SYNTHESIS OF TRITIUM-LABELED FOSFOMYCIN

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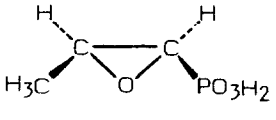
SUMMARY

Tritium gas was used as a labeling agent for the preparation of [1,2-^{3}H]fosfomycin. Introduction of tritium into a precursor, the synthesis including resolution of the intermediate racemic 1,2-epoxypropylphosphonic acid, and preparation of both amine and calcium salts of the labeled antibiotic are described.

Keywords: [1,2-^{3}H]Fosfomycin, Synthesis/Isotopes, Tritium Labeling

INTRODUCTION

Studies requiring a radiolabeled tracer have become a standard part of the development of most new medical substances. Discovery of the new antibiotic fosfomycin (I), (2R-cis)-(3-methyloxiranyl)phosphonic acid (1), followed by determination of its structure and chemical synthesis (2), prompted the request for synthesis of a labeled sample of the material in our laboratory.



Fosfomycin (1)

DISCUSSION

A survey of available routes to fosfomycin (2,3, and unpublished reports) suggested that a practicable process for generating the needed tracer would

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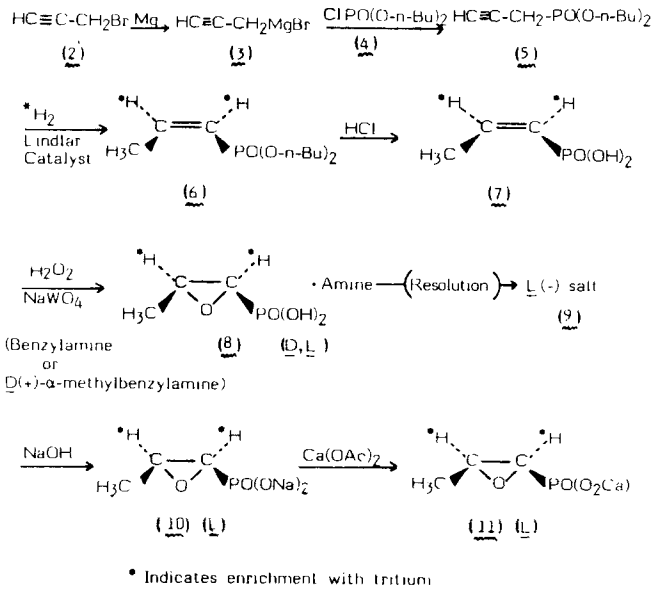
commence with propargyl alcohol. Labeled propargyl alcohol was not commercially available, however, nor was there any obvious or attractive route for its synthesis. Accordingly, a decision was made to employ tritium as the labeling agent for this assignment. (The short half-life of phosphorus-32 rendered this isotope unsuitable for many of the intended studies.)

Treatment of di-(n-butyl) 1-propynylphosphonate (5) (from propargyl alcohol, see (4)) in benzene with hydrogen diluted tritium gas over a presaturated Lindlar catalyst ($Pd/BaSO_4$ + quinoline) led to di-(n-butyl) cis-1-[1,2-^{3}H]propenylphosphonate (6). In order that formation of the undesired, labeled propanyl analogue through overreduction would be avoided, the labeling reduction was carried only to partial completion. The resulting tritium-labeled intermediate (6) was purified by dilution with carrier and distillation. Hydrolysis of the ^{3}H-labeled di-(n-butyl) phosphonate (6) in conc. hydrochloric acid gave cis-1-[1,2-^{3}H]propenylphosphonic acid (7).



• Indicates enrichment with tritium

In our initial synthesis the epoxidation of (7) was done in the presence of benzylamine, yielding the benzylammonium salt. The acidic strength of the free phosphonic acid is sufficient to catalyze opening of the oxirane ring. Accordingly, the isolation, resolution, and other necessary manipulations were done to the extent possible in the salt form. To convert the benzylammonium salt to the optically active D(+)-α-methylbenzylammonium salt for resolution, the benzylamine was removed by passing a solution of (8) through a cold-jacketed column containing resin on the acid cycle. Eluate from the column was led directly into a cold solution of the optically active amine in water. The combination of low temperature and short residence time as free acid served to suppress the formation of 1,2-glycol.

Resolution was then readily accomplished by dilution of the salt with a ten-fold amount of optically active carrier and recrystallization (2X) from isopropyl alcohol. That the resulting product was optically pure was demonstrated by extensive dilution with optically pure carrier, followed by five-fold recrystallization. The observed constancy and distribution of radioactivity indicated that resolution was complete after two recrystallizations.

Subsequent epoxidation of the olefinic acid (7) was done in the presence of an equivalent amount of D-α-methylbenzylamine, leading directly to its salt of D,L-1,2-epoxy[1,2-^{3}H]propylphosphonic acid (8). Resolution was then achieved by dilution with resolved carrier and crystallization. Success of resolution, again, was indicated by the gross distribution of radioactive material and by the specific radioactivity of the product.

A slightly alkaline solution of fosfomycin sodium salt was obtained by treatment of a solution of (8) with sodium hydroxide followed by removal of the liberated amine with chloroform. The calcium salt was then formed by the addition of a concentrated solution of calcium acetate.

Preparation of these salts of the labeled antibiotic, with the concomitant demonstration of optical purity completed our objectives for the labeling of this substance.

EXPERIMENTAL

Radioactivity measurements were made with a Packard Instrument Co. Tri Carb® Series 3000 liquid scintillation spectrometer, using a phosphor comprised of PPO and POPOP in toluene-ethanol 7:3. Melting points were measured in open capillaries in a modified Hershberg apparatus with totally immersed, calibrated thermometers and were not corrected. Analytical TLC was done with silica gel and with cellulose plates with adsorbent layer 250 microns in thickness. Tritium gas was supplied by New England Nuclear; measurement of its specific activity has been previously described (5).

Di-(n-butyl) Cis-1-propenyl[1,2-^{3}H]phosphonate (6) -- A suspension of 230 mg of Lindlar catalyst (Pd-$BaSO_4$/quinoline) in 40 ml of benzene was shaken under tritium/hydrogen (^{3}H) at 760 Torr, 22° until there was no further uptake. ^{3}H was displaced with nitrogen, and 1.162 g, 5 mmoles, of di-(n-butyl) propynylphosphonate (5) was added along with 10 ml more benzene. The ^{3}H atmosphere was restored, and shaking was continued until the uptake slowed -- 16.1 cc (STP), approx. 0.8 mmole, had been absorbed at this point. The ^{3}H atmosphere was again removed, replacing with nitrogen, and 230 mg of fresh catalyst was added. ^{3}H was again returned to the system and shaking was continued until a total of 81.7 cc was absorbed. Assuming that the additional catalyst required 35.3 ml of this, the total uptake by the substrate was 62.5 cc (STP, 2.79 mmoles for the 5 mmoles of (6)).

After standing overnight under nitrogen the mixture was filtered from catalyst. An aliquot of the solution was removed for determination of the specific activity. The amount of radioactive material in the aliquot indicated a total tritium uptake by substrate of 750 millicuries.

Most of the benzene was distilled off through a short Vigreaux column, under nitrogen sweep and at atmospheric pressure. The residual oily product was transferred to a small distilling unit with a Vigreaux side arm, completing the transfer with 3 grams of pure di-(n-butyl) 1-propenylphosphonate (6) carrier. After

removal of residual benzene under vacuum, product was distilled and collected, b.p. 80-89°/0.2 mm, leaving about 1 g of residue. Four additional 1 g portions of carrier were added and distilled in the same manner, leaving a final residue of approx. 1 g. Total carrier added: 6.95 g. Total distilled product: 6.73 g. The specific activity of this product was found to be 107 μCi/mg, 25 mCi/mmole.

Cis-1-[1,2-^{3}H]propenylphosphonic Acid (7) -- The diluted, distilled di-(n-butyl) propenylphosphonate (6) 6.7 g, 18.7 mmoles, was suspended in 67 ml of conc. hydrochloric acid. This mixture was stirred and heated such that distillate was slowly removed through a small Vigreaux column. After five hours of slow distillation the residual mixture was allowed to cool and stand. Any remaining ester or other neutral material was removed by extraction (4 x 10 ml) with chloroform. The aqueous phase was then concentrated under vacuum on the rotary evaporator, leaving the water soluble product (7). This residual material was freed of residual HCl and water by addition and distillation of 2 x 25 ml of water followed by 2 x 25 ml of benzene, and finally 25 ml of chloroform, leaving 3.26 g, 26.7 mmoles, 93% of (7).

D,L-1,2-Epoxy[1,2-^{3}H]propylphosphonic Acid (8) D(+)-α-Methylbenzylamine Salt --Crude 1-[1,2-^{3}H]propenylphosphonic acid (7), 3.26 g, 26.7 mmoles, was suspended in 13.4 ml of 1-propanol and 3.71 g, 30.7 mmoles of D(+)-α-methylbenzylamine was added. The pH of the resulting mixture was 5.5. The 100 ml, four-necked flask containing this mixture was fitted with a stirrer, a pH electrode, a thermometer, and an addition funnel. After the addition of 146 mg of sodium tungstate and 2.9 mg of disodium versenate the mixture was warmed to 50-55°C and 5.1 ml of 30% hydrogen peroxide was added from the dropping funnel over a 20 minute period. Stirring was continued at 50-55°C, with additional amine being added as needed to maintain the pH above 5.0. After 45 minutes an additional 1 ml of hydrogen peroxide was introduced. After a total of 80 minutes from the start of the peroxide addition the excess peroxide was quenched by the addition of saturated aqueous sodium sulfite with stirring and cooling to keep the temperature below 60°C.

Water was removed by displacement distillation (moderate vacuum) with 1-propanol to leave the product (8-salt) as a solution in this solvent. Inorganic salts were removed by filtration, and the cake was washed with 100 ml of methanol which was combined with the filtrate.

(2R-Cis)-(3-methyloxiranyl)[1,2-^{3}H]phosphonic Acid D(+)-α-Methylbenzylamine Salt (9) Carrier Resolution -- The alcoholic solution containing crude D,L-product (8) was concentrated under vacuum to an estimated 30 ml volume. A slurry of 3.000 g of pure unlabeled (2R-cis)-(3-methyloxiranyl)phosphonic acid D(+)-α-methylbenzylamine salt (fosfomycin) in 8 ml of water was added, and the mixture was warmed by stirring briefly in a 50° water bath to complete the solution of the carrier. This mixture was seeded (with resolved carrier) as the solution cooled, and separation of crystals was well advanced as the mixture came to room temperature. After aging overnight (cold) the product was collected and washed 3 x 5 ml with cold isopropyl alcohol-water 4:1. The air-dried product weighed 3.0865 g. This material was twice recrystallized from isopropyl alcohol-water 4:1, 35 ml and 24 ml respectively (minimum volumes for complete solution at reflux) to obtain 2.266 g of final, resolved product. Radioactive count of first isolated material: 8.4×10^3 cpm/μg; after one recrystallization the value came to 7.32×10^3; and after the second recrystallization the activity was 7.77×10^3 (no further decrease). At the observed counting efficiency of 18% the count value leads to an activity of 19.6 μCi/mg, or 5.43 mCi/mmole, for a total of 44.3 mCi in the product.

Purity of this product was determined by radio-GLC and by thin layer chromatography. For the GLC analysis a sample of the salt was trimethylsilylated with bis-trimethylsilylacetamide (BSA) and then injected into an instrument fitted with both a mass detector (flame) and a radioactivity detector suitably arranged for tritium activity measurement. (Carrier gas: helium, 75 ml/min; column: dual 4 ft. with 3.8% SE-30; 4 min. at 105°, 5°/min to 150°). The main mass peak (ca. 92% by peak area) corresponded to the main radioactivity peak (ca. 99.6%). The

minor, impurity peaks in the mass trace (total ca. 8%) were shown to be due to products from interaction of the phenethylamine with BSA. TLC analysis: a. methanol/pyridine/water, 30/5/15, SiO_2, 99.2% of the total plate radioactivity in the product spot, R_f 0.73; b. methanol/pyridine/water, 30/5/15, cellulose, R_f 0.8, 99.55% of the plate radioactivity in the product spot. The melting point, which is dependent on the degree of hydration, was 136.5-138°C, compared with 138-140°C for the best available reference sample.

D,L-1,2-Epoxy[1,2-^{3}H]propylphosphonic Acid - Benzylamine Salt (8) -- As described for the example with α-methylbenzylamine, crude (7), benzylamine salt, was treated with 30% of hydrogen peroxide (4.2 ml) in the presence of sodium tungstate (0.165 g of the dihydrate). Product in this case was isolated from 95% ethanol. After a recrystallization from 95% ethanol (10 ml for 628 mg of crude DL) 430 mg of (8) with a specific activity of 80 μCi/mg was obtained. This product showed an excellent 1:1 correlation of benzylamine and the phosphonic acid by NMR] [60 mhz. d_2O, c=5 molar: 5H (phenyl), s, 7.56 ppm; 2H (benzylic), s, 4.27 ppm; 2H (methine), multiplet, 2.7-3.6 ppm; 3H (methyl), d,d, centered at 1.56 ppm],TLC analysis revealed a single radioactive spot.

Resolution via the Free Phosphonic Acid -- A solution of 400 mg of (8) (1.63 mmoles, 31.5 mCi) in 5 ml of water (deoxygenated by N_2 bubbling -- as was all the water used in this particular experiment) was passed through a column (jacketed, cooled with circulating water maintained at 1°C) containing 25 ml of Dowex® 50 x 4 resin on the acid cycle, prewashed to neutrality. The solution required about 3 min to pass through the column, and was discharged into a cold, stirred solution/suspension of D(+)-α-methylbenzylamine (200 mg) as it came from the column. Elution was completed with 30 ml of water (combined with the product). Water was removed from the resulting salt by concentration under vacuum, and the residual material was slurried with acetone (2 x 5 ml) to obtain a residual product,

439 mg. This material was again slurried with acetone, leaving 389 mg. This sample was then recrystallized two times from isopropyl alcohol/water 4:1, with a final recovery of 145 mg. This material had a specific activity of 83 μCi/mg, m.p. 139:5-141°C. Recovery over the two recrystallations was 37.3%. Total radioactivity in the resolved salt: 12:03 mCi. The small numerical magnitudes reported (6) for the D(+)-α-methylbenzylamine salts of the L and D isomers of (S) [L (fosfomycin): $[\alpha]_{405}^{280}$ -2.6° (C = 5% in water); D: $[\alpha]_{405}^{280}$ + 15.6° (C = 5%) in water)] with the resulting small difference from racemic material made direct measurement of rotation of the radioactive sample of little value and such was not done. Distribution of radioactivity across the resolution [16.8 mCi (recovery adjusted) from 31.5 mCi] indicated clearly that resolution had been accomplished.

Check for Completeness of Resolution -- In separate 15 ml centrifuge tubes were weighed a. 0.686 and b. 0.750 mg of the salt just described. To these were added 399.0 and 400.0 mg respectively of pure L carrier. These samples were then recrystallized six times each, withdrawing a sample for radioactive count after each of cycles 4-6. The accumulated samples were simultaneously dried out and counted, giving for a. 27.3, 27.3 and 27.3; and for b. 29.6, 30.5 and 30.0 CPM/μg. Based on the CPM (remeasured contemporaneously for this experiment) and dilution of the starting sample the calculated values were a. 27.7, and b. 30.3 CPM. This excellent agreement and constancy thus confirm that the resolution was complete.

[1,2-^{3}H]Fosfomycin Calcium Salt -- In a 15 ml centrifuge tube was placed 40.4 mg of (S α-methylbenzylamine salt) and 755 mg of unlabeled carrier, and 3.5 ml of water. Sodium hydroxide (50% aqueous, 0.18 ml) was added (subsurface),

from a micrometer pipette, at the rate of 0.01 ml/min) with stirring, cooling and under N_2, bringing the pH to 9.0. Centrifuging and decanting removed traces of precipitated impurity. The pH was brought up to 11.4 by the addition of 0.13 ml more NaOH in the same manner. The liberated phenethylamine separated as an oil. The aqueous layer was decanted to a clean tube, and the final traces of amine were removed by 3 x 1 ml extractions with chloroform. The pH was adjusted to 9.0 with acetic acid. A freshly filtered solution (1.95 ml) of calcium acetate (8.8 g of $Ca(OAc)_2 \cdot H_2O$ in 28 ml of water) was then introduced (subsurface and from a micrometer pipette) with stirring, cooling and seeding. Crystallization was prompt. After aging in an ice bath for 40 min, the product was collected on a small sintered funnel and washed 3 x 5 ml with water, taking care not to allow the filter cake to collapse. After removal of the final wash the product was air dried briefly, then dried overnight in vacuo at 56° to obtain 432.2 mg (2.22 mfw - monohydrate), 77.8% of the theory. The 60 mhz proton NMR spectrum (4.7 molar solution in versene-d_2O at pH 9.2) showed no detectable glycol, which would have been detected if present to the extent of 5% or more by the appearance of a doublet (6 hz splitting, and possibly exhibiting long-range splitting by P of ca. 0.5 hz) situated 0.23 ppm upfield from the methyl 3H doublet-doublet of the fosfomycin calcium salt.

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