

Antifungal Activity of Valinomycin, a Cyclodepsipeptide from *Streptomyces padanus* TH-04

Tae Heon Lim, Hyuncheol Oh¹, Soon Youl Kwon, Jin-Ho Kim, Hyo-Won Seo², Jeong-Hun Lee²,
Byung-Sun Min^{2*}, Jin-Cheol Kim³, Chi-Hwan Lim⁴, and Byeongjin Cha⁵

Regional Innovation Center, Sangju University, Sangju 742-711, Korea

¹MCBI & College of Natural Sciences, Silla University, Busan 617-736, Korea

²College of Pharmacy, Catholic University of Daegu, Gyeongbuk 712-702, Korea

³Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea

⁴Department of Bio Environmental Chemistry, Chungnam National University, Daejeon 305-764, Korea

⁵Department of Plant Medicine, Chungbuk National University, Cheongju 361-763, Korea

Abstract – Using antifungal activity-guided fractionation, an actinomycete, *Streptomyces padanus* strain TH-04, which was isolated from mummified peaches infected with *Monilinia fructicola*, was found to produce the valinomycin (**1**). The structure of **1** was established using spectroscopic data, which including one- and two-dimensional NMR experimental and mass spectroscopy. Valinomycin (**1**) showed antifungal activity against *Phytophthora capsici*, with an IC₅₀ value of 15.9 µg/mL.

Keywords – *Streptomyces padanus* TH-04, valinomycin, structural determination, antifungal activity

Introduction

Genus *Streptomyces* is the source of the vast majority of actinomycete secondary metabolites that have been discovered to date. Substantial numbers of *Streptomyces* species produce medically useful agents, including a wide array of antifungal, antibacterial, and antitumor agents (Jensen *et al.*, 2007; Jeong *et al.*, 2006; Miyadoh, 1993). Accordingly, *Streptomyces* strains, as sources of a larger and wider variety of novel drugs, have been continuously noted more frequently than any of the other actinomycete genera (Lee *et al.*, 2005). In the course of our screening program for bioactive natural compounds, we isolated a number of actinomycete strains from mummified peaches infected with *Monilinia fructicola* in various regions of the Republic of Korea (Lim *et al.*, 2000). *Streptomyces padanus* strain TH-04 was identified in the actinomycete strains showing antifungal activity against several pathogenic fungi, including *M. fructicola*, *Colletotrichum gloeosporioides*, *Magnaporthe grisea*, *Rhizoctonia solani*, *Phytophthora capsici*, *Alternaria kikuchiana*, *Fusarium solani*, *Fusarium oxysporum* (Lim *et al.*, 2000). Activity-guided fractionation by solvent partitioning and purification

with silica gel column chromatography yielded a pure active compound. The structure of this compound was found to be that of a dodeca-cyclodepsipeptide, valinomycin, based upon the results of combined spectral analysis. This compound is known to be an antibiotic substance. However, the plant antifungal activity of valinomycin has not been reported thus far. In this paper, we describe the fermentation of *Streptomyces padanus* TH-04 and isolation, physico-chemical properties, structural determination, and antifungal activity of valinomycin (**1**).

Experimental

General experiments – ¹H-NMR (600 MHz) and ¹³C-NMR (150 MHz) spectra were measured with a Bruker DMX 600 FT-NMR spectrometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. Optical rotation was measured on a JASCO DIP-370 polarimeter. ESI-MS spectrum was recorded on a HP5989A DIP mass spectrometer. Column chromatography was carried out using Kieselgel 60 (63-200 µm, Merck Co.). Thin layer chromatography (TLC) was carried out using plates coated with silica gel 60 F₂₅₄ (Merck Co.) and spots were visualized under UV light and by 10% sulfuric acid (in H₂O) following by heating.

Actinomycete strain – Actinomycete strain TH-04

* Author for correspondence

Fax: +82-53-850-3602; E-mail: bsmin@cu.ac.kr

was isolated from mummified peaches infected with *Monilinia fructicola* collected at Jochiwon, Chungcheongnam-do, Republic of Korea. The samples were heated for 30 min at 80 °C, and were then used as isolation sources after they were washed three times with sterilized distilled water and dried. Actinomycete colonies were isolated after 2 - 4 weeks incubation on starch-nitrate agar. The colonies were freeze-dried and stored at -40 °C until use.

For identification of selected strain, 16S rDNA sequences were analyzed. Target DNA was amplified with a PCR kit using universal primers (27F 5'-AGA GTT TGA TCA TGG CTC AG-3', 1492R 5'-GGA TAC CTT ACG ACT T-3'). The conditions of the thermal cycles were as follow; pre-denaturation at 94 °C for 5 min; 40 cycles of denaturation for 1 min at 94 °C, annealing at 60 °C for 1 min, extension at 72 °C for 1.5 min, further extension at 72 °C for 10 min, and cooled to 4 °C. After purification of PCR product, it was sequenced with DNA analyzer (ABI PRISM 3700).

Fermentation – For isolation of active substance in liquid culture, fermentation of actinomycete strain TH-04 was performed in a 1 L flask containing 500 mL of YMB (yeast extract 3 g, malt extract 3 g, peptone 5 g, dextrose 10 g, and H₂O 1 L). The inoculum consisted of culture broth (10⁸ cfu/mL) that was incubated for 7 days at 150 rpm and 25 °C on YMB. Inoculant was added at a rate of 2 × 10⁵ cfu per 100 mL of medium. Incubation was performed with shaking at 150 rpm and 25 °C for 7 days. The conditions for incubation have been reported previously (Lim *et al.*, 2000).

Extraction and isolation – For extraction, culture broth was dried with a vacuum freeze dryer (Samwon, SFDSM24L, Korea). Dried powder containing bacteria was successively extracted three times at 4 °C with MeOH in order to create sub-antifungal powder without bacteria, and was evaporated under reduced pressure to obtain a residue (88.5 g). The MeOH extract was suspended in H₂O and partitioned with hexane, EtOAc, BuOH and water, successively, to yield the hexane-, EtOAc-, and BuOH-soluble fractions, respectively. The EtOAc-soluble fraction (2.5 g) was chromatographed on a silica gel column (85 g). Elution was started with hexane-EtOAc (8 : 1, 8 : 2, 8 : 3), CHCl₃ : MeOH (100 : 0, 95 : 5, 10 : 1, 7 : 1) and then CHCl₃-MeOH-H₂O (8 : 2 : 0.5) yielded 7 fractions (Fr. 1 - 7). Repeated column chromatography of fraction 2 (510 mg), which was showed antifungal activity against *P. capsici*, on silica gel (75 g) and recrystallization with MeOH yielded compound **1** (35 mg).

Compound 1 – Colorless plates; mp 186 - 188 °C; [α]_D²⁰ + 30.8° (c 0.5, MeOH); ESI-MS *m/z*: 1111.5 [M + H]⁺;

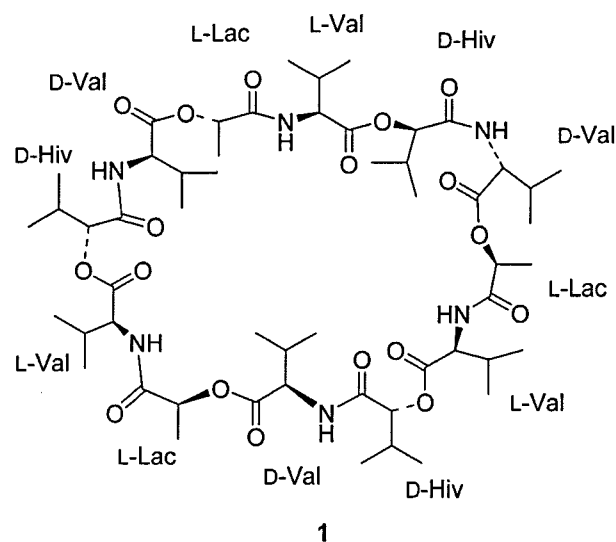


Fig. 1. Structure of compound **1** from *S. padanus* TH-04.

Table 1. NMR spectral data of compound **1** (MeOH-*d*₄, ¹H; 600 MHz, ¹³C; 150 MHz)

	¹ H-NMR	¹³ C-NMR	HMBC (C to H)
Hiv			
1-CO		172.1	2-H, 7-H
2-CH	4.82 (1H, d, 4.1)	80.2	4-H, 5-H
3-CH	2.28 (1H, m)	31.8	2-H, 4-H, 5-H
4-CH ₃	0.98 (3H, m)	19.4	2-H, 3-H, 5-H
5-CH ₃	0.98 (3H, m)	17.6	2-H, 3-H, 4-H
Val			
6-CO		171.6	7-H, 12-H
7-CH	4.35 (1H, d, 8.5)	59.9	8-H, 9-H, 10-H
8-CH	2.28 (1H, m)	30.7	7-H, 9-H
9-CH ₃	0.98 (3H, m)	19.8	7-H, 8-H, 10-H
10-CH ₃	0.98 (3H, m)	19.2	7-H, 9-H
Lac			
11-CO		173.2	12-H, 13-H, 15-H
12-CH ₃	5.08 (1H, q, 7.0)	71.9	13-H
13-CH ₃	1.40 (3H, d, 6.5)	17.9	12-H
Val			
14-CO		172.6	15-H, 2-H
15-CH	4.20 (1H, d, 9.0)	59.9	16-H, 17-H, 18-H
16-CH	2.25 (1H, m)	31.0	15-H, 17-H
17-CH ₃	0.98 (3H, m)	19.6	15-H, 18-H
18-CH ₃	0.98 (3H, m)	18.8	15-H, 17-H

¹H- and ¹³C-NMR data: see Table 1.

Antifungal activity test – Fungal strain used in the antifungal activity test is *P. capsici* as a representative fungus of plant pathogen. Test compound was dissolved in EtOH and added to potato dextrose agar. The concentration of test compound in medium was adjusted to 0.1, 1, 10, 100, and 1000 µg/ml and final concentration of EtOH was 0.5%. Agar-plugs (dia. 5 mm) with mycelium of *P. capsici* grown on PDA as inoculum were placed on test medium. The cultivation was carried out at 25 °C for

5 days. The antifungal activity was measured by the diameter of growth inhibitory zone around the paper disc (Bang *et al.*, 2000).

Results and Discussion

Streptomyces padanus TH-04 was harvested from mummified peaches infected with *Monilinia fructicola* and incubated at 150 rpm and 25 °C in YMB broth. The culture broth was dried with a freeze dryer and extracted with MeOH. The MeOH extract was partitioned with hexane, EtOAc, and BuOH, respectively. Bioassay-guided fractionation of the EtOAc-soluble components via silica gel column chromatography followed by recrystallization with MeOH yielded pure crystals of the compound (1).

Compound (1) was obtained in the form of optically active crystals, $[\alpha]_D^{25} +30.8^\circ$, that gave a molecular ion in the ESI-MS at m/z 1111.5, appropriate for molecular formula of $C_{54}H_{90}N_6O_{18}$. The compound exhibited four α -methine protons at δ 5.08 (q, $J=7.0$ Hz), 4.82 (d, $J=4.1$ Hz), 4.35 (d, $J=8.5$ Hz), and 4.20 (d, $J=9.0$ Hz), three methine protons at δ 2.28 (2H, m) and 2.25 (m), and seven methyl protons at δ 1.40 (d, $J=6.5$ Hz) and 0.98 (18H, m), which were assignable to a tetradepsipeptide in the 1H -NMR spectrum, compared with that of montanastatin isolated from a soil actinomycete *Streptomyces anulatus* (Pettit *et al.*, 1999). In addition, the ^{13}C -NMR and DEPT spectra displayed typical signals of depsipeptide, such as four carbonyl carbons at δ 173.2, 172.6, 172.1, and 171.6, two α -methine carbons of amino acid residues at δ 59.9 ($\times 2$), two oxygenated methine carbons at δ 80.2 and 71.9, and seven methyl carbons at δ 19.8, 19.6, 19.4, 19.2, 18.8, 17.9, and 17.6. Assignments of the cross-peaks in the COSY, HMQC, HMBC and TOCSY spectra suggested that compound 1 was composed of four units, a α -hydroxyisovaleryl unit ($(CH_3)_2CHCHOCO$ (Hiv), two valines ($(CH_3)_2CHCHNHCO$ (Val), and a lactoyl unit CH_3CHOCO (Lac) (Zeggaf *et al.*, 1989). The sequence was established by analysis of the HMBC spectrum (Rukachaisirikul *et al.*, 2006). Long-range correlations between Val α -H (δ_H 4.35) and Hiv CO (δ_C 172.1), together with Val α -H (δ_H 4.35)/Lac α -H (δ_H 5.08) and Val CO (δ_C 171.6) confirmed the linkages of the Hiv CO/Val NH and Val CO/Lac CHO. The Lac CO (δ_C 173.2) further connected with the Val CH (δ_H 4.20) in the HMBC spectrum. Furthermore, the ESI mass spectral data found for compound 1 established the molecular formula as $C_{54}H_{90}N_6O_{18}$, corresponding to a trimetric molecule of the tetradepsipeptide (D-Hiv-D-Val-L-Lac-L-Val). The absolute configuration was assigned by HPLC

analysis of the acid hydrolysate using a ligand-exchange type chiral column (Fukuda *et al.*, 2004) and by comparison with the 1H - and ^{13}C -NMR data of those in the relevant literature (Pettit *et al.*, 1999). Thus, compound 1 was determined to be valinomycin; *cyclo*-(-D-Hiv-D-Val-L-Lac-L-Val)₃.

Valinomycin (1) exhibited antifungal activity against *P.capcisi*, with an IC₅₀ value of 15.9 μ g/ml. Valinomycin has been reported to have apoptosis-inducing activity against Chinese hamster ovary cells (Abdalah *et al.*, 2006), cytotoxic activity against human and murine cancer cells (Ryoo *et al.*, 2006; Pettit *et al.*, 1999), and antiviral activity (De Clercq, 2006). This may be the first report to indicate that valinomycin has antifungal activity against *P.capcisi*.

References

- Abdalah, R., Wei, L., Francis, K., and Yu, S.P., Valinomycin-induced apoptosis in Chinese hamster ovary cells. *Neurosci. Lett.* **11**, 68-73 (2006).
- Bang, K.H., Kim, Y.K., Min, B.S., Na, M.K., Rhee, Y.H., Lee, J.P., and Bae, K.H., Antifungal activity of magnolol and honokiol. *Arch. Pharm. Res.* **23**, 46-49 (2000).
- De Clercq E., Potential antivirals and antiviral strategies against SARS coronavirus infections. *Expert. Rev. Anti. Infect. Ther.* **4**, 291-302 (2006).
- Fukuda, T., Arai, M., Tomoda, H., and Omura, S., New beauvericins, potentiators of antifungal miconazole activity, produced by *Beauveria* sp. FKI-1366 II. Structure elucidation. *J. Antibiot.* **57**, 117-124 (2004).
- Jensen, P.R., Williams, P.G., Oh, D.C., Zeigler, L., and Fenical, W., Species-specific secondary metabolite production in marine actinomycetes of the genus *Salinispora*. *Appl. Environ. Microbiol.* **73**, 1146-1152 (2007).
- Jeong, S.Y., Shin, H.J., Kim, T.S., Lee, H.S., Park, S.K., and Kim, H.M., Streptokordin, a new cytotoxic compound of the methylpyridine class from a marine-derived *Streptomyces* sp. KORDI-3238. *J. Antibiot.* **59**, 234-240 (2006).
- Lee, J.Y., Lee, J.Y., Moon, S.S., and Hwang, B.K., Isolation and antifungal activity of 4-phenyl-3-butenic acid from *Streptomyces koyangensis* strain VK-A60. *J. Agric. Food Chem.* **53**, 7696-7700 (2005).
- Lim, T.H., Lee, J.M., Chang, T.H., and Cha, B., Antifungal activity and identification of an actinomycetes strain isolated from mummified peaches. *Kor. J. Appl. Microbiol. Biotechnol.* **28**, 161-166 (2000).
- Miyadoh, S., Research on antibiotic screening in Japan over the last decade: A producing microorganisms approach. *Actinomycetologica* **9**, 100-106 (1993).
- Pettit, G.R., Tan, R., Melody, N., Kieilty, J.M., Pettit, R.K., Herald, D.L., Tucker, B.E., Mallavia, L.P., Doubek, D.L., and Schmidt, J.M., Antineoplastic agents. Part 409: isolation and structure of montanastatin from a terrestrial actinomycete. *Bioorg. Med Chem. Lett.* **7**, 895-899 (1999).
- Rukachaisirikul, V., Chantaruk, S., Tansakul, C., and Saithong, S., Chaicharem-wimonkoon, L., Pakawatchai, C., Isaka, M., and Intereya, K., A cyclopeptide from the insect pathogenic fungus *Cordyceps* sp. BCC 1788. *J. Nat. Prod.* **69**, 305-307 (2006).
- Ryoo, I.J., Park, H.R., Choo, S.J., Hwang, J.H., Park, Y.M., Bae, K., Shin-

Ya, K., and Yoo, I.D., Selective cytotoxic activity of valinomycin against HT-29 human colon carcinoma cells via down-regulation of GRP78. *Biol. Pharm. Bull.* **29**, 817-820 (2006).

Zeggaf, C., Poncet, J., Jouin, P., Dufour, M.N., and Castro, B., Isopropenyl chlorocarbonate (IPCC) in amino acid and peptide

chemistry: esterification of N-protected amino acids; application to the synthesis of the depsipeptide valinomycin. *Tetrahedron* **45**, 5039-5050 (1989).

(Accepted May 7, 2007)