KIJANIMICIN (Sch 25663), A NOVEL ANTIBIOTIC PRODUCED BY *ACTINOMADURA KIJANIATA* SCC 1256

FERMENTATION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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A novel antibiotic complex has been isolated from the fermentation broth of a new species of *Actinomadura*, *A. kijaniata* SCC 1256. The complex was separated from the broth by a solvent extraction procedure and consists of 1 major component, designated kijanimicin, and 3 minor components. Kijanimicin was isolated from the complex by column chromatography and/or preparative high pressure liquid chromatography. Structurally the compound is a unique, large acid enol antibiotic and possesses an unusual *in vitro* spectrum of activity against some Gram-positive and anaerobic microorganisms. *In vivo* it has also shown interesting activity against malaria.

In the course of our screening for novel antibiotic producing organisms, a culture was noted that appeared distinct in pigmentation from cultures previously observed. This strain, designated SCC 1256, was found to be producing a complex of novel antibiotics from which the major component, designated kijanimicin (Sch 25663) was separated. These antibiotics were active against an unusual spectrum of microorganisms including anaerobes, particulary *Propionibacterium acnes*. *In vivo* activity against *Plasmodium berghei* and *P. chabaudi* in mice was also observed.

This paper details the fermentation and isolation of the antibiotic complex, as well as purification, identification, characterization, ^{14}C labelling, and biological activity of the major component, kijanimicin. Details of the taxonomic evaluation of the producing organism as well as chemical structure elucidation appear elsewhere[1~3].

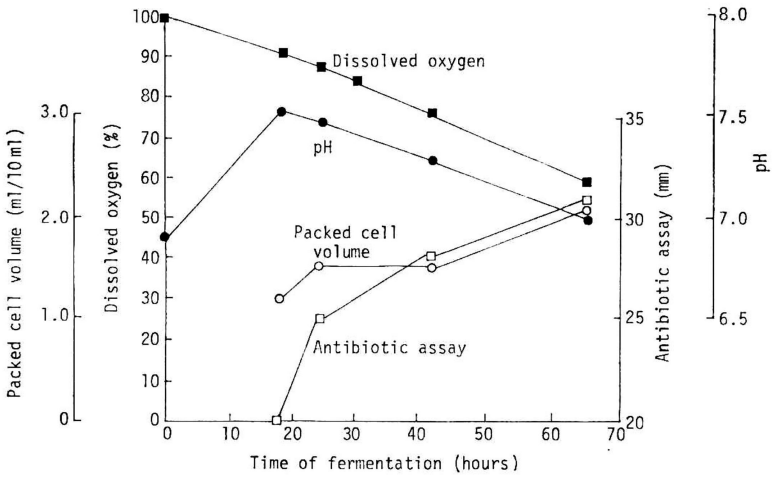
Producing Culture

The producing culture was isolated from a soil sample collected in Kenya. The culture has been identified as a new species of the genus *Actinomadura*, designated *A. kijaniata*[1]. The strain has been deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A. and has been assigned accession number ATCC 31588.

Production of Kijanimicin

The medium used for the preparation of the inoculum for antibiotic production consisted of 0.3% beef extract, 0.5% tryptose, 0.1% dextrose, 2.4% potato starch and 0.2% calcium carbonate. The pH of this medium was adjusted to 7.2 prior to sterilization. A 300 ml Erlenmeyer flask containing 70 ml of this medium was inoculated with 5 ml of the stock suspension of the producing strain maintained at −20°C. The flask was incubated at 30°C on a rotary shaker at 250 rpm for 48 hours.

Fig. 1. Time course study of kijanimicin fermentation.



The resulting seed culture (25 ml) was transferred to a 2-liter Erlenmeyer flask containing 500 ml of the same seed medium and incubation was carried out under identical conditions for 48 hours. This secondary seed culture was used to inoculate 10 liters of the fermentation medium composed of 0.5% yeast extract, 1% dextrose, 2% soluble starch, 0.5% NZ-Amine A, 0.4% calcium carbonate and 0.024% cobalt chloride. Fermentation was carried out in a 14-liter New Brunswick Scientific Laboratory Fermentor at 30°C with 3.5 liters of air per minute and agitation at 350 rpm. The initial pH of the fermentation medium was adjusted when necessary to 6.8.

The course of a typical 10-liter fermentation is illustrated in Fig. 1. The pH and dissolved oxygen levels were continuously monitored during the entire fermentation by means of probes submerged in the vessel. Microbial growth was determined by packed cell volume. Determinations of antibiotic activity were made by disc assays against *Sarcina lutea* on agar plates. Peak kijanimicin production was reached after about 66 hours of fermentation.

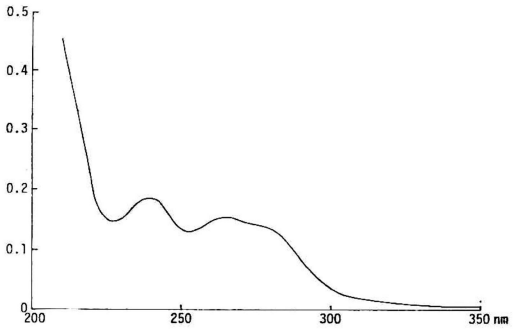
Extraction and Isolation of Kijanimicin

A 60-liter aliquot of the fermentation broth was extracted twice with ethyl acetate. After separation the solvent phases were combined and concentrated to dryness. The dry residue was dissolved in acetone, added to a mixture of ethyl ether - hexane (6: 4) and the resulting buff colored precipitate (antibiotic complex) filtered and dried over vacuum. Silica gel thin-layer chromatography of the complex in chloroform - methanol (93: 7) visualized by bioautography against *Sarcina lutea* resulted in four biologically active components. The components were labelled 1 through 4 based on decreasing Rf values. Component two which accounts for the major portion of the activity has been designated kijanimicin.

Of 58 g of the crude kijanimicin thus obtained, 10 g was dissolved in methanol and chromatographed on a 1 kg column of silica gel and eluted with chloroform - methanol (93: 7). The desired active fractions as determined by chromatography were then combined, evaporated to dryness, redissolved in methanol and precipitated with an ethyl ether - hexane mixture. The precipitate was filtered, dried in vacuum and 3 g of kijanimicin was obtained as an off-white powder.

Isolation of kijanimicin was also obtained by preparative high pressure liquid chromatography using a Waters Prep LC/System 500 fitted with two 300 g silica gel columns. The columns were eluted by gradient elution using methanol - methylene chloride, 8: 92, 16: 84, 50: 50. The desired fractions were combined and precipitates yielded 3.1 g of kijanimicin from 10 g of complex.

Fig. 2. UV absorption spectra of kijanimicin.



Physico-chemical Characteristics of Kijanimicin

Kijanimicin obtained as an amorphous off-white powder showed the following properties: The compound is soluble in ethanol, methanol and acetone but insoluble in ether, petroleum ether and water. The UV absorption spectrum in methanol (Fig. 2) shows peaks at 239, 264, and 274 nm with

Fig. 3. Infrared spectrum of kijanimicin ($CHCl_3$).

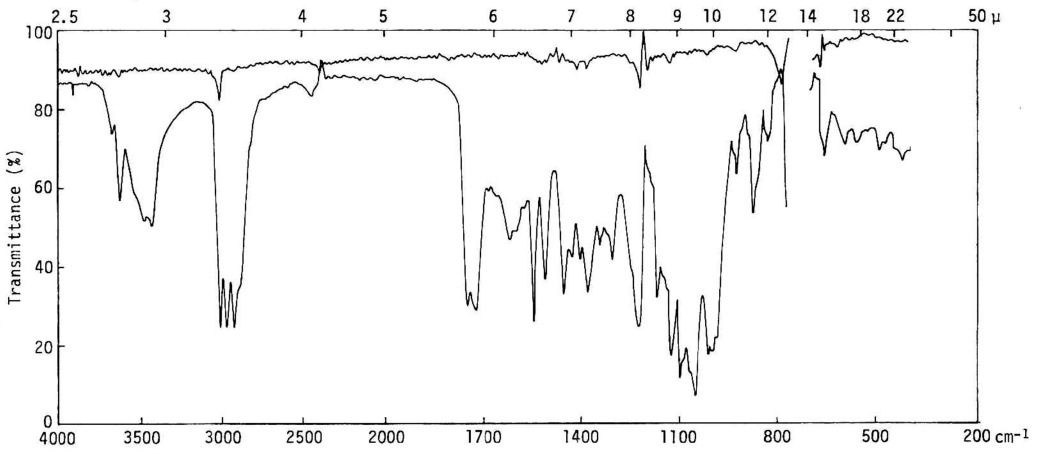


Table 1. Chromatographic migration of kijanimicin.

Paper chromatography system*	Rf**	Thin-layer chromatography system****	Rf
n-Butanol - water - acetic acid (4: 5: 1) upper phase used, ascending	0.96	Acetone - toluene (3: 2)	0.15
		Chloroform	0.00
Benzene - methanol (9: 1), descending	0.78	Methanol	0.74
Propanol - water - acetic acid (4: 5: 1), descending	1.00	Toluene - methanol (96: 4)	0.00
		Toluene - methanol (94: 6)	0.00
Propanol - pyridine - acetic acid - water (6: 4: 1: 3), ascending	0.91	Toluene - methanol (88: 12)	0.05
80% Methanol plus 3% sodium chloride, descending***	0.96		
Toluene - petroleum ether - acetone (3: 2: 3), descending	0.88		

* Whatman No. 1 paper

** Detection: bioautography *vs Sarcina lutea*

*** Paper buffered with 0.95 M Na_2SO_4 plus 0.05 M $NaHSO_4$.

**** Analtech Silica Gel GF TLC plates

an $E^{1\%}_{1cm}$ of 92, 75, and 65 respectively. The infrared spectrum (Fig. 3) revealed sharp peaks at 1230, 1510, 1545, 1625, 1730, 1755 cm^{-1}.

Elemental analysis shows the compound contains 60.14% C, 7.78% H and 2.12% N, and has a specific optical rotation of ($[\alpha]_D^{26}$ −124.2° (*c* 0.2, MeOH)).

The Rf values of kijanimicin on Whatman No. 1 paper and on Analtech Silica Gel GF thin-layer plates in several solvent systems are given in Table 1.

Kijanimicin is stable in buffered solution from pH 6~8 at room temperature and up to 100°C for at least 30 minutes. It loses activity immediately at room temperature at pH 2~3 and pH 9~10. The total structure and absolute configuration of kijanimicin is reported separately by MALLAMS *et al.*[2,3] It has been shown by chemical degradation, spectroscopic studies and X-ray crystallographic studies to have a novel tetronic acid structure. The molecule has a branched tetrasaccharide unit as well as a novel nitrosugar moiety glycosidically linked to the aglycone.

From the above data it is concluded that kijanimicin is a new antibiotic belonging to the tetrocarcin[4,5] and antlermicin[6,7] family.

^{14}C-Labelling of Kijanimicin

Incorporation of [1-^{14}C]sodium acetate, [2-^{14}C]sodium acetate, [1-^{14}C]sodium propionate and [methyl-^{14}C]-L-methionine into kijanimicin in shake-flask fermentations were 25, 6, 51 and 6%.

Table 2. *In vitro* activity of kijanimicin against various bacteria and fungi.

Organism		No. of strains	Average MICs (mcg/ml) (24 hours incubation)
Anaerobes	*Propionibacterium acnes*	24	0.86[a,b]
	Bacteroides corrodens	1	>32
	Bacteroides fragilis	2	>32
	Clostridium novyi	1	>32
	Clostridium septicum	1	32
Gram-positive aerobes	*Bacillus subtilis*	1	<0.13
	Staphylococcus sp.	16	64
	Streptococcus sp.	4	24
Gram-negatives	*Escherichia coli*	5	>128
	Klebsiella sp.	3	>128
	Enterobacter sp.	4	64
	Proteus sp.	2	>128
	Pseudomonas aeruginosa	3	>128
	Salmonella schottmuelleri	1	>128
	Serratia sp.	2	>128
Yeasts	*Candida* sp.	7	>25
Dermatophytes	*Epidermophyton floccosum*	1	>25[c]
	Trichophyton sp.	2	17.5[c]
	Microsporum sp.	2	17.5[c]

[a] MIC tetracycline 2.46 mcg/ml, clindamycin <0.26 mcg/ml.
[b] 48 hours incubation.
[c] 72 hours incubation.

Table 3. *In vivo* activity of kijanimicin against *Plasmodium berghei* KBG in mice. Subcutaneous treatment starting 3 days post-infection.

Compound	mg/kg	No. of days treated	Survivors	Survival time (Average)		Parasitemia at sacrifice
			Total	No. of mice	Days	
Controls	—	—	0 / 33	33	8.6	
Kijanimicin Na	100	1	0 / 10	10	15.3	
〃	250	1	9 / 10	1	23	
				9	>31	0
〃	50	5	9 / 10	1	20	
				9	>31	0
Daraprim	25	1	0 / 10	10	15.9	
〃	100	1	8 / 10	2	20	
				8	>31	0

A 10-liter fermentation, using [1-^{14}C]sodium propionate as radiolabelled precursor was performed to prepare ^{14}C-kijanimicin. The same fermentation procedures as described above were used, except for addition of 8.7 mCi [1-^{14}C]sodium propionate at 48 hours and harvesting at 72 hours. Crude antibiotic isolated from the fermentation broth weighed 7.8 g. ^{14}C-Kijanimicin was isolated following silica gel column chromatography of the crude antibiotic complex. The final product weighed 1.73 g, and showed a total radioactivity of 646 μCi and a specific radioactivity of 0.24 μCi/mg.

Biological Properties

Kijanimicin was shown to have good activity against *Propionibacterium acnes* and generally weaker activity against other organisms tested. *In vivo* it showed interesting activity against malaria.

In vitro dilution tests to determine minimum inhibitory concentrations (MICs) were done using thioglycollate broth for Gram-positive, Gram-negative, and anaerobic organisms and SABOURAUD-dextrose broth for fungi. The results shown in Table 2 indicate that kijanimicin has good activity against the 24 strains of *P. acnes* studied. It was three times more potent than tetracycline and approximately 1/3 as active as clindamycin *in vitro*. The antibiotic has weak activity against other anaerobes, aerobic Gram-positive bacteria, and dermatophytes but shows good activity against *B. subtilis*. It was not active against Gram-negative bacteria and yeasts at the concentrations tested.

The antibiotic was found to be effective *in vivo* against rodent malaria. A series of experiments were done using three different strains of *Plasmodium berghei* and one strain of *P. chabaudi* utilizing the sodium salt or free acid of kijanimicin. Mice were infected intraperitoneally and treated subcutaneously or orally either before or after infection for up to 5 days. A representative experiment is shown in Table 3.

The antibiotic was as active as daraprim when the mice were treated 3 days post-infection with a single dose (250 mg/kg) or with multiple doses (50 mg/kg for five days). Other experiments showed kijanimicin to have activity similar to chloroquine and primaquine but less than that of quinacrine and mefloquine. The compound was not effective orally.

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