NEOMYCIN PRODUCTION BY *MICROMONOSPORA* SPECIES 69-683*

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Micromonospora sp. 69-683 produces neomycin B and except for an early report of production of actinomycin from a culture not well defined, is presently the only other known example of a major *Streptomyces*-produced antibiotic also produced by a member of the genus *Micromonospora*. A variety of carbon and nitrogen sources were found to be suitable substrates for *Micromonospora* sp. 69-683. The antibiotic is removed from the fermentation broth with a cationic-exchange resin and is purified by passing the mixture through a Dowex 1×2 resin column. Chemical, physical and biological data confirm that it is identical to neomycin B.

The isolation of neomycin in 1948 resulted from a comprehensive screening of streptomyces which have yielded many important chemotherapeutic agents. In 1963 WEINSTEIN *et al*[4] reported on gentamicin, the first important antibiotic produced by a member of the genus *Micromonospora*. This report concerns the production of neomycin by *Micromonospora* sp. 69-683 and is presently the only known example of an important streptomyces-produced antibiotic which is also produced by a member of the genus *Micromonospora*.

Materials and Methods

Preliminary Taxonomy

Colonies of *Micromonospora* sp. 69-683 on an organic agar medium are plicate (folded) and initially are a reddish orange turning a deep dark brown as abundant sporulation develops in the colony. A light yellowish diffusible pigment is typically produced.

An electron-micrograph of a thin section of the spore of *Micromonospora* sp. 69-683 attached to its sporophore is shown in Figure 1. The sporophore has a diameter of 0.5 micron. The spore surface appears minutely warty. Rupture of the thin cell wall will facilitate release of the mature spore.

Detailed taxonomic studies will be reported elsewhere.

Antibiotic Production

The medium used for the inoculum preparation is detailed in Table 1. The culture was grown for 72 hours at 28°C on a rotary shaker. A 5% inoculum was then used for all fermentation studies. The initial soil isolate produced only 20 mcg/ml but by a series of mutation steps utilizing exposure to UV light and natural selection the potency was increased to approximately 400 mcg/ml.

The medium used for fermentation of the culture is detailed in Table 2. Flask fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of medium. Maximum antibiotic yields of 300~400 mcg/ml were reached after 96-hour fermentation at 28°C on a rotary shaker run at 280 rpm. Other carbohydrate and nitrogen sources were studied and several can be used which

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Table 1. Composition of inoculum medium

Component	g/liter
Starch	24
Yeast extract	5
Tryptose	5
Beef extract	3
Calcium carbonate	2
Dextrose	1

Table 2. Composition of fermentation medium

Component	g/liter
Soluble starch	20
Yeast extract	10
Dextrose	10
Casein hydrolysate	5
Calcium carbonate	4
Cobalt chloride	0.00013

Table 3. Effect of various carbohydrates on production of antibiotic 69-683*

Carbohydrate	Antibiotic production (mcg/ml)**
Starch, soluble (control)	248
Galactose	396
Dextrose	364
Dextrin	346
Lactose	344
Maltose	341
Fructose	310
Cellobiose	306
Raffinose	304
Starch, potato	299
Sucrose	285
Mannitol	207
Arabinose	189
Fucose	175
Sorbose	138
Ribose	< 16
Xylose	< 16

* Control medium as shown in Table 2. Carbohydrates substituted on a weight basis for soluble starch in medium shown in Table 2.

** Average of duplicate fermentations.

will provide similar antibiotic yields as that obtained with this medium. The effect of carbohydrate sources on antibiotic production is shown in Table 3. Other nitrogen sources which can be used include corn steep, peptones, fish solubles and NZ Amine type A (Sheffield).

Isolation and Purification

The extraction and isolation of the antibiotic mixture was readily achieved by an ion-exchange procedure. The pH of the whole broth was adjusted to 2 with strong mineral acid in order to release the major part of the antibiotic activity from the mycelium. The mycelial cake was discarded and oxalic acid added to the neutral filtrate to precipitate calcium ions. The precipitated calcium oxalate was separated by filtration. The filtrate was neutralized and charged to Amberlite IRC 50 ion-exchange resin in the ammonium cycle. The resin was washed and the inactive spent broth and washings were discarded. The antibiotic mixture was eluted from the resin with 2 N ammonium hydroxide. The elute was concentrated and its pH adjusted to 4.5 with sulfuric acid, treated with Darco G 60, and the crude antibiotic mixture precipitated as its sulfate salt with methanol. The mixture consisted of at least 3 components.

A bioautogram of a typical paper chromatographic pattern of the complex is shown in Fig. 2. The solvent system consists of 2-butanone - tertiary butanol - methanol - 6.5 N ammonium hydroxide in a ratio of 16:3:1:6 and is referred to as BBMN. Whatman No. 1 paper was used. The papers were developed descending for 24 hours and the detection of the material carried out by the usual bioautogram technique using agar plates seeded with *Staphylococcus aureus* ATCC 6538P. One major and two minor zones were present; the major component was designated as 683 B.

Isolation of the major component was achieved by column chromatography using a Dowex 1×2 resin bed in the hydroxyl cycle and eluting with water. The column was monitored by chromatographing the fractions and identifying like components by the previously described technique. Similar fractions were combined and lyophilized.

Results and Discussion

The growth and antibiotic production in fermentations carried out in 14-liter New Brunswick fermentors at 28°C are shown in Fig. 3. Antibiotic production increased sharply from 48 to 72

hours. During this time period the pH of the fermentation broth also rose while growth, which is measured as packed-cell volume (PCV), appeared to level off. Peak antibiotic production occurred from 72 to 120 hours, after which time the titer decreased.

The described fermentation and isolation methods afforded a material that has a chromatographic pattern as shown in Figs. 4 and 5. Fig. 4 shows a comparison between antibiotic 69-683 B and neomycin B in the BBMN solvent system.

Fig. 5 shows the same materials in the same order in a solvent system consisting of propanol-pyridine-acetic acid-water in a ratio of 15:10:3:12 (PPAW). Both chromatograms were plated against *S. aureus* as previously described. These two figures indicate the chromatographic identity of antibiotic 69-683 B with neomycin B, as did numerous other chromatographic systems used, but not illustrated.

Fig. 6 shows the comparative hydrolytic patterns of 69-683 B and neomycin B. The compounds were hydrolyzed with 6 N hydrochloric acid at 100°C for 2 hours and chromatographed on Whatman No. 1 paper developed ascending using solvent system PPAW. Hydrolysis products were detected with 0.25% ninhydrin reagent in pyridine-acetone (1:1). The hydrolytic products of antibiotic 69-683 B are seen in the top lane, those of neomycin B on the bottom and both are shown after spotting on top of each other in the center lane. This chromatogram illustrates the similarity of the two compounds.

The N-acetyl derivatives of both 69-683 B and neomycin B were made by reacting their respective bases with acetic anhydride in methanol until the reaction was negative to ninhydrin. The resulting N-acetyl derivatives were then chromatographed descending on Whatman No. 1 paper using butanol-pyridine-water (6:4:3). The PAN-DUTCHER method[3], consisting of sodium hypochlorite treatment followed by starch-potassium iodide staining was used for the detection of the biologically inactive derivatives. Both amides had identical R_f values of 0.37.

In 1959 LEACH and TEETERS[2] reported the cleavage of neomycin with aqueous hydrochloric acid; DUTCHER and DONIN[1] also performed this reaction using methanolic HCl which

Fig. 1. Section of spore of *Micromonospora* sp. 69-683. Electron micrograph ×66,000. Legend: C=cortex; Cw=septum; Hw=vegetative cell wall; Osc=outer spore coat(s); Pm=plasma membrane; Sph=sporophore; Wr=wart

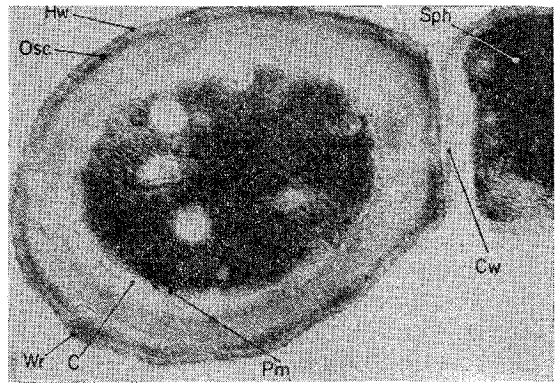


Fig. 2. Bioautograph of antibiotic 69-683 complex. Whatman No. 1 paper using 2-butanone-*tert.*-butanol-methanol-6.5 N ammonium hydroxide (16:3:1:6); plated against *S. aureus* 6538 P.

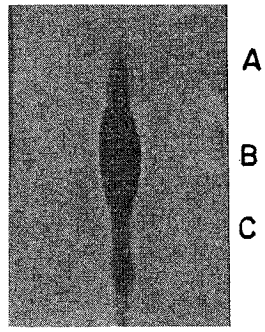


Fig. 3. Growth and antibiotic production of culture in 14-liter fermentor.

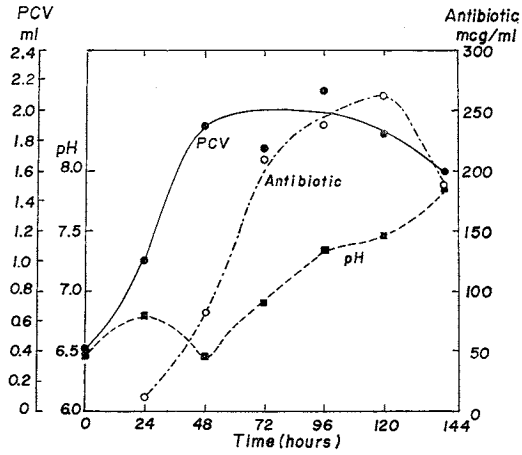


Fig. 4. Comparison of antibiotic 69-683 B and neomycin B on Whatman No. 1 paper using 2-butanone-*tert.*-butanol-methanol-6.5 N ammonium hydroxide (16:3:1:6); plated against *S. aureus* ATCC 6538 P.

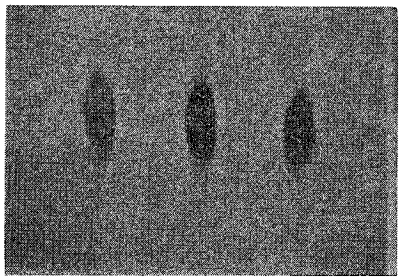


Fig. 5. Comparison of antibiotic 69-683 B and neomycin B on Whatman No. 1 paper using propanol-pyridine-acetic acid-water (15:10:3:12); plated against *S. aureus* ATCC 6538 P.

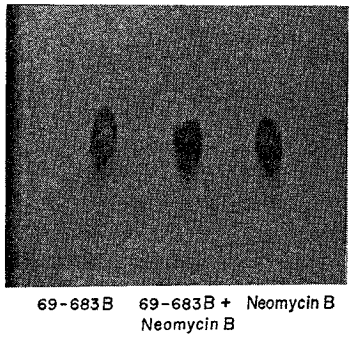


Fig. 6. Comparison of hydrolysis products of antibiotic 69-683 B and neomycin B on Whatman No. 1 paper using propanol-pyridine-acetic acid-water (15:10:3:12). Compounds visualized by ninhydrin.

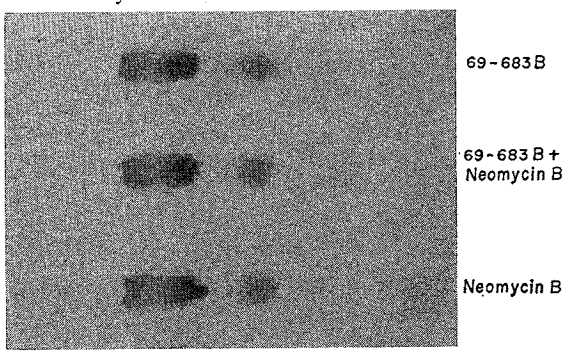


Fig. 7. Bioautographic comparison of neamines after chromatography using 2-butanone-*tert.*-butanol-methanol-6.5 N ammonium hydroxide (16:3:1:6).

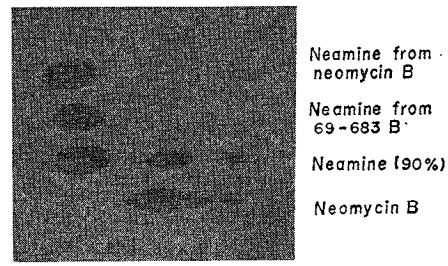
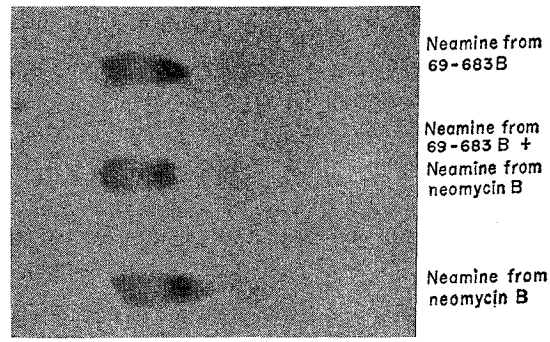


Fig. 8. Hydrolysis patterns of neamines chromatographed on Whatman No. 1 paper using propanol-pyridine-acetic acid-water (15 : 10 : 3 : 12). Compounds visualized by ninhydrin.



yielded a fragment called neamine. A bioautogram of an authentic neamine sample chromatographed in the BBMN system is seen in Fig. 7. The sample is approximately 90% pure. Chromatographed alongside are methanolysis products of neomycin B and antibiotic 69-683 B as performed by a modification of the described procedures using a longer methanolysis period to insure complete reaction. Chromatographically, this hydrolysis product from both 69-683 B and neomycin B is identical with neamine.

The hydrolytic pattern of neamine isolated from the methanolysis products of 69-683 B and neomycin B is shown in Fig. 8. These products were spotted along the top and bottom lanes respectively and together in the center lane, clearly demonstrating the identity between both compounds.

Antibiotic 69-683 B and neomycin B were compared in a number of biological test systems. *In vitro* tests against a variety of sensitive and resistant bacteria showed the two preparations to have identical potency and spectrum including their inactivity against an *Escherichia coli* strain carrying a neomycin-kanamycin phosphorylating R-

factor. *In vivo* tests demonstrated similar acute toxicity and protective activity in mice. This data is thus consistent with the view that 69-683 B and neomycin B are the same antibiotic.

A comparison of chemical and physical data shown in Table 4 such as carbon, hydrogen and nitrogen values, optical rotation and pK as well as mass spectra and NMR data all demonstrate the identity of 69-683 B with neomycin B.

In summary, the fermentation and isolation of an antibiotic produced by *Micromonospora* sp. 69-683 has been described. Chromatographic patterns before and after hydrolysis, chemical and physical data, and biological spectrum, have demonstrated that antibiotic 69-683 B is identical with the streptomyces-produced antibiotic, neomycin B.

Table 4. Comparison of some chemical and physical properties of antibiotic 69-683 B and neomycin B

Elemental analysis	Antibiotic 69-683 B	Neomycin B
C	40.87 %	41.06%
H	7.15 %	7.15%
N	11.75 %	11.95%
O (by diff.)	40.23 %	39.84%
$[\alpha]_D^{20}$	+55.6°	+58.7°
pKa	8.1	8.1

Samples dried under high vacuum at a temperature of 60°C.

Acknowledgements

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References

1) DUTCHER, J. D. & M. N. DONIN: The identity of neomycin A, neamine and the methanolysis product of neomycin B and C. J. Am. Chem. Soc. 74: 3420~3423, 1952
2) LEACH, B. E. & C. M. TEETERS: Neamine, an antibacterial degradation product of neomycin. J. Am. Chem. Soc. 73: 2794~2797, 1951
3) PAN, S. C. & J. D. DUTCHER: Separation of acetylated neomycins B and C by paper chromatography. Anal. Chem. 28: 836~838, 1956
4) WEINSTEIN, M. J.; G. M. LUEDEMANN, E. M. ODEN & G. H. WAGMAN: Gentamicin, a new broad-spectrum antibiotic complex. Antimicr. Agents & Chemoth.-1963: 1~7, 1964