# MICROBIAL PRODUCTION OF AMPHOTERICIN B-$^{3}$H AND THE SYNTHESIS OF ITS SODIUM DESOXYCHOLATE (CARBOXYL-$^{14}$C) COMPLEX AND METHYL-$^{14}$C-ESTER

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Amphotericin B-$^{3}$H with a specific radioactivity in excess of 4 $\mu$Ci/mg was produced by fermentation of *Streptomyces nodosus* IMRU 3694 in a complex medium, using acetate-$^{3}$H as a precursor. The medium employed gave a production yield of 2.5~3.5 mg/ml of amphotericin B. The most efficient incorporation of acetate-$^{3}$H occurred when the precursor was added at 24 hours after inoculation. It was observed, that the amount of amphotericin A co-produced in the fermentation broth with amphotericin B was significantly reduced by the addition of ethanol to the production medium. Complete inhibition of amphotericin A production was achieved at a level of 2% ethanol in the fermentation medium without an appreciable effect on the yield of amphotericin B. Methanol, 1-propanol, 2-propanol and 1-butanol lowered the yield of both antibiotics indiscriminately. The syntheses of amphotericin B-$^{3}$H-Na desoxycholate (carboxyl-$^{14}$C) complex and amphotericin B-$^{3}$H methyl-$^{14}$C-ester are also described.

In the course of pharmacological investigations with amphotericin B and its methyl ester[1], it was found desirable to employ tritium labeled compounds. The production of carbon-14 labeled amphotericin B has been previously reported[2,3]. However, no report has yet described the production of the tritium-labeled amphotericin B and the subsequent preparation of its carbon-14 labeled methyl-$^{14}$C-ester and its sodium desoxycholate (carboxyl-$^{14}$C) complex (Fungizone). The present study deals with the preparation of these doubly labeled amphotericin B derivatives.

## Materials and Methods

Radioactive compounds: Sodium acetate-$^{3}$H (550 mCi/mM) and N-methyl-$^{14}$C-N-nitroso-*p*-toluenesulfonamide (10 mCi/mM) were obtained from New England Nuclear Co., Boston, Massachusetts. Crystalline desoxycholic acid (carboxyl-$^{14}$C) (3.1 mCi/mM) was purchased from Mallinkrodt, St. Louis, Missouri.

Fermentation, extraction and isolation of amphotericin B-$^{3}$H: *Streptomyces nodosus* IMRU 3694 was used as the amphotericin B producing organism. A complex fermentation medium composed of 5% glucose, 1% $CaCO_3$ and 3% Pharmamedia (cotton seed flour) at pH 7.7 before sterilization was used for the production. The methods of LINKE *et al.*[2] were employed for the maintenance of the organism, fermentation, analysis, extraction and isolation of amphotericin B.

Purification of amphotericin B-$^{3}$H: The isolated radioactive antibiotic was further purified by counter current distribution (CCD) followed by fractional precipitation. A solvent system, chloroform-methanol (with 3% $CaCO_3$)-$H_2O$-1-butanol (20:20:10:1, v/v), was used in a 30 cell CRAIG CCD apparatus. The main fraction of the antibiotic distributed in the lower and

upper phases of tubes No. 16~22 after 30 transfers was isolated and further purified by fractional precipitation using the method of VANDEPUTTE *et al.*[4]

The preparation of amphotericin B-$^{3}$H-sodium desoxycholate (carboxyl-$^{14}$C) complex: A method similar to that described by BARTNER *et al.*[5] was used. Here, 12.9 mg of desoxycholic acid (carboxyl-$^{14}$C) (0.1 mCi) was dissolved in 4 ml of alkaline water (NaOH, pH 11.6) containing 27.4 mg of unlabeled sodium desoxycholate. The solution was cooled to +7°C. To this, 50 mg of amphotericin B-$^{3}$H (0.135 mCi) was added and the mixture stirred until complete dissolution occurred. The pH of the solution was then brought down to 7.6 by careful addition of aqueous HCl. Stirring was continued at room temperature for one hour followed by lyophilization. The powder thus obtained gave a clear solution when reconstituted in 5% aqueous dextrose when needed for injection.

Synthesis of amphotericin B-$^{3}$H methyl-$^{14}$C-ester: A modified method of MECHLINSKI *et al.*[6] was employed. For the production of diazomethane-$^{14}$C a special small distillation apparatus with no joints was constructed to trap the liberated diazomethane as quickly as possible in cooled tetrahydrofuran (THF). Using this apparatus the yield of diazomethane produced from 125 mg of N-methyl-N-nitroso-*p*-toluenesulfonamide was 50%. The concentration of diazomethane THF was about 0.3 molar. For the production of radiolabeled diazomethane 7.05 mg of N-methyl-$^{14}$C-N-nitroso-*p*-toluenesulfonamide (0.3 mCi) and 117.95 mg of unlabeled compound as carrier were dissolved in 1.5 ml THF. This resultant solution was then slowly added to a 0.5 ml of a mixture consisting of 1.0 g KOH in 0.3 ml $H_2O$, 2.0 ml ethanol and 4.0 ml THF. A total of 0.8~1.0 ml of THF containing 0.3 molar radioactive diazomethane was collected by distillation.

For the preparation of amphotericin B-$^{3}$H methyl-$^{14}$C-ester, 15 mg of amphotericin B-$^{3}$H (68 $\mu$Ci) and 35 mg of unlabeled amphotericin B were dissolved in 1 ml of dimethylsulfoxide and 0.2 ml of methanol and the resultant solution cooled in ice to +6°C. To this, the ice-cooled diazomethane-$^{14}$C reagent was added dropwise. The production of the methyl ester derivative was monitored by thin-layer chromatography (Silica gel G) using a solvent system of chloroform - 95% ethanol - 20% acetic acid (1:1: saturation, v/v). After completion of the esterification process, the product was precipitated from the reaction mixture by the addition to 30 ml of absolute diethyl ether. The resultant precipitate was collected by centrifugation at 10,000 rpm for 10 minutes. After washing 3 times with 20-ml aliquots of absolute diethyl ether, the product was dried under vacuum.

## Results

### Studies on Acetate-$^{3}$H Incorporation into Amphotericin B-$^{3}$H

Table 1 shows the relationship between time of addition and efficiency of radioactive acetate

Table 1. Incorporation of acetate-$^{3}$H into amphotericins A and B complex in *S. nodosus* fermentations

| Time of addition of precursor (hours) | Average specific activity (dpm/$\mu$ mole) | |
|---|---|---|
| | 200 $\mu$Ci acetate-$^{3}$H/50 ml | 1,000 $\mu$Ci acetate-$^{3}$H/50 ml |
| 0 | $4.3\times10^{4}$ | $3.0\times10^{5}$ |
| 24 | $7.0\times10^{4}$ | $3.9\times10^{5}$ |
| 48 | $2.2\times10^{4}$ | $2.2\times10^{5}$ |
| 72 | $1.9\times10^{4}$ | |

Duplicate flasks were used for each time interval. The antibiotics produced were isolated 8 days after the inoculation of the fermentation broth and the average level of radioactivity incorporated determined.

incorporation by *S. nodosus* into the mixture of coproduced amphotericins. The highest efficiency of incorporation of acetate-$^3$H was achieved when the precursor was added at 24 hours after inoculation. The specific activity of the amphotericin A and B mixture produced was correspondingly higher with increasing levels of the radioactive precursor employed at 24 hours after the inoculation. This is illustrated in Table 2. Amphotericin B-$^3$H with a specific radioactivity of more than 4 $\mu$Ci/mg was obtained by a single addition of the precursor to the fermentation broth at 24 hours after the inoculation. The presence of radioactive product and the purity of this material was examined by TLC. The bioactivity of the purified isolated amphotericin B-$^3$H, as revealed by a minimum inhibitory concentration (MIC) of 0.25 $\mu$g/ml against *Saccharomyces cerevisiae* #216 as determined by agar dilution method, was found to be comparable to that of an authentic antibiotic reference sample[7].

Table 2. Effect of precursor level added on its incorporation into the amphotericin A and B complex in *S. nodosus* fermentations

| Amount of acetate-$^3$H added ($\mu$Ci/50 ml) | Average specific activity (dpm/$\mu$ mole) |
|---|---|
| 200 | $6.1\times10^4$ |
| 400 | $1.0\times10^5$ |
| 600 | $1.8\times10^5$ |
| 1,000 | $3.6\times10^5$ |

The precursors were added 24 hours after the inoculation. Duplicate flasks were used for each experiment. The antibiotics produced were isolated 8 days after the inoculation and the average level of radioactivity incorporated determined.

## Effect of Aliphatic Alcohols on Amphotericins A and B Production

During the radioactivity incorporation studies it was noted that the production of amphotericin A in the mixture with amphotericin B was significantly influenced by the addition of a small amount of ethanol into the medium. Table 3 shows the effects of the time of addition and the concentration of ethanol on the production of the antibiotics. The ratio of ampho-

Table 3. Effect of ethanol on the production of amphotericins in *S. nodosus* fermentations

| | | Amphotericin B (mg/ml)[c] | Amphotericin A (mg/ml[c]) | Ratio B/A |
|---|---|---|---|---|
| Time of addition[a] (hours) | 0 | 2.2 | 0.3 | 7.3 |
| | 24 | 2.0 | 0.1 | 20.0 |
| | 48 | 1.9 | 1.3 | 1.5 |
| | 72 | 2.4 | 2.0 | 1.2 |
| | Control | 2.8 | 2.6 | 1.1 |
| Ethanol added[b] (%) | 0.0 | 2.5 | 2.5 | 1.0 |
| | 1.0 | 2.2 | 0.6 | 3.6 |
| | 2.0 | 2.1 | 0.0 | only amphotericin B |
| | 4.0 | 1.4 | 0.0 | only amphotericin B |
| | 8.0 | 0.0 | 0.0 | no growth of organism |

[a] At the indicated time interval 1.0 ml 95% ethanol per 50 ml medium was added. Triplicate flasks were used for each time interval.

[b] At 24 hours after inoculation the different levels of 95% ethanol were added to 50 ml medium. Triplicate flasks were used for each group.

[c] The production of the amphotericins was examined after 8 days of fermentation. The quantities of amphotericins B and A were measured spectrophotometrically at a wavelength of 383 nm and 305 nm, respectively[2].

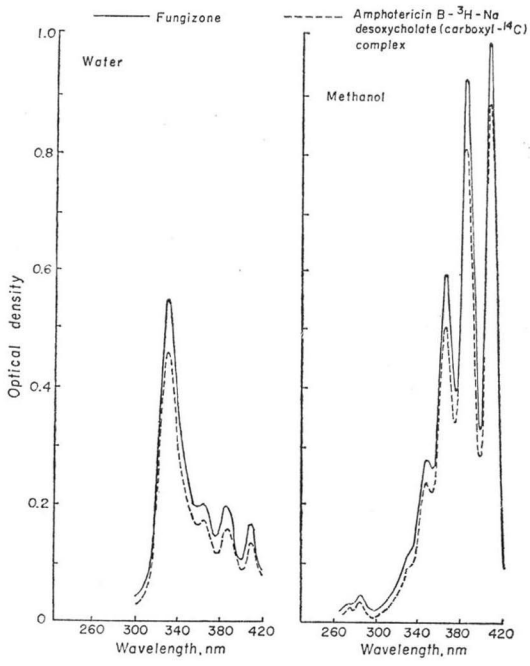
Table 4. Effect of alcohols on the production of amphotericins in *S. nodosus* fermentations

| Alcohol added[a)] | Yield and ratio of amphotericin produced (mg/ml[b)]) | | | | | |
|---|---|---|---|---|---|---|
| | 1% alcohol | | | 2% alcohol | | |
| | B | A | B/A | B | A | B/A |
| Control | 2.4 | 1.6 | 1.5 | 2.6 | 2.0 | 1.3 |
| Methanol | 2.0 | 1.1 | 1.8 | 2.0 | 1.0 | 2.0 |
| Ethanol | 2.4 | 0.6 | 3.8 | 2.2 | 0.0 | only amphotericin B |
| 1-Propanol | 2.1 | 1.0 | 2.0 | 0.6 | 0.0 | only amphotericin B |
| 2-Propanol | 2.6 | 1.7 | 1.5 | 1.8 | 1.0 | 1.8 |
| 1-Butanol | 0.2 | 0.0 | only amphotericin B | 0.0 | 0.0 | no production |

a) The alcohol was added 24 hours after inoculation. Triplicate flasks were employed.
b) The production of amphotericins B and A were examined spectrophotometrically after 8 days of fermentation[2].

tericin B to A produced was the highest (20:1) when 1 ml of ethanol was added to 50 ml of the inoculated medium 24 hours after the inoculation. The ethanol concentration affected the yield of amphotericin A much more than that of amphotericin B. Various other aliphatic alcohols were also examined under similar fermentation conditions, and the results are presented in Table 4. Ethanol was shown to be the most effective agent in interfering with amphotericin A production, whereas 2% of 1-propanol and 1 and 2% of 1-butanol severely interfered with the growth of *S. nodosus* in the fermentation.

Fig. 1. Absorption spectra of commercial Fungizone® and amphotericin B-$^{3}$H complexed with sodium desoxycholate (carboxyl-$^{14}$C) in water and in methanol




## Amphotericin B-$^{3}$H-Sodium Desoxycholate (Carboxyl-$^{14}$C) Complex

In Fig. 1 we compare the UV spectra of the amphotericin B desoxycholate complex and authentic Fungizone® in methanol and distilled water. The specific radioactivity of $^{3}$H and $^{14}$C was 2.7 and 2.4 $\mu$Ci/mg, respectively. The bioactivity of the complex produced (MIC=2.25 $\mu$g/ml, based on amphotericin B content) was comparable to that of authentic Fungizone®[7].

## Amphotericin B-$^{3}$H Methyl-$^{14}$C-Ester

The specific radioactivities of $^{3}$H and $^{14}$C in the compound produced were 1.36 and 0.55 $\mu$Ci/mg, respectively.

The esterification of amphotericin B-$^{3}$H was confirmed by IR and TLC examination. The

presence of $^{14}C$ in the compound synthesized was also confirmed by radioactivity measurement of $^{14}C$ on the TLC spot.

## Discussion

Amphotericin B, a heptaene macrolide antifungal antibiotic, was originally described by GOLD *et al.*[8] Its molecular structure is distinguished by the presence of a large macrolactone ring containing seven conjugated double bonds. This lactone ring is synthesized *via* the polyketide pathway from acetate and propionate units by a mechanism common to many antibiotics[9]. Production by microbial fermentation of labeled polyene antibiotics, using radioactive acetate as a precursor, has been frequently employed. PERLMAN *et al.*[3] showed that addition of acetate-1-$^{14}C$ at a time between 48 and 72 hours after inoculation resulted in optimum incorporation of the radioactive precursor in amphotericin B. Likewise LINKE *et al.*[2] reported that the optimum uptake of acetate-1-$^{14}C$ in amphotericin B was on the second day after inoculation. In a study of the biogenesis of candicidin, the aromatic heptaene macrolide antibiotic, LIU *et al.*[10], however, showed that there was practically no difference in the efficiency of incorporation if the precursor was added any time between 10 to 50 hours after the inoculation.

The highest efficiency of incorporation of acetate-$^{3}H$ in our studies was achieved when the precursor was added at 24 hours after inoculation. The differences in the time course of radioactivity incorporation observed by us and others may be a result of some experimental differences such as aeration, inoculum size, inoculum age, *etc.*, as well as structural differences of the antibiotic produced such as is recognized between amphotericin B and candicidin.

The specific activity of the amphotericin B produced (Table 2) was proportional to the dosage of radioactivity supplied with the addition of the precursor and correlated well with previous findings of PERLMAN[3] and LIU[10]. LINKE *et al.*[2], however, found no relationship to the precursor radioactivity dosage.

In our studies, the precursor, sodium acetate-$^{3}H$ was dissolved in 95% ethanol in order to avoid possible contamination of the fermentation medium during addition. It was found that both the total amount and the ratio of amphotericin B to A were significantly affected by the added ethanol. Some effect was also observed with other alcohols (Table 4), however, ethanol showed the highest degree of selectivity towards suppressing amphotericin A content in the fermentation product without a serious effect on the amphotericin B production.

Variations in the amphotericin B to A ratio in different experiments did not appear to influence significantly the specific radioactivity of amphotericin A and B in the isolated mixture. This may be a result of an indiscriminate incorporation of the precursor into both antibiotics since the structures of amphotericin B and A are closely related, and the antibiotics are produced by a similar polyketide biosynthetic pathway. The cause of the effect of ethanol on the production ratio of amphotericins, however, is not clear. Utilization of low levels of the alcohol in secondary metabolic pathways or selective inhibition of certain enzymes cannot be ruled out.

Isolation of pure amphotericin B from a fermentation mixture containing amphotericin A has been troublesome and often led to a considerable decrease in amphotericin B yield. We circumvented this problem in the present studies by a selective inhibition of amphotericin A production without affecting amphotericin B yield through the addition of ethanol to the fermentation medium.

This approach to simplify the isolation procedure of amphotericin B may well have potential application with other polyene macrolide antibiotics known to exist as co-produced mixtures and found to be difficult to separate.

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