 and H-4.

These experiments concluded the synthesis of the deuterated caged ATP **2**, but two related deuterated alcohols **11** and **12** were prepared to assist with interpretation of the mass spectrum of the [^{18}O]-labelled compound **14** (see below). Thus reduction of the deuterated ketone **5** with $NaBH_4$ gave the ring-labelled alcohol **11**, while reduction of unlabelled 2-nitroacetophenone **4** with $NaBD_4$ gave the alcohol **12** with deuterium specifically in the side chain.



11 R^1 = D, R^2 = H, X = ^{16}O
12 R^1 = H, R^2 = D, X = ^{16}O
13 R^1, R^2 = H, X = ^{16}O
14 R^1, R^2 = H, X = ^{18}O



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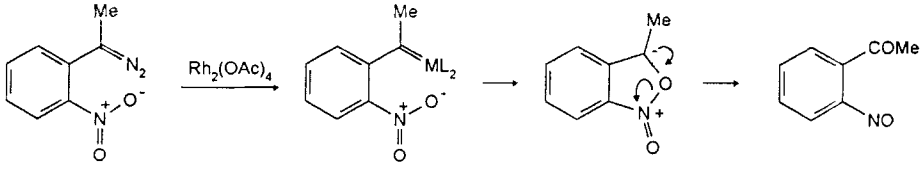
The preparation of the labelled caged ATP **3** bearing ^{18}oxygen in the bridge between the photolabile group and the terminal phosphate was planned to proceed via [^{18}O]1-(2-nitrophenyl)ethanol **14**. Initial attempts to prepare the alcohol **14** aimed to achieve efficient isotope

incorporation by reaction of the unlabelled diazo compound **6** with only a slight excess of $[^{18}O]H_2O$. Baumann and MacLeod [9] have described a similar procedure with ethyl diazoacetate and rhodium acetate catalysis [10] to obtain ^{18}O-labelled ethyl glycolate. When a THF solution of 1-(2-nitrophenyl)diazoethane **6** was added to a slight excess of H_2O in THF in the presence of a catalytic amount of $Rh(OAc)_2$, the orange colour of the diazo compound was rapidly discharged and nitrogen was evolved. However the principal product was 2-nitrosoacetophenone, together with smaller amounts of unidentified materials: none of the anticipated 1-(2-nitrophenyl)ethanol was detectable. Evidently the initially generated carbenoid is efficiently intercepted by the neighbouring nitro group, as shown in the suggested mechanism (Scheme 1). When the diazo compound **6** was added to an approx. stoichiometric quantity of H_2O in THF solution in the presence of catalytic amounts of strong acids (camphorsulphonic or hydrochloric acid), decolourisation and gas evolution proceeded smoothly until the amount of diazo compound added to the reaction mixture exceeded the quantitiy of acid present, whereupon the reaction virtually ceased. When the amount of either acid was increased to a stoichiometric quantity, complete decolourisation of the diazo compound ensured, but the required alcohol **13** was barely detectable in the crude reaction mixture. Apparently the intermediate cation formed by protonation of the diazo compound was preferentially neutralised by the camphorsulphonate or chloride counterion of the added acid.



Scheme 1

In the light of these results we abandoned the attempt to achieve near-stoichiometric isotope utilisation. Since the previous reactions suggested that the diazo compound **6** efficiently trapped a variety of acids, it seemed likely that an $[^{18}O]$-labelled carboxylic acid could be used to form an ester which after acyl-oxygen cleavage would give the labelled alcohol **14**. Accordingly, acetic acid was

equilibrated with excess [^{18}O]H_2O [11] and a THF solution of the diazo compound **6** (equal to the molar amount of acetic acid) was added to the exchange solution. *In situ* alkaline hydrolysis of the labelled acetate **15** then gave the labelled alcohol **14**, with ^{18}O-enrichment of 85.6% (see below). This material was phosphorylated and the product coupled with ADP as previously described [8] to give the ^{18}O- labelled caged ATP derivative **3**. In a minor variant of the procedure previously described [8] we used an improved reverse phase HPLC purification [cf. ref 12] of the product which was particularly effective for removal of free nucleotides. Details are given in the Experimental section. The isotopic enrichment of the labelled site in the ^{18}O-labelled caged ATP was not measured directly but is expected to be identical to that of the labelled alcohol **14**, since there is no opportunity for isotopic exchange during the synthesis.

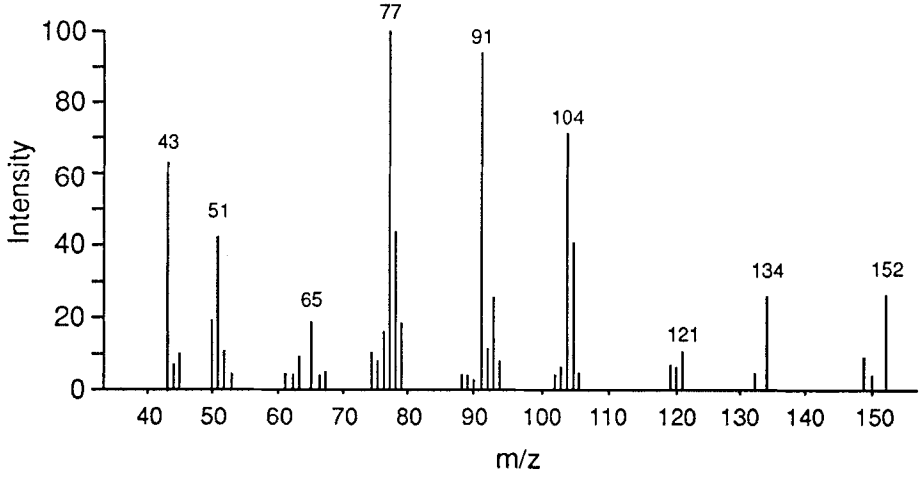


Figure 1. Mass spectrum of 1-(2-nitrophenyl)ethanol **13** at normal isotopic abundance.

The isotopic enrichment of the alcohol **14** was measured by GCMS, but the molecular ion (m/e 167 for the unlabelled alcohol) was not observed in the EI-mass spectrum. The EI-mass spectrum of the unlabelled alcohol **13** is given in Figure 1, which shows an ion at m/z 152, corresponding to the loss of $CH_3^{\cdot}$ from the molecular ion. To confirm that this ion was derived from the alcohol **13**, the ring- and sidechain-deuterated alcohols **11** and **12** were also examined. Both showed an ion at the expected m/z 153. Furthermore, in the [^{18}O]-labelled alcohol **14** the ion

appeared at m/z 154. Full analysis of the fragmentation pattern in the mass spectrum of the alcohol **13** is beyond the scope of the present work although the spectrum shown in Fig. 1 poses intriguing questions. For example the strong peaks at m/z 77 and 91 would normally be diagnostic of an unsubstituted benzyl derivative. Atypical fragmentation has been reported [12] for 2-nitrotoluene but the alcohol **13** shows still more complex behaviour. The effects of the various isotopic substitutions on the principal fragment ions are summarised in Table 1.

Table 1. Effects of isotopic substitution on the principal fragment ions in the EI-mass spectrum of 1-(2-nitrophenyl)ethanol **13**.

Fragment ions of alcohol **13** m/z	Labelled Compound		
	[ring-D] (**11**)	[Side chain-D] (**12**)	[^{18}O] (**14**)
152	+1	+1	+2
149	+1	n.c	+2
134	+1	n.c	+2
121	+1	n.c	+2
105	+1	n.c	+2
104	+1	n.c	+2
93	+1	n.c	n.c
91	+1	n.c	n.c
77	+1	b	n.c
65	+1	n.c	n.c
51	a	n.c	n.c
43	n.c	n.c	+2

n.c., no change; a, 2 equal peaks at m/z 51 and 52; b, 2 equal peaks at m/z 77 and 78.

The methods described above provide the required labelled caged ATP compounds in adequate amounts for mechanistic studies. The results of these investigations will be published elsewhere.

EXPERIMENTAL

General Details. – ^{1}H NMR spectra were recorded in $CDCl_3$ unless otherwise specified on JEOL FX90Q or Bruker AM400 spectrometers. Thin layer chromatography was on 0.2 mM Merck GF_{254} plates and flash chromatography was on Merck 40-63 μm silica gel (Type 9385). Petroleum ether was the fraction boiling in the range 40 - 60°C. All deuterated reagents were from Aldrich,

Gillingham, Dorset, U.K. and [^{18}O]water (97.2% enrichment) was from Isotec Inc., Miamisberg, Ohio, U.S.A. Analytical HPLC was as previously described [1,8]. Gas chromatography – mass spectrometry was performed on a Hewlett-Packard instrument comprising a Model 5890 gas chromatograph and Model 5971 mass selective detector fitted with a 12.5 m x 0.2 mm capillary column with a 0.33 μm coating of 5% phenyl methyl silicone. The head pressure of helium carrier gas was 20 p.s.i. The injector temperature was 180°C and the oven programme from the time of injection was as follows: 0.3 min at 80°C, then 32°C.min^{-1} to 120°C, isothermal at 120°C for 2.5 min, then 20°C.min^{-1} to 180°C and isothermal at 180°C for 4.5 min. The retention time of 1-(2-nitrophenyl)ethanol was 8.1 min.

Nitration of 3-acetamidoacetophenone. – Finely powdered 3-acetamidoacetophenone (63 g) was added over 30 min to fuming nitric acid (d 1.52; 315 ml) stirred at -10°C. The solution was stirred for a further 15 min at 0°C and poured onto crushed ice (1 kg). The mixture was basified by careful addition of solid Na_2CO_3 and the precipitate was collected, washed thoroughly with water and dried. Analytical TLC (EtOAc-petroleum ether 8:2) showed 3 principal spots, R_f 0.53, 0.39 and 0.26. The total material was crystallised once from EtOH (500 ml) to give 3-acetamido-2-nitroacetophenone **8a** as buff crystals (28 g), m.p. 166-168°C (lit., [5] 168-169°C), corresponding to the middle spot on TLC; λ_{max}/nm (EtOH) 306 (ε/M^{-1}cm^{-1} 1500); δ_H 9.10 (1 H, br s , NH), 8.55 (1 H, d/d, $J_{4,5}$ 8.0, $J_{4,6}$ 1.4, H-4), 7.60 (1 H, t, H-5), 7.22 (1 H, d/d, $J_{5,6}$ 7.5, H-6), 2.56 (3 H, s, ArCOC*H*$_3$) and 2.25 (3 H, s, NHCOC*H*$_3$).

The mother liquor from this crystallisation was concentrated to *ca.* 125 ml and allowed to cool. A solid (18.3 g) was obtained which clearly contained two crystal forms, and by TLC was enriched in the highest R_f compound. Small-scale crystal picking followed by crystallisation from EtOH gave pure 3-acetamido-4-nitroacetophenone **9a** as orange plates, m.p. 124-124.5°C (lit., [3] 121°C); λ_{max}/nm (EtOH) 236 (ε/M^{-1}cm^{-1} 20550), 270 sh (5700) and 344 (2600); δ_H 9.35 (1 H, d, $J_{2,6}$ 1.8, H-2), 8.26 (1 H, d, $J_{5,6}$ 8.3, H-5), 7.71 (1 H, d/d, H-6), 2.66 (3 H, s, ArCOC*H*$_3$) and 2.32 (3 H, s, NHCOC*H*$_3$). A portion (14.2 g) of the remaining mixed solid was heated at 100°C for 45 min

with 5 M hydrochloric acid, cooled and neutralised with conc. aq. NH_3. The precipitated solid was filtered, washed with water, dried and crystallised twice from MeOH to give 3-amino-4-nitroacetophenone **9b** as dark red prisms (1.8 g), m.p. 163-165°C (lit; [3] 163°C); λ_{max}/nm (EtOH) 239 (ε/$M^{-1}cm^{-1}$ 20600) 280 (9000) and 422 (5500); δ_H 8.20 (1 H, d, $J_{5,6}$ 8.8, H-6), 7.40 (1 H, d, $J_{2,6}$ 1.7, H-2), 7.19 (1 H, d/d, H-5), 6.13 (2 H, br s, NH_2) and 2.66 (3 H, s, $COCH_3$).

Finally, the mother liquor which remained from the original mixture after the first two crystal crops was evaporated under reduced pressure. A portion of the residue was flash chromatographed (EtOAc-petroleum ether 65:35) and crystallised from EtOAc-petroleum ether to give 5-acetamido-2-nitroacetophenone **10a**, corresponding to the spot of lowest R_f, as buff needles, m.p. 150-151°C (lit., [5] 149-150°C); λ_{max}/nm (EtOH) 319 (ε/$M^{-1}cm^{-1}$ 1200); δ_H 8.10 (1 H, d, $J_{3,4}$ 9.2, H-3), 8.01 (1 H, br s, NH), 7.74 (1 H, d/d, $J_{4,6}$ 2.2, H-4), 7.52 (1 H, d, H-6), 2.54 (3 H, s, $ArCOCH_3$) and 2.22 (3 H, s, $NHCOCH_3$). The remaining mixture (9 g) from the mother liquor was dissolved in EtOH (50 ml) under reflux, treated dropwise with conc. hydrochloric acid (15 ml) and heated under reflux for a further 1.5 h. The solution was diluted with H_2O (25 ml), allowed to cool and neutralised with conc. aq. NH_3. The solution was concentrated under reduced pressure until a precipitate began to form then diluted with water and filtered. The solid was washed with water and dried, and a portion was flash chromatographed (EtOAc-petroleum ether 45:55) to give 5-amino-2-nitroacetophenone **10b** as orange plates, m.p. 150-151°C (lit., [5] 152-153°C) from aq. MeOH; λ_{max}/nm (EtOH) 379 (ε/$M^{-1}cm^{-1}$ 16000); δ_H 8.02 (1 H, d, $J_{3,4}$ 8.8, H-3), 6.64 (1 H, d/d, $J_{4,6}$ 2.7, H-4), 6.40 (1 H, d, H-6), 4.56 (2 H, br s, NH_2) and 2.49 (3 H, s, $COCH_3$).

3-Amino-2-nitroacetophenone **8b**. – A suspension of 3-acetamino-2-nitroacetophenone **8a** (26 g) in MeOH (145 ml), water (31 ml) and conc. HCl (15.5 ml) was heated under reflux for 2 h, then diluted with water (25 ml), cooled to room temp. and neutralised with conc. aq. NH_3. The solution was concentrated under reduced pressure at 25°C until a solid began to form, then allowed to crystallise. The solid was collected, washed with H_2O, dried and recrystallised from benzene to give the amine **8b** as brown prisms (15.2 g), m.p. 91-92°C (lit., [5] 93-93.5°C); λ_{max}/nm (EtOH) 224

($\varepsilon/M^{-1}cm^{-1}$ 16250) and 407 (5400); δ_H 7.34 (1 H, m, H-5), 6.85 (1 H, d/d, $J_{5,6}$ 8.3, $J_{4,6}$ 1.3, H-6), 6.55 (1 H, d/d, $J_{4,5}$ 7.1, H-4), 6.09 (2 H, br s, NH_2) and 2.47 (3 H, s, $COCH_3$).

[Ar3-D]2-Nitroacetophenone **5**. – 3-Amino-2-nitroacetophenone (2.34 g, 13 mmol) was ground to a fine powder and added to a mixture of concentrated DCl (5.2 ml) and D_2O (6.5 ml). The mixture was cooled in an ice-bath, treated dropwise with a solution of $NaNO_2$ (0.936 g) in D_2O (2.25 ml) and stirred in ice for 0.5 h. Cold 50% D_3PO_2 (7.6 ml) was added dropwise and the mixture was stirred for a further 1 h in ice, then kept overnight at 4°C. The dark solution was neutralised by dropwise addition of 4 M NaOH and extracted with ether. The ether extract was washed with water, dried (Na_2SO_4) and evaporated under reduced pressure and the residue was purified by flash chromatography (EtOAc-light petroleum 30:70) followed by Kugelrohr distillation at 1 mmHg (oven temperature 200°C) to give the deuterated ketone **5** as a yellow oil (0.95 g, 44%). The ^1H NMR spectrum is described in the Discussion section.

[Ar3-D]1-(2-nitrophenyl)ethanol **11**. – A stirred solution of the deuterated ketone **5** (0.45 g) in EtOH (11 ml) was cooled in an ice bath and $NaBH_4$ (0.2 g) was added. After 1 h the solution was treated dropwise with glacial acetic acid (0.2 ml), concentrated under reduced pressure, diluted with water and extracted with ether. The ether extract was washed with aq. $NaHCO_3$, dried (Na_2SO_4) and evaporated and the residue was purified by Kugelrohr distillation at 0.8 mmHg (oven temperature 190°C) to give the alcohol **11** as a yellow oil (0.41 g); δ_H ($CDCl_3$; 400 Mhz) 7.82 (1 H, d/d, $J_{5,6}$ 1.2, H-6), 7.64 (1 H, t, J_{ortho} 7.5,H-5), 7.41 (1 H, br d, H-4), 5.39 (1 H, q, J 6.3, ArC*H*) and 1.54 (3 H, d, CHCH_3). The unlabelled alcohol **13** under the same conditions showed 7.86 (1 H, d/d, $J_{3,4}$ 8.2, $J_{3,5}$ 1.2, H-3), 7.81 (1 H, d/d, $J_{5,6}$ 7.9, $J_{4,6}$ 1.3, H-6), 7.62 (1 H, t/d, H-5), 7.40 (1 H, t/d, H-4), 5.39 (1 H, q, J 6.3, ArC*H*) and 1.54 (3 H, d, CHCH_3).

[side chain 1-D]1-(2-nitrophenyl)ethanol **12**. – The unlabelled ketone **4** was reduced as above using $NaBD_4$ to give the labelled alcohol **12**, which had ^1H NMR spectrum identical to that for the unlabelled alcohol **13**, with the exception that the methyl resonance appeared as a singlet at δ1.54.

[Ar3-D]P^3-1-(2-Nitrophenyl)ethyl adenosine triphosphate **2**. – A solution of the labelled ketone **5** (450 mg) in EtOH (5.45 ml) was mixed with 95% hydrazine hydrate (306 mg) and glacial acetic acid (175 µl) and heated under reflux for 3 h. The solvent was evaporated under reduced pressure and the residue was dissolved in ether and washed with aq. $NaHCO_3$ and brine, dried (Na_2SO_4) and evaporated. The residue was kept *in vacuo* (0.5 mm Hg) for 2 h to remove traces of water, then dissolved in $CHCl_3$ (10 ml) and stirred vigorously for 10 min with activated MnO_2 (1 g; Merck 805958). The suspended solid was filtered and washed with $CHCl_3$ and the combined filtrates were evaporated under reduced pressure. The residual crude diazo compound **7** was dissolved in Et_2O (10 ml). A solution of disodium ATP (550 mg) in water (15 ml) was adjusted to pH 4.0 and added to the ethereal diazo solution and the mixture was stirred vigorously at room temp overnight. The ether layer was removed and the aqueous layer was washed twice with ether. In two equal runs the aqueous material was applied to a preparative reverse phase HPLC column (2 x 30 cm; Waters C_{18} packing material, Cat. No. 20594) equilibrated in 10 mM Na phosphate, pH 5.5 and the column was eluted at 2.5 ml min^{-1} with the same buffer for *ca* 2.5 h, by which time the free nucleotide had eluted and the absorbance had remained at baseline for at least 1 h. The mobile phase was changed to distilled water which eluted the caged nucleotide **2** as the conductivity of the emergent mobile phase fell to that of the distilled water. Final desalting by ion-exchange chromatography on DEAE-cellulose was as described previously [1,8]. The recovered [Ar3-D]caged ATP **2** (160 µmol) was identical on analytical reverse phase and anion exchange HPLC [1,8] with the unlabelled species **1**. The ^{1}H NMR spectrum was identical with that of the unlabelled species **1** [8] except for the signals from the nitro-substituted ring, as described in the Discussion section.

[alcohol - ^{18}O]1-(2-Nitrophenyl)ethanol **14**. – A solution of glacial acetic acid (120 mg, 2 mmol) in [^{18}O]water (1 ml) was heated at 100°C for 16 h in a sealed tube and cooled to room temp. Tetrahydrofuran (2 ml) was added followed by dropwise addition of a THF solution (2 ml) of the diazo compound **6** [prepared as described [1] from the hydrazone of 1-(2-nitrophenyl)acetophenone (358 mg, 2 mmol)]. The solution was stirred until gas evolution ceased (*ca.* 10 min) then diluted

with MeOH) (1.35 ml) and treated with 6 M aq. KOH (0.67 ml). After 0.5 h the solution was partitioned between ether and water and the ether phase was washed with water, dried (Na_2SO_4) and evaporated. The residue was purified by flash chromatography (EtOAc-light petroleum 1:3) to give the labelled alcohol **14** as a yellow oil (183 mg, 55%).

[C,γ-^{18}O]P^3-1-(2-Nitrophenyl)ethyl adenosine triphosphate **3**. – The labelled alcohol **14** was phosphorylated and the product coupled with ADP as previously described [8] for the unlabelled species **1**. The crude reaction mixture was purified first by anion exchange chromatography on DEAE-cellulose [8], then by reverse-phase preparative HPLC and anion exchange chromatography as described above. Compound **3** was obtained in 38% yield after purification.

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