SYNTHESIS OF [8-^{14}C] N^6-(Δ^2-ISOPENTENYL) ADENOSINE

M.H. Fleysher.

Department of Experimental Therapeutics
Roswell Park Memorial Institute
Buffalo, New York 14203
U.S.A.

Received on February 19, 1972

SUMMARY

N^6-(Δ^2-isopentenyl)adenosine, known as IPA or IPAdo, is a normal component of t-RNA which has growth stimulatory effects in plants and growth inhibitory effects in mammalian cells, including human leukemia. 8-^{14}C-IPA was synthesized by alkylating 8-^{14}C adenosine with Δ^2-isopentenyl bromide. The purity of the radioactive product was greater than 99%. A procedure was devised for isolating this compound and preserving its purity.

Introduction

N^6(Δ^2-isopentenyl)adenosine (IPAdo) is a natural component of mammalian and plant t-RNA. This compound has kinetin-like effects in plant systems and growth inhibitory effects on mouse and human tumor cells in culture and in vivo (1). The availability of isotope-labelled IPAdo with high specific activity was required to investigate the mode of action of the drug in mammalian systems. In this study 8-^{14}C IPAdo was synthesized by direct alkylation of adenosine, followed by conversion of the N^1 isomer to the N^6 isomer by treatment with a base. The conversion procedure of Martin and Reese[2] was adopted in view of its efficiency at low temperature and because it permits recycling of unreacted labeled adenosine. The preparation of an

IPA of low specific activity has been described previously by Hall et al.[3]

Unlabelled IPA is a relatively unstable compound which cleaves in aqueous media and at room temperature at a rate of about 3.5% per month, adenosine being the main product. The radioactive [8^{14}C]IPA decomposes under the same conditions at the rate of about 11% per day. A technique was therefore developed to isolate and preserve the labelled compound at >99% purity under conditions in which there is no appreciable decomposition.

A. Materials and Methods. Chemicals

[8^{14}C]Adenosine was purchased from Amersham-Searle. The specific activity was 50 mCi/mMol. Radiochemical purity was stated to by >98%. Δ^2-Isopentenylbromide (1 bromo-3-methyl-but-2 ene) was synthesized for this work by the process of Hall and Fleysher.[4] Freshly distilled material was used. N,N-Dimethylformamide (Eastman Kodak Reagent Grade) was dried over Drierite and redistilled, the fresh fraction boiling at 151-152° was collected. Dimethylamine -25% aqueous solution was obtained from Eastman Kodak and Ethanol (200° Proof), from Publicker Industries. The markers used, adenosine and adenine were purchased from Nutritional Biochemicals, Inc. Unlabelled N^6-Isopentenyladenosine was obtained from Starks Associates Inc., Buffalo, N.Y., and was recrystallized twice from ethanol, dried, and then stored at -70° to avoid degradation.

Chromatography

The system, ethylacetate/nPropanol/H_2O, 4:1:2 (upper phase) was used for both paper and TLC chromatography. Whatman 3MM paper was prewashed with N-acetic acid, water and ethanol. TLC glass plates, coated with Silica gel GF to a thickness of 500 microns, were obtained from Analtech, Inc., Newark, Delaware.

Instrumentation

All UV determinations were done on a Cary Model 14 recording spectrophotometer. Strip chromatograms were scanned for radioactivity on a Tracerlab 4π scanner. Measurements of radioactivity on segments of the marked strips and on aliquots of

solutions were obtained on a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3310, using the "PPO-POPOP" and Bray's solution respectively.

Preparation of [8-^{14}C]-N^6-(Δ^2-Isopentenyl)adenosine

One mC of [8-^{14}C]adenosine, containing 5.38 mg (0.02 mMol) in aqueous solution, was evaporated to dryness in a flash evaporator at 25° and azeotroped 3 times with 2 ml EtOH to remove water. It was then dried in the dark, in a vacuum dessicator over P_2O_5 for several days. To the ado was added 100 µl of anhydrous N,N-dimethyl-formamide and 5 µl (6.5 mg, 0.043 mMol) of Δ^2-isopentenylbromide. The reaction flask was stoppered and swirled until a homogeneous solution resulted. This solution was stored in the dark, at room temperature for 2 days. To effect conversion of the N^1 to the N^6 analog, 0.1 ml EtOH and 0.1 ml of 25% aqueous 25% dimethylamine (5.55 mMol) were added, and the reaction mixture was kept in the dark for two days with occasional swirling. The mixture was then evaporated to dryness in vacuo and azeotroped twice with 0.2 ml EtOH and then with water (2 x 0.2 ml). The solid was suspended in 1 ml EtOH and 3 ml of ethylacetate added, whereupon a clear solution was obtained.

Aliquots of the solution were applied to the baseline of 2 acid washed Whatman papers (17.7 cm x 57 cm), with markers on the margins, and chromatographed for 3.5 hrs at room temperature (descending) in a presaturated tank. Under UV light the dried sheets revealed: 1) A heavy band of unreacted radioactive adenosine (Rf = 0.16); 2) two faint radioactive bands (unidentified) at Rf 0.41 and 0.58 respectively; and 3) a heavy band of [8-^{14}C]IPA (Rf = 0.79). The IPA bands (15.6 x 6 cm) were cut out and the product eluted (descending) with 50% EtOH. The elution volume of 200 ml was collected. Counts on the last 35 ml of eluate indicated a content of only 0.16 mµMol of IPA per ml. The eluate was evaporated and dissolved in a volume of 20 ml water, and stored in a freezer (-20°). As determined by UV spectrometry this solution (absorption max. 268 mµ) contained 2.878 mgs (0.00854 mMol) of IPA (42.7% of theory). By scintillation analysis the compound contained 1.12×10^{11}

DPM/mMol, while the original labelled adenosine contained 1.10×10^{11} DAM/mMol. Hence it is concluded that the original radioactivity of Ado was transferred intact to the labelled IPA. The yield calculated from the scintillation analysis is 43.0% which agrees with the 42.7% obtained by UV spectrophotometry. The chemical purity of the labelled IPA, when assayed by chromatography and following scintillation counting on paper strips (3 x 57 cm), showed that the material contained [8-^{14}C]IPA, 87.04%; [8-^{14}C]Ado, 10.28%; and [8-^{14}C]Ade (adenine), 2.66%. Since the highest specific activity IPA was sought, (>99%) the material was frozen at -70° prior to final purification.

Recycling of Unreacted 8-^{14}C Ado

The [8-^{14}C]Ado bands were cut out and eluted with 32 ml water, and 2.20 mg of the material was obtained (41% recovery) as measured by UV spectrometry (259 mμ max). After evaporation, the Ado was dried and reacted with isopentenyl-bromide in the above manner, and yielded a second crop 32 ml containing 0.683 mg [8-^{14}C]IPA which when assayed as described above contained 87.3% [8-^{14}C]IPA; 10.8% [8-^{14}C]Ado and 1.8% [8-^{14}C]Ade. It was stored at -70°. It was noted at this time that some aqueous control samples of [8-^{14}C]IPA, stored at -20°, when reassayed had undergone noticeable degradation to [8-^{14}C]Ado. A diluted sample originally containing 87% [8-^{14}C]IPA and 10.3% [8-^{14}C]Ado was allowed to stand at room temperature for 3 days, and reassayed, contained 58.2% IPA* and 38.3% Ado*, thus indicating a decomposition rate of approx. 11% per day. This compares with a freshly crystallized sample of non radioactive IPA purified by chromatography which decomposed at a rate of 3.5% per month. (Freshly crystallized unlabelled IPA stored in the dry state at -70° shows no appreciable degradation over a 6 month period). It was also noted by two dimensional chromatography in a slower moving solvent system (iPrOH-NH_4OH-H_2O - 7:1:2) and strip assay, that [8-^{14}C]IPA kept at room temperature, was cleaved at the rate of over 3.5% per day, whereas if the sample was eluted, an 11% decomposition rate per day was observed.

A second recycling of recovered [8-^{14}C]Ado (0.95 mg) to [8-^{14}C]IPA was purified on TLC glass plates (20 x 20 cm) precoated with Silica gel GF using the above solvent, at 4°, for 90 minutes, and yielded 3 bands 1) adenine, Rf 0.085; 2) Ado, Rf 0.25; and 3) IPA, Rf 0.63. The bands were cut out and extracted, the IPA with absolute EtOH and the Ado with water. The IPA extract (7 ml) showed 99.6% [8-^{14}C]IPA and Ado and Ade, 0.2% each. The Ado band was comprised of 10.9% IPA, 88.3% Ado, and 0.7% Ade; the Ade band comprised 80% Ade, 6.0% Ado and 14% IPA, all radioactive.

While this last purification method furnishes [8-^{14}C]IPA of >99% purity there is some overlapping of bands because of the relatively short length of the chromatographic plate (20 cm); and this results in a loss of product, and in a failure to detect any side reactions. It therefore appears preferable to carry out the original purification of the reaction mixture on paper, as above, for better resoltuion, and the final purification conducted on TLC plates followed by extraction with absolute ethanol.

Purification of [8-^{14}C]IPA on TLC Plates

All of the preparations of [8-^{14}C]IPA which contained about 88% IPA were pooled, evaporated and lyophilized. The residue was dissolved in 3 ml ethylacetate and 1 ml EtOH in the cold room (4°). All subsequent operations were conducted at 4°. Aliquots of the material were applied to four 20 x 20 cm Silica gel GF coated glass plates and chromatographed in a TLC tank with the ethylacetate-propanol-water system for 90 minutes, in the dark. The solvent front was 16 cm from the baseline and the Rf's were 0.70, 0.30 and 0.18 for IPA, Ado and Ade, respectively.

The IPA bands were removed, combined, and extracted repeatedly with absolute EtOH, in the cold room, by mixing with a magnetic stirrer for 0.5 hr. The first extract furnished 20 ml of solution containing (by UV) 2.09 mgs IPA as a 0.310 mMolar solution. The second extraction furnished 28 ml of solution containing 0.437 mgs IPA as 0.046 mMolar solution and the final 3 extractions were combined to 69.0 ml of solution containing 0.297 mgs of IPA as a 0.013 mMolar solution. Assayed for purity the [8-^{14}C] IPA contained 99.7% IPA; 0.2% Ado and 0.1% Ade all of specific acitivty, 50 mC/mMol.

The yield of purified [8 ^{14}C] IPA (including control samples) was 3.00 mg (0.008 mMol) or 45% of theory. Prior to final purification the yield of radioactive IPA was 3.63 mg (0.0108 mMol) or 54% of theory.

The product preserved in absolute ethanol appears to be stable and does not undergo the breakdown observed in aqueous solutions. Storage is at -70°. For experimental work requisite amounts are pipetted, the EtOH quickly evaporated, and the material diluted and quickly used.

Acknowledgement

This work was supported by Research Grants Nos. CA-11047, CA-04175 and CA-12825 from the National Cancer Institute, U.S. Public Health Service. The author wishes to express his thanks to Drs. E. Mihich and M.T. Hakala for their interest in the work.

References

(1) R.H. Hall, In "Progress in Nucleic Research and Molecular Biology", edited by J.N. Davidson and W.E. Cohn, Vol. 10, p. 57, Academic Press, New York, N.Y., 1970.

(2) D.M.G. Martin and C.B. Reese, J. Chem. Soc. 1968, (c), 1731.

(3) V. Paces, E. Werstiuk and R.H. Hall, Plant. Physiol. 48, 775 (1971). We are indebted to Professor Hall for a preprint of the method.

(4) R.H. Hall and M.H. Fleysher in "Synthetic Procedures in Nucleic Acid Chemistry", Vol. 1, W.W. Zorbach and R.S.Tipson, Eds., Interscience Publishers, New York, N.Y., 1968.