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Research Article

Synthesis of ^{32}P-labeled intermediates on the purine biosynthetic pathway

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Summary

A fast and efficient route to ^{32}P-labeled intermediates on the purine biosynthetic pathway is described. Adenosine kinase catalyzes the phosphorylation of 5-aminoimidazole-4-carboxamide ribonucleoside (AICARs) to give [^{32}P]-5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). This was then hydrolyzed to [^{32}P]-4-carboxy-5-aminoimidazole ribonucleotide (CAIR). Treatment of CAIR with N5-CAIR mutase gave [^{32}P]-5-aminoimidazole ribonucleotide (AIR) in milligram quantities. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: preparation; ^{32}P-labeled; AIR; CAIR; AICAR; adenosine kinase

Introduction

In the course of our studies on thiamine biosynthesis, we required radiolabeled 5-aminoimidazole ribonucleotide (AIR) (**1b**), an intermediate on the purine and thiamine biosynthetic pathways (Figure 1).[1] While AIR (**1b**) has been previously synthesized using a chemical route,[2] this route is not readily amenable to the incorporation of a radiolabel because of low yields and the need to purify intermediates. An

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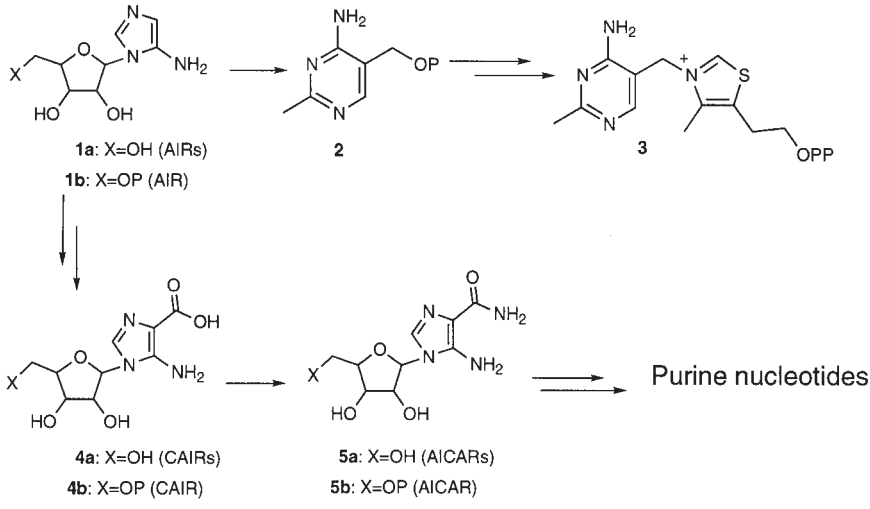


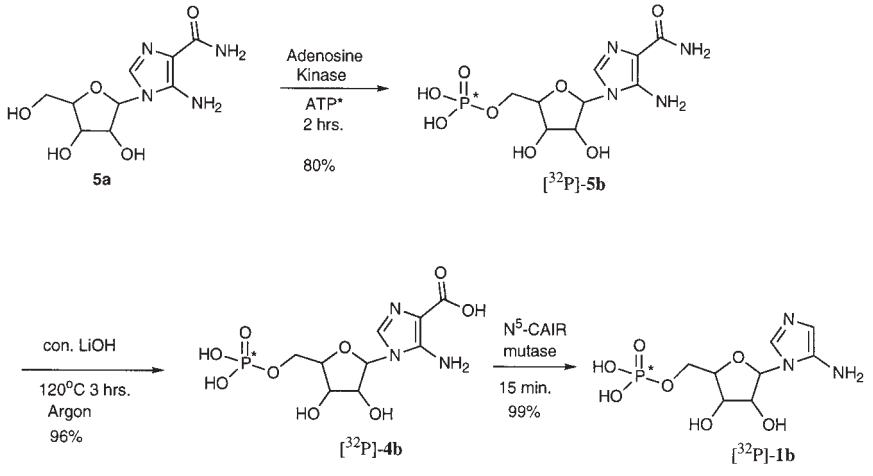
Figure 1. AIR (1b) is an intermediate on the thiamine pyrophosphate (3) and the purine nucleotide biosynthetic pathways.

enzymatic route, using the first five enzymes on the purine nucleotide biosynthetic pathway, has been used to synthesize [^{13}C]AIR and could be adopted to the synthesis of radiolabeled AIR.[3] However, this route is relatively complex. The wide substrate tolerance of adenosine kinase[4–6] and the structural similarity between 5-aminoimidazole ribonucleoside (AIRs) (**1a**) and adenosine suggested to us that this enzyme might be able to catalyze the phosphorylation of AIRs (**1a**) or one of its close biosynthetic analogs. In this paper, we demonstrate that this prediction is correct and utilize adenosine kinase as a synthetic tool to provide a facile synthetic route to ^{32}P-labeled intermediates on the purine biosynthetic pathway.

Results and discussion

While adenosine kinase did not catalyze the phosphorylation of AIRs (**1a**), or 4-carboxy-5-aminoimidazole-ribonucleoside (CAIRs) (**4a**) it did efficiently catalyze the phosphorylation of 5-aminoimidazole-4-carboxamide ribonucleoside (AICARs, **5a**). Thus, treatment of an excess of AICARs (**5a**) and terminally labeled [^{32}P]-ATP with adenosine kinase resulted in the complete consumption of the ATP in 2.5 h. HPLC purification yielded pure [^{32}P]-5-aminoimidazole-4-carboxamide

ribonucleotide (AICAR) (**5b**) in 80% yield (based on ATP as the limiting reagent, Scheme 1).



Scheme 1.

[^{32}P]-AICAR (**5b**) was saponified to [^{32}P]-4-carboxy-5-aminoimidazole ribonucleotide (CAIR) (**4b**) using concentrated lithium hydroxide. The use of the lithium counter ion greatly facilitated the purification because the lithium chloride or lithium acetate, generated by neutralization with hydrochloric acid or acetic acid, was soluble in ethanol while CAIR (**4b**) was not. This difference in solubility allowed the salt to be extracted leaving pure CAIR (**4b**). Sodium or potassium hydroxide could also be used for the saponification but were less satisfactory because neutralization followed by passage through an acidic cation exchange resin caused about a third of the [^{32}P]-CAIR (**4b**) to decarboxylate to [^{32}P]-AIR (**1b**).

AIR (**1b**) is a moderately reactive compound and was therefore prepared immediately before use by the enzymatic decarboxylation of [^{32}P]-CAIR (**4b**) using N^5-CAIR mutase.[3] This reaction was rapid (<15 min) and quantitative. CAIR (**4b**) has also been converted to AIR (**1b**) under acidic conditions but removal of salts from the product is then necessary, making this method unsuitable for the radiochemical scale synthesis because of product loss and decomposition during purification.

Overall, the reaction sequence outlined in Scheme 1 provides a facile route to [^{32}P]-AIR (**1b**) in good yield (76%) on 0.25 – 2.0 mg scale from a readily available starting material. While our interests were in

acquiring labeled AIR (**1b**), the strategy described here should also be applicable to the preparation of the later radiolabeled intermediates on the purine nucleotide biosynthetic pathway.

Experimental

A dehydrated form of LB broth was purchased from Gibco/BRL (Gaithersburg, MD). Ampicillin and IPTG were from Jersey Lab and Glove Supply (Livingston, NJ). Tris, agarose, EDTA, DTT, X-Gal and SDS-PAGE molecular weight markers were from Sigma (St Louis, MO). All starting materials, reagents and solvents were purchased from Aldrich-Sigma Chemical Co. (St Louis, MO) and used without further purification, unless otherwise indicated. Acrylamide/Bis (37.5:1) was purchased from BioRad Laboratories (Hercules, CA). All radioactive materials were purchased from ICN Pharmaceuticals. Adenosine kinase and N^5-CAIR mutase are readily purified from overexpression strains as previously described.[6,7]

All high pressure liquid chromatography (HPLC) was performed on a Hewlett Packard 1100. Reactions and purifications were monitored at 254 nm with a Hewlett Packard 1100 diode array detector.

Reaction mixtures were analyzed using a Supelcosil LC-18-T, 15 cm × 4.6 mm, 3 μm column (Supelco, Bellefonte, PA). Isocratic elution using 100 mM KH_2PO_4 buffer pH 6.6 (1 ml/min) gave retention times as shown (minutes, detected at 254 nm with a 380 nm reference) CAIR (1.85), AIR (2.10), AIRCA (3.14), ATP (4.16), ADP (4.98), AICARs (12.18).

Radiolabeled compounds were checked for purity using the above HPLC conditions and detected by absorbance at 254 nm and by in line scintillation counting using a Packard 500TR Flow Scintillation Analyzer (LQTR 300 μl flow cell, Ultima-Flow AP Packard LSC-cocktail).

[^{32}P]-5-aminoimidazole 4-carboxamide ribonucleotide (AICAR, **5b***)*

ATP (3.5 mg, 20 μCi, 3.1 mCi/mmol) and AICARs (**5a**, 2.1 mg) were added to 100 mM Tris-HCl buffer pH 7.8 containing 1.25 mM $MgCl_2$ and 0.1% BSA (0.5 ml). Adenosine kinase[6] (100 μg) was added to the reaction mixture which was then incubated at 37°C until all the ATP had been consumed (2–2.5 h). The reaction was stopped by freezing the

mixture which was stored at −80°C. After thawing, the reaction mixture was filtered through a Microcon regenerated cellulose filter (10 000 mw cut-off) to remove the proteins and purified by HPLC (Supelcosil SPLC-18-DB, 25 cm × 10 cm, 5 μm column, A: water, B: 25 mM diisopropylethylamine acetate (aq.) C: methanol. Elution conditions: 2:3 A:B 0–20 min, 2:3 A:B to 9:1 A:C 20–22 min, 9:1 A:C for 22–30 min. Flow rate = 3 ml/min). Under these conditions the AICAR (**5b**) eluted at 23.0–24.5 min. HPLC analysis demonstrated >99% conversion of AICARs (**5a**) to AICAR (**5b**). The product-containing fractions were collected and dried by rotary evaporation. The resulting solid was dissolved in 1–2 ml 1:1 methanol:water and then dried. This produced 2.4 mg of (**5b**) (16 μCi, 3.1 mCi/mmol 80% yield) as the bisdiisopropylethylamine salt. To obtain the free acid, the sample was dissolved in 100 μl ethanol and dried three times. ^{1}H NMR (500 MHz, D_2O 4.65) 7.26 (s, 1 H), 5.40 (d, 1 H, $J = 7$), 4.39 (t, 1 H, $J = 5.5$), 4.15 (m, 1 H), 4.06 (m, 1 H), 3.48 (m, 2 H), 3.46 (m, 4 H), 2.95 (q, 4 H, $J = 7$), 1.04 (m, 15 H). ESMS (negative ion) 337, 97, 89 (analytical data on cold sample).

[^{32}P]-4-carboxy-5-aminoimidazole ribonucleotide (CAIR, **4b***)*

Lithium hydroxide (1 ml, 1 M) was added to (**5b**) (2.4 mg) in a 5-ml pear shaped flask. This mixture was stirred at 120°C while blowing argon over the surface. When the mixture reached near dryness, it was sealed under argon and heated at 120°C for 3 h. The reaction was cooled and opened under argon. HPLC analysis demonstrated >99% conversion of AICAR (**5b**) to CAIR (**4b**).

Purification method 1: dH_2O (0.5 ml) was added and the pH was adjusted to 7 with 1 N acetic acid. The solution was then lyophilized and the resulting solid was transferred to a micro-centrifuge tube with 1 ml 100% ethanol. The tube was vortexed for 1 min and centrifuged at 6000 rpm for 5 min and the ethanol was removed. This extraction procedure was repeated four times. The remaining white solid (pure CAIR, **4b**) was dried under vacuum (starting AICAR 16 μCi, 3.1 mCi/mmol, produced 11.7 μCi, 3.1 mCi/mmol of CAIR, 73% yield). ^{1}H NMR (500 MHz, D_2O 4.62) 8.16 (s, 1 H), 5.62 (d, 1 H, $J = 6$), 4.38 (t, 1 H, $J = 6$), 4.21 (m, 1 H), 3.90 (m, 2 H). ESMS (negative ion) 338, 294, 97, 89 (analytical data on cold sample).

Purification method 2: The reaction was diluted to 3 ml with water and loaded onto a small ion exchange column (0.4 mg (2 meq) of well-washed Dowex 50Wx8, 50–100 mesh H form resin (Supelco)). The

product was eluted with water (25 ml) and lyophilized to give a white solid consisting of a 7:3 mixture of CAIR:AIR (starting AICAR 16 μCi, 3.1 mCi/mmol, produced 15.4 μCi, 3.1 mCi/mmol of CAIR, 96% yield). ^{1}H NMR (500 MHz, D_2O 4.60) 8.40 (s, 0.3 H), 8.16 (s, 0.7 H), 6.57 (s, 0.3 H), 5.69 (d, 0.3 H, $J = 4.4$), 5.62 (d, 0.7 H, $J = 5.2$), 4.38 (m, 1 H), 4.21 (m, 1 H), 3.96 (m, 2 H) (analytical data on cold sample).

[^{32}P]-aminoimidazole ribonucleotide (AIR, **1b***)*

A solution of CAIR (**4b**) (15.4 μCi, 3.1 mCi/mmol in 25 μl water) and N^5-CAIR mutase[7] (2.5 μl, 8 mg/ml in 25 mM Tris–HCl pH 8.5, 3 mM $MgCl_2$) was added to 75 μl of the same buffer. The reaction was incubated at 37°C for 30 min and filtered through a Microcon regenerated cellulose filter (10 000 mw cut-off). The filter was washed twice with 25 μl water, the washings were combined with the filtrate and lyophilized to give AIR (15.2 μCi, 3.1 mCi/mmol 99% yield). ^{1}H NMR (500 MHz, D_2O 4.52) 7.47 (s, 1 H), 6.22 (s, 1 H), 5.46 (d, 1 H, $J = 7$), 4.45 (t, 1 H, $J = 6.5$), 4.22 (m, 1 H), 4.05 (m, 1 H), 3.73 (m, 2 H). ^{13}C NMR (400 MHz, D_2O) 138.2, 130.8, 90.1, 86.1 (d, $J = 33.2$), 75.9, 71.3, 64.6 (d, $J = 18.4$), 60.4. ESMS (negative ion) 294, 97, 89 (analytical data on cold sample).

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