RK-397, A NEW OXO PENTAENE ANTIBIOTIC

KIMIE KOBINATA, HIROYUKI KOSHINO, TAKUJI KUDO, KIYOSHI ISONO†, and HIROYUKI OSADA*

The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-01, Japan
†Department of Marine Science, Tokai University, Orido, Shizuoka 424, Japan

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In the course of the screening for new inhibitors against tumor cells from microorganisms, we isolated a new antibiotic, RK-397 (Fig. 1). This antibiotic is active against tumor cells, bacteria and fungi. The present paper describes taxonomy and fermentation of the producing microorganism and isolation, physico-chemical properties, and biological activities of the antibiotic.

The producing strain, 87-397, was isolated from a soil sample collected in Saku city, Nagano prefecture, Japan. The strain has been deposited under accession number FERM P-13195 at the National Institute of Bioscience and Human-Technology (formerly Fermentation Research Institute), Agency of Industrial Science and Technology, Japan. Taxonomic characterization was done according to the method and media recommended by ISP[1]. Microscopic observation showed that aerial mycelium formed spirales and the mature spore chain comprised more than 50 spores each. The spore surface was smooth. Soluble pigment was not produced. The cell wall of strain 87-397 in submerged-cultures contained LL-diaminopimelic acid. The major menaquinones were MK-9 (H6), MK-9 (H8) and MK-9 (H4). According to these taxonomic studies, strain 87-397 belongs to the genus *Streptomyces*[2], but the species was not determined.

The producing strain, *Streptomyces* sp. 87-397, was cultured at 28°C for 72 hours in a jar fermentor containing 18 liters of a medium consisting of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soybean flour 2.5%, sodium chloride 0.2% and dipotassium hydrogen phosphate 0.005%. The pH was adjusted to 7.0 before sterilization. The production titer of RK-397 substance was monitored by the cytotoxicity against human leukemia K-562 cells and the conventional paper disk agar method using *Pyricularia oryzae* as an indicator microorganism. The activity in the broth reached maximum after 72 hours fermentation.

The fermentation broth (pH 7.6, 18 liters) was filtered with an aid of Celite, and the mycelial cake was extracted with 80% aqueous acetone. After removal of acetone *in vacuo*, the mycelium extract was combined with the filtrate and extracted twice with 15 liters of ethyl acetate. The organic layer was concentrated *in vauco*. One liter of hexane was added to the concentrated extract and the precipitate was collected by centrifugation. The precipitate was dissolved in a small amount of methanol and applied to a silica gel column (5 × 40 cm) which was equilibrated with chloroform. The column was eluted stepwise with chloroform-methanol (10:1, 1:1 and 1:3). The active principle was eluted with a mixture of chloroform-methanol (1:1) and concentrated to dryness *in vacuo*. A yellowish orange powder (4.1 g) was obtained after lyophilization. Half of the powder was subjected to chromatography on a silica gel column (3 × 30 cm) which was successively developed with the same solvent systems as the previous column. The active fractions were combined and concentrated *in vacuo* to yield a yellowish orange powder (300 mg). The powder was dissolved and applied to an ODS column (3 × 40 cm) equilibrated with 60% aqueous methanol. The stepwise elution was done with methanol-water (6:4, 7:3 and 8:2). The active principle was detected by TLC (Merck Silica gel plate F_{254}, chloroform-methanol 2:1). Active fractions were combined and the methanol was evaporated *in vacuo*, followed by lyophilization to give a yellow powder (84 mg). Final purification was achieved by preparative HPLC on a column (2 × 30 cm) of Senshu Pak ODS-H with 80% aqueous methanol (flow rate, 6.0 ml/minute; photodiode array detector, 200 ~ 400 nm; retention time, 28 minutes). The active fractions were combined and concentrated *in vacuo* to remove methanol in a dark brown flask to shut off light. Finally, 40 mg of a pure powder was obtained.

Fig. 1. Structure of RK-397.

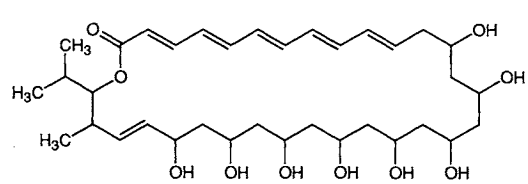


Table 1. Physico-chemical properties of RK-397.

Appearance	Yellow powder
Nature	Neutral substance
MP	157~163°C (dec)
$[\alpha]_D^{20}$	−21° (*c* 0.3, MeOH)
High resolution FAB-MS *m/z*	637.3930 (M+H): Calcd for $C_{35}H_{56}O_{10}$+H (637.3951)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	260 (104), 360 (540)
IR ν_{max} (KBr) cm^{-1}	3350, 2900, 1420, 1250, 1105
TLC, Rf value	0.45
	Silica gel plate F_{254} (Merck), $CHCl_3$-MeOH (2:1)

Fig. 2. ^1H NMR of RK-397 in methanol-d_4, 600 MHz (ppm from TMS an internal standard).

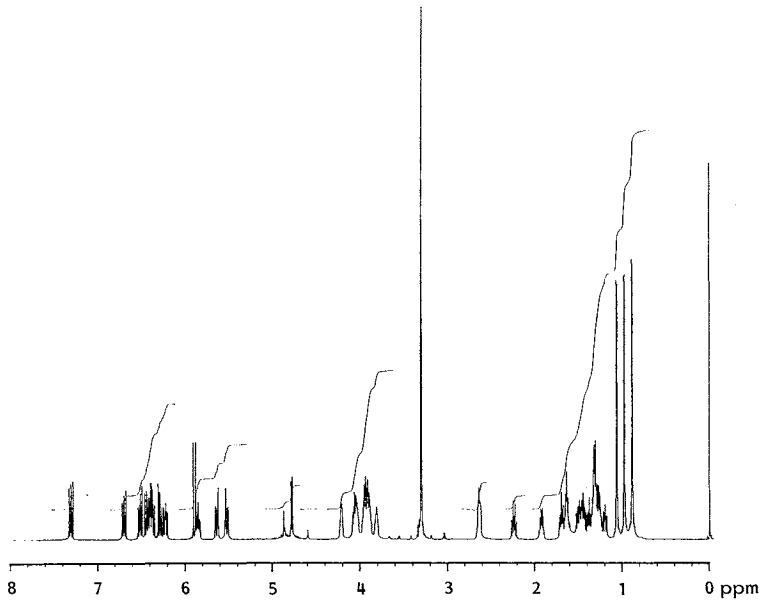
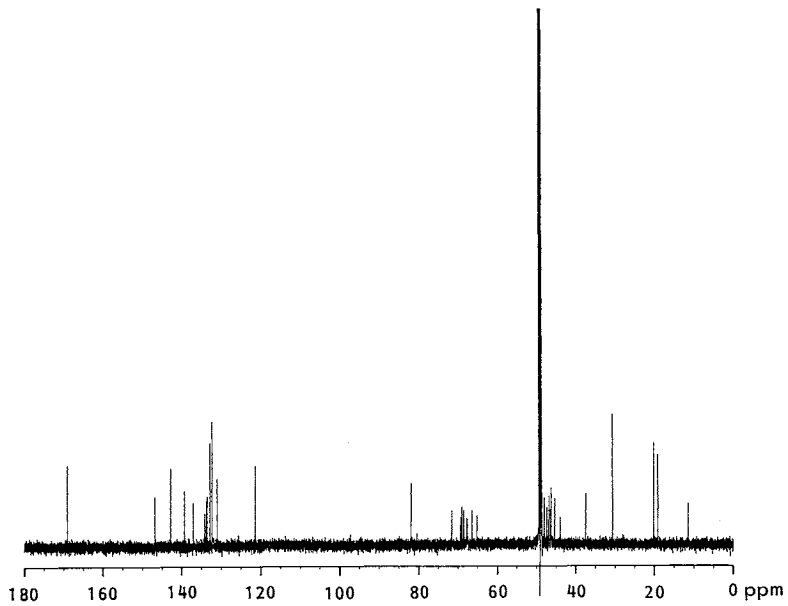


Fig. 3. ^{13}C NMR of RK-397 in methanol-d_4, 150 MHz (ppm from TMS an internal standard).



RK-397 is soluble in methanol, ethanol, and ethyl acetate, but insoluble in chloroform and water. The physico-chemical properties of RK-397 are summarized in Table 1. The ^{1}H and ^{13}C NMR spectra are shown in Figs. 2 and 3, respectively. The molecular formula $C_{35}H_{56}O_{10}$was established by high resolution FAB-MS. The structure (Fig. 1) was determined as a pentaene polyol by the UV, IR and NMR spectroscopies[3]. Mycoticin[4], flavofungin[5], roflamycoin[6] and roxaticin[7] are known members of the oxo pentaene group and it was concluded that RK-397 is a new member of this group.

RK-397 was cytotoxic against human leukemia, HL-60 and K-562 cells at a concentration of 50 μg/ml. The antibiotic induced the bleb-formation on K-562 cells at the concentrations over 0.05 μg/ml. We have reported that the blebs on K-562 cells were induced by inhibitors of protein phosphatase and activators of protein kinases[8], however, the mode of action of RK-397 was different from the protein phosphorylation (data not shown). RK-397 showed broad antimicrobial activities against filamentous fungi, yeast and bacteria at 50 to 100 μg/ml (Table 2). Another group of polyene antibiotics, amphotericin B and nystatin, is known to interact with ergosterol in eukaryotic cell membranes and inhibits fungal but not bacterial growth[9]. Faerifungin, which is structurally related to RK-397, showed a broad spectrum of antimicrobial activity[10], which corroborates that the mechanism of action of RK-397 is different from that of amphotericin B and nystatin. The mechanism of action of oxo polyene polyol antibiotics against bacteria needs to be resolved. Acute toxicity of RK-397 to mice was not observed up to 40 mg/kg (ip).

Table 2. Antimicrobial spectrum of RK-397.

Microorganisms	MIC (μg/ml)
Pyricularia orizae IFO5994	50
Botryotinia fuckeliana IFO5365	50
Candida albicans IFO1594	100
Cryptococcus neoformans KC-201	>100
Escherichia coli NIHJ	>100
Pseudomonas aeruginosa L-form N-10	50
Staphylococcus aureus 209P	100
S. aureus Smith	50
S. aureus JS-1 (MRSA)	>100

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