K-582, A NEW PEPTIDE ANTIBIOTIC. I

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A new basic peptide antibiotic designated as K-582 was isolated, purified and characterized. When K-582 was applied to a column of Al_2O_3 or Bio-Gel P-2 or CM Sephadex, two major peaks which were named Fraction I (K-582 A) and Fraction II (K-582 B) were obtained. The nitrogen content, the behavior in color reaction, the absorption bands of amide linkages in the infrared absorption spectrum, ^1H NMR spectrum and C-13 NMR spectrum indicated the peptide nature of K-582 A and K-582 B. K-582 was effective against yeasts, but inactive against other Gram-positive bacteria, Gram-negative bacteria and *Mycobacterium*. The toxicity was low in mice.

During our screening program for substances with antibiotic activity, we found a new substance named K-582 produced by the strain 582 M which was isolated from a soil sample collected in Sendai and from the results of detailed taxonomical studies the strain was identified as *Metarhizium anisopliae* (METSCH.) SOROK. var. *anisopliae*.

The antibiotic, K-582, exhibited a significant growth inhibition of *Candida* in liquid media, of viruses in tissue culture and of ascites tumor in mice.

This paper deals with the characterization of *Metarhizium anisopliae* (METSCH.) SOROK. var. *anisopliae* 582 M, the fermentation process, the isolation procedure and the properties of K-582.

Taxonomical Studies

Strain 582 M was isolated by SHIGEJI KONDO from a soil sample collected in the vicinity of Sendai City, Miyagi Prefecture, Japan. The taxonomical characterization was carried out according to the methods described by BARNETT[1], BARRON[2] and TULLOCH[3] and the strain 582 M was classified as *Metarhizium anisopliae* (METSCH.) SOROK. var. *anisopliae*.

1. Macroscopic description

Colonies on CZAPEK-peptone agar grew rapidly, attaining a diameter of 4.5 ~ 5.0 cm in 2 weeks at 28°C. The colonies are more or less fasciculated, plane or sometimes lightly furrowed radially. They have white margins with olivaceous to dark olivaceous colored sporulating areas in center. Colonies are often intermixed or zonate. Transformation to light yellow to dark olivaceous colors occurs. Exudate is lacking. The odor was conspicuously pungent similar to that of streptomyces (Tables 1, 2 and 3.).

Table 1. Growth of colony (mm) of *Metarhizium anisopliae* (METSCH.) SOROK. var. *anisopliae* 582 M on different media.

Temperature (°C)	CZAPEK peptone agar			CZAPEK-DOX agar			SABOURAUD agar		
	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
20	13×15	19×18	27×25	7× 7	17×17	28×30	12×13	19×20	23×24
30	16×19	23×24	30×23	7×10	18×18	28×29	16×19	23×23	30×31
37	—	—	4× 4	—	2× 2	3× 4	—	1.5× 2	3× 3

Table 2. Details of cultural characteristics of *Metarhizium anisopliae* (METSCH.) SOROK. var. *anisopliae* 582 M on three different media.

Characteristics of colony		CZAPEK peptone agar	CZAPEK-DOX agar	SABOURAUD agar
Growth		Moderate	Moderate	Moderate
Depth in submerged growth	(7da)	200~700 nm	200~700 nm	200~700 nm
	(10~12da)	800~2,000 nm	800~2,000 nm	800~2,000 nm
Elevation		Convex	Umbonate	Convex
Surface texture	(21da)	Fasciculate by heavily sporing	Fasciculate by heavily sporing	Fasciculate by heavily sporing
Color (surface)		Yellow green to dark green	Yellow green to dark green	Yellow green to dark green
Color (reverse)		Olivaceous buff	Olivaceous buff	Olivaceous buff
Color changes in medium		Pale yellow	Pale yellow	Pale yellow
Wrinkle		Present	Present	Present
Exudate		Absent	Absent	Absent
Odor		Pungent	Pungent	Pungent

Table 3. Cultural characteristics of *Metarhizium anisopliae* (METSCH.) SOROK. var. *anisopliae* 582 M on different media after 2-week cultivation at 28°C.

Medium	Growth	Morphology of colony					Color changes in medium
		Texture	Color	Elevation	Wrinkle	Exudate	
CZAPEK peptone agar	Moderate	Compact Fasciculate	Yellow green to dark green	Convex	Present	Absent	Present
CZAPEK-DOX agar	Moderate	Compact Fasciculate	Yellow green to dark green	Umbonate	Present	Absent	Present
SABOURAUD agar	Moderate	Compact Fasciculate	Yellow green to dark green	Convex	Present	Absent	Present
Corn meal agar	Very slow	Thin	Dark green	Flat, mycelia thin	Absent	Absent	Absent
Malt extract agar	Slow, spread	Thin	Dark green to gray green	Raised, mycelia thin	Absent	Absent	Present
Potato dextrose agar	Very slow	Thin	Dark green	Raised, mycelia thin	Absent	Absent	Present
Gelatin agar	Very slow	Thin	Dark green	Flat, mycelia thin	Absent	Absent	Present
Nutrient agar	Slow	Floccose	No sporulation	Capitate, mycelia dense	Absent	Absent	Absent

2. Microscopic description

A sporulating layer gave an appearance similar to a sporodochium. Mycelium consisted of hyaline, branched hyphae. Hyphae were 50~150×1~1.5 μ. Conidiophores were short, hyaline, septate, erect,

simple or branched, terminating singly or more often closely branched in penicillate fashion, ultimately forming a cluster of phialides. A columnar structure with conidial masses developed on a loose bymenium-like layer. Phialides were cylindrical, hyaline and taper to narrow apex.

Phialospores were nonseptate, smooth, light yellow green, cylindrical to elliptiocal with rounded ends, 4.0~8.0×2.0~2.5 μ, with a mean of 5.0~7.0 μ long. They produced inbasipetal chains and were closely adhering in tall columns of a grey-green color.

Production and Isolation

The strain 582 M was cultivated in CZAPEK-peptone medium which contained 3.0% glucose, 1.0% Polypepton, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$.

K-582 was produced in a shaken flask-culture on a rotary shaker at 200 r.p.m. or surface-culture incubated at 28°C. Maximum production of K-582 was attained after 14~16 days incubation at 28°C on a rotary shaker or in a 500-ml ROUX flask containing 130 ml of the same medium.

After incubation, the culture filtrate (5 liters) was adjusted to pH 7.0 with 1 N HCl. To remove cationic impurities, the culture filtrate was applied to the IRC-50 (H-type) column. The antibiotic was eluted with 1 N HCl. After concentration of the eluate, the aqueous solution of the concentrate was

Fig. 1. Chromatography of K-582 on Bio-Gel P-2 column.

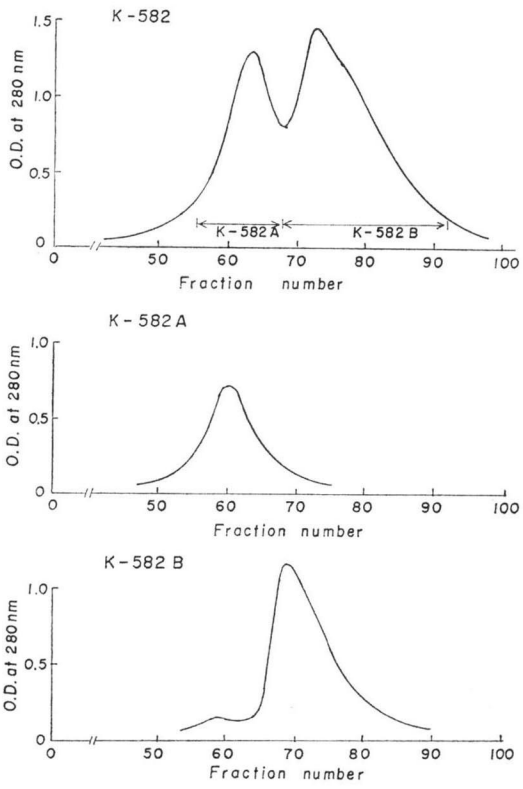
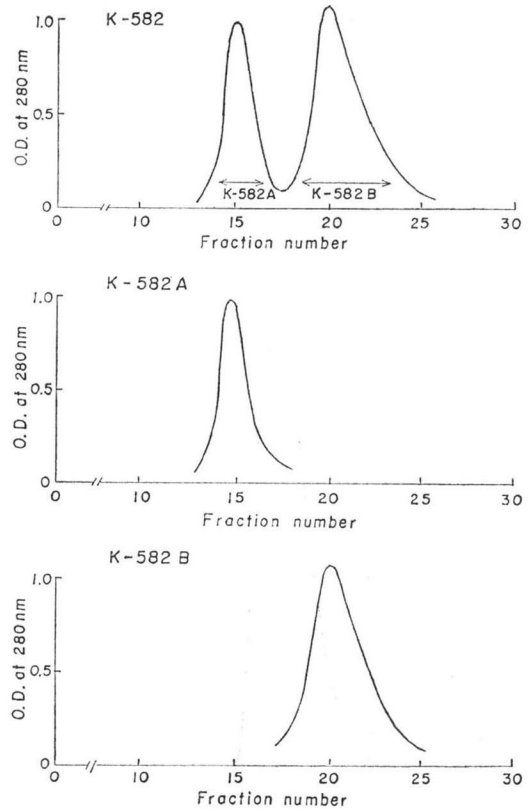


Fig. 2. Chromatography of K-582 on CM Sephadex.



precipitated with acetone - ethanol (4: 1) and then the precipitate was dissolved in methanol. Methanol was evaporated and a white powder (5.0 g) was obtained.

This powder was named K-582. When this powder was dissolved again in methanol, washed with acetone, dissolved in water, and applied to a column of Al_2O_3 or Bio-Gel P-2 or CM Sephadex, two major peaks which were named K-582 A (0.41 g) and K-582 B (0.35 g) were obtained (Figs. 1, 2).

Physicochemical Properties

K-582 is a white powder with basic properties. It is freely soluble in water and methanol, but insoluble in ethanol, acetone, ether, benzene, chloroform, *n*-hexane, cyclohexane and petroleum ether. Paper chromatographic mobility of K-582 in solvent system (*n*-BuOH - AcOH - H_2O, 4: 1: 2) revealed 0.07 of Rf. The Rf value was detected by bioautography against *Candida albicans*. Ninhydrin could also be used for its detection. K-582 was applied to paper chromatography or TLC furthermore, but both K-582 A and K-582 B were not isolated.

The optical rotation of K-582 A was $[\alpha]_D^{22}$ +0.2 (*c* 1, H_2O). The elementary analysis gave the following composition; C, 39.35; H, 7.24; N, 18.03; Cl, 14.83 (%). The molecular weights estimated by means of gel filtration, freezing point or vapor pressure were 1300, 1300 and 1200, respectively. It melted at 192~198°C with decomposition (Table 4). The ultraviolet absorption spectrum in methanol exhibited a maximum absorption at 278 nm ($E_{1cm}^{1\%}$ 1175) (Fig. 3). Fig. 4 shows the IR spectrum in KBr. Hydrolysis in 6 N HCl at 110°C for 24 hours in a sealed tube and subsequent

Fig. 3. Ultraviolet absorption spectrum of K-582 A.

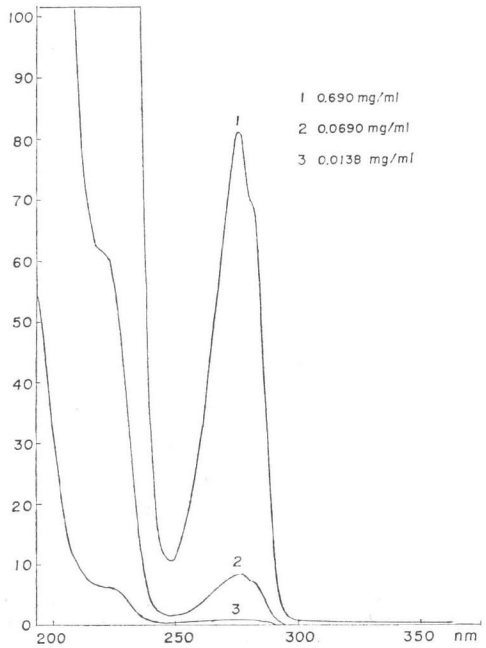


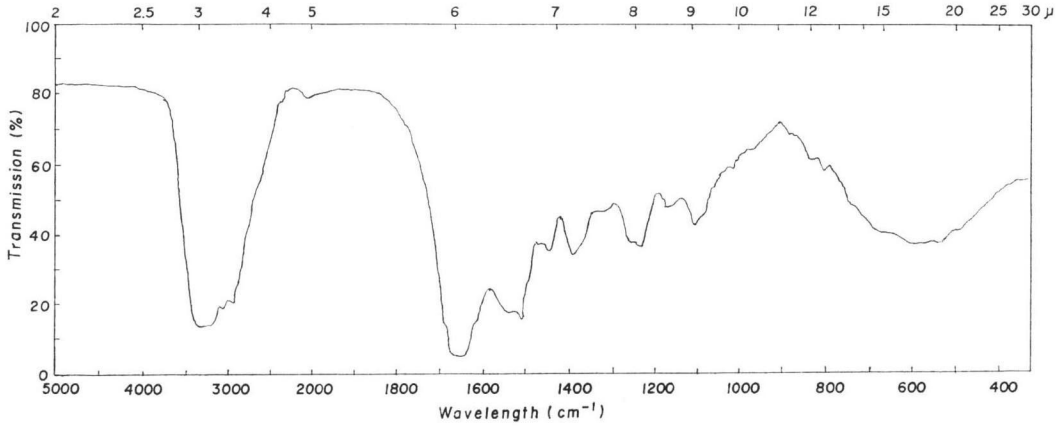
Table 4. Physical and chemical properties.

	K-582 A·HCl	K-582 B·HCl
$[\alpha]_D^{22}$	+0.2 (*c* 1, H_2O)	+0.6 (*c* 1, H_2O)
Anal. (Found) %	C: 39.35 H: 7.24 N: 18.03 Cl: 14.83	C: 36.25 H: 6.61 N: 18.62 Cl: 14.06
Decomp.	192~198°C	197~203°C
M.W.		
1, Gel filtration	*ca.* 1,300	*ca.* 1,200
2, Freezing-point	*ca.* 1,300	*ca.* 1,220
3, Vapor pressure	*ca.* 1,200	*ca.* 1,200
UV λ_{max}	278 nm	278 nm
$E_{1cm}^{1\%}$	1,175	1,175

Table 5. Amino acid composition of K-582 A or K-582 B.

K-582 A		K-582 B	
Amino acid	mol ratio	Amino acid	mol ratio
Arginine	1	Arginine	1
Threonine	1	Threonine	1
Hydroxyarginine	1	Hydroxyarginine	2
Tyrosine	1	Tyrosine	1
Ornithine	2	Ornithine	2
Lysine	1	----------	—

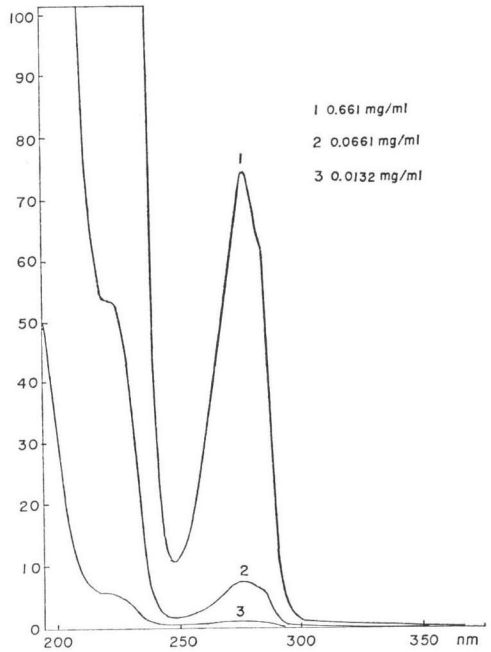
Fig. 4. Infrared spectrum of K-582 A.



amino acid analysis revealed the presence of arginine, threonine, hydroxyarginine, tyrosine, ornithine and lysine in K-582 A (Table 5).

The optical rotation of K-582 B was $[\alpha]_D^{22}+0.6$ (c 1, H_2O). The elementary analysis gave the following composition; C, 36.25; H, 6.61; N, 18.62; Cl, 14.06 (%). The molecular weight determined by gel filtration, freezing point and vapor pressure were 1200, 1220 and 1200, respectively. It melted at 197~203°C with decomposition (Table 4). The ultraviolet absorption spectrum in methanol exhibited a maximum absorption at 278 nm ($E_{1cm}^{1\%}$ 1175) (Fig. 5). The infrared spectrum in KBr tablet is shown in Fig. 6. Hydrolysis in 6 N HCl at 110°C for 24 hours in a sealed tube and subsequent amino acid analysis revealed the presence of arginine, threonine, hydroxyarginine, tyrosine and ornithine in K-582 B (Table 5).

Fig. 5. Ultraviolet absorption spectrum of K-582 B.



^1H NMR spectra of K-582 A and B are shown in Fig. 7. These data are consistent with the amino acid composition that was obtained by amino acid analysis.

The ^1H NMR spectrum (100 M Hz, D_2O) of K-582 A indicates the presences of one methyl group of threonine in the region δ 1.05~1.30 (3H, d) and of four aromatic protons of tyrosine in the region δ 6.30~6.85 (4H, q). The number of protons of K-582 B is similar to that of K-582 A, except that the number of protons is less than K-582 A in the region δ 1.30~2.05 due to the methylene group. These data are consistent with the amino acid composition obtained by amino acid analysis.

The noise-decoupled and off-resonance-decoupled CMR spectra of K-582 in D_2O (500 mg/ml, both K-582 A and B) were obtained with Varian CFT-20 spectrometer at room temperature (Fig. 8). The

Fig. 6. Infrared spectrum of K-582 B.

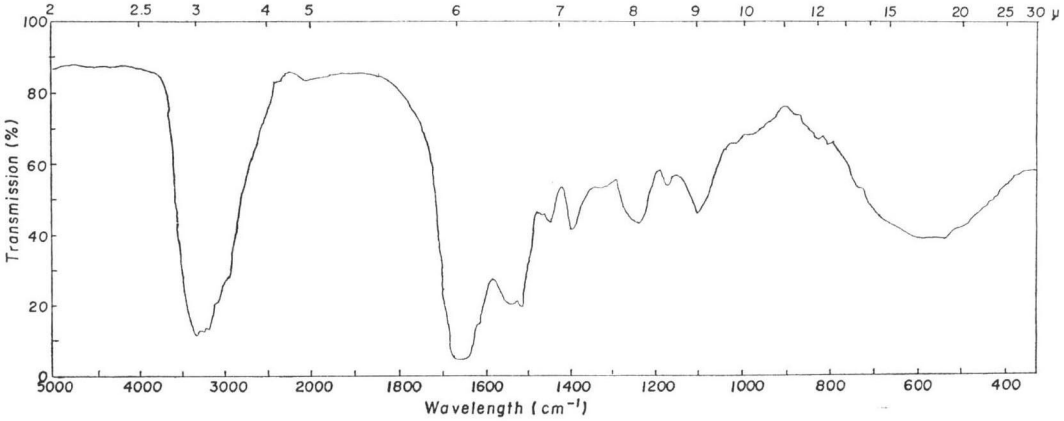


Fig. 7. ^{1}H NMR spectra of K-582.

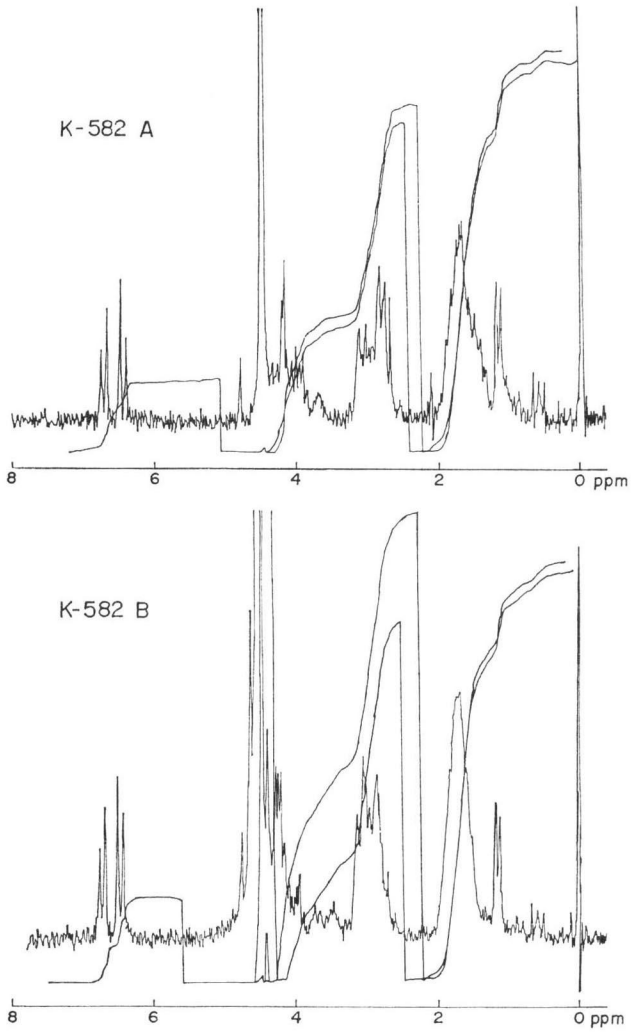
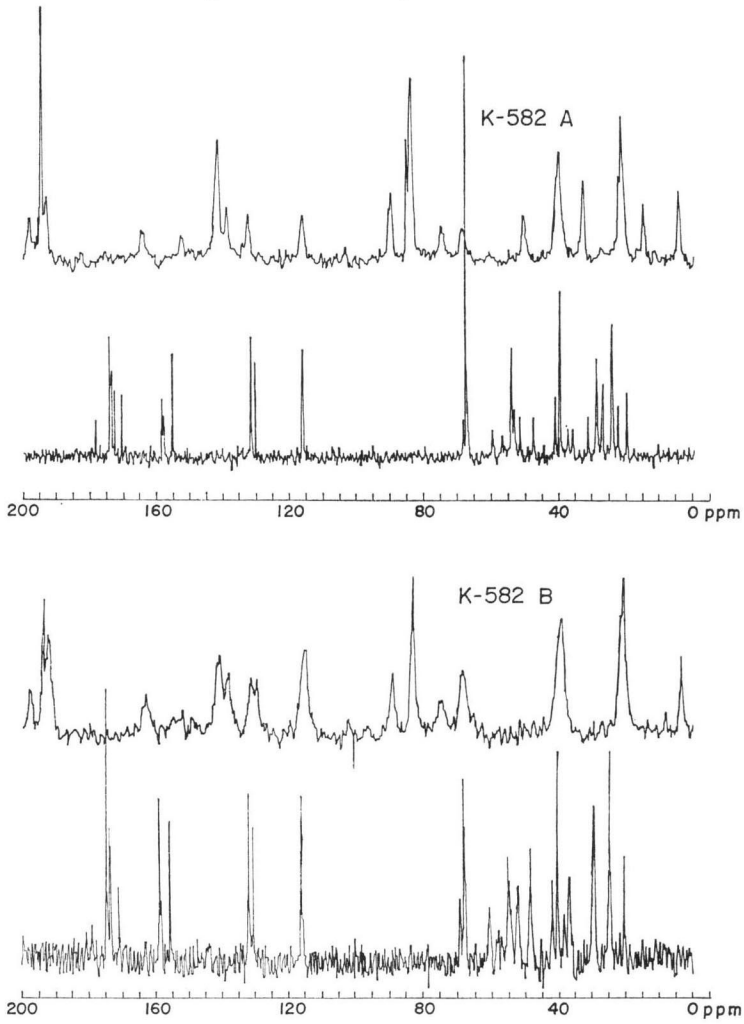


Fig. 8. C-13 NMR spectra of K-582.



chemical shifts (relative to TMS) were measured relative to the resonance of internal dioxane ($\delta_{\text{dioxane}} =$ 67.4 ppm). The assignment of the resonance to specific carbons was made on the basis of the chemical shifts of the free amino acids* and peptides[4~8].

The results supported the amino acid composition obtained by amino acid analysis, and suggested that K-582 B did not contain lysine, but contained two (or more than one) γ-hydroxy arginine residues as well as ornithine comparing with K-582 A.

The behaviors of K-582 A or K-582 B towards chemical test in color reaction were exactly the same. Namely, they gave positive reactions to ninhydrin, xanthoprotein, MILLON, SAKAGUCHI and PAULI, but negative to MOLISCH, TOLLENS and sodium nitroprusside. Also in precipitation reaction they gave positive formations to phosphotungustic acid, picric acid, flavianic acid, pentachlorophenol and benzaldehyde. They are soluble in water and methanol, and insoluble in ethanol, acetone, ether, benzene, chloroform, *n*-hexane, cyclohexane and petroleum ether.

* Standard amino acids including -OH-Arg, isolated from K-582 A and B in the research laboratory of Kakenyaku Kako Co., Ltd., were measured for this study (unpublished).

Table 6. Antimicrobial spectrum of K-582.

Test organism	MIC (mcg/ml)	
	K-582 A	K-582 B
Candida albicans	0.2	0.4
Candida tropicalis	0.2	0.4
Candida pseudotropicalis	0.2	0.4
Candida utilis	0.2	0.4
Candida guilliermondii	0.2	0.4
Candida krusei	0.2	0.4
Saccharomyces cerevisiae Br-60	0.2	0.4
Saccharomyces rouxii Boutroux	0.2	0.4
Zygosaccharomyces salsus	0.2	0.4
Willia anomala	0.2	0.4
Hansenula anomala	0.2	0.4
Torulaspora delbrueckii	0.2	0.4
Rhodotorula rubra	0.2	0.4
Mycotorula japonica	0.2	0.4
Debaryomyces klockeri	0.2	0.4
Pullularia pululans	0.2	0.4
Proteus OX-19	20	40
Bacillus subtilis	>100	>100
Staphylococcus aureus 209-P	>100	>100
Escherichia coli	>100	>100
Shigella sonnei	>100	>100
Sarcina lutea Hata	>100	>100
Bacillus mycoides	>100	>100
Mycobacterium timothee	>1,000	>1,000
Mycobacterium smegmatis	>1,000	>1,000
Mycobacterium $H_{37}Rv$	>1,000	>1,000
Trichophyton asteroides	>1,000	>1,000
Trichophyton rubrum	>1,000	>1,000
Trichomonas vaginalis	>1,000	>1,000

Table 8. Cytotoxicity of K-582.

Cell	ID_{50} (mcg/ml)
3T3	125
3T3 SV_{50}	200
RK-13	200
Daudi	>200
P_3HR 1	>200
NC-37 C6	>200
Human lymphocytes	10

Table 7. Antiviral effect of K-582 against polio virus, NDV, influenza virus (WSN) and VSV growth.

Concentration (mcg/ml)	Cytotoxicity	Cytophathic effect (CPE)			
		Polio	NDV	WSN	VSV
1,000	+	−	−	−	−
500	−	−	−	−	−
250	−	−	++	−	−
125	−	−	+++	++	−
62	−	+	+++	++	++
31	−	++	+++	++	++
Control	−	+++	+++	++	+++

Table 9. Acute toxicity of K-582.

Route of administration	Test substance	Sex	LD_{50} (mg/kg)
iv	K-582 A	M	120 ~144
		F	144 ~172.8
	K-582 B	M	24.1~ 34.8
		F	24.1~ 34.8
	K-582	M	144 ~172.8
		F	144 ~172.8
ip	K-582 A	M	>300
		F	≧300
	K-582 B	M	43.2~ 57.2
		F	43.2~ 57.2
	K-582	M	360 ~432
		F	≧300
sc	K-582 A	M	≧320
		F	≧320
	K-582 B	M	86.8~104
		F	104 ~125
	K-582	M	320 ~400
		F	400 ~500
po	K-582 A	M	>6,000
		F	>6,000
	K-582 B	M	2,400 ~3,000
		F	2,400 ~3,000
	K-582	M	>6,000
		F	>6,000

Mice (ICR-JCL)
Dose form: Suspended in sodium chloride solution

Fig. 9. Tumor inhibitory effect of K-582 on SN-36.

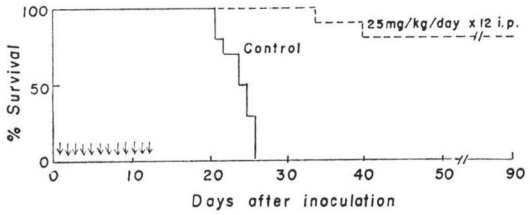


Fig. 10. Tumor inhibitory effect of K-582 on EHRLICH ascites carcinoma.

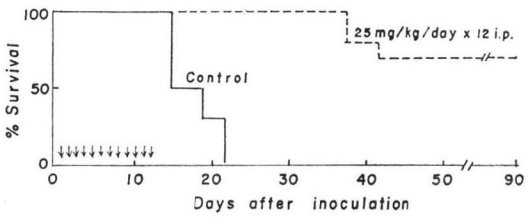


Fig. 11. Tumor inhibitory effect of K-582 on Sarcoma 180.

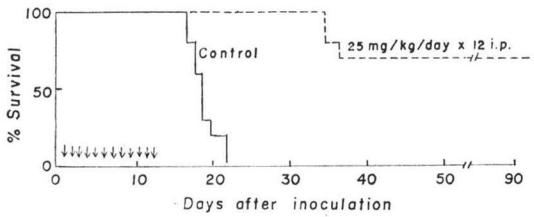
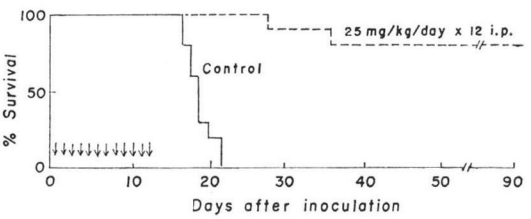
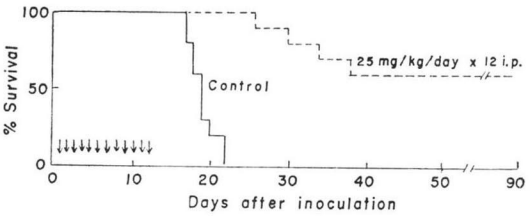


Fig. 12. Tumor inhibitory effect of K-582 A on Sarcoma 180.



The nitrogen content, the behavior in color reaction and the absorption bands of amide linkages in the infrared absorption spectrum indicate the peptide nature of K-582 A and K-582 B.

Fig. 13. Tumor inhibitory effect of K-582 B on Sarcoma 180.



Biological Properties

Antimicrobial, Antiviral and Cytotoxicity Test

In vitro antimicrobial and antiviral spectrum of K-582 was determined by employing 29 strains of microbes and 4 strains of viruses. As shown in Table 6, K-582 A and B were effective against yeasts in the concentration of 0.2 mcg/ml, and 0.4 mcg/ml, respectively, but inactive against other Gram-positive bacteria, Gram-negative bacteria and *Mycobacterium*.

In vitro activity of K-582 (complex) against 4 strains of viruses was tested using an agar-plaque diffusion test. K-582 was found to be active against polio virus, NDV, influenza virus (WSN) and VSV as shown in Table 7.

The cytotoxicity of K-582 was examined in various tissue culture cells including $3T3SV_{50}$, RK-13 (rabbit kidney cells), Daudi, P_3HR 1 (Burkitt lymphoma cells), NC-37 C6 (human lymphoblastoid cells) and human lymphocytes.

A stock of these cells was grown in EAGLE's minimum essential medium supplemented with 10% calf serum. After 48 hours of cultivation of these cells (1×10^5 ml) inserted in Leighton tubes with a coverslip, K-582 dissolved in growth medium was added to the tube. Morphological changes of these cells were observed under a light microscope after 72 hours cultivation from the addition of K-582. As shown in Table 8, ID_{50} (mcg/ml) of K-582 against these cells was very weak.

Effect on Transplanted Tumors

K-582 was tested for its antitumor effects in several transplanted tumors in mice. The tumors used were as follows: EHRLICH ascites carcinoma, CROCKER sarcoma 180 (ascites form), mouse lymphatic leukemia SN-36 (ascites form) in *dd* mice. For the test on the tumors of mice, a suspension of tumor cells (10^6) in ascitic form was inoculated intraperitoneally. For all therapeutic tests, K-582 was dissolved in saline and intraperitoneal injection was started 24 hours after tumor implantation.

The effects of K-582 on survival time of mice are presented in Figs. 9, 10 and 11. When 25 mg/kg/day of K-582 was injected intraperitoneally once daily for 12 days, the highest survival rate was obtained.

Furthermore, K-582 A and K-582 B were tested for these antitumor effects in CROCKER sarcoma 180 (ascites form) in *dd* mice. For the test on the tumor of mice, a suspension of tumor cells (10^6) in ascitic form of CROCKER sarcoma 180 was inoculated intraperitoneally. K-582 A or K-582 B was dissolved in saline and the intraperitoneal injection was started 24 hours after tumor implantation. When 25 mg/kg/day of K-582 A or K-582 B was injected intraperitoneally once daily for 12 days, the increase of ascites was inhibited and the survival period of tumor-bearing mice was markedly prolonged (Figs. 12, 13).

Toxicity of K-582 for Mice

As shown in Table 9, the acute toxicity of K-582, K-582 A and K-582 B was determined in mice (ICR-JCL) by a single administration given by intravenous, intraperitoneal, subcutaneous and oral routes. In particular a single administration by the oral route indicated a very weak toxicity.

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