

TRITIUM INCORPORATION AT SPECIFIC POSITIONS IN BENZO[a]PYRENE

D. Warshawsky* and M. Calvin

Laboratory of Chemical Biodynamics and Lawrence Berkeley Laboratory,
University of California, Berkeley, California 94720

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Summary: The carcinogen, benzo[a]pyrene, was tritiated by two methods: exchange reaction with T_2SO_4 at the 1, 3 and 6 positions and quenching of a Grignard reagent prepared from 6-iodo-benzo[a]pyrene with T_2O which gave almost exclusively 6-tritiated material. The exchange reaction incorporated 118 mc/mole of which 60% was determined to be in the 6 position and 40% in the 1 and 3 positions. The Grignard reaction produced a specific activity of 56 mc/mole of which 97.5% was incorporated in the 6 position.

Introduction

Polycyclic aromatic hydrocarbons (PAH), and benzo[a]pyrene (BaP) in particular, randomly labeled with tritium (1,2,3,4,5,6) have been used for in vitro (7,8) and in vivo (9,10,11) studies of binding of PAH to cellular components, and in studies of the metabolism of these compounds in cell cultures (12,13,14,15, 16,17). However, the positions of BaP involved in the binding to cellular components are still unknown.

Pullman (18) has suggested that the K region (e.g., 4,5 positions of BaP) is involved in the binding to DNA, RNA and protein. Recently, BaP 4,5 epoxide has been shown to be formed during the metabolism of BaP (12,19). On the other hand, Cavalieri and Calvin (20,21) and others (22,23) have shown that the 6-position is the most nucleophilic position, followed closely by the 1 and 3 positions. Therefore, they have proposed that these may be the sites involved in the binding (20,21).

This work was undertaken to specifically label BaP at the six position since it is known that this position is the region of highest electron density (23).

* Present address: University of Cincinnati Medical Center, Department of Environmental Health, Kettering Labs., 3223 Eden Ave., Cincinnati, Ohio 45219.

Furthermore, the 1 and 3 positions are known to undergo NIH shifts (24) and thus a label in those positions may not be as useful. BaP exclusively labeled at the 6 position may answer a basic question as to whether that position is involved in binding to biopolymers by enzymatic activation and may also be helpful in studies of PAH metabolite formation.

Materials and Methods

The radioactive samples were dissolved in toluene and counted in scintillation fluid made up of 40 ml Packard Permafluor per 1 liter toluene and counted on a Packard TriCarb liquid scintillation spectrometer, Model 3375. UV spectra were recorded on a Cary 118. All melting points were recorded on a Thomas Hoover Capillary Melting Point Apparatus. One percent T_2O (27.9 Ci/ml) and a special glove box in which all reactions were done were supplied by the Environmental Control-Safety Services Department of the Lawrence Berkeley Laboratory. Baker and Adamson reagent grade concentrated sulfuric acid was used in the tritium exchange reactions. BaP was obtained from Aldrich and chromatographed on a neutral alumina column (Woelm activity I) with benzene and then crystallized from benzene-isopropanol (m.p. 178-9°C).

Iodine Dipyrindine Nitrate (25): To a solution of 2.57 g of iodine in 150 ml of chloroform cooled below 15°C was added a solution containing 1.75 g of $AgNO_3$ (J. T. Baker Co.), 3.2 g of pyridine and 5 ml of chloroform also cooled below 15°C. The mixture was stirred for 30 min and the silver nitrate precipitate removed by filtration. Addition of ether caused the product of iodine dipyrindine nitrate to separate out as an oil which solidified. A yield of 2.1 g or 60% was obtained.

6-Iodobenzo[a]pyrene (24): To 595 mg (2.35 mmole) of BaP in 100 ml of chloroform was added 904 mg (2.46 mmole) of iodine dipyrindine nitrate. After stirring for 30 min at room temperature the solvent was evaporated and the residue chromatographed on a neutral alumina column with benzene. The product appeared as a yellow spot on tlc and did not fluoresce. Recrystallization from benzene-isopropanol gave yellow crystals; m.p. 214-15°C, lit.

214-15°C (26); m/e 378 (parent ion). Anal. calcd., $C_{20}H_{11}I$: C, 63.49; H, 2.91; I, 33.60. Found: C, 63.56; H, 2.89; I, 33.71.

Exchange Reaction: One ml of conc H_2SO_4 was added dropwise to a flask containing 0.1 g T_2O (2.8 Ci) at ice bath temperature. One hundred mg of benzo[a]pyrene was then added and the reaction stirred for 2 min with a magnetic stirrer. The deep red solution was then poured into water (10 ml) and chloroform (5 ml) previously chilled, while the temperature was maintained below 17°C. The chloroform layer was separated and the aqueous layer extracted again with 5 ml of chloroform. The total organic layer was washed with 5 ml of H_2O and 5 ml of saturated sodium chloride.

The chloroform layer was evaporated and the residue was dissolved in hexane and chromatographed on a neutral alumina column with benzene. The benzene was evaporated, and the resulting yellow solid recrystallized from benzene-isopropanol, 61.9 mg (62% yield); m.p. 176-77°C. Tlc of the BaP in both benzene and benzene-EtOH (19:1) on silica gel chromatograms (Eastman 6066) gave one fluorescent spot with better than 99.3% of the radioactivity and a specific activity of 118 mCi/mmole.

Grignard Reaction: All the standard precautions were taken to maintain anhydrous conditions during the reaction. The solvent, tetrahydrofuran (THF), was refluxed for 2 hr in lithium aluminum hydride and then distilled and stored over molecular sieves. Three hundred fifty mg of iodobenzo[a]-pyrene, dried under vacuum, and 133 mg of phenylbromide distilled at 155-6°C were added together in 40 ml of THF to 900 mg of magnesium in a carefully dried three-neck flask equipped with a dry nitrogen inlet. Some difficulty was encountered at this stage in initiating the reaction. Therefore, a crystal of iodine was added and the flask was heated with gentle stirring. After 30 min the non-fluorescent yellow solution turned gray and eventually purple in color. Heating was continued for 15 min and the reaction flask gently stirred until the deep purple color persisted. The reaction was quenched with 50 μ l of T_2O (1.6-fold excess) with total activity of 1.3

curie, and the solution turned clear yellow in color and fluoresced blue under a UV lamp. To the reaction mixture was added 5 ml of chloroform and 5 ml of water. The chloroform was separated and the water layer washed twice more with 5 ml of chloroform. The total organic layer was then washed twice with 5 ml of H₂O and twice with 5 ml of saturated sodium chloride.

The chloroform layer was evaporated and the residue dissolved in benzene and chromatographed on a neutral alumina column. The first blue fluorescent fractions were collected, evaporated, giving a light yellow residue, and recrystallization from benzene-isopropanol giving 156 mg (67% yield) of yellow crystals; m.p. 176-77°C. Tlc of the BaP in both benzene and benzene-EtOH (19:1) on silica gel chromatograms (Eastman 6066) gave one fluorescent spot with better than 99.1% of the radioactivity and a specific activity of 56 mCi/mmole.

Iodination of Tritiated BaP from Exchange Reaction: To 37 mg (100 mmole) of iodine dipyridine nitrate in 10 ml of chloroform was added 5.38 mg (0.023 mmole) of unlabeled BaP plus 1 ml of a stock solution (1 mg/10 ml chloroform) of tritiated BaP determined both by weight and absorption spectroscopy. The reaction mixture was stirred for 30 min at room temperature, the chloroform evaporated, and the residue chromatographed three times on 0.5 mm silica gel plates (EM Reagents, Cat. 7748; silica gel, PF254+366) with benzene as a solvent. The non-fluorescent yellow band was removed from silica gel plates and extracted into chloroform, filtered, and the residue, after evaporation, was recrystallized twice from benzene-isopropanol giving 3 mg (36.8%) of 6-iodo-BaP, m.p. 214-15°C, m/e 378 (parent ion). The UV spectrum in toluene was identical with a pure sample of 6-iodo-BaP in toluene. The specific activity was 51.2 mCi/mmole which, after taking into account the dilution factor of 55, indicates that only 57% of the tritium was at the 6 position.

Iodination of Tritiated BaP from Grignard Reaction: To 4.5 mg of iodine dipyridine nitrate in 10 ml of chloroform was added 2.64 mg of tritiated BaP. After stirring for 45 min at room temperature, the chloroform was evaporated

and the residue chromatographed three times on 0.5 mm silica gel plates (EM Reagents, Cat. 7748, silica gel, PF254+366) in benzene. The non-fluorescent yellow band was removed from silica gel plates and extracted into chloroform, filtered, and the residue after evaporation was recrystallized three times from benzene-isopropanol giving 3.2 mg (82%) of IBaP, m.p. 214-15°C. The UV spectrum in toluene was identical with a pure sample of 6-iodobenzo[a]pyrene in toluene. The specific activity was 1.43 mCi/mmole, indicating that 97.5% of the tritium was at the 6 position.

Results and Discussion

Cavalieri and Calvin (21) used nmr to study the kinetic isotope exchange at the 1,3 and the 6 positions of BaP. Further deuterium studies(28) using both nmr and mass spectral analysis, and some tritiation work using low levels of T_2O (2.2 mCi/ml) indicated the best conditions for the tritium exchange at these three positions were concentrated sulfuric acid at room temperature for 5 min followed by quenching with H_2O . This procedure, using T_2O at 27.9 mCi/ml, gave labeled BaP with 40% of the tritium in the 6 position, 60% in the 1 and 3 positions, and a recovery of only 33% of the BaP.

The purpose of the work described here, however, was to synthesize BaP tritiated specifically at the 6 position. Preliminary studies of deuterium exchange using concentrated sulfuric acid (28) indicated that treatment with the acid at 5-10°C for 2 min before quenching with water should give the highest ratios of tritium at the 6 as compared to the 1 and 3 positions, as shown in Table I. The specific activity of the BaP tritiated under these conditions was 118 mCi/mmole with an increase in the recovery of BaP to 62%. Iodination at the 6 position decreased the specific activity (corrected for dilution) to 51.2 mCi/mmole, indicating that the 6 position contained 57% of the tritium, an increase of 17% over the results previously indicated at room temperature for 5 min.

The second approach involved the synthesis of 6-iodo-BaP followed by formation of the Grignard reagent and quenching with T_2O . The results

TABLE I. TRITIATION OF BENZO[a]PYRENE

	Specific activity mCi/mmole	After iodination at 6	% T at 6 position
Exchange reaction	118	51.2	57
Grignard	56	1.43	97.5

are listed in Table I. A specific activity of 56 mCi/mmole was obtained with a recovery of 67%. Iodination of the 6 position reduced the specific activity to 1.43 mCi/mmole, indicating that more than 97% of the tritium was at the 6 position.

Work is presently underway using the [6-³H] BaP for studies of binding BaP to biopolymers under the influence of microsomal enzymes and for studies of the metabolism of BaP in cell cultures. These efforts should indicate whether or not the 6, the 1 and/or 3 positions are involved in binding of the carcinogen to biopolymers and help unravel the metabolism of this carcinogen.

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