PRODUCTION OF AMPHOTERICIN B-^{14}C BY *STREPTOMYCES NODOSUS* FERMENTATION, AND PREPARATION OF THE AMPHOTERICIN B-^{14}C-METHYL-ESTER

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A selected strain of *Streptomyces nodosus* capable of producing 4,500 μg amphotericin B per ml in a complex medium was able to produce 3,500 μg amphotericin B per ml in defined medium. Amphotericin A was coproduced in both fermentation media. An improved method was developed for their quantitative determination directly from the culture broth, and a satisfactory isolation procedure for amphotericins A and B was worked out. Final separation of the two compounds was accomplished by countercurrent distribution. Several ^{14}C-precursors were used to optimize the specific activity of amphotericin B, and depending on the time of addition to the inoculated medium and the precursor concentration, different rates of incorporation were obtained. Amphotericin B-^{14}C-methyl-ester was synthesized by chemical conversion of purified amphotericin B with freshly prepared ^{14}C-diazomethane.

Streptomyces nodosus, originally isolated from soil of the Orinoco River region in Venezuela, coproduces two polyene antibiotics, the tetraene amphotericin A and the heptaene amphotericin B[1,2]. Both antibiotics are active against yeasts and yeastlike fungi[3], with the latter having found use in the treatment of the deep-seated and superficial mycoses[4]. Amphotericin B and other polyene macrolides are characteristic in complexing with membrane sterols of sensitive organisms[5,6]. In addition they exhibit a remarkable *in vivo* effect on sterol and steroid metabolism in animals[7,8] and humans[9]. For *in vivo* and *in vitro* mode-of-action tracer studies, the need of a radioactive amphotericin B was evident[10]. An attempt had been made before to produce amphotericin B-^{14}C in a complex medium[11]. We intended in the present work to produce this antibiotic with high specific activity also in a defined medium, utilizing improved and more convenient methods for its quantitative determination, isolation, purification and separation from the coproduced amphotericin A.

Methods

A strain of *Streptomyces nodosus* IMRU 3694 was used and maintained by monthly inoculation of a spore suspension to slants composed of 10 g glucose, 10 g yeast extract, 15 g agar and 1,000 ml distilled water. The slants were incubated up to 10 days at 28°C until appearance of spores and then stored in a refrigerator. For transfers the spores of one slant were suspended in 5 ml distilled water and inoculated into 50 ml liquid glucose-yeast extract-medium in 300-ml Erlenmeyer flasks with cotton plugs. The flasks were incubated at 28°C on a 245 rpm shaker. Usually after 5~8 days the spores developed into spheres of vegetative cells, and this suspension was used to inoculate the production medium. For the production of amphotericin B (and amphotericin A) either a complex medium composed of 50 g glucose, 10 g $CaCO_3$, 30 g Pharmamedia (cottonseed flour) and 1,000 ml distilled water at pH 7.7 or a defined medium composed of 50 g glucose, 10 g $CaCO_3$, 1 g asparagine (Na-salt), 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot$

$4H_2O$ and 1,000 ml distilled water at pH 7.6 was utilized. These media, after sterilization in an autoclave for 15 minutes at 15 lb./sq. in. and 121.6°C, were generally inoculated with 10% of a freshly grown culture. The yields of amphotericin B were variable; in complex medium up to 4,560 μg/ml were produced whereas the yield in defined medium was up to 3,500 μg/ml. Since the fermenting conditions are very important for optimum antibiotic production[12], all experiments were carried out in 300-ml deep baffled flasks (Bellco Glass, Inc.) filled with 50 ml of the appropriate medium and closed with a cotton-layered cheese cloth. The flasks were incubated at 28°C on a 245 rpm shaker. For optimum antibiotic yields the flasks were usually harvested 7~8 days after inoculation.

A high producing strain of *S. nodosus* IMRU 3694 was obtained by preparing a spore suspension dilution series in dsitilled water. For this purpose the aqueous spores suspensions were plated on glucose-yeast extract-agar petri dishes, and after incubation the colonies were selected according to size. Each single colony was tested in complex medium for performance in amphotericin B production.

Quantitative determination of amphotericin B (and amphotericin A) directly from culture broth: To 1 ml of culture broth, or any other unknown solution of the amphotericin B and/or A, 9 ml of dimethyl sulfoxide (DMSO) were added, vigorously mixed, incubated at room temperature for 30 minutes and then centrifuged. The clear yellow-orange supernatant was diluted 1 : 10 with methanol, and an occasionally appearing fine precipitate was removed by centrifugation. In presence of higher antibiotic concentration 1 ml of this clear light-yellow solution was diluted to 10 ml with methanol. The absorption of the appropriate methanol dilution was recorded in 1-cm quartz cuvettes with a Cary 14 Spectrophotometer in the wave length range from 420 nm to 280 nm. Amphotericin B showed the three specific heptaene U. V. absorption peaks at 408, 383 and 364 nm; its concentration was calculated on the basis of the absorption at 383 nm assuming a ΔE_1^1 of 1.650 (10 μg amphotericin B/ml methanol). The UV spectrum of amphotericin A showed the three specific tetraene peaks at 320, 305 and 291 nm, and its concentration was determined on the basis of the absorption at 305 nm assuming a ΔE_1^1 of 0.850 (10 μg amphotericin A/ml methanol).

Isolation of amphotericin B and amphotericin A from culture broth: 100 ml culture broth, containing the amphotericins B and A, was adjusted to pH 6.5 with 1 N HCl, and then extracted with 100 ml *n*-butanol. This mixture was vigorously shaken and after 10 minutes centrifugated for 10 minutes at 25,000 rpm. The sediment was repeatedly extracted with 100 ml of *n*-butanol. The *n*-butanol phases were combined, filtered through a folded Whatman No. 2 filter, and then vacuum-evaporated at 35°C with an aspirator followed by oil pump vacuum to about 5 ml volume. This *n*-butanol concentrate was cooled in a refrigerator and diluted into 30 ml of dry ethyl ether at 4°C. The precipitate, composed of the crude amphotericins B and A, was washed twice with dry ether at 4°C, dried in a desiccator, and finally stored at −20°C in a freezer for further purification.

Final separation and purification of amphotericin B (and amphotericin A) by countercurrent distribution (CCD): Several solvent systems producing two phases were examined with regard to the specific partition ratio K for amphotericins B and A to obtain a satisfactory separation. The chosen CCD-system was composed of chloroform - methanol (with 3% $CaCl_2$) - H_2O-*n*-butanol (20 : 20 : 10 : 1), and this system had a separation factor β of 1.60 for the two amphotericins. In comparison to amphotericin B, amphotericin A displayed a much better solubility in the upper phase of this system. An excess of *n*-butanol lowered the K value of this system significantly. In one example 2.2 g crude amphotericin B and amphotericin A were dissolved in 120 ml lower and 120 ml upper phases of the above solvent system. After filtration through a Whatman No. 2 folded filter the first 3 tubes of a 200-tube CCD apparatus were filled with 40 ml lower and 40 ml upper phases each. The remaining tubes were filled with the plain solvent system. The CCD apparatus was adjusted to 20 shaking cycles, 5-minute settling time, and the separation was completed after 97 transfers in a recycling mode of operation. The

amphotericin content of each 5th tube was determined in the lower and upper phases. For this purpose 20 μl of lower and upper phase each were diluted with 5 ml of methanol; the antibiotic concentrations were determined spectrophotometrically at the appropriate UV absorption peaks. The main concentration peak of the purified amphotericin B was distributed in the lower and upper phases of tubes No. 40 to 64. The amphotericin A peak was distributed beyond tube 65. Both CCD solvent phases containing the major portion of amphotericin B were diluted with 25% H_2O(v/v) and the methanol and chloroform were then removed by vacuum evaporation at 35°C. The resulting aqueous phase was cooled in a refrigerator over night at +4°C. The precipitated amphotericin B was collected by centrifugation for 10 minutes at 15,000 rpm, then washed twice with cold water and finally dried by lyophilization, yielding 154 mg of purified antibiotic. A similar procedure may be used to obtain amphotericin A from the appropriate CCD fractions.

Preparation of Amphotericin B-^{14}C-Methyl-ester

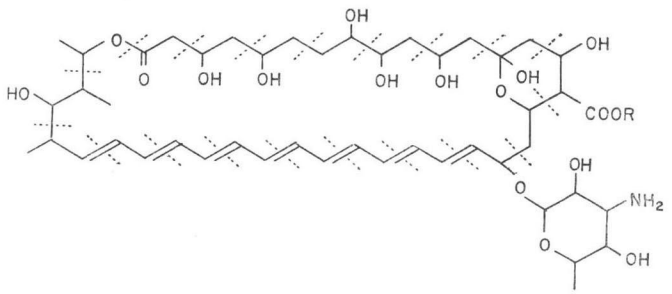
^{14}C-Diazomethane: 2.5 g N-methyl-N-nitroso-*p*-toluenesulfonamide and 14.5 mg N-methyl-^{14}C-N-nitroso-*p*-toluensulfonamide (0.25 mc, NEN Corp.) dissolved in 10 ml tetrahydrofurane were slowly added to a stirred solution of 1 g KOH in 0.3 ml H_2O, 2 ml ethanol and 4 ml tetrahydrofuran. A total of 8 ml tetrahydrofurane including the main fraction of liberated radioactive diazomethane was collected by distillation.

Fig. 1. Structure of amphotericin B

I: R=H, Amphotericin B

II: R=$^{14}CH_3$, Amphotericin B-^{14}C-methyl-ester

····: indicates division of the macrolide ring into 16 C_2 and 3 C_3 units



Amphotericin B-^{14}C-Methyl-ester (Fig. 1, II): The ice-cooled diazomethane-reagent was added dropwise to a cooled solution of 1 g amphotericin B in 10 ml DMSO and 1 ml methanol[13]. The reaction was completed after 5 minutes, and the mixture was diluted into 200 ml absolute ethyl ether. The precipitated amphotericin B-^{14}C-methyl-ester was collected by centrifugation (10 minutes at 15,000 rpm), washed with absolute ethyl ether, dried in a vacuum desiccator and then stored in a freezer. The specific activity of the obtained product, as determined with a Packard Tricarb Liquid Scintillation Spectrometer (Model 3003), was 25 mc/Mol or 50,000 cpm (58,000 dpm) per 1 mg.

Cofactor Studies to Improve Amphotericin B Production

Several cofactor studies were conducted to improve the yields of amphotericin B in complex and defined medium. Optimum yields of amphotericin B (4,560 μg/ml) were obtained in a complex medium with 5% glucose as carbon source; the same medium with only 0.5% glucose produced 80% less antibiotic. Similar effects of various glucose concentrations on amphotericin B and A production were observed by other investigators[14]. With decreasing glucose concentrations (5~0.5%) we observed a slight increase of the pH value (up to pH 8.5) toward the end of fermentation in the complex medium. Substitution of the 5% glucose by fructose or mannose produced 35% less amphotericin B with the former and 48% less antibiotic with the latter.

Soybean meal produced in our system approximately 20% less amphotericin B than cottonseed flour.

In the defined medium compounds with a polyenic moiety, *i.e.* D, L-α-tocopherol, vitamin K and β-carotene as well as shikimic acid, palmitic acid and riboflavin had no significant effect upon amphotericin B production, whereas 0.01% gossypol under the same conditions increased the yield up to 100%. Although some investigators reported a stimulation in production of other polyene antibiotics by β-carotene[15] and palmitic acid[16] we could not confirm these results in the case of amphotericin B fermentation. Inorganic phosphate and/or nitrogen in the defined medium did not stimulate amphotericin B production. Also methionine did not serve as a suitable nitrogen source. As found by other investigators during studies on candicidin fermentation[17], asparagine proved to be a nitrogen source of choice also for the production of amphotericin B. While a supplement of 0.01% of iron and manganese salts to the defined medium stimulated amphotericin B yields dramatically, salts of copper, boron, molybdenum, nickel and cobalt produced no significant yield improvements under the same conditions.

Studies on ^{14}C-Incorporation into Amphotericin B

Since the total structure of amphotericin B was elucidated[18], it is possible to draw conclusions on its biosynthesis (Fig. 1, I). The macrolide ring of polyene antibiotics derives from a polyacetate biosynthetic sequence[19], and in the case of amphotericin B (Fig. 1) the ring is composed of three propionate units (C_3) and sixteen acetate units (C_2). Therefore, even if amphotericin B is a product of the secondary metabolism of *S. nodosus*, the precursors of choice would be glucose or its metabolites propionate and acetate. The use of U-^{14}C-glucose as precursor was avoided, because of a probable dilution effect due to the high glucose content of the available media.

The incorporation experiments were carried out as described above in 300-ml deep baffled flasks containing 50 ml of the appropriate medium with 10% inoculum. The flasks of each series, including controls, were incubated at 28°C in a confined shaker box (New Brunswick Scientific Corp., Model G-25) at 245 rpm. The desired precursors, dissolved in no more than 0.5 ml of ethanol, were added to the growing culture with a disposable syringe. Air was pulled through the shaker box by means of an aspirator, and the liberated $^{14}CO_2$ was absorbed in a trap filled with 10% KOH. The flasks were harvested after 8 days, and the crude radioactive antibiotics isolated as described above. The radioactivity was determined with a Packard Tricarb Liquid Scintillation Spectrometer (Model 3003): cpm values were calculated immediately into dpm to unify all experimental data. For thin-layer chromatography on silica gel G plates the lower phase of the solvent system chloroform - methanol - borate buffer

Table 1. Incorporation of U-^{14}C-acetate into amphotericin B depending on time of addition*

Day of addition after inoculation	dpm/M Ampho. B	mc/M Ampho. B
Control	6.89×10^{7}	31.3 μc
Same day	2.17×10^{10}	9.8
1	1.13×10^{10}	5.1
2	7.22×10^{10}	32.8
3	5.26×10^{9}	2.3
4	1.88×10^{9}	0.8
5	1.66×10^{9}	0.7
6	1.98×10^{9}	0.9
7	1.91×10^{9}	0.8

* Conditions: 50 ml medium with 10% inoculum; 100 μc U-^{14}C-acetate added.

(0.025 M, pH 8.5) (2 : 2 : 1) was used. The plates were developed by spraying with 17% sulfuric acid in ethanol after heating the plate at 100°C for 10 minutes. The R_f value for amphotericin A was 0.18 and the R_f for amphotericin B was found to be 0.21.

The incorporation of U-^{14}C-acetate into amphotericin B is time dependent (Table 1). Optimum uptake of the radioactive acetate was accomplished on the second day after inoculation; under the used experimental conditions the obtained specific activity at this time was 32.8 mc/M amphotericin B. Addition of the same amount of precursor on day 4~7 after inoculation had no significant effect upon incorporation of ^{14}C. This result confirms other experiments, indicating that polyene synthesis as a function of the secondary metabolism usually reaches an optimum a few days after inoculation[20].

Table 2. Incorporation of different ^{14}C-precursors into amphotericin B*

Precursor	μc added	dpm/M Ampho. B	mc/M Ampho. B
Control	—	6.73×10^{7}	30.6 μc
U-^{14}C-Acetate, (Na-salt)	100	2.77×10^{9}	1.3
	200	9.06×10^{9}	4.1
	300	1.07×10^{10}	4.8
	400	2.32×10^{10}	10.5
2-^{14}C-Propionate, (Na-salt)	100	8.29×10^{9}	3.8
	200	7.24×10^{10}	32.9
U-^{14}C-Propionate, (Na-salt)	100	1.92×10^{9}	0.8
U-^{14}C-Ringlabeled *p*-aminobenzoic acid	50	1.32×10^{10}	6.0

* Conditions: 50 ml medium with 10% inoculum; precursors added on the 3rd day after inoculation.

Table 3. Comparison of U-^{14}C-acetate incorporation into amphotericin B in complex and defined medium*

μc U-^{14}C-Acetate (Na-salt) added	Complex medium		Defined medium	
	dpm/M Ampho. B	mc/M Ampho. B	dpm/M Ampho. B	mc/M Ampho. B
Control	3.55×10^{8}	0.1	3.74×10^{8}	0.1
100	9.06×10^{10}	41.2	2.27×10^{10}	10.3
200	2.87×10^{10}	13.0	4.73×10^{10}	21.5
400	—	—	8.39×10^{10}	38.1
600	1.99×10^{10}	9.0	—	—

* Conditions: 50 ml medium with 10% inoculum; precursor added on the 2nd day after inoculation.

2-^{14}C-Propionate was the most efficient precursor of all ^{14}C-compounds tested (Table 2); this compound yielded the highest specific activity of amphotericin B (32.9 mc/M amphotericin B) while U-^{14}C-propionate produced much lower specific activities of the antibiotic than U-^{14}C-acetate. A major part of the U-^{14}C-propionate radioactivity was probably lost by decarboxylation during its passage through the biosynthetic pathways. In the complex medium (Table 3) the incorporation of ^{14}C into the amphotericin B declines with increasing precursor concentration, whereas the specific activity of the polyene in the defined medium increases under the same conditions and an amphotericin B of relative high specific activity was obtained. This phenomenon is probably due to precursor adsorption effects in the colloidal complex medium. The weak radioactivity found in the controls (Tables 1~3) is possibly caused by refixation of liberated $^{14}CO_2$ in the closed experimental system.

Although amphotericin B possesses no aromatic moiety, we observed incorporation of radioactivity derived from U-^{14}C-ringlabeled *p*-aminobenzoic acid into this heptaene. Under experimental conditions as described in Table 2, 50 μc U-^{14}C-ringlabeled *p*-aminobenzoic acid added

to 50 ml of growing culture yielded a specific activity of 6.0 mc/M amphotericin B. It is possible that *p*-aminobenzoic acid in a first stage may have undergone biodegradation by *S. nodosus*, and, in a second stage, the produced metabolites may have been immediately incorporated into amphotericin B.

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