ANTIBIOTIC 6640.* II

FERMENTATION, ISOLATION, AND PROPERTIES

GERALD H. WAGMAN, RAYMOND T. TESTA and JOSEPH A. MARQUEZ

Microbiology Department, Schering Corporation,
Bloomfield, New Jersey 07003, U. S. A.

(Received for publication October 3, 1970)

Antibiotic 6640 is produced by aerobic submerged fermentation of a new species of organism, *Micromonospora inyoensis* (NRRL 3292). The antibiotic is produced substantially as a single component and is extracted from the broth by a cationic exchange procedure. It is purified by use of an anionic resin. Chromatographic studies indicate that it most nearly resembles, but is different from gentamicin C_1a. This report describes the production, isolation, purification, and chemical and physical properties of antibiotic 6640.

A new broad-spectrum, aminoglycoside antibiotic, named antibiotic 6640, has been isolated from the fermentation broth of a new species of the genus *Micromonospora*, *M. inyoensis* (NRRL 3292) (WEINSTEIN *et al.*, 1970). The antibiotic is produced substantially as a single component and is extracted from the broth by an ion-exchange procedure along with minor quantities of other active compounds. Chromatographic and biological studies indicate that it most nearly resembles, but is different from gentamicin C_1a. This report describes the production, isolation, purification, and chemical and physical properties of antibiotic 6640.

Experimental

Tank Fermentation of *M. inyoensis*

1. Germination Stage: The germination is carried out as in I (WEINSTEIN *et al.*, 1970), but in two stages. A 25-ml portion of inoculum from the first stage is aseptically transferred to a two-liter Erlenmeyer flask containing 500 ml of the sterile germination medium. The flask and its contents are incubated for 3 days at 28°C on a rotary shaker (280 rpm, 2-inch (*ca.* 5 cm) stroke).

2. Fermentation Stage: A 500-ml portion of the germinated culture is transferred to a 14-liter fermentor containing 9.5 liters of the following sterile medium:

Dextrin	50 g	Cobalt chloride	0.00013 g
Dextrose	5 g	Tap water	1,000 ml
Soybean meal	35 g	Antifoam (Dow-Corning B)	10 ml
Calcium carbonate	7 g		

The pH is adjusted to 8.0, which drops to 7.2 following sterilization. The fermentation is carried out while agitating at 250 rpm with an air flow of 4.5 liters per

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

minute at 25 psi. Peak potency is reached at 66~90 hours.

At the end of this period, the potency of the broth reaches a peak of 150~225 mcg/ml by the assay described by WEINSTEIN *et al.* (1970). Samples were assayed after acid extraction of the whole broth at pH 2. The pH of the culture medium remains essentially unchanged during the fermentation. The packed cell volume (PCV) reaches a peak value of approximately 2.5 to 3.0 ml (PCV is determined by centrifuging 10 ml of broth in a graduated centrifuge tube at 2,000 rpm in a clinical centrifuge for 20 minutes and reading the volume of packed cells).

Isolation of the Antibiotic

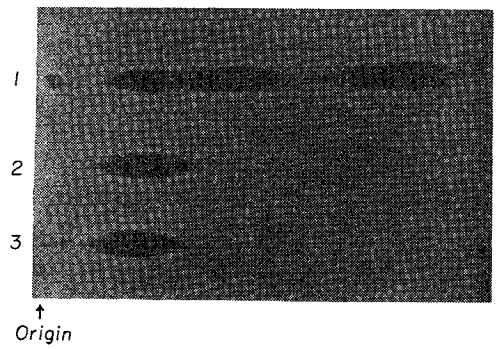
The antibiotic is isolated from the fermentation broth by an ion-exchange procedure. The pH of the whole broth is adjusted to 2.0 with 12 N sulfuric acid which releases a large portion of the antibiotic from the mycelium. The broth is filtered, adjusted to pH 7.0 with concentrated ammonium hydroxide and calcium is precipitated by addition of a saturated solution of oxalic acid. Following filtration, the filtrate is again brought to neutrality with concentrated ammonium hydroxide and passed through an IRC 50 (NH_4^+) ion-exchange resin column. The column is washed with water and the antibiotic fraction is eluted from the resin with 2 N ammonium hydroxide. The eluate is concentrated and evaporated to dryness. The resulting crude antibiotic 6640 base has a potency of about 500 mcg/mg and contains small quantities of minor components.

Purification of the antibiotic is carried out by dissolving the crude base in distilled water and charging it to a Dowex 1×2 (OH^-) anion-exchange absorption column. The column is eluted with distilled water and monitored (1) with a conductivity bridge to determine when the eluate is salt-free and (2) by assaying the eluted fractions against *Staphylococcus aureus*.

Fractions containing only the antibiotic 6640 component are combined. This is determined by chromatographic behaviour on Whatman No. 1 paper using a solvent system composed of the lower phase of a mixture of chloroform - methanol - 17 % ammonia (2 : 1 : 1). Zones are detected by ninhydrin spray or by bioautography against *S. aureus*. The pooled fractions are lyophilized to yield antibiotic 6640 base which assays, by definition, 1,000 mcg/mg. The sulfate derivative was prepared by titrating the base with sulfuric acid to pH 4.5 and precipitating the sulfate by addition of excess methanol.

Fig. 1. Bioautograph of antibiotic 6640 compared with gentamicins.

Chromatogram run on Whatman No. 1 paper using lower phase of chloroform - methanol - 17 % ammonia (2 : 1 : 1) and plated against *S. aureus* ATCC 6538 P. Lane 1, L to R, gentamicins C_1a, C_2, C_1 ; lane 2, gentamicin C_1a, lane 3, antibiotic 6640.



Characterization and Properties of the Antibiotic

Antibiotic 6640 was compared, by bioautography of paper chromatograms, with a variety of antibiotics including gentamicin (WEINSTEIN *et al.*, 1970). In

Table 1. Comparative Rf values of hydrolysis products of the N-acetyl and sulfate derivatives

Paper chromatographic solvent system	Rf Values of hydrolysis products		
	N-Acetyl antibiotic 6640	N-Acetyl gentamicin C_1a	N-Acetyl gentamicin complex
Butanol - pyridine - water - acetic acid, 6 : 4 : 3 : 1	0.09	0.09	0.09
	0.18 trace	0.15	0.15
	no zone	0.19	0.22
	0.35	0.39	0.34
	0.49	0.47	0.48
	Antibiotic 6640 sulfate	Gentamicin C_1a sulfate	Gentamicin complex sulfate
Propanol - pyridine - water - acetic acid, 6 : 4 : 3 : 1	no zone	0.17	0.18
	0.20	0.20	0.20
	no zone	0.31	0.31
	no zone	0.43	0.43
	0.53	0.53	0.53
	0.64	0.65	0.65

Table 2. Chemical and physical properties of antibiotic 6640

	Base	Sulfate [a]		Hydrochloride [a]	
		Preparation I	Preparation II	Preparation I	Preparation II [b]
Elemental analysis	Average of 2	Average of 2	Average of 2	Average of 2	Average of 2
C	49.40	31.28	32.52	36.25	37.70
H	8.05	6.55	6.33	7.34	7.15
N	14.71	9.60	9.32	10.52	10.61
O (by diff.)	27.84	—	—	—	—
SO_4 [c]	—	30.21	33.90	—	—
Cl [c]	—	—	—	27.94	25.60
Rotation	+183.4	+101.4	+105.1	+115.9	+112.2
	C = 1 %	C = 0.3 %	C = 1 %	C = 0.3 %	C = 1 %
Equivalent weight	94.3	—	—	—	—
pK_a	8.0	—	—	—	—

a) Contains 1 mole solvated methanol per mole antibiotic.
b) Dried in an Abderhalden apparatus over ethanol, b.p. 78.5℃.
c) Single determination.

a series of solvent systems, it was clearly differentiated from other related antibiotics except gentamicin C_1a. After development with the lower phase of a chloroform - methanol - 17 % ammonium hydroxide system (2 : 1 : 1) on Whatman No. 1 paper, the Rf was identical with gentamicin C_1 a (as detected by heating after treatment with 0.25 % ninhydrin in pyridine - acetone) but the the colors were distinctly different. The color produced during the early stages of heating (100°C for 3 minutes) was yellow as opposed to light gray of gentamicin C_1a. The final ninhydrin color is dark gray while that of gentamicin C_1a is purple. A photograph of a representative bioautogram is shown in Fig. 1.

Comparisons of the respective hydrolysis products were made in order to distinguish this antibiotic from gentamicin C_1a. The antibiotics were differentiated by paper chromatographic comparisons of the acid hydrolysis products of their respective bases, N-acetyl and sulfate derivatives. The inactive N-acetyl derivative was prepared according to the procedure described by WEINSTEIN *et al.*, 1964. The compounds were

hydrolyzed with 6 N hydrochloric acid at 100°C for 2 hours in sealed tubes and chromatographed on paper in a solvent system consisting of *n*-butanol – pyridine – water – acetic acid in a ratio of 6 : 4 : 3 : 1 (v/v). As illustrated in Table 1, ninhydrin sprayed chromatograms of the hydrolysate of the antibiotic 6640 N-acetyl derivative indicates the presence of one product, positive to the reagent, which has the same migration rate and pattern as 2-deoxystreptamine, and which is also contained in gentamicin C_1a. However, a major hydrolysis product and two minor products are missing which are characteristic in the gentamicin C_1a hydrolysis pattern. Comparisons of the sulfate hydrolysates also were made using a propanol – pyridine – water – acetic acid system in a ratio of 6 : 4 : 3 : 1 (v/v). The differences are also detailed in Table 1 and indicate that three of the spots present in the gentamicin C_1a sulfate hydrolysate (at Rf's 0.17, 0.31 and 0.43) are absent in the corresponding antibiotic 6640 mixture.

Table 3. Solubility of antibiotic 6640 and its salts

Solvent	Base	Hydrochloride	Sulfate
Methanol	Sparingly soluble	Soluble	Insoluble
Acetone	Insoluble	Very slightly soluble	Insoluble
Chloroform	Slightly soluble	Very slightly soluble	Insoluble
Ether	Insoluble	Insoluble	Inosluble
Benzene	Insoluble	Insoluble	Insoluble
Water	Very soluble	Very soluble	Very soluble

Terminology is according to U. S. Pharmacopeia XVII, p. 8.

The 2-deoxystreptamine, whose presence was indicated by chromatography, was isolated from the antibiotic 6640 N-acetyl derivative and was shown to be chromatographically identical to an authentic sample. Also, a comparison of its elemental analysis with that of authentic 2-deoxystreptamine confirmed their identity.

Antibiotic 6640 solutions in McIlvane's buffers in the pH range of 2~8, and in borate buffers up to pH 10 were stable at 100°C (boiling water bath) for at least 30 minutes. The chemical and physical characteristics of the base and two of its salt derivatives are listed in Table 2. The antibiotic exhibits no absorption peaks in the ultraviolet range (200~400 mμ). Solubilities of the base, hydrochloride and sulfate are given in Table 3.

Discussion

Antibiotic 6640, a new aminoglycoside antibiotic isolated from fermentation broths of a new species of the genus *Micromonospora*, specifically, *M. inyoensis*, has been differentiated from other known related antibiotics by a variety of chemical and biological methods. Chromatographic and analytical data indicate that it contains 2-deoxystreptamine and very closely resembles gentamicin C_1a. Further chemical investigations, to be reported elsewhere, show that antibiotic 6640 probably differs from gentamicin C_1a only by the presence of a double bond in one of the amino sugar constituents.

Acknowledgements

We acknowledge the capable technical assistance of Mr. Alphonse Murawski, Mr. Walter Reiblein, and Mrs. Janet Bailey in these studies.

References

1) Weinstein, M. J.; J. A. Marquez, R. T. Testa, G. H. Wagman, E. M. Oden & J. A. Waitz: Antibiotic 6640, a new *Micromonospora*-produced aminoglycoside antibiotic. J. Antibiotics 23 : 551~554, 1970

2) Weinstein, M. J.; G. H. Wagman & R. I. Taber: Toxicity of acetylated gentamicin and neomycin. Antimicr. Agents & Chemoth.-1965 : 227~231, 1966