VOL. 52, NO. 1, JAN. 1999 THE JOURNAL OF ANTIBIOTICS pp. 61 — 64

NOTES

Isolation and Structure of a New Antibiotic Viridomycin F Produced by *Streptomyces* sp. K96-0188

SATOSHI ŌMURA*, YUMI ENOMOTO, MAYUMI SHINOSE, YŌKO TAKAHASHI, YUZURU IWAI and KAZURO SHIOMI

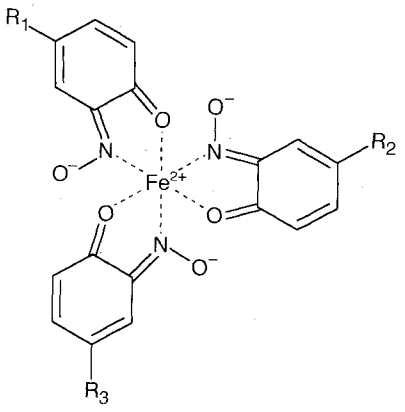
Research Center for Biological Function, The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan

(Received for publication September 11, 1998)

In the course of our screening for new antibiotics, a new compound, viridomycin F (**1**, Fig. 1), was isolated from a culture broth of *Streptomyces* sp. K96-0188. Compound **1** is a complex of iron and *p*-substituted *o*-nitrosophenol trimer and showed moderate insecticidal and nematocidal activities. Several iron complexes of *p*-substituted *o*-nitrosophenols, viridomycin A (**2**)[1~3] and E[4], actinoviridin (**3**)[5], 4-hydroxy-3-nitrosobenzamide ferrous chelate (**4**)[6], and ferroverdin (**5**)[7,8], have been isolated as green pigments from actinomycetes. As for biological activities, only moderate antibacterial activity of **2** against some Gram-positive bacteria has been reported[1,3]. In this report, we describe the characteristics of the producing strain and the fermentation, isolation, physico-chemical properties, structure elucidation, and biological activities of **1**.

The strain K96-0188 was isolated from a soil sample collected at Bizen City, Okayama prefecture, Japan. The vegetative mycelia grew abundantly on yeast extract-malt extract agar and inorganic salts-starch agar, and did not show fragmentation into coccoid forms or bacillary elements. The aerial mycelia grew moderately on inorganic salts-starch agar and glycerol-asparagine agar. The spore chains were *Rectiflexibiles* type, and each had more than 20 spores per chain. The spores were cylindrical in shape, 1.1 × 0.8 μm in size and had a smooth surface (Fig. 2). Whirls, sclerotic granules, sporangia, and flagellated spores were not observed. The isomer of DAP in whole-cell hydrolysates of strain K96-0188 was determined to be LL-type. The major menaquinones were MK-9 (H_6) and (H_8).

Fig. 1. Structures of iron complexes of *p*-substituted-*o*-nitrosophenols produced by *Streptomyces*.



	R_1	R_2	R_3
Viridomycin F (**1**)	CHO	CHO	CH_2OH
Viridomycin A (**2**)	CHO	CHO	CHO
Actinoviridin (**3**)	COOH	COOH	COOH
4-Hydroxy-3-nitrosobenzamide ferrous chelate (**4**)	$CONH_2$	$CONH_2$	$CONH_2$

Ferroverdin (**5**)

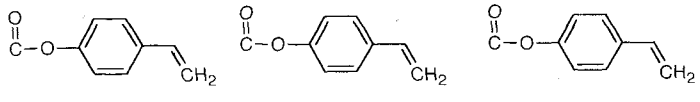
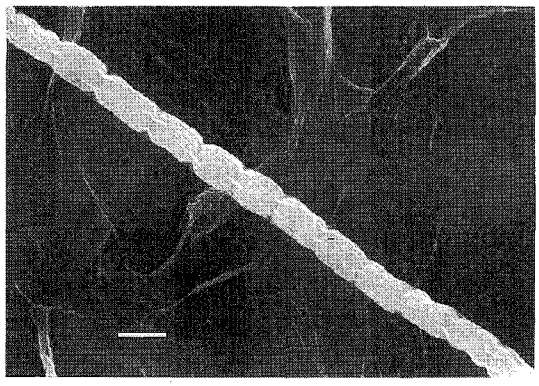


Fig. 2. Scanning electron micrograph of spore chain of *Streptomyces* sp. K96-0188 grown on inorganic salts-starch agar for 14 days.

Bar represents 1.0 μm.



The vegetative mycelia showed brown color. The aerial mass showed white to gray on inorganic salts-starch agar and glycerol-asparagine agar. Melanoid pigments were produced on glucose-peptone-gelatin and tryptone yeast extract broth, and soluble green pigment was produced on tyrosine agar. The strain utilized D-glucose, L-arabinose, raffinose, melibiose, D-fructose, and sucrose well, but did not utilize D-mannitol and *i*-inositol.

From the above characteristics, the strain K96-0188 is considered to belong to the genus *Streptomyces*[9]. The strain was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the name of *Streptomyces* sp. K96-0188 and the accession No. of FERM P-16398.

The slant culture of the strain K96-0188 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 0.1%, starch 1.0%, peptone 0.3%, meat extract 0.3%, yeast extract 0.1%, and $CaCO_3$ 0.3% (pH 7.0 before sterilization). After this seed culture was incubated on a rotary shaker at 27°C for 3 days, 2 ml of the culture was transferred into 500-ml Erlenmeyer flasks containing 100 ml of a producing medium consisting of soluble starch 4.0%, defatted soybean meals 2.0%, $FeSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%, KCl 0.03%, and 32 μl/liter of 0.1 N sodium thiosulfate (pH 6.5 before sterilization). The fermentation was carried out at 27°C for 6 days on a rotary shaker.

The fermentation broth (5 liters) was centrifuged at 3,000 rpm for 10 minutes. The mycelial cake was extracted with MeOH and the solvent was evaporated.

Table 1. Physico-chemical properties of **1**.

Appearance	Green powder
MP (°C)	>300
$[\alpha]_D^{25}$	+6.0 (*c* 0.1, MeOH)
Molecular formula	$C_{21}H_{14}N_3O_9Fe$
FAB-MS (*m/z*)	508 (M^-)
HR-FAB-MS	Calcd 508.0079 ($C_{21}H_{14}N_3O_9Fe$)
	Found 508.0081 (M^-)
UV_{max}^{MeOH} nm (ε)	207 (21800), 226 (sh, 13700),
	288 (7620), 435 (2290), 680 (2790)
IR ν_{max} (KBr) cm^{-1}	3400, 1589, 1494, 1429, 1083

Then the residual aqueous solution was adjusted to pH 2.0 with HCl and extracted with ethyl acetate. The organic layer was concentrated *in vacuo* to give a green oil (1.3 g). The oil was charged on a column of silica gel and developed with $CHCl_3$-MeOH (5:1~2:1). The fractions showed activity against *Artemia salina* were combined and concentrated *in vacuo* to give a green oil (134 mg). The oil was further purified by centrifuged partition chromatography in descending mode with a solvent system of ethyl acetate, BuOH, EtOH, and water (10:5:2:8) to yield a green powder of **1** (10.7 mg).

The physico-chemical properties of **1** are summarized in Table 1. Negative HR-FAB-MS revealed the molecular mass ion (M^-) of 508 and the molecular formula $C_{21}H_{14}N_3O_9Fe$ for **1**. This compound exhibited visible absorption maxima at 435 nm and 680 nm, which was comparable to the spectra of iron complexes of *p*-substituted *o*-nitrosophenols[1~7]. The structure of **1** was considered to be similar to these compounds.

The structure of **1** was deduced on the basis of 1D and 2D NMR experiments and comparison with chemical shifts of **2**~**4**[4,6]. The ^{13}C NMR and DEPT spectra of **1** showed the presence of one methylene, eleven methines including two aldehydes (δ 190.9 and 191.0), and nine quaternary carbons. Some carbon signals overlapped, and they were assigned by their intensities and long-range ^{13}C-^1H COSY. The ^{13}C and ^1H NMR spectral data of **1** are summarized in Table 2. The ^1H NMR and ^1H-^1H COSY of **1** showed three sets of three aromatic methine protons, which suggested three 1,2,4-trisubstituted benzene residues. They were confirmed by HMBC experiment (Fig. 3). Two aldehydes and one hydroxymethylene (δ_C 63.5, δ_H 4.46 (CH_2), and δ_H 4.30 (OH)) were assigned to connect with each benzene residue by HMBC. The remaining three nitrogens and six oxygens were suggested to be arranged as in Fig. 3 by

Table 2. The ^{1}H and ^{13}C NMR data of **1**.

Position	^{13}C	^{1}H
1	124.9 s	
1-CHO	191.0 d	9.84 s (1H)
2	115.6 d	7.64 d (1H, J=1.8 Hz)
3	160.33 s	
4	181.0 s	
5	123.2 d	7.13 d (1H, J=8.4 Hz)
6	134.7 d	7.92 dd (1H, J=1.8, 8.4 Hz)
1′	124.9 s	
1′-CHO	190.9 d	9.82 s (1H)
2′	115.4 d	7.60 d (1H, J=1.8 Hz)
3′	160.33 s	
4′	181.5 s	
5′	122.9 d	7.05 d (1H, J=8.4 Hz)
6′	134.7 d	7.88 dd (1H, J=1.8, 8.4 Hz)
1″	131.1 s	
1″-CH_2	63.5 t	4.46 s (2H)
2″	110.2 d	6.95 d (1H, J=1.8 Hz)
3″	160.27 s	
4″	179.7 s	
5″	123.0 d	7.03 d (1H, J=8.4 Hz)
6″	138.9 d	7.47 dd (1H, J=1.8, 8.4 Hz)
OH		4.30 br.s (1H)

The acetone-d_6 signals (2.06 ppm of ^{1}H and 29.8 ppm of ^{13}C) were used as references.

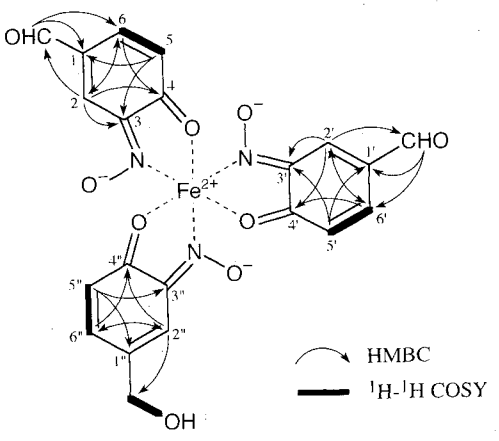
comparing carbon chemical shifts of **1** with those of **4**[6].

Among aromatic residues of **2**~**4**, those of **2** and **3** were shown as *o*-nitrosophenols[4,5]. However, those of **4** were shown as quinone-oxime forms by the chemical shifts of C-3 (δ 158.7) and C-4 (δ 179.9)[6], and the quinone-oxime forms seems more reasonable than *o*-nitrosophenols. Though some reports described viridomycins as dimers of *o*-nitrosophenols[1,4], X-ray crystallography of **5** revealed that it was a trimer[8]. The negative FAB-MS and NMR spectra of **1** also suggested that **1** was a trimer. *o*-Nitrosophenols were also studied as analytical reagents to detect iron ions and some synthetic derivatives have been reported. Analytical studies of their iron complex revealed that most of them were trimers (the others were tetramers)[10].

Therefore, the structure of **1** was elucidated as shown in Fig. 1. One of three aldehyde groups in viridomycin A is replaced by hydroxymethylene and forms a heterotrimer in **1**.

Insecticidal and nematocidal activities of **1** were evaluated in a microplate assay with brine shrimp *Artemia salina* and free-living nematode *Caenorhabditis elegans*. The nauplii larvae hatched from eggs were used for the brine shrimp assay. *C. elegans* was cultivated on an agar plate covered with *Escherichia coli* for 3~4 days at 20°C and the grown nematodes were used for the assay. These organisms and **1** were incubated in 96-well microplate at 20°C. After 48 hours, the motilities were assessed visually under microscope (×40) in comparison with controls.

Fig. 3. Structure of **1** elucidated by ^{1}H-^{1}H COSY and HMBC.



Compound **1** affected the motilities of *A. salina* at 200 μg/ml and *C. elegans* at 500 μg/ml.

Though **2** was reported to have some antibacterial activity, **1** inhibited only the growth of *Piricularia oryzae* and *Acholeplasma laidlawii* weakly among the tested bacteria and fungi.

Experimental

NMR spectra were obtained with JEOL JNM-EX270 spectrometers using acetone-d_6 as a solvent. Mass spectrometry was conducted on a JEOL JMS-AX500 HA spectrometer. UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively. Optical rotation was recorded on a JASCO model DIP-181 polarimeter. Melting point was measured with a Yanaco micro melting point apparatus MP-S3.

Acknowledgment

The authors are grateful to Ms. T. SAKABE, School of Pharmaceutical Sciences, Kitasato University, for measurements of mass spectra. Thanks are also due to Mr. Y. KASE, Ms. M. HIGASHIMURA, and Ms. S. MORIKAWA for excellent technical assistance. This work was supported in part by a grant from Japan Keirin Association.

References

1) KHOKHLOV, A. S. & I. N. BLINOVA: Structure of the new

iron-containing antibiotic viridomycin A. Dokl. Acad. Nauk SSSR 215: 1493～1496, 1974

2) BLINOVA, I. N.; S. A. EGOROVA, I. V. MARCHENKO, L. I. SAULINA, N. O. BLINOV & A. S. KHOKHLOV: New iron-containing antibiotic. Isolation and properties of viridomycins A, B, and C. Khim. Prirod. Soedin. 11: 490～498, 1975
3) YANG, C.-C. & J. LEONG: Mode of action of 4-hydroxy-3-nitrosobenzaldehyde from *Streptomyces viridans*. Antimicrob. Agents Chemother. 20: 558～562, 1981
4) KUROBANE, I.; P. L. DALE & L. C. VINING: Characterization of new viridomycins and requirements for production in cultures of *Streptomyces griseus*. J. Antibiotics 40: 1131～1139, 1987
5) BLINOV, N. O.; I. N. BLINOVA & A. S. KHOKHLOV: Nitrosophenol system of iron metabolism regulation in actinomycetes. Izv. Akad. Nauk SSSR, Ser. Biol.: 70～78, 1989
6) CONE, M. C.; C. R. MELVILLE, J. R. CARNEY, M. P. GORE & S. J. GOULD: 4-Hydroxy-3-nitrosobenzamide and its ferrous chelate from *Streptomyces murayamaensis*. Tetrahedron 51: 3095～3102, 1995
7) CHAIN, E. B.; A. TONOLO & A. CARILLI: Ferroverdin, a green pigment containing iron produced by streptomycete. Nature 176: 645, 1955
8) CANDELORO, S.; D. GRDENIC, N. TAYLOR, B. THOMPSON, M. VISWAMITRA & D. C. HODGKIN: Structure of ferroverdin. Nature 224: 589～591, 1969
9) WILLIAMS, S. T.; M. GOODFELLOW & G. ALDERSON: Genus *Streptomyces* Waksman and Henrici, 1943. *In* BERGEY's Manual of Systematic Bacteriology. Volume 4. *Ed.*, S. T. WILLIAMS *et al.*, pp. 2452～2492, Williams & Wilkins, Baltimore, 1989
10) TÔEI, K.; S. MOTOMIZU & T. KORENAGA: Nitrosophenol and nitrosonaphthol derivatives as reagents for the spectrophotometric determination of iron and determination of micro-amounts in waters with 2-nitroso-5-dimethylaminophenol. Analyst 100: 629～636, 1975