A NEW ANTIBIOTIC VICTOMYCIN (XK 49-1-B-2)

II. ISOLATION, PURIFICATION AND PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES

SEIGO TAKASAWA, ISAO KAWAMOTO, RYO OKACHI, MASAHIRO KOHAKURA*, RYOSUKE YAHASHI and TAKASHI NARA

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.
3-6-6 Asahimachi, Machidashi, Tokyo, Japan
*Fuji Factory, Kyowa Hakko Kogyo Co., Ltd. Suntogun,
Shizuokaken, Japan

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A new antibiotic, victomycin, is active against a variety of Gram-positive and Gram-negative bacteria, and also has *in vivo* activity against solid Sarcoma 180 and EHRLICH ascites carcinoma. It belongs to the phleomycin-bleomycin group of antibiotics and has been differentiated from all known phleomycins and bleomycins by its physicochemical properties and thin-layer chromatograms.

In the previous paper[1], it was reported that antibiotic XK 49-1-B-2, designated victomycin, was produced by *Streptosporangium violaceochromogenes* MK 49, and also some cultural conditions were reported. This microorganism was found to produce at least 2 groups of antibiotics, the one (XK 49-2) active against only Gram-positive becteria and the others (XK 49-1-B) active against Gram-positive and Gram-negative bacteria. Purification by CM-Sephadex chromatography showed the presence of at least three antibiotics in XK 49-1-B of which XK 49-1-B-2 was the main component. This paper deals with the isolation, purification, and physicochemical and biological properties of victomycin (XK 49-1-B-2).

Isolation and Purification

A medium containing 2% glucose, 3% corn steep liquor and 0.1% $CaCO_3$, pH 7.2 (before sterilization) was used for antibiotic production. The strain was cultured in 1,000 liters medium in 2,000-liter tank. The fermentation was run at 30°C for 12 days with 300 liters/min aeration and continuous agitation at 150 r.p.m. The fermentation broth was adjusted to pH 4.0 with HCl, filtered with Radiolite No. 600 (Showa Kagaku Kogyo Co., Ltd.) and adjusted to pH 6.8. The filtrate (900 liters) was passed through a 50 liter-column of Amberlite IRC-50 (H^+). The column was washed with water and the antibiotics were eluted with 0.5 N HCl. Antibiotic XK 49-2, active only against Gram-positive bacteria, was not absorbed on IRC-50 resin. The eluate containing XK 49-1-B-2 was adjusted to pH 7.0 with Amberlite IR-4B (OH^-), and passed through a 500 ml-column of IRC-50 (NH_4^+). After washing with 0.3 N NH_4OH and water, XK 49-1-B was eluted with 0.5 M ammonium formate. The XK 49-1-B fraction was diluted with 5 volumes of water, adjusted to pH 6.8 and passed through a 200-ml column of IRC-50 (H^+) for desalting. After washing with water, XK 49-1-B was eluted with 0.5 N HCl, neutralized with Dowex 44 (OH^-) and concentrated to dryness *in vacuo*. The crude preparation was dissolved in 50% methanol, passed through a 500-ml column of Sephadex LH-20

and eluted with 50% aqueous methanol. The active fraction was concentrated *in vacuo* and precipitated with 10 volumes of acetone to obtain 1.2 g of a dark green powder. Five hundred mg of this powder was dissolved in 50 ml of 0.05 M aqueous ammonium formate solution, passed through a 50-ml column of CM Sephadex C-25, washed with 500 ml of 0.1 M aqueous ammonium formate and eluted by the gradient elution from 0.1 to 1.0 M ammonium formate. On the CM Sephadex column, XK 49-1-B was separated into 3 compounds designated XK 49-1-B-1, XK 49-1-B-2 and XK 49-1-B-3, eluted at 0.18~0.24 M, 0.3~0.37 M and 0.45~0.53 M of ammonium formate, respectively. XK 49-1-B-2 was the major component. XK 49-1-B-2 fractions were combined, diluted with 5 volumes of water and passed through a 50 ml-Amberlite CG-50 (H^+) column. After washing with water, XK 49-1-B-2 was eluted with 0.5 N HCl, adjusted to pH 6.0 with Dowex 44 (OH^-) and concentrated to dryness *in vacuo*. The preparation was dissolved in a small amount of 50% aqueous methanol, applied to the top of a Sephadex LH-20 column and eluted with 50% aqueous methanol. The active fractions were adjusted to pH 5.0 with HCl, concentrated to a small volume and treated with 10 volumes of acetone to obtain 56 mg of blue powder, after filtration and drying *in vacuo*. This powder of XK 49-1-B-2 is victomycin hydrochloride.

Characterization of Victomycin

Victomycin was isolated as a blue-colored, amorphous hydrochloride and is highly soluble in water, soluble in methanol, slightly soluble in ethanol and insoluble in such organic solvents as higher alcohols, acetone, chloroform, ethyl acetate, butyl acetate, ethyl ether and benzene. The melting point was not sharp and decomposed at above 190°C. The elemental analysis was: C, 40.36; N, 15.36; H, 6.04; S, 1.98; Cl, 7.74 and Cu, 4.0%. As seen in Fig. 1, victomycin hydrochloride had UV maxima at 244 nm ($E^{1\%}_{1cm}$ 117) and 293 nm ($E^{1\%}_{1cm}$ 86). The ratio of the intensity of the absorption at 244 nm to that at 293 nm was 1.36. The IR spectrum of victomycin hydrochloride in KBr tablet is presented in Fig. 2. The optical rotatory dispersion (ORD) curve in water (*c*, 0.5) of victomycin shows the positive COTTON effect at 558 nm, peak $[\alpha]^{30}_{610}$ −144.0, $[\alpha]^{30}_{D}$ +107.2 and trough $[\alpha]^{30}_{500}$ −100.0. The UV absorption prevented obtaining the ORD curve under 350 nm. Victomycin gave positive SAKAGUCHI, PAULI and EHRLICH reactions and negative ninhydrin reaction.

In paper chromatography, Rf values of this antibiotic were 0.75 with 20% aqueous ammonium chloride, 0.72 with 10% aqueous ammonium chloride, 0.40 with 1% aqueous ammonium chloride, 0.15 with 0.5% aqueous ammonium chloride, 0.00 with water-saturated *n*-butyl alcohol, 0.08 with *n*-butyl alcohol-acetic acid-water (3:1:1) and 0.02 with water-saturated *n*-butyl alcohol containing 2% of *p*-toluene sulfonic acid and 2% of piperidine. Rf values on silicagel TLC of victomycin and

Fig. 1. UV spectrum of victomycin in aqueous solution

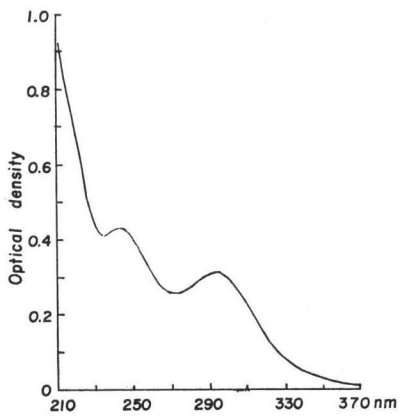


Fig. 2. IR spectrum of victomycin in KBr Tablet

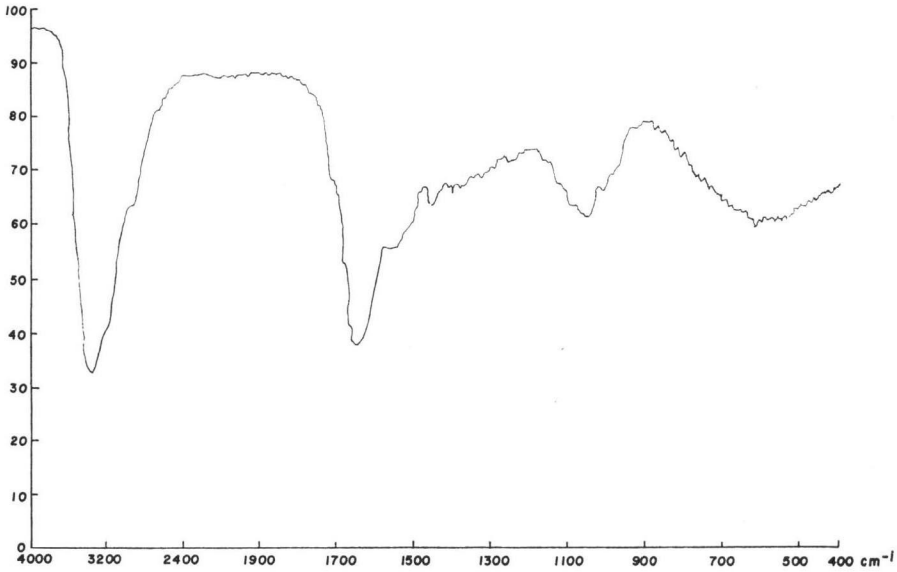


Table 1. Rf values of victomycin and related compounds in silica gel TLC

	Solvent system			
	1	2	3	4
Victomycin	0.20	0.75	0.35	0.40
Bleomycin A_2	0.28	0.46	0.55	0.08
Bleomycin A_5	0.05	0.60	0.25	0.08
Bleomycin B_2	0.54	0.80	0.78	0.10
Bleomycin B_4	0.14	0.75	0.68	0.10
Zorbonomycin B	0.35	0.77	0.83	0.29

Solvent 1: chloroform - methanol - 17 % ammonia water (2:1:1 upper layer)
2: 10 % ammonium acetate - methanol (1:1)
3: methanol - 10 % ammonium acetate - 10 % ammonia water (10:9:1)
4: 0.05 M citrate buffer (pH 6.9)

Antibiotics were detected by bioautography on agar trays seeded with *Bacillus subtilis* KY 4273.

Table 2. Stability of victomycin in aqueous solution

	pH	Activity remaining (%)			
		0	30 min.	2 hrs.	4 hrs.
30°C	2.5	110	110	100	100
	7.0	100	100	80	100
	9.5	100	103	80	80
60°C	2.5	110	115	70	70
	7.0	100	100	95	95
	9.5	100	55	17	8

The aqueous solutions of victomycin at 50 μg/ml were adjusted to pH 2.5, 7.0 and 9.5 and kept at 30°C and 60°C. After storage for the specified hours, a part of each solution was taken out, adjusted to pH 7.0 and measured for activity against *B. subtilis* KY 4273

some bleomycin components (A_2, A_5, B_2, B_4 and zorbonomycin B) are shown in Table 1. As seen in Table 2, victomycin was stable in acidic and neutral solution but somewhat unstable in alkaline solution.

Biological Activity

As seen in Table 3, victomycin has potent antibacterial activity against Gram-positive and Gram-negative bacteria and also is active against many bacteria which are resistant to some antibiotics. Furthermore, victomycin has potent antitumor activity against solid Sarcoma 180

Table 3. Antibacterial spectrum of victomycin

Organism	MIC* (μg/ml)
Streptococcus faecalis ATCC 10541	>41.7
Staphylococcus aureus ATCC 6538p	0.066
S. aureus KY 8942 (R-SM, KM, PM)	>4.2
S. aureus KY 8950 (R-SM, TC, PC, SA)	0.26
S. aureus KY 8953 (R-SM, KM, NM, TC, EM)	>4.2
S. aureus KY 8956 (R-SM, KM, PM, TC, EM)	>4.2
S. aureus KY 8957 (R-SM, KM, PM, TC, CP)	>4.2
Bacillus subtiliis KY 4273	0.001
Bacillus cereus ATCC 9634	0.066
B. cereus var. *mycoides* ATCC 9463	<0.001
Escherichia coli ATCC 26	0.004
E. coli KY 8310 (R-SM, KM, GM, TC, CP)	0.13
E. coli KY 8302 (R-SM, KM, TC, CP)	0.26
E. coli KY 8314 (R-SM)	0.26
E. coli KY 8315 (R-SM, KM, PM, NM)	0.033
Proteus vulgaris ATCC 6897	1.32
Pseudomonas aeruginosa BMH No. 1	10.4
Shigella sonnei ATCC 9290	0.053
Salmonella typhosa ATCC 9992	0.053
Klebsiella pneumoniae ATCC 10031	0.053

* Assayed with agar dilution method at pH 8.0 R: Resistant, SM: Streptomycin, KM: Kanamycin, PM: Paromomycin, NM: Neomycin, GM: Gentamicin, TC: Tetracycline, CP: Chloramphenicol, SA: Sulfonamide

Table 4. Activity against Sarcoma 180 (Solid)

	Dosage (mg/kg)×days (ip)	Average tumor weight (g)
Victomycin	3×8	0.36±0.20
	2×8	0.72±0.23
None		3.13±1.03

At 24 hours after the subcutaneous transplantation of about 2 mm^3 fragments of tumor solid into the axillary region of mice (dd strain, male, 19±1 g of body weight), a solution containing 2 and 3 mg/kg of victomycin was administrated intraperitoneally once daily for 8 days

Tumor solids were taken out and weighed at 10 days after transplantation

Table 5. Activity anainst EHRLICH ascites carcinoma

	Dosage (mg/kg) ×days (ip)	Average survival days	Survival for 60 days
Victomycin	3×6	>35.1	2/10
	2×6	>34.0	1/10
None		15.6	0/10

The daily intraperitoneal injection of 2 and 3 mg/kg of victomycin for 6 days to mice (dd strain, male, body weight; 22±1 g) was started 24 hours after the intraperitoneal transplantation of 5×10^5 tumor cells

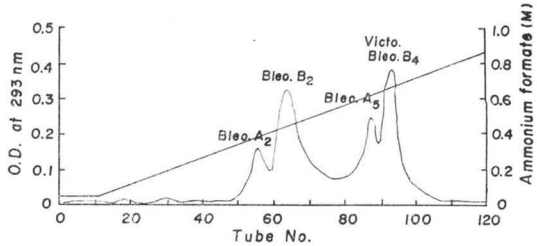
and EHRLICH ascites carcinoma, as shown in Table 4 and Table 5, respectively. It was however inactive against Leukemia L 1210. The LD_{50} of victomycin was about 25 mg/kg, intravenous, in mice.

Discussion

From the physicochemical and biological properties described above, victomycin seems to belong to the phleomycin-bleomycin group antibiotics. Since the ratio of the intensities of

the absorptions at 244 and 293 nm is 1.36, victomycin is similar to the bleomycins[3] and zorbonomycin B[5] (ratio of 1.1~1.4) and different from the phleomycins[4], zorbamycin[5], zorbonomycin C[5], YA-56X[6] and YA-56Y[6] (ratio of 2.7~3.0). The A group of the bleomycins, for example, A_1, demethyl A_2, A_2, A_2'-a, A_2'-b, A_3, A_4, A_5 and A_6 and bleomycin B_1 give negative SAKAGUCHI reactions, while the group of bleomycins, B_2, B_3, B_4, B_5 and B_6 give positive SAKAGUCHI reactions[2,7,8]. Since victomycin gives a positive SAKAGUCHI reaction, it may be related to the bleomycin B group. A mixture of victomycin, bleomycin A_2, A_5, B_2 and B_4 was dissolved in a small amount of 0.05 M aqueous ammonium formate and chromatographed over a CM Sephadex C-25 column (1.2×50 cm). The column was washed with 50 ml of 0.05 M aqueous ammonium formate and then eluted gradiently by 0.05~1.0 M of aqueous ammonium formate. As seen in Fig. 3, four peaks were observed by UV scan at 293 nm. Each peak was collected and desalted with IRC-50 (H^+) and Sephadex LH-20. Each fraction was analyzed by silicagel TLC. The first peak was bleomycin A_2 (0.36 M), the second B_2 (0.46 M), the third A_5 (0.65 M) and the last was the mixture of victomycin and bleomycin B_4 (0.72 M). Since victomycin was eluted together with bleomycin B_4, it is different from bleomycin B_2, B_3, B_5 and B_6 which are eluted with different concentrations of ammonium formate from bleomycin B_4[3,7,8]. Victomycin is distinctly different from bleomycin B_4, since its Rf values are 0.35 and 0.40 on silicagel TLC with solvent 3 and solvent 4 while those of bleomycin B_4 are 0.68 and 0.10, respectively (Table 1). Victomycin is also different from zorbonomycin B on silicagel TLC.

Fig. 3. CM sephadex chromatography of victomycin and relatied compounds.
Column: CM-sephadex C-25 (1.2×50 cm)
Elction: 0.05~1.0 M ammonium formate
Flow rate: 40 ml/hour



Platomycins, a new phleomycin-bleomycin-like group of antibiotics, were also discovered by NARA *et al*[9]. Victomycin has been differentiated from the platomycins and details will be reported in the near future.

These data clearly show that victomycin is different from any of the known bleomycin components.

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References

1) KAWAMOTO, I.; S. TAKASAWA, R. OKACHI, M. KOHAKURA, I. TAKAHASHI & T. NARA: A new antibiotic victomycin (XK 49-1-B-2). I. Taxonomy and production of the producing organism. J. Antibiotics 28: 358~365, 1975
2) UMEZAWA, H.; K. MAEDA, T. TAKEUCHI & Y. OKAMI: New antibiotics, bleomycin A and B. J. Antibiotics, Ser. A 19: 200~209, 1966
3) UMEZAWA, H.; Y. SUHARA, T. TAKEUCHI & K. MAEDA: Purification of bleomycins. J. Antibiotics, Ser. A 19: 210~215, 1966
4) IKEKAWA, T.; F. IWAI, H. HIRANAKA & H. UMEZAWA: Separation of phleomycin components and their properties. J. Antibiotics, Ser. A 17: 194~199, 1964
5) ARGOUDELIS, A. D.; M. E. BERGY & T. R. PYKE: Zorbamycin and related antibiotics. I. Production, isolation and characterization: J. Antibiotics 24: 543~557, 1971

6) ITO, Y.; Y. OHASHI, S. KAWABE, M. SAKURAZAWA, T. OZAWA, Y. EGAWA & T. OKUDA: The antibiotic YA-56 complex: Isolation, purification and physicochemical properties of the main components. J. antibiotics 26: 77~83, 1973
7) FUJII, A.; T. TAKITA, K. MAEDA & H. UMEZAWA: New components of bleomycin. J. Antibiotics 26: 396, 1973
8) FUJII, A.: Study on antitumor antibiotics, bleomycin. (Thesis for the doctorate awarded by Tokyo University) 1971
9) NARA, T.; S. TAKASAWA, R. OKACHI, I. KAWAMOTO, S. SATO, M. YAMAMOTO, T. SATO & A. MORIKAWA: Antibiotics platomycin A and B. German Patent 2,408,121: 1974