Natural Product Sciences
19(1) : 61-65 (2013)

Viriditoxin, from a Jellyfish-derived Fungus, is Antibiotic to Fish Pathogens

Juan Liu[1,4], Famei Li[2], Eun La Kim[1], Jongki Hong[3], and Jee H. Jung[1,*]

[1]*College of Pharmacy, Pusan National University, Busan 609-735, Korea*
[2]*Shenyang Pharmaceutical University, Shenyang 110016, China*
[3]*College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea*
[4] *South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China*

Abstract – A bioassay-guided fractionation of the extract of the fungus *Paecilomyces variotii*, which was derived from the giant jellyfish *Nemopilema nomurai*, led to the isolation of antibacterial compounds viriditoxin and its monomeric subunit semi-viriditoxin. Viriditoxin showed significant antibacterial activity against several marine fish and human pathogens including MDR strains. Significant potencies against resistant pathogens such as VRE *Enterococcus faecium*, VRE *Enterococcus faecalis*, and MRSA were highly interesting. Viriditoxin also showed notable antibacterial activity against the fish pathogen *Streptococcus iniae*. Its potency was over 100-fold higher than oxytetracycline which is employed as a general antibiotic for aquaculture.
Keywords – *Paecilomyces variotii*, Viriditoxin, Antibacterial activity, Jellyfish-derived fungus.

Introduction

In nature, the 6,6'-binaphthopyran-1-ones are rare metabolites and so far only five analogues have been reported (Suzuki *et al*., 1990; Suzuki *et al*., 1992; Arnone *et al*., 1995; Elix *et al*., 2004). Most of 6,6'-binaphthopyran-1-ones were isolated as fungal metabolites except pigmentosin A, which was obtained from the lichen *Hypotrachyna immaculata* (Elix *et al*., 2004). Talaroderxines A and B are atropisomers isolated from a heterothallic ascomycetous fungus, *Talaromyces derxii*, and have strong antibacterial activity against *Bacillus subtilis* (Suzuki *et al*., 1990). Asteromine, a metabolite from a strain of *Mycosphaerella asteroma*, exhibits phytotoxic effects on *Cucumis sativus*, mild antibacterial and antifungal activity, and lethality to the brine shrimp *Artemia salina* (Arnone *et al*., 1995). Of these analogues, viriditoxin was the most interesting due to its broad-spectrum antibiotic activity against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE). The mechanism of action and broad-spectrum antibacterial properties of viriditoxin was studied and identified as an inhibitor of bacterial cell division by blocking FtsZ, which presents an excellent novel target for antibacterial drug discovery (Wang *et al.*, 2003).

Viriditoxin was first isolated from a mycelia extract of *Aspergillus viridi-nutans* (Lillehoj and Ciegler, 1972), and the structure was revised in 1990 (Suzuki *et al*., 1990). This compound was also isolated from other fungi, such as *A. brevipes* (Lillehoj and Milburn, 1973), *A. fumigatus* (Mukhopadhyay *et al*., 1987), and *Paecilomyces variotii* (Jiu and Mizuba, 1974).

In our study on the antibacterial components of the fungus *P. variotii* derived from the giant jellyfish *Nemopilema nomurai*, viriditoxin (**1**) and its monomeric subunit semi-viriditoxin (**2**) were isolated as causative components.

Experimental

General – ^{1}H and ^{13}C NMR spectra were recorded on UNITY 400 and Varian INOVA 500 instruments. Chemical shifts were reported with reference to the respective residual solvent or deuterated solvent peaks. FABMS data were obtained on a JEOL JMS SX-102A. HRFABMS data were obtained on a JEOL JMS SX-101A. CD spectra were recorded on a JASCO J-715 instrument in $CHCl_3$; HPLC was performed with an YMC ODS-H80 column (250 × 10 mm i.d., 4 μm, 80 Å) using a Shodex RI-71 detector.

*Author for correspondence
College of Pharmacy, Pusan National University, Busan 609-735, Korea.
Tel: +82-51-510-2803; E-mail: jhjung@pusan.ac.kr

Fungal materials – The fungus *Paecilomyces variotii* was isolated from the jellyfish *Nemopilema nomurai* collected off the southern coast of Korea in June 2007. The specimen was deposited at the Marine Natural Product Laboratory, PNU. Following a rinse with sterile sea water, the jellyfish tissue was homogenized and then inoculated on malt extract agar (MEA), which was prepared with 75% sea water, containing glucose (20 g/L), malt extract (20 g/L), agar (20 g/L), peptone (1 g/L), and antibiotics (10,000 units/mL penicillin and 10,000 μg/mL streptomycin, 5 mg/L). Fungi growing out of the jellyfish tissue were separated on the same MEA medium until a pure culture was obtained. Twelve pure fungal strains (J08NF-1~J08NF-12) were isolated from the jellyfish. The fungal strain J08NF-1 was selected on the basis of significant antibacterial activity against the gram-positive strain *Staphylococcus aureus* SG 511 (zone of inhibition 14 mm at 400 μg/disc), and it was identified as *Paecilomyces variotii* by Dr. K. S. Bae using morphological and biochemical analyses. *P. variotii* was then cultured in MEA medium (prepared with 75% sea water) containing glucose (20 g/L), malt extract (20 g/L), and peptone (1 g/L) at 30 ºC on a shaker platform at 155 rpm for 21 days, in total of 22 L.

Extraction and isolation – The culture medium and mycelia were extracted with EtOAc at room temperature. The antibacterial activity of the crude EtOAc extract of *Paecilomyces variotii* was tested against a panel of human pathogens (*Staphylococcus aureus* SG 511, *Salmonella typhimurium*, *Klebsiella aerogenes* 1522 E, *Escherichia coli* 078, and *Enterobacter cloacae* 1321 E) and marine pathogens (*Edwardsiella tarda* FP 5060, *Listonella anguillarum* FP 5208, *Streptococcus iniae* FP 5228, and *Vibiro ichthyoenteri* FP 4004) by disc diffusion method. The results showed that *S. aureus* SG 511 and 2 marine strains, *S. iniae* FP 5228 and *V. ichthyoenteri* FP 4004 were sensitive at an exact concentration of 400 μg/disc. Guided by the lethality to brine shrimp larvae (LD_{50} 2 μg/mL) and antibacterial activity against these strains, the EtOAc extract (10.2 g) was partitioned between aqueous MeOH and *n*-hexane, whose zones of inhibition against *S. aureus* SG 511 were 13 and 7 mm at 30 μg/disc, respectively. Some yellow precipitate cannot be dissolved in both aqueous MeOH and *n*-hexane, and exhibited significant antibacterial activity against *S. aureus* SG 511 (Inhibition zone: 22 mm at 30 μg/disc). By filtration of the yellow precipitate, compound **1** (1.8 g) was obtained. The aqueous MeOH layer was subjected to a step-gradient MPLC (ODS-A, 120 Å, S-30/50 mesh) eluting with 50% to 100% MeOH to afford 21 fractions. Each fraction was tested for lethality to brine shrimp larvae and for antibacterial activity against *S. aureus* SG 511, *S. iniae* FP 5228, and *V. ichthyoenteri* FP 4004. Fraction 5, one of the bioactive fractions (Inhibition zone: 20 mm at 30 μg/disc), was subjected to reversed-phase HPLC (YMC ODS-H80, 250 × 10 mm i.d., 4 μm, 80 Å) eluting with 75% MeOH + 0.4% HCOOH (v/v) to afford 9 subfractions. Compound **2** (2.6 mg) was obtained by purification of subfraction 3 on reversed-phase HPLC (YMC ODS-H80, 250 × 10 mm i.d., 4 μm, 80 Å) eluting with 60% ACN.

Viriditoxin (**1**) – Light yellow, amorphous powder; $[\alpha]_D^{25}$ –163.6 (c = 0.51, $CHCl_3$); CD ($c = 5.5 \times 10^{-5}$ M, $CHCl_3$): $\Delta\varepsilon$ (nm) + 7.7 (325.0), 0 (308.0), – 109.2 (275.5), 0 (264.5), 100.6 (255.5), 0 (227.0). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.84 (H, s, H-8), 6.16 (H, s, H-5), 4.90 (H, m, H-3), 3.69 (3H, s, 7-OC<u>H</u>$_3$), 3.61 (3H, s, COC<u>H</u>$_3$), 2.84 (2H, m, H-4), 2.77 (2H, m, H-11); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 170.6 (C-1), 170.5 (<u>C</u>OCH$_3$), 163.5 (C-10), 160.8 (C-7), 159.1 (C-9), 139.4 (C-5a), 133.9 (C-4a), 113.5 (C-5), 110.0 (C-6), 108.5 (C-9a), 99.6 (C-10a), 98.8 (C-8), 76.4 (C-3), 56.6 (7-O<u>C</u>H$_3$), 52.3 (CO<u>C</u>H$_3$), 39.4 (C-11), 32.4 (C-4). HRFABMS *m/z* 661.1574 $[M - H]^-$ (calcd for $C_{34}H_{29}O_{14}$, 661.1557).

Semi-viriditoxin (**2**) – Light yellow, amorphous powder; $[\alpha]_D^{25}$ –67.4 (c = 0.25, $CHCl_3$); ^{1}H NMR (pyridine-d_6, 500 MHz) δ 6.89 (H, s, H-5), 6.87 (H, d, J = 1.5 Hz, H-8), 6.78 (H, d, J = 1.5 Hz, H-6), 5.19 (H, m, H-3), 3.80 (3H, s, 7-OC<u>H</u>$_3$), 3.64 (3H, s, COC<u>H</u>$_3$), 3.08 (H, m, H-4$_a$), 3.03 (H, m, H-4$_b$), 3.01 (H, m, H-11$_a$), 2.91 (H, m, H-11$_b$); ^{13}C NMR (Pyridine-d_6, 100MHz) δ 169.2 (<u>C</u>OCH$_3$), 169.1 (C-1), 163.4 (C-9), 162.0 (C-7), 158.8 (C-10), 140.0 (C-5a), 132.8 (C-4a), 114.5 (C-5), 108.5 (C-10a), 100.5 (C-8), 99.0 (C-9a), 98.3 (C-6), 74.8 (C-3), 54.2 (7-O<u>C</u>H$_3$), 50.6 (CO<u>C</u>H$_3$), 38.4 (C-11), 31.6 (C-4).

Bacterial strains and antibiotics – The human pathogens, *S. aureus* SG 511, *S. aureus* SG 503, *Salmonella typhimurium*, *Klebsiella aerogenes* 1522 E, *Escherichia coli* DC 0, *E. coli* TEM, *Pseudomonas aeruginosa* 1771, *Enterobacter cloacae* P 99, and *E. cloacae* 1321 E, were donated by the Korea Institute of Science and Technology (KIST). The marine strains, *Edwardsiella tarda* FP 5060, *Listonella anguillarum* FP 5208, *S. iniae* FP 5228, and *V. ichthyoenteri* FP 4004, were provided by National Fisheries Research & Development Institute, Korea. The methicillin-resistant *S. aureus* 3089 (MRSA), multidrug-resistant (MDR) *P. aeruginosa* 2200, MDR *S. typhimurium* 8173, MDR *E. cloacae* 0252, MDR *E. coli* 1137, VRE *Enterococcus faecium* 5207, VRE *Enterococcus faecalis* 5210 and MDR *Vibrio parahaemolyticus* 7001 were purchased from Korea National Reasearch Resource Bank

(KNRRB). All standard antibiotics were purchased from the Sigma Aldrich Co.

Antibacterial assay – MIC values of the compounds were determined by the modified 0.5 Mcfarland standard method. Two-fold dilutions of the compounds in the range of (40 - 0.16 μg/mL) were prepared in 0.5% DMSO. Antibiotics were similarly diluted in 0.5% MeOH to generate a series of concentrations ranging from 40 to 0.16 μg/mL per well. The turbidity of the bacterial suspensions was measured at 600 nm, and adjusted with medium to match the 0.5 McFarland standards (10^5 - 10^6 colony forming units/mL). Subsequently, 180 μL of bacterial culture was inoculated into each well, and the test solutions (20 μL) were added to 96-well plates. Finally, the plates were incubated at 36 °C for 24 h, and the MIC values were determined in triplicates and re-examined at appropriate times. To ensure that these vehicles had significant effect on the bacterial growth, each bacterial species was additionally cultured in a blank solution containing LB or BHI broth media at concentrations equivalent to those of the test solutions.

Result and Discussion

The culture medium and mycelia were extracted with EtOAc. The EtOAc extract (10.2 g) was further partitioned between aqueous MeOH and *n*-hexane. Yellow precipitate which cannot be dissolved in both solvents was obtained and exhibited significant antibacterial activity against *Staphylococcus aureus* SG 511 (Inhibition zone: 22 mm at 30 μg/disc). After filtration, the compound (**1**) showed high purity. The high yield (81.8 mg/L) and significant antibacterial activity of compound **1** were of interest.

Compound **1** was a yellow, amorphous powder. The exact mass of the $[M - H]^-$ ion at *m/z* 661.1574 matched well with the expected formula $C_{34}H_{29}O_{14}$ (Δ +1.7 mmu). The NMR data of compound **1** was almost identical to that of viriditoxin (Suzuki *et al*., 1990). The chirality of the axis between the naphtha-α-pyrone moieties in compound **1** was confirmed by exciton chirality method. The CD spectrum exhibited a strong negative ($\Delta\varepsilon$ −109.2 at 275.5 nm) and a positive second ($\Delta\varepsilon$ +100.6 at 255.5 nm) Cotton effects, which was similar to the reported (Fig. 2). This was due to the coupling between the ^1B_b transitions of two naphthalene chromophores, the long axis of naphtho-α-pyrone moieties in the compound was twisted in a counter-clockwise manner. Thus, the chirality of compound **1** was defined as R_a-configuration. Based on biogenetic consideration and specific rotation ($[\alpha]_D^{25}$ −163.6) of **1** (Park *et al*., 2011; Weisleder and Lillehoj, 1971), the configuration at C-3 was defined to be *S*, which is the same as that of the co-isolated compound **2** (*vide infra*).

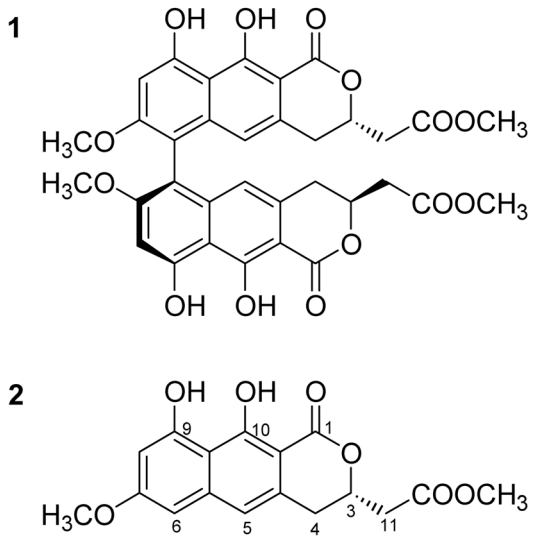

Fig. 1. Viriditoxin (**1**) and semi-viriditoxin (**2**).

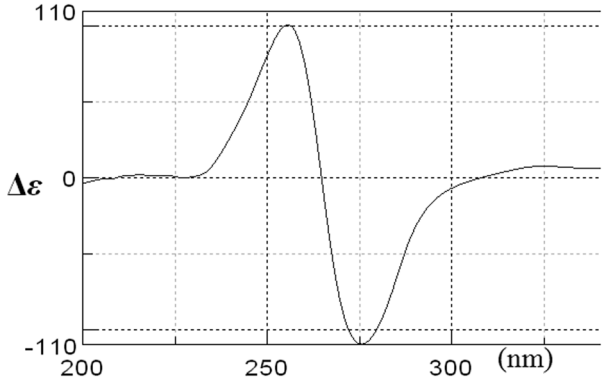

Fig. 2. CD spectrum of viriditoxin.

Various bioactivities, especially antibacterial activity, of viriditoxin (**1**) were reported. Viriditoxin has been shown to block FtsZ polymerization leading to an arrest of cell division, and finally to cell death. Viriditoxin showed broad-spectrum antibiotic activity against Gram-positive pathogens, including methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci* (Wang *et al.*, 2003). Viriditoxin was lethal to mice with LD_{50} of 2.8 mg/kg (Weisleder and Lillehoj, 1971). Viriditoxin can activate ATP hydrolysis (ATPase) and swelling in rat liver mitochondria (Wong and Hamill, 1976).

Considering possible biological role of compound **1** in marine ecology, it was evaluated for antibacterial activity against marine fish pathogens in addition to human

Table 1. MICs (μg/mL) of viriditoxin (**1**) and antibiotics

Strains	1	A	B	C	D
Edwardsiella tarda FP 5060 [a]	> 40		0.63		
Vibrio ichthyoenteri FP 4004	0.63		0.16		
***Streptococcus iniae* FP 5228** [a]	0.31		40		
Staphylococcus aureus SG 511	0.16	0.16			
Staphylococcus aureus SG 503	40	0.16			
Escherichia coli DC 0	40	0.63			
Escherichia coli DC TEM	> 40	1.25			
Pseudomonas aeruginosa 1771	0.16	0.16			
Salmonella typhimurium	> 40	1.25			
Klebsiella aerogenes 1522 E	> 40	1.25			
Enterobacter cloacae P 99	> 40	40			
Enterobacter cloacae 1321 E	> 40	1.25			
MDR *Pseudomonas aeruginosa* 2200	> 40			20	
MDR *Salmonella typhimurium* 8173	> 40				0.31
MRSA 3089	1.25			0.63	
MDR *Vibrio parahaemolyticus* 7001	> 40				0.63
MDR *Enterobacter cloacae* 0252	> 40				0.16
MDR *Escherichia coli* 1137	> 40				0.63
VRE *Enterococcus faecium* 5207	1.25				1.25
VRE *Enterococcus faecalis* 5210	1.25				1.25

[a] The strains *Edwardsiella tarda* FP 5060, *Vibrio ichthyoenteri* FP 4004, and *Streptococcus iniae* FP 5228 were derived from marine fishes. **A**: tetracycline, **B**: oxytetracycline, **C**: vancomycin, **D**: levofloxacin

pathogens (Table 1). Compound **1** showed significant antibacterial potency against fish pathogens *Streptococcus iniae* FP 5228 and *Vibrio ichthyoenteri* FP 4004. It was especially notable that compound **1** inhibited the fish pathogen *S. iniae* FP 5228 with potency over 100-fold higher than that of oxytetracycline. Oxytetracycline is one of most frequently employed antibiotic in aquaculture. Compound **1** inhibited growth of human pathogens *S. aureus* SG 511 and *Pseudomonas aeruginosa* 1771, with MIC values of 0.16 μg/mL, which is comparable to that of tetracycline. Viriditoxin also inhibited the growth of three MDR strains, MRSA 3089, VRE *Enterococcus faecium* 5207, and VRE *Enterococcus faecalis* with similar potency (MIC 1.25 μg/mL) to that of levofloxacin.

Compound **2** was isolated as a yellow, amorphous powder, and it was identified as defined semi-viriditoxin by comparison of NMR data those reported. The absolute configuration at C-3 was defined as *S* by comparison of the optical rotation with the reported one (Tan and Donner, 2009). Semi-viriditoxin was previously isolated from the fungus *Paecilomyces variotii* and weak antibacterial activity against a number of bacteria was reported (Ayer *et al.*, 1991). Compound **2** showed weak inhibitory activity against the human pathogen *S. aureus* SG 511 with a MIC value of 40 μg/mL.

Viriditoxin (**1**), from a jellyfish-derived fungus, showed potent antibacterial activity, especially against the fish pathogen *S. iniae* FP 5228. The potency was over 100-fold higher than that of oxytetracycline, and this suggest a potential ecological role of **1** in the jellyfish *N. nomurai*. Compound **1** also showed antibacterial activity against VRE with potency comparable to that of levofloxacin. Further study on antibacterial activity of **1** against fish pathogens and VRE would yield valuable informations.

Acknowledgments

This study was supported by a 2- year research grant of Pusan National University. The authors thank Dr. K. S. Bae for the identification of the fungus.

References

Arnone, A., Assante, G., Montorsi, M., and Nasini, G., Asteromine, a bioactive secondary metabolite from a strain of *Mycosphaerella asteroma*. *Phytochemistry* **38**, 595-597 (1995).

Ayer, W.A., Craw, P.A., and Nozawa, K., Two 1H-naphtho[2,3-*c*]pyran-1-one metabolites from the fungus *Paecilomyces variotii*. *Can. J. Chem.*

69, 189-191 (1991).

Elix, J.A. and Wardlaw, J.H., Pigmentosin A, a new naphthopyrone from the lichen *Hypotrachyna immaculata*. *Aust. J. Chem*. **57**, 681-683 (2004).

Jiu, J. and Mizuba, S., Metabolic products from *Spicaria divaricata* NRRL 5771. *J. Antibiot*. **27**, 760-765 (1974).

Lillehoj, E.B. and Ciegler, A., A toxic substance from *Aspergillus viridi-nutans*. *Can. J. Microbiol.* **18**, 193-197 (1972).

Lillehoj, E.B. and Milburn, M.S., Viriditoxin production by *Aspergillus viridi-nutans* and related species. *Appl. Microbiol*. **26**, 202-205 (1973).

Mukhopadhyay, T., Roy, K., Coutinho, L., Rupp, R.H., Ganguli, B.N., and Fehlhaber, H.W., Fumifungin, a new antifungal antibiotic from *Aspergillus fumigatus* Fresenius 1863. *J. Antibiot*. **40**, 1050-1052 (1987).

Park, Y.S., Grove, C.I., Gonzalez-Lopez M, Urgaonkar S, Fettinger J.C., and Shaw J.T., Synthesis of (−)-viriditoxin: A 6,6'-binaphthopyran-2-one that targets the bacterial cell division protein FtsZ. *Angew. Chem. Int. Ed.* **50**, 3730-3733 (2011).

Suzuki, K., Nozawa, K., Nakajima, S., and Kawai, K., Structure revision of mycotoxin, viriditoxin, and its derivatives. *Chem. Pharm. Bull*. **38**, 3180-3181 (1990).

Suzuki, K., Nozawa, K., Nakajima, S., Udagawa S., and Kawai, K., Isolation and Structures of antibacterial binaphtho-α-pyrones, talaroderxines A and B, from *Talaromyces derxii*. *Chem. Pharm. Bull*. **38**, 1116-1119 (1992).

Tan, N.P.H. and Donner, C.D., Total synthesis and confirmation of the absolute stereochemistry of semiviriditoxin, a naphthopyranone metabolite from the fungus *Paecilomyces variotii*. *Tetrahedron* **65**, 4007-4012 (2009).

Wang, J., Galgoci, A., Kodali, S., Herath, K.B., Jayasuriya, H., Dorso, K., Vicente, F., González, A., Cully, D., Bramhill, D., and Singh, S., Discovery of a small molecule that inhibits cell division by blocking FtsZ, a novel therapeutic target of antibiotics. *J. Biol. Chem*. **278**, 44424-44428 (2003).

Weisleder, D. and Lillehoj, E.B., Structure of viriditoxin, a toxic metabolite of *Aspergillus viridi-nutans*. *Tetrahedron Lett*. **48**, 4705-4706 (1971).

Wong, D.T. and Hamill, R.L., Viriditoxin induces swelling and ATPase by activation of calcium transport in liver mitochondria. *Biochem. Biophys. Res. Commun*. **71**, 332-338 (1976).

Received March 11, 2013
Revised March 20, 2013
Accepted March 21, 2013