A NEW ANTIBIOTIC XK-62-2 (SAGAMICIN)

I. ISOLATION, PHYSICOCHEMICAL AND ANTIBACTERIAL PROPERTIES

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A new aminoglycoside antibiotic XK-62-2 (Sagamicin) produced by *Micromonospora sagamiensis* var. *nonreducans* nov. sp. MK 62 was isolated from its fermentation beer by use of a cationic exchange resin and silica-gel column chromatography. The purified antibiotic showed a close similarity to gentamicin C complex in physical, chemical, and antimicrobial properties, but a significant difference from gentamicin in its more remarkable activity against some *Pseudomonas aeruginosa* strains resistant to gentamicin C_{1a}.

Our previous work showed that a new antibiotic XK-62-2 produced by *Micromonospora sagamiensis* var. *nonreducans* MK-62 is a water-soluble, basic aminoglycoside probably closely related to gentamicin[1]. Several methods for gentamicin separation have been reported by ROSSELET *et al.*,[2] MAEHR[3], MAEHR and SCHAFFNER[4], and WAGMAN *et al*[5]. These authors showed that the gentamicin C complex consists of gentamicin C_1, C_2 and C_{1a}. The use of various chromatographic systems such as thin-layer plates[6,7], papergrams[8] and silicic acid-glass fiber sheets[9] also leads to the same conclusion. Very recently the presence of some minor components was reported in the fermentation broth of gentamicin-producers and in gentamicin C complex by WAGMAN *et al.*[10], KERSHNER[11], WILSON *et al.*[12], and RINEHART[29].

The present report describes the isolation, purification, physicochemical properties and antimicrobial activities of XK-62-2.

Materials and Methods

Microorganisms *Micromonospora sagamiensis* var. *nonreducans* nov. sp. MK-62 was used for the production of XK-62-2. Test organisms used for antibiotic assay were type cultures preserved in this laboratory as well as fresh clinical isolates.

Chemicals Gentamicin C_1 sulfate (645 mcg/mg), gentamicin C_{1a} sulfate (764 mcg/mg), gentamicin C_2 sulfate (622 mcg/mg) and gentamicin C complex were kindly supplied by Dr. G. H. WAGMAN, Schering Res. Div., Schering Corp.

Thin-layer and paper chromatography Separation of gentamicin C complex was made by chromatographic bioassay and ninhydrin assay methods as reviewed by ODEN *et al.*[13]

Antibacterial spectra Minimal inhibitory concentrations of XK-62-2 were determined by agar dilution assay.

Fermentation The technique was described in the succeeding paper.[1]

Isolation and purification To the whole broth harvested from a tank fermentor, oxalic acid was added to precipitate calcium ions and the pH of the whole broth was adjusted to 2.0 with 6 N sulfuric acid. Acidified whole broth was stirred for 30 minutes to release the antibiotic activity from the mycelium. The mycelium and solid cake in the fermentation

broth was separated by filtration. The filtrate was neutralized to pH 6.8 with 6 N sodium hydroxide and charged to an IRC-50 cation-exchange resin in the ammonium cycle. The resin was washed with deionized water and the spent broth and washings were discarded. Another antibiotic active against only gram-positive bacteria was contained in the spent and washings. The antibiotics adsorbed on IRC-50 resin were eluted with 1 N ammonium hydroxide. The eluate was collected by a fraction collector and each fraction was assayed by paper disc on agar plates seeded with *Bacillus subtilis* KY 4273 to locate the active fractions, which were combined, concentrated *in vacuo*, and passed through an anion-exchange resin (Dowex 1×2) column in the hydroxyl cycle. The resin was washed with distilled water and active fractions were collected. The active fractions were concentrated and acetone was added to precipitate inorganic salts. The precipitate were removed by filtration, the filtrate was concentrated and adjusted to pH 4.5 with 6 N sulfuric acid. The crude antibiotic mixture, sulfate salt, was precipitated with methanol. The preparation consisted of at least two components. A typical paper chromatographic bioautogram of the complex showed two main inhibition zones on an agar plate seeded with *B. subtilis* KY 4273. One component, XK-62-1, was later identified as gentamicin C_{1a}. The other main component, XK-62-2, was considered to be a new antibiotic close to the gentamicin-type antibiotics. Isolation of XK-62-2 was achieved by column chromatography using a silicic acid AR, 200 mesh (Mallinckrodt Chemical Works, St. Louis, Mo., U. S. A.) bed and eluting with the lower layer of chloroform - iso-propanol - 28% ammonium hydroxide (2 : 1 : 1, v/v).[14,27] The column was chromatographed to locate XK-62-2 by the above-mentioned technique. Active fractions were combined and concentrated repeatedly with addition of benzene to remove the organic solvents completely. The residue was dissolved in water and lyophilized to obtain the free base of XK-62-2. To obtain the sulfate salt of XK-62-2, the concentrated solution was adjusted to pH 4.5 with 6 N sulfuric acid and adding methanol.

Results and Discussion

Isolation and Physico-chemical Properties

Five grams of the crude antibiotic mixture precipitated by methanol was charged on the silicic acid bed in a glass column (25 mm in diameter and 500 mm in length), and eluted with the solvent at a flow rate of 30 ml/hour. Ten-ml fractions were collected and monitored chromatographically. XK-62-2 was eluted in the fractions 53~75 and XK-62-1 (gentamicin C_{1a}) in fractions 85~120. Under these conditions of fermentation and isolation, the C_1 and C_2 components of gentamicin were not detected. The yield was 900 mg of XK-62-2 free base.

The physico-chemical properties of XK-62-2 base are shown in Table 1. Its ultraviolet absorption spectrum shown in Fig. 1 exhibits no characteristic peaks in the ultraviolet range (220~360 nm). The infrared spectrum of XK-62-2 is given in Fig. 2. XK-62-2 shows Rf values given in Table 2 on thin-layer chromatography.

Table 1. Physico-chemical properties of XK-62-2

Elemental analysis	C 51.90	White amorphous powder
	H 8.81	Basic
	N 15.18	Soluble in water, methanol
Empirical formula	$C_{20}H_{41}N_5O_7$	Insoluble in chloroform, ethylacetate,
Melting point	260°C(dec.)	benzene, petroleum ether
Molecular weight	463	XK-62-2 sulfate is soluble only in water
$[\alpha]_D^{20}$	+116°(*c* 1, H_2O)	

Fig. 1. Ultraviolet absorption spectrum of XK-62-2

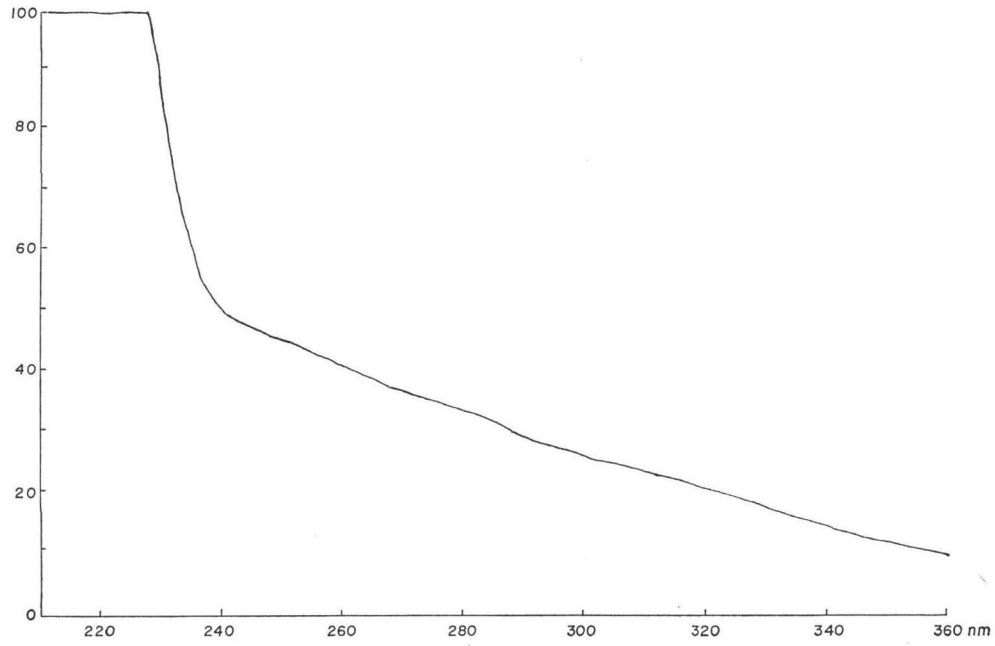

Fig. 2. Infrared spectrum of XK-62-2

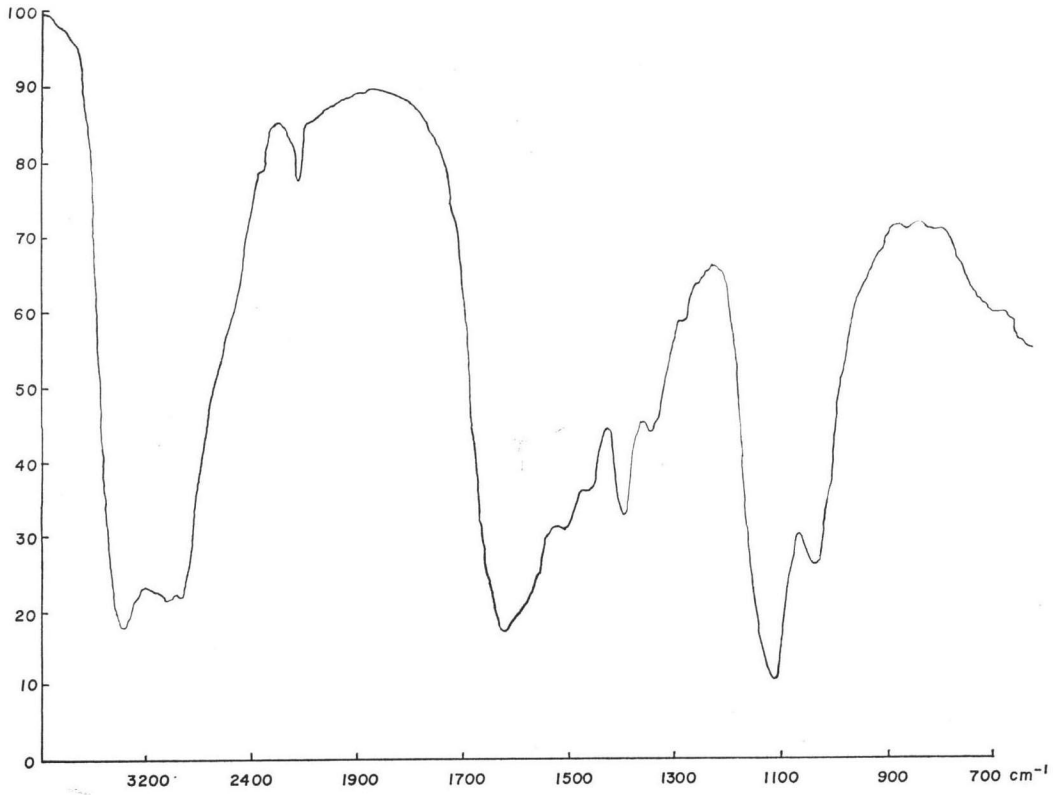

Table 2. Rf Values of XK-62-2 on thin-layer chromatography

Antibiotics		Rf	
		I[a]	II[b]
XK-62-2		0.86	0.27
Gentamicin C complex		0.85	0.24 ~0.30
Streptomycin		0.04	0.87
Neomycin B		0.37	0.50
Kanamycin A		0.77	0.76
Nebramycin factor	2	0.80	0.35
	4	0.80	0.50
	5	0.75	0.73
	6	0.54	0.54
Lividomycin B		0.63	0.81
Ribostamycin		0.75	0.72
Spectinomycin		0.70	0.63
Butirosin A		0.30	0.70

a) I. Upper layer of chloroform-methanol-17% ammonium hydroxide (2:1:1, v/v).
b) II. 10% Ammonium acetate-methanol (1 : 1, v/v).
Silica-gel (Silica-Rider 5B, Daiichi Pure Chemicals Co., Ltd.) sheet (200 mm×200 mm).
Ascending development at room temperature.
Bioautographed on *B. subtilis* KY 4273.

As a result of these studies, it was revealed that this new antibiotic XK-62-2 was a new water-soluble basic antibiotic and might be a member of the gentamicin group. Table 3 shows a comparison between XK-62-2 and other water-soluble basic aminoglycoside antibiotics including the gentamicin C complex by paper chromatography developed with the lower layer of chloroform - methanol - 17% ammonium hydroxide (2 : 1 : 1, v/v).[8] The first aminoglycoside antibiotic produced by genus *Micromonospora* was the gentamicin complex as reported by WEINSTEIN *et al.*[15] The Schering investigators later reported the production of sisomicin[16~18] and neomycin[19] by *M. inyoensis* and *Micromonospora* sp. 69-683, respectively. XK-62-2 isolated from the fermentation broth of *M. sagamiensis* MK-62 was clearly differentiated from those *Micromonospora*-produced antibiotics, as shown in Table 3. Its Rf value was also different from

Table 3. Comparison between XK-62-2 and other water-soluble basic aminoglycoside antibiotics on paper chromatography

Antibiotics		Rf	Antibiotics		Rf
Gentamicin	A	0.00	Nebramycin factor	2	0.00
	B	0.02		4	0.00
	C_{1a}	0.18		5	0.00
	C_2	0.38		6	0.00
	C_1	0.59	Streptomycin		0.00
Sisomicin		0.18	Mannosidostreptomycin		0.00
Antibiotic	460[a]	0.00	Hydroxystreptomycin		0.00
Neomycin	B	0.00	Lividomycin	A	0.00
	C	0.00		B	0.00
Neamine		0.00		D	0.00
Kanamycin	A	0.00	Butirosin	A	0.00
	B	0.00		B	0.00
	C	0.00	Ribostamycin		0.00
Spectinomycin		0.39	XK-62-1		0.18
Paromomycin		0.00	XK-62-2		0.49

Ascending development for 8 hours at room temperature in the lower phase of chloroform-methanol-17% ammonium hydroxide (2 : 1 : 1, v/v).
Bioautographed on *B. subtilis* KY 4273.
Filter paper: Whatman No. 1 (10 mm×400 mm).
a) Antibiotic 460 complex was prepared in this laboratory from the fermentation broth of *Micromonospora chalcea* var. *flavida* NRRL 3222.[28]

other related aminoglycoside antibiotics produced by *Streptomyces* and *Bacillus*. In fact further investigations on its structure indicated that XK-62-2 is a new member of the gentamicin C

Table 4. *In vitro* antibacterial activities of XK-62-2 and gentamicin C complex.

Test organisms	MIC (mcg/ml)	
	XK-62-2	Gentamicin C complex
Staphylococcus aureus		
KY 4279	0.004	0.004
KY 8942 (KM, PM, SM^r)	0.065	0.065
KY 8950 (SM, TC, PC, SA^r)	0.008	0.004
KY 8953 (SM, KM, PM, KM-B, TC, NM, EM^r)	0.004	0.004
KY 8956 (SM, PM, TC, EM, OLM^r)	0.001	0.002
KY 8957 (CM, SM, KM-B, TC, PM^r)	0.002	0.002
Bacillus subtilis KY 4273	0.004	0.002
cereus KY 3308	0.016	0.016
cereus var. *mycoides* KY 3312	0.008	0.008
Escherichia coli		
KY 4271	0.008	0.016
KY 8302 (CM, SM, KM, PM, TC, SPCM^r)	0.033	0.033
KY 8310 (CM, SM, KM, GM, KM-B, PM, TC, SPCM^r)	1.05	1.05
KY 8314 (SM^r)	0.008	0.008
KY 8315 (SM, KM, PM, NM^r)	0.016	0.016
KY 8327	8.3	8.3
KY 8321	2.15	2.15
KY 8348	5.25	5.25
Klebsiella pneumoniae KY 4275	0.008	0.008
Shigella sonnei KY 4281	0.07	0.07
Salmonella typhosa KY 4278	0.016	0.016
Proteus vulgaris KY 4277	0.03	0.03
KY 4296	0.021	0.04
mirabilis KY 4293	0.04	0.04
KY 4290	0.021	0.04
morganii KY 4298	0.04	0.04
rettgerii KY 4288	0.021	0.021
KY 4289	0.021	0.021
Serratia marcescens KY 4299	0.017	0.017
Providencia sp. 164 KY 8464	8.3	8.3
Herellea vaginicola KY 3637	0.13	0.13
KY 3640	0.26	0.13
Neisseria catarrharis KY 4282	0.002	0.002
Aerobacter aerogenes KY 4272	0.008	0.008
Candida albicans KY 5011	>10	>10
Aspergillus niger	>10	>10

Assayed by agar dilution assay at pH 8.0.

KM; kanamycin A, NM ; neomycin B, KM-B; aminodeoxykanamycin,
PM ; paromomycin, EM ; erythromycin A, SA ; sulfonamide,
SM ; streptomycin, OLM; oleandomycin, PC ; penicillin G,
TC ; tetracycline, CM ; chloramphenicol, SPCM; spectinomycin.

complex having a methyl group on the 6'-amino position in purpurosamine of gentamicin C_{1a}.[20]

In Vitro Antibacterial Activities of XK-62-2

The antibacterial activity of XK-62-2 *in vitro* against a selected group of gram-positive and gram-negative bacteria is shown in Table 4. The activity is broad-spectrum and almost equal to that of the gentamicin C complex. XK-62-2 exhibits particularly high activity against penicillin-macrolide sensitive or resistant *Staphylococcus*, kanamycin sensitive or resistant

Table 5. *In. Vitro* antibacterial activities of XK-62-2 against clinically isolated *Pseudomonas aeruginosa*

	MIC (mcg/ml)				
	XK-62-2	Streptomycin	Kanamycin A	Gentamicin C complex	Gentamicin C_{1a}
KY 4276	0.33	2.6	1.63	0.33	0.33
KY 8520	0.33	5.2	104	0.65	0.33
KY 8510	0.33	0.65	13	1.3	2.6
KY 8511	42	2500	13	42	42
KY 8512	0.33	42	3.3	0.65	0.33
KY 8562	42	156	167	62	42
KY 8514	0.65	156	208	1.3	0.33
KY 8516	0.33	0.65	26	1.3	5.2

Assayed by agar dilution assay at pH 8.0.

Escherichia, and against gram-negative rods *Pseudomonas*, *Proteus*, *Klebsiella*, *Salmonella etc.* *E. coli* KY 8321 is known as a gentamicin-resistant strain having the 2''-OH nucleotidylating enzyme initially reported by BENVENISTE *et al.*[21] *Providencia* sp. 164 is also a gentamicin-resistant strain which has a 2'-N-acetylating enzyme recently reported by the same authors.[22,23] XK-62-2 and gentamicin C complex showed a cross-resistance against these resistant microorganisms. This antibiotic, like other aminoglycoside antibiotics, displays very little activity against *Streptococcus*, yeasts and filamentous fungi. The *in vitro* antibacterial activity against clinically isolated *Pseudomonas aeruginosa* in comparison with streptomycin, kanamycin, gentamicin C complex and gentamicin C_{1a} is shown in Table 5. Some of the gentamicin-resistant strains (KY 8511 and KY 8562) showed a cross-resistance against XK-62-2. *P. aeruginosa* KY 8511 is a clinical isolate which has a gentamicin 3-N-acetylating enzyme resembling that of *P. aeruginosa* 130 described by BRZEZINSKA *et al.*[24]

The most significant property of the antibacterial activity of XK-62-2 was however its high activity against gentamicin C_{1a}-resistant *Pseudomonas* such as KY 8510 and KY 8516. Our preliminary tests show that *P. aeruginosa* KY 8510 and 8516 isolated from clinical patients appear resistant to gentamicin C_{1a} by an enzymatic acetylation of the 6'-NH_2 group in the purpurosamine moiety. This inactivation mechanism was initially reported by BENVENISTE *et al.*[25] Gentamicin C_{1a} and sisomicin are inactivated by these microorganisms. The gentamicin C complex has reduced activity against these resistant strains. The higher activity of XK-62-2 against these two strains can be taken for granted from its structure,[20] because the presence of 6'-N-methyl in purpurosamine should protect it from enzymatic 6'-N-acetylation by these strains. Since

gentamicin C_1 also possesses 6'-N-methyl in purpurosamine, it is indeed more potent than gentamicin C_{1a}, against organisms having this inactivating mechanism.[25]

In Vivo Activities of XK-62-2

XK-62-2 appears to be also highly active against various bacterial infections in mice, and has an intravenous acute LD_{50} in mice of 93 mg/kg.

WEINSTEIN *et al.*[26] reported the chronic toxicity of gentamicin, and MARQUEZ *et al.* recently noted that the presence of 6'-N-methyl in purpurosamine of gentamicin C components reduces the degree of ataxia and impairment of the righting reflex caused by that antibiotic.[27] Since XK-62-2 also has a 6'-N-methyl group, it too may be expected to show less chronic toxicity than gentamicin. Further studies on the activity *in vivo* of XK-62-2 will be reported elsewhere.

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