ISOLATION AND PROPERTIES OF VALANIMYCIN, A NEW AZOXY ANTIBIOTIC

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Valanimycin, a new azoxy antibiotic, was isolated from culture broths of a streptomycete. Valanimycin is an unstable oil at room temperature and active against some Gram-positive and Gram-negative bacteria, mouse leukemia L1210 cells in cultures, and prolongs the life span of mice inoculated with Ehrlich carcinoma or L1210.

During the course of prescreening of potential antitumor antibiotics by use of a *recA*-deficient mutant of *Escherichia coli* as a test organism, an active substance isolated from culture filtrates of a streptomycete was characterized chemically and biologically. The active substance was named valanimycin on the basis of its possible biosynthesis from valine and alanine. This paper deals with isolation procedures, structure determination and biological activities of valanimycin, as well as the taxonomy of the producing organism.

Materials and Methods

Instruments

IR, UV and NMR spectra were recorded on Hitachi IR spectrophotometer Model 260-10, Hitachi UV spectrophotometer Model 220S, and Jeol GX400 FT-NMR spectrometer, respectively. A secondary ionization mass spectrum (SI-MS) was obtained with Hitachi M-80H mass spectrometer.

Taxonomy

The taxonomy of the producing organism was performed by the method of SHIRLING and GOTTLIEB[1]. Diaminopimelic acid was determined according to BECKER, *et al.*[2].

Assay of Antibiotic Activity

Production and purification of valanimycin were followed by the antibiotic activity determination by the paper disc-agar diffusion method with *Escherichia coli* BE1121†† as the test organism. See the legend to Table 6 for the genetic markers.

Culture Conditions

Spores from a slant culture of the producing organism were inoculated into 500-ml flasks each containing 100 ml of the culture medium which consisted of maltose 2%, peptone 0.5%, meat extract

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0.5%, yeast extract 0.3%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1% and water to volume, the pH being adjusted to 7.2 before sterilization. The flasks were then incubated at 30°C with shaking at 220 rpm for approximately 40 hours.

Animals

Female CDF_1 mice weighing 19 to 21 g and female ICR/S weighing 23 to 25 g were used for determining the antitumor activity of valanimycin.

Biological Activities

The antibacterial spectrum of valanimycin was determined by the agar dilution method in Mueller-Hinton agar medium (Difco). Cytotoxicities *in vitro* to leukemia L1210, P388/S (doxorubicin-sensitive) and P388/ADR (doxorubicin-resistant) cells were determined as follows. L1210 cells were suspended at a density of 1.0×10^5 cells/ml in Eagle's MEM supplemented with 10% calf serum. One ml aliquots of the cell suspension, including a desired amount of a valanimycin sample, were distributed into wells of Falcon Petri dishes and incubated at 37°C for 2 days. Cells of each run were counted with a Coulter Counter. Cells of P388/S and P388/ADR were cultured likewise except that they were suspended at a density of 5×10^4 cells/ml in RPMI 1640 medium supplemented with 10% calf serum and 10 μM mercaptoethanol. The IC_{50} was defined as the concentration at which the cell growth was inhibited by 50%. To determine the antitumor effect on tumor-bearing mice, 1×10^5 cells of L1210 were inoculated intraperitoneally into each CDF_1 mouse and valanimycin was administered intraperitoneally once every day for 10 days, starting on the day of the tumor inoculation. The possible elongation of the life span by valanimycin was determined with these mice. Six mice were used for each determination. Effects on ICR/S mice, each inoculated intraperitoneally with 2×10^6 Ehrlich carcinoma cells, were determined likewise.

Results

Taxonomy of the Producing Organism

The strain was isolated from a soil sample collected at Sado Island, Niigata Prefecture, Japan, and numbered MG456-hF10 in this Institute. A transmission electron micrograph of this strain is represented in Fig. 1. Strain MG456-hF10 grows on various agar media containing sucrose, glucose, starch or glycerid as the carbon source and asparagine, nitrate or yeast extract as the nitrogen source. Optimum growth was observed at temperatures ranging from 27°C to 37°C. Morphological and physiological characteristics of strain MG456-hF10 resemble those of *Streptomyces viridifaciens* IMC S-0679 (ISP 5239) except for utilization of D-mannitol, D-xylose and sucrose (Table 1). The whole cell hydrolysate contained L,L-diaminopimelic acid. As a result of the comparison, the producing organism was designated as *Streptomyces viridifaciens* MG456-hF10. It has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan, with the accession number of FERM-P 8146.

Production of Valanimycin

The time course of cell growth and valanimycin production is shown in Fig. 2. Cell growth was monitored by determining packed cell volumes. Concentration of the antibiotic in the culture broth reached the maximum (135 μg/ml) at about 35 hours of incubation and decreased rapidly after 40 hours of incubation. Most of the antibiotic activity was found in the culture filtrate, while a limited amount is in the mycelia.

Purification and Isolation

Isolation procedures are summarized in Fig. 3. In brief, 5 liters of the broth filtrate, after the

Table 1. Comparison of MG456-hF10 and *Streptomyces viridifaciens* IMC S-0679 (ISP 5239).

Characteristic	MG456-hF10	*S. viridifaciens*
Spore chain morphology	Straight~retinaculum-aperpum	Straight~retinaculum-aperpum
Spore surface	Smooth	Smooth
Color of growth	Pale yellowish brown	Pale yellowish brown
Color of aerial mycelium	Brownish gray	Brownish gray
Soluble pigment	Yellowish	Faint yellowish
Melanoid pigment production	−	−
Gelatin liquefaction	+	+
Skim milk hydrolysis	+	+
Starch hydrolysis	+	+
Carbon source Utilization		
Positive:	D-Glucose, L-arabinose, D-fructose, D-mannitol*	D-Glucose, L-arabinose, D-fructose, D-xylose, sucrose
Negative:	D-Xylose*, sucrose*, inositol, L-rhamnose, raffinose	Inositol, L-rhamnose, raffinose, D-mannitol

* The asterisked carbon sources were distinguished by the two strains.

Fig. 1.

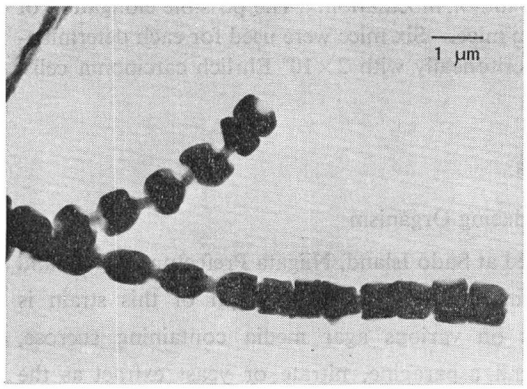
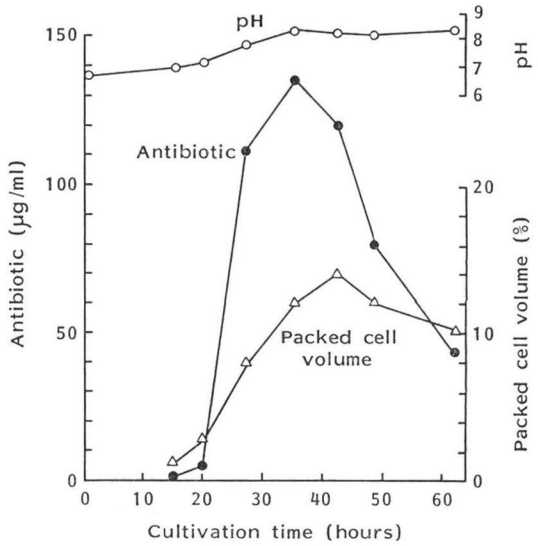


Fig. 2. Time course of antibiotic production.



pH was adjusted to 3.0, were vigorously mixed with 2.5 liters of butyl acetate and the mixture was left standing. The antibiotic distributed in the upper layer was then transferred into 1 liter of 10 mM sodium phosphate solution, pH 7.0 (PBS). The PBS fraction, after a brief evaporation to remove butyl acetate, was passed through a column of Dowex 1X8 (Cl^-, 50 ml). The column was washed with PBS and eluted with 500 ml of a linear gradient of 0 to 1.0 M sodium chloride in PBS. Active fractions were collected (200 ml) and extracted with 100 ml of ethyl acetate at pH 3. The ethyl acetate layer was concentrated to about 5 ml and placed on a column of Sephadex LH-20 (2.5 cm×90 cm). The column was eluted with 50% methanol in PBS. Active fractions were collected, methanol was evaporated *in vacuo*, and the remaining aqueous solution (50 ml) was extracted with 50 ml of ethyl acetate at pH 3. After removal of ethyl acetate by evaporation *in vacuo*, about 30 mg of valanimycin remained as a colorless oil.

Physico-chemical Properties and Structure Determination

The physico-chemical properties of valanimycin are summarized in Table 2 and the IR spectrum

Fig. 3. Isolation procedures.

Broth filtrate
↓ pH adjusted to 3.0
Extracted with BuOAc
↓
Transferred into phosphate buffered solution, pH 7.0 (PBS)
↓
Dowex 1X8 (Cl^-, 100~200 mesh)
↓ washed with PBS
↓ eluted with 0~1.0 M NaCl in PBS
↓ pH adjusted to 3.0
Extracted with EtOAc
↓ concd *in vacuo*
Sephadex LH-20
↓ eluted with 50% MeOH in PBS
↓ pH adjusted to 3.0
Extracted with EtOAc
↓ concd *in vacuo*
Colorless oil (unstable)

Table 2. Physico-chemical properties of valanimycin.

Appearance	Colorless oil
Stability	Unstable if dried especially in the absence of salt
Solubility:	
Soluble in	H_2O, MeOH, EtOH, EtOAc
Insoluble in	$CHCl_3$, *n*-hexane
Color reactions:	
Positive	$KMnO_4$, Mo-H_2SO_4, ninhydrin, bromocresol green, Rydon-Smith
Negative	2,4-Dinitrophenylhydrazine
UV	230 nm (shoulder)
pKa	*ca.* 4.7 (solvent extraction)
Reactivity	Amines

Table 3. NMR spectral data ($\delta^{D_2O}_{ppm}$) of valanimycin.

^1H (400 MHz)	^{13}C (100 MHz)
1.00 (6H, d, J=7.3 Hz)	20.3 (q)
2.38 (1H, m)	29.2 (d)
4.14 (2H, d, J=7.3 Hz)	78.1 (t)
5.40 (1H, s)	115.7 (t)
5.72 (1H, s)	149.4 (s)
	170.5 (s)

CH_3 / CH_3 $>CHCH_2-$, $CH_2=$, $-\underset{|}{C}=$

and the NMR chemical shifts are shown in Fig. 4 and Table 3, respectively. These data, together with the results of proton-decoupling experiments, suggested the presence of $(CH_3)_2CHCH_2$, $=CH_2$ and $=C$ in the structure. However, further structural analysis, such as mass spectrometry and elemental analysis, was impossible due to the instability of valanimycin. Therefore, attempts were made to obtain some stable derivatives. Valanimycin was found to readily react with various amines to form amine adducts. The ammonia adduct was prepared by the procedures outlined in Fig. 5. In brief, a portion of a partially purified valanimycin fraction, the methanolic eluate of the Sephadex LH-20 column chromatography (about 15 mg of valanimycin in 50 ml), was mixed with 28% ammonia water to make 2 N ammonium hydroxide. The solution was stirred for 1 hour at room temperature and, after evaporation of methanol and ammonia *in vacuo*, the remainder was passed through a column of Dowex 50WX8 (H^+, 5 ml). The column was washed with 50 ml of water and eluted with 50 ml of 2 N ammonium hydroxide. The eluate was concentrated *in vacuo* and freeze-dried. The ammonia adduct was obtained as a white powder (12 mg) which was found to be most stable among various amine adducts. The physico-chemical properties, IR spectrum and NMR chemical shifts of the ammonia adduct are shown in Table 4, Table 5 and Fig. 6, respectively. These data suggested the presence of $(CH_3)_2CHCH_2$ and $CHCH_2$ in the structure. The UV absorption maximum at 223 nm (ε 4,950) and the characteristic band near 1500 cm^{-1} in the IR spectrum implied the presence of an azoxy group. All these tentative structures were confirmed by platinum oxide-catalyzed reduction of the ammonia adduct yielding isobutylamine and D,L-α,β-diaminopropionic acid, both were identified by thin-layer chromatography and ^1H NMR. Comparison of the ^{13}C NMR spectrum of the ammonia adduct with that of the methylamine adduct revealed that (a) the signal of a triplet carbon at 41.1 ppm in the former shifted downfield to 50.3 ppm in the latter, (b) the signal of

Fig. 4. IR spectrum of valanimycin (KBr disc).

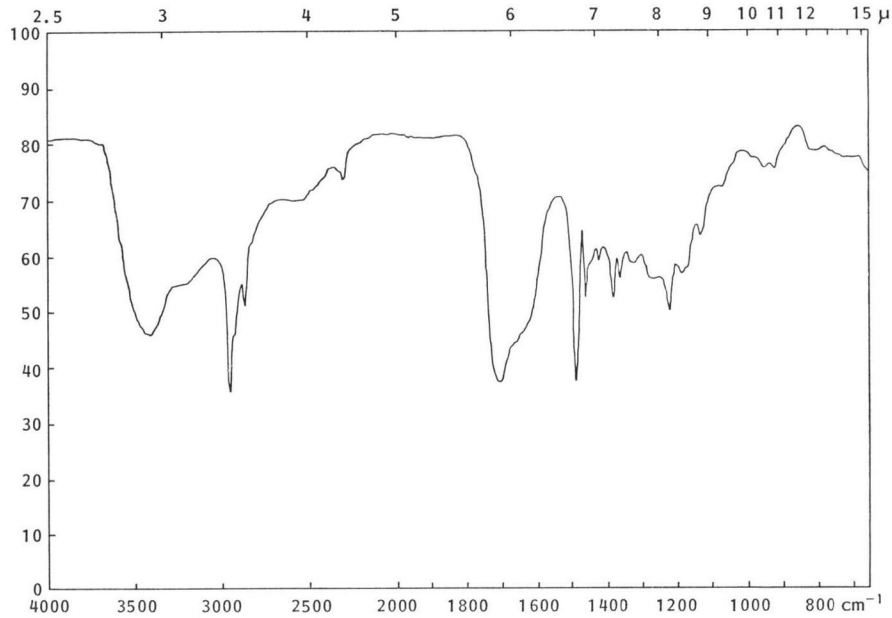


Table 4. Physico-chemical properties of the ammonia adduct.

Appearance	White powder
Elemental analysis	
Found:	C 44.29, H 7.93, N 22.33
Calcd for $C_7H_{15}N_3O_3$:	C 44.43, H 7.99, N 22.21
SI-MS (*m/z*)	190 $(M+H)^+$
Molecular formula	$C_7H_{15}N_3O_3$
MP	150.5°C (dec)
UV $\lambda_{max}^{H_2O}$ nm (ε) Acidic:	220 (5,400)
Neutral:	223 (4,950)
Alkaline:	220 (shoulder)
Color reactions: Positive:	Ninhydrin, Tollens, triphenyltetrazolium chloride, Mo-H_2SO_4, Rydon-Smith
Negative:	2,4-Dinitrophenylhydrazine
Solubility: Soluble in	H_2O, MeOH
Insoluble in	Acetone, EtOAc, DMSO, $CHCl_3$, n-hexane

Fig. 5. Preparation of the ammonia adduct.

Sephadex LH-20 fraction
- NH_4OH added to 2 N
- stirred for 1 hour at room temp
- NH_3 evaporated

Dowex 50WX8 (H^+, 50~100 mesh)
- washed with H_2O
- eluted with 2 N NH_4OH
- concd *in vacuo*
- freeze-dried

White powder (relatively stable)

Table 5. NMR spectral data ($\delta_{ppm}^{D_2O}$) of the ammonia adduct.

^1H (400 MHz)	^{13}C (100 MHz)
0.98 (3H, d, J=7 Hz)	19.7 (q)
1.01 (3H, d, J=7 Hz)	28.5 (d)
2.41 (1H, m)	41.1 (t)
3.49 (2H, m)	64.8 (d)
4.13 (2H, m)	77.7 (t)
4.49 (1H, dd, J=6, 8 Hz)	173.1 (s)

CH_3, CH_3 $\rangle$ $CHCH_2$–, –$\underset{|}{C}HCH_2$–

Fig. 6. IR spectrum of the ammonia adduct (KBr disc).

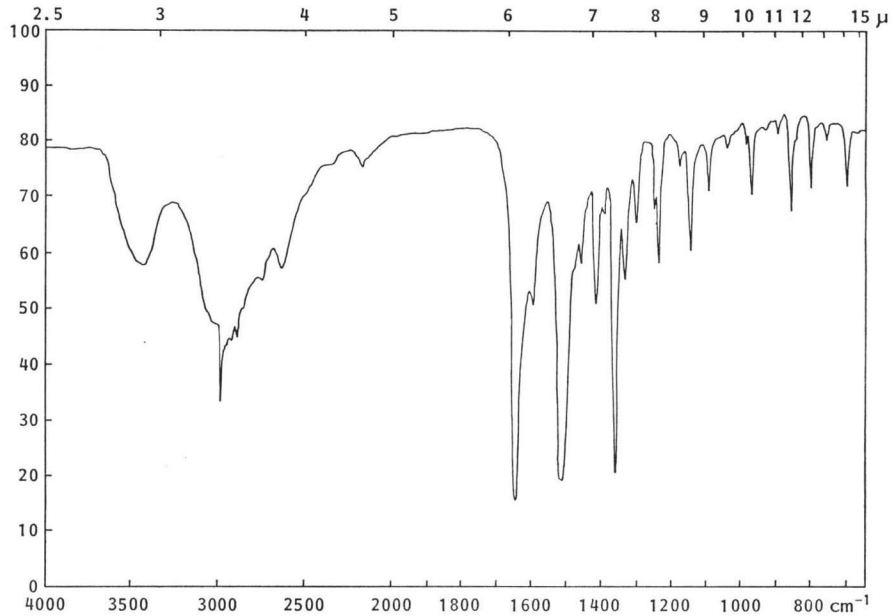
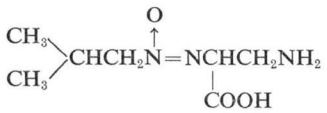
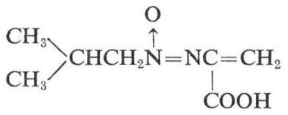


Fig. 7. Structures of valanimycin and its ammonia adduct.

(a)



(b)



a quartet carbon appeared at 34.3 ppm in the latter, and (c) the two spectra were otherwise similar to each other. From these results, the structure of the ammonia adduct was determined as shown in Fig. 7(a). The azoxy oxygen was assigned to the nitrogen attached to the isobutyl moiety based on comparison of the chemical shift of the methylene proton (4.13 ppm) with those of the corresponding methylene protons of some azoxy compounds[3~5]. Since valanimycin gave isobutylamine and D,L-α-alanine on catalytic reduction, its structure was determined as shown in Fig. 7(b). The UV spectrum of valanimycin has a shoulder at 230 nm (Table 2) which should be due to overlap of the azoxy band (about 220 nm) and that of the acrylic moiety (end absorption). The 275 nm band characteristic to azoxy seems to be too weak to be detected in the valanimycin spectrum.

Biological Activities

Valanimycin is active against both some Gram-positive and Gram-negative bacteria, as shown in Table 6. It should be noted that valanimycin is especially active against *E. coli* BE1121, a DNA-repair deficient mutant of *E. coli* K12. This mutant strain had been the test organism for finding and isolation of valanimycin. As shown in Table 7, valanimycin was toxic to *in vitro* cultures of cells of mouse leukemia L1210, P388/S (doxorubicin-sensitive), and P388/ADR (doxorubicin-resistant) with the IC_{50} values of 0.79, 2.65, and 1.44 μg/ml, respectively. Valanimycin prolonged the life span of mice inoculated with Ehrlich carcinoma or L1210, as shown in Table 8.

Table 6. Antibacterial spectrum of valanimycin.

Test organism	MIC (μg/ml)
Staphylococcus aureus 209P	10
S. aureus Smith	10
Micrococcus flavus FDA16	10
M. luteus IFO 3333	10
M. luteus PCI 1001	5
Bacillus anthracis	10
B. subtilis NRRL B-558	10
B. subtilis PCI 219	10
B. cereus ATCC 10702	>10
Corynebacterium bovis 1810	10
Escherichia coli NIHJ	2.5
E. coli K12	1.25
E. coli ML1629	2.5
Shigella dysenteriae JS11910	2.5
S. flexneri 4b JS11811	2.5
S. sonnei JS11746	2.5
Salmonella typhi T-63	2.5
S. enteritidis 1891	2.5
Proteus vulgaris OX19	5
P. mirabilis IFM OM-9	2.5
P. rettgeri GN311	2.5
P. rettgeri GN466	5
Serratia marcescens	10
Pseudomonas aeruginosa A3	10
Klebsiella pneumoniae PCI 602	10
Mycobacterium smegmatis ATCC 607	>10
Escherichia coli BEM11	1.25
E. coli BE1121	0.078

Genetic markers of BEM11 are F^-, Thr^-, Leu^-, Arg^-, Pro^-, Str^r, His^- and *tolC* and those of BE1121 are *recA*, and otherwise the same as BEM11.

Table 7. Inhibition of the growth *in vitro* of mouse leukemia cells by valanimycin.

Leukemia cells	IC_{50} (μg/ml)
L1210	0.79
P388/S	2.65
P388/ADR	1.44

Table 8. Antitumor activity of valanimycin to mice inoculated with Ehrlich carcinoma or L1210.

Dose (mg/mouse)	Elongation of life span (T/C, %)	
	Ehrlich carcinoma (2×10^6 cells/mouse)	L1210 (1×10^5 cells/mouse)
2.50	62	54
1.25	147	142
0.625	155	122
0.312	97	108
0.156	101	101
0.078	81	108

T/C stands for test run/control run.

Discussion

There are only a few naturally occurring azoxy compounds reported so far. Among them are macrozamine[6], cycasin[7], elaiomycin[8], and LL-BH872α[9]. Macrozamine and cycasin are toxic substances of plant origin. Elaiomycin, an antibiotic produced by *Streptomyces hepaticus*, is reported to be effective *in vitro* against *Mycobacterium tuberculosis*, but carcinogenic and inactive *in vivo*. LL-BH872α, another antibiotic produced by *Streptomyces hinnulinus*, is reported to have antifungal activity. Valanimycin is a new member of this class. Studies of the mode of action of valanimycin are in progress. High sensitivity of the DNA-repair deficient mutant of *E. coli* to valanimycin suggests that the primary target is DNA. It is likely that valanimycin acts as an alkylating agent, as does cycasin. We are interested in studying the biosynthesis of valanimycin, also. Preliminary data suggest that both valine and alanine are incorporated into the corresponding moieties of valanimycin (to be published).

Acknowledgment

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References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
2) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell wall preparations for strains of various form genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
3) GREENE, F. D. & S. S. HECHT: Oxadiaziridines, the cyclic form of an azoxy group. Synthesis, valance isomerism and reactivity. J. Org. Chem. 35: 2482~2486, 1970

4) McGAHREN, W. J. & M. P. KUNSTMAN: Revision of azoxy assignments in LL-BH872α and elaiomycin based on circular dichroism studies on synthetic azoxy compounds. J. Org. Chem. 37: 902~906, 1972
5) Moss, R. A. & G. M. LOVE: α-(Proximal)-azoxyalkyl carbanions — New synthesis of a proximal α,β-unsaturated azoxyalkane. Tetrahedron Lett. 1973: 4701~4703, 1973
6) LANGLEY, B. W.; B. LYTHGOE & N. V. RIGGS: Macrozamine. II. The aliphatic azoxy structure of the aglycone part. J. Chem. Soc. 1951: 2309~2316, 1951
7) RIGGS, N. V.: Glucosyloxyazoxymethane, a constituent of the seeds of Cycas circinalis L. Chem. Ind. 1956: 926, 1956
8) STEVENS, C. L.; B. T. GILLIS, J. C. FRENCH & T. H. HASKELL: Elaiomycin. An aliphatic α,β-unsaturated azoxy compound. J. Am. Chem. Soc. 80: 6088~6092, 1958
9) McGAHREN, W. J. & M. P. KUNSTMANN: A novel α,β-unsaturated azoxy-containing antibiotic. J. Am. Chem. Soc. 91: 2808~2810, 1969