THE SYNTHESIS OF TRITIUM-LABELLED AZIDOTHYMIDINE

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SUMMARY

The synthesis of ^{3}H-labelled azidothymidine (AZT) from [methyl-^{3}H]thymidine is described. Purification of labelled AZT was accomplished by column chromatography. The purity of the labelled compound was determined by high performance liquid chromatography connected with a radioactive flow detector. The labelled AZT was found to be stable at pH ranging from 2.3 to 7.4 at 37°C and at hyperthermic temperature of 42°C.

Key Words: 3′-Azido-3′-deoxy[methyl-^{3}H]thymidine, ^{3}H-AZT, AZT, AIDS, HIV

INTRODUCTION

Azidothymidine (3′-azido-3′-deoxythymidine, AZT, zidovudine, Retrovir) is the only approved drug at present for the treatment of patients suffering from acquired immunodeficiency syndrome (AIDS) and AIDS related complex (ARC). AIDS is a debilitating and fatal disease caused by human immunodeficiency virus (HIV). AZT is initially phosphorylated by cellular enzymes to its triphosphate form which then competes with thymidine triphosphate and leads to the inhibition of the reverse transcriptase, a critical enzyme for replication of retroviruses[1]. AZT acts as a chain terminator due to the lack of the 3′-OH function, thus inhibiting the formation of 5′ to 3′-phosphodiester linkages.

AZT was initially synthesized by Horwitz et al[2]. The synthetic procedure was later modified by Glinski et al[3]. and then by Lin et al[4]. Intracellular metabolism of AZT to AZT-5′-triphosphate has been recently reported[1]. Although 3′-azido-3′-deoxy[methyl-^{3}H]thymidine (^{3}H-AZT) was utilized in these biochemical experiments, a synthetic procedure of ^{3}H-AZT has not been published. Since there is a significant upsurge in the research

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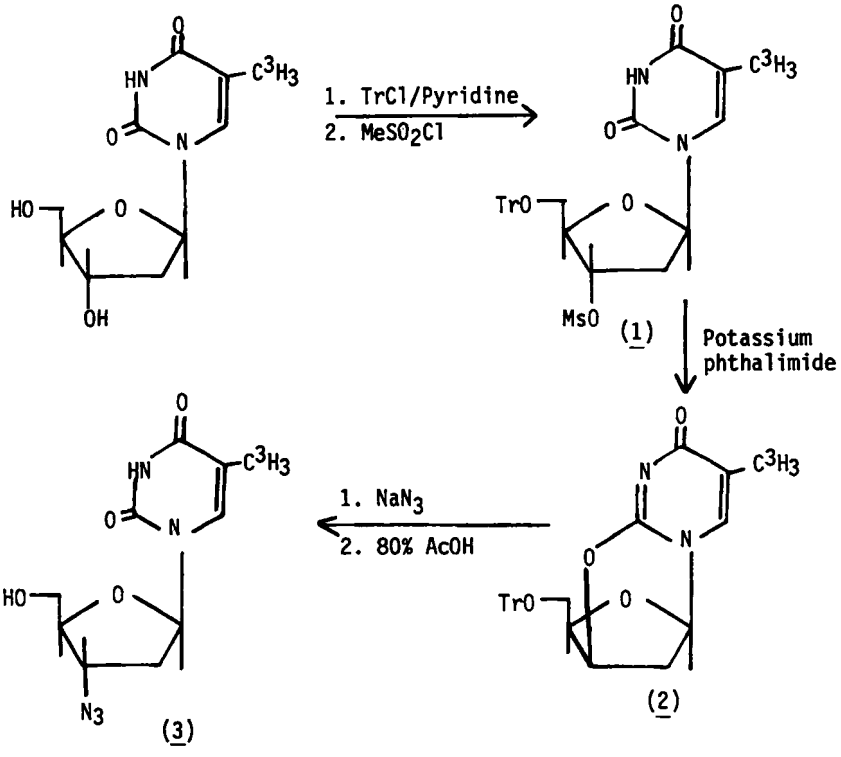
involving AZT, it was deemed appropriate to report the synthesis of ^{3}H-AZT. We have synthesized ^{3}H-AZT by modifying the published procedure of Glinski et al[3]. [Methyl-^{3}H]Thymidine was utilized as the starting material and a series of steps as shown in the following scheme were followed sequentially.



METHODS

The radiopurity was determined by employing a Beckman (Model 340) high performance liquid chromatographic system equipped with an on-line Flow-One Beta radioactive flow detector (Model CR, 2.5 ml cell type, Radiomatic Instruments). A reverse phase Econosphere C18 column (150 x 4.6 mm, 5 μ) was used with a mobile phase containing 15% acetonitrile in 25 mM ammonium phosphate buffer (pH 2.2). AZT was detected at 267 nm with a peak retention time of 3.2 min with a flow rate of 2 ml/min. For radiodetection, a simultaneous flow of scintillation cocktail (Flo-Scint II) was used at 6 ml/min.

Infra-red spectra were obtained in KBr pellets on a Beckman IR-10 spectrophotometer. Reactions were monitored by TLC (silica gel) and column

chromatography was performed by using silica gel (70-230 mesh, 60 Å). [Methyl-^{3}H]Thymidine (100 mCi/100 ml water, specific activity 2 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA, U.S.A).

Synthesis of 3'-azido-3'-deoxy[methyl-^{3}H]thymidine (3)

A solution of [methyl-^{3}H]thymidine (100 mCi, 2Ci/mmol) was evaporated to dryness on a rotary evaporator at 40°C and final traces of moisture were removed by evaporating it with 1:1 mixture of benzene:ethanol. The solid thus obtained was mixed with 472 mg of thymidine to provide a total of 2 mmol of the starting material which was dissolved in dry pyridine (4.5 ml) and 730 mg (2.6 mmol) of trityl chloride was added to the solution. The reaction mixture was heated at 100°C in an oil bath with stirring for 0.5 hr. It was then cooled to room temperature and 85 ml of ice cold water was added to the solution with stirring. The resulting paste was filtered, dissolved in ethanol (25 ml) and evaporated to dryness under vacuum. The crude 5'-O-trityl[methyl-^{3}H]thymidine was dissolved in dry pyridine (4.5 ml), and 338 mg (2.34 mmol) of methanesulfonyl chloride was added in drops with stirring at 4°C. The reaction mixture was further stirred in a cold room overnight and the ester (1) was then precipitated by adding 85 ml of ice cold water. The resulting solid was filtered, dissolved in ethanol (25 ml) and the solvent removed under vacuum. The residue of crude 3'-O-methanesulfonyl-5'-O-trityl[methyl-^{3}H] thymidine (1) was dissolved in dimethylformamide (15 ml) and was mixed with a solution of 1.75 g (9.4 mmol) of potassium phthalimide in water (5 ml). The reaction mixture was heated at 95°C in an oil bath for 0.5 hr. It was then cooled and cold water (60 ml) was added to the mixture with stirring. The resulting paste was filtered, dissolved in ethanol and evaporated to dryness. The residue of crude 5'-O-trityl-2,3'-anhydro[methyl-^{3}H]thymidine (2) was dissolved in dimethylformamide (10 ml) and a solution of 513 mg (7.9 mmol) of sodium azide in 1.5 ml of water was added. The mixture was heated under reflux for 13 hr. The solution was then brought to room temperature and to the stirred solution 65 ml of ice cold water was added. The solid formed was filtered and dissolved in ethanol (25 ml) and then evaporated to dryness under vacuum. The crude 3'-azido-3'-deoxy-5'-O-trityl[methyl-^{3}H)-thymidine was

dissolved in 12 ml of 80% aqueous acetic acid and was heated in an oil bath at 100°C for 0.5 hr. The solution was then cooled and after addition of 25 ml of water, the precipitated trityl alcohol was removed by filtration. The filtrate was evaporated to dryness under vacuum at 50°C. The residue of crude AZT was then purified by column chromatography (silica gel) using a hexane:ethyl acetate (1:1) mixture followed by ethyl acetate as eluent to give 180 mg (overall yield, 33.7%) of pure 3'-azido-3'-deoxy[methyl-^{3}H]thymidine (3). The ^{3}H-AZT was identical to a sample of similarly prepared non-labelled AZT[3] with respect to Rf, m.p., UV, IR and retention time on HPLC. The ^{3}H-AZT (3) was dissolved in 100 ml of methanol and stored at -20°C. The total radioactivity of this solution was 26.7 mCi and the specific activity was 35.9 mCi/mmol.

To determine the radiochemical purity of 3, a 10 µl aliquot was added to 990 µl of 0.02 M sodium phosphate buffer (pH 7.4). Five µl of this solution was mixed with 2.5 µl of unlabelled AZT (0.1 mg/ml) and then injected onto the HPLC column. The radioactivity peak in the Flo-One detector was at the same retention time as the peak of the unlabelled AZT detected by UV at 267 nm. The radioactivity peak of 3 was 97.7% radiochemically pure.

The stability of the radiolabelled 3 was determined at various temperatures and pH to ensure the appropriateness of the label for drug metabolism studies. A 10 µl sample of 3 was diluted with 990 µl of the appropriate phosphate buffer (adjusted to a pH of 2.3, 5.0 and 7.4) and was then incubated for 4 hr at either 37°C or hyperthermic temperature of 42°C. The ^{3}H-AZT solutions were then analyzed for the determination of radiochemical purity. The results of these experiments are listed in Table 1.

DISCUSSION

The synthesis of ^{3}H-AZT (3) has been accomplished from commercially available [methyl-^{3}H]thymidine by modifying the published procedure[3]. To allow a continuous flow of synthetic sequence, the intermediates in each step were reacted further without purification. This enabled us to obtain a 33.7% overall yield of ^{3}H-AZT with a radiochemical purity of 97.7%. To ensure that there was no significant exchange of radioactivity between [methyl-^{3}H]thymidine

and water during the synthetic procedure at reaction temperatures of up to 100°C, we heated a sample of [methyl-^{3}H]thymidine in water for up to 13 hr and in aqueous acetic acid for 0.5 hr. The radiochemical purity of [methyl-^{3}H] thymidine remained greater than 95%.

Since ^{3}H-AZT would be expected to be utilized in a variety of biochemical and pharmacological studies, a series of experiments were conducted to determine the stability of radiolabelled AZT. The results shown in Table 1 indicate that ^{3}H-AZT remained radiochemically pure at pH 2.3, 5.0 and 7.4 at 37°C for up to 4 hr. The radiochemical purity of ^{3}H-AZT at these pH remained intact even at hyperthermic temperature of 42°C. No attempts were made to determine the stability of ^{3}H-AZT in buffer at higher temperatures and at longer durations of time. The solution of ^{3}H-AZT in methanol can be conveniently stored in the refrigerator for an extended time with no loss of activity.

TABLE 1. The effects of temperature and pH on the radiochemical purity of 3'-azido-3'-deoxy [methyl-^{3}H]thymidine (3).

No.	pH	Temperature (°C)[a]	%Radiochemical Purity[b]
1	7.4	37	97.1
2	7.4	42	96.5
3	5.0	37	97.0
4	5.0	42	96.2
5	2.3	37	94.8
6	2.3	42	97.2

[a]Time for incubation was 4 hr for each run.

[b]The initial radiochemical purity was 97.7%

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