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# Synthesis of High Specific Activity Tritium Labelled [2-$^3$H]-Adenosine-5'-Triphosphate §

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## Summary

A procedure for high level tritium labelling at the C2-H position of adenosine 5'-triphosphate ([2-$^3$H]-ATP, **1**), based on the tritiodehalogenation reaction of 2-bromoadenosine 5'-triphosphate (**2**) has been elaborated. This precursor was prepared in a six-step synthesis from guanosine. The tritiodehalogenation of (**2**) for three hours over palladium oxide in phosphate buffer yielded tritium labelled ATP with high specific activity, in good chemical yield.

**Keywords:** Tritiodehalogenation, ATP, tritium gas, tritium NMR, HPLC

## Introduction

Tritium labelling has long been established as an effective means for the study of molecular structure by $^3$H NMR spectroscopy.[1] The method is especially attractive for investigating biological systems where the large size (and vast number of hydrogen atoms) and slow isotropic tumbling of the molecules makes interpretation of proton NMR spectra intractable. We required tritium labelled adenosine 5'-triphosphate ([2-$^3$H]-ATP, **1**) with high specific activity for two such studies: the binding of ATP to the S1 fragment of myosin in relation to chemomechanical energy transduction vital to muscular function,[2] and incorporation of ATP as adenosine into a self-cleaving RNA molecule for characterization of RNA structure in solution using $^3$H NMR spectroscopy.[3]

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§ A brief description of this synthesis was included in the Experimental section of Ref. 2.

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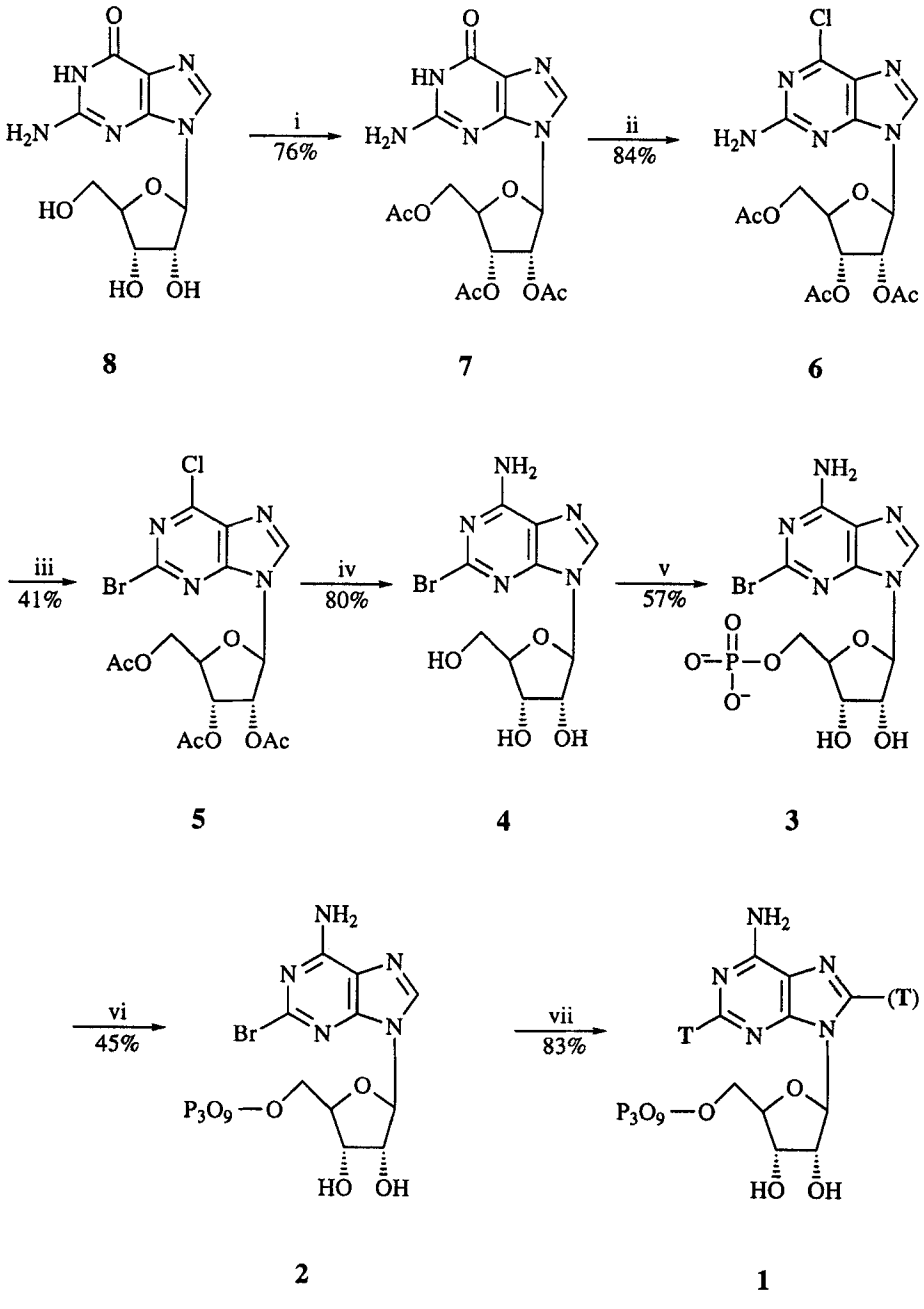
The adenine portion of ATP may be labelled with tritium at the C2-H and C8-H positions. Tritiation at the C8-H position is achieved by heterogeneous metal catalyzed exchange of ATP in solution with $T_2$ gas[4-6] or by tritiodehalogenation of 8-bromo-ATP,[7] and $^3H$ NMR analyses have shown that some tritium is also incorporated in the C2-H position.[1,5] While the precursors for these reactions are commercially available, a major disadvantage of labelling in the C8-H position is that this triton exchanges rapidly; *e.g.* kinetic data on adenosine derivatives[8,9] have shown that the C8 exchanges *ca.* 2000 times faster than the C2 triton. We pursued labelling in the C2-H position as better suited to our biochemical studies, and investigated the synthesis of an appropriate 2-halo-substituted ATP molecule as a precursor. The synthesis and tritiodehalogenation of 2-bromo-adenosine 5'-triphosphate (2-bromo-ATP, **2**) for the preparation of high specific activity [2-$^3$H]-ATP (**1**) are now described.

## Results and Discussion

2-Bromo-ATP (**2**) was selected as our preferred precursor for catalytic tritiodehalogenation to ATP (**1**), and the full synthetic route is shown in Scheme 1. Guanosine (**8**) was readily acetylated by the action of acetic anhydride in the presence of pyridine and N,N-dimethylformamide (DMF), giving a 76% yield. The product, guanosine 2',3',5'-triacetate (**7**), was reacted with phosphoryl chloride[10] to form the 6-chloro derivative (**6**) in 62% yield. A minor modification involving the addition of phosphoryl chloride to the reaction mixture at –20°C instead of at room temperature improved the yield to 84%. The subsequent deaminative bromination of (**6**) with t-butyl nitrite and bromoform[11] resulted in 2-bromo-6-chloro-9-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl) purine (**5**), which on ammonolysis[12] gave 2-bromoadenosine (**4**). 2-Bromo-adenosine 5'-monophosphate (**3**) was obtained as its barium salt from (**4**) by reaction with phosphoryl chloride in triethyl phosphate, as described for the general synthesis of nucleoside 5'-monophosphates.[13] The monophosphate (**3**) was converted to the tributylammonium salt and reacted with anhydrous tributylammonium pyrophosphate in the presence of 1,1'-carbonyldiimidazole[14] to form the tributylammonium salt of 2-bromo-ATP. 2-Bromo-ATP tetrasodium salt (**2**) was obtained by further treatment with sodium perchlorate in acetone.

2-Bromo-ATP (**2**) in phosphate buffer was exposed to carrier free tritium gas in the presence of PdO catalyst to yield tritium labelled ATP (**1**). The labelled product was analyzed by HPLC (Figure 1), and the chromatogram shows quantitative conversion of (**2**) [retention time ($t_R$) = 22 min] to ATP, which was observed as the major peak ($t_R$ = 4.5 min). A minor peak




**Scheme 1:** Synthesis of 2-$^3$H-Adenosine 5'-triphosphate. Reaction conditions as follows: i). $Ac_2O$ / Pyridine / DMF / 75°C / 3.75 h, ii). $Et_4NCl$ / N,N-DMA / $CH_3CN$ / $POCl_3$, iii). t-BuONO / $CHBr_3$, iv). $NH_3$ / $CH_3OH$, v). $POCl_3$ / $(EtO)_3P{=}O$, vi). $Bu_3NH^+H_3P_2O_7$ / $(C_3H_3N_2)_2C{=}O$, vii). PdO / $T_2$ / Phosphate buffer pH 8.6 / 3 h. More complete detail is given in the Experimental section and References.

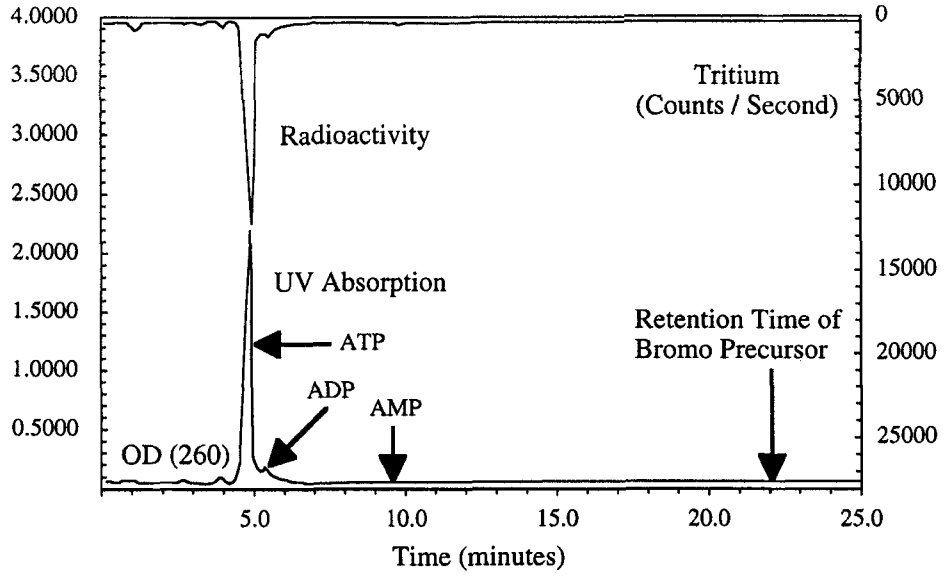



**Figure 1**: HPLC chromatogram of the tritiated ATP obtained on tritiodehalogenation of 2-bromo-ATP (**2**). HPLC conditions given in the Experimental section. The UV and Radioactivity detectors were connected in series, resulting in a small offset of the radioactivity trace to longer retention times.

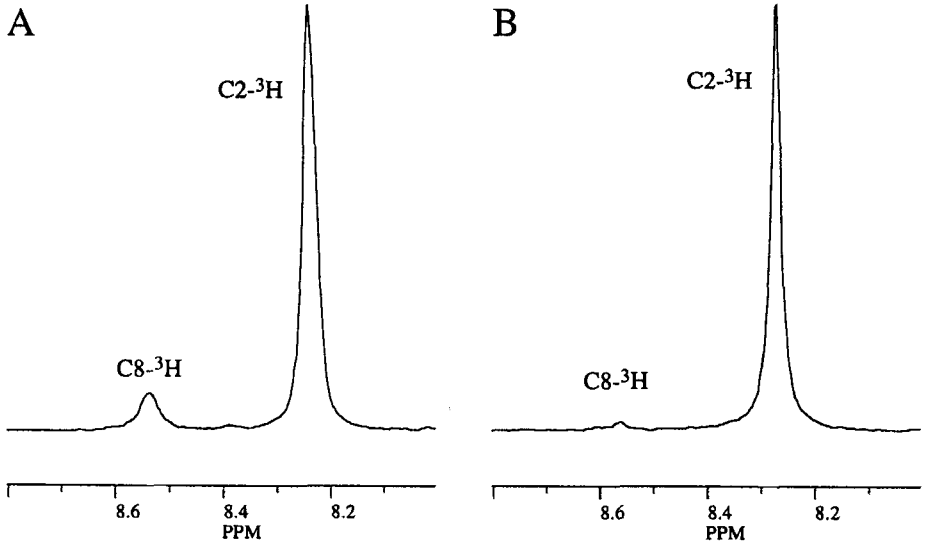



**Figure 2**: 320 MHz tritium NMR spectra of 2-$^3$H-ATP in $D_2O$, over the region δ 8.0-8.8 ppm. A). Sample tritiated using the method given in the Experimental section. The integrals show a relative incorporation for C8:C2=10:90. B). Sample labelled by first exposing the catalyst to tritium gas, then adding the exposed catalyst to the solution.

corresponding to adenosine 5'-diphosphate ($t_R$ = 5.4 min) was observed, presumably arising from hydrolysis of ATP during the tritiation reaction. No adenosine 5'-monophosphate ($t_R$ = 9.3 min) was detected. The specific activity of the product ATP was calculated by the combination of UV absorption ($\lambda$ = 258 nm, $\varepsilon$ = 1.58 x $10^4$) and radioactivity measurements, and was found to be 26.2 Ci/mmol. The $^3$H NMR spectrum of the crude product in Figure 2A shows the major resonance at $\delta$ 8.24 due to the C2 triton, and a small peak at $\delta$ 8.54 from metal catalyzed exchange of the C8 proton. Using the integrals in the $^3$H spectrum the specific activity of the C2 triton may be calculated as 23.6 Ci/mmol (*ca.* 90% incorporation of $^3$H at C-2). The $^3$H NMR spectrum of labelled ATP from another preparation is shown in Figure 2B. The specific activity of this sample was slightly lower (21.4 Ci/mmole), but essentially all the tritium was at the 2-position. The tritiation procedures for the samples analyzed in Figures 2A and 2B differed in only one small detail. In sample 2A the PdO catalyst was added to the reaction mixture prior to introduction of tritium gas, but in sample 2B the catalyst was exposed to $T_2$ on the catalyst spoon, and subsequently added to the thawed solution. This difference in incorporation has been duplicated over a number of ATP samples. The fact that the specific activity at the 2-position is comparable for the two approaches is reassuring, and the low C8 exchange contribution for the second approach suggests a different catalyst site for that type of labelling activity.

A probable mechanism for the dehalogenation reaction involves reduction of PdO to $\alpha$-Pd [palladium without a support], which catalyzes the dehalogenation. This is supported by the observations of Gordeeva *et al*[7] who found that $\alpha$-Pd, prepared by the reduction of $PdCl_2$ with sodium borohydride, catalyzed tritiodehalogenation of 8-bromo-nucleotides with the added advantage of enabling specific activities increased by a factor of 1.2–1.3 over that using 5% $Pd/BaSO_4$, a catalyst commonly used in metal catalyzed exchange reactions of nucleosides and nucleotides.[4-6]

## Conclusion

The method described here gives [2-$^3$H]-ATP with a specific activity far exceeding that obtainable by the heterogeneous catalyzed exchange approach.[4-6] We expect that this specifically labelled ATP will be useful in many other biochemical studies using $^3$H NMR spectroscopy, in addition to the recent applications in our own laboratory.[2,3] After completing this work we discovered a commercial source of 2-chloro-ATP, another possible precursor for [2-$^3$H]-ATP. As expected from previous experience comparing chloro- and bromo- substituted molecules,

preliminary reactions with 2-Cl-ATP gave incomplete dehalogenation under the conditions reported here, and the specific activity of the labelled product was lower.[15]

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## Experimental

Tritium gas was purchased from EG&G Mound Laboratories, Ohio, and contained 97.9% $T_2$, with the largest contaminant being DT (1.76%). Guanosine and other chemicals were obtained from Aldrich Chem. Co. and were used without further purification. Phosphoryl chloride and acetonitrile were carefully dried and freshly distilled before use.

Melting points were taken on a hot-stage microscope with a Fisher-Jones melting point apparatus and are uncorrected. HPLC was performed using a Waters model 510 pump and a 1/4" x 25 cm Supelco LC-18 column with a mobile phase of 0.1M diammonium phosphate adjusted to pH 6.0 with phosphoric acid, at a flow rate of 2 mL/min. A Hewlett Packard 1040A diode array spectrophotometer was used for UV analyses, and radioactivity was recorded by a IN/US Ramona-5-LS radiometric detector. NMR spectra were obtained on an IBM Instruments Inc.AF-300 NMR spectrometer ($^1$H at 300 MHz, $^3$H at 320 MHz and $^{31}$P at 121 MHz). Samples were made to a volume of 250 µL in NMR solvent in Teflon tubes (Wilmad, 507-TR-8"). Spectra were acquired at 297 K and the referencing of chemical shifts in $^3$H NMR spectra was achieved by generation of a ghost $^3$H signal from internal TMS or DSP in the $^1$H NMR spectrum.[16] Mass spectrometry was performed by the Analytical Laboratory, College of Chemistry, University of California, Berkeley, CA, U.S.A.

2',3',5'-Tri-O-acetylguanosine (**7**)

This synthesis was as previously described for 2',3',5'-tri-O-acetylguanosine.[10] A mixture of guanosine (**8**, 14.2 g, 50 mmol), acetic anhydride (30 mL, 318 mmol), pyridine (15 mL, 185 mmol) and DMF (40 mL) was heated at 75°C for 3.75 h. The mixture was filtered while hot,

cooled to room temperature, and evaporated to dryness. Recrystallization in isopropanol and drying *in vacuo* for 2 days at 80°C over $P_2O_5$ gave 15.6 g (76%) (**7**); mp 230-232°C (softening from 195°C, lit.[10] 230-233°C); $^1$H NMR (DMSO-$d_6$) δ 2.04 (s, 6H, 2 x $CH_3$), 2.11 (s, 3H, $CH_3$), 4.23-4.39 (m, 3H, C5'-H + C4'-H), 5.49 (br s, 1H, C3'-H), 5.78 (t, 1H, J = 5.9 Hz, C2'-H), 5.98 (d, 1H, J = 5.9 Hz, C1'-H), 6.54 (s, 2H, $NH_2$), 7.93 (s, 1H, C8-H), 10.74 (s, 1H, NH); mass spectrum (70 eV) m/z (relative intensity) 409 ($M^+$, 11), 259 (74), 194 (51), 193 (46) 157 (37), 151 (79), 139 (100), 97 (96).

2-Amino-6-chloro-9-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl) purine (**6**)

Compound (**7**) (12.28 g, 30 mmol), tetraethylammonium chloride (9.95 g, 60 mmol, dried overnight under vacuum at 80°C), and N,N-dimethylaniline (3.84 mL, 30 mmol) were dissolved in acetonitrile (70 mL), and cooled to –20°C ($CCl_4$/dry ice bath). Phosphoryl chloride (16.44 mL, 180 mmol) was added dropwise to the vigorously stirred reaction mixture. The solution was stirred for a further 20 min. at 0°C, and then heated at 100°C for 10 min. The reaction mixture was processed[10] to yield 10.8 g (84%) of (**6**): mp 150-151°C (lit.[10] 152-153°C); $^1$H NMR ($CDCl_3$) δ 2.09, 2.11, 2.15 (each s, 3H, $CH_3$), 4.34-4.49 (m, 3H, C4'-H + C5'-H), 5.31 (s, 2H, $NH_2$), 5.73-5.77 (m, 1H, C3'-H), 5.96 (t, 1H, J = 5.0 Hz, C2'-H), 6.02 (d, 1H, J = 5.0 Hz, C1'-H), 7.89 (s, 1H, C8-H); mass spectrum (70 eV) m/z (relative intensity) 429 ($M^+$, 5), 427 ($M^+$, 15), 259 (90), 170 (30), 169 (27), 139 (100), 134 (31), 97 (92).

2-Bromo-6-chloro-9-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl) purine (**5**)

Compound (**6**) (10.5 g, 24.5 mmol) was placed under nitrogen in a round-bottomed flask fitted with a water-cooled reflux condenser. While cooling the flask in an ice bath, t-butyl nitrite (45 mL, 378 mmol), and then bromoform (50 g, 198 mmol) were added dropwise with stirring. The mixture was stirred under argon at room temperature for 30 min., and then at 85°C for 4 hr. The residue obtained on evaporation of this reaction mixture *in vacuo* was recrystallized from methanol to give (**5**); yield 5.0 g (41%); mp 158-159°C (lit.[11] 155-156°C); $^1$H NMR ($CDCl_3$) δ 2.10, 2.14, 2.15 (each s, 3H, $CH_3$), 4.41-4.43 (m, 2H, C5'-H), 4.47-4.51 (m, 1H, C4'-H), 5.57-5.60 (m, 1H, C3'-H), 5.79 (t, 1H, J = 5.5 Hz, C2'-H), 6.23 (d, 1H, J = 5.5 Hz, C1'-H), 8.27 (s, 1H, C8-H); mass spectrum (70 eV) m/z (relative intensity) 494 ($M^+$, 0.03), 492 ($M^+$, 0.27), 490 ($M^+$, 0.19), 313 (5), 259 (75), 237 (12), 235(44), 233 (38), 157 (52), 139 (100), 97 (93).

2-Bromoadenosine (**4**)

2-Bromoadenosine (**4**) was prepared from (**5**) (1.7 g, 3.45 mmol) by ammonolysis using the procedure described for its synthesis from the corresponding 2,6-dibromo derivative;[12] yield 0.96

g (80%), $^1$H NMR ($CD_3OD$) δ 3.72-3.91 (m, 2H, C5'-H), 4.13-4.16 (m, 1H, C4'-H), 4.30-4.33 (m, 1H, C3'-H), 4.67 (t, 1H, J = 5.6 Hz, C2'-H), 5.91 (d, 1H, J = 5.6 Hz, C1'-H), 8.26 (s, 1H, C8-H); mass spectrum (FAB, glycerol matrix), m/z (relative intensity) 348 ($MH^+$, 60), 346 ($MH^+$, 63), 216 (65), 214 (64).

2-Bromoadenosine 5'-monophosphate (**3**)

2-Bromoadenosine 5'-monophosphate (**3**) was prepared as the barium salt by the reaction of (**4**) (345 mg, 1.0 mmol) dissolved in triethyl phosphate (2.5 mL) with phosphoryl chloride (110 μL, 1.2 mmol), as described in a general procedure for the synthesis of nucleoside 5'-phosphates;[13] yield 320 mg (57%); $^1$H NMR ($D_2O$) δ 4.0-4.01 (m, 2H, C5'-H), 4.35 (m, 1H, C4'-H), 4.47-4.49 (m, 1H, C3'-H), 4.72 (t, 1H, J = 5.5 Hz, C2'-H), 6.01 (d, 1H, J = 5.5 Hz, C1'-H), 8.52 (s, 1H, C8-H); $^{31}$P NMR ($^1$H decoupled) δ 4.28; mass spectrum (FAB, glycerol matrix), m/z (relative intensity) 564 ($MH^+$, 20), 562 ($MH^+$, 20), 429 (47), 307 (46), 215 (100).

2-Bromoadenosine 5'-triphosphate (**2**)

2-Bromoadenosine 5'-monophosphate barium salt (**3**, 53.6 mg, 0.2 mmol) was dissolved in water (5 mL) containing a small amount of Dowex 50 ($H^+$) resin and then passed through a 1x5 cm column of the same resin. The column was washed further with 30 mL water. Pyridine (1 mL) was added to the eluate and the solution was evaporated to dryness *in vacuo*. The residue was rendered anhydrous by three further evaporations with 5 mL portions of anhydrous pyridine. The product was converted to the tributylammonium salt by addition of tributylamine (48 μL, 0.2 mmol), followed by repeated evaporation of anhydrous pyridine and DMF. This salt was dissolved in DMF (2 mL) and reacted with 1,1'-carbonyldiimidazole (160 mg, 1.0 mmol) and anhydrous tributylammonium pyrophosphate (363 mg, 1.0 mmol), following the procedure described for synthesis of deoxyribonucleoside 5'-triphosphates.[14] The product was purified by chromatography on DEAE-Sephadex A-25 (3x25 cm column) with a linear gradient of triethylammonium bicarbonate (pH 7.5, 0-1 M in 3.5 L, flow rate 5 mL/min, UV detection at 260 nm). Five well separated UV absorbing fractions were obtained; the fourth (major) fraction corresponded to the tributylammonium salt of 2-bromo-ATP (as shown by $^1$H and $^{31}$P NMR). This fraction was evaporated to dryness, and freed from residual triethylammonium bicarbonate by co-evaporations with ethanol (4x10 mL). The residue was dissolved in methanol (4 mL) and converted to the desired tetrasodium salt by reaction with sodium perchlorate (366 mg, 3.0 mmol) in acetone;[14] yield 60 mg (45%); $^1$H NMR ($D_2O$, TSP) δ 4.22-4.34 (m, 2H, C5'-H), 4.41-4.43 (m, 1H, C4'-H), 4.60-4.62 (m, 1H, C3'-H), 4.78-4.82 (m, 1H, C2'-H), 6.07 (d, 1H, J = 6.0

Hz, C1'-H), 8.50 (s, 1H, C8-H); $^{31}$P NMR ($D_2O$) δ –6.67 to –7.00 (m, $P_\alpha$), –8.95 (d, $P_\gamma$, J=19.8 Hz), –20.30 (t, $P_\beta$, J=19.8 Hz); mass spectrum (FAB, glycerol matrix), m/z (relative intensity) 676 ($MH^+$, 15), 674 ($MH^+$, 14), 654 (23), 652 (21), 630 (15), 628 (14).

2-[$^3$H]-Adenosine 5'-triphosphate (**1**)

The tritiodehalogenation reaction has been completed more than six times at various chemical scales using the 2-bromo-ATP precursor synthesized above, with very similar results. The following description is representative: PdO catalyst (32 mg) on a spoon in a side-arm reaction flask was placed on the vacuum line overnight. The vacuum was broken with dry nitrogen, and 2-bromo-ATP (**2**, 10 mg, 0.015 mmol) dissolved in phosphate buffer (pH 8.6, 1 mL) was injected into the flask through the side-arm, and frozen with liquid nitrogen. The reaction flask and substrate were exhaustively degassed by the application of several freeze-pump-thaw cycles. The catalyst was dropped from the spoon into the reaction flask, and tritium gas was then introduced to *ca.* 80 kPa. The substrate was thawed and the $T_2$ gas pressure adjusted to around 95 kPa. The reaction mixture was then stirred at room temperature. After 3 h the substrate was frozen (liquid $N_2$) and the residual $T_2$ pumped away. The flask was extensively flushed with $N_2$, followed by addition of methanol (2x1 mL) to remove labile or dissolved tritium. After the sample volume was reduced by pumping away methanol, the flask was removed from the vacuum line. The catalyst was filtered, washed with water (2x2 mL) and the filtrate lyophilized. HPLC analysis of the residue showed complete conversion of 2-bromo-ATP to ATP (**1**) (Figure 1); total activity 320 mCi (83% yield), specific activity 26.2 Ci/mmol (by HPLC); $^1$H NMR ($D_2O$, TSP) δ 4.22-4.34 (m, 2H, C5'-H), 4.41-4.43 (m, 1H, C4'-H), 4.60-4.62 (m, 1H, C3'-H), 4.78-4.82 (m, 1H, C2'-H), 6.13 (br. s, 1H, C1'-H), δ 8.24 (s, 0.1H, C2-H), 8.54 (s, 0.9H, C8-H); $^3$H NMR ($D_2O$) δ 8.24 (s, 89.9%, C2-$^3$H), 8.54 (s, 10.1%, C8-$^3$H).

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