MICROSYNTHESIS OF PHOTOLABILE 8-[2-^{3}H]AZIDOADENOSINE NUCLEOTIDES

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1. Introduction

The discovery [1] that 8-azidoadenosine-triphosphate could be used to photoaffinity label the ($Na^+ + K^+$)ATPase of erythrocyte ghosts has opened up a whole new area of endeavor in photoaffinity labeling. Comparable analogs of cyclic AMP [2–4], NAD^+ [5], FAD [5], co-enzyme A [6] and GTP [7] have now been synthesized and used in a variety of labeling studies. The 8-azido-ATP or ADP analogs have also been used to photoaffinity label the mitochondrial F_1-ATPase [8] and the ADP transport carrier of mitochondria [9], respectively. The photoaffinity labeling of glutamate dehydrogenase [10] has been studied with 8-azido ADP as well. A definitive review of photoaffinity labeling has appeared in [11].

It is essential in such studies to have available appropriately labeled radioactive analogs. Toward this end we have devised an improved method of microsynthesis of both 8-N_3[2-^{3}H]ATP and 8-N_3[2-^{3}H]-AMP-PNP in overall radiochemical yields of 30–40%. The synthesis of 8-N_3AMP-PNP has not previously been reported.

2. Materials and methods

AMP was purchased from P. L. Biochemicals; [2-^{3}H]AMP from Amersham/Searle; tetraethylammonium hydroxide from Aldrich; periodic acid from Sigma; potassium azide from Eastman Organic Chemicals; DEAE-Sephacel (particle size 40–150 μm) from Pharmacia Chemicals; Reacti-Therm metal heating block and Reacti-Vials were from Pierce Chemical Corporation. Absorbance scans were done on a Cary-14. Radioactivity was measured with a liquid scintillation counter (Beckman LS 230) using Scinti-Verse (Fisher Scientific).

Dimethylformamide and methanol were stored over molecular sieves. Chloroform and acetone were dried with activated aluminum oxide. Pyridine was stored over CaH_2.

2.1. *Synthesis of 8-bromo[2-^{3}H]adenosine-5'-monophosphate*

The solution of 59 nmol [2-^{3}H]AMP (ammonium salt in 50% aqueous ethanol; total vol. 1 ml, 1 mCi; spec. act. 17 Ci/mmol) was evaporated in a small titration vessel (total vol. 3 ml) with a dry nitrogen stream. After addition of 5 μmol AMP in 1.55 ml H_2O (pH 3.80) and 0.2 M sodium acetate buffer, pH 3.9 (0.250 ml), the titration vessel was stopped and protected from light. This solution was adjusted to pH 3.90 using 0.1 N NaOH delivered below the solution surface via a glass capillary from a Radiometer pH stat. Freshly-prepared saturated bromine water (200 μl) was added dropwise through a small Teflon tube to the magnetically stirred solution. The rate of addition of bromine water was such that the pH maintained by the pH stat did not drop below pH 3.90. After addition of all the bromine water the pH stat was set to maintain pH 3.95.

After 1.5 h at room temperature the reaction mixture was directly applied to a DEAE-Sephacel column

Abbreviations: 8-BrAMP, 8-bromoadenosine-5'-monophosphate; 8-N_3AMP, 8-azidoadenosine-5'-triphosphate; 8-N_3AMP-PNP, 8-azidoadenyl-5'-yl-imidodiphosphate; (Et) $_3NH^+HCO_3^-$, triethylammonium bicarbonate; (Et) $_4N^+N_3^-$, tetraethylammonium azide; DMF, dimethylformamide; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography

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Table 1
Chromatographic properties of nucleotide analogs

	R_F[a]		t_R[b] (min)
	A	B	
AMP	0.38	–	21.3
8-BrAMP	0.43	–	23.1
8-N_3AMP	0.48	–	22.5
ATP	–	0.33	16.9
8-N_3ATP	–	0.44	17.5
8-N_3AMP-PNP	–	0.49	18.4

[a] TLC analyses run on Cellulose F plates (Baker Chem. Co.): Solvent A, *n*-butanol: acetic acid: H_2O; 5:2:3 (v/v/v); Solvent B, isobutyric acid: H_2O: conc. NH_4OH; 66:33:1 (v/v/v)

[b] HPLC analyses used a DuPont Model 830 equipped with a Whatman Partisil-10 SAX column. A 2%/min gradient of 0–50% 0.75 M KH_2PO_4 pH 4.0 at 600 p.s.i. was used to separate the monophosphates and a 0–100% 0.75 M KH_2PO_4 (pH 4.0) at 10%/min gradient at 600 p.s.i. to separate the triphosphates

(1.5 × 70 cm) and eluted using a linear gradient of 0–0.3 M triethylammonium bicarbonate [$(Et)_3NH^+HCO_3^-$] pH 7.5 (4°C), 2 liter total vol. Fractions were collected at a 1.0 ml/min flow rate, maintained with a peristaltic pump. Unreacted [2-^{3}H]-AMP was fully separated at 0.11 M $(Et)_3NH^+HCO_3^-$ from the major peak of 8-Br[2-^{3}H]AMP which eluted at 0.14 M $(Et)_3NH^+HCO_3^-$. The 8-Br[2-^{3}H]AMP gave the characteristic A_{265} max (ϵ = 15 100, pH 7.0) with an A_{232} min [12] and was judged to be free of [2-^{3}H]-AMP by TLC and HPLC (table 1).

On occasion a small amount of a side product (λ_{max} = 263 nm) was eluted at the front of the 8-BrAMP peak. This side product affected the azide displacement reaction by yielding a non-photolabile compound with an absorbance A_{278} max. Therefore only those fractions having an A_{265} max were pooled which resulted in ~85% yield of 8-Br[2-^{3}H]AMP. These combined fractions were evaporated to dryness under reduced pressure in a 300 ml pear-shaped flask (Kontes, Vineland, NJ), which facilitated the transfer step. The residue was redissolved and evaporated several times with anhydrous methanol to remove excess $(Et)_3NH^+HCO_3^-$ and water. The final residue was dissolved in 1 ml anhydrous methanol, transferred to a 0.3 ml Reacti-Vial (Pierce) stepwise with drying by a stream of dry nitrogen gas.

2.2. *Synthesis of 8-azido[2-^{3}H]adenosine-5′-monophosphate*

The gum-like triethylammonium salt of 8-Br[2-^{3}H]-AMP (5 μmol) was stored for 12 h in a vacuum desiccator over P_2O_5. The residue was then dissolved in 55 μl dry DMF and heated to 70°C in a thermostatically controlled metal heating block. $(Et)_4N^+N_3^-$, 25 μl 2 M solution, in dry DMF (70°C) was added and the reaction temperature maintained at 70°C for 7 h. The yellow reaction mixture was applied directly to a DEAE–Sephacel column (1.5 × 70 cm) and eluted using a linear gradient of 0–0.4 M $(Et)_3NH^+HCO_3^-$, pH 7.5 (4°C), 1 liter total vol. The appropriate fractions of 8-N_3[2-^{3}H]AMP (eluted at 0.23 M $(Et)_3NH^+HCO_3^-$; 80% yield) were pooled and treated as for 8-Br[2-^{3}H]AMP.

8-N_3[2-^{3}H]AMP was identified and characterized by the observed shift in the A_{max} from 265–281 nm (ϵ = 13 300, pH 7.0). The photolability of the analog was demonstrated spectrophotometrically by the decay of the 281 nm peak with time on exposure to ultraviolet light [3]. The analog was also shown to be pure by TLC and HPLC (cf. table 1).

2.3. *Synthesis of 8-azido[2-^{3}H]adenyl-5′-yl-imidodiphosphate and 8-azido[2-^{3}H]adenosine-5′-triphosphate*

8-N_3[2-^{3}H]AMP (triethylammonium salt) 5 μmol, was applied to a Dowex 50 WX8 column (50–100 mesh, 0.5 × 20 cm; pyridinium form prepared by treating the H^+ form with an excess of pyridinium chloride made by bubbling HCl-gas into a 50/50, v/v, pyridine–ether mixture). The pyridinium salt of 8-N_3[2-^{3}H]AMP was eluted with H_2O, dried several times with anhydrous pyridine under reduced pressure and redissolved in 2 ml dry DMF. The conversion to the tri-*n*-octylammonium salt was achieved by addition of 2.2 μl tri-*n*-octylamine and repeated evaporation with dry DMF.

The tri-*n*-octylammonium salt of 8-N_3[2-^{3}H]AMP in 2 ml dry DMF was activated [13] by dropwise addition of a freshly prepared solution of 4.1 mg 1,1′carbonyldiimidazole (stored under vacuum over P_2O_5) in 1 ml dry DMF under vigorous stirring. The reaction mixture was stirred under vacuum for 24 h before excess 1,1′carbonyldiimidazole was destroyed by addition of 2.0 μl anhydrous methanol.

Coupling of the imidodiphosphate or pyrophos-

phate group to the activated nucleoside-5′-monophosphate was done according to the following procedure: using an equimolar amount of tri-*n*-octylamine (11 μl) the tetrasodium salt of pyrophosphate (or imidodiphosphate) [14] (25 μmol) was converted into the corresponding tri-*n*-octylammonium salt as described for 8-N_3[2-^{3}H]AMP, dissolved in 2 ml DMF and added dropwise under vigorous stirring to the nucleoside-5′-phosphoimidazolide. The reaction mixture was stirred under vacuum for 72 h. After centrifugation to remove the precipitated imidazolium pyrophosphate or imidazolium imidodiphosphate, the sediment was washed twice with 1 ml dry DMF. The combined supernatants were evaporated under reduced pressure to a small volume (2 ml) and directly applied to a DEAE-Sephacel column (1.5 × 70 cm). 8-N_3[2-^{3}H]-ATP (eluted at 0.37 M $(Et)_3NH_4^+HCO_3^-$) or 8-N_3[2-^{3}H]-AMP-PNP (eluted at 0.33 M; 60–70% yield) were fully separated from side products using a linear gradient of 0–1.0 M $(Et)_3NH^+HCO_3^-$, pH 7.5 (4°C), 2 liter total vol. The triphosphate fractions were identified by an acid labile phosphate to adenylate ratio of 2:1. Further characterization was done with TLC and HPLC (cf. table 1). The triethylammonium salts of 8-N_3[2-^{3}H]AMP-PNP or 8-N_3[2-^{3}H]ATP were converted to the sodium salts using a Dowex 50 WX8 column (Na^+ form). Specific activities centered around 37 μCi/μmol nucleotide.

2.4. *Synthesis of tetraethylammonium azide*

Periodic acid (10 mmol, 2.28 g $HIO_4 \cdot 2\ H_2O$) was mixed with 10 ml *p*-dioxane. Tetraethylammonium hydroxide (10 mmol, 7.1 ml 20% solution in water) was added with stirring until all periodic acid crystals had dissolved and an apparent pH 7.0 had been reached. Potassium azide (10 mmol, 0.8 g) was dissolved in 2 ml water, added to the tetraethylammonium periodate solution, and stirred for 30 min. The precipitated potassium periodate was removed by filtration. Any remaining potassium periodate was precipitated by the addition of 5 vol. chloroform and again removed by filtration. The filtrate was evaporated to dryness under reduced pressure. The resulting $(Et)_4N^+N_3^-$ salt was repeatedly suspended in chloroform and evaporated as before until the salt changed from a clear gum to a white crystalline form. The crystals were then dissolved in 100 ml hot chloroform and any insoluble material (tetraethylammonium bicarbonate) was removed by filtration. The chloroform was evaporated as before. The $(Et)_4N^+N_3^-$ was then recrystallized from a minimal amount of hot acetone, filtered quickly because of the hygroscopic properties of the crystals, and stored either in a vacuum desiccator at 22°C or dissolved in dry DMF and stored sealed at −20°C. Both methods have been used and there was no evidence of decomposition after 4 months.

The average yield was 75% and a molecular weight of 172 g was assumed to determine concentrations. Infrared analysis showed the characteristic azide stretch at 2170 cm^{-1}.

3. Results and discussion

The described synthetic procedure leads to satisfactory yields as well as to high specific activities of 8-N_3[2-^{3}H]ATP and 8-N_3[2-^{3}H]AMP-PNP. It is even possible to increase the amount of radioactivity in the starting reaction by a factor of 100 as long as 2.5–5 μmol total AMP is used in the bromination step. If smaller quantities of radioactive product are desired the use of adenosine as a carrier as suggested [3] in the preparation of cyclic [^{32}P]AMP is recommended.

The bromination reaction was a modification of the procedure in [4]. The pH of the reaction was controlled by a pH stat to reduce the amount of sodium acetate buffer required, since it adversely affects the chromatographic resolution of 8-BrAMP and AMP on a DEAE-Sephacel column. Furthermore, by adding the freshly prepared bromine water mixture slowly while maintaining pH 3.90–3.93, side reactions could be almost quantitatively eliminated. Using the pH stat without the addition of any buffer decreased the yield about 10%.

The displacement of the 8-bromo group by $(Et)_4N^+N_3^-$ was done in DMF at 70°C. The solubility problem of the nucleotide analog and azide salts in DMF was solved by using the thoroughly dried triethylammonium salt of 8-BrAMP and tetraethylammonium azide. The main advantages of the tetraethylammonium azide are that:

1. It is easily prepared in crystalline form.
2. Stock standard solutions can be made.
3. It is readily soluble in DMF at 70°C.

For the coupling reaction both reactants 8-N_3AMP

and pyrophosphate or imidodiphosphate were converted to the corresponding tri-*n*-octylammonium salts because of the higher solubility of these salts in DMF at room temperature. Using the method in [15] the excess imidodiphosphate or pyrophosphate used nearly coelutes from DEAE-Sephacel with the corresponding nucleoside-5′-triphosphates, a situation normally requiring a second chromatographic separation. On the other hand, in the method of [13] excess imidodiphosphate or pyrophosphate precipitates as the imidazolium salts as the reaction proceeds and a single chromatographic step will yield pure nucleoside triphosphate derivatives.

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