ANTIBIOTIC 6640, A NEW *MICROMONOSPORA*-PRODUCED AMINOGLYCOSIDE ANTIBIOTIC

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Antibiotic 6640 is a new aminoglycoside antibiotic isolated from fermentation broths of a new species of the genus *Micromonospora*, named *Micromonospora inyoensis*. It has been differentiated from other known related antibiotics by a variety of chemical and biological methods. Its spectrum of activity appears to be quite similar to that of gentamicin *in vitro* and *in vivo*. The acute toxicity is approximately twice that of gentamicin in mice.

A new broad-spectrum aminoglycoside antibiotic, named antibiotic 6640, has been isolated from the fermentation broth of a new species of the genus *Micromonospora* (WEINSTEIN *et al.* 1970). This report presents initial data concerning the taxonomy of the producing organism, as well as chemical and biological characteristics of the novel antibiotic produced. Reports of more detailed biological and chemical studies follow.

Materials and Methods

The organism which produces the antibiotic is a new species of the genus *Micromonospora*, named *Micromonospora inyoensis*, which was isolated from soil obtained from the Inyo National Forest in California. Taxonomic studies establishing the validity of this new species on the basis of biochemical and morphological characteristics will be published elsewhere. A culture of *M. inyoensis* has been deposited in the collection of the U.S. Department of Agriculture, Northern Utilization Research and Development Division, in Peoria, Illinois, where it has been designated as NRRL 3292. The colonial morphology of the 14-day old culture was examined after incubation at 24~26°C on an agar medium consisting of N-Z amine type A, 3%; dextrose, 1%; and agar, 1.5%. Macroscopically, there are no aerial mycelia, the colony is viscid to poorly plicate, and growth is fair to poor with a few well-developed colonies appearing late in the inoculation area. A faint reddish-brown diffusable pigment is associated with colonies on some plates. Microscopically, the mycelium is long, branched, regular and nonseptate when examined by phase contrast, and averages about 0.5 μ in diameter. Spores are occasionally born singly on simple sporophores, 1.0~1.5 μ in diameter, ovoid to spherical in shape and rough-walled.

For laboratory production of antibiotic 6640, a lyophilized culture of *M. iyoensis* is added to a 300-ml Erlenmeyer flask containing 100 ml of the germination media as described in Table 1. The flask and its contents are incubated for 3 days at 35°C on a rotary shaker. Five ml of inoculum from the germination stage is transferred to a 500-ml Erlenmeyer flask containing 100 ml of the fermentation medium as described in Table 1. This flask and its contents are incubated for 3~6 days at 28°C on a rotary shaker at 250~300 rpm.

Antibiotic 6640 was formerly known as rickamicin and has now been named sisomicin.

Antibiotic potencies were determined by means of a cylinder cup-agar diffusion assay similar to that described for gentamicin (ODEN *et al.*, 1963) for which *Staphylococcus aureus* ATCC 6538P was the test organism. A unit of antibiotic 6640 activity is the amount of material which produces a zonal response of 16.3±0.9 mm under the conditions of this assay and has been defined as 1 mcg. For determinations of *in vitro* sensitivity, all test organisms were incubated in yeast beef broth at 37°C for 18~24 hours except where indicated. The volume in the tubes was 3 ml and the inoculum was 0.05 ml of a 1 : 1000 dilution of an 18-hour broth culture. Animal studies were carried out in CF-1 male albino mice weighing approximately 20 g each. Drug solutions were prepared in sterile distilled water after correction for base content.* In therapeutic tests, animals were treated once, 1 hour after intraperitoneal infection with approximately 10^7 organisms per mouse. Control infected mice died in 18~24 hours; survivors in treated groups were determined 48 hours after infection. Generally, groups of 7 mice each at 5 dose levels in addition to 10 controls were used for each test. PD_{50} and LD_{50} values were determined by probit procedures. The reference lot of gentamicin sulfate used was from Schering Corporation, and had a potency of 612 mcg/mg.

Table 1. Media for growth of *Micromonospora inyoensis* for production of antibiotic 6640

Germination medium

Component	Amount
Beef extract	3 g
Tryptose	5 g
Yeast extract	5 g
Dextrose	1 g
Potato starch	24 g
Calcium carbonate	2 g
Tap water	1,000 ml

pH adjusted to 7.5 before sterilization

Fermentation medium

Component	Amount
Soybean meal	30 g
Corn steep solids	5 g
Soluble starch	30 g
Dextrose	5 g
Calcium carbonate	7 g
Cobalt chloride	0.13 mg
Tap water	1,000 ml

pH adjusted to 7.2 before sterilization

Results and Discussion

Isolation and Characterization

Antibiotic 6640 is isolated from the fermentation broth by an ion-exchange procedure. The pH of the whole broth is adjusted to 2.0 with acid (which releases that proportion of the antibiotic which is found in the mycelium). After filtration the broth is neutralized and oxalic acid is added to precipitate calcium. Following filtration, the filtrate is again brought to neutrality with base and adsorbed to an IRC50 ion-exchange resin column and eluted from the resin with ammonium hydroxide. The eluate is concentrated and is evaporated to dryness. Material produced in this manner has a potency of approximately 500 mcg/mg according to the assay described above and contains antibiotic 6640 as the base with small quantities of minor components. Further purification of antibiotic 6640 is carried out on a Dowex 1×2 anion exchange adsorption column in the hydroxyl form. Fractions containing antibiotic 6640 are combined and the pooled fractions are lyophilized to yield antibiotic 6640 base, assaying approximately 1,000 mcg/mg.

Antibiotic 6640 was compared by bioautography of paper chromatograms with a variety of antibiotics, including gentamicin, and was differentiated from other possibly related antibiotics in a series of solvent systems shown in Table 2. The antibiotic showed slight but consistent differences between its migration and that of gentamicin

* The antibiotic 6640 used in these studies was the sulfate derivation having a potency of 640 mcg/mg in terms of base.

Table 2. Comparative Rf values of antibiotic 6640 and other aminoglycoside antibiotics

Paper chromatographic systems	Rf's of antibiotics						
	Antibiotic 6640	Gentamicin components			Neomycin	Kanamycin	Paromomycin
		C_1	C_2	C_1a			
80 % Methanol plus 3 % sodium chloride (w/v) 1 : 1 descending*	0.49	0.61 (Complex,	0.60 0.51 ~	0.51 0.61)	0.0, 0.17	0.0, 0.28	0.0, 0.28
Propanol - pyridine - acetic acid - water (6 : 4 : 1 : 3) ascending (v/v)	0.29	0.43 (Complex,	0.39 0.29 ~	0.29 0.43)	0.05	0.08	0.07
80 % Phenol ascending (v/v)	0.45	0.45 (Complex,	0.45 0.45)	0.45	0.0 ~ 0.12	0.0 ~ 0.17	0.0 ~ 0.2
Benzene - methanol (v/v) 9 : 1 descending	0.0	0.0 (Complex,	0.0 0.0)	0.0	0.0	0.0	0.0
n-Butanol - water - acetic acid (40 : 50 : 100) (upper phase used) ascending	0.0	0.0 (Complex,	0.0 0.0)	0.0	0.0	0.0	0.0
		Rt for 26 hours**					
2 % *p*-Toluene sulfonic acid in water-saturated *n*-butanol	0.51	0.56 (Complex,	0.57 0.57)	0.57			
		Rt for 6 hours**					
Chloroform - methanol - 17 % ammonium hydroxide, (2 : 1 : 1)	0.21	0.67	0.40	0.21			

* Paper buffered with 0.95 molar Na_2SO_4 + 0.05 molar $NaHSO_4$.

** $Rt = \frac{\text{distance of zone from origin}}{\text{distance from origin to end of paper}}$

C_1a in two of the paper chromatography systems described. Consequently further comparisons were made to distinguish antibiotic 6640 from gentamicin C_1a. These differences were found in regard to the color produced with the two antibiotics after ninhydrin spray and as to the chromatographic pattern of the acid hydrolysis products of the N-acetyl and sulphate derivatives. 2-Deoxystreptamine was isolated from the N-acetyl derivative of antibiotic 6640 and has been shown to be identical to an authentic sample. Antibiotic 6640 is stable to boiling for at least 30 minutes in the pH range of 2~10. Other chemical and physical characteristics of the base and of its salts are given in WAGMAN *et al.* (1970).

Table 3. *In vitro* activity of antibiotic 6640 and gentamicin

Organism	No. Strains	MIC (mcg/ml)* Range	
		Antibiotic 6640 a)	Gentamicin b)
Baciilus subtilis	1	0.08	0.08
Diplococcus pneumoniae	5	0.08~0.3	0.03~0.3
Sarcina lutea	1	0.75	0.75
Staphylococcus aureus	9	0.01~0.3	0.03~0.3
Streptococcus faecalis	1	0.8	0.8
Streptococcus pyogenes	11	0.08~0.3	0.03~0.3
Aerobacter sp.	12	0.01~0.75	0.01~0.3
Escherichia coli	11	0.08~0.3	0.03~0.3
Klebsiella sp.	6	0.3 ~0.75	0.08~0.3
Proteus sp.	18	0.08~0.75	0.08~0.3
Pseudomonas aeruginosa	17	0.03~0.75	0.03~0.75
Salmonella sp.	6	0.03~0.3	0.08~0.3
Pasteurella multocida c)	2	7.5	7.5
Vibrio coli d)	1	2.4	1.2

a) Antibiotic 6640 sulfate, 640 mcg/mg
b) Gentamicin sulfate, 612 mcg/mg
c) Brain heart infusion 10 % serum
d) Fluid thioglycolate
* Yeast beef broth pH 7.4

Table 4. Therapeutic activity of antibiotic 6640 and gentamicin against bacterial infections in mice. All mice given a single subcutaneous dose one hour post infection.

Infecting organism	PD_{50} mg/kg	
	Antibiotic 6640 [a]	Gentamicin [b]
Staphylococcus aureus Gray	1.8	2.4
Streptococcus pyogenes C203	6.0	14.0
Escherichia coli sc.	1.7	3.2
Klebsiella pneumoniae sc.	0.8	0.8
Proteus vulgaris 120.4	1.7	1.5
Pseudomonas aeruginosa sc.	1.1	1.7
Salmonella paratyphi B sc.	0.5	2.5

a) Antibiotic 6640 sulfate, 640 mcg/mg
b) Gentamicin sulfate, 612 mcg/mg

Table 5. Acute toxicity of antibiotic 6640 and gentamicin in mice

Route	LD_{50} (mg/kg)	
	Antibiotic 6640 [a]	Gentamicin [b]
I. V.	34 (33～ 35)*	69 (63～ 75)
I. P.	221 (207～234)	440 (402～478)
S. C.	288 (246～341)	478 (428～528)

* 95% confidence limits
a) Antibiotic 6640 sulfate, 640 mcg/mg
b) Gentamicin sulfate, 612 mcg/mg

Chemical investigations to be reported elsewhere indicated that antibiotic 6640 differs from gentamicin C_1a by the presence of a double bond in one of the amino sugar constituents.

In Vitro Antimicrobial Activity of Antibiotic 6640

The *in vitro* antimicrobial activity of antibiotic 6640 sulfate was studied against a variety of organisms in comparison with the reference gentamicin sulfate. The results of these tests against a selected group of gram-positive and gram-negative bacteria are shown in Table 3. The activity appears to be equal to that of gentamicin. Both antibiotics exhibit particularly high activity against penicillin sensitive and resistant *Staphylococcus*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Salmonella*, and *E. coli*. Antibiotic 6640, like gentamicin, is inactive against cellular and filamentous fungi. Bactericidal levels were the same or slightly higher, depending upon the organism, than the bacteriostatic levels. Antibiotic 6640 was active *in vitro* against kanamycin-resistant but not gentamicin-resistant strains of bacteria.

In Vivo Activity of Antibiotic 6640

The therapeutic activity of antibiotic 6640 and gentamicin were compared against a variety of gram-positive and gram-negative bacterial infections in mice. The results of these tests are given in Table 4. Antibiotic 6640 shows a high degree of activity in these mouse protection tests, and was found to be 1～5 times as active as gentamicin depending upon the organism studied. The acute toxicity in mice (Table 5) is approximately twice that of gentamicin. Antibiotic 6640, at a dose of 50 mg/kg subcutaneously, provides 100% protection against experimental *Rickettsia akari* infections in mice.

References

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