44-HOMOOLIGOMYCINS A AND B, NEW ANTITUMOR ANTIBIOTICS FROM *Streptomyces bottropensis*

PRODUCING ORGANISM, FERMENTATION, ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL PROPERTIES

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Oligomycin antibiotics, 44-homooligomycin A (NK86-0279 II) and B (NK86-0279 I) are newly discovered antitumor antibiotics with the substitution of ethyl for methyl at carbon 26. They were isolated from the culture broth of *Streptomyces bottropensis* NK86-0279. The structure of these two compounds was deduced by spectroscopic and X-ray crystallographic analyses. These antibiotics showed potent antitumor activities against various tumor cells *in vitro*, and were active against Colon 26 carcinoma *in vivo*. Although they showed no activity at 1,000 μg/ml against Gram-positive and Gram-negative bacteria and yeast, they have antifungal activity.

In our continuing search for new microbial metabolites with antitumor activity, *Streptomyces bottropensis* NK86-0279, isolated from a soil sample collected at Funabashi, Chiba, Japan, was found to produce new antibiotics with potent antitumor activity against various tumor cells. The active compounds, NK86-0279 I and II, were recovered from the mycelium with acetone extraction and purified by chromatography. Structural studies revealed that these are new oligomycins (44-homooligomycins A and B), with substitution of ethyl for methyl groups at the C-26 position.

NK86-0279 I and II showed potent growth inhibition against various tumor cells and fungi, but no inhibitory activity was found against bacteria. Compound I induced a prolongation of survival time of mice transplanted with Colon 26 carcinoma.

In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structure and biological properties of these compounds.

Materials and Methods

Taxonomic Studies

The producing organism, strain NK86-0279, was isolated from a soil sample collected at Funabashi City, Chiba Prefecture, Japan. The media and procedures used for cultural and physiological characterization of strain NK86-0279 were described by SHIRLING and GOTTLIEB[1]. Each culture was incubated at 27°C for 2 to 3 weeks before observation. The color names used in these studies were based on the Color Standard of Nihon Shikisai Co., Ltd. Cell wall analysis was performed by the methods of BECKER *et al.*[2] and YAMAGUCHI[3]. Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[4].

Fermentation

A loopful of the slant culture of strain NK86-0279 was inoculated to a 500-ml flask containing 110 ml of the seed medium composed of soluble starch 2.0%, glucose 0.5%, peptone 0.5%, yeast extract 0.5%, soybean meal 0.5%, K_2HPO_4 0.05%, $MgSO_4$ 0.05% and $CaCO_3$ 0.2% (pH 7.2). The flasks were shaken on a rotary shaker (190 rpm) for 2 days at 27°C. A 200-liter tank fermenter containing 120 liters of production medium composed of glycerol 4%, Polypepton 0.5%, yeast extract 0.3%, meat extract 0.5%, NaCl 0.05%, and $MgSO_4$ 0.05% (pH 7.0) was inoculated with 3 liters of the seed broth and cultured for approximately 48 hours at 27°C, aerated at 120 liters per minute and agitated at 300 rpm.

In Vitro Cytotoxicity

HeLa S_3 (human cervix epitheloid carcinoma), PC-3 (human lung adenocarcinoma), G361 (human malignant melanoma), LL (murine Lewis lung carcinoma), B16 (murine melanoma) cells were grown in RPMI-1640 medium (Gibco) supplemented with fetal calf serum (FCS, 10%) and kanamycin (60 μg/ml), and SW1116 (human colonic carcinoma) cells were grown in RPMI-1640 medium containing 1% of non-essential amino acid supplemented with FCS (10%), beznylpenicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C under humidified atmosphere in a CO_2 incubator. The exponentially growing cells were harvested, counted and suspended in the culture media at 1.5×10^3, and 3×10^5 cells/ml, respectively. After planting into wells of 96-well tissue culture plate with test materials, they were incubated for 72 hours. The cytotoxic activities against HeLa S_3 cells were colorimetrically determined at 660 nm after staining viable cells with a methylene blue solution.

Results and Discussion

Identification of Strain NK86-0279

Morphological observations were made with a light and a scanning electron microscope (Fig. 1) on cultures grown at 27°C for 2 to 3 weeks on oatmeal agar, yeast extract-malt extract agar and inorganic salts-starch agar. This strain showed spiral hyphae from branched aerial hyphae and no whirl. No fragmentation of vegetative mycelia, sporangia, zoospores, vegetative mycelium spore or synneamata were observed. A matured spore chain comprised 20 or more spores ($0.6 \sim 0.8 \times 1.2 \sim 1.4$ μm) with a smooth surface. The characteristics of strain NK86-0279 are summarized in Table 1. The aerial mass color was brownish white to brownish gray. A slight brown soluble pigment was observed. The physiological characteristics and utilization of carbon sources of strain NK86-0279 are summarized in Tables 2 and 3. Hydrolyzed whole cell of strain NK86-0279 contained LL-diaminopimelic acid. Accordingly, the cell wall of this strain was concluded to be Type I.

Based on the taxonomic properties described above, strain NK86-0279 is considered to belong to the genus *Streptomyces*, and to be a strain of the gray series of the PRIDHAM and TRESNER grouping[5]. The strain NK86-0279 was compared with *Streptomyces* species described in the literatures[4~10]. A known strain which closely relates to the strain NK86-0279 is *Streptomyces bottropensis*. These two strains were completely coincident with each other in, for example, the colors of aerial hyphae on various media, utilization of sugars and formation of melanin-like pigment. Therefore, this strain was

Fig. 1. Scanning electron micrograph of a spore chain of strain No. NK86-0279 on starch agar, 5 days culture.

Scale: 1 μm.

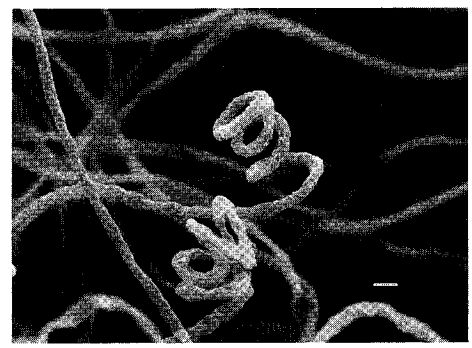


Table 1. Cultural characteristics of strain No. NK86-0279.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Pale yellowish brown	White	Faint, yellowish
Glycerol-nitrate agar	Pale yellowish	White	Faint, brownish
Yeast extract-malt extract agar (ISP medium 2)	Pale yellowish brown	Grayish white ~brownish gray	None
Oatmeal agar (ISP medium 3)	Colorless	Grayish white ~light brownish gray	None
Inorganic salts-starch agar (ISP medium 4)	Pale yellowish brown	Light brownish gray ~brownish gray	Faint, brownish
Glycerol-asparagine agar (ISP medium 5)	Pale yellow~cream	Brownish white ~light brownish gray	None
Glucose-asparagine agar	Pale yellow ~pale yellowish brown	Brownish white ~light brown	None
Tyrosine agar (ISP medium 7)	Dark brownish gray	Grayish white ~light brownish gray	Dark brown
Calcium-malate agar	Colorless ~pale yellowish brown	Thin, white	None

Table 2. Physiological properties of strain No. NK86-0279.

	NK86-0279
Temperature range for growth (°C)	10~37
Optimum temperature (°C)	24~32
Nitrate reduction	Negative
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Negative
Melanin production	Positive
Gelatin liquefaction	Positive

Table 3. Utilization of carbohydrates by strain No. NK86-0279.

D-Glucose	+	L-Rhamnose	+
L-Arabinose	+	Inositol	+
D-Xylose	+	D-Mannitol	+
D-Fructose	+	Raffinose	+
Sucrose	+	D-Galactose	+

Symbols: +, Growth.

designated as *S. bottropensis* NK86-0279. The strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan under the accession No. FERM P-9626.

Production of NK86-0279 Compounds

A typical fermentation profile for the production of NK86-0279 is shown in Fig. 2. The production of active compounds during the fermentation was monitored by *in vitro* cytotoxic activity against HeLa S_3 cells and assessed from the standard curve of the purified NK86-0279 I. The production of NK86-0279 compounds in mycelium started on day 1 and reached a maximum of about 11.1 μg/ml on day 3.

Isolation and Purification of NK86-0279 I and II

The procedure of isolation of NK86-0279 compounds is outlined in Fig. 3. The mycelial cake (6.1 kg), separated from the cultured broth (240 liters) by use of a Sarples-type centrifuge, was extracted twice with acetone (20 liters $\times$ 2). The extract was concentrated under reduced pressure giving a brownish oily residue (220 g). The residue was extracted with chloroform after washing with *n*-hexane to yield a crude antibiotic complex (19 g). This solid was dissolved in chloroform and applied to a column of silica gel (4.4 $\times$ 52.8 cm) which was developed with a chloroform-methanol gradient (40:1~20:1). The eluate was monitored by the cytotoxicity against HeLa S_3 cell and also UV absorption on TLC plate (silica gel F254). The active fractions were concentrated and chromatographed on a Sephadex LH-20 column with methanol elution. The mixture of NK86-0279 I and II obtained was further purified by reverse phase HPLC on an ODS-column

Fig. 2. Time course of 200-liter fermentater by *Streptomyces bottropensis* NK86-0279.

■ pH, ● NK86-0279, □ mycelium, ▲ glycerol.

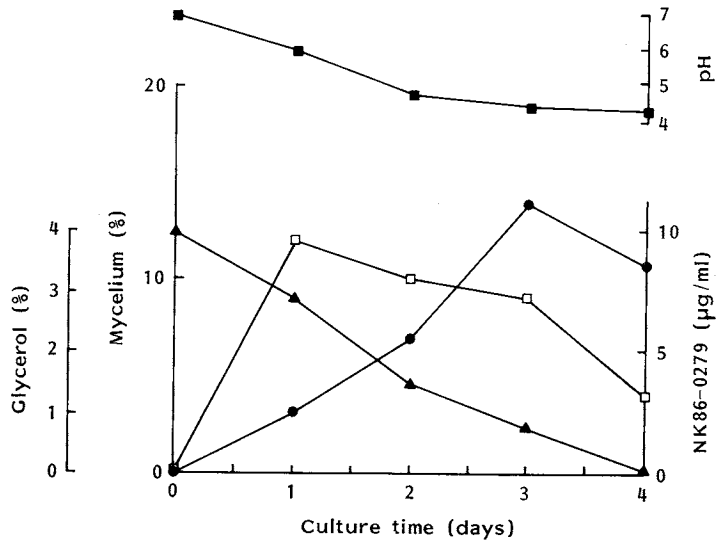


Fig. 3. Isolation procedure of NK86-0279 I and II.

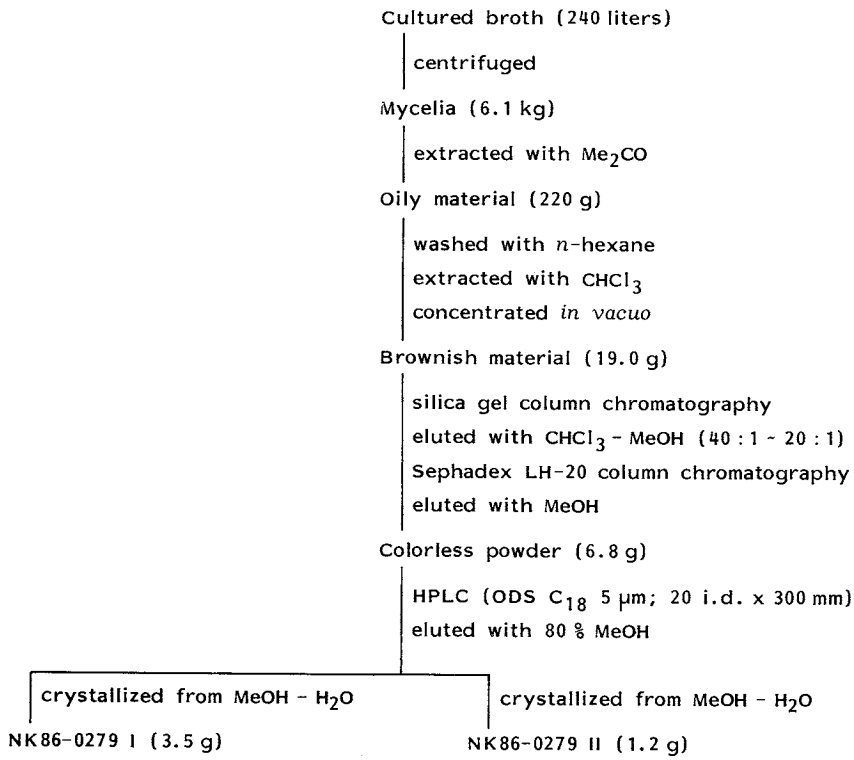
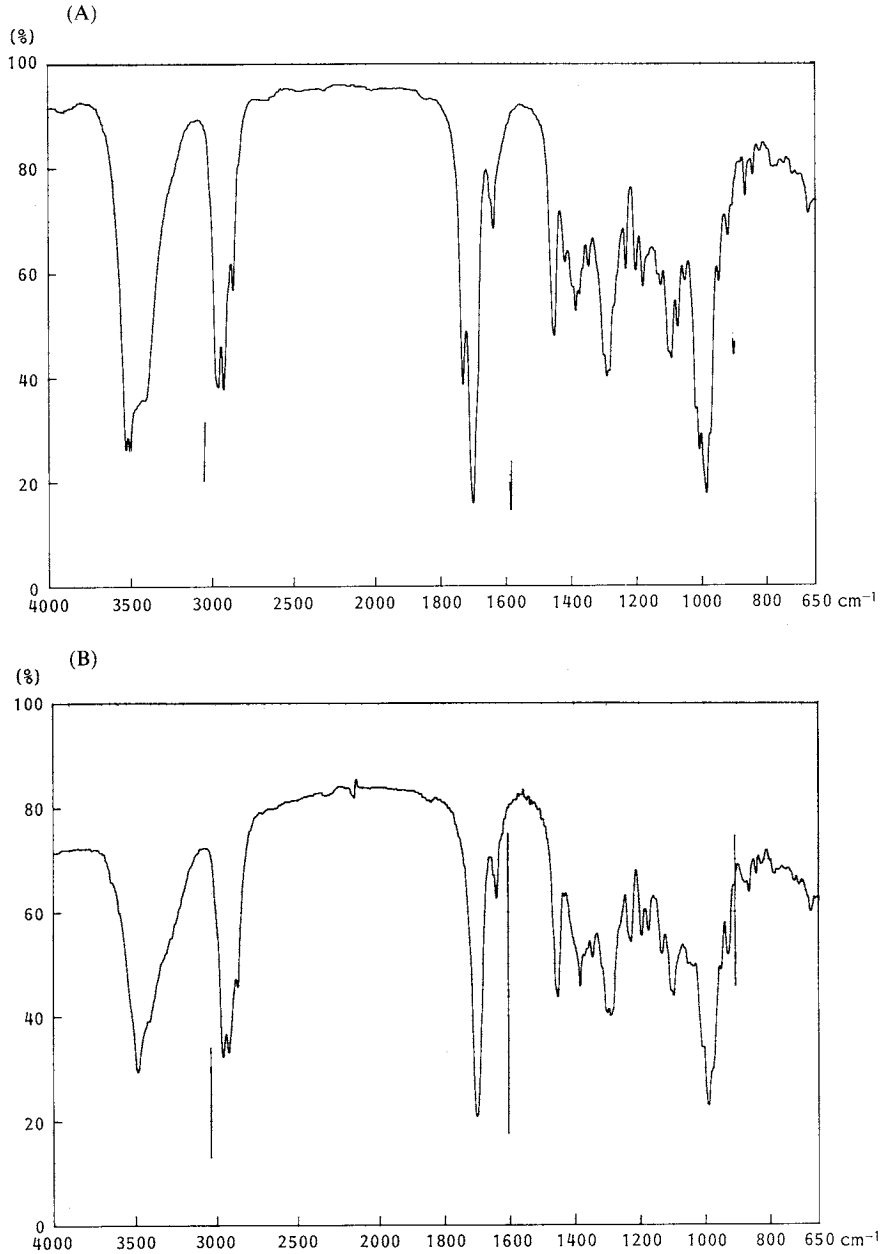


Fig. 4. IR spectra of NK86-0279 I and II (KBr).

(A) NK86-0279 I, (B) NK86-0279 II.



(Nucleosil 5C18, 20 × 300 mm, 80% MeOH, flow rate; 10 ml/minute). The compounds I and II were eluted with retention times of 26.8 and 32.5 minutes, respectively. The two active fractions were concentrated *in vacuo* resulting in crude crystals, then recrystallized from aqueous methanol to yield 3.5 g (NK86-0279 I) and 1.2 g (NK86-0279 II) of colorless crystals, respectively.

Physico-chemical Properties

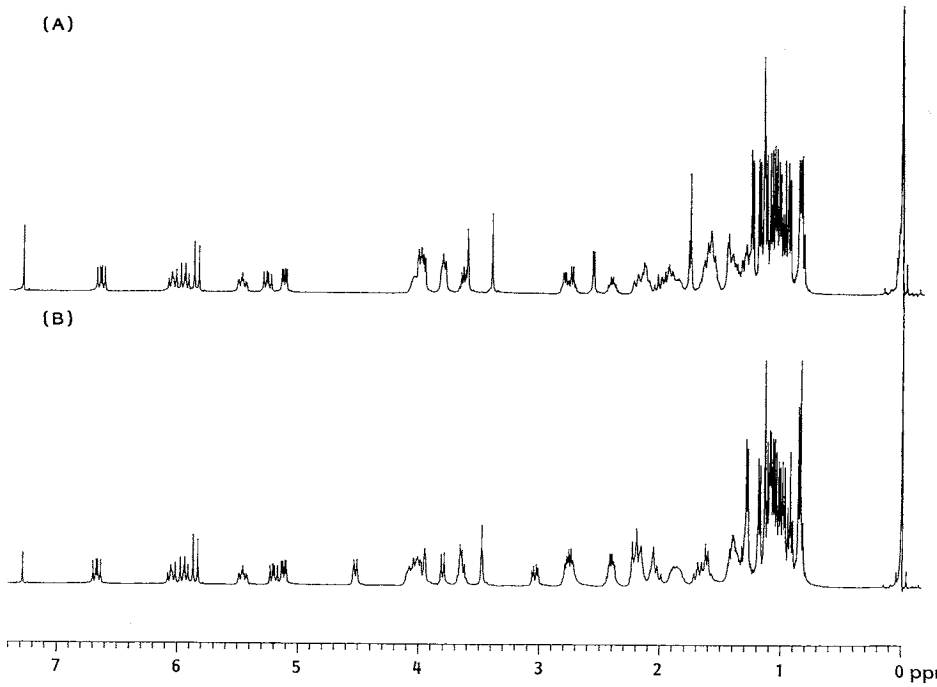
The physico-chemical properties of NK86-0279 I and II are summarized in Table 4. The two compounds

Table 4. Physico-chemical properties of NK86-0279 I and II.

	I	II
Appearance	Colorless prism	Colorless prism
MP (°C)	157~159	161~165
$[\alpha]_D^{20}$	−47.0° (*c* 1.0, MeOH)	−39.5° (*c* 1.0, MeOH)
Elemental analysis	Calcd for $C_{46}H_{74}O_{12}$: C 66.32, H 9.24	Calcd for $C_{46}H_{76}O_{11}$: C 68.63, H 9.52
	Found: C 66.54, H 9.29	Found: C 68.34, H 9.67
Molecular formula	$C_{46}H_{74}O_{12}$	$C_{46}H_{76}O_{11}$
FD-MS ($(M+H)^+$) *m/z*	819	805
UV λ_{max}^{MeOH} nm (ε)	225 (33,040), 232.5 (30,790), 242.5 (sh)	225 (40,280), 232 (35,890), 242.5 (sh)
IR ν_{max} (KBr) cm^{-1}	3450, 2960, 1730, 1705, 1640, 1460, 1395, 990	3470, 2960, 1705, 1640, 1450, 1390, 990
Rf value[a]		
(1)	0.37	0.41
(2)	0.53	0.59

[a] Silica gel TLC (Merck Art. No. 5715), solvent (1): *n*-hexane-acetone (2:1), (2): chloroform-methanol (30:1).

Fig. 5. ^1H NMR spectra of NK86-0279 I and II (400 MHz, $CDCl_3$).
(A) NK86-0279 I, (B) NK86-0279 II.



are soluble in methanol, ethanol, dimethyl sulfoxide, acetone, ethyl acetate and chloroform, slightly soluble in ethyl ether, and insoluble in water and *n*-hexane. They give positive color reactions for molybdatophosphoric acid, potassium permanganate and iodine vapor reagents, but not for Dragendorff and Elrich reagents. The IR and ^1H NMR spectra of two antibiotics are shown in Figs. 4 and 5, respectively. The molecular formulas of NK86-0279 I and II were determined to be $C_{46}H_{74}O_{12}$ and $C_{46}H_{76}O_{11}$,

Table 5. ^{13}C NMR data of NK86-0279 I (**1**), II (**2**), oligomycin A (**3**) and oligomycin B (**4**) (100 MHz, $CDCl_3$).

Position	1	2	3	4	Position	1	2	3	4
1	164.98	164.98	165.11	165.19	24	35.99	36.06	35.86	35.82
2	122.58	122.82	122.75	122.46	25	76.01	76.33	76.21	75.83
3	149.11	148.55	148.38	148.92	26	36.79	44.15	38.46	31.31
4	40.34	40.25	40.16	40.22	27	101.13	100.13	99.23	100.81
5	72.99	73.01	72.99	73.04	28	203.12	26.36	26.01	203.00
6	46.60	46.67	46.56	46.50	29	43.95	26.53	26.53	44.08
7	220.26	219.97	219.95	220.11	30	36.79	30.52	30.52	37.06
8	45.96	45.71	45.71	45.96	31	67.10	67.28	67.27	67.21
9	72.67	72.64	72.66	72.72	32	41.74	42.66	42.63	41.72
10	41.87	41.95	41.96	41.81	33	64.60	64.70	64.70	64.63
11	220.26	220.35	220.26	220.26	34	25.04	24.81	24.78	25.04
12	83.10	83.02	83.01	83.09	35	17.91	17.92	17.94	17.88
13	72.04	72.26	72.28	71.99	36	9.44	9.35	9.28	9.39
14	33.63	33.54	33.52	33.63	37	8.40	8.31	8.30	8.40
15	38.38	38.46	38.46	38.41	38	13.98	14.11	14.09	13.91
16	129.87	129.41	129.36	129.84	39	21.06	21.02	21.02	21.03
17	132.24	132.43	132.43	132.25	40	14.55	14.51	14.49	14.55
18	130.63	130.31	130.29	130.60	41	28.68	28.53	28.53	28.68
19	137.20	137.76	137.80	137.31	42	12.11	12.09	12.08	12.12
20	46.14	46.17	46.03	46.06	43	5.99	6.10	6.07	5.95
21	31.20	31.55	31.46	31.14	44	21.88	21.61	11.79	11.70
22	30.70	31.02	30.97	30.70	44′	13.11	14.37	—	—
23	71.02	69.08	69.06	71.12	45	13.08	11.46	11.23	12.79

Chemical shift in ppm from TMS.

Table 6. The antimicrobial spectra of NK86-0279 I and II.

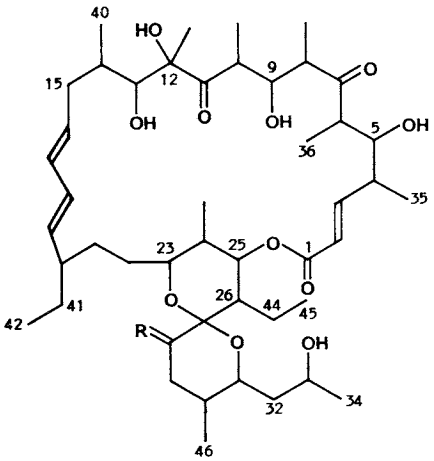
Test organism	MIC (μg/ml)		Test organism	MIC (μg/ml)	
	I	II		I	II
Staphylococcus aureus FDA 209P	>100	>100	*Enterobacter aerogenes* ATCC 13048	>100	>100
Bacillus subtilis PCI 219	>100	>100	*Serratia marcescens* GN 6485	>100	>100
Micrococcus luteus ATCC 9341	>100	>100	*Shigella sonnei*	>100	>100
Mycobacterium smegmatis ATCC 607	>100	>100	*Mucor javanicus*	>100	>100
Candida albicans NIH 3147	>100	>100	*Aspergillus niger*	1.56	6.25
Saccharomyces cerevisiae	>100	>100	*A. oryzae*	1.56	3.13
Sporobolomyces salmonicolor	>100	6.25	*Penicillium chrysogenum*	0.78	1.56
Escherichia coli NIHJ	>100	>100	*Fusarium roseum* f. sp.	0.39	6.25
Klebsiella pneumoniae PCI 602	>100	>100	*Rhizopus hangchao*	>100	6.25
Proteus mirabilis IFO	>100	>100	*Torula herbarum*	—	3.13
Pseudomonas aeruginosa IFO 3445	>100	>100	*Glomerella cingulata*	—	6.25
Salmonella typhi	>100	>100			

respectively, by FD-MS (*m/z* 819 $(M+H)^+$ and 805 $(M+H)^+$) and elemental analysis.

The IR spectra of compounds I and II indicated the existence of hydroxyl (3450 or 3470 cm^{-1}), methyl (2900 cm^{-1}) and carbonyl (1705 cm^{-1}) groups. The conjugated diene system was indicated by the IR (1640 cm^{-1}) and UV (λ_{max} 225 nm) spectra. These spectral properties suggested to be an oligomycin.

In the ^1H and ^{13}C NMR spectra, similar signal patterns were observed between compound I and oligomycin B, or compound II and oligomycin A[11~15]. The comparison of the ^{13}C NMR data of NK86-0279 I (**1**) and oigomycin B (**4**), or NK86-0279 II (**2**) and oligomycin A (**3**) (see Table 5), especially

Fig. 6. Structures of NK86-0279 I and II.



I R=O
II R=H_2

Table 7. *In vitro* cytotoxicities of NK86-0279 I (**1**), II (**2**), oligomycin A (**3**) and oligomycin B (**4**).

	IC_{50} (μg/ml)[a]			
	1	**2**	**3**	**4**
Hela S_3	5.63	2.80	3.28	4.29
PC-3	1.56	1.99	7.85	8.75
SW1116	0.031	2.80	0.105	0.127
G361	5.66	1.63	—	—
LL	0.091	0.0085	2.95	2.12
Colon 26	0.026	0.025	0.0202	0.0492
B16	4.79	0.64	0.115	0.114

[a] Concentration causing 50% inhibition of cell growth.

the following signals, led us to determine the structure of NK86-0279 I as 44-homooligomycin B and II as 44-homooligomycin A (Fig. 6): I, C-44′, 13.11 in **1** vs. no signals in **4**; C-44 21.88 in **1** vs. 11.70 in **4**; C-26 36.79 in **1** vs. 31.31 in **4**. II, C-44′ 14.73 in **2** vs. no signals in **3**; C-44 21.61 in **2** vs. 11.79 in **3**; C-26 44.15 in **2** vs. 38.46 in **3**. Comparative analysis of on H-H and C-H COSY spectra between I and oligomycin B or II and oligomycin A, indicated the existence of an ethyl residue at the C-26 position for the NK86-0279 compounds. The oligomycins have a methyl residue at the same position. X-Ray crystallographic analysis of NK86-0279 I confirmed the complete structure[16].

Biological Properties

The antimicrobial spectra of NK86-0279 I and II were determined by the agar dilution method: The antibiotics were active against fungi, but inactive against yeast and bacteria. The minimum inhibitory concentration (MIC) of the antibitotics are summarized in Table 6.

The antibiotics were tested for *in vitro* cytotoxicity against various tumor cells. The results are summarized in Table 7. Both antibiotics showed similarly potent cytotoxicity against murine and human cells as compared with oligomycins.

The antibiotics gave moderate antitumor activity against Colon 26 carcinoma *in vivo* (manuscript in preparation).

The acute toxicities (LD_{50}) of compounds I and II were 1.67 and 1.05 mg/kg, respectively, when administered intraperitoneally to mice.

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