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Anti-dormant Mycobacterial Activity of Viomellein and Xanthomegnin, Naphthoquinone Dimers Produced by Marine-derived *Aspergillus* sp.

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In the course of a search for anti-dormant mycobacterial substances from marine-derived microorganisms, viomellein (**1**) and xanthomegnin (**2**) were re-discovered from the active fraction of the culture of a marine-derived *Aspergillus* sp. together with rubrosulphin (**3**) and asteltoxin (**4**) on the guidance of bioassay-guided separation. In particular, compound **1** showed higher activity against the dormant than against actively growing *Mycobacterium bovis* BCG and weak activity against *M. smegmatis*. Furthermore, evidence that compound **1** did not directly bind to plasmid DNA suggests its anti-mycobacterial activity differs from its direct chelating effect on the mycobacterial genome.

Keywords: Viomellein, Marine-derived *Aspergillus* sp., Antibiotics, Tuberculosis, Dormant.

Tuberculosis is one of the most common causes of morbidity and mortality in adults who are HIV-positive and living in poverty. The World Health Organization (WHO) estimated that there were 1.5 million tuberculosis-related deaths in 2015. Similar to other infectious diseases, the appearance of multi-drug resistant strains is a problem associated with tuberculosis treatment. Moreover, the long-term chemotherapy required to cure effectively the disease is a considerable problem. One of the major reasons for the extended chemotherapeutic regimens and widespread epidemicity of tuberculosis is that the causative agent, *M. tuberculosis*, has an ability to revert to a state of dormancy [1].

The marine environment is a rich source of drug leads because of its chemical and biological diversity. Numerous natural products or their derivatives have been reported as anti-mycobacterial substances. However, most previous studies focused on growth-inhibitory activity against *M. tuberculosis* under active growing conditions [2]. Therefore, new lead compounds that are effective against *M. tuberculosis* in both its active and dormant states are urgently needed.

Although the pathology of the latent *M. tuberculosis* infection is still unclear, a hypoxic condition was found to induce the dormancy of *Mycobacterium* sp., which has a drug susceptibility profile resembling that of the latent *M. tuberculosis* infection [3]. Based on these findings, we have established a screening system to search for substances with antimicrobial activity against dormant mycobacteria. In this assay system, the minimum inhibitory concentration (MIC) values of isoniazid against *M. smegmatis* (a non-pathogenic strain of a fast growing type) and *M. bovis* Bacille Calmette-Guérin (BCG, a vaccine strain of a slow growing type) were 2.5 and 0.05 μg/mL, respectively under aerobic conditions, whereas the corresponding values were > 25 μg/mL under hypoxic conditions, as shown in Table 1. To date, we have discovered some marine natural products such as trichoderins (new aminolipopeptides) [4], neamphamide B (new cyclic depsipeptide) [5], and 2-methoxy-3-oxoaaptamine (new aaptamine class alkaloid) [6], as anti-dormant mycobacterial substances using the assay system

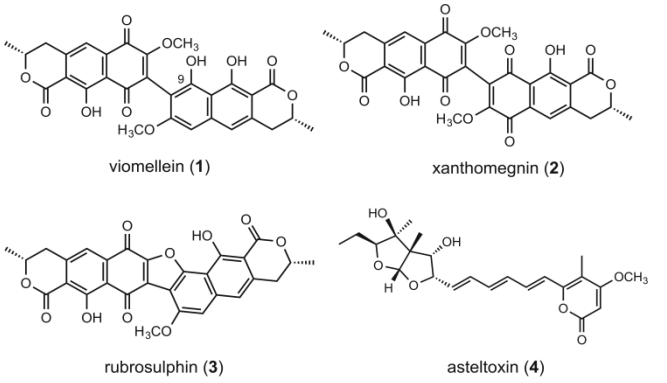

Figure 1: Chemical structures of compounds **1**-**4**.

system. The continuous screening of marine sponges and marine-derived microorganisms led to the re-discovery of viomellein (**1**) [7] and xanthomegnin (**2**) [8, 9] as anti-dormant mycobacterial substances from the active fraction of the culture of a marine-derived *Aspergillus* sp., together with rubrosulphin (**3**) [8, 10, 11] and asteltoxin (**4**) [12] following the bioassay-guided separation (Figure 1). Although compound **3** has been reported as a natural product, compound **1** was easily transformed into compound **3** in MeOH solution.

Compound **1** exhibited potent anti-microbial activity against *M. bovis* BCG under both actively growing aerobic and dormancy-inducing hypoxic conditions with MICs of 6.25 and 1.56 μg/mL, respectively, while the compound showed weak activity against *M. smegmatis* (Table 1). Compound **1** has a tendency to affect *M. bovis* BCG under dormant rather than actively growing conditions. In contrast, compound **2** showed weak anti-microbial activity against both *M. smegmatis* and *M. bovis* BCG with MIC values of 12.5~50 μg/mL, while compounds **3** and **4** showed no effects at up to 200 μg/mL (Table 1). These results suggest that the phenolic hydroxy group at C-9 of compound **1** is important for its activity against *M. bovis* BCG. On the other hand, compound **1** was reported to show

Table 1: Anti-mycobacterial activity of compounds **1**-**4** and isoniazid.

	MIC (μg/mL)			
	M. smegmatis		*M. bovis* BCG	
Compds	Aerobic	Hypoxic	Aerobic	Hypoxic
1	25	50	6.25	1.56
2	12.5	12.5	25	50
3	>200	>200	>200	>200
4	>200	>200	>200	>200
Isoniazid	2.5	25	0.05	>100

activities such as insecticidal [13], inhibition of the addition of a small ubiquitin-like modifier (SUMOylation) [14], and antimicrobial against several kinds of bacteria and fungi [9], whereas no anti-mycobacterial activity has been reported for the compound.

In addition, compound **1** is also known as a mycotoxin, and to have weak genotoxic activity, as evaluated using the SOS chromotest against *Escherichia coli* K12 [15]. Therefore, we speculate that the anti-mycobacterial activity of compound **1** is caused by its effect on the mycobacterial genome. To confirm this speculation, we next investigated whether compound **1** binds to DNA using plasmid pMV206 (Figure 2). Compound **1** was detected on the agarose gel at concentrations ranging from 3 to 30 μg/10 μL with a retention factor (Rf) of 0.58 (Figure 2A and C), while that of the plasmid pMV206 was calculated as 0.51 and 0.41 (Figure 2B and C). Then, no significant reduction and band shift were observed in the DNAs (Figure 2B). This result suggests that compound **1** did not bind to the plasmid DNA directly and, therefore, its anti-mycobacterial activity might be different from its direct chelating effect on the mycobacterial genome. A study of the detailed mode of action of compound **1** is currently in progress.

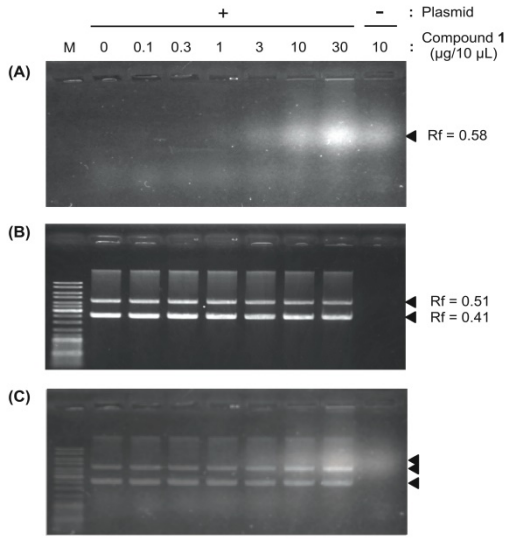

Figure 2: Binding of compound **1** to plasmid DNA. (**A**) Agarose gel was observed under UV conditions at 312 nm to detect compound **1**. (**B**) Resolved DNA samples were visualized using ethidium bromide staining. (**C**) Merged gel images of **A** and **B**. Retention factor (Rf) values of compound **1** and pMV206 were determined by analyzing each gel image using Adobe Photoshop.

Experimental

Anti-mycobacterial activity under aerobic and hypoxic conditions: *M. smegmatis* mc^2155 and *M. bovis* BCG Pasteur were grown in Middlebrook 7H9 broth containing 10% oleic albumin dextrose catalase (OADC, both BD, Franklin, NJ, USA), 0.5% glycerol, and 0.05% Tween 80. The MIC values of the test compounds against *M. smegmatis* and *M. bovis* BCG under aerobic and hypoxic conditions were determined using the established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [4]. Mid-log phase bacilli [*M. smegmatis* (1 x 10^4 CFU/0.1 mL) or *M. bovis* BCG (1 x 10^5 CFU/0.1 mL)] were inoculated in a 96-well plate, and then, serially diluted samples were added to the 96-well plate. In the case of the aerobic conditions, the bacteria were incubated at 37°C for 36 h (for *M. smegmatis*) or for 7 days (for *M. bovis* BCG). In the case of the hypoxic conditions, the mycobacterial bacilli were grown in Middlebrook 7H9 broth at 37°C under a nitrogen atmosphere containing 0.2% oxygen until the optical density reached 0.8 at 600 nm. Subsequently, the bacilli were inoculated to the 96-well plate at the same density under aerobic conditions and incubated at 37°C under a nitrogen atmosphere containing 0.2% oxygen for either 96 h (for *M. smegmatis*) or 14 days (for *M. bovis* BCG). After incubation, 50 μL of MTT solution (0.5 mg/mL) was added to each well and incubated at 37°C for an additional 12 h under aerobic or hypoxic conditions. The optical density at 560 nm was measured to determine the MIC value.

Isolation of compounds 1-4 from marine-derived* Aspergillus *sp.: The marine-derived *Aspergillus* sp. 02E28_2-2 was isolated from an unidentified marine sponge, which was collected at Sabang Island, Indonesia in 2014. The strain was cultured in rice medium (totally 250 g of unpolished rice and 500 mL of artificial sea water) under static condition at 30°C for 2 weeks. The culture was extracted with acetone and a mixed organic solvent of acetone/MeOH/EtOAc (4:2:1), and then the organic solvents were combined and evaporated under reduced pressure to obtain a crude extract. This was partitioned into a water/EtOAc mixture. The active EtOAc soluble portion (9.3 g) was further partitioned into an *n*-hexane/90% aq MeOH mixture. On the guidance of bioassay, the 90% MeOH soluble portion [5.2 g, minimum inhibitory concentration (MIC) against *M. bovis* BCG (3.13 μg/mL in aerobic conditions, 6.25 μg/mL in hypoxic conditions)] was fractionated by silica gel column chromatography [eluted with $CHCl_3$:MeOH:H_2O (lower phase)] to obtained 8 fractions (Fr.M1~Fr.M8). Among these, Fr.M5 [eluted with $CHCl_3$:MeOH:H_2O = 100:3:1 (lower phase)] showed potent anti-mycobacterial activity [1.3 g, (MIC: 2.5 μg/mL in aerobic conditions, 2.5 μg/mL in hypoxic conditions)] against *M. bovis* BCG. The active Fr.M5 was separated by normal-phase HPLC [Cosmosil 5SL-II (10 mm id × 250 mm); eluted with $CHCl_3$/toluene/AcOH = 70/30/1] to give a viomellein (**1**) containing fraction (Fr.M5-1, 30 mg), a rubrosulphin (**3**) containing fraction (Fr.M5-2, 13 mg) and a xanthomegnin (**2**) containing fraction (Fr.M5-3, 17 mg). Fr.M5-1 was subjected to Sephadex LH-20 column chromatography eluted with $CHCl_3$ to provide viomellein (**1**, 11 mg) [7]. Fr.M5-3 was purified by reversed-phase HPLC [Cosmosil $5C_{18}$-AR-II (10 mm id × 250 mm), eluted with MeCN/H_2O = 60/40] to give xanthomegnin (**2**, 4.8 mg) [8, 9]. Fr.M5-2 was recrystallized with $CHCl_3$-MeOH to obtain rubrosulphin (**3**, 1.5 mg) [8, 10, 11]. On the other hand, Fr.M6 [eluted with $CHCl_3$:MeOH:H_2O = 100:3:1 (lower phase)] showed moderate anti-microbial activity against *M. bovis* BCG under both aerobic and hypoxic conditions with a MIC of 20 μg/mL. Then, Fr.M6 (490 mg) was separated by ODS-HPLC [Cosmosil $5C_{18}$-AR-II (10 mm id × 250 mm); eluted with MeOH/H_2O = 60/40] to give asteltoxin (**4**, 90 mg) [12] and a trace amount of compound **1**. Compounds **1**-**4** were identified by ESI-TOF-MS and 2D-NMR analyses and comparison with authentic spectral data [7-12].

DNA binding analysis of compound 1: The binding of compound **1** to pMV206 of plasmid DNA was evaluated as described below. The indicated amounts of compound **1** were added to 10 μL TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) in the presence or absence of pMV206 (500 ng). Then, each reaction mixture was incubated at room temperature for 1 h; the samples were mixed with

a DNA loading dye (NEB, #B7024S), and then subjected to agarose gel electrophoresis without boiling. After electrophoresis, the agarose gel was observed under UV conditions at 312 nm to detect compound **1** using the ImageQuant LAS4010 Digital Imaging System (GE Healthcare Life Sciences). In addition, the resolved DNAs were visualized using ethidium bromide staining. The retention factor (Rf) values of compound **1** and pMV206 were determined by analyzing each gel image using Adobe Photoshop.

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References

[1] Wayne LG, Sohaskey CD. (**2001**) Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annual Review of Microbiology*, ***55***, 139-163.
[2] Copp BR, Pearce AN. (**2007**) Natural product growth inhibitors of *Mycobacterium tuberculosis*. *Natural Product Reports*, ***24***, 278-297.
[3] Wayne LG, Hayes LG. (**1996**) An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infection and Immunity*, ***64***, 2062-2069.
[4] Pruksakorn P, Arai M, Kotoku N, Vilchèze C, Baughn AD, Moodley P, Jacobs Jr., WR, Kobayashi M. (**2010**) Trichoderins, novel aminolipopeptides from a marine sponge-derived *Trichoderma* sp., are active against dormant mycobacteria. *Bioorganic & Medicinal Chemistry Letters*, ***20***, 3658-3663.
[5] Yamano Y, Arai M, Kobayashi M. (**2012**) Neamphamide B, new cyclic depsipeptide, as an anti-dormant mycobacterial substance from a Japanese marine sponge of *Neamphius* sp. *Bioorganic & Medicinal Chemistry Letters*, ***22***, 4877-4881.
[6] Arai M, Han C, Yamano Y, Setiawan A, Kobayashi M. (**2014**) Aaptamines, marine spongean alkaloids, as anti-dormant mycobacterial substances. *Journal of Natural Medicines*, ***68***, 372-376.
[7] Stack ME, Mazzola EP, Eppley RM. (**1979**) Structures of xanthoviridicatin D and xanthoviridicatin G, metabolites of *Penicillium viridicatum*: Application of proton and carbon-13 NMR spectroscopy. *Tetrahedron Letters*, ***52***, 4989-4992.
[8] Simpson TJ. (**1977**) ^{13}C Nuclear magnetic resonance spectra and biosynthetic studies of xanthomegnin and related pigments from *Aspergillus sulphureus* and *melleus*. *Journal of the Chemical Society, Perkin Transactions 1*, 592-595.
[9] Zeeck A, Russ P, Laatsch H, Loeffler W, Wehrle H, Zähner H, Holst H. (**1979**) Isolation of the antibiotic semi-vioxanthin from *Penicillium citreo-viride* and synthesis of xanthomegnin. *Chemische Berichte*, ***112***, 957-978.
[10] Durley RC, MacMillan J, Simpson TJ, Glen AT, Turner WB. (**1975**) Fungal products. Part XIII. Xanthomegnin, viomellin, rubrosulphin, and viopurpurin, pigments from *Aspergillus sulphureus* and *Aspergillus melleus*. *Journal of the Chemical Society*, *Perkin Transactions 1*, 163-169.
[11] Stack ME, Eppley RM, Dreifuss PA, Pohland AE. (**1977**) Isolation and identification of xanthomegnin, viomellein, rubrosulphin, and viopurpurin as metabolites of *Penicillium viridicatum*. *Applied and Environmental Microbiology*, ***33***, 351-355.
[12] Kruger GJ, Steyn PS, Vleggaar R, Rabie CJ. (**1979**) X-ray crystal structure of asteltoxin, a novel mycotoxin from *Aspergillus stellatus* Curzi. *Journal of the Chemical Society, Chemical Communications*, 441-442.
[13] Ondeyka JG, Dombrowski AW, Polishook JP, Felcetto T, Shoop WL, Guan Z, Singh SB. (**2003**) Isolation and insecticidal activity of mellamide from *Aspergillus melleus*. *Journal of Industrial Microbiology and Biotechnology*, ***30***, 220-224.
[14] Hirohama M, Kumar A, Fukuda I, Matsuoka S, Igarashi Y, Saitoh H, Takagi M, Shin-ya K, Honda K, Kondoh Y, Sait, T, Nakao Y, Osad, H, Zhang KY, Yoshida M, Ito A. (**2013**) Spectomycin B1 as a novel SUMOylation inhibitor that directly binds to SUMO E2. *ACS Chemical Biology*, ***8***, 2635-2642.
[15] Auffray Y, Boutibonnes P. (**1987**) Genotoxic activity of some mycotoxins using the sos chromotest. *Mycopathologia*, ***100***, 49-53.