SEPTAMYCIN, A POLYETHER ANTIBIOTIC

TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

CAMILLA KELLER-JUSLÉN, HAMILTON D. KING, ZOLTAN L. KIS and ALBERT VON WARTBURG

Sandoz Ltd., Pharmaceutical Division, Chemical Research, Basel, Switzerland

(Received for publication July 7, 1975)

Septamycin is a metal complexing polyether antibiotic produced by a strain of *Streptomyces hygroscopicus* NRRL 5678. The metabolite, a monocarboxylic acid, was isolated as the sodium salt $C_{48}H_{81}NaO_{16}$. The crystal structure and absolute configuration were established by X-ray analysis of the *p*-bromophenacyl derivative. Septamycin has a thirty-carbon backbone and contains seven heterocyclic rings. Supported by direct comparison septamycin proved to be identical with antibiotic A28695 A isolated from *Streptomyces albus* NRRL 3883. The metabolite is active against gram-positive bacteria and *Eimeria tenella* (chicken coccidiosis).

In the course of our search for new antibiotics from soil actinomycetes a strain of *Streptomyces hygroscopicus* (NRRL 5678) was isolated which produced a metabolite effective against gram-positive bacteria. The active compound designated as septamycin is a member of the polyether antibiotic group.

This report presents the taxonomy of the producing strain, as well as the fermentation, isolation, characterization and biological activities of septamycin.

Taxonomic Study

The septamycin-producing strain NRRL 5678 was isolated from a fresh soil sample collected in the vicinity of Madison, Wisconsin, U.S.A. in 1968. The microorganism was identified as a strain of *Streptomyces hygroscopicus*.[1] It had the fundamental characteristics of the organism, namely, the sporophores have narrow compact spirals that are often bourne in dense clusters (Fig. 1) and give a pseudoverticillate appearance; the brownish-grey spores are oval, 0.8~1.0 by 1.0~1.2 nm; the spore surfaces are smooth to warty as determined from electron microscopic observation (Fig. 2), and the distinctive hygroscopic character on most media.

However, in contrast to the reference strain[1], our strain NRRL 5678 did not decompose cellulose and there was doubtful utilization of arabinose and xylose.

Fermentation and Isolation

Fermentation was performed on a 2-liter scale in shake flasks. One milliliter of a dense spore suspension of the culture NRRL 5678 was used to inoculate 100 ml of the vegetative growth medium, consisting of 2.5% Pharmamedia (Trader's Protein Division, Fort Worth, Texas) and 2.5% Cerelose (glucose technical grade) with a pH 6.7. Incubation of the inoculated vegetative flasks was made at 27°C for 2 days on a rotary shaker machine (200 rpm), then

Fig. 1. Photomicrograph of *Streptomyces hygroscopicus*, strain NRRL 5678 (Malt-Yeast extract agar ×850)

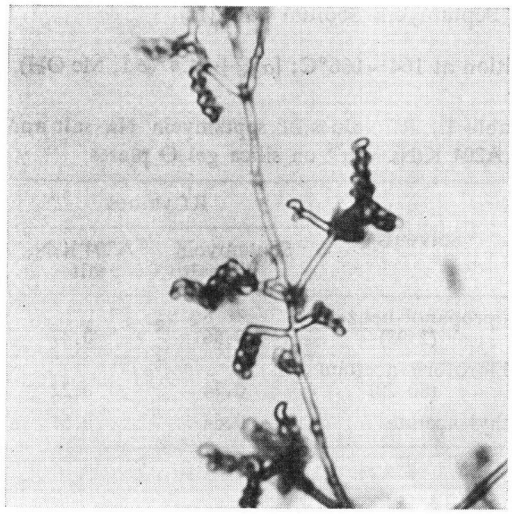
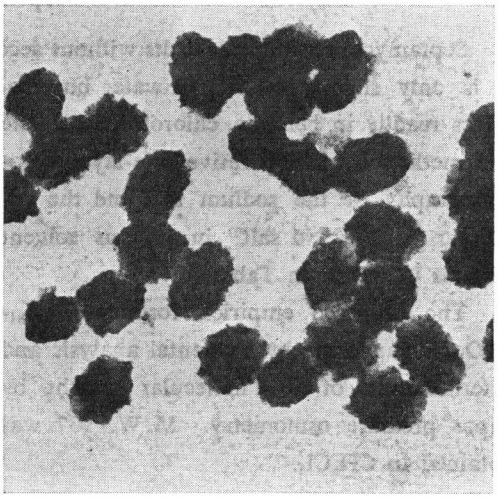


Fig. 2. Electronmicrograph of spores of *Streptomyces hygroscopicus*, strain NRRL 5678 (Malt-Yeast extract agar ×5,350), showing smooth and warty surfaces.



this vegetative stage was used to inoculate 2 liters of the fermentation medium composed of 3% malt extract and 0.5% Pharmamedia; pH 6.8. Media were sterilized at 121°C for 20 minutes. The inoculate fermentation flasks were incubated for 4~7 days at 27°C on a rotary shaker at 200 rpm. The fermentation broth was tested for antibiotic activity by a paper disc agar diffusion assay using *Staphylococcus aureus* and *Escherichia coli* as test organisms. The fermentations were harvested at the time of their highest production of the antibiotic.

Isolation

The isolation procedure for septamycin sodium salt from the broth is outlined in Chart 1.

Chart 1. Isolation procedure for septamycin

Fermentation broth
↓
solvent extraction
↓
chromatography on silica gel
↓
gel filtration chromatography on Sephadex LH 20 in MeOH
↓
crystallization from hexane
↓
Septamycin

The fermentation broth (1.8 liters) was homogenized at pH 7 with an Ultra-Turrax homogenizer and then centrifuged at 3,000 rpm. The supernatant was extracted three times with 1,2-dichloroethane and the organic layer was concentrated under reduced pressure. The crude extract (1.9 g) was dissolved in chloroform and chromatographed on 100 g silicagel (Merck, 0.05~0.2 mm) using chloroform with increasing amounts of methanol as solvents. The fractions eluted with chloroform-methanol (97 : 3) containing the main activity were pooled and the solvents removed *in vacuo*. The resulting solid, 388 mg, was further purified by Sephadex LH 20 column chromatography using methanol as the eluant. Each fraction was estimated by bioassay and checked by tlc on silica gel plates (silica gel G Merck) in the system isopropanol-benzene (2:98). Detection was made by spraying with a solution of Ceric-sulfate (0.2%) in 50% H_2SO_4 followed by heating at 110~130°C. Active and chemically

pure fractions were combined and the solvents removed *in vacuo*. By adding hexane to the residue, septamycin sodium salt crystallized spontaneously to yield 103 mg white crystals.

Physical and Chemical Properties of Septamycin Sodium Salt (II)

Septamycin sodium salt melts without decomposition at 164~166°C; $[\alpha]_D^{20}$+14.4°(*c* 1, Me OH). It is only slightly soluble in water but dissolves readily in benzene, chloroform, acetone and methanol. Comparative thin-layer chromatography of the sodium salt and the antibiotic A 204 K/Na salt[2] in various solvent systems is shown in Table 1.

Table 1. Rf values of septamycin Na salt and A204 K/Na salt[2] on silica gel G plates

Solvents	Rf values	
	Septamycin Na salt	A204 K/Na salt
Isopropanol-benzene (2:98)	0.56	0.47
Chloroform-acetone (80:20)	0.34	0.22
Ethyl acetate	0.64	0.51

The proposed empirical formula $C_{48}H_{81}NaO_{16}$ is supported by elemental analysis and determination of the molecular weight by vapor pressure osmometry. M. W. 937 was obtained in CH_2Cl_2.

Calcd. for $C_{48}H_{81}NaO_{16}$ (M.W. 937): C, 61.5; H, 8.7; Na, 2.5
Found: C, 61.7; H, 9.0; Na, 2.4

Fig. 3. IR spectrum of septamycin sodium salt in CH_2Cl_2

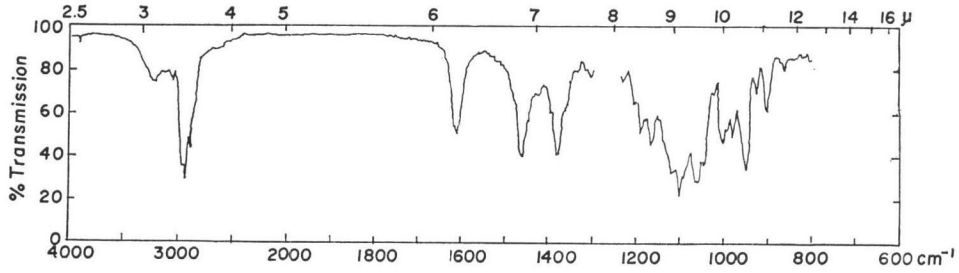
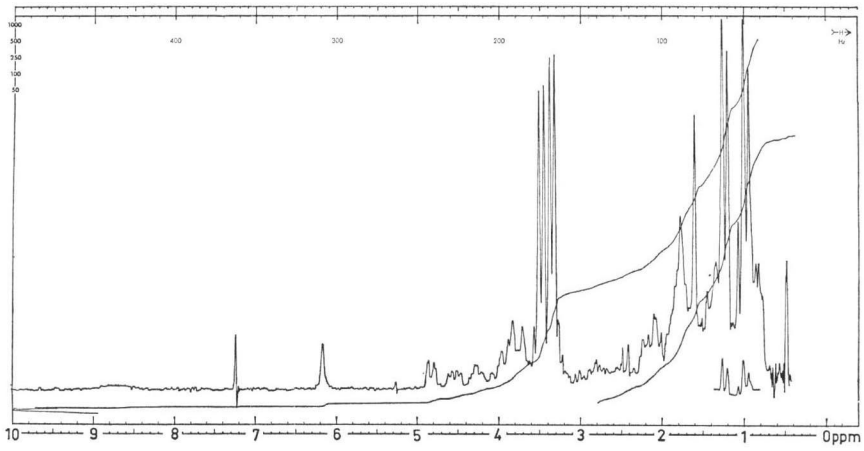


Fig. 4. 100 MHz NMR spectrum of septamycin sodium salt in $CDCl_3$ (TMS as internal standard)



Septamycin sodium salt has no characteristic UV maximum in methanol except end absorption. The IR spectrum (Fig. 3) showed typical bands at 1609 cm^{-1} accounting for carboxylate ion and at 1165/1100 cm^{-1} indicating the etheral character of several oxygen atoms.

In the NMR spectrum (Fig. 4) peaks in the range of δ 0.7 ~ 1.7 indicate the presence of 9~10 C-methyl groups and signals at δ 3.32~3.51 correspond to 4 methoxy groups.

Septamycin is very sensitive to acids; however, the acid form could be obtained as a white amorphous powder by shaking a chloroform solution of the sodium salt at 0°C with an equivalent of 1 N HCl. The free acid (**I**) formed a white, amorphous solid with m.p. 100 ~ 103°C; $[\alpha]_D^{20}$ +24.4° (*c* 1.24, methanol). Methylation of the free acid with an excess of ethereal diazomethane gave the amorphous methylester m.p. 78~83°C; $[\alpha]_D^{20}$ +21.3° (*c* 0.76, chloroform).

Calcd. for $C_{49}H_{84}O_{16}$ (M.W. 929):	C, 63.3; H, 9.1; O, 27.6
Found:	C, 62.9; H, 9.0; O, 27.4

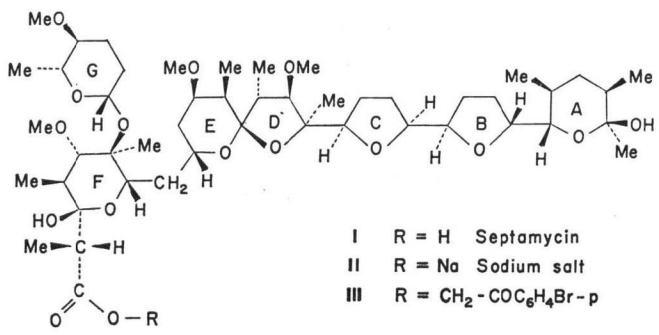
Its IR spectrum in CH_2Cl_2 showed absorption at 1755/1700 cm^{-1}. The NMR spectrum ($CDCl_3$) suggested the presence of 10-C-methyl groups at δ 0.8~1.4, 4 methoxy groups at δ 3.28, 3.35, 3.43 and 3.55 and methylester at δ 3.81. On silica gel plates septamycin methylester gave the same Rf value in chloroform-methanol (97:3) as the free acid or the sodium salt.

Structure of Septamycin

On the basis of the physico-chemical data and the biological properties, septamycin was considered to represent a member of the polyether antibiotic group. Septamycin has a close resemblance to A 204A.[2] The latter metabolite is the largest of the known polyether antibiotics, it contains seven cyclic ether moieties and, in contrast to septamycin, an additional methoxy group.

Since purely chemical methods failed to establish the full structure of septamycin we attempted the preparation of a derivative with a heavy atom suitable for X-ray crystallographic studies. As a convenient compound we used the *p*-bromophenacylester of septamycin which was prepared as follows: A mixture of 375 mg septamycin, 33 mg $NaHCO_3$ and 111 mg of *p*-bromophenacylbromide in 3 ml tetrahydrofuran-dimethylformamide (1 : 1) was refluxed for 2 hours. The solvents were removed *in vacuo* and the resulting solid was dissolved in chloroform and washed with water. The organic layer was dried, evaporated to dryness and the residue chromatographed on silica gel (Merck). The main fractions, eluted with benzene-

Fig. 5. Structural formula of septamycin and derivatives



ethylacetate (7:3), yielded 282 mg crystalline septamycin-*p*-bromophenacylester (**III**) m.p. 157~160°C (from ether).

Calcd. for $C_{56}H_{87}BrO_{17}$ (M.W. 1112): C, 60.5; H, 7.8; O, 24.5; Br, 7.2
Found: C, 60.1; H, 7.8; O, 25.0; Br, 7.4

Recrystallization from acetonitrile furnished orthorhombic crystals of septamycin-*p*-bromophenacylester monohydrate. X-ray analysis[3] revealed the structure and the relative and absolute configuration of this derivative (Fig. 5). The bromophenacylester (**III**) could be reconverted to the sodium salt (**II**) identical in all respects with the septamycin Na salt isolated from the culture broth.

Biological Properties

Septamycin possesses antibacterial activity as well as substantial activity against Newcastle Disease and Herpes simplex viruses. The antimicrobial spectrum *in vitro*, obtained by conventional broth-dilution assay, is shown in Tables 2 and 3.

Table 2. Antimicrobial spectrum of septamycin Na salt

Organism	Minimum inhibitory concentration (mcg/ml)
Staphylococcus aureus	0.31
Streptococcus pyogenes	0.1
Bacillus subtilis	0.1
Bacillus stearothermophilus	0.01
Escherichia coli	0.1
Candida albicans	>100
Clostridium pasteurianum	0.1

Table 3. Activity of septamycin Na salt against antibiotic-resistant variants of strains

Organism	Minimum inhibitory concentration (mcg/ml)
Staphylococcus aureus resistant to penicillin	0.31
Sarcina lutea resistant to macrolides	0.31
Streptococcus faecalis resistant to aminoglycoside	0.1
Micrococcus sp. resistant to tetracycline	0.31

The cell proliferation of Mastocytoma cells P-815 is inhibited (50% inhibition) by septamycin at a concentration of 0.1 mcg/ml. The acute toxicity of septamycin (LD_{50}) by intravenous injection is in dogs 5 mg/kg and 10~30 mg/kg p.o. in rats. Septamycin has an anticoccidial activity in chickens similar to other polyether antibiotics. It is effective in reducing mortality and increasing average bond weight of chickens infected with *Eimeria tenella* and other *Eimeria* species. Septamycin shows herbicidal properties against cress but not against oats.

Having completed our work on septamycin the DOS Patent No. 2262501 of the Eli Lilly & Co. describing two novel polyether antibiotics, A 28695 A and B,[4] came to our attention. An exchange of samples and direct comparison of them in both laboratories revealed septamycin to be identical with A 28695 A as judged by paper and thin-layer chromatographic movement, physical chemical data, antimicrobial spectrum and X-ray diffraction powder pattern.

Acknowledgement

We are grateful to Dr. J. G. WHITNEY and Dr. R. L. HAMILL, The Lilly Res. Labs., Eli Lilly & Co., Indianapolis, for reference samples of A204, A28695 A and B resp. We are also indebted to Dr.

H. MIETH, Sandoz Forschungsinstitut G. m. b. H. A-1235 Vienna, Austria, for the *in vivo* assays and to Dr. W. PACHE, Sandoz Basel, for the MIC tests.

References

1) BERGEY's Manual of Determinative Bacteriology, 8th Ed., pp. 771~776. Williams & Williams Co., 1974
2) JONES, N. D.; M.O. CHANEY, J. W. CHAMBERLIN, R. L. HAMILL & S. CHEN: Structure of A204 A, a new polyether antibiotic. J. Am. Chem. Soc. 95: 3399~3400, 1973
3) PETCHER, T. J. & H. P. WEBER: X-Ray crystal structure and absolute configuration of *p*-bromophenacyl-septamycin monohydrate, a polyether antibiotic. J.C.S. Chem. Comm. 1974-17: 697~698, 1974
4) HAMILL, R.L. & M.M. HOEHN: Antibiotics A28695 A and A28695 B and a process for production thereof. U.S. Patent 3,839,558, Eli Lilly and Company, October 1, 1974